# Elucidation of the physical and functional interactions between the MEIS1A and the CREB and CRTC transcription factors

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# ELUCIDATION OF THE PHYSICAL AND FUNCTIONAL INTERACTIONS BETWEEN THE MEIS1A AND THE CREB AND CRTC TRANSCRIPTION FACTORS

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# TABLE OF CONTENTS

# LIST OF FIGURES AND TABLES

13

# CONTRIBUTIONS OF AUTHORS

Mark Featherstone is the thesis supervisor and he supervised the work in all three chapters.

**CHAPTER 3: To elucidate the mechanism behind the transcriptional activity of MEIS1A at the C-terminus**

Goh* SL, Looi* Y, Shen H, Fang J, Bodner C, Houle M, Ng AC, Screaton RA, Featherstone M. *co-first authors (2009) (published in J Biol. Chem.)

I performed the experiments in figures 1-3. Figure 4-6, 8 and 11 were carried out by Dr Goh Siew Lee and were presented in the paper. Figures 7, 9 and 10 were performed by Dr Shen hui. I also contributed to the writing of the paper.

**CHAPTER 4: The effects of CREB and its mutants (ACREB and ACREBR314A), CBP and other protein interactions at the MEIS1A C-terminus**

I performed all the experiments in this chapter.

**CHAPTER 5: To determine the nature of the MEIS-CRTC-CREB complex and the structural changes accompanying the interactions**

I performed the experiments in figures 1-5 and 8-13 in this chapter. I performed figures 6-8 together with the expertise of Dr Cornelia Hunke from the lab of our collaborator Dr Gerhard Gruber. Figure 12 was performed by Dr Shen hui.

17

# ABSTRACT

Myeloid ecotropic viral integration site 1 (MEIS1), a member of the TALE class of homeodomain-containing transcription factors, is a co-factor for HOX and PBX proteins. Together, these regulators are involved in patterning the anteroposterior axis and limbs of developing embryos, organogenesis, hematopoiesis and cancer. Recent findings revealed that the MEIS1A C-terminus harbors a transcriptional activation domain that is responsive to protein kinase-A (PKA) signaling and dependent on the co-activator CREB-binding protein (CBP). This same C-terminus that harbors the transcriptional activation domain and is responsive to PKA signaling has also been shown to mediate MEIS1A oncogenicity. This thesis seeks to extend our current understanding of the mechanisms by which the MEIS1A C-terminus exerts its transactivation function. We describe here the involvement of CREB and its coactivators CBP and CRTC in contributing to the PKA responsiveness at the MEIS1A C-terminus. Our studies revealed an ability of CRTC to bypass PKA for transactivation at the MEIS1A C-terminus. We have also demonstrated physical interaction between CRTC and MEIS proteins and have mapped the domain in CRTC that interacts with MEIS to the N-terminal coiled-coil region. Further chromatin immunoprecipitation confirmed co-occupancy of MEIS1, CRTC2 and PBX1 proteins on MEIS1 target genes. With the use of CREB mutants ACREB and ACREBR314, I determined the importance of CREB to the activity of MEIS1A. By means of proximity ligation assay, I have further established endogenous interactions between MEIS-CRTC, MEIS-CREB and MEIS-CBP. These findings strongly implicate the MEIS1A C-terminus in binding

and functionally interacting with CREB and CRTC. I have also attempted to elucidate the structure of the MEIS and CRTC proteins in the hope of understanding how the proteins interact to function during development. I have in this thesis identified a cooperative role of MEIS, CREB and CRTC interaction, where CRTC with the help of CREB physically cooperates with MEIS to achieve PKA-inducible transactivation through the MEIS1A C-terminus, implicating the concerted action of these proteins in developmental processes.

# LIST OF ABBREVIATIONS

ABD-B  abdominal-B

ALL  acute lymphoblastic leukemia

AML  acute myeloid leukemia

ARE  auto-regulatory element

ATF-1  activating transcription factor-1

bZIP  basic-leucine-zipper

CAD  constitutive activation domain

CBD  CREB binding domain

CBP  CREB binding protein

CRE  cyclic AMP response element

CREB  cyclic AMP response element binding protein

ChIP  chromatin immunoprecipitation

CREM  cAMP response element modulator

CRTC  cAMP-regulated transcriptional co-activators

CTD  C-terminal domain

DBD  DNA binding domain

E14.5  embryonic day 14.5

EXD  extradenticle

FSK  Forskolin

HD  homeodomain

HSC  hematopoietic stem cells

| HEK293T | human embryonic kidney 293T |
|---------|------------------------------|
| hpf | hours post fertilization |
| HR | homologous region |
| HTH | homothorax |
| HTLV-1 | human T-cell leukemia virus type 1 |
| KID | kinase inducible domain |
| KIX | KID interaction domain |
| LMB | Leptomycin B |
| MEIS | myeloid ecotropic viral integration site |
| MLL | mixed lineage leukemia |
| NTD | N-terminal domain |
| NLS | nuclear localization signals |
| NES | nuclear exporter sequences |
| PBX | pre-B-cell leukemia homeobox |
| PEPCK | phosphoenolpyruvate carboxykinase |
| PKA | protein kinase A |
| PLA | proximity ligation assay |
| PP | phosphatase |
| PREP | PBX-regulating protein |
| r | rhombomere |
| RA | retinoic acid |
| RARE | retinoic acid response elements |
| Rlu | relative light units |

| | |
|---|---|
| SIK | salt-induced-kinase |
| TAD | transcriptional activation domain |
| TALE | three-amino-acid-loop extension |
| TAX | transforming protein of human T-cell leukemia virus type 1 |
| TSA | Trichostatin A |
| Ubx | ultrabithorax |
| HAT | histone acetylasetransferase |
| G6Pase | glucose-6-phosphatase |
| PGC-1α | peroxisome proliferator-activated receptor γ coactivator 1α |

# CHAPTER 1

# GENERAL INTRODUCTION

# 1  **MEIS**

The *Meis1* (<u>m</u>yeloid <u>e</u>cotropic viral <u>i</u>ntegration <u>s</u>ite 1) homeobox gene was first identified as the site of viral integration in 15% of myeloid tumors arising in BXH-2 mice (Moskow et al, 1995). Insertional mutation caused by this virus produced misexpression of proto-oncogenic truncated *Meis1* (Moskow et al, 1995). The gene is located on chromosome 11 in mouse and on chromosome 2p13–p14 in human (Moskow et al, 1995; Smith et al, 1997). Subsequent DNA cross-hybridization studies revealed two highly conserved *Meis1*-related genes residing on separate chromosomes, *Meis2* and *Meis3* (Nakamura et al, 1996a; Oulad-Abdelghani et al, 1997; Steelman et al, 1997; Yang et al, 2000). *Meis* orthologs were subsequently identified in a number of species: *homothorax (hth)* in *Drosophila*, *Meis* in chick and mammals, *meis* in zebrafish, *Xmeis* in *Xenopus laevis* and *unc-62* (a.k.a. *ceh-25*) in *C. elegans* (Pai et al, 1998; Salzberg et al, 1999; Steelman et al, 1997; Van Auken et al, 2002; Waskiewicz et al, 2001).

## 1.1  **MEIS and TALE**

*Meis*, together with the *Pbx (<u>P</u>re-<u>B</u>-cell leukemia homeobox)* and *Prep (<u>P</u>BX-regulating protein)* families, encodes members of the three-amino-acid-loop extension (TALE) class of homeodomain-containing proteins (Mann & Abu-Shaar, 1996; Moens & Selleri, 2006; Moskow et al, 1995; Nakamura et al, 1996a). The TALE homeoproteins, as the name suggests, contain an extra three

highly conserved residues (Pro-Tyr-Pro) at positions 24–26 in the loop between helix 1 and 2 in the typical homeodomain (Bürglin, 1995; Burglin, 1997). Other members of the TALE family in animals include TGIF, and IRO (Iroquois) (Burglin, 1997). In plants: KNOX and BEL, and in fungi: M-ATYP (atypical mating type genes), and CUP (Burglin, 1997). Table 1 shows the members of the TALE class proteins and their corresponding ortholog within each family and the species in which they were first described. Apart from the homeodomain, the MEIS protein shares two homology regions with other members of the MEINOX TALE-homedomain-containing proteins, namely PREP1 and PREP2. The homology regions reside within the N-terminal regions termed HR1 and HR2 (residues 58–137) (Figure 1) (Berthelsen et al, 1998c; Pai et al, 1998). The N-terminal HR1 domain of MEIS/HTH/PREP allows the formation of a heterodimer via the PBC-A domain within the PBX/EXD N-terminus (Figure 1) (Chang et al, 1997; Haller et al, 2002; Shanmugam et al, 1999).

Tale class proteins

| Sub-family name | Protein name | Species[a] |
|---|---|---|
| PBC | PBX1 | human |
| | PBX2 | human |
| | PBX3 | human |
| | PBX4 | mouse |
| | lazarus | zebrafish |
| | EXD | fly |
| | CEH-20 | nematode |
| MEIS[b] | MEIS1 | mouse |
| | MEIS2 | mouse |
| | MEIS3 | mouse |
| | HTH | fly |
| | CEH-25 | nematode |
| PREP[b] | PREP1 | human |
| | PREP2 | human |

**Table 1 Members of the vertebrate TALE class protein namely PBC, MEIS and PREP**

Listed are the corresponding ortholog under each family and the species in which they were first described in. Adapted from (Featherstone, 2003).
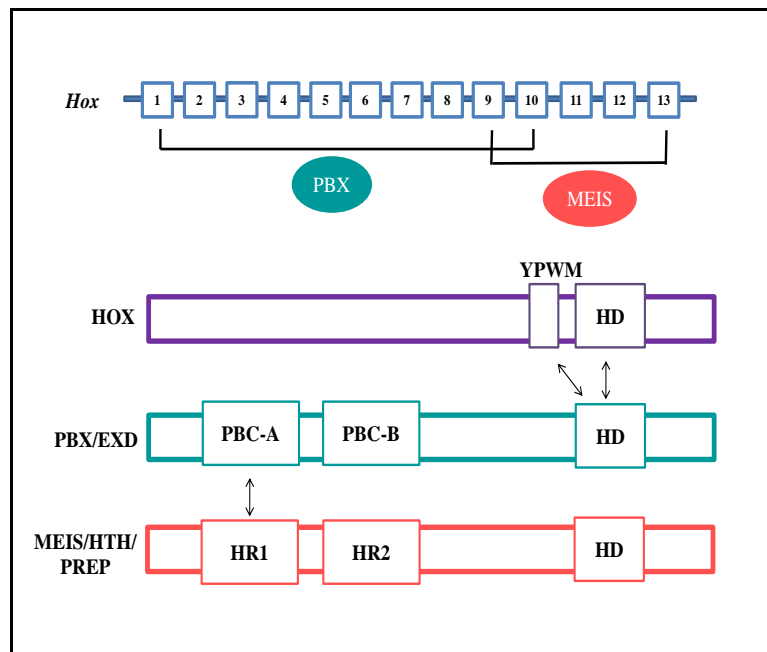
**Figure**

**1 HOX and its cofactors**

Upper panel, PBX interacts with and binds DNA cooperatively with HOX proteins from paralog group 1 to 10, while MEIS interacts with and binds DNA cooperatively with HOX proteins from paralog group 9 to 13. HOX paralog group 9 and 10 interacts with MEIS and PBX.

Lower panel, A schematic of HOX proteins and their DNA binding partners from the PBC and MEIS/PREP families. Functional domains are indicated. HD represents the homeodomain which are present in all three protein families. HOX and PBC family interaction involves the HOX conserved YPWM motif from paralog group 1 to 8. A conserved residue at the N-terminal of the homeodomain mediates HOX group 9 and 10 interaction with PBX. Interaction between PBX and MEIS family is mediated through the PBX-A domain of PBX and the HR1 domain of MEIS. In addition, the N-terminus of HOX and the HD and C-terminus of MEIS mediates the HOX and MEIS binding (interaction not shown). Domains involved in protein-protein interactions are shown by arrows. Adapted from (Goh 2007).

## 1.2 MEIS paralogs and their expression in normal development

There are five isoforms of *Meis1* as a result of alternative splicing into *Meis1a-e* (Geerts et al, 2005; Knoepfler et al, 1997; Moskow et al, 1995; Wermuth & Buchberg, 2005). Expression of *Meis1* has been found in the liver, pancreas, appendix and brain, with a high level within the cerebellum (Smith et al, 1997). Some identified roles of *Meis1* during embryonic development include the establishment of hematopoietic stem cells, cell cycle regulation, proximo-distal limb growth, embryonic implantation as well as in the development of the eye (Azcoitia et al, 2005; Bessa et al, 2008; Mercader et al, 1999; Xu et al, 2008; Zhang et al, 2002).

*Meis1* is highly expressed in the adult bone marrow and E14.5 fetal liver rich in hematopoietic stem cells (HSC), indicating a role of *Meis1*, together with *Hox* and *Pbx1* in proliferation and self-renewal of the HSC (Hisa et al, 2004; Pineault et al, 2002). A recent paper, in support of the finding has also showed an indirect effect of *Hoxb4* in targeting the expression of *Meis1* in HSC (Irving & Mason, 2000). In addition, MEIS, along with other TALE family proteins PBX and PKNOX have also found to play a role in regulating regional epidermal barriers both in human and mice skin, a region where a dermal HOX protein code has also been implicated (Jackson et al, 2011; Rinn et al, 2008).

MEIS2, with 85% protein sequence identity to MEIS1, has its gene expression pattern in the developing nervous system, limbs, face, and in viscera of lung, gut , kidney as well as in rhombomeres 2 and 3 of wild-type embryos (Cecconi

28

et al, 1997; Niederreither et al, 2000; Oulad-Abdelghani et al, 1997). *Meis2* has also been shown to mark proliferating striatal precursors/neurons for striatal neuron differentiation, as well as muscle and neural tissue histogenesis (Cecconi et al, 1997; Toresson et al, 1999). In the adult mouse, however, most of the *Meis2* expression is detected in the brain and female genital tract, though it is interesting to note the different distribution of the alternative splice forms in these organs. *Meis2c* and *d* are more abundant in the female genital tract, while *Meis2a* and *b* are more abundant in the brain (Oulad-Abdelghani et al, 1997).

*Meis1* and *2* have been shown to act upstream of and directly regulate *Pax6* expression in vertebrate lens formation in the eye (Heine et al, 2008; Zhang et al, 2002). Together with *Pax6*, *Meis2* has been shown to be required in human neocortex development (Larsen et al, 2010). MEIS3, a MEIS family member with 67% identity with MEIS1, with gene expression abundantly expressed in pancreatic islets and β-cells and has been shown to regulate pancreatic cell survival (Liu et al, 2010). The involvement of *Meis1*, *2* and *3* have also recently been seen at the peripheral nervous system which were differentially expressed during dorsal root ganglion (DRG) and sympathetic chain formation both in mice and chick (Bouilloux, 2010).

## 1.3  HTH in normal development

In *Drosophila*, HTH expression is high in the endoderm cells of the embryonic midgut, where the PBX homolog encoded by *extradenticle* (*exd*) is nuclear

(Mann & Abu-Shaar, 1996). *hth* is both responsible and necessary for cell fate specification of inner photoreceptors (Wernet et al, 2003). Together with *exd*, *hth* is also required for salivary gland formation (Henderson & Andrew, 2000). In other roles, *hth* also plays the part of an antenna selector gene and acts with *distal-less*, a gene associated in proximodistal patterning of the *Drosophila* limbs (Casares & Mann, 1998; Dong et al, 2000). A recent finding provided evidence in the regulation of *meis* on the *Prod1* promoter during axolotl limb regeneration, further extending the role of *meis* in specifying proximodistal axis (Shaikh et al, 2011).

The role of *hth* can also be seen in inhibition of wing development where, together with *teashirt,* act to repress the *vestigial* gene and hence inhibits wing blade formation (Casares & Mann, 2000). In contrast, at the wing hinge, *hth* is instead activated by the *wingless* gene for wing hinge development (Casares & Mann, 2000).

In the developing eye, HTH has been shown to act with EXD to repress eye formation and to prevent inappropriate eye development (Pai et al, 1998; Zhang et al, 2002). In addition, *hth* has been shown to be activated by *wingless* (*wg*) and repressed by *decapentaplegic* (*dpp*) and a complex of EYELESS (PAX6 homolog), HOMOTHORAX and TEASHIRT has been found, suggesting a cooperative role of these proteins in promoting cell proliferation in the eye disc and a control of premature expression of other downstream transcription factors (Bessa et al, 2002; Mann & Chan, 1996; Peng et al, 2009). Two alternative splice forms of *hth* were found: one encodes the DNA-binding homeodomain

(*hthFL*) while one does not (*hDless*) (Noro et al, 2006). The *hDless* form was also identified in the mouse *Meis1* (Noro et al, 2006). Both isoforms induce nuclear localization of *exd* but each carries out unique functions *in vivo* (Noro et al, 2006).

### 1.4    MEIS expression in chicken

Chicken *Meis1* and *Meis2* have also been identified to play a role during early embryogenesis and organogenesis during chicken development with an indication of the loss of the *Meis3* ortholog in birds during evolution (Sanchez-Guardado et al, 2011b). *Meis1* and *2* expression have also been observed during early otic specification suggesting a role of *Meis* in the developing inner ear (Sanchez-Guardado et al, 2011a).

### 1.5    MEIS expression in xenopus

*Xenopus* homolog of M*eis1* consists of at least three forms, *Xmeis1*, *Xmeis2* and *Xmeis3*. Two alternative splice variants were detected for *Xmeis1*, namely *Xmeis1a* and *Xmeis1b* with divergence at the C-terminus (Moskow et al, 1995; Steelman et al, 1997). *Xmeis1b* has been shown to play a significant role in neural crest development and its misexpression induces ectopic of neural crest marker expression in developing embryos (Maeda et al, 2001). *Xmeis1a*, on the other hand, exhibits a less obvious effect (Maeda et al, 2001). *Xmeis3* has been found in neural development in *Xenopus* and activates transcription of posteriorly expressed neural markers (Salzberg et al, 1999). Expression was

also observed in the hindbrain from rhombomere 2 to 4 and anterior spinal cord of developing *Xenopus* (Salzberg et al, 1999). Misexpression of *Xmeis3* resulted in loss of cement gland, forebrain, eyes and midbrain structure (Salzberg et al, 1999). MEIS has also been shown to caudalize and dorsalize the CNS of the *Xenopus* embryos and in cultures (Maeda et al, 2001; Salzberg et al, 1999). In support of this, ectopic *Xmeis3* expression lead to caudalization of *Xenopus* embryos (Salzberg et al, 1999). Synergistic regulation has also been shown between *Xmeis3* and *Hoxd1* during gastrulation (In der Rieden et al, 2011).

### 1.6 **MEIS expression in zebrafish**

In zebrafish, *meis1.1* is detected in three domains along the anteroposterior axis: the presumptive forebrain, the anterior midbrain and the hindbrain/spinal cord from rhombomere 3 onwards at the 2 somite stage (10.5 hpf) (Figure 2) (Waskiewicz et al, 2001). At the 10 somite stage (14 hpf), *meis1.1* expression expands to the anterior to include rhombomere 2, as well as appearing in the developing eye fields, while expression in the presumptive forebrain persists (Figure 2) (Waskiewicz et al, 2001). At the 20 somite stage (19 hpf), the expression of *meis1.1* broadens, peaking in the hindbrain rhombomeres 2 to 4 and in the developing eye. At 24 hpf, the expression of *meis1.1* is expressed broadly in the neural tube, with high levels of expression within the hindbrain, retina and anterior midbrain-hindbrain boundary (Figure 2) (Waskiewicz et al, 2001).

*Meis2.2* is expressed in the forebrain and from rhombomere 4 posteriorly in the hindbrain as well as the spinal cord at 2 somite stage (Figure 2) (Waskiewicz et al, 2001). At the 10 somite stage, *meis2.2* can be detected within the presumptive eye fields, the forebrain and from rhombomere 2 posteriorly throughout the spinal cord (Waskiewicz et al, 2001). By 24 hpf, *meis2.2* has extended towards the ventral forebrain, midbrain, hindbrain and spinal cord (Figure 2) (Waskiewicz et al, 2001).

Within the developing eye, *meis1.1* has a role in cell cycle control by regulating *cyclin D1* and c-myc expression (Bessa et al, 2008; Heine et al, 2008). Expressions of *meis1.1* and *meis2.2* have also been detected in the olfactory epithelium, *meis2.1* and *meis2.2* in the branchial arches of the developing zebrafish (Santos et al, 2010).

*meis1* has also been implicated as a regulator of endothelial cell development and *meis1* knockdown exhibit defects on arterial development thus revealing the role of *meis* in differentiation of endothelial cells and the formation of vascular network (Minehata et al, 2008). Knockdown of *meis1.1* in zebrafish causes a delay in the G1 to S phase transition, correlating with significant reduction of cyclin D3 levels, resulting in severely reduced eyes and smaller lenses (Bessa et al, 2008; Hisa et al, 2004). Such phenotype has been recapitulated in *Meis1* mutant mice (Argiropoulos et al, 2010; Hisa et al, 2004).

*meis3.1* can be detected in the posterior hindbrain up to rhombomere 3/4 (Waskiewicz et al, 2001). By 24 hpf, expression is reduced in the hindbrain while the expression in the spinal cord and somites remains high (Figure 2)

(Waskiewicz et al, 2001). *meis3* expression overlaps expression of *sonic hedgehog* (*shh*) in the endoderm of zebrafish embryos (diIorio et al, 2007). Knockdown of *meis3* revealed its role in restriction of insulin expression in the anterior endoderm by acting upstream of *shh* (diIorio et al, 2007).
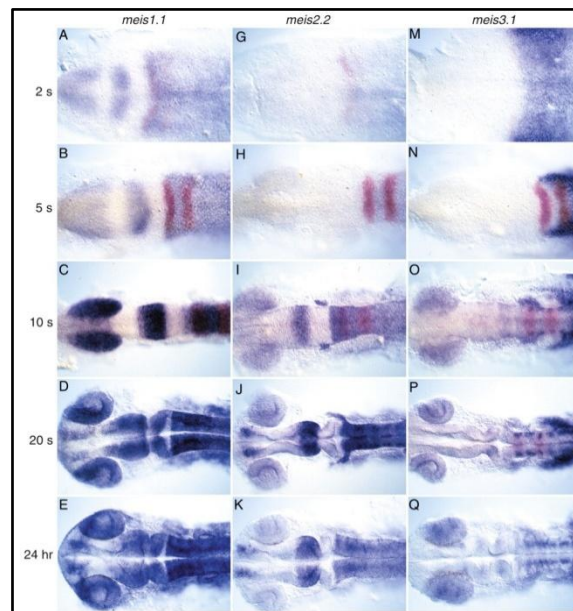


**Figure 2 Expression patterns of *meis1.1*, *meis2.2* and *meis3.1* at various stages during zebrafish development.**

RNA *in situ* hybridization of *meis1.1* (A-E), *meis2.2* (G-K) and *meis3.1* (M-Q) at various developmental stages. *meis* expression is in blue, *krox20* in r3 and r5 is in red. Each panel is oriented such that anterior is towards the left. Adapted from (Waskiewicz et al, 2001).

### 1.7   MEIS and hematopoiesis

During embryogenesis, waves of hematopoietic activity from different sites of the embryo contribute to both embryonic and adult hematopoiesis (Galloway & Zon, 2003). Vertebrate hematopoiesis occurs in two successive waves: the

primitive and the definitive wave. Implications of *Meis1* and *Pbx* were detected in hematopoiesis as *Meis1* deficient and *Pbx* knockdown mice were shown to exhibit profound embryonic anemia (Azcoitia et al, 2005; DiMartino et al, 2001; Hisa et al, 2004). Despite the evolutionary differences between mammals and zebrafish, their hematopoietic pathways as well as the transcription factor genes that govern the pathways, are highly conserved. Given the similarities, both the mammalian and zebrafish hematopoietic pathway will be discussed here.

### 1.7.1 Primitive wave

The first wave of hematopoiesis, the primitive/embryonic wave in the mammalian embryos initiates within the yolk sac between embryonic days 7.25 and 9.0, generating erythrocytes (Takahashi et al, 1989). In zebrafish development, the primitive wave is also transient and predominantly produces erythrocytes and primitive macrophages to supply blood cells for the initial stages of embryonic development (Galloway & Zon, 2003). The anatomical location for the primitive wave is the intermediate cell mass between 10-26 hpf and the rostral blood island (Jagadeeswaran et al, 1999). Some of the markers expressed during this wave include: *scl, gata1, gata2, fli1a, imo2, hhex* (Liao et al, 2000; Ransom et al, 2004; Thompson et al, 1998). The role of *meis1* and *pbx1* during zebrafish primitive hematopoiesis have also been noted, where inhibition of the proteins led to defects in erythropoietic gene expression (Goh 2007; Pillay et al, 2010). *meis* and *pbx*, in association with certain *hox* genes, have been proposed to function upstream of *gata1* to specify erythropoietic cell lineage and to inhibit myelopoiesis (Pillay et al, 2010).

### 1.7.2  Definitive wave

The second wave, the definitive/fetal-adult hematopoiesis is responsible for maintaining long-term HSC renewal potential throughout the life of the organism. The HSC population is initially found in the aorta-gonad-mesonephros at 24-48 hpf and finally residing at the liver and bone marrow around 4-5 dpf (Galloway & Zon, 2003; Zon, 1995). Some of the markers expressed in this wave include: *c-myb, imo2, ikaros, and runx1* (Burns et al, 2002; Kalev-Zylinska et al, 2003; Kalev-Zylinska et al, 2002; Thompson et al, 1998). In mammals, *Meis* expression has been detected in the adult bone marrow and fetal liver and its requirement during differentiation of the megakaryocytic lineage and  correct patterning of the vascular network suggesting its role during definitive hematopoiesis (Azcoitia et al, 2005; Di Rosa et al, 2007; DiMartino et al, 2001; Ferretti et al, 2006; Hisa et al, 2004; Pineault et al, 2002). In support of this role, *Meis1* deficient mice die during midgestation as a result of failure of definitive hematopoiesis (Azcoitia et al, 2005).

### 1.8  Regulation of expression by retinoic acid

Vitamin A (retinol) adopts various roles during embryogenesis as well as in various adult physiologies. Its primary metabolite, retinoic acid (RA) acts as ligand that binds to retinoid receptors (RARs or RXRs). These receptors bind specifically to retinoic acid response elements (RAREs) within the nucleus, and their regulatory role in transcription of gene targets are governed by altering the

binding of corepressors and coactivators (Duester, 2008). Loss of RA signaling by deficiency in vitamin A or by compound RAR mutations revealed the importance of RA in the development of various organs including hindbrain, spinal cord, heart, skeleton, forelimb buds, eyes, lungs, pancreas and genitourinary tract (Clagett-Dame & DeLuca, 2002; Dersch & Zile, 1993; Dickman et al, 1997; Lohnes et al, 1994; Mendelsohn et al, 1994). As a morphogen (a molecule that emits signal from a localized source, forming a concentration gradient across a developing tissue), retinoic acid has been indicated to play a role in the anterior-posterior patterning, particularly in the development of the hindbrain and specifying the left-right symmetry (Irving & Mason, 2000; Mercader et al, 2000; Schneider et al, 1999).

### 1.9    Meis induction by retinoic acid

Expression of *Meis1* and *Meis2* is restricted to the proximal domain of the developing limb where RA acts upstream of *Meis1* and *Meis2* to regulate proximal limb development (Capdevila et al, 1999; Mercader et al, 1999; Mercader et al, 2000). The importance of *Meis* as a proximal regulator can be observed when overexpression of either *Meis1* or *Meis2* leads to inhibition or truncation of distal limb compartments (Capdevila et al, 1999; Mercader et al, 1999). RA administration activates ectopic *Meis1/Mei*s2 expression in the distal limb and promotes proximalization of limb cells and inhibits distalization during limb development and regeneration (Capdevila et al, 1999; Mercader et al, 1999; Mercader et al, 2005). Upon limb-bud emergence, fibroblastic growth

factor (FGF) then restricts RA synthesis and in turn *Meis* signaling to the proximal limb in the chick embryo (Mercader et al, 1999). The conservation of the effect of RA and *Meis* on hindbrain patterning can be seen in developing *Xenopus laevis* embryos, where *Xmeis3* plays a role in posteriorizing cell fate (Dibner et al, 2001).

In P19 cells, *Meis2a* to *Meis2d* are induced upon induction by retinoic acid, consistent with the *in vivo* studies observed (Oulad-Abdelghani et al, 1997). Mouse forelimb and hindlimb show up to four fold enhancement in the mRNA levels of *Meis1, 2 and 3* after RA treatment (Qin et al, 2002). In addition, levels of *Pbx* mRNA and proteins (PBX1, 2 and 3) were also elevated post transcriptionally upon RA induction, possibly due to stabilization by MEIS protein (Ferretti et al, 2000; Knoepfler & Kamps, 1997; Oulad-Abdelghani et al, 1997; Qin et al, 2002).

## 1.10  Meis loss of function/mutant

*hth* mutants are embryonic lethal and display defects in head involution, abdominal and thoracic segmentation, patterning of sensory organs and the central nervous system (Kurant et al, 1998; Rieckhof et al, 1997). In the adult, *hth* mutants causes antenna to leg transformation, ectopic eye growth in the ventral head and defect in leg segmentation whose phenotype resembles those of *exd* (Casares & Mann, 1998; Gonzalez-Crespo et al, 1998; Pai et al, 1998; Yao et al, 1999). The phenotype resulting from mutation of *Drosophila hth* resembles that of *exd* mutants and multiple *hox* gene mutants, signifying its

participation in most *hox* and *exd* functions (Kurant et al, 1998; Pai et al, 1998; Rieckhof et al, 1997). In addition, cooperative role for *Meis1* and *Pbx* in congenital heart disease is suggested by the observation that mice bearing mutations in either gene display similar cardiac anomalies (Stankunas et al, 2008). *Meis1* deficient mice die around midgestation of cardiac anomalies, hemorrhage and liver hypoplasia, signifying the importance of *Meis1* in hematopoiesis (Azcoitia et al, 2005). *Meis1* null embryos fail to separate the blood and lymphatic vasculature, show extensive hemorrhaging, and completely lack megakaryocyte/platelets during angiogenesis and die by E14.5 (Carramolino et al, 2010; Hisa et al, 2004).

### 1.11  MEIS in oncogenesis

*Meis* was originally detected due to its activation by retroviral insertion in mice myeloid tumors, identifying *Meis* as a leukemogenic gene (Moskow et al, 1995; Nakamura et al, 1996a). Supporting this finding, high *Meis* expression is also seen in a subset of human myeloid leukemia cell lines (Smith et al, 1997). MEIS1 in collaboration with PBX and HOX has been shown to accelerate and promote leukemic transformation in mice (Chang et al, 1997; Lawrence et al, 1999; Schnabel et al, 2000; Thorsteinsdottir et al, 2001; Wermuth & Buchberg, 2005). In human, *Meis1,* in concert with various *Hox* genes, is upregulated in most acute myeloid leukemias (AML) and mixed-lineage leukemias (MLL) (Afonja et al, 2000; Azcoitia et al, 2005; Fujino et al, 2001; Lawrence et al, 1999; Moskow et al, 1995; Nakamura et al, 1996b; Wang et al, 2005).

There is a 16% overlap between the target genes regulated by MEIS and MN1, a negative prognostic factor in patients with AML (Heuser et al, 2011; Heuser, 2010). Their common target genes reveal identical binding sites, suggestive of the dependence of MN1 on the transcriptional activity of MEIS1 for its transforming potential (Heuser et al, 2011; Heuser, 2010). A dominant trans-active fusion of VP16-Meis1 renders *Meis1* spontaneously oncogenic, suggestive of MEIS functioning as an autonomous oncoprotein and mimics combined activity of MEIS1-HOXA9 without co-expression of exogenous or endogenous *Hox* genes (Mamo et al, 2006; Wang et al, 2006). HOXA9 and MEIS1A are found to be co-expressed in human AML and have been shown to induce acute myeloid leukemia in mice (Calvo et al, 2001; Kroon et al, 2001; Thorsteinsdottir et al, 2001; Wermuth & Buchberg, 2005). Given that HOXA9 and MEIS1 are down-regulated during normal myeloid differentiation, it is suggested that the pair acts to regulate progenitor abundance by suppressing differentiation and maintaining self-renewal (Calvo et al, 2001). With regards to their leukemogenic effects, overexpression of neither MEIS1A, HOXA9 nor PBX1 on its own leads to leukemogenesis, further demonstrating a collaborative effect in transformation of hematopoietic cells (Kroon et al, 1998). Apart from its requirement for initiating AML progenitors, *Meis1* also plays a role in transcribing HSC genes like *Cd34, Flt3* and *Erg1* and complexes of HOXA7/A9, MEIS1 and PBX1/2 were found on the *Flt3* promoter, giving rise to AML (Nicholas Zorko, 2010; Palmqvist et al, 2006; Wang et al, 2005; Wang et al, 2006). The *c-myb* gene plays a role in leukemia, highlighting a function of

c-myb in mediating HSC development by acting as an essential downstream target in mediating HOXA9-MEIS1 transformation of hematopoietic cells (Hess et al, 2006). Evidence of *Meis1*, together with *c-myb,* was found to be upregulated in neuroblastoma and is important for cell growth and proliferation of neuroblastoma cells (Geerts et al, 2005; Geerts et al, 2003; Thiele et al, 1988).

In the context of MLL, *Meis1* is also found to be an essential and rate-limiting regulator, and *Meis1* overexpression strongly induces caspase-dependent apoptosis in both hematopoietic and non-hematopoietic cells (Wermuth & Buchberg, 2005; Wong et al, 2007). In contrast coexpression of both *Meis1* and *Hoxa9* protect cells from various apoptosis signals, indicating a cooperative effort in modulating leukemogenesis (Wermuth & Buchberg, 2005). In other roles, *Meis1* expression along with that of certain *Hox* genes and *Pbx*, has been detected in ovarian cancer (Crijns et al, 2007). An even more recent paper has also implicated *Meis1* in binding and regulation of mitochondrial promoters and a role in pancreatic cancer cells (Tomoeda et al, 2011).

Many studies have implicated MEIS1 in limb development. Its importance in limb development was further strengthened through a strong association between *MEIS1* and restless leg syndrome (RLS) (Walters & Rye, 2009; Winkelmann et al, 2007; Xiong et al, 2009; Young et al, 2009). Peripheral and brain iron deficiency has been shown previously in RLS, and recent findings have revealed an increase in ferritin levels following a knockdown of *MEIS1 in*

*vitro*, thus providing an important association between RLS, MEIS1 and iron metabolism (Catoire et al, 2011).

*Bmi-1* is a gene that is essential in regulation and maintenance of the proliferative activity of both normal and leukemic stem cells (Lessard & Sauvageau, 2003). Its role in malignant hematopoiesis can be seen as HOXA9-MEIS1-induced mouse leukemia is dependent on the expression of *Bmi-1* (Lessard & Sauvageau, 2003). *In vivo* co-occupancy of *Meis1* is seen on regulatory sequences of *Flt3*, *Trib2* and *Ccl3*, thus revealing the presence of other pathways employed by *Meis1* for leukemogenesis (Argiropoulos et al, 2008). These studies have therefore provided evidences of the cooperative capability of *Meis1* with various (proto-) oncogenes in normal physiology and oncogenesis.

# 2   Cooperative interaction between MEIS with HOX

## 2.1   Homeodomain-DNA complex

The *Hox* genes are known as the master regulatory genes which play important role in regulating the anterior-posterior body axis development (Favier & Dolle, 1997; Veraksa et al, 2000). The HOX homeobox consists of a conserved 180 nucleotide sequence which encodes a 60 amino acid sequence known as the homeodomain (Gehring et al, 1994a; Gehring et al, 1994b; Wolberger, 1996). The homeodomain consist of an N-terminal arm and three α helices held by hydrophobic interactions. Helix 2 and 3 resemble a helix-turn-helix motif, with the recognition helix, helix 3, and making contacts with the major groove of

DNA. The flexible N-terminal arm of the homeodomain contacts the minor groove and determines the specificity for HOX monomer binding (Joshi et al, 2007; Phelan & Featherstone, 1997; Ryoo et al, 1999). All HOX homeodomain recognize a core binding site of 5' TAAT 3' sequence on the DNA. The first two bases (<u>TA</u>AT) are specified by the flexible N-terminal arm which often bears an arginine or a lysine at position 3 and an arginine at position 5 (Featherstone, 2003). The two 3' bases of the core binding site (TA<u>AT</u>) are specified by helix 3 and are contacted in the major groove (Billeter et al, 1993; Pabo & Sauer, 1992; Wolberger et al, 1991). Asparagine occupies position 51 within all homeodomain within the helix 3 and contacts DNA at (TA<u>A</u>T) (Featherstone, 2003). Position 50 on the homeodomain plays a major role in DNA recognition by contacting the two bases 3' to the core (5' TAAT<u>NN</u> 3') (Featherstone, 2003). A glutamine at position 50 (Gln50), such as is found in all HOX homeodomains, recognizes binding site TAATTG, TAATGG, or TAATTA, while a lysine at position 50 (Lys50), such as is found in the Bicoid homeodomain, favors TAAT<u>CC</u> (Featherstone, 2003; Grant et al, 2000; Hanes & Brent, 1989; Treisman et al, 1989; Tucker-Kellogg et al, 1997).

Since all HOX homeodomains bear a glutamine at position 50, the opportunities for differential sequence recognition with the HOX family are constrained (Featherstone, 2003). Having said that, there have been instances which demonstrate regulatory roles of HOX monomer *in vivo* (Galant & Carroll, 2002; Li & McGinnis, 1999; Li et al, 1999; Pinsonneault et al, 1997). However, collective work has established that the homeodomain alone is not likely to be

able to dictate HOX-DNA binding specificities (Affolter et al, 1990; Desplan et al, 1988; Ekker et al, 1994; Ekker et al, 1991; Hoey & Levine, 1988; Mann et al, 2009).

Thus one of the mysteries of HOX specificity is how different HOX proteins which possess similar *in vitro* DNA binding specificity as monomers direct cells into distinctly different developmental pathways to execute distinct highly specific functions *in vivo* (Brooke et al, 1998; Dessain et al, 1992; Ekker et al, 1994; Laughon, 1991). The identification of HOX cofactors helped explain how HOX functions can be discriminated by increasing specificity and affinity of HOX-DNA binding. X-ray crystal structures suggest that the HOX-cofactor-DNA complex not only increase the size of the binding sites but also help enforce additional structure to the otherwise unstructured homeodomain (Mann et al, 2009).

## 2.2    Cooperative DNA binding model

It is hence revealed that one way for HOX proteins to achieve functional diversity and specificity *in vivo* is by association with other proteins. The DNA binding ability of HOX transcription factors dramatically increases by forming heterodimeric complexes with other homeodomain-containing proteins (Asahara et al, 1999; Mann & Affolter, 1998; Mann & Chan, 1996; Mann et al, 2009; Phelan & Featherstone, 1997; Sprules et al, 2003). A well characterized HOX cofactor is the transcription factor PBX, which binds to HOX proteins in paralog groups 1 to 10. The HOX/PBX heterodimer shows a significantly

longer recognition site hence a higher binding affinity and specificity than a HOX monomer (Figure 3) (Chang et al, 1995; Medina-Martinez & Ramirez-Solis, 2003; Shen et al, 1997b).



**Figure 3 Comparison of consensus DNA binding sites of HOX monomer**
The sequences recognized by the HOX homeodomain in the monomeric and heterodimeric sites are aligned. HOX/PBX heterodimer shows a significantly longer recognition site than the HOX monomer. Adapted from Figure 1A (Phelan & Featherstone1997).

### 2.3 PBC group of TALE proteins

The PBC-group of TALE proteins includes Pre-B cell leukemia homeobox (PBX) in vertebrates, EXTRADENTICLE (EXD) in *Drosophila* and CEH-20 in C. *elegans* (Burglin, 1998; Burglin & Ruvkun, 1992; Flegel et al, 1993; Monica et al, 1991; Rauskolb et al, 1993). The mammalian PBX1 was first identified due to its involvement in t(1;19) chromosomal translocation in 25% of childhood pre-B-cell acute leukemia (Kamps et al, 1990; Nourse et al, 1990). The translocation results in the formation of a fusion transcript which codes for the oncogenic E2A-PBX1 chimeric protein, where the C-terminal DNA binding domain of E2A is replaced by the homeodomain containing sequences of PBX1

45

(Kamps et al, 1990; Nourse et al, 1990). Isoforms for PBX1 and 3 have been identified and result from post-transcriptional alternative splicing (Kamps et al, 1990; Milech et al, 2001; Monica et al, 1991; Nourse et al, 1990). Through alternative splicing, *PBX1* encodes PBX1A and PBX1B isoforms with distinct biological properties (Asahara et al, 1999; Di Rocco et al, 1997). A C-terminal activation domain was mapped in PBX1A but is not present in the shorter PBX1B (Di Rocco et al, 1997). While PBX1B is predominant during mouse embryogenesis, PBX1A expression is primarily restricted to developing neural tissues and both forms E2A-PBX1A and E2A-PBX1B are detected in human primary tumor cells (Kamps et al, 1991; Schnabel et al, 2001).

Subsequently, PBX2 and PBX3 were isolated based on their homology to PBX1. A fourth member of the PBX family, PBX4, was also identified in mouse, human and zebrafish (Monica et al, 1991; Popperl et al, 2000; Vlachakis et al, 2000; Wagner et al, 2001). PBX2 and PBX3 showed extensive sequence homology of 92 to 94% outside the homeodomain to PBX1, covering over 266 amino acids of sequence identity (Monica et al, 1991). Beyond the 266 amino acids, all three PBX proteins are distinguished by extensive divergence at their extreme C- and N-termini (Monica et al, 1991). All three *Pbx* transcript can be detected in most fetal and adult tissues and tested cell lines, except for PBX1 which is absent in lymphoid cell lines (Monica et al, 1991; Roberts et al, 1995). Members of the same subfamily of homeodomain containing proteins appear to have latent oncogenic potential as demonstrated

by the transformation competency when PBX2 and 3 are fused with E2A (Monica et al, 1994).

The widespread expression domain of *Pbx1* overlaps that of many *Hox* genes, allowing their protein products to act in concert to activate downstream target genes. The expression of *Pbx1* also extends beyond the regions of *Hox* expression, an indication of *Hox*-independent functions in transcriptional regulation (DiMartino et al, 2001; Kim et al, 2002; Kmita et al, 2005; Krumlauf, 1993; Roberts et al, 1995; Schnabel et al, 2001; Selleri et al, 2001).

### 2.4 Domains of PBX

The PBC domain consist of 2 regions of homology N-terminal to the homeodomain namely PBC-A and PBC-B (Figure 4) (Burglin, 1998). Upon DNA-binding, a fourth alpha helix (Figure 4) consisting of a 16 amino acid residues at the C-terminus of the PBX homeodomain contributes to a hydrophobic binding pocket for the HOX YPWM peptide which is crucial in stabilizing interactions with DNA (Jabet et al, 1999; Lu & Kamps, 1996; Passner et al, 1999; Piper et al, 1999; Sprules et al, 2000; Sprules et al, 2003). Physical interaction studies showed that intramolecular contacts between the PBX1 N-terminus and the HD masked the two nuclear localization signals (NLS) residing within the PBX homeodomain (Figure 4 asterisks) (Abu-Shaar et al, 1999; Ryoo et al, 1999; Saleh et al, 2000). The PBC-A domain of PBX1A, PBX1B and PBX2 has been shown to mediate interactions with other TALE proteins like PREP1 and MEIS1 (Berthelsen et al, 1998c; Chang et al, 1997).

Two nuclear exporter sequences (NES) have been found within the PBC-A domain across residues 45-72 and another across residues 73-90 (Berthelsen et al, 1999; Kilstrup-Nielsen et al, 2003). PBC domain of PBX1 also acts to controls the balance between nuclear export and import (Kilstrup-Nielsen et al, 2003). A 25 residue α-helix inhibitory domain within the PBC-B domain binds to the homeodomain thereby inhibiting intrinsic PBX-DNA binding and heterodimerization with HOX proteins (Calvo et al, 1999; Neuteboom & Murre, 1997). The folding of the inhibitory helix over the position 28 (E28) (Figure 4) within the homeodomain by intramolecular interactions represses activity either by masking or destabilizing the homeodomain-DNA complex (Calvo et al, 1999). In addition to the 39 residues sequence N-terminal to that inhibitory helix which mediates PBX dimerization, the PBC-B domain also contains four conserved serine residues (187, 193, 202 and 290) within the PBC-B domain with a nuclear localization signal (Calvo et al, 1999; Kilstrup-Nielsen et al, 2003). In another scenario, nuclear localization of PBX1 independent of MEIS protein has also been found. In certain cell context specific cases, protein kinase A (PKA) phosphorylates the serine residues with the PBC-B domain *in vitro*, and the activation of PKA was thus shown to block nuclear export of PBX1 in distal chick limb buds *in vivo* (Kilstrup-Nielsen et al, 2003). PREP1, another TALE homeodomain protein, has also been found to bind to the N-terminus of PBX1 during cooperative DNA-binding and de-represses the inhibitory helix function within PBX1 (Calvo et al, 1999).
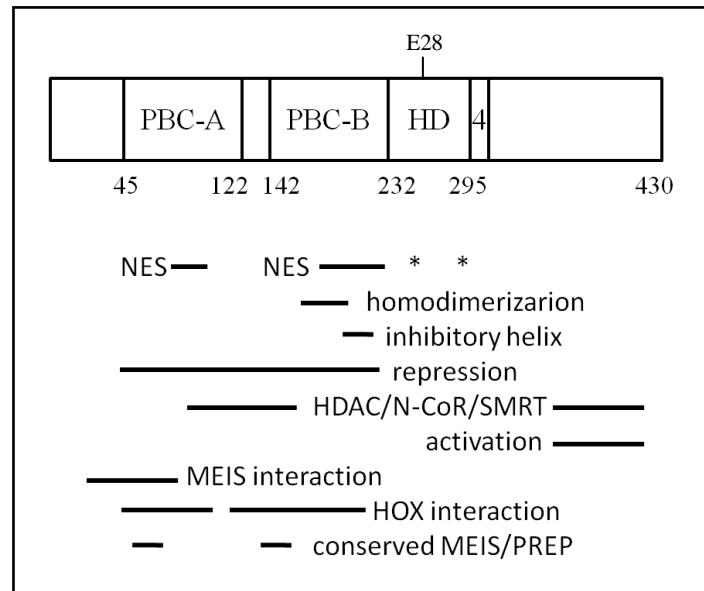
**Figure 4 Domains of human PBX1A**

PBC-A. PBC-B domains, the homeodomain (HD), the fourth helix (4), E28 represents glutamate at position 28 within the homeodomain. Nuclear localization signals (NLS) are indicated in asterisks. Adapted from (Featherstone, 2003).

## 2.5   HOX-PBX cooperative DNA binding

HOX-PBX cooperative interactions are modulated by two regions. In the HOX partner, interaction with PBX is mediated by a short conserved motif lying upstream of homeodomain called the "hexapeptide" or YPWM motif (Chang et al, 1995; Green et al, 1998; Lu & Kamps, 1996; Phelan et al, 1995; Shanmugam et al, 1997). In the PBX partner, interaction with HOX is mediated by the *Hox* cooperative motif or GKFQ domain residing C-terminal to the homeodomain (Green et al, 1998).The GKFQ motif is dispensable for cooperative interaction but acts to stabilize the HOX-PBX complex (Green et al, 1998; Lu & Kamps, 1996). On the other hand, the YPWM motif interacts with the PBX

homeodomain and is strictly required for cooperative interactions both *in vitro* and *in vivo*, even in the absence of the GKFQ domain (Green et al, 1998).

PBX1-HOX heterodimers bind the consensus sequence 5'-ATGATTNAT[G/T/A][G/T/A]3' site and thereby displays greater DNA-binding specificity than HOX or PBX monomers (Figure 3) (Lu et al, 1994; Van Dijk, 1993). HOX proteins recognize and bind to the 3' half site (T[A/T]AT[G/T/A][G/T/A]) while PBX recognizes and binds the 5' ATGAT half site. The recognition of A vs T at the second position of the HOX half site is specified by the N-terminal arm of the HOX homeodomain while the last two positions (G, T or A) are specified by a glutamine at position 50 of the homeodomain (Chan & Mann, 1996; Chang et al, 1996; Phelan & Featherstone, 1997). This suggests an important role of the N-terminal arm of the homeodomain in the DNA-binding specificity of PBX-HOX heterodimers (Featherstone, 2003; Phelan & Featherstone, 1997; Ryoo et al, 1999).

Interactions of HOX proteins from paralog groups 1 through 8 with PBX are mediated by a conserved YPWM sequence located N-terminal to the homeodomain, while this purpose is served by three amino acids (ANW) in the same region the HOX proteins encoded by paralog groups 9 and 10 (Chang et al, 1996; Johnson et al, 1995; Neuteboom et al, 1995; Phelan et al, 1995; Shen et al, 1996). Cooperative binding between PBX-HOX heterodimers is restricted to HOX proteins from paralog group 1 through 10, thereby excluding proteins from paralog group 11-13 (Chang et al, 1996; Shen et al, 1997b).

Dimerization between PBX/EXD and HOX cooperatively binds DNA and stabilizes the DNA bound complex (Chan et al, 1994; Chang et al, 1995; Passner et al, 1999; Peltenburg & Murre, 1996; Phelan et al, 1995; Piper et al, 1999). The exception to the case can be seen during the regulation of the limb-promoting gene *distalless*, where HOX proteins seem to bypass the requirement of classical YPWM motif for the recruitment of *Drosophila extradenticle* (Chan et al, 1994; Merabet et al, 2007). Thus, in most cases, the flexibility in HOX-PBC contact contributes to the specificity of HOX protein function (Mann & Chan, 1996; Merabet et al, 2007).

A previous finding by the lab has established that the formation HOX-PBX complex can lead to two outcomes. Depending on the context, HOX-PBX complex can repress or activate transcription through differential regulation with coregulators. Transcriptional activation can be mediated by binding of the HOX-PBX complex to coactivators (eg: CBP) while transcriptional repression can be brought about by associating with repressor complex such as histone deacetylases (Saleh et al, 2000). Importantly, PKA signaling has been shown to act as a switch that converts HOX-PBX from repressor to activator establishing that HOX-PBX functions in patterning *via* cell signaling (Saleh et al, 2000).

## 2.6 PBX expression in normal development

*Pbx* genes have been shown to be essential in early limb bud formation, limb bud positioning, establishment of limb axis as well as in pelvic girdle development (Capellini et al, 2011a; Capellini et al, 2011b). Apart from its

collaboration with HOX proteins to regulate cell fate specificity, cooperative interactions between PBX and other homeodomain-containing proteins has also been shown. Such partners include ENGRAILED (Erickson et al, 2007; Peltenburg & Murre, 1996), the pancreatic and duodenal homeobox 1 transcription factor (PDX1) which regulates somatostatin and insulin expression has been implicated (Goudet et al, 1999; Peers et al, 1995) and EMX2 which has a role in central nervous system and urogenital development (Capellini et al, 2010). In another non-HOX related context, PBX has been found to bind to the myogenin promoter to mark muscle-specific genes for MyoD-mediated activation (Berkes et al, 2004).

### 2.7 PBX mutant

*Pbx* mutant die at E15/16 with severe hypoplasia or aplasia of multiple organs and defects of the axial and appendicular skeleton (Selleri et al, 2001). The malformation affected the proximal skeletal patterning but not affecting distal structures (Selleri et al, 2001). *Pbx* mutants in mice recapitulate the *Hox* loss of function phenotypes, supporting the dependence of PBX and HOX proteins function in the control of developmental processes (Moens & Selleri, 2006). Evidence can be seen in the inactivation of *Pbx1*, also in *Pbx2* and *Pbx3*, leads to cardiac anomalies, a defect which has been observed in disruption of *Hoxa3* gene expression (Chisaka & Capecchi, 1991; Stankunas et al, 2008). In addition, *Pbx* null mice exhibited similar phenotype as *Hoxa3* single mutants and other *Hox* compound mutants in pharyngeal development (Manley et al, 2004).

## 2.8 **PBX expression in Drosophila**

The *Drosophila* EXD bears 71% resemblance to its human ortholog PBX1 (Rauskolb et al, 1993). Expression of *Pbx1* in the mouse limb resembles that of its ortholog in the *Drosophila* leg (Gonzalez-Crespo et al, 1998). The *exd* gene patterns the proximal region of the leg by antagonizing the distal gene *wingless* and *decapentaplegic*, while in the distal domain it is suppressed by *distalless*, which prevents nuclear import of *exd* (Diaz-Benjumea et al, 1994; Gonzalez-Crespo et al, 1998; Lecuit & Cohen, 1997; Rauskolb et al, 1995). In *Drosophila*, *exd* is required for proper patterning of the peripheral nervous system (between stages 8-10), a time where EXD and HTH start to accumulation in the nuclei (Rauskolb et al, 1993). The activity of EXD is regulated post-translationally by nuclear translocation and it is only active in the nucleus (Aspland & White, 1997; Mann & Abu-Shaar, 1996). Reduction in *exd* expression leads to homeotic transformation in the embryo where thoracic segments adopt an anterior fate while abdominal segments adopt a more posterior fate (Peifer & Wieschaus, 1990). Complete removal of maternally and zygotically expressed *exd* leads to specific alterations in segmentation, a result of loss of ENGRAILED function and gene expression (Kobayashi et al, 2003; Peifer & Wieschaus, 1990).

Cooperative interactions were also observed between UBX (in *Drosophila*) and EXD and acts to stabilize the DNA-bound form of UBX (Chan et al, 1994). Interestingly, mutation in *exd* alters segmental identity without changing either the domains of *Hox* gene expression (Peifer & Wieschaus, 1990). Thus, these

findings suggest that *exd* and the *Hox* genes function in parallel during development (Rauskolb et al, 1993).

## 2.9 PBX expression in zebrafish

The PBX ortholog *lazarus/pbx4* in zebrafish is required for segmentation of the hindbrain and anterior trunk development and has also been shown to be required for multiple *hox* functions as defects in the *lazarus* mutant phenocopies *hox* loss of function mutants in mice (Arenkiel et al, 2004; McClintock et al, 2002; Popperl et al, 2000; Studer et al, 1996). *Meis3* has been shown to interact cooperatively with *Pbx4* and *Hoxb1b* in promoting hindbrain fates in the zebrafish (Salzberg et al, 1999; Vlachakis et al, 2001; Vlachakis et al, 2000). Expression of *hoxb1b* and *hoxa2* were suppressed in the context of a *lazarus* mutant (Cooper et al, 2003). Knockdown of *Meis1.1* displayed a similar phenotype to *lazarus* mutant, where it is suggested to function in similar pathway as *pbx* in zebrafish hindbrain (Waskiewicz et al, 2001). In other functions, *meis3*, together with *pbx4,* acts upstream of *shh* and plays a role in foregut development *via* insulin restriction (diIorio et al, 2007).

## 2.10 The MEINOX and PBX

TALE class proteins encoded by *Meis* genes in animals and products of the *Knox* genes in plants contain a conserved domain residing N-terminal to the homeodomain. This domain was proposed to be derived from an ancestral domain, termed MEINOX, and must have evolved prior to the split between

54

animals and plants (Burglin, 1997). PREP1 and 2, additional TALE class homeodomain proteins closely related to MEIS, have also been placed in the MEINOX subfamily on the basis of these conserved domains (Fognani et al, 2002). The complete MEINOX sequence was also found within the PBC domain which implied that PBX was also derived from the ancestral MEINOX gene.

# 3 PREP1

*PREP1* (PBX-regulating protein), also known as PKNOX1 due to its resemblance to the *Knotted-1* gene in plants, was purified along with *PBX* as a component of the urokinase enhancer factor 3 involved in tissue remodeling and cell migration (Berthelsen et al, 1998b; Burglin, 1998). It was also independently identified during a search for genes on human chromosome 21 (Berthelsen et al, 1998c; Chen et al, 1997). Murine PREP1 shares 100% identity with human PREP1 in the homeodomain and 95% similarity throughout the whole protein (Ferretti et al, 1999). Members of the PREP family include human and mouse *PREP/PKNOX1* and *2* (vertebrate) and zebrafish *prep1.1, 1.2 and 2* genes (Bernardi et al, 2010; Berthelsen et al, 1998a; Chen et al, 1997; Choe et al, 2002; Deflorian et al, 2004; Fognani et al, 2002; Haller et al, 2002; Imoto et al, 2001; Vaccari et al, 2010). Expression of *Prep1* and *Prep2* can be detected early in mouse development (Ferretti et al, 1999). PREP1 is expressed ubiquitously in the adult and is highest in the testis and thymus, while PREP2, though not as broadly expressed, displayed strong

expression in the brain, heart, skeletal muscle, ovaries and testis (Ferretti et al, 1999; Fognani et al, 2002; Haller et al, 2002). The differences in relative levels between *prep1 and prep2* varies between organs, suggesting a tissue-specific control (Haller et al, 2002).

PREP1 shares two homology regions with MEIS proteins within the N-terminal regions termed HR1 and HR2 (residues 58–137) (Berthelsen et al, 1998c) . The N-terminal MEINOX domain (HR1) of PREP/MEIS mediates heterodimer formation through interaction with the PBC-A domain within the PBX N-terminus (Haller et al, 2002). Interaction studies show that PREP and PBX1 form strong complexes in solution independent of DNA-binding (Berthelsen et al, 1998b; Berthelsen et al, 1998c). Heterodimerization of PREP1 and PBX promotes PREP1 binding to DNA at a higher affinity to a 5'TGACAG3' motif and overexpression of PREP1 increases the stability of PBX2 by preventing its proteasomal degradation (Berthelsen et al, 1998c; Longobardi & Blasi, 2003).

### 3.1 PREP-PBX-HOX Heterotrimer

Given the similarity between PREP and MEIS and their affinity for PBX, PBX-HOX dimer has been found to activate and enhance gene transcription through interactions with PREP or MEIS (Ferretti et al, 2000; Jacobs et al, 1999; Popperl et al, 1995; Samad et al, 2004). PREP1 has been shown to form a stable ternary structure with PBX and HOX on DNA *in vitro* (Berthelsen et al, 1998b), and evidence suggest that such heterotrimers function in the autoregulation of *Hoxb1* (Ferretti et al, 1999). *In vitro* studies have also

identified the PREP1-PBX1-HOXB1 complex within protein extracts from P19 cells upon retinoic acid induction (Ferretti et al, 2000). Expression of all three proteins are found in the mouse rhombomere 4 *in vivo*, suggestive of a regulatory role of PREP1 during development (Berthelsen et al, 1998b; Ferretti et al, 2005; Ferretti et al, 2000; Ferretti et al, 1999).

Evidence of cooperativity of the HOX-PBX-PREP heterodimer can be seen where HOXD10-PBX1B-PREP1 complex are found to activate the rat renin promoter cooperatively in COS-7 cells (Pan et al, 2005). Involvement of HOX-PBX1-PREP1 complex has also been shown in *in vitro* fetal adrenal-specific enhancer of the *Ad4BP/SF-1* gene (Zubair et al, 2006). However in certain contexts, cooperative interaction was identified between PBX1-PREP1 or PBX1-HOXB1 but no HOXB1-PBX1-PREP1 heterotrimer was detected at the human $\alpha2$(V) collagen COL5A2 promoter (Penkov et al, 2000), suggesting that trimer formation may be binding site specific and dependent on the context and constitution of the binding sites (Penkov et al, 2000).

### 3.2   prep expression in zebrafish

The zebrafish *prep1.1* gene was found to display ubiquitous expression starting from the 2 cell stage, (indicating maternal deposition of *prep1.1* mRNA) up to 24 hpf. Strong expression is then restricted to the head from 48 hpf onwards (Deflorian et al, 2004). Low levels of expression are detected throughout the embryo during segmentation (examined from 13-25 hpf) (Choe et al, 2002). The expression pattern of *prep1.1* at gastrula and segmentation stages

resembles that of *lazarus* (Popperl et al, 2000; Vlachakis et al, 2000). *prep1.1* has established essential functions in hindbrain development and neural crest cell differentiation (Deflorian et al, 2004).

Zebrafish *prep1.1* loss-of-function resulted in a significant defect in hindbrain segmentation and patterning (Deflorian et al, 2004). Downregulation of *prep1.1* causes a significant reduction of pbx2 and lazarus proteins and the overall PREP/MEIS/PBX DNA-binding activity was also strongly reduced (Choe et al, 2002; Deflorian et al, 2004; Ferretti et al, 2006; Mercader et al, 1999; Waskiewicz et al, 2001).

*prep1.2* has much more resemblance to *prep1.1* than *prep2,* contains 10 exons as well as an exon-intron organization identical to *prep1.1(Vaccari et al, 2010).* Both prep1.1 and prep1.2 harbors a glutamic acid rich region, though it resides at the C-terminal region in the former and at the N-terminal region in the latter (Vaccari et al, 2010). Maternal and zygotic expression of the paralog gene *prep1.2* is detected ubiquitously up to early somitogenesis when expression begins to restrict to the head and trunk mesenchyme (Vaccari et al, 2010; Waskiewicz et al, 2001). *prep1.2* has established a role during pharyngeal endoderm segmentation as well as pectoral fin bud development (Vaccari et al, 2010). Supporting this data, a loss of function displays malformed pharyngeal cartilage and a lack of pectoral fin, a phenotype closely resembling the loss of RA (Grandel et al, 2002; Vaccari et al, 2010). Thus, the different features between *prep1.1* and *prep1.2* appears to confer specific function in regulating independent processes (Vaccari et al, 2010).

# 4   Heteromeric interactions involving MEIS

## 4.1   HOX and MEIS

Functional synergy between *Meis* and *Hox* genes was first indicated in BXH2 mice with leukemia: 19 out of 20 leukemias presenting a retroviral insertion at the *Meis1* gene also harbored a second insertion at *Hoxa7* or *Hoxa9* (Nakamura et al, 1996b). DNA binding by ABD-B class HOX proteins HOXA9 to 13 and MEIS has been demonstrated and heterodimerization enhanced DNA binding stability (Chang et al, 1997; Crans-Vargas et al, 2002; Ferretti et al, 2000; Shen et al, 1997a). Deletion studies have mapped the interaction domains of ABD-B HOX proteins and MEIS to the N-terminal amino acids 1 to 61 in HOXA9 and a region C-terminal to the homeodomain of MEIS (Shen et al, 1997b). ChIP studies demonstrated that HOXA9 and MEIS1 specifically bind to the conserved distal enhancer at the *Meis1* locus which correlates with active MEIS expression (Wang, 2008). Thus overexpression of MEIS1 in acute leukemia is possibly sustained via an autoregulatory loop mediated through a distal enhancer element (Wang, 2008). In addition, *Meis1* was found to be recruited to the MEIS1 target promoter *in vivo*, further establishing its autoregulatory function (Goh et al, 2009). The degree of requirement for MEIS by different HOX proteins (for example between zebrafish Hoxb1a and Hoxb1b) are different and this preference could be assigned to the N-terminus of HOX, where a transcription activation domain resides (Choe & Sagerstrom, 2005).

The DNA binding complex formed by HOX-MEIS-DNA displayed a slower dissociation than MEIS-DNA alone, suggesting that HOX protein stabilizes the MEIS-DNA interactions (Shen et al, 1997b). Despite not forming DNA complex with ANTP class HOX proteins, MEIS is still able to form a trimer as a non-DNA-binding partner with DNA bound PBX-HOXD4 (Shanmugam et al, 1999). In another scenario, a different trimer class can form which involves non-DNA-bound PBX and DNA-bound MEIS-HOXD9 or D10 heterodimers (Shanmugam et al, 1999).

ChIP has shown the recruitment of MEIS together with PBX1, HOXA1 and HOXB1 to the *Hoxb1* autoregulatory element (ARE) which directs expression in rhombomere 4 of the hindbrain (Huang et al, 2005; Jacobs et al, 1999; Popperl et al, 1995). The *Hoxb2* enhancer, where the MEIS1-PBX1-HOXB1 binds, functions to direct appropriate expression of *Hoxb2* gene in response to *Hoxb1* cross-regulation to maintain r4 identity (Maconochie et al, 1997). In addition, putative binding sites have been identified for HOX-MEIS1 within the conserved *Hoxa3* hindbrain enhancer (Manzanares et al, 2001).

## 4.2  MEIS and PBX

The first identified *cis*-acting target element for members of both the *Pbx* and *Meis1* family is the cAMP-responsive sequence (CRS) (Bischof et al, 1998a; Bischof et al, 1998b). Binding site for PBX1 and MEIS1 can be detected on the CRS in the bovine *CYP17* gene, and the sequence is conserved in *Drosophila* and *C. elegans* (Bischof et al, 1998a; Bischof et al, 1998b). This thus provided a

first example of cooperative interaction between MEIS1 and PBX1 as neither protein can bind to the element alone (Bischof et al, 1998a).

*In vivo* and *in vitro* studies have identified heterodimerization of endogenous MEIS1 and PBX1 in the absence and presence of DNA binding (Chang et al, 1997). MEIS1-PBX1 complex binding to DNA has identified a core sequence of 5' TGATTGACAG 3' recognition site revealed two half sites, consisting of the 5' TGAT PBX site and a 3' TGACAG MEIS1 site (Chang et al, 1996; Chang et al, 1997; LeBrun & Cleary, 1994; Lu et al, 1995; Van Dijk et al, 1993).

In *Drosophila*, physical interaction has been shown between the HR domains of HTH and the PBC-A domain of EXD, thereby inducing nuclear localization of EXD *in vivo* (Abu-Shaar et al, 1999; Jaw et al, 2000; Pai et al, 1998; Ryoo et al, 1999). In the absence of MEIS/HTH, PBX/EXD is directed to the cytoplasm by the export receptor CRM1, an exporter which is specifically inhibited by the Leptomycin B (LMB) cytotoxin (Sakamoto & Frank, 2009; Zhang et al, 2005). EXD is thus exported out of the nucleus due to the dominant net nuclear export signal over the nuclear import signal (Abu-Shaar et al, 1999; Berthelsen et al, 1999). In the presence of HTH, the interaction shifts the balance between the nuclear export and import signals to favor nuclear localization of EXD (Abu-Shaar et al, 1999). An additional factor modulating the subcellular distribution is the nonmuscle myosin II heavy chain B (NMHCB) which has been shown to compete with MEIS to promote cytoplasmic retention of PBX/EXD *in vivo* (Huang et al, 2003).

61

A dominant negative form of MEIS protein induces a phenocopy of the *lazarus/pbx4* phenotype, and in addition, the E2A-PBX1 fusion protein produces a myeloid leukemia phenotype similar to that induced by MEIS1 suggesting a common pathway for these gene functions (Dedera et al, 1993; Kamps & Baltimore, 1993; Waskiewicz et al, 2001). In addition, the vertebrate *Meis1* gene is able to rescue the *hth* mutant phenotype and replace the role of *hth* in inducing nuclear translocation of *exd* in cell culture and *Drosophila* embryos suggesting an evolutionarily conserved mechanism for regulating *Hox* activity via the nuclear localization of *exd* (Rieckhof et al, 1997). The role of MEIS and PBX as well as their *Drosophila* homolog HTH and EXD was established where nuclear import of EXD by HTH is the main role of HTH in patterning the embryonic peripheral nervous system (PNS) (Kurant et al, 2001; Kurant et al, 1998). The observation of extremely low levels of the HTH protein found in embryos deficient in EXD further supports their cooperative and mutual stabilizing role (Kurant et al, 1998). Exclusion to this is the scenario when certain imaginal disc cells are also capable of localizing *exd* to the nucleus independent of *hth* (Ferretti et al, 2000; Rieckhof et al, 1997). In the case of the developing female genital tract at certain phases of cell cycle, PBX1 was found to be cytoplasmic even in the presence of MEIS, indicating another exception to the case (Dintilhac et al, 2005). An HTH-EXD complex has also been implicated in pattering of the thorax in *Drosophila* (Aldaz et al, 2005). MEIS-PBX complex plays a role in the activation of megakaryocytic gene expression and regulating expression at the dorsal neural tube of *Xenopus*

tailbud embryos and tadpoles (Kelly et al, 2006; Okada et al, 2003). This interaction has also been observed in related *C. elegans* MEIS/HTH homolog UNC-62 and the PBX/EXD homolog CEH-20 in specification and differentiation of the mesodermal M lineage (Jiang et al, 2009). Apart from acting as cofactors to HOX, other functions of the MEIS-PBX complex have been implicated which include complex formation with other homeodomain-containing transcription factors such as *Engrailed* and *Pdx1*, as well as interaction with bHLH proteins like *MyoD* to regulate *myogenin* gene expression, with recently described cooperative interactions with Kruppel-like factor, *Klf4* in tumorigenesis and maintanence of stem cell pluripotency (Berkes et al, 2004; Bjerke et al, 2011; Knoepfler et al, 1999; Liu et al, 2001).

### 4.3 HOX-PBX-MEIS heterotrimer

Ternary complexes of HOX-PBX-MEIS have been shown to play a role in the regulation of HOX function (Jacobs et al, 1999; Kurant et al, 1998; Ryoo et al, 1999; Shanmugam et al, 1999; Shen et al, 1999). MEIS has been shown to bind DNA cooperatively with products of HOX groups 9-13, while PBX-HOX complexes have been described for the products of HOX groups 1-8 (Chang et al, 1996; Shen et al, 1997a). Hence, MEIS and PBX bind to different sets of HOX proteins, with the exception of HOX paralog group 9 and 10, which interact with both MEIS and PBX. Two types of trimers have been demonstrated where MEIS can either be DNA-bound or non-DNA-bound (Jacobs et al, 1999; Shanmugam et al, 1999; Shen et al, 1999). A third

permutation involving the formation of a MEIS-HOXA9-PBX trimer in the absence of DNA has also been observed *in vitro* (Shen et al, 1999). Heterotrimeric complexes have been observed to augment DNA-binding affinity and specificity (Fujino et al, 2001; Huang et al, 2005; Moskow et al, 1995; Nakamura et al, 1996a; Shanmugam et al, 1999). The resulting complexes are highly oncogenic and greatly reduce the latency of HOX-induced leukemia, implying a role for MEIS as a cooperating oncoprotein (Calvo et al, 2001; Calvo et al, 2002; Fujino et al, 2001; Kroon et al, 1998; Nakamura, 2005; Schnabel et al, 2000; Wang et al, 2005). Using chimeric NUP98-homeodomain fusions, mouse NUP98-HOXA9 confers potent transcriptional activity mediated through CREB binding protein (CBP)/p300 and recapitulate phenotypes observed in human diseases (Kasper et al, 1999; Kroon et al, 2001). *Meis1* has been shown to accelerate transformation of NUP98-HOXA9 in acute myeloproliferative disease, which eventually develops into AML (Kroon et al, 2001). The involvement of *Meis* in cell cycle regulation can be established by the observation where HOXA10-PBX1-MEIS1 trimer activates the cyclin dependent kinase inhibitor p21 to regulate cell cycle arrest and differentiation (Bromleigh & Freedman, 2000). These assorted DNA-binding complexes are suggestive of additional roles in regulating transcription.

### 4.4   **PBX and MEIS expression independent of HOX**

*Meis* expression in zebrafish exhibits similar patterns to the expression of *Pbx* and *Hox* within the hindbrain. However, *Meis* is also expressed in non-*Hox* expressing tissues, suggesting a role in *Hox*-independent functions (Waskiewicz et al, 2001). In *Drosophila* development, the EXD-HTH dimer is able to regulate transcription independent of HOX protein binding (Jaw et al, 2000). *hth* and *exd* expression has also been detected in the absence of *Hox*, and is required for correct positioning of antenna as well as leg and wing development (Abu-Shaar & Mann, 1998; Azpiazu & Morata, 2000; Casares & Mann, 1998; Casares & Mann, 2000; Casares & Mann, 2001; Dong et al, 2000; Dong et al, 2001; Dong et al, 2002; Wu & Cohen, 1999; Wu & Cohen, 2000). In other cases, MEIS-PBX complexes in the absence of detectable HOX participation were shown to play an important role in regulating transcription of the platelet factor 4 gene, thus implicating MEIS-PBX in megakaryocytic gene expression (Okada et al, 2003).

# 5   **Transcriptional activation by MEIS1**

The *Meis1* transcript undergoes alternative splicing and encodes a number of isoforms including MEIS1A and MEIS1B which are highly conserved apart from their extreme C-termini (Figure 5) (Huang et al, 2005; Steelman et al, 1997). Studies by the Featherstone lab revealed a strong PKA-inducible transcriptional activation function to the MEIS1A C-terminus consisting of residues 335-390 (Figure 5) (Goh et al, 2009; Huang et al, 2005). Further to that,

MEIS1A was also found to promote transcriptional activity with histone-deacetylase inhibitor trichostatin A (TSA) treatment (Huang et al, 2005).

On its own, the MEIS1A is unable to cause leukemia, while a fusion to the transactivation domain of VP16 renders it spontaneously oncogenic VP16-MEIS1 protein (Mamo et al, 2006; Wang et al, 2006). This VP16-MEIS1 fusion requires the MEIS1 binding to PBX and DNA but not its C-terminal domain (Mamo et al, 2006; Wang et al, 2006). In addition, the fusion retains the aggressiveness of Hoxa9 and Hoxa7 and enhances its oncogenic potential (Mamo et al, 2006; Wang et al, 2006). The HOXA9 N-terminal domain (NTD) is essential for cooperative transformation with MEIS1 which is dispensable in VP16-Meis1 progenitors (Wang et al, 2006).

This ability of the transactivation domain to restore essential function of both N-terminus of HOXA9 and C-terminus of MEIS1 suggest the presence of an inherent transactivation function within the C-terminus of MEIS1A (Huang et al, 2005; Mamo et al, 2006; Wang et al, 2006). Thus, the co-localization of transactivation and oncogenic functions to the same C-terminal domain, in addition to the ability of the VP16 transactivation domain to replace the MEIS1 C-terminus in oncogenic assays, supported the notion that the MEIS1A C-terminus exerts its oncogenic functions *via* transcriptional activation of target genes (Huang et al, 2005; Mamo et al, 2006; Wang et al, 2006).

The MEIS1B C-terminus was found to display a strong constitutive transactivation function that could be enhanced by PKA. By contrast the MEIS1A C-terminus appeared inert in the absence of PKA or TSA (Huang et al,

2005). Thus, the distinctive response of MEIS1A was selected as the candidate for further investigation of the activity of MEIS1 rather than the compound activity of MEIS1B.
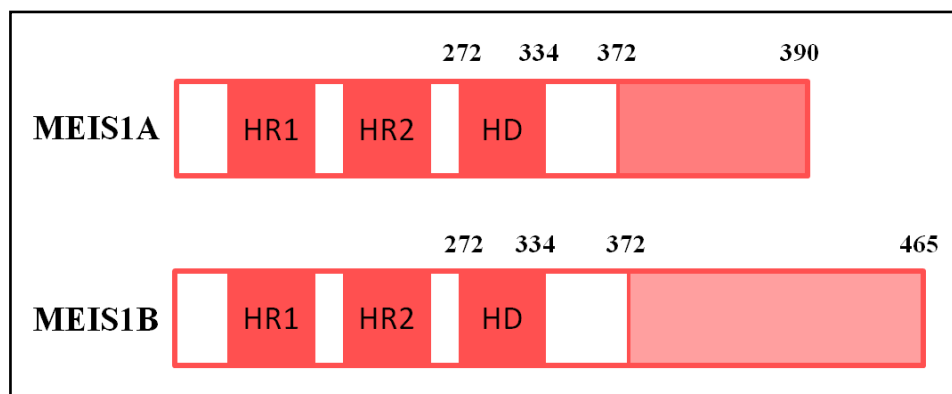


**Figure 5 Schematic diagram of the domain structures of the MEIS1A and MEIS1B homeoproteins**

HD represents the homeodomain. HR1 and HR2 indicate the PBX interacting domains. Colored block area from amino acids 372 onwards correspond to the unique C-terminal region of MEIS1A and MEIS1B.

# 6 Cyclic adenosine monophosphate response element binding protein (CREB)

The cyclic adenosine monophosphate response element binding protein, CREB, is a basic leucine zipper (bZIP) transcription factor that activates gene expression in response to increased levels of intracellular cAMP.

Genes responsive to cAMP harbor one or more so-called cAMP-response elements (CRE) within their regulatory regions which serve as recognition sites for binding by CREB, CREM and ATF (Mayr & Montminy, 2001; Shaywitz &

67

Greenberg, 1999; Yamamoto et al, 1988). The full CRE has the consensus sequence 5'TGACGTCA3' while the half-site is 5'TGACG3' (or 5'CGTCA3' on the other strand). The 43 kd CREB protein binds the CRE as a homodimer or heterodimer with other bZIP family proteins.

Heterodimerization and DNA binding are mediated by a leucine zipper motif at its C-terminus (Comb et al, 1986; Dwarki et al, 1990; Montminy et al, 1986; Shankar et al, 2005a; Short et al, 1986; Yamamoto et al, 1988). Mutations which hamper dimer formation also inhibit DNA binding (Dwarki et al, 1990). Two major domains with transactivation function reside within the N-terminal part of CREB. The central kinase inducible domain (KID) contains a cluster of phosphorylation sites that regulates transactivation (Figure 6) (Parker et al, 1996). A second domain flanking the KID consists of two glutamine-rich domains Q1 and Q2 (or the constitutive activation domain, CAD) and provides surfaces for interaction with and stabilization of the basal transcriptional complex (Figure 6) (Felinski & Quinn, 1999; Ferreri et al, 1994; Laoide et al, 1993; Nakajima et al, 1997). Together, the KID and the Q2 domain make up the transactivation domain (TAD).
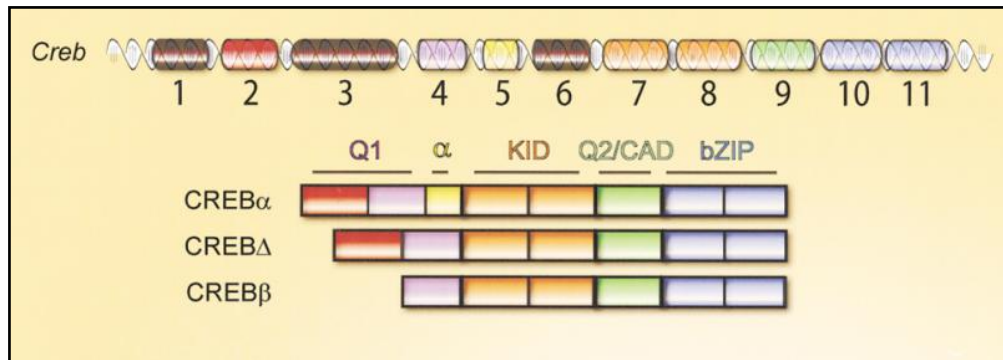
**Figure 6 Structure of *creb* gene**

Upper panel, The genomic structure of *creb* gene with the coding exons indicated in various colors.

Lower panel, Alternative splicing result in three isoforms of CREB: CREBα, CREBβ and CREBΔ (Blendy et al, 1996; Hoeffler et al, 1990; Hoeffler et al, 1988).

## 6.1 CREB antagonists

Transcription factors related to CREB include the cAMP response element modulator (CREM) and activating transcription factor-1 (ATF-1). These factors are related to CREB by homology, particularly within the bZIP domain (De Cesare et al, 1999; Foulkes et al, 1991; Hai et al, 1989; Mayr & Montminy, 2001; Rehfuss et al, 1991; Shaywitz & Greenberg, 1999). In addition to the homodimers, heterodimers between CREB and other bZIP family proteins like ATF-1, c-JUN and CREM have been identified as well (Benbrook & Jones, 1990; Foulkes et al, 1991; Kobayashi & Kawakami, 1995; Loriaux et al, 1994). Alternative splicing generate CREM isoforms, which binds CRE specifically and efficiently but encodes for different exons and function as transcriptional activators or repressors (Foulkes et al, 1991; Walker & Habener, 1996).

## 6.2 **CREB regulation**

The activity of CREB is known to be upregulated by extracellular signal-regulated kinase (ERK) also known as mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) via phosphorylation at Serine 133 residing within the KID (Chrivia et al, 1993; Gonzalez & Montminy, 1989; Kwok et al, 1994; Montminy & Bilezikjian, 1987; Yamamoto et al, 1988). In the absence of cAMP stimulus, PKA resides in the cytoplasm and exists as an inactive heterotetrameric protein of two catalytic subunits and two regulatory subunits bearing cAMP binding sites (Figure 7).

Upon cAMP binding, the regulatory subunits change conformation, dissociate from the complex and render the catalytic subunits free to diffuse into the nucleus (Figure 7) (Alberts et al, 2004; Hagiwara et al, 1993). The nuclear PKA then phosphorylates CREB at Serine 133 within its KID domain (Hagiwara et al, 1993; Harootunian et al, 1993).

CREB is then activated upon phosphorylation enabling it to recruit the CREB binding protein (CBP) and activate transcription (Cardinaux et al, 2000; Parker et al, 1996). The role of CBP in the activation of CREB is critical as dimerization and phosphorylation alone is not sufficient for activation (Chrivia et al, 1993). The solution structure revealed that upon phosphorylation, the KID domain of CREB assumes a more ordered structure and associates with the KID interaction (KIX) domain at the N-terminus of CBP (Radhakrishnan et al, 1997). The CREB-mediated transcription function is contributed by the intrinsic and associated histone acetyltransferase (HAT) activity and the bridging properties

with RNA polymerase II via RNA helicase A of the coactivators CBP/p300 (Johannessen et al, 2004; Nakajima et al, 1997; Ogryzko et al, 1996). Forskolin is a direct activator of adenylate cyclase and has been used to study responses to increases in cAMP levels.

It was long thought that the response of CREB to increased cAMP was entirely mediated by recruitment of CBP following phosphorylation at Ser133. More recently, however a second major pathway responsible for CREB's cAMP responsiveness has been revealed which involves the coactivator of CREB, cAMP-regulated transcriptional co-activators (CRTCs). Recruitment of CRTC enhances CRE-dependent transcription and activates CREB in a phosphorylation independent pathway.

The position Arginine 314 within the bZIP/dimerization domain of CREB plays a role in CBP/p300 recruitment and KIX-independent CREB transactivation function. In addition, this residue is also critical to mediate CRTC binding to CREB, and thus binding to CBP/p300 (Xu et al, 2007). Following activation, transcriptional activity of CREB can be attenuated by dephosphorylation of the same Serine 133 residue. Dephosphorylation of Serine 133 may be carried about by phosphatases: protein phosphatase 1 (PP-1) and PP-2A (Bito et al, 1996; Hagiwara et al, 1992; Wadzinski et al, 1993). In another study, phosphorylation of Serine 142 by (CAMK) has also been found to inactivate CREB activity (Sun et al, 1994; Sun & Maurer, 1995). Repressors of CREB include isoforms of CREM: α, β and γ isoforms as well as the Inducible cAMP early repressor (ICER) that binds CRE sequences and represses activity via

autoregulatory loop (Foulkes et al, 1991; Molina et al, 1993). Another study has identified a nuclear factor known as the Regulator of G protein Signaling (RGS) protein that suppresses CREB-mediated gene expression by binding to the phosphorylated CREB-CBP complex thereby reducing its interaction with CRE sites (Xie et al, 2008).

### 6.3 Calcium and CREB

As mentioned, apart from intracellular cAMP levels, CREB levels can also be modulated by elevated intracellular calcium/calmodulin-dependent kinases (Cam II and IV kinase) level (Ghosh & Greenberg, 1995). Calcium release in response to intracellular signaling via the endoplasmic reticulum (ER) or extracellular signaling via receptor or voltage calcium channels binds to the protein calmodulin and the complex can then activate the PKA pathway and result in phosphorylation of CREB, ATF-1 and CREM (de Groot et al, 1993; Shaywitz & Greenberg, 1999; Sun et al, 1996). The calcium cascade, like the cAMP pathway, phosphorylates CREB on Serine 133 to activating CRE containing genes (Dash et al, 1991; Montminy et al, 1990; Sheng et al, 1991). All three cAMP, calcium and CREB have been implicated in memory mechanisms in both vertebrates and invertebrates, converging these signaling pathway at CREB (Bartsch et al, 1995; Bourtchuladze et al, 1994; Dash et al, 1991; Impey et al, 1996; Kaang et al, 1993; Kandel & Abel, 1995; Yin et al, 1995; Yin et al, 1994).
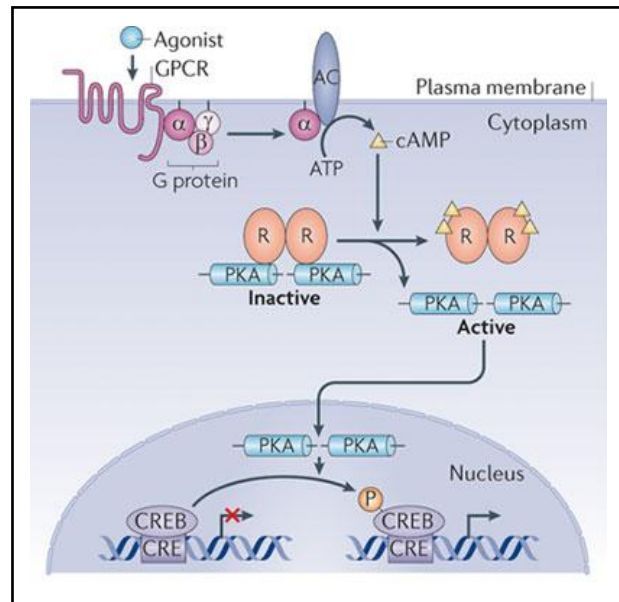
**Figure 7 cAMP signals CREB phosphorylation**

Binding of G-protein coupled receptors (GPCRs) leads to activation of adenylyl cyclase (AC) and catalyses the synthesis of cyclic AMP. Increase in cellular cAMP stimulate protein kinase A (PKA) signaling and phosphorylates the cAMP-responsive element (CRE)-binding protein (CREB) at Ser133 in the nucleus thereby promoting target gene expression at CRE promoters. Adapted from (Altarejos & Montminy, 2011).

## 6.4 CREB dominant negative mutants: ACREB, KCREB and M1 CREB

The dimerization characteristics of CREB proteins have lead to the construction of dominant negatives of CREB to investigate the role CREB plays in various pathways. The constructed CREB mutants M1 CREB, ACREB and KCREB function to inhibit activity of endogenous CREB (Ahn et al, 1998; Gonzalez & Montminy, 1989; Walton et al, 1992). The unphosphorylatable M1 CREB mutant, though mutated at the Serine 133 phosphorylation site, still retains

73

binding to CRE sequences and works by preventing access by CREB and other factors that binds CRE (Gonzalez & Montminy, 1989; Shaywitz & Greenberg, 1999). The dominant negative ACREB consists of the CREB bZIP domain artificially fused with an acidic extension replacing the usual basic residues at the N-terminus, thereby allowing the acidic extension to form a coiled-coil extension upon heterodimerization, thus preventing DNA binding of the endogenous CREB (Figure 8) (Ahn et al, 1998). The ACREB mutant forms a stable ACREB-CREB heterodimer that is 3,300-fold more stable than a CREB b-ZIP homodimer, thus preferentially and efficiently preventing endogenous CREB DNA binding (Ahn et al, 1998; Walton et al, 1992). The ACREBR314A mutant, an ACREB construct with a point mutation at the CRTC binding site of Arginine 314, disabling its ability to bind CRTC. KCREB consist of a full length CREB sequence with a single point mutation within its DNA-binding domain which abolishes binding to DNA while retaining its dimerization ability, thus preventing its interaction with the CRE (Shaywitz & Greenberg, 1999; Walton et al, 1992).
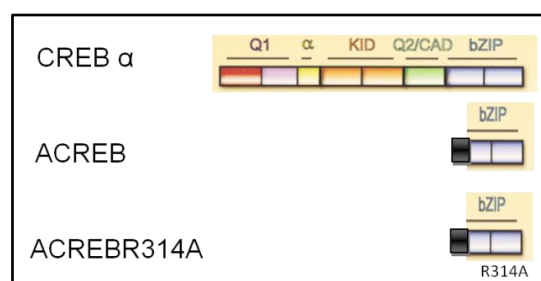


**Figure 8 Diagram of full length CREB vs CREB mutants**

Domains of ACREB consist of the bZIP domain and an acidic extension (black bar). Adapted and modified from (Lonze & Ginty, 2002).

## 6.5 CREB in normal development

CREB is ubiquitously expressed and controls a variety of functions in response to various physiological signaling pathways from normal hematopoiesis, cardiac gene expression, spermatogenesis, circadian rhythms, cell survival in pancreatic β-cells to regulation of body weight (Cheng et al, 2008; Chiappini et al, 2011; Don & Stelzer, 2002; Ginty et al, 1993; Huggins et al, 2007; Husse & Isenberg, 2005; Liu et al, 2002; Mayr & Montminy, 2001; Ruppert et al, 1992).

Within the liver, CREB has been shown to bind to the liver-enriched phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) promoter, where CREB is suggested to mediate tissue-specific response to cAMP in basal as well as PKA-induced expression by activating transcription (Herzig et al, 2001; Liu et al, 1991; Quinn & Granner, 1990; Roesler et al, 1995; Wynshaw-Boris et al, 1986; Xing & Quinn, 1993). In another related study, CREB was found to mediate expression of the gluconeogenic program *via* interaction with the nuclear receptor coactivator PGC-1 and nuclear receptor subfamily 4 group A (NR4A) in response to prolonged fasting, which in turn potentiates induction and amplifies gluconeogenic gene expression, further corroborating the role of CREB in the liver (Herzig et al, 2001; Pei et al, 2006). In addition, CREB deficient mice displayed a phenotype of fatty liver with elevated levels of nuclear hormone receptor PPAR-γ further establishing its role in liver metabolism (Herzig et al, 2003).

CREB plays a role in modulating a variety of forms of learning; memory in particular has been well studied. Studies on CREB in *Aplysia*, *Drosophila* and mice have demonstrated CREB's profound role in long-term, but not short term memory (Alberini et al, 1994; Bartsch et al, 1995; Bourtchuladze et al, 1994; Dash et al, 1990; Kogan et al, 1997; Yin et al, 1995; Yin et al, 1994). Not limited to memory, a role of CREB has been shown in regulating development and neurogenesis and cell survival in neurons in the adult brain while mice brains with *Creb* knockdown displayed in neurodegeneration, implicating CREB as an important modulator in brain function (Dworkin & Mantamadiotis, 2010; Lonze & Ginty, 2002; Mantamadiotis et al, 2002).

### 6.6    *Creb* knockouts and transgenic mice

Mice with targeted disruption of genes encoding one isoform of CREB have a compensatory increase in other CREB isoforms as well as an increase in levels of CREM, suggesting compensatory mechanisms within the CREB/ATF family of transcription factors (Blendy et al, 1996; Bourtchuladze et al, 1994; Hummler et al, 1994).  Mice null for the *Creb* gene exhibit excessive apoptosis and impairment in axonal growth and projections of their sensory neurons while transgenic mice expressing unphosphorylatable CREB (CREBM1) display a block in proliferation resulting in a dwarf phenotype with atrophied pituitary glands (Lonze & Ginty, 2002; Struthers et al, 1991).

6.7 **CREB in oncogenesis**

Recently, overexpression of CREB has also been detected in patients with acute lymphoid and myeloid leukemia and correlates with poor clinical outcome (Cheng et al, 2007; Kinjo et al, 2005; Pigazzi et al, 2007; Shankar et al, 2005a). In addition, CREB transgenic mice develop myeloproliferative disease after 1 year but not leukemia, suggesting that CREB contributes to, but is not sufficient for leukemogenesis (Cheng et al, 2007; Cheng et al, 2008). Evidence of oncogenic cooperativity can be seen between CREB and the oncoprotein TAX, a transforming protein of human T-cell leukemia virus type 1 (HTLV-1) believed to be a leading cause to HTLV-1 dependent leukemogenesis (Adya et al, 1994; Colgin & Nyborg, 1998). In support of CREB's role in oncogenesis, CREB protein is overexpressed in 94% of patients with ALL and 76% in AML (Pigazzi et al, 2007). The oncogenic effect of overexpressed CREB has been ascribed to an increase in phosphorylated CREB which causes hyperactivation of target genes involved in cell proliferation and survival (Desdouets et al, 1995). Expression of the dominant negative KCREB inhibits tumor growth and metastasis in human melanoma cells, thereby implicating CREB in metastasis-promoting pathways (Xie et al, 1997). In addition, studies using rat cardiac fibroblasts have shown that abnormal activation of CREB contributes to increased proliferation, further establishing the role of CREB in maintaining homeostasis (Leicht et al, 2000).

## 6.8 CREB and MEIS

*Meis1* has been found to be a candidate significantly upregulated during a microarray analysis of genes in CREB-overexpressing myeloid cells (Esparza et al, 2008). CREB has also been implicated in AML, suggesting a possible cooperative association with MEIS in leukemogenesis (Conkright & Montminy, 2005; Esparza et al, 2008; Kinjo et al, 2005; Shankar et al, 2005a; Shankar & Sakamoto, 2004). In support of these speculations, a recent work has identified physical interaction between MEIS1 and CREB proteins in which interaction depends on the phosphorylation state of CREB (Wang et al, 2010b). These newly reveled data hint at a cooperative association of CREB and MEIS, alongside other factors, to facilitate transcription and oncogenesis.

# 7 CREB-regulated transcription coactivator (CRTC)

CREB-regulated transcription coactivator 1 (CRTC1), previously known as Transducer Of Regulated CREB activity 1 (TORC1) belongs to a family of co-activators which enhances CRE-dependent transcription via a phosphorylation-independent way (Conkright et al, 2003). Prior to its association with CREB, CRTC1 was also known as mucoepidermoid carcinoma translocated-1 (MECT1) and was first identified as part of the MECT1-MAML2 fusion protein that comprises the N-terminal CREB regulator MECT1 and the C-terminal transcriptional activation domain of the Notch coactivator Mastermind-like 2 (MAML2) (Enlund et al, 2004; Tonon et al, 2003). Subsequently, through two independent screenings, CRTC1 was identified as a co-activator of CREB

signaling that enhances CRE-dependent transcription by binding to the bZIP DNA binding domain of CREB (Conkright et al, 2003; Iourgenko et al, 2003).

There are three CRTC family members in mammals: CRTC1, CRTC2 and CRTC3. Expression of CRTC1 is highest in the human brain and very low in all other tissues while expression of CRTC2 and 3 are detected ubiquitously in most tissues (Wu et al, 2006b). CRTC2 level is found highest in the liver compared to CRTC1 and 3 (Koo et al, 2005). CRTC1 and 2 are latently cytoplasmic under basal condition whilst CRTC3 is constitutively nuclear (Conkright et al, 2003; Screaton et al, 2004). A single functional homolog of CRTC has been indentified in *Drosophila* and in *C. elegans*, suggesting an evolutionarily conserved role (Mair et al, 2011; Wang et al, 2008).

Immunofluorescence microscopy using truncated CRTC2 identified a nuclear localizing sequence (NLS) (aa 56-144 of CRTC2) and two nuclear export sequences (aa145-320) and the motifs were shown to be conserved within the CRTC family (Screaton et al, 2004). Other domains of CRTCs include a conserved N-terminal coiled coil domain, the CREB binding domain (CBD) with transactivation potential and bind as tetramers to CREB dimers in the nucleus (Bittinger et al, 2004; Conkright et al, 2003; Iourgenko et al, 2003; Screaton et al, 2004). In addition, CRTCs also contain a central regulatory region (REG) harboring the nuclear localization and export signals as well as calcineurin binding sites, a splicing domain (SD) as well as a C-terminal domain (Screaton et al, 2004). The C-terminus comprises a constitutively active transactivation domain (TAD) that functions in coordinating assembly of the

transcriptional apparatus by associating efficiently with TAFII130 (Figure 9) (Conkright et al, 2003; Iourgenko et al, 2003). CRTC2 is phosphorylated by AMPK and SIK2 at Serine 171, by microtubule affinity-regulating kinase 2 (MARK2) at Serine 275, and by SIK2 at Serine 307. Phosphorylation at this latter site promotes 14-3-3 binding and cytoplasmic retention of CRTC2 (Figure 9) (Altarejos & Montminy, 2011).

Deletion mutants have mapped the CREB-interaction domain of CRTC1 to the N-terminal 56 amino acids within the coiled-coil domain, while the cognate interface on CREB is in the bZIP domain (Conkright et al, 2003). Recruitment of CRTC does not modulate CREB DNA binding activity, but rather enhances interaction and access of CREB to the components of the transcription machinery (Conkright et al, 2003). CRTC1 binds CREB via position Arginine 314 within the bZIP domain of CREB, a position which has also been shown to contribute to the recruitment of CBP/p300 (Xu et al, 2007). In addition, the direct binding of CRTC to CREB helps recruit and stabilize CBP occupancy over the promoter (Ravnskjaer et al, 2007; Xu et al, 2007). How CRTC does so is only beginning to be unveiled. Studies have shown that in the nucleus, the dephosphorylated CRTC2 undergoes acetylation at Lys628 by CBP, thereby blocking CRTC2 from ubiquitination and resulting stabilization (Dentin et al, 2007; Liu et al, 2008; Ravnskjaer et al, 2007).

## 7.1 CRTC regulation (activation/inactivation)

Under basal conditions, CRTCs are anchored in the cytoplasm *via* phosphorylation-dependent interaction with 14-3-3 protein. This is dependent on phosphorylation at Ser307 by salt-induced-kinase 2 (SIK2) and members of the AMPK family of Ser/Thr kinases (Katoh et al, 2006; Koo et al, 2005; Screaton et al, 2004; Shaw et al, 2005). CRTC and SIK have been found to be highly expressed in the brain, an area where CREB is known to mediate learning and memory function, providing a hint at their cooperative regulation (Screaton et al, 2004).

Stimuli such as calcium and cAMP dephosphorylates CRTC2 interactions at distinct 14-3-3 interaction sites (Ser 171, Ser 275 and Ser 307), with a dominant role at Ser 171, resulting in nuclear translocation of CRTCs (Jansson et al, 2008). Synergistic activity between calcium and cAMP signals have been observed to promote CRTC2 nuclear localization (Screaton et al, 2004). Within the nucleus, the unphosphorylated CRTC associates with CREB over target promoters, activating gene expression (Katoh et al, 2004; Screaton et al, 2004; Wang et al, 2008). In addition to the stimulus, dephosphorylation of CRTC2 at Ser 171 may act in parallel to expose the nearby nuclear localization signal, promoting nuclear access to CRTC (Screaton et al, 2004).

## 7.2 CRTC in gluconeogenesis

Glucose is the major energy source in mammals and energy homeostasis is maintained during fasting by fat burning and by mobilizing glucose for glucose-

dependent tissues like the brain and the red blood cells. Glucose homeostasis is maintained in the liver by promoting the storage or production of glucose in response to insulin and glucagon signaling (Pilkis & Granner, 1992). Liver cells contain specific enzymes specific for the regulation of hepatic gluconeogenesis and glycolysis such as phosphoenolpyruvate (PEPCK), fructose 1,6-bisphosphatase (Fruc1,6-Pase) and glucose-6-phosphatase (G6Pase) (Pilkis & Granner, 1992).

### 7.3    CRTC in fasting/ starvation

During fasting, levels of plasma insulin begin a descent which relieves the inhibition on gluconeogenic enzymes like PEPCK while allowing hormones like glucagon to enhance gluconeogenic gene expression by increasing cAMP levels (Koo et al, 2005; Pilkis & Granner, 1992). In parallel, this increase in gluconeogenic gene expression is further enhanced by the concerted effect of CRTC and the forkhead box domain protein within the FOXO family (FOXO1) (Dentin et al, 2007; Haeusler et al, 2010). Collectively, the net increase in the level of gluconeogenic genes results in an increased rate of gluconeogenesis. Upon prolonged fasting, the body begins to decrease gluconeogenesis in an attempt to prevent muscle wasting and glucose dependent tissues like the brain begins to use liver-derived ketone bodies as fuel. The hepatic CRTC2 level then begins to fall due to deacetylation and ubiquitination. Deacetylation of CRTC2 can be regulated by the upregulated NAD+ dependent deacetylase sirtuin 1 (SIRT1) (Liu et al, 2008). In parallel, SIRT1 acts to enhance the activity of

FOXO1 and PGC1α, thus maintaining the expression of the gluconeogenic program (Puigserver et al, 2003).

## 7.4 CRTC in feeding

During feeding, the increase in circulating insulin levels induces SIK2 phosphorylation of CRTC2 and begins to turn down the gluconeogenic program (Dentin et al, 2007). At the same time, deacetylation of CRTC2 by SIK2 disrupts the CRTC-CBP interactions (Yang et al, 2001; Yuan & Gambee, 2000). In addition, the rise in insulin has also been implicated in the disruption of CREB-CBP-CRTC interaction, further impeding gluconeogenesis (He et al, 2009; Zhou et al, 2004). Type 2 diabetes is characterized by a high blood glucose level in the context of insulin resistance, resulting in an increase in gluconeogenic gene expression. Another mechanism contributing to the increase in blood glucose is via the O-glycosylation of intracellular proteins (Altarejos & Montminy, 2011). O-glycosylation is an enzymatic reaction in which a glycan is added to Ser/Thr residues in proteins, disabling phosphorylation at the same site (Altarejos & Montminy, 2011). O-glycosylation of CRTC2 at Ser171 was observed in mice which enhance the activity of CRTC2 by preventing phosphorylation, resulting in nuclear translocation of CRTC2 and activation of CREB (Dentin et al, 2007).

## 7.5    CRTC in energy sensing pathway (AMPK)

An additional modulator which could override cAMP as well as hormonal effects is the modulation of CRTC2 by calcineurin and AMPK levels, which increases upon stress and disrupts hepatic glucose production by catalyzing Serine 171 phosphorylation on CRTC2 (Koo et al, 2005). CRTC has been shown to function downstream of calcineurin and AMPK signalling in response to nutritional stimulus. Calcineurin/PP2B (CnA) is a calcium/calmodulin-dependent Ser/Thr phosphatase that is directly activated by calcium influx, and physical interaction between CRTC2 and CnA has been observed (Screaton et al, 2004). AMPK has been shown to target and decrease CRTC activity in C. *elegans* (Mair et al, 2011). A high nutrient state activates calcineurin resulting in dephosphorylation of CRTC and subsequent CREB gene expression (Brunet, 2011). In the absence of nutrients, AMPK is activated which then keeps CRTC in the cytoplasm via binding to 14-3-3 protein (Brunet, 2011).

Thus, the link between the energy-sensing (AMPK) pathway and the hormonal pathway (insulin and glucagon) to modulate glucose output converges on CRTC, making CRTC a critical target in regulation of gluconeogenesis (Koo et al, 2005; Shaw et al, 2005).

## 7.6    CRTC role in development and interacting partners of CRTC

Apart from acting as the glucose sensor in the liver, the role of CRTC has been increasingly elaborated in various systems, shedding light onto the multi-faceted role of this CREB co-activator. Involvement of CRTC ranges from

84

maintaining energy balance to fertility as *Crtc* deficient mice were found to be obese and infertile (Altarejos et al, 2008). A role of CRTC in maintenance of energy balance in the brain can also be seen in *Drosophila* where *crtc* mutants displayed reduced glycogen and lipid stores and are rendered vulnerable to starvation and oxidative stress (Choi et al, 2011; Wang et al, 2008). Disruption of CRTC1 has also been implicated in synaptic and memory dysfunction and an impairment of *CRTC1* gene transcription has been observed in Alzheimer's disease as well as in several other psychiatric disorders (Espana et al, 2010; Kovacs et al, 2007; Rabheru & Persad, 1997). Given the implication of CREB in age-related disorders such as Alzheimer's, it is no surprise that one of the many roles associated with CRTC involves ageing. Studies in *C. elegans* have shown how AMPK and calcineurin promote longevity by regulation and inhibition of CRTC (Mair et al, 2011). Life span extension can thus be brought about *via* the phosphorylation-induced inactivation of CRTC mediated by AMPK activation and calcineurin inactivation (Mair et al, 2011).

Nuclear CRTCs have also been found to play a role in muscle mitochondrial biogenesis *via* activation of the peroxisome proliferator-activated receptor γ coactivator 1α (*PGC-1α*), upregulating the expression of nuclear and mitochondrial encoded genes (Koo et al, 2005; Screaton et al, 2004; Than et al, 2011; Wu et al, 2006b). Physical interaction has been shown between CRTC2 and a spliceosome factor NONO (p54nrb), suggesting a role of CRTC in splicing (Amelio et al, 2007). Other implicated roles of CRTC include the regulation of steroidogenesis in the adrenal cortex in rats (Spiga et al, 2011). In

addition, CRTC can also be phosphorylated and activated by the Mitogen activated/extracellular signal-regulated kinase kinase 1 (MEKK1) implicating CRTC in mitogenic signaling program (Siu et al, 2008). Supporting evidence for the role of CRTC as a target of mitogenic kinases can be deduced from its role in promoting cellular growth and proliferation in association with the mitogen AP-1, a mediator of mitogenic response (Canettieri et al, 2009b).

Other evidence of CRTC in human T-cell leukemia lies in its association with the oncoprotein TAX and initiating the activation of HTLV-1 transcription (Siu et al, 2006). There has been implication on the t(11;19)(q21;p13) translocation of CRTC in mucoepidermoid carcinoma which act via disruption of the Notch signaling pathway (Conkright et al, 2003; Ellisen et al, 1991; Tonon et al, 2003). The same chromosomal rearrangement resulting in a CRTC1-MAML2 fusion has also been found in a lung mucoepidermoid carcinoma, benign Warthin's tumors (Johansson et al, 1995; Nordkvist et al, 1994; Stenman et al, 1998). Transcripts of the fusion have subsequently been identified in the cervix, breast, primary thyroid and sweat glands thus implicating the fusion as prognostic marker in tumors arising from mucous gland (Achcar Rde et al, 2009; Behboudi et al, 2005; Camelo-Piragua et al, 2009; Enlund et al, 2004; Kaye, 2009; Kazakov et al, 2007; Komiya et al, 2010; Tirado et al, 2007). Recent study has identified a conditional associating complex of MEIS-CBP-CRTC with the Ser/Thr kinase GSK-3 to facilitate HOX-mediated transcriptional and transformation in leukemia (Wang et al, 2010b). A finding which places CRTC alongside MEIS in HOX-dependent leukemic program.

Together, these data suggest at least two general pathways promoted by activation of CRTC: a metabolic pathway (a role in gluconeogenic gene expression) and a proliferative pathway (a role in cancer) (Canettieri et al, 2009a). The preferential expression levels of CRTC in certain tissues could help explain the strong constitutive function of CREB in certain types of cells and why CREB phosphorylation is not sufficient to activate gene expression in certain cases thus providing a hint at the role of CRTC as the point of converging signals in controlling the magnitude of CREB responses during development (Altarejos & Montminy, 2011; Canettieri et al, 2009a; Conkright et al, 2003; Iourgenko et al, 2003).
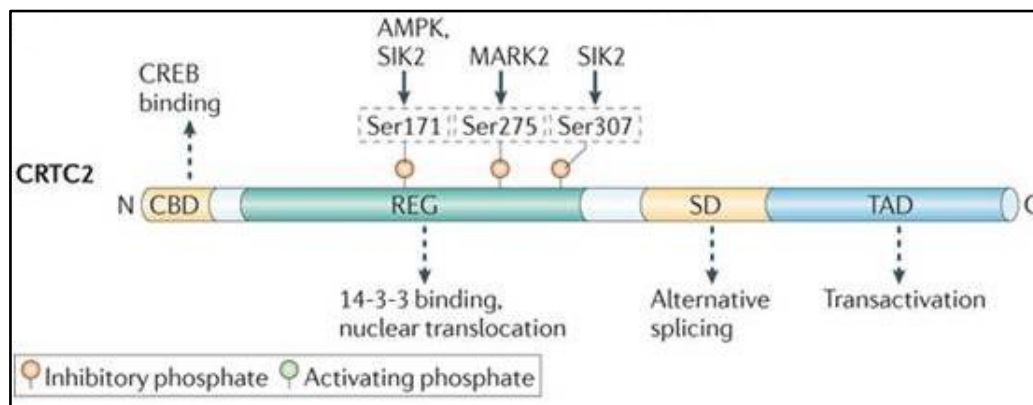


**Figure 9 Schematic diagram of CRTC2**

CBD: CREB binding domain REG: regulatory region. SD: splicing domain. TAD: transactivation domain. Various phosphorylation sites were indicated. Adapted from (Altarejos & Montminy, 2011).

# RATIONALE

MEIS1, a cofactor of HOX and PBX proteins, has been implicated in embryonic patterning and oncogenesis. Previous studies by our lab established that MEIS1A harbors a C-terminal transcriptional activation domain that responds to PKA signaling. Supporting studies have also mapped a conserved transcriptional function to the C-terminus of MEIS1A which is required for accelerating leukemogenesis, particularly in collaboration with HOXA9 (Mamo et al, 2006; Wang et al, 2005). Of interest to our lab, and the subject of this thesis, is molecular basis for the PKA-responsiveness of the MEIS1A C-terminus. Results in Chapter 3 discuss how this mechanism involves the CREB coactivator CRTCs. Chapter 4 assesses the effects of CREB using CREB mutants, CBP and other protein interactions at the MEIS1A C-terminus. Finally in Chapter 5, I discuss my attempts to determine the nature of the MEIS-CRTC-CREB complex in order to reveal the structural changes accompanying their interactions. Lastly, I propose future experiments to further understand the role of MEIS1 and CRTC in transcriptional regulation in the hope of extending this knowledge to physiological and disease models.

# CHAPTER 2

# MATERIALS AND METHODS

*Plasmid constructs-* Expression plasmids for MEIS1A, PKAα, PBX1A, HOXA1, GAL-MEIS1A(335-390) and GAL-MEIS1A-(GQWHYM) have been described previously (Huang et al., 2005; Saleh et al., 2000b, Shanmugam et al., 1999) . pML5XUAS is a luciferase reporter with five copies of GAL4 binding sites. (Huang et al., 2005). pRL Renilla is a luciferase control reporter vector. pMLHoxb1ARE is another luciferase reporter contains a 150bp Hoxb1 ARE upstream of the adenovirus major late promoter (Saleh et al., 2000). CRTC2, control shRNA plasmids, Flag-CRTC2 and Flag-CRTC2 (Wobble) expression plasmids have been previously reported (Conkright et al, 2003; Screaton et al, 2004). Flag-CRTC1 coding sequence was PCR-amplified from template pCMV-SPORT6-CRTC1 purchased from Open Biosystems (catalogue number MHS1010–7507865; accession number BC028050), and cloned into BamHI and XhoI sites of pcDNA3.1(+) that had already been inserted with a FLAG tag. FLAG-CRTC1-(47–634), FLAG-CRTC1-(47–290), and FLAG-CRTC1-(148–290) were subcloned as EcoRI-NotI fragments. FLAG-CRTC1-(1–431), FLAG-CRTC1-(1–518), Flag-CRTC1-(1–493), and FLAG-CRTC1-(1–627) were generated by removal of ClaI-XhoI, BsrGI-XhoI, SfiI-XhoI, and BspEI-XhoI fragments, respectively, and ligated following a blunt ending treatment by T4DNA polymerase (Fermentas). Flag-CRTC1, Flag-CREB, Flag-CREB (R314A) and Flag-CREB (S133A) expression plasmids have been previously reported (Conkright et al., 2003; Screaton et al., 2004; Goh et al., 2009). CRE-luc is a luciferase reporter assays construct courtesy of Walter Born. CREB-341 expression plasmid was a generous gift by Richard Goodman (Oregon Health &

Science University). ACREB was a generous gift of Charles Vinson (National Institutes of Health, Bethesda) and has been described previously (Ahn et al., 1998). Flag-ACREBR314A was generated with reverse primer overlapping position 314 of CREB mutated to alanine. GSK3β, GSK3β (S9A) and DN-GSK3β expression plasmids were purchased from Addgene. Expression plasmids for pcDNA3-HA-GSK3β wildtype, pcDNA3-HA-GSK3 βS9A and pcDNA3-HA-GSK3β K85A were purchased from Addgene with plasmids number: 14753, 14754 and 14755 respectively. Expression plasmid for pXJ-GFP was a kind gift from Dr Koh Cheng Gee's lab in School of Biological Science, NTU. pXJ-GFP-MEIS1a was constructed by PCR-amplified construct of MEIS1A into BamHI and XhoI sites of pXJ-GFP plasmid. CRE-luc is a luciferase construct encodes the firefly luciferase reporter gene with a cAMP response element (CRE) incorporated. To construct the expression plasmid for pSG5-MEIS1A Δ334-390, MEIS1A (1-1002 basepairs) coding sequencing was PCR-amplified from constructed and cloned into EcoRI and SacI sites of the pSG5 plasmid. DNA region coding for MEIS1A, CRTC1 and CREB341 were amplified by PCR and cloned into Ndel and BamH1 sites of pET15b and Bsa1 and Xho1 sites of pSUMO that had already a 6XHis tag at the N-terminus of pET15b and pSUMO. Flag-CRTC1 (1-290) was amplified by PCR and cloned into Bsa1 and Xho1 sites of pSUMO.

*Cell culture and transfections-* HEK293T cells were cultured in Dulbecco's modified Eagle's medium (cat no. 12100-046), supplemented with 10% fetal

bovine serum from Gibco, L-glutamine, and penicillin/streptomycin. HEK293T cells were seeded at 75% to 90% confluence in 6-well plates for immunoprecipitation and in 12-well plates at a concentration of $1.5 \times 10^5$ cells/ml for luciferase assay. Cells were allowed to attached overnight and then transfected by Lipofectamine 2000 reagent (catalog no. 11668-019, Invitrogen).

*Chemical-* MG132 (Merck, catalogue number 474790) was used at 10 uM for 5 h. Forskolin (Sigma-Aldrich Product Number F6886). Dithiothreitol (DTT) was purchased from Biorad (510610), Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from BST (12481C25) and Lithium chloride (LiCl) was purchased from Sigma (3650-1008).

*Antibodies-* Anti-MEIS NT is an affinity purified rabbit polyclonal antibody raised in-house against amino acid residues 1–34 of MEIS1 (Huang et al, 2003; Huang et al, 2005). Anti-MEIS1/2/3 mouse monoclonal antibody was purchased from Upstate Biotechnology (catalog no. 05-779). Anti-Flag mouse monoclonal antibody (catalog no. F3165) and anti-actin mouse antibody (A1978) were from Sigma. The anti-CRTC1 (sc-46268) and anti-CRTC2 (catalog no. sc-46273) antibodies were purchased from Santa Cruz Biotechnology. The anti-PBX1 (catalogue number sc-889) and anti-Gal4 (catalog no. sc-577) polyclonal antibodies were purchased from Santa Cruz Biotechnology. The anti-Gal4 (catalog no. sc-577) polyclonal antibodies were purchased from Santa Cruz Biotechnology. Vectashield mounting medium for

fluorescence with DAPI was purchased from Vector Laboratories Inc (H-1200). The secondary antibodies used in this study were horseradish peroxidase-conjugated rabbit anti-mouse IgG (A9044 Sigma), horseradish peroxidase-conjugated anti-rabbit IgG (P0448 DakoCytomation) and horseradish peroxidase-conjugated anti-goat IgG (A5420 Sigma). Anti-Flag antibody was purchased from Sigma (catalog no. F1804). All secondary antibodies were purchased from Molecular Probes (Invitrogen): Alexa-Fluor 488, 546 rabbit anti-goat IgG (catalog no. A-11078, A-21085), Alexa-Fluor 488, 546 goat anti-mouse IgG (catalog no. A-11039, A-11040), Alexa-Fluor 594 chicken anti-mouse IgG (catalog no. A21201), Alexa-Fluor 488, 546 goat anti-rabbit IgG (catalog no A-11034, A11010). We purchased the anti-FLAG M2 affinity agarose (catalogue number A2220) and anti-actin (catalogue number A3853) from Sigma. The anti-CBP (catalog no. ab50702) and rabbit polyclonal anti-GFP antibody (ad290) were purchased from Abcam.

*Luciferase assays-* A total of 800 ng of DNA consisting of 150 ng of pML5XUAS luciferase reporter or CRE luciferase reporter and 200 ng of each expression plasmid, 50ng of Renilla luciferase reporter were included in all reactions as an internal control value in which the pML5XUAS luciferase reporter gene may be normalized. All reactions were topped up to 800 ng with PSKII empty vector. Media was changed after 24 h and at 48 h post-transfection, cell lysates were harvested with passive lysis buffer (PLB) from the Dual luciferase assay kit (catalog no. E1960 Promega). 250 ul of PLB we

93

dispensed into each well for 12-well culture plate to completely cover the cell monolayer. Culture plates were rocked gently at room temperature for 15 min. Lysate samples were cleared for 30 s by centrifugation at top speed and 50 ul aliquot of supernatant was added to a 96-well luminometer plate (Thermo Scientific cat: 9502887). Samples were quantified for luciferase activity using Fluoroskan Ascent FL luminometer (Thermo Electron Corporation) that dispense 100 ul per reaction of luciferase assay reagent and 100 ul of stop and glow solution. The Renilla reporter was co-transfected to normalize transfection efficiency. Data were normalized and compared as Relative luciferase relative fold induction (Rlu). Titration of increasing amount of plasmids (0-200ng) with a fix amount of reporter and control plasmids have been attempted to determine the saturation point. A linear readout was observed with increasing amount of plasmid.

*Immunoflorescence staining-* An indirect immunofluorescence approach against endogenous levels of MEIS1/2/3, CRTC1 and CRTC2 as well as overexpressed Flag-tagged protein levels of CRTC1 were carried out. 1.0 x $10^5$ cells/ml HEK293T cells were seeded in 40-mm-diameter tissue culture dishes lined with ethanol- and acid-washed coverslips and allowed to attach overnight. Medium was changed the following day after transfection. 24 h after transfection cells were washed twice in pre-warmed Phosphate Buffer Saline (PBS) and fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) for 20 min at room

temperature, followed by boiling with citrate buffer. Cells were washed thrice in PBS, blocked with 10% goat serum (Hyclone) in 0.1% Triton/PBS. Antibodies used were goat polyclonal anti-CRTC1 (sc-46268) and anti-CRTC2 (sc-46274) (Santa Cruz Biotechnology, Inc., USA). Alexa Fluor dye 546 (on MEIS1A) and Alexa Fluor dye 488 (on CRTC1) from Invitrogen were used as the fluorescent label. Secondary antibodies were purchased from Molecular Probes (Invitrogen,USA): Alexa-Fluor 488, 546 rabbit anti-goat IgG (catalog no. A-11078, A-21085), Alexa-Fluor 488, 546 goat anti-mouse IgG (catalog no. A-11039, A-11040). Cells were mounted in Vectorshield DAPI (Vector Laboratories, USA). Anti-Flag antibody was purchased from Sigma (catalog no. F1804).

*Immunoprecipitation and Western Blot analysis-* Cells were washed twice in ice-cold PBS 48 h post-transfection and harvested in 500 ul of Buffer B ( 150 mM KCl, 0.1% NP-40, 20 mM Tris-Cl, pH 8.0, 5 mM MgCl$_2$, 10% (w/v) added with protease inhibitor cocktail (catalog no. 11873580001, Roche). Following two freeze thaw cycles, cells were spun down at 4°C for 10 min. For Immunoprecipitation, the supernatant was then incubated with appropriate antibody for 5 h to overnight at 4°C, followed by a 3 h incubation at 4°C with 30 ul of a 50% slurry of Protein A agarose (catalog no. 16-156, Upstate Biotechnology). The precipitates were washed three times, each with 500 ul of Buffer B. Protein A agarose precipitate were then eluted with 1X sodium dodecyl sulfate (SDS) sample buffer and boiling. Elution from anti-Flag M2

affinity agarose was done by adding a 7.5 ug of Flag-peptide (catalog no. F3290, Sigma) for 1 h at 4˚C. Protein samples were separated by SDS-PAGE and transferred to 0.45 um  PVDF membrane. The membranes were blocked with 5% non-fat milk powder in 0.1% Tween 20 in PBST for 1 h at room temperature to reduce non specific background, followed by primary antibody incubation for 2 h at room temperature or overnight at 4˚C. The membranes were then washed six times, 5 min each with PBST, and incubated with secondary antibody conugated with horseradish peroxidase for 45 min at room temperature. Subsequent to six 5 min PBST washes, bound antibodies were detected with a chemiluminescent kit (catalog no. WBKLS0100 Millipore).

*Proximity ligation assay-* Cells were seeded and grown on 6 well-plates for transfection and re-seeded in 8-well lab-tek chamber slides (cat no. 177402 Thermo Scientific) of 200 ul media per well with a confluency of 2500 cells/ml. Transfected cells were washed with PBS at room temperature, fixed, and permeabilized using 0.2% Triton/PBS the following day. Samples were blocked in 1X blocking stock (10% FBS in 0.1%Triton/PBS) for 10 min at room temperature and washed with 0.1% Triton/PBS for 1 min, twice. Primary antibodies were diluted 100 times in 1%Triton/PBS and co-incubated overnight at 4°C. Slides were washed with PBST (1XPBS with 0.1% Tween) for 5 min, twice. PLA was performed using a Duolink II Detection Kit (catalog no. 92007-0030, Genome Holdings). Slides were then incubated with a mixture of two diluted PLA probes in 1XPBS in a pre-heated humidity chamber for a

maximum of 2 h at 37˚C. Cells were washed with clean PBS 5 min, twice and incubated with diluted Duolink Hybridization stock in filtered water for 15 min at 37˚C for rolling circle amplification (RCA). To complete the rolling-circle priming template, T4 DNA ligase and samples were incubated 15 min at 37°C in a humidified chamber. RCA of the ligated oligonucleotide template was initiated by addition of DNA polymerase and incubation for 60 min at 37°C in a humidified chamber. Finally, Texas red–labeled oligonucleotide detection probes were incubated as 1× detection stock and incubated for 60 min at 37°C in a humidified chamber. Washing procedures with 2x saline-sodium citrate (SSC), 1x SSC, 0.2x SSC and 0.02x SSC for 2 mins each followed by a wash with 70% Ethanol for 1 min. Samples were fitted with coverslips and mounted with Vectashield and examined with a Zeiss LSM710 META Confocal Microscope under a 63× objective.

*Chromatin immunoprecipitation (ChIP) Assay*—ChIP assays were performed according to the protocol from Upstate Biotechnology with minor changes as reported previously (Huang et al, 2005; Rastegar et al, 2004). P19 cells induced to differentiate down the neural pathway by aggregation in the presence of retinoic acid were treated with 20 uM forskolin for 2 h, cross-linked with 1% formaldehyde for 10 min at 37 °C, collected, and washed twice with ice-cold PBS containing protease inhibitor mixture. A 200-ul aliquot of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, protease inhibitor mixture) was added to each $1 \times 10^6$ cells and incubated on ice for 10 min. The 200-ul

lysates were sonicated at 4 °C with 10 sets of 10-s pulses at 30% amplitude of a Betatec Sonics Vibra Cell sonicator to an average DNA length of 200 bp and centrifuged for 10 min at 4 °C. Each 100-ul sonicated cell supernatant was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.0, 167 mM NaCl, protease inhibitor mixture) and pre-cleared with 40 ul of a 50% slurry of salmon sperm DNA/Protein A-agarose (catalogue number 16-157, Upstate Biotechnology) for 30 min at 4 °C with rotation. After an overnight incubation with anti-MEIS NT, anti-PBX1, anti-CRTC2, or anti-rabbit IgG antibodies, 30 ul of salmon sperm DNA/Protein A slurry was added for 1 h at 4 °C, along with a no antibody as control. To remove nonspecific DNA from the protein A-antibody-histone complex, we performed extensive washes with 500 ul of each buffer in the following sequences: once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.0, 150 mM NaCl), once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.0, 500 mM NaCl), once with lithium chloride buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-Cl, pH 8.0), and twice with TE buffer (1 mM EDTA, 10 mM Tris-Cl, pH 8.0). Each wash was done by first pipetting up and down for 10 times, and then 8–10 min incubation on a rotating platform at 4 °C. Subsequently, the histone complex was eluted from the antibody by incubating twice with 125 ul of elution buffer (1% SDS, 0.1 M NaHCO3) for 15 min at room temperature. Cross-links were reversed at 65 °C for 4 h in the presence of 0.2 M NaCl. DNA was phenol-chloroform-extracted, ethanol

98

precipitated, and resuspended in 40 ul of distilled water (catalogue number 15230-147, Invitrogen). Five percent (by volume) of the immunoprecipitated DNA was served as template in quantitative real-time PCR by a SYBR Green JumpStart Taq ReadyMix kit (catalogue number S1816, Sigma) with a Roche LightCycler. The sequences of ChIP primers used in this study were as follows: for *Hoxb1* ARE, 5'-CTCTGGTCCCTTCTTTCC and 5'-GGCCAGAGTTTGGCAGTC; for *Hoxb2* r4 enhancer, 5'-AGGCCTTTTTAAGGGATATGC and 5'-AGGCCTCAAAGCTGAAAATGA; for *Meis1* promoter, 5'-TTAGGACTGATTCAAGGAAAGC and 5'-GCCCCTCAGACCCAACTAC; and for *gapdh*, 5'-AACGACCCCTTCATTGAC and 5'-TCCACGACATACTCAGCAC. The primers for the murine *Meis1* gene flank a consensus PBX-MEIS binding site having the sequence 5'-TGATTGACAG-3'.

*Knock down studies*- Knock down of human CREB1 was performed with ON-TARGETplus siRNA Reagents (Thermo Scientific) using a SMARTpool reagent of 4 siRNAs which target both human CREB1 isoform A and B. HEK293T cells were first seeded on a 24-well plate on day 0 followed by siRNA knockdown using DharmaFECT general transfection protocol on day 1 using Thermo Scientific DharmaFECT transfection reagents. Fresh media was replaced on day 2. Transfection of plasmids was done on day 3 following the knockdown. Cells were harvested at 96 hrs at >80% viability for transcriptional assay and western blot on day 4. CREB shRNA was purchased from

Dharmacon RNAi Technologies (On-TARGET plus SMART pool L-003619-0005, Human CREB1).

*Protein expression-* A 2 ml overnight cell culture was inoculated to 1 l of LB broth at 37°C. Cells were grown to A600 = 0.4-0.6. IPTG was added to a final concentration of 1 mM and protein expression induced for 2.5 h at 37°C. Cells from one litre culture were resuspended in 30-ml of buffer (50 mM Tris, pH 8.5, 200 mM NaCl, EDTA, DTT) and lysed via sonication (BANDELIN, Sonopuls HD 2200, cone tip KE76). Lysate was centrifuged at 10,000 g for 35 min. The supernatant was passed through a 0.45 um membrane (Pall Cooperation).

*His-Tag purification-* The supernatant was allowed to bind to the Ni-NTA-Agarose (Nickel-Nitrilotriacetic Acid Agarose, Qiagen) and spin for 2 h at 4°C. After binding, elution was started by applying an increasing concentration of Imidazole from 0 mM to 300 mM.  Protein elution profile was determined with a 17.5% SDS-PAGE gel.

*Superdex-* The sample was loaded onto a Superdex G-75 HR 10/30 as well as Superdex G-200 HR 10/30 gel-size exclusion chromatography column (GE Healthcare) using a buffer of 50 mM Tris–HCl (pH 8.5) and 200 mM NaCl. The protein was concentrated using Centricon YM-3 spin concentrators (Millipore).

*Ion exchanger*-The protein sample was loaded onto a column carrying positively-charged functional groups, which attracted negatively charged proteins. Because the pI of MEIS1A is 6.13, at a pH of 8.5, the MEIS1A protein should be negatively charged and should be binding to the column. Unbound proteins will then be washed off as flow through. Bound proteins were gradually eluted with an increasing ionic strength.

*Baculoviral expression and insect cell culture*- Cloning and expression of Flag-CRTC1 was performed using the Bac-to-bac expression system and step by step guide can be found in the manual by Invitrogen (catalog no. A11101). *Spodoptera frugiperda* Sf9 insect cells were used as the host for the baculovirus transfer vector. Sf9 cells were cultured under serum-free condition in sf-900 II Serum free media (SFM). Cells were maintained in 27 ˚C incubator with an active controlled oxygenated system at 10-50% of air saturation.

*Native gel*- 15% native gel (30% Acrylamine, 5XTBE, 10% APS, TEMED) was set to pre-run at a voltage of 300 for 1 h in transfer buffer sans methanol. Protein bands were then visualized by staining in commassie blue.

*Dynamic light scattering studies*- The homogeneity of the purified PREP2 protein was measured using a Zetasizer by Malvern Instruments. Zetasizer measurement was performed according to the standard operating procedure (SOP) with a concentration of 1 mg/ml purified protein. Details of the

procedure were explained in the Zetasizer Nano Series User Manual (MAN0317).

# CHAPTER 3

# To elucidate the mechanism behind the transcriptional activity of MEIS1A at the C-terminus

# 1   ABSTRACT

MEIS1, a cofactor of HOX and PBX proteins, has been implicated in embryonic patterning as well as in oncogenesis. Previous studies by the lab have established that the MEIS1A C-terminus harbors a transactivation domain that is responsive to PKA signaling which is dependent on CBP. Supporting studies have also mapped a conserved transcriptional function to the C-terminus of MEIS1A which is required for accelerating leukemogenesis. Our studies investigated the involvement of the CREB coactivator CRTCs in the PKA-mediated induction of the transcriptional functions of the MEIS1A C-terminus. Overexpression studies revealed the ability of CRTC to bypass PKA for transactivation at the MEIS1A C-terminus. Knockdown studies of CRTC further supported the role of CRTC1 in mediating MEIS1A transactivation. We also established the physical involvement of the CREB coactivator CRTC1 and CRTC2 in mediating the transcriptional function at the MEIS1A C-terminus, a result that was further supported by proximity ligation studies. Authenticity of the effect of CRTC1 was also observed strongly in the well-established CRE-reporter assay while chromatin immunoprecipitation revealed recruitment of MEIS1, PBX1, and CRTC2 on MEIS1 target genes *Hoxb1* and *Hoxb2*. The CRTC1 interaction domain on MEIS1A appears to involve the MEIS1A C-terminus since deletion of the MEIS1A C-terminus prevents co-immunoprecipitate with CRTC. The MEIS1 interaction domain on CRTC was mapped to the N-terminus, a region which has also been shown to mediate CREB binding. These results imply the physical collaborative effort of CREB

104

and CRTC with MEIS1 to achieve a PKA inducible activation at the MEIS1A C-terminus, pointing at the convergent action of these proteins in normal development as well as in oncogenic processes.

# 2   RESULTS

**Transcriptional activation by MEIS1A in response to PKA signaling**

Previous results from the Featherstone lab have indicated that the MEIS1A C-terminus harbors a transactivation domain that is responsive to PKA (Huang 2005). Deletion studies have mapped the region of PKA-responsiveness to residues 335-390 of MEIS1A (Huang 2005). To reveal the mechanism behind the transcriptional activity of the MEIS1A C-terminus (residues 335-390), I employed a fusion of this domain to the GAL4 DBD-binding domain. Transcriptional activity was then reported by five tandem copies of GAL-DBD (5XUAS) driving expression from a firefly luciferase cassette.

The functionality of our PKA and CRTC1 expression vectors was validated using a luciferase reporter driven by cAMP response elements (CREs) HEK293T cells. The results show a robust transcriptional response to both PKA and CRTC1 expression, confirming the functionality of our reagents (Figure 1). Consistent with previous data from the lab, a strong transcription of the luciferase gene was detected in response to PKA signaling in HEK293T cells (Figure 2) (Goh et al, 2009; Huang et al, 2005).

## 2.1   CRTCs bypass the need for PKA to activate transcription by MEIS1A

Recent studies have identified a family of PKA-responsive CREB coactivators called CRTCs (Conkright et al, 2003; Screaton et al, 2004). To test whether the effect of PKA on the MEIS1A C-terminus might be mediated by CRTC1, expression vectors for CRTC1, GAL-DBD with 5XUAS and MEIS1A C-

terminus were tested in HEK293T cells in the presence and absence of PKA (Figure 2). Luciferase assays showed that CRTC1 was able to bypass PKA signaling to activate transcription of the luciferase gene driven by the GAL-MEIS1A C-terminus. The absence of synergistic activation upon co-expression of PKA and CRTC1 suggest the activity seen at the GAL-MEIS C-terminus is mediated by CRTC. Similar activation was seen by CRTC2 at the GAL-MEIS1A C-terminus (SL. Goh, data not shown), which shares a 32% similarity with CRTC1 (Iourgenko et al, 2003).
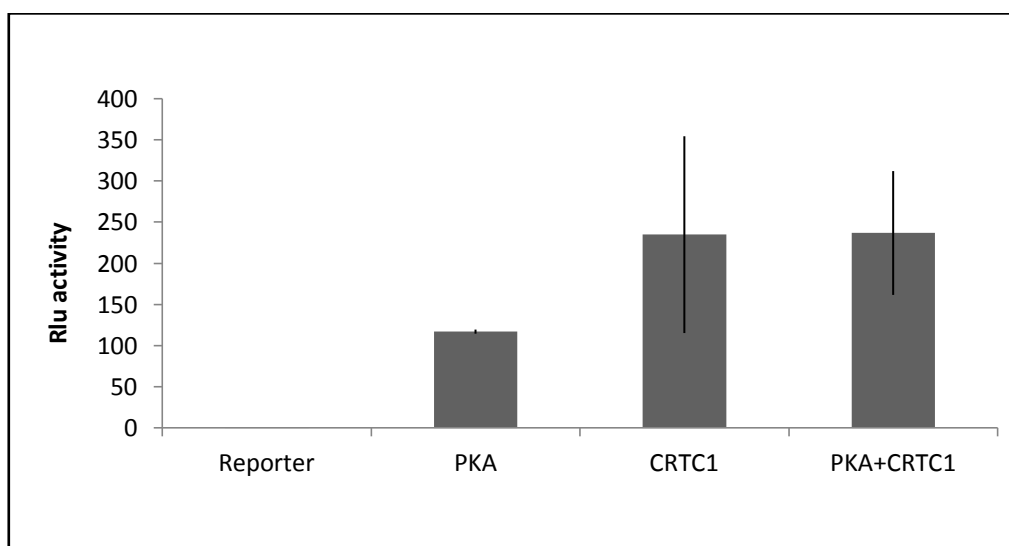
**Figure 1 Effect of PKA and CRTC1 at the CRE-luc promoter**

HEK293T cells were transiently transfected with the cAMP response element (CRE) luciferase reporter ("Reporter") and expression plasmids for PKA and CRTC1 as indicated. The effect of the PKA catalytic domain and/or CRTC1 is shown. Luciferase activities were measured at 48 h post-transfection. Error bars represent the standard deviation of three independent experiments. Rlu: Relative luciferase.
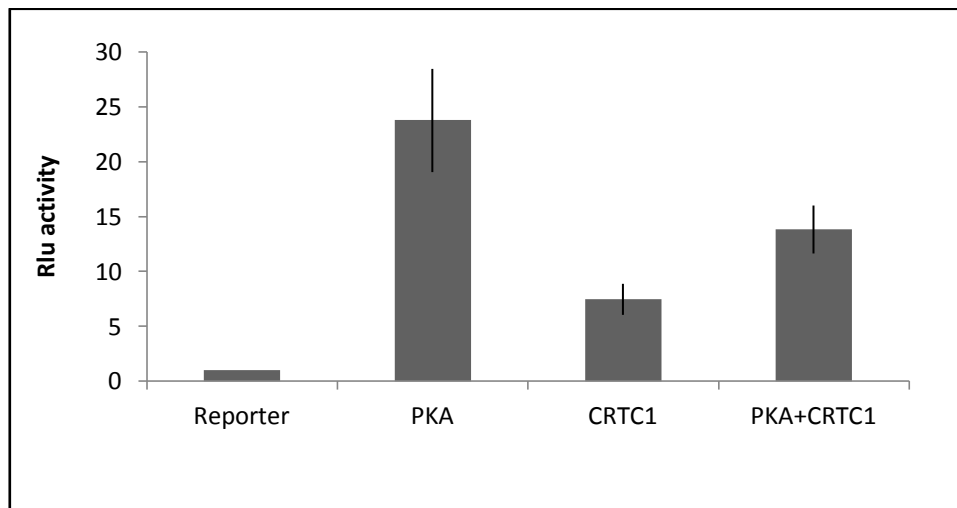
**Figure 2 CRTC1 bypasses the need for PKA to activate transcription through the MEIS C-terminus**

HEK293T cells were transfected with a pML5xUAS luciferase reporter ("Reporter") and expression vectors for GAL-MEIS1A-(335–390) in the presence of. The effect of the PKA catalytic domain and/or CRTC1 is shown. Luciferase activities were measured at 48 h post-transfection. Error bar represent the standard deviation of three independent experiments. Rlu: Relative luciferase.

## 2.2 Localization of endogenous and overexpressed CRTCs

Under basal conditions, CRTC proteins are phosphorylated by SIK2 and anchored in the cytoplasm *via* binding with 14-3-3 protein. PKA inhibits SIK2 and shifts CRTCs to the unphosphorylated state resulting in accumulation of CRTCs in the nucleus. This suggests that the ability of CRTC to bypass PKA observed in Figure 2 could be due to the accumulation of CRTC protein within the nucleus. Immunofluorescence experiments were performed on endogenous as well as Flag-tagged CRTC constructs to verify their subcellular location. Immunofluorescence detection with an antibody recognizing all endogenous MEIS products (MEIS1/2/3) showed cytoplasmic and nuclear staining in HEK293T cells, with a slightly higher proportion of MEIS visualized in the nucleus (Figure 3). Both the endogenous CRTCs showed an even accumulation both within the nucleus and cytoplasm (Figure 3). Following overexpression, strong CRTC2 was quantitated and observed strongly in the nucleus (Figure 3) whilst no significant nuclear accumulation was observed with overexpressed CRTC1 nucleus (Figure 3).

| Localization | Endogenous | Over-expressed |
|---|---|---|
| Nucleus | 0 | 99 |
| Nucleus > cytoplasm | 98 | 1 |
| Nucleus = cytoplasm | | |
| Nucleus < cytoplasm | 0 | 0 |
| Cytoplasm | 0 | 0 |
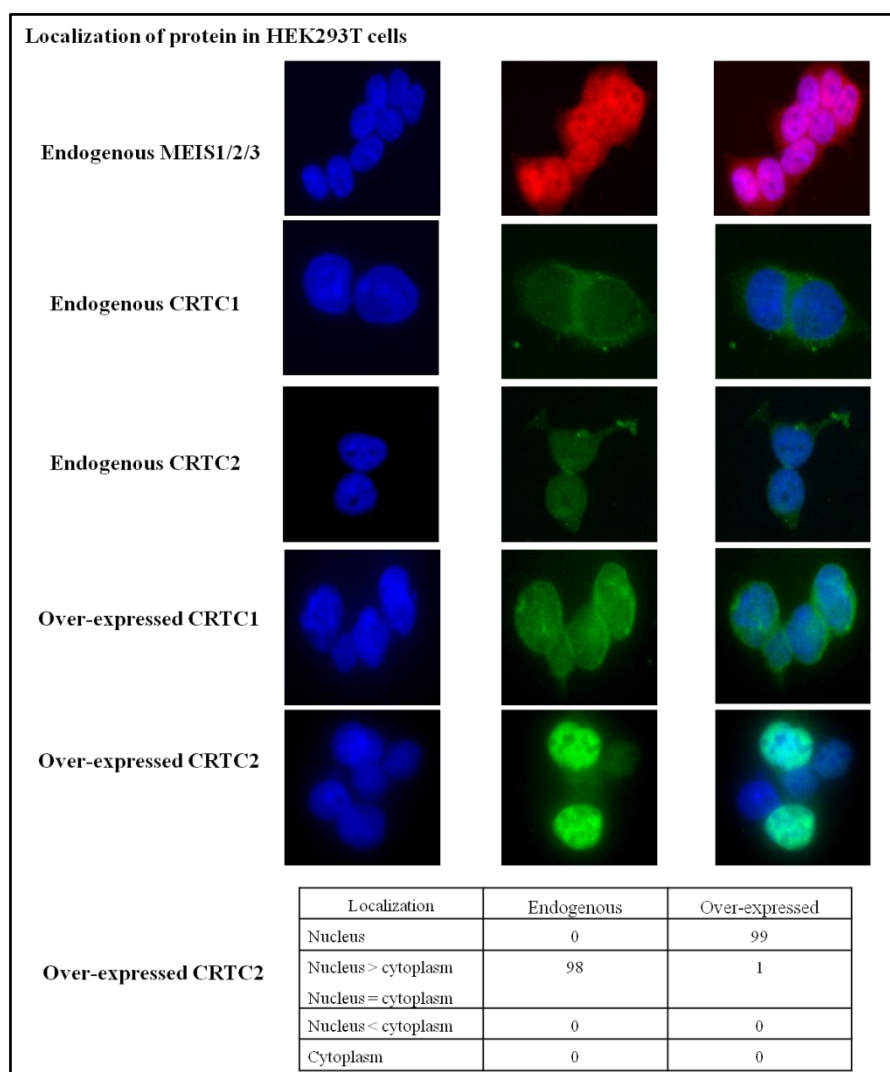
**Figure 3 Cellular localization and quantification of endogenous and overexpressed proteins**

Upper panel: Localization of CRTCs and MEIS proteins in HEK293T cells with anti-CRTC1, anti-CRTC2 and anti-MEIS1/2/3 antibodies. DAPI staining served to visualize nuclear staining.

Lower panel: Quantification of overexpressed CRTC2 in HEK293T cells

*Nuclear*, nuclear signal, Cytoplasm, cytoplasmic signal; *N=C*, signals in the nucleus and cytoplasm approximately equal; *N<C*, nuclear signal weaker than cytoplasmic; *C*, exclusively cytoplasmic signal. 100 cells each were scored for the distribution of endogenous *versus* overexpressed CRTC2.

## 2.3    Contribution of CRTC1 at an authentic MEIS1 target promoter

The contribution of CRTC1 to the transcriptional activity of MEIS1A was accessed on an authentic MEIS1 target promoter consisting of the 150 bp autoregulatory element (ARE) of the *Hoxb1* gene driving luciferase gene expresion (Figure 4). As shown in the luciferase reporter assay, co-expression of MEIS, PBX and HOX strongly activated transcription in the presence of PKA signaling (Lane 2, darker bar. Diagram in Figure 4). As seen at the GAL-MEIS1A C-terminus reporter, CRTCs alone activate transcription by MEIS-PBX-HOX complex as strongly as PKA (Lane 3 and 6. Diagram in Figure 4). Deletion of the N-terminal 46 amino acid fragment within CRTC1 and the C-terminal 203 amino acid region significantly reduced the transcriptional activation initiated by CRTC1 (Lane 4 and 5. Diagram in Figure 4). A deletion of the MEIS1A C-terminus (MEIS1AΔ334-390) impaired the transcriptional activity of the ternary complex in response to both CRTCs (Lanes 8-11. Diagram in Figure 4). This suggests that the MEIS1A C-terminus fulfills its role as a transactivation domain by recruitment of CRTCs. Note that the deletion of MEIS1A C-terminus would not completely abolish reporter gene activity as the endogenous MEIS1 can bind and recruit endogenous as well as overexpressed CRTC1 (Lanes 7-11 control. Diagram in Figure 4).

## 2.4    The role of CRTC in MEIS1A transactivation using shRNA

The effect of CRTC at mediating MEIS1A transactivation was accessed using shRNA against CRTC2. CRTC2 shRNA plasmid (2.5-10 ng) exhibited an

inhibition of transcriptional level at the MEIS1A C-terminus comparable to that without co-expression of Flag-CRTC2 (Top: Lanes 5-7 vs Lane 3. Diagram in Figure 5).This reduction in activity seen at Lanes 5-7 (Top) correlated with a depletion of Flag-CRTC2 protein level at Lane 2 (Lower panel in Figure 5). Specificity of the shRNA was validated when the control shRNA as well as a resistant version of Flag-CRTC2 (Wobble) did not show a reduction in activity nor protein levels Lanes 8-10 (Top panel in Figure 5) and Lanes 4-6 (Lower panel in Figure 5).

## 2.5 Role of CRTC on transcriptional activation at the MEIS1A C-terminus in response to PKA signaling

With respect to the PKA response at the MEIS1A C-terminus, knockdown of CRTC2 impaired response of the MEIS1A C-terminus to PKA signaling, suggesting that the specific transcriptional activity seen at the C-terminus is mediated by the endogenous levels of CRTC2 (Lane 4 vs Lane 3 Figure 6). A Gal-DBD harboring only the Gal DNA binding domain served as the control did not activate transcription in response to PKA signaling (Lane 1 and 2 Figure 6).
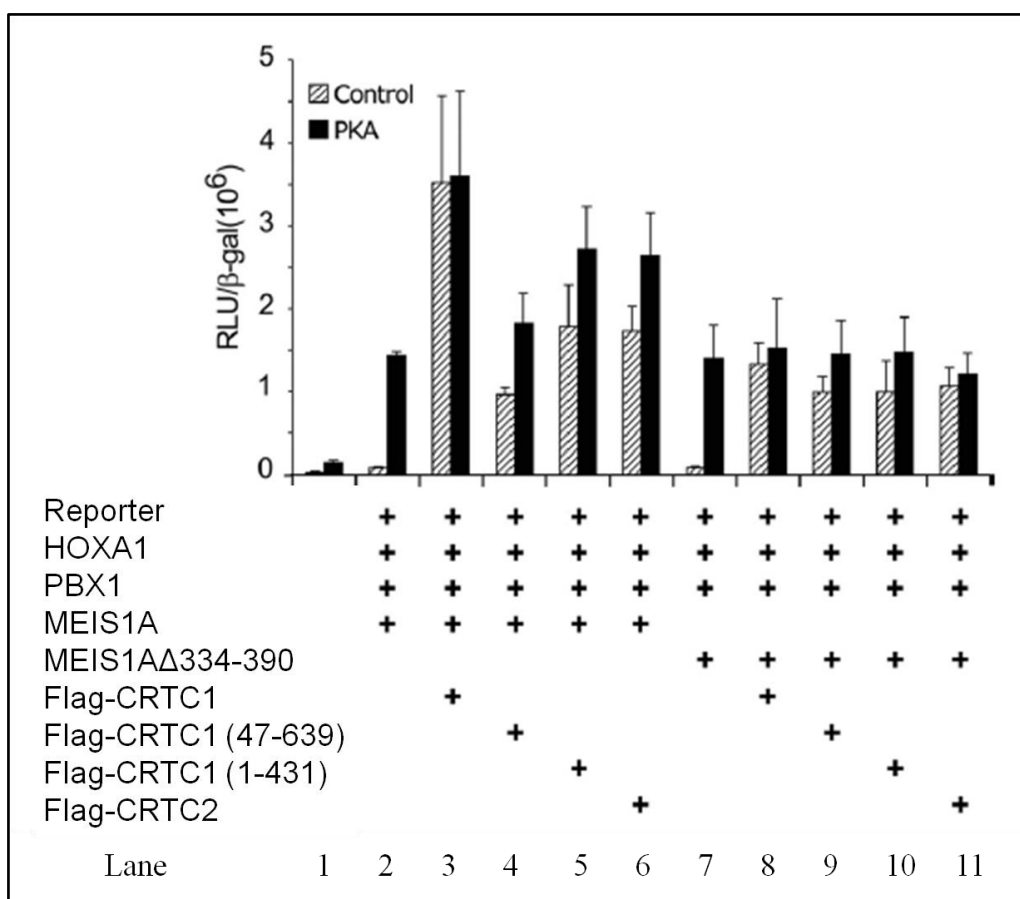
**Figure 4 Contribution of CRTC1 to MEIS1A transcriptional activity on MEIS1 enhancer**

HEK293T cells were transiently transfected with the pMLHoxb1ARE luciferase reporter ("Reporter") and expression plasmids as indicated. Luciferase activities in the absence or presence of the PKA catalytic domain (control or PKA) were measured at 48 h post-transfection. Error bar represent the standard deviation of three independent experiments. Rlu: Relative luciferase. Adapted from Goh et al 2009.
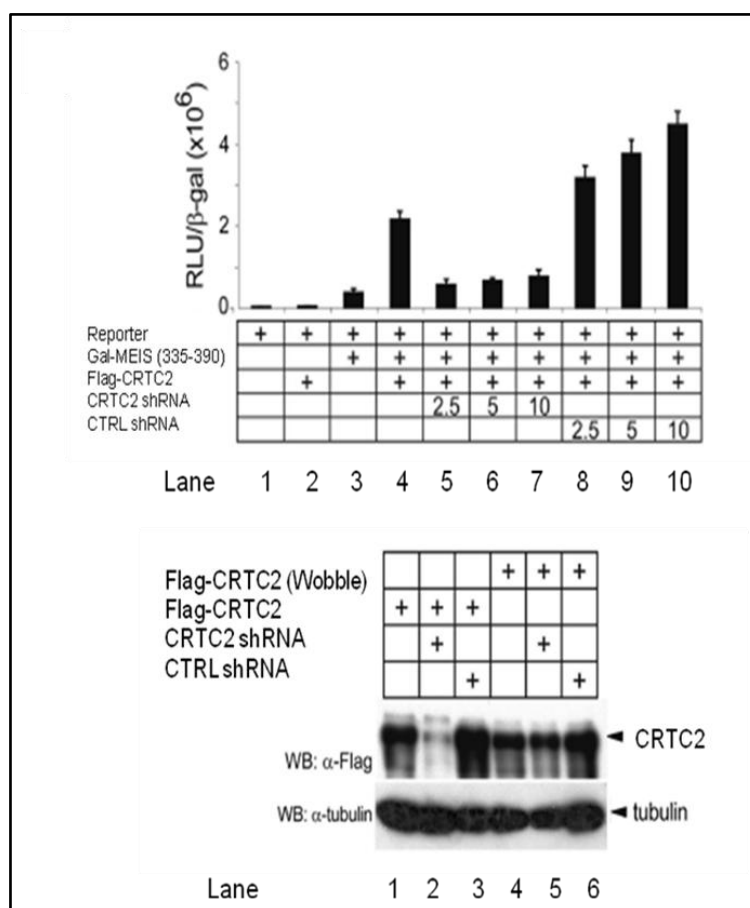
**Figure 5 Knockdown of CRTCs prevents PKA-mediated activation of the MEIS1A C-terminus**

*Upper panel*, effect of CRTC2 shRNA or control (*CTRL*) shRNA on GAL-MEIS1A-(335–390) luciferase transcription augmented by CRTC2. The indicated plasmids were co-transfected with the pML5xUAS reporter in HEK293T cells. Error bar represent the standard deviation of three independent experiments. Rlu: Relative luciferase.

*Lower panel*, knockdown of FLAG-CRTC2 protein levels in CRTC2 or control shRNA treated cells was verified by immunoprecipitation with M2 beads followed by Western blot (*WB*) analysis with an anti-FLAG antibody. Cell extracts were probed for tubulin, confirming equivalent protein concentrations in each sample. FLAG-CRTC2(Wobble) served as an RNA interference-resistant control. Adapted from Goh et al 2009. Adapted from Goh et al 2009.

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| GAL-DBD | + | + | | |
| GAL-MEIS(335-390) | | | + | + |
| CTRL shRNA | + | | + | |
| CRTC2 shRNA | | + | | + |
| Lane | 1 | 2 | 3 | 4 |
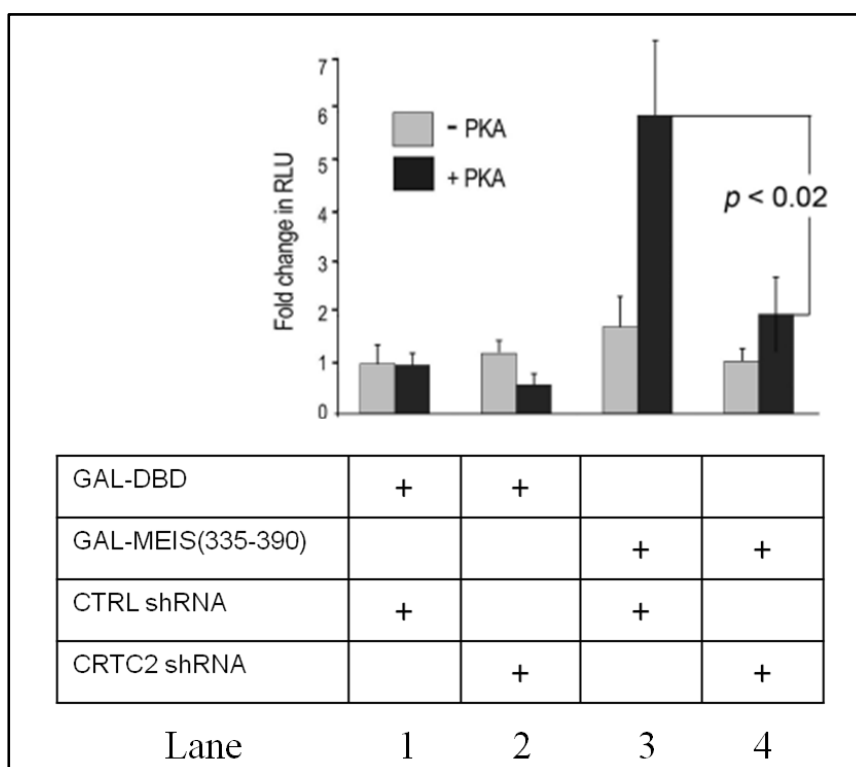
**Figure 6 Role of CRTC2 on transcriptional activation at the MEIS1A C-terminus in response to PKA signaling**

Cells were transfected with the pML5xUAS reporter and expression vectors for either the GAL DBD or GAL-MEIS1A-(335–390), along with a PKA expression vector or empty plasmid. Transcriptional activation by PKA through the MEIS1A C-terminus was abrogated by coexpression with the CRTC2-specific shRNA but not the control (CTRL) shRNA. Error bar represent the standard deviation of three independent experiments. Rlu: Relative luciferase. Adapted from Goh et al 2009.

## 2.6    Association of MEIS1A and CRTCs

Given the role of CRTCs in transcriptional activation through the MEIS1A C-terminus, it would be of interest to determine if CRTC1 binds to MEIS1A *in vivo*. Co-immunoprecipitation was performed using whole cell extracts from HEK293T cells overexpressing MEIS1A and/or Flag-CRTC1. Figure 7 shows a co-precipitation of MEIS1A with Flag-tagged CRTC1 using anti-Flag M2 agarose beads (Lane1. Diagram in Figure 7). The specificity of the co-immunoprecipitation is supported when no MEIS1A co-immunoprecipitation was seen from cells transfected with MEIS1A or Flag-CRTC1 alone (Lane 2 and 3. Diagram in Figure 7). Neither MEIS nor CRTC1 protein were detected in the control immunoprecipitation in the absence of overexpression or stimulus (Lane 4. Diagram in Figure 7).

The MEIS-CRTC complex was further examined *in vivo* with HEK293T cells using antibody against endogenous CRTC2 proteins (Lanes 8-13. Diagram in Figure 8). MEIS1A was also detected from extracts of CRTC2 from cells overexpressing MEIS1A (Lanes 8-10. Diagram in Figure 8). Importantly, we found a 10% co-precipitation of endogenous MEIS1A protein with endogenous CRTC2 (Lanes 11-13. Diagram in Figure 8). Once again, specificity was verified as no MEIS1A protein was detected in the control immunoprecipitation using an anti-GAL4 antibody (Lane 14. Diagram in Figure 8). The MEIS1A mutant contains a mutation where the extreme last six residues (GQWHYM) are changed to alanine (AAAAAA). Such mutation renders it non-responsive to PKA signalling as well as CRTC1 signalling. Stable complexes between this

117

MEIS1A mutant and Flag-CRTC1 were also detected suggesting that while these six residues are required for the PKA responsiveness of the MEIS1A C-terminus, they are dispensable for interaction with CRTC1 (H. Shen, data not shown).

In addition, co-immunoprecipitation was performed using wild type MEIS1A and a MEIS1A mutant lacking the entire C-terminus (Δ334-390). Figure 9 Lane 6 shows that in the absence of the C-terminus, MEIS1A is unable to co-immunoprecipitate with CRTC1. The same was shown for MEIS1A (Δ334-390) and CRTC2 (H. Shen, data not shown). A possible explanation for such observation is that the C-terminus act to stabilize the MEIS protein as well as its binding to other protein. Experiments were thus set out to verify this possibility. Figure 9 has identified that the destabilization of the MEIS1A (Δ334-390) protein and its interaction with CRTC1 was due to a result of proteasome-mediated degradation, since the addition of proteasome inhibitor MG132 was able to recover the mutant protein levels, though its interaction with CRTC is still impaired (H. Shen, data not shown). This result further strengthens the importance of the MEIS1A C-terminus for interaction with the CRTC family.

However, the MEIS C-terminus was found required but not sufficient for binding with CRTC1 as co-immunoprecipitation complex was not detected for Flag-CRCT1 with either GAL-MEIS C-terminus alone nor with GAL-MEIS C-terminus with the last six residues mutated to alanine (H. Shen, data not shown). These data by far are suggestive of the requirement of MEIS1A C-terminus, including though not only restricted to, at mediating interactions with CRTC1.

## 2.7 Characterizing the MEIS1A binding domain on CRTCs

To characterize the MEIS1A binding domain on CRTCs, multiple CRTC1 deletion mutants were constructed and their associations with MEIS1A *in vivo* were accessed. Whole cell lysates from HEK293T cells were used and co-transfected with plasmids expressing MEIS1A or various Flag-tagged deletion mutants. Associations were seen with MEIS1A and Flag-CRTC1 (1-627) (Lane 4 Figure 10), Flag-CRTC1 (1-518) (Lane 10 Figure 10), Flag-CRTC1 (1-493) (Lane 14 Figure 10), and Flag-CRTC1 (1-431) (Lane 8. Diagram in Figure 10). These interaction studies provided an indication that the CRTC1 C-terminus is dispensable for MEIS1A binding.

A CRTC1 fragment consisting of amino acid 47-634 was able to complex with MEIS1A, however with weaker affinity due to the lack of its conserved N-terminal coiled coil domain (Lane 6 vs Lane 1 Figure 10). Reducing the fragment to 47-290 still retains its binding to MEIS1A, albeit at a weaker affinity (Lane 16 Figure 10). A further truncation of the N-terminal end of CRTC1 to 148-290 abolishes binding to MEIS1A (Lane 18 Figure 10). A deletion mutant consisting of residues 1-147 mediates weak binding of CRTC1 (H. Shen, data not shown). Strong binding requires a minimum of residues 1-290, which includes, but is more extensive than, the coiled coil domain at the CRTC1 N-terminus (residues 1-45) (H. Shen, data not shown).
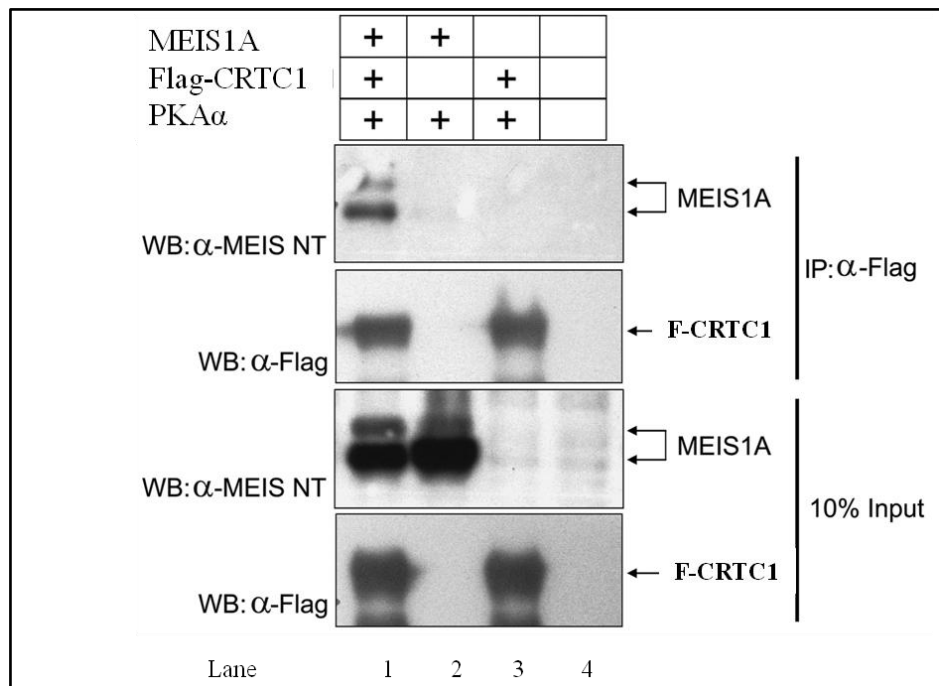
**Figure 7 Physical interactions of MEIS1A and Flag-tagged CRTC1**

Co-immunoprecipitation assay of FLAG-tagged CRTC1 and untagged MEIS1A in transfected HEK293T cells. Anti-MEIS NT and anti-FLAG Western blot (*WB*) analyses were performed on FLAG-CRTC1 immunoprecipitates (*IP*) prepared with anti-FLAG M2 affinity agarose. 10% input levels of MEIS1A and FLAG-CRTC1 are indicated. Adapted from Goh et al 2009.
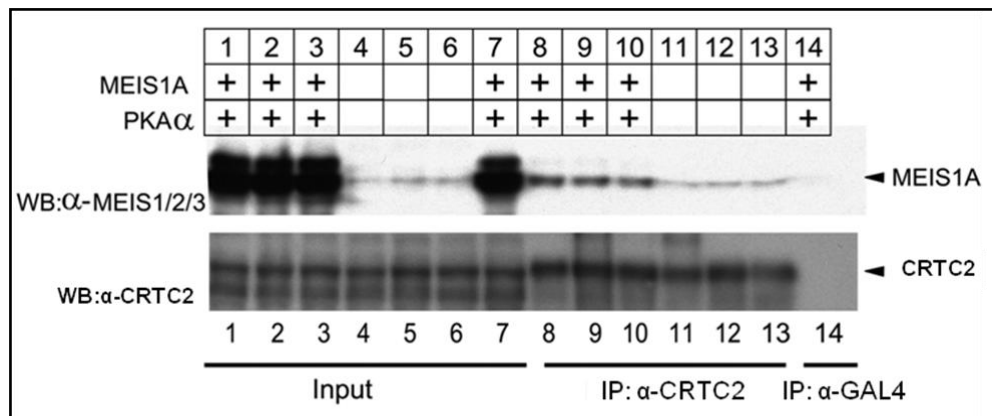
**Figure 8 Physical interactions of endogenous MEIS1A and CRTC2**

*Upper panel*, Western blot analysis of transfected and endogenous MEIS1A detected in immunoprecipitates of endogenous CRTC2 from HEK293T cells.

*Lower panel*, anti-CRTC2 Western blot analysis showing immunoprecipitated CRTC2 by the anti-CRTC2 antibody but not the control anti-GAL4 antibody. 10% input levels of MEIS1A and CRTC2 are shown. Adapted from Goh et al 2009.

| | | | | | | |
|---|---|---|---|---|---|---|
| Flag-CRTC1 | | + | | | + | + |
| MEIS1A | | | + | | + | |
| MEIS1AΔ334-390 | | | | + | | + |

WB: Anti-MEIS NT                IP: Anti-Flag

WB: Anti-MEIS NT

WB: :Anti-Flag              10% input
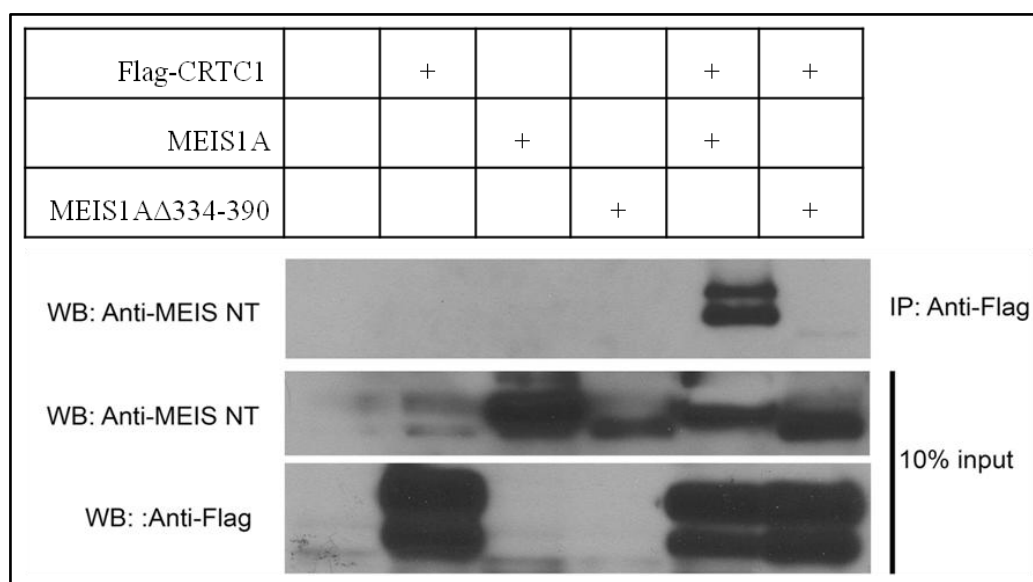
**Figure 9 MEIS1A mutant lacking the C-terminus fails to co-immunoprecipitate with CRTC1**

HEK293T cells were co-transfected with a FLAG-tagged CRTC1 expression vector and a vector encoding either wild-type MEIS1A or a mutant lacking the CRTC-responsive C-terminus (MEIS1A-(Δ334–390)). Adapted from Goh et al 2009.
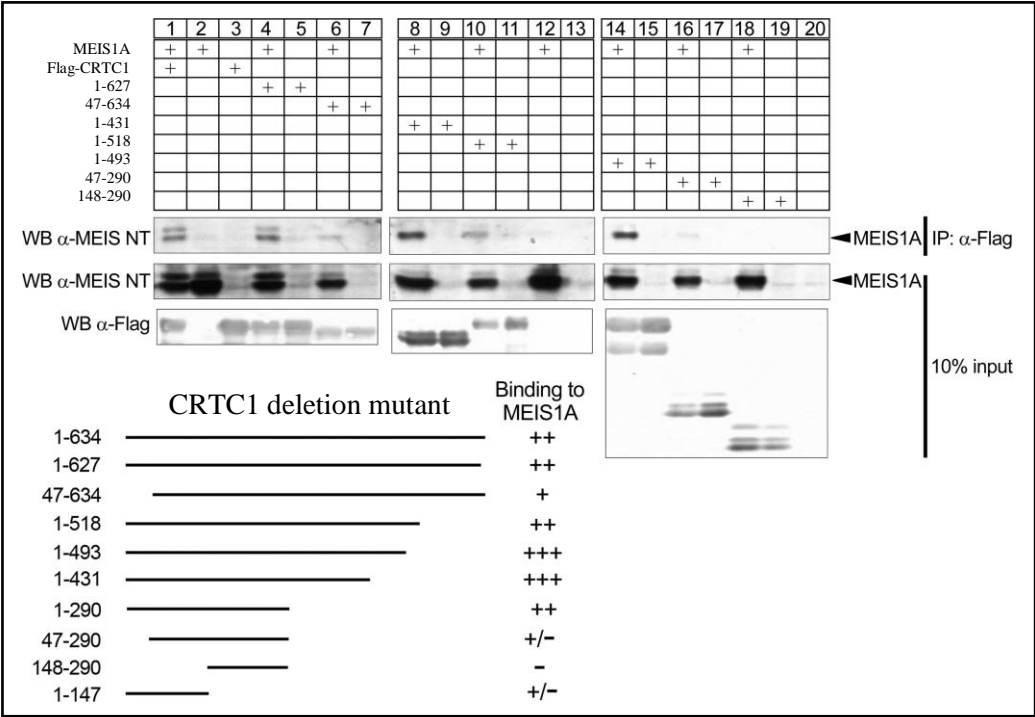
**Figure 10 MEIS1 interaction maps to the N-terminus coiled coil region of CRTC1**

*Upper panel*, co-immunoprecipitation between untagged MEIS1A and full-length FLAG-tagged CRTC1 (*Flag-CRTC1*) or its deletion derivatives in transfected HEK293T cells. MEIS1A proteins co-precipitated with FLAG-CRTC1 derivatives prepared using anti-FLAG M2 affinity agarose were revealed by Western blot (*WB*) analysis with anti-MEIS N-terminus antibody. The *bottom two panels* show inputs of MEIS1A and FLAG- CRTC1 derivatives, respectively.

*Lower panel*, schematic diagram of CRTC1 constructs and their MEIS1A binding activities. *WB*, Western blot; *IP*, immunoprecipitation. The plus and minus signs below *Binding to MEIS1A* correlate with the extent of binding to MEIS1A by the various CRTC1 mutants. Adapted from Goh et al 2009.

## 2.8    Recruitment of MEIS1, PBX1, and CRTC2 to MEIS1 targets

The involvement of CRTC for transcriptional activation at the MEIS1 C-terminus was then assessed at the *in vivo* level on its recruitment to various MEIS1 target genes alongside with MEIS and PBX. ChIP assay on differentiating mouse P19 embryonic carcinoma cells with or without Forskolin treatment were performed. Real-time PCR was carried out on immunoprecipitated DNA using primers spanning the *Hoxb1* ARE and *Hoxb2* r4 enhancer and *Meis1* promoter. Values obtained were normalized and expressed as relative occupancy.  Immunoprecipitates of the forskolin treated cells revealed a higher recruitment to all three enhancers (Figure 11a-c). Endogenous proteins were found to occupy the *Meis1* promoter (Figure 11a). Upon stimulation, CRTC2 was also found to be recruited to the *Meis1* promoter and the *Hoxb2* r4 enhancer (Figure 11a and b). Housekeeping gene *gapdh* served as a control and showed no significant recruitment on any targets (Figure 11d). These results revealed the co-recruitment of CRTC2 and MEIS1 on some MEIS1 target genes *in vivo*, supporting a physical and functional role of these proteins in transcriptional regulation.

124
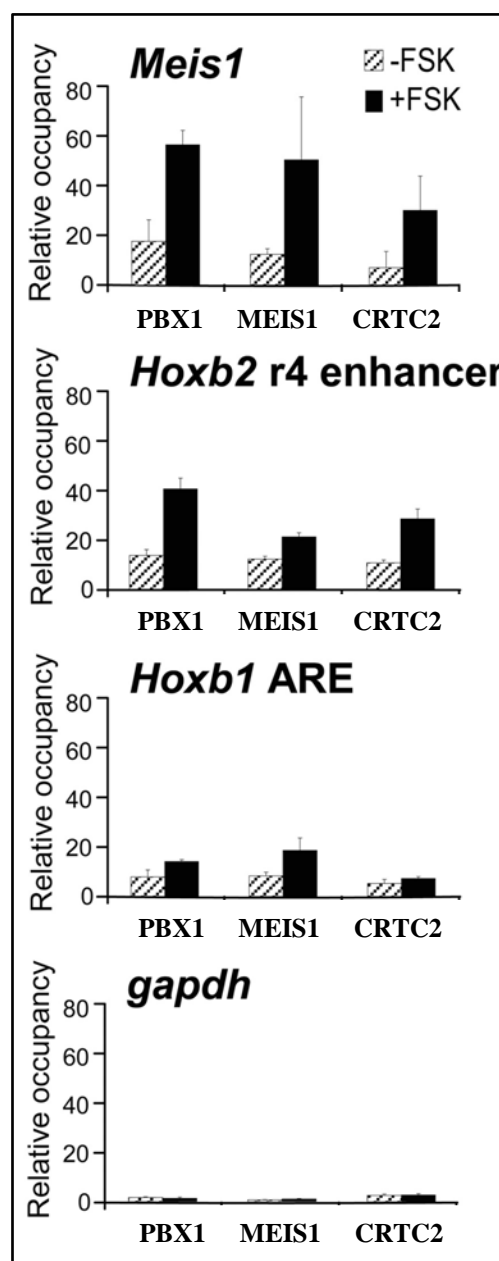
**Figure 11 Recruitment of MEIS1, PBX1, and CRTC2 to endogenous MEIS1 targets**

The results of ChIP assays in untreated (*-FSK*) or forskolin-treated (*+FSK*) mouse P19 cells were presented. Values obtained by LightCycler quantification were normalized and expressed as relative occupancy. *A*, the *Meis1* promoter, which harbors a consensus PBX-MEIS binding site. *B*, the *Hoxb2* r4 enhancer. *C*, the *Hoxb1* ARE. *D*, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)

served as an internal control. Error bar represent the standard deviation of three independent experiments. Adapted from Goh et al 2009**.**


# 3 DISCUSSION

Previous studies by the lab have established that the MEIS1A C-terminus harbors a transactivation domain that is responsive to PKA signaling. Supporting studies have also mapped a conserved transcriptional function to the C-terminus of MEIS1A which is required for accelerating leukemogenesis (Mamo et al, 2006; Wang et al, 2005). In this chapter we have further supported these data to establish the physical involvement of the CREB coactivator CRTC1 and CRTC2 in mediating the transcriptional function at the MEIS1A C-terminus. The authenticity of the effect of CRTC1 has also been observed strongly in the well-established CRE-reporter assay as well as in *in vivo* assays.


## 3.1 CRTCs bypass the need for PKA to activate transcription by the MEIS1A via physical association

I first tested with success the functionality of the PKA and CRTC1 expression vectors using the CRE luciferase reporter (Figure 1). The mechanism behind the transcriptional activity of the MEIS1A C-terminus (residues 335-390) was then tested using a 5XUAS luciferase reporter. Results from the assay revealed that the CREB coactivator CRTC1 and CRTC2 bypass PKA signaling to activate transcription of the luciferase gene driven by the 5XUAS luciferase reporter (Figure 2). Since activity of CRTC is determined by its dephosphorylation state which in turns determines its nuclear localization. This suggests that the ability

of the overexpressed CRTCs to bypass PKA for activation through MEIS1A could be due to the accumulation of CRTC protein within the nucleus. Immunofluorescence was thus performed to examine if the localization of CRTC correlates with its ability to bypass PKA for activation through MEIS1A. Both endogenous CRTCs showed even accumulation within the nucleus and cytoplasm (Figure 3). Following overexpression, CRTC2 was observed strongly in the nucleus while CRTC1 was found to be both nuclear and cytoplasmic. Thus, the quantity of nuclear CRTC2 may constitute its activation in the nucleus, whereas for CRTC1, a low level of CRTC1 may be sufficient for binding.

Multiple truncated mutants of CRTC1 have identified two regions contributing to the effect on MEIS1A (Figure 10). The first region resides within the N-terminal 46 residues of the CRTC1 conserved coiled coil domain required for tetramer formation and binding to CREB, without which impedes CRTC1 function (Lane 6. Figure 10) (Conkright et al, 2003). In addition, the CRTC1 C-terminal harboring an overlapping transcriptional activation domain has been previously suggested to coordinate assembly of the transcriptional apparatus was also shown to be required for MEIS1A activity (Conkright et al, 2003).

## 3.2 Role of CRTC on transcriptional activation at the MEIS1A C-terminus in response to PKA signaling

CRTC2 shares 32% similarity with CRTC1 and have been shown to emulate CRTC1 in potentiating the activity of MEIS1A (Iourgenko et al, 2003). Results

from the lab have observed notably weaker activity generated by CRTC2 than CRTC1, an observation that was consistent with previous findings (Figure 4) (Conkright et al, 2003). Thus, most of the experiments performed by me are performed using CRTC1 if not both CRTCs. The knockdown of CRTC2 and ChIP were performed by previous PhD student Siew Lee Goh. Knockdown of overexpressed and endogenous CRTC2 with shRNA confirmed a specific inhibition of activity at the MEIS1A C-terminus (Figure 5). Such impairment was also observed in response to PKA signaling, further supporting the role of CRTC in mediating MEIS1A transactivation (Figure 6).

Co-immunoprecipitation results demonstrate for the first time physical interaction of MEIS1A with Flag-tagged CRTC1 (Figure 7). Significantly, we have also identified co-precipitation of endogenous MEIS1A protein with endogenous CRTC2 in HEK293T cells (Figure 8). Endogenous interactions between CRTC1 and MEIS should have been tested as well. It would be a complete approach to have both CRTC1 and 2 tested in parallel to these sets of data.

### 3.3  Contribution of CRTC1 to MEIS1A on authentic MEIS1 target promoter

Previous results have reported strong activation of the MEIS-PBX-HOX ternary complex in the presence of PKAα (Figure 4). Reporter assay have identified that CRTC1 and also CRTC2 confers transcriptional activation by the ternary complex to the same extend as PKAα alone (Figure 4).

## 3.4 Characterizing MEIS1A binding domain on CRTC

The interaction domain on CRTC1 has been narrowed to residues 1-147 which mediate binding albeit weak to MEIS1A, this domain prove to be critical for without which binding was not feasible (Figure 10). Strong binding requires a minimal of residues 1-290, which includes, but is more extensive than the coiled coil domain N-terminus of CRTC1 (residues 1-45).

## 3.5 Characterizing CRTC1 interaction domain on MEIS1A

MEIS1A mutant with the last six residues extreme C-terminal mutated to alanine abolishes its response to PKA. Stable complexes were detected between this MEIS1A mutant and Flag-CRTC1. While co-immunoprecipitation was performed using wild type MEIS1A and a MEIS1A mutant lacking the C-terminus ($\Delta$334-390) showed that in the absence of the C-terminus, MEIS1A is unable to co-immunoprecipitate with CRTC1 (Figure 9). Results thus hinted that the MEIS1A C-terminus is required for interaction with CRTC1 while the PKA responsive residues at the C-terminus of MEIS1A are dispensable in CRTC1-MEIS1A interaction.

Further observations suggest for the importance of the MEIS1A C-terminus in mediating CRTC1 interaction. The GAL-MEIS1A C-terminus reporter consisting of just the C-terminal 56 residue fragment of MEIS1A was sufficient to activate transcription in the presence of CRTC1 and CRTC2 while mutations within the MEIS1A C-terminus abolished activation by CRTC1 and CRTC2 (SL. Goh, data not shown). By comparison with the full length MEIS1A,

MEIS1A (Δ334-390) mutant lacking the C-terminus showed impaired activation of a Hoxb1 ARE reporter in the presence of CRTC1 and CRTC2 (Figure 4). This reduction in activity can be supported by binding impairment of the proteins as a result of a lack of binding between MEIS1A (Δ334-390) with CRTC1/CRTC2 (Figure 9 and H. Shen, data not shown).

### 3.6    Contribution of CRTC1 on authentic MEIS1 target enhancers *in vivo*

The involvement of CRTC for transcriptional activation at the MEIS1 C-terminus was then accessed at the *in vivo* level on its recruitment to various MEIS1 target genes alongside with MEIS and PBX. ChIP assay demonstrate co-occupancy of endogenous MEIS1, CRTC2 and PBX1 on the *Hoxb2 r*4 enhancer and *Meis1* promoter upon cAMP stimulus, confirming the biological relevance of the findings (Figure 11). These results revealed the recruitment CRTC2 and its role with MEIS1 on MEIS1 target genes *in vivo*, providing a strong physical and functional role of these proteins in transcriptional regulation. The *in vitro* studies showed that the MEIS1A C-terminus fulfils its key role as a transactivation domain by recruitment of CRTCs and stabilizing the interaction. These results presented here as well as results from other previous studies suggest that the CRTC component of a CRTC-MEIS complex may act to stabilize the interaction and promote the assembly of transcription complex by recruitment of the TFIID-associated factor TAFII30 and CBP (Conkright et al, 2003; Johannessen et al, 2004). These data together with the *in vivo* results

imply that HOX, MEIS, PBX and CRTC collaborate and converge in pathways that induce subsets of genes involved in embryonic development as well as in normal hematopoiesis or in leukemogenesis.

# CHAPTER 4

# THE EFFECTS OF CREB AND ITS MUTANTS (ACREB AND ACREBR314A), CBP AND OTHER PROTEIN INTERACTIONS AT THE MEIS1A C-TERMINUS

# 1  ABSTRACT

Given the previously identified role of CREB in the PKA responsiveness of the MEIS1A C-terminus, we further explored CREB's role through the use of non-DNA binding dominant negative CREB: ACREB and a derivative of ACREB, ACREB R314A, that cannot bind CRTCs. Results showed a reduction in the PKA responsiveness of the MEIS1A C-terminus reporter in the presence of ACREBR314A, supporting the contribution of CREB to PKA-induced transcriptional activation through the MEIS C-terminus. However, in my hands, the CREB mutants ACREB and ACREBR314A still showed activation at the MEIS1A C-terminus, an indication of a nonspecific effect at the reporter. Knockdown studies as well as proximity ligation assay were used to access and validate the effect of CREB at mediating MEIS1A transactivation and interaction. In my hands, no observable effect was observed for serine/threonine kinase GSK3 at the MEIS1A C-terminus, contrary to published data (Wang et al 2010). Studies with the CREB mutants CREBS133A and CREBR314A hinted at a crucial role of the CREB-CBP and CREB-CRTC interactions in stimulating transcriptional activity by the MEIS1A C-terminus. These results bolstered a role for CREB in regulating MEIS1A C-terminus activity in a manner that depends on its association with CRTC and CBP but independent of its DNA binding activity. The interaction found between MEIS and the CREB coactivator CBP as well as MEIS with the CREB coactivator CRTC were further verified using proximity ligation assays. No conclusive data could be

drawn from the PLA interaction studies with the MEIS1A and MEIS1A (Δ334)

(MEIS1A lacking the C-terminus) expression plasmids.

# 2   RESULTS

## 2.1   CRE reporter is modestly responsive to overexpressed CREB, but strongly responsive to PKA and CRTC1

Two approaches were taken to investigate the role for CREB in PKA- and CRTC- induced transcriptional activation by the MEIS1A C-terminus. The first approach was to interfere with wild type CREB through the use of the dominant negative mutants of CREB. The second approach involved testing the effects of reduced wild type CREB expression.

The first CREB mutant is the ACREB construct which consist of an acidic amphipathic sequence that replaces the N-terminus of the CREB dimerization domain thus masking the N-terminus of the leucine zipper domain of the wild-type CREB that binds DNA (Ahn et al, 1998). The second dominant negative CREB mutant is the ACREBR314A, a construct of ACREB with a point mutation at the CRTC binding site of Arginine 314, disabling its ability to bind CRTC.

In order to validate the inhibitory activity of the dominant negative constructs of CREB, the robust cAMP response element (CRE) reporter was first used. Co-transfection of expression vectors for CRE-luciferase reporter and wild type CREB in HEK293T cells did not alter the activity in the absence or presence of PKA (Figure 1). In contrast, a strong PKA response was detected for the CRE luciferase reporter. The effect of CRTC was also validated for the CRE reporter as, when overexpressed, CRTC1 alone generated a strong response for the

135

CRE-reporter (Figure 2). Thus, these tests validated the appropriate responsiveness to PKA and CRTC1.
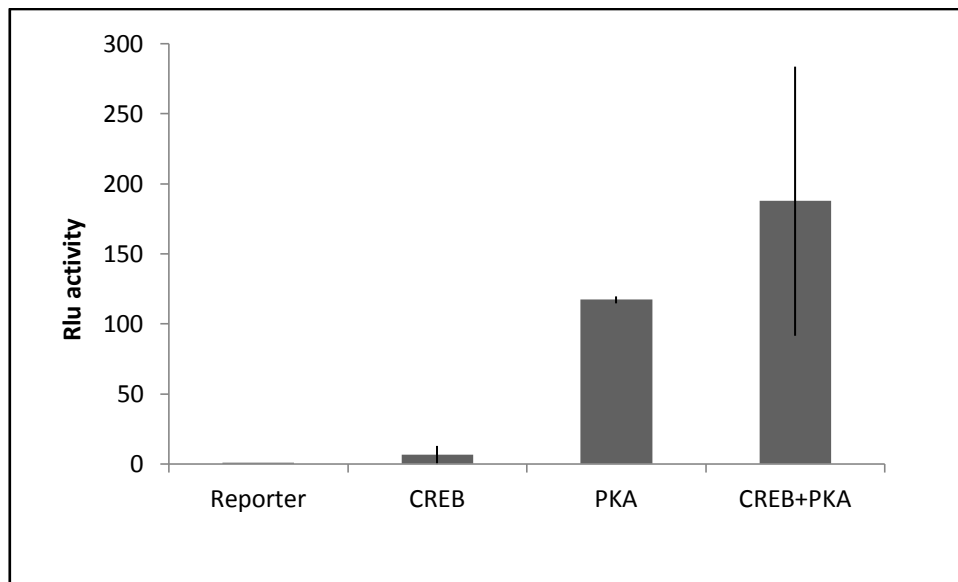


**Figure 1 CRE reporter is modestly responsive to overexpressed CREB, but strongly responsive to PKA**

HEK293T cells were transiently transfected with the cAMP response element (CRE) luciferase reporter and the effect of the CREB and PKA catalytic domain were shown. Luciferase activity was measured at 48 h post-transfection. Error bar represent the standard deviation of three independent experiments. Rlu: Relative luciferase activity.

## 2.2 CREB mutants ACREB and ACREBR314A inhibit activation of a CRE reporter by PKA and CRTC1

Having validated the effect of CRE reporter at mediating the activities of CREB, PKA and CRTCs, the ability of the dominant negatives constructs to inhibit these functions were subsequently determined. An inhibitory effect of ACREB was observed by CRTC1 overexpression, and ACREBR314A overexpression inhibited activation by both PKA and CRTC1 (Figure 2). In parallel, physical properties of the dominant negative constructs were determined. As expected, both ACREB and ACREBR314A physically interact with wild type CREB in co-immunoprecipitation assay (Figure 3).

## 2.3 CREB mediates PKA-responsiveness of the 5XUAS luciferase reporter

Given the responsiveness of MEIS1A C-terminus to PKA and the PKA dependent activation of CREB via the cAMP signaling pathway, the mechanism by which the MEIS1A C-terminus responds to PKA signaling was investigated. Consistent with the observations for the CRE reporter, expression of wild type CREB341 did not alter the activity for the MEIS1A C-terminus in the absence or presence of PKA suggesting a high endogenous level of CREB in the HEK293T cells tested (Figure 4).
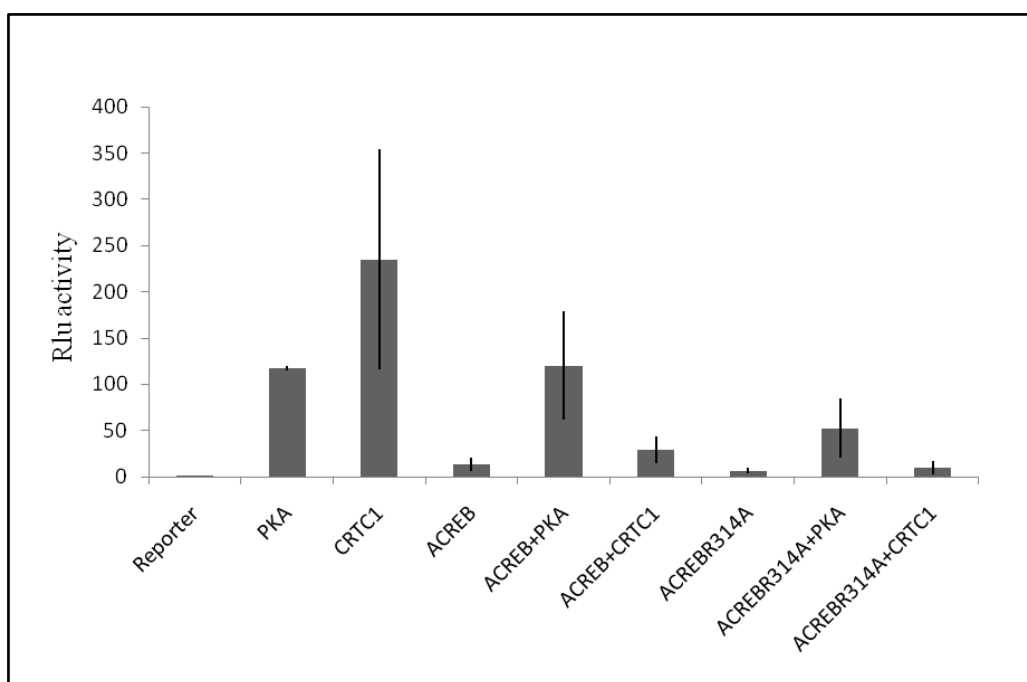
**Figure 2 CREB mutants ACREB and ACREBR314A inhibit activation of a CRE reporter by PKA and CRTC1**

HEK293T cells were transiently transfected with the cAMP response element (CRE) luciferase reporter and the effects of the CREB mtuants (ACREB an ACREBR314A), CRTC1 and PKA were shown. Luciferase activity was measured at 48 h post-transfection. Error bar represent the standard deviation of three independent experiments. Rlu: Relative luciferase activity.
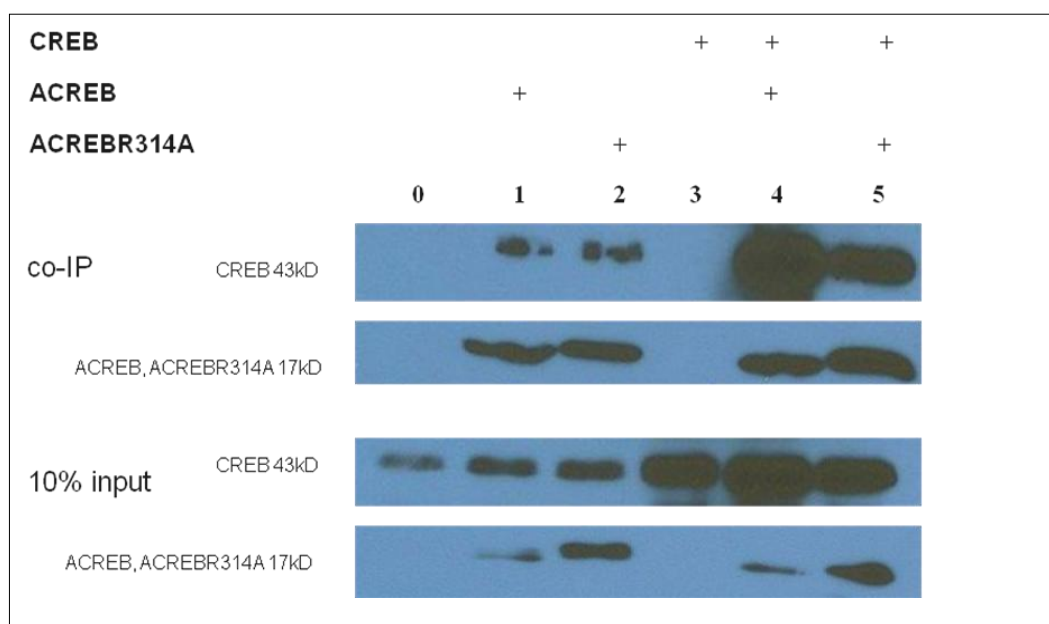
**Figure 3 CREB mutants ACREB and ACREBR314A physically interact with wild type CREB**

Co-immunoprecipitation assay of FLAG-tagged ACREB and FLAG-tagged ACREBR314A and untagged CREB in transfected HEK293T cells. Anti-CREB and anti-FLAG Western blot (*WB*) analyses were performed on FLAG-ACREB and ACREBR314A immunoprecipitates (*IP*) prepared with anti-FLAG M2 affinity agarose. 10% input levels of all three forms of CREB were indicated.
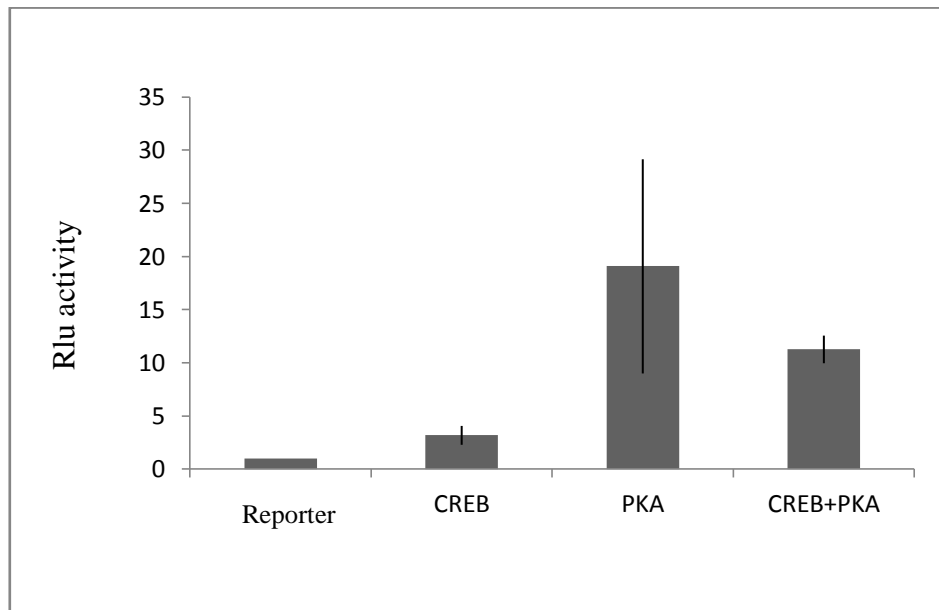
**Figure 4 CREB mediates PKA-responsiveness of the MEIS1A C-terminus**
HEK293T cells were transiently transfected with expression vectors for GAL-MEIS1A-C-terminus (335-390) alongside the pML5xUAS luciferase reporter. The effect of CREB and the PKA catalytic domain were shown. Luciferase activity was measured at 48 h post-transfection. Error bars represent the standard deviation of three independent experiments.

2.4 **ACREB and ACREBR314A strongly and non-specifically activated the 5XUAS luciferase reporter**

Both ACREB and ACREBR314A strongly induce transcription activation through a mutated MEIS1A C-terminus (GQWHYM) that is non-responsive to PKA (data not shown) and CRTC1 (Huang et al, 2005) (Figure 5). This strongly suggests a non-specific effect at the 5XUAS luciferase reporter and hence no insights into the role of CREB can be obtained through the use of these dominant negative constructs.

140

## 2.5    Efficacy of CREB knockdown using siRNA

Next we investigated whether the role of CREB in PKA- and CRTC- mediated activation through the MEIS1A C-terminus can be revealed by decreasing levels of wild type CREB expression. This was assessed using small interfering RNA-induced gene knockdown (siRNA) against CREB1. siRNA were delivered into HEK293T cells, which were transfected with expression plasmids before the cells were harvested to determine the efficacy and results of the knockdown. Western blot analysis was used to verify the efficacy of the knockdown in CREB protein levels using endogenous, overexpressed and negative control for siRNA as comparison. Results showed that the siRNA efficiently knocks down endogenous CREB expression (Figure 6).

## 2.6    Efficacy of CREB knockdown on CRE luciferase reporter

Next, the functionality of the knockdown was validated by examining the effect of CREB on a known CREB dependent transcriptional mechanism, the activation of a CRE reporter by PKA and CRTC. Indeed results showed that CREB knockdown impaired PKA- and CRTC-mediated transcriptional activation of the CRE reporter, though with modest effect (Figure 7). This could be due to the compensatory effect of ATF family for CREB function.
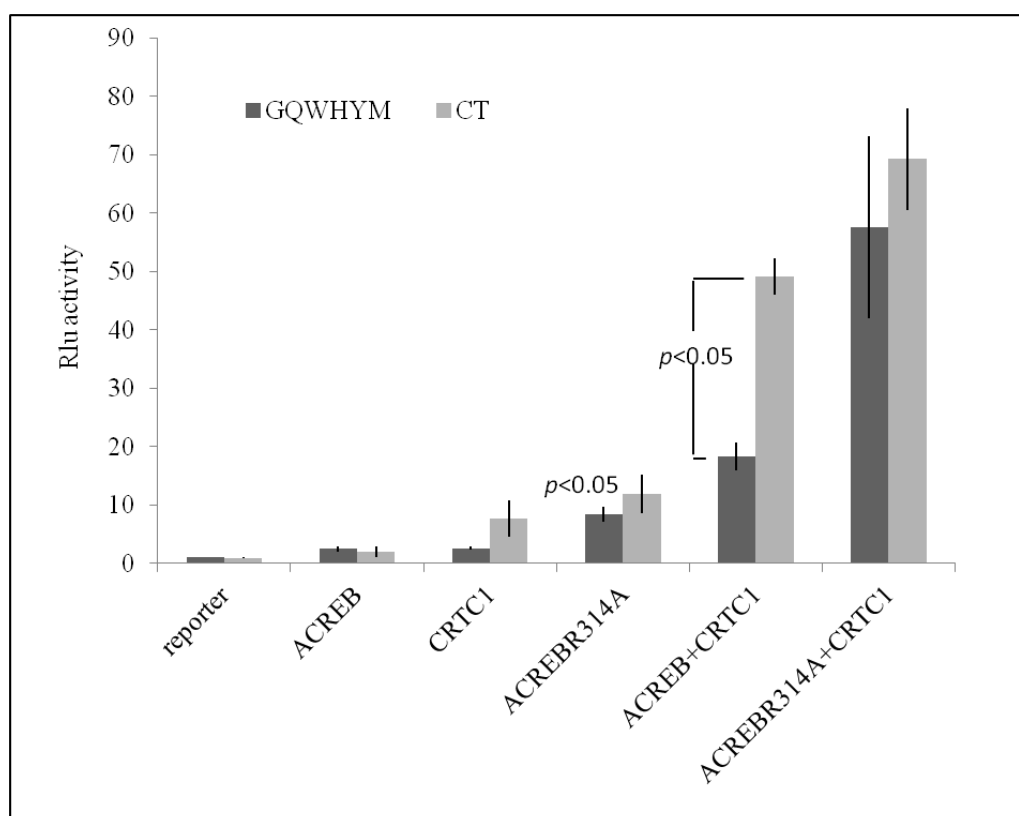
**Figure 5 Strong activation seen at MEIS C-terminus reporter with ACREB and ACREBR314 in the presence of CRTC1**

HEK293T cells were transfected with expression vectors for GAL-MEIS1A(GQWHYM), GAL-MEIS1A-(335–390) shown as CT, alongside the pML5xUAS luciferase reporter. The effects of the CREB mutants (ACREB an ACREBR314A) in the presence and absence of CRTC1 are shown. Luciferase activity was measured at 48 h post-transfection. Error bar represent the standard deviation of three independent experiments and p signifies the results of the Student's t test applied to values through the MEIS1A C-terminus versus the MEIS1A C-terminus mutant in the presence of the ACREB mutants.
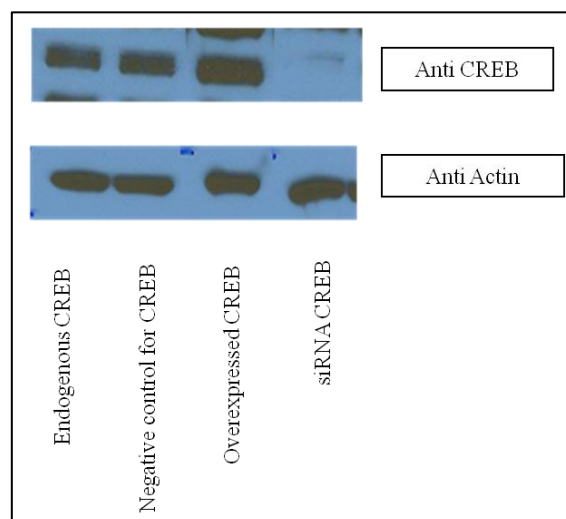
**Figure 6 Efficacy of CREB knockdown using siRNA**

Protein levels of siRNA CREB knockdown were verified in HEK293T cells, alongside with endogenous and overexpressed CREB341 using Western blot analyses with Anti-CREB antibody and Anti actin as control.
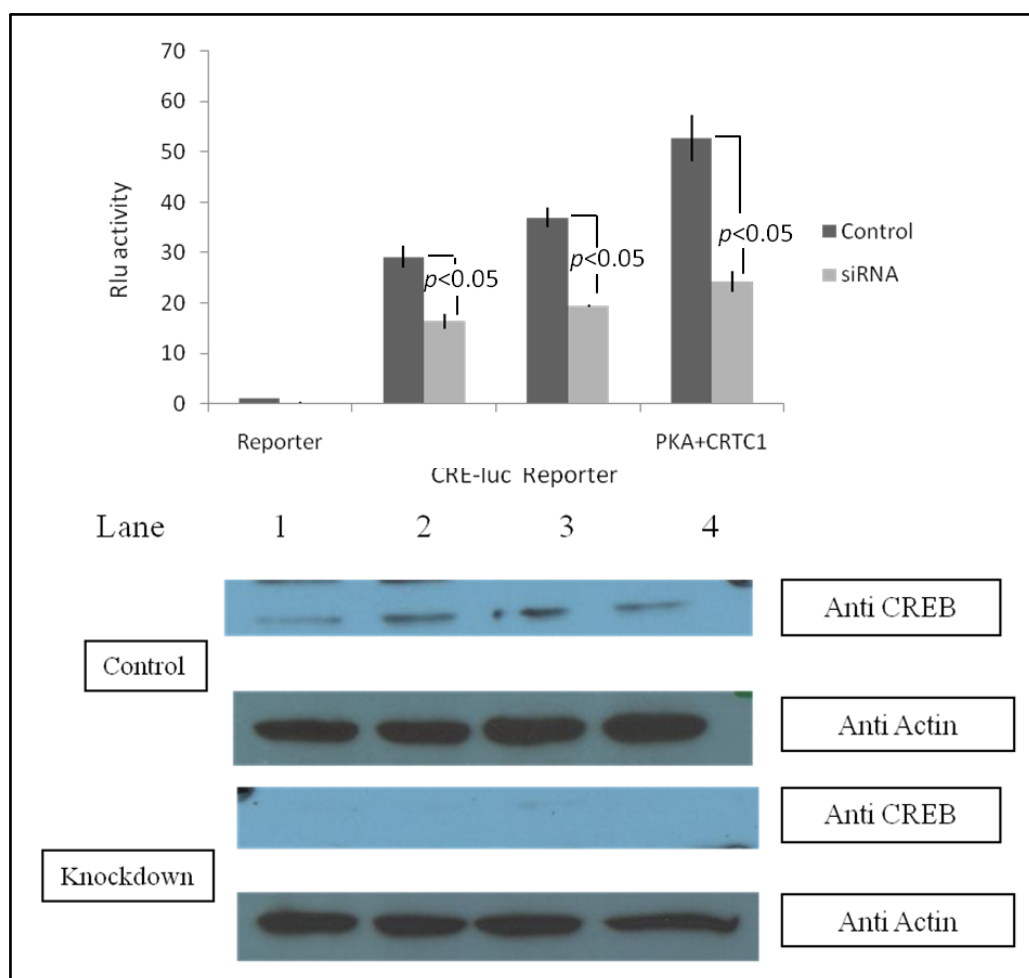
**Figure 7 Effect of CREB knockdown at the CRE luciferase reporter**

Upper panel, HEK293T cells were transfected with expression vectors for CRE-luciferase reporter in control cells or cells with CREB siRNA. The effect of and the PKA catalytic domain and/or CRTC1 are shown. Luciferase activities were measured at 48 h post-transfection. Error bar represent the standard deviation of three independent experiments. Rlu: Relative luciferase activity.

Lower panel, following transfection, protein levels of control and siRNA CREB knockdown cells were verified. Western blot analysis was performed using anti-CREB antibody and anti-actin serves as control.

## 2.7 CREB knockdown impairs PKA-induced transcriptional activation through the MEIS1A C-terminus

Having validated the appropriate controls in the system, I now test the effect of CREB knockdown on transcriptional activation through the MEIS1A C-terminus. Results in Figure 8 showed that transcriptional activation by the MEIS1A C-terminus in response to PKA is indeed dependent on CREB. However, for CRTC1 the effect appeared weak.

## 2.8 No observable effect was observed for the serine/threonine kinase GSK3 at the MEIS1A C-terminus

Given a recent report about the interaction between MEIS and the multifunctional serine/threonine kinase GSK3, the effect of GSK3 at the MEIS1A C-terminus was also accessed (Wang et al 2010). Three forms of GSK3 were tested, the wild type GSK3, which had been reported to promote transcription of the MEIS containing complex; two GSK mutants: GSKS9A, a mutant version of GSK3 that lacks the inhibitory phosphorylation site, as well as the kinase-dead mutant GSKK85A. GSK3beta had been reported to promote the conditional association of CREB and its coactivators CRTC and CBP with MEIS1, thus wild type GSK was expected to increase activity at the MEIS1A C-terminus while the mutant should reduce the association and hence reduce activity at the reporter. No change in transcription at the MEIS1A C-terminus was observed following expression of any of the three GSK plasmids (Figure 9 Upper panel). To verify the protein expression levels of the plasmids, western

blotting was performed to determine the levels of all three GSK derivatives in the HEK293T cells (Figure 9 Lower panel). A faint endogenous GSK3 band was detected in the control (untransfected) lane and distinct bands of various GSK proteins were detected upon overexpression, which validated the identity and efficacy of the plasmids (Figure 9 Lower panel). Actin served as a loading control for all lanes (Figure 9 Lower panel). This discrepency could possibly stem from a result of a different cell line.

## 2.9 Additional evidence of MEIS and CRTC interactions

Interactions between MEIS1 and CRTC1 were further supported using immunofluorescence staining. Figure 10 showed the localization of the MEIS1 and CRTC1 proteins in HEK293T cells. Both CRTC1 and MEIS1/2/3 antibody revealed a nuclear and cytoplasmic localization of both proteins, with a high degree of overlap, a prerequisite for their cooperative activity (Figure 10).

## 2.10 Validation of protein-protein interactions between MEIS, CREB, CRTC and CBP using proximity ligation assay

Proximity ligation assay (PLA) was used as an independent assay to quantify interactions down to single protein-protein level. Controls were performed where negative control consisted of only the MEIS1 antibody as the primary probe, while known interactions between MEIS and PBX proteins served as positive control (Figure 11).

146

## 2.11 Validation of PLA using controls and known CREB-CRTC interactions

The physical association between MEIS and CREB coactivator CRTC prompted us to independently validate the interactions by means of the proximity ligation assay (PLA) (Figure 11). Cells were treated with forskolin for 1 h at a concentration of 3 uM just prior to fixing. Having validated the PLA techniques with the recommended positive and negative controls, known interaction were tested as controls. As expected, interactions were observed between CREB and CRTC (Figure 11).

## 2.12 Association of MEIS and CRTC using proximity ligation assay

Having validated the PLA assay, I then proceeded to use this technique as an independent test of the novel protein-protein interactions involving MEIS-CREB, MEIS-CRTC and MEIS-CBP. Interactions between MEIS and CRTC1, MEIS and CRTC2 were detected by PLA as shown in a representative image in Figure 11, where each interaction corresponds to one red dot in the image.

Forskolin (FSK), which has been shown to activate the CREB signaling pathway, was used to stimulate CREB activity. Exposure to forskolin has been shown to promote forskolin stimulated cAMP accumulation of nuclear CRTCs (Conkright et al, 2003). However, no noticeable increase between endogenous MEIS1 and CRTC1 or CRTC2 interaction were observed in the nucleus upon stimulus treatment with forskolin. Having failed to observe increase in nuclear MEIS-CRTC interactions in forskolin stimulated HEK293T cells, effect of forskolin was tested on other cell lines with known response to forskolin.

147

Endogenous nuclear CRTC1 was successfully detected in CAD neuroblastoma cells, validating the efficacy of the FSK used (Figure 12).

### 2.13  Association of MEIS-CBP using PLA

The Featherstone lab has previously shown that transcriptional activation through the MEIS1A C-terminus is dependent on the co-activator CBP (Huang et al, 2005). This prompted us to assess the interactions between MEIS and CBP independently *via* PLA. Using PLA, I have identified interaction between endogenous MEIS and CBP in HEK293T cells (Figure 11). Consistent with its inability to promote nuclear CRTC in HEK293T, no increase in interactions were observed upon FSK stimulus (Figure 11).
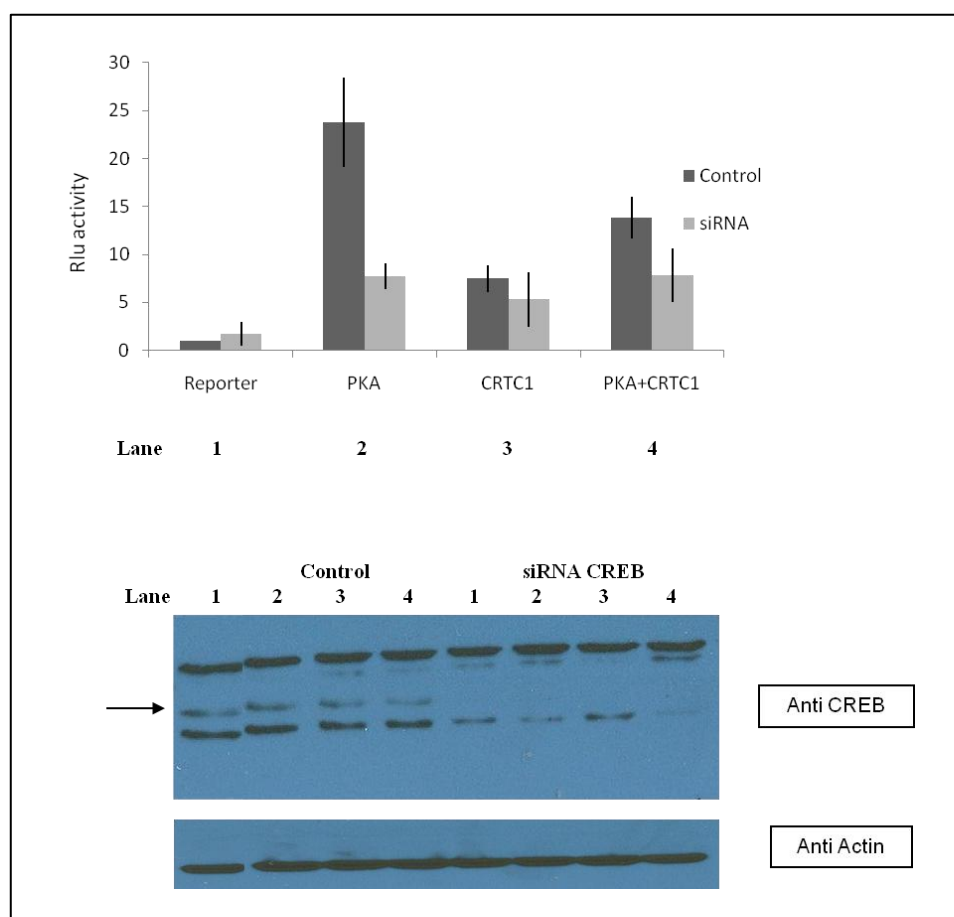
**Figure 8 Transcriptional activation generated by PKA and CRTC1 at the GAL-MEIS1A C-terminus reporter was hampered by CREB knockdown**

Upper panel, HEK293T cells were transfected with expression vectors for GAL-MEIS1A-(335–390) plus the pML5xUAS luciferase reporter in control cells or cells with CREB siRNA. The effects of and the PKA catalytic domain and/or CRTC1 were shown. Luciferase activities were measured at 48 h post-transfection. Error bar represent the standard deviation of three independent experiments.

Lower panel, following transfection, protein levels of control and siRNA CREB knockdown cells were verified. Western blot analysis was performed using anti-CREB antibody and anti-actin serves as control.
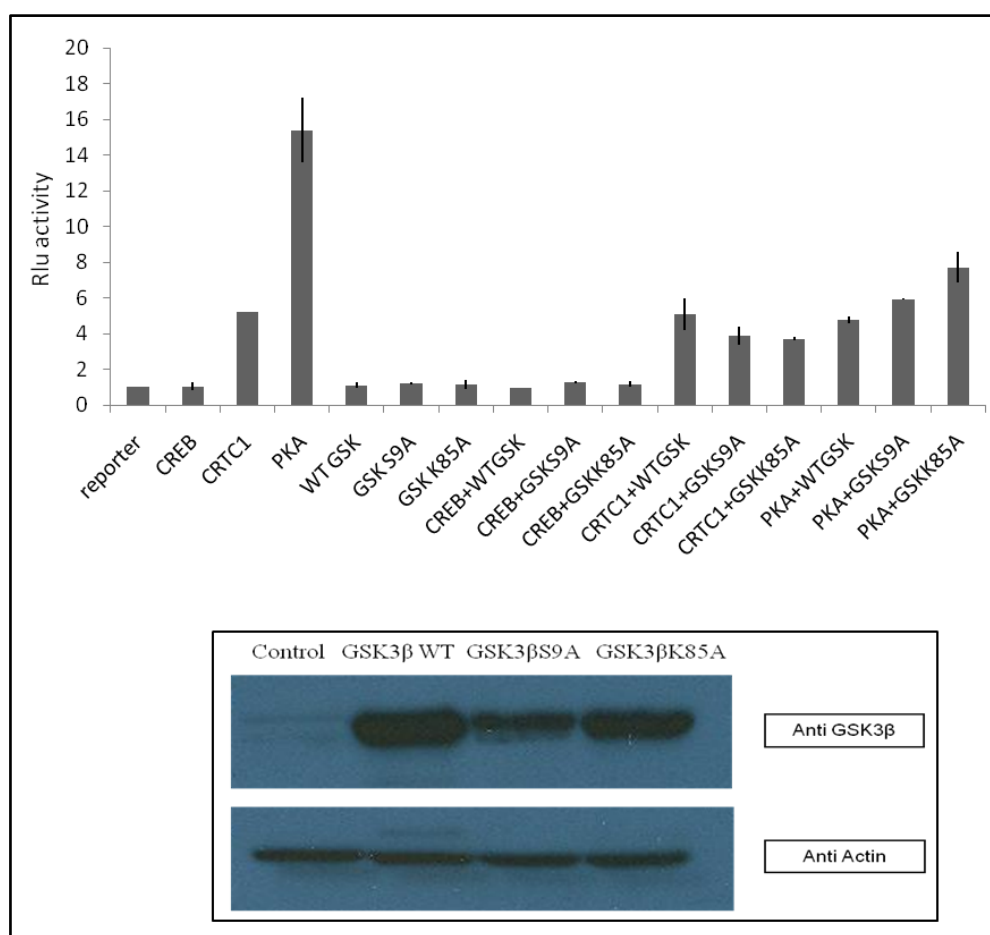
**Figure 9 Effect of GSK at the GAL-MEISCT reporter**

Upper panel, HEK293T cells were transfected with expression vectors for GAL-DBD, GAL-MEIS1A-(335–390) plus the pML5xUAS luciferase reporter. The effect of various GSK expression vectors in the presence/absence of CREB, PKA and CRTC1 are shown. Luciferase activities were measured at 48 h post-transfection. Error bar represent the standard deviation of three independent experiments. Rlu: Relative luciferase activity.

Lower panel, Protein levels of all three GSK3β alongside the endogenous GSK3β level (control) in HEK293T cells were verified using Western blot analyses using anti- GSK3β antibody and anti-actin as control.
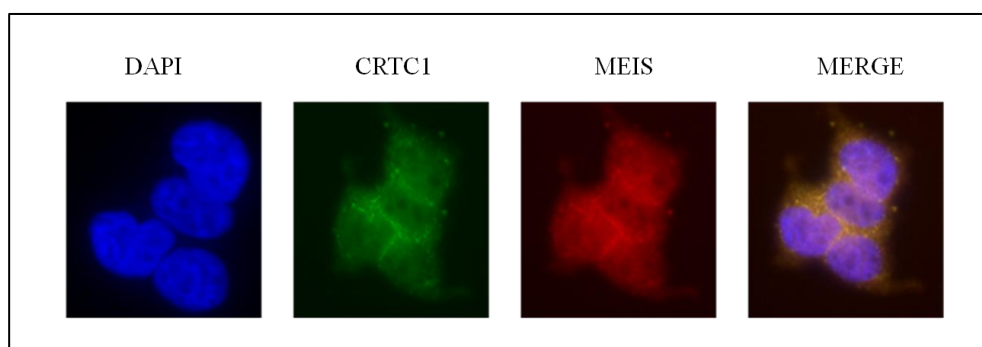
**Figure 10 Co-localization of endogenous CRTC1 and MEIS1/2/3 proteins in HEK293T cells**

Immunofluorescence assay showing cellular co-localization of endogenous CRTC1 and MEIS proteins in HEK293T cells with anti-CRTC1 and anti-MEIS1/2/3 antibodies. DAPI staining served to visualize nuclear staining.
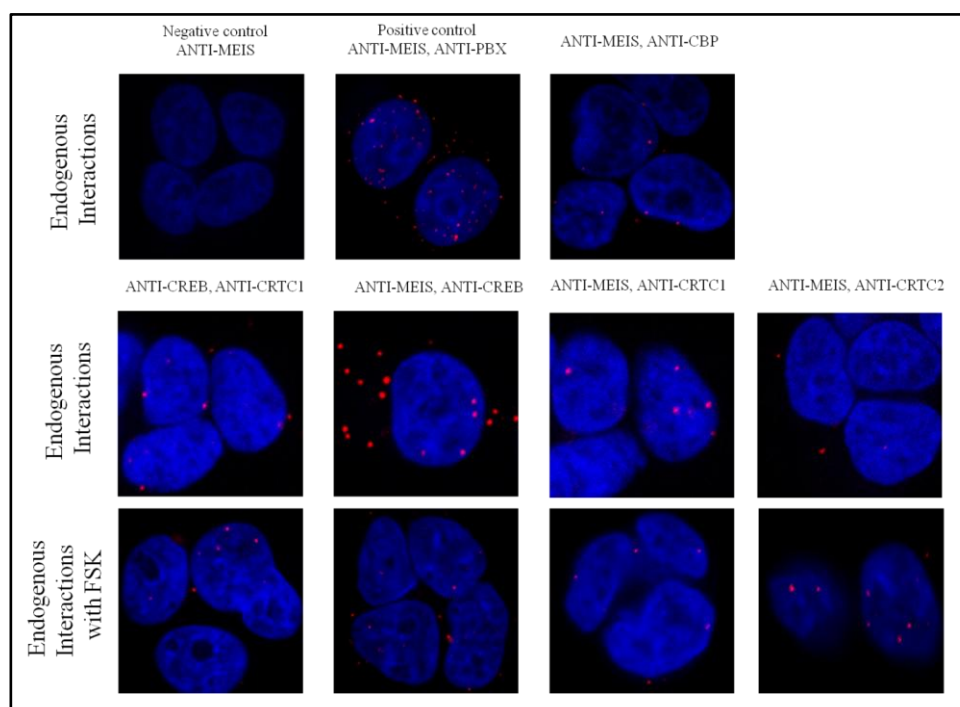
**Figure 11 Protein interactions in wild type and FSK stimulated HEK293T cells using PLA**

Confocal microscopy in combination with PLA, which detects protein-protein complexes, was used to explore interactions between MEIS, CREB, CRTC and CBP. Interactions between forskolin stimulated cells were indicated. A representation of the interactions are shown, where DAPI staining served to visualize nuclear staining.
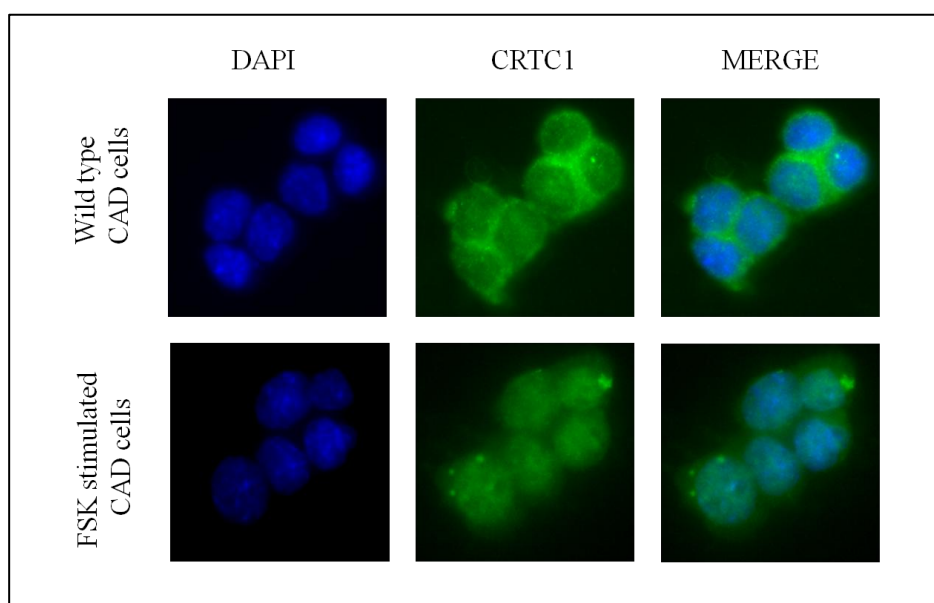
**Figure 12 Localization of endogenous CRTC1 in wild type and FSK stimulated CAD cells**

Immunofluorescence assay showing cellular localization of endogenous CRTC1 proteins in wild type CAD cells and forskolin (100um for 1 h) stimulated CAD cells using anti-CRTC1 antibodies. DAPI staining served to visualize nuclear staining.

## 2.14 **The role of the MEIS1A C-terminus in mediating protein interactions**

Various GFP-tagged MEIS1A constructs were made by me and as control, cells were transfected with GFP which showed an even distribution of the green fluorescent protein throughout the transfected cells. Expression o the full length GFP-tagged MEIS1A expression plasmid was included as a control with regards to a MEIS1A plasmid lacking the C-terminus MEIS1A (Δ 334). Nuclear expression of GFP-MEIS1A and GFP-MEIS1A (Δ 334) protein were observed (Figure 13).

Having distinguished the population of transfected HEK293T cells using the auto-fluorescence property of GFP, PLA interactions between endogenous protein and the GFP-tagged MEIS1A protein was subsequently identified (Figure 14). Having validated the expression of the plasmids, the role of MEIS1A and its C-terminus in mediating protein interactions between CREB, CRTC and CBP was further assessed using PLA. A similar set of assays was performed to investigate the role of the C-terminus in endogenous interactions with CRTC and CBP, as well as its role in mediating interactions between CREB-CBP and CBP-CRTC proteins (Figure 14). Interactions were detected in all GFP-tagged MEIS1A and GFP-tagged MEIS1A (Δ 334) expressing cells with CRTC1 and CBP. Endogenous interactions were also observed for CREB-CBP and CBP-CRTC1 within the GFP-tagged MEIS constructs. Given that interactions were observed in all scenarios, this set of PLA data thus cannot at this point shed much further light on the mode of interactions regarding the

154

MEIS1A C-terminus.
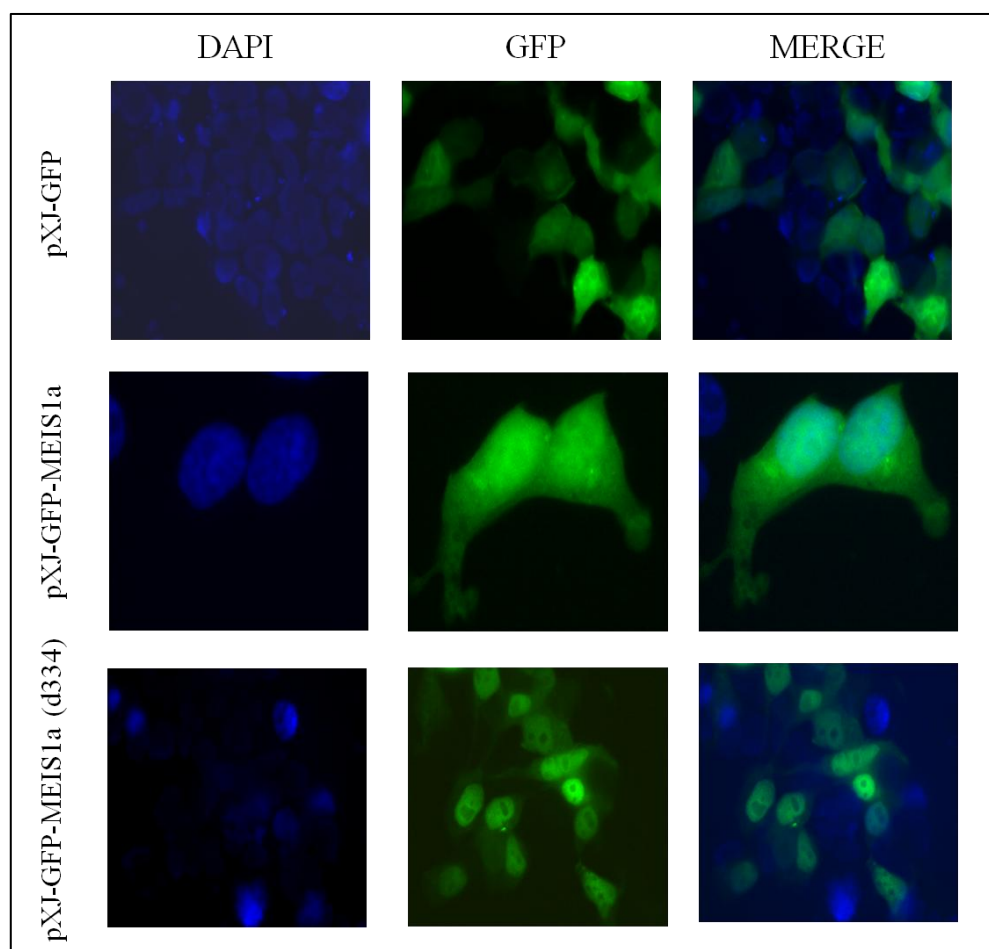


**Figure 13 Localization of GFP containing expression plasmids in HEK293T cells**

Cells were shown alongside GFP as control. Green signals correspond to the GFP expressing proteins and MERGE corresponds to an overlay with the nuclear DAPI stain. All images were captured at 63X magnification; image for pXJ-GFP-Meis1a was captured in 63X magnification and enlarged digitally for a clearer view.
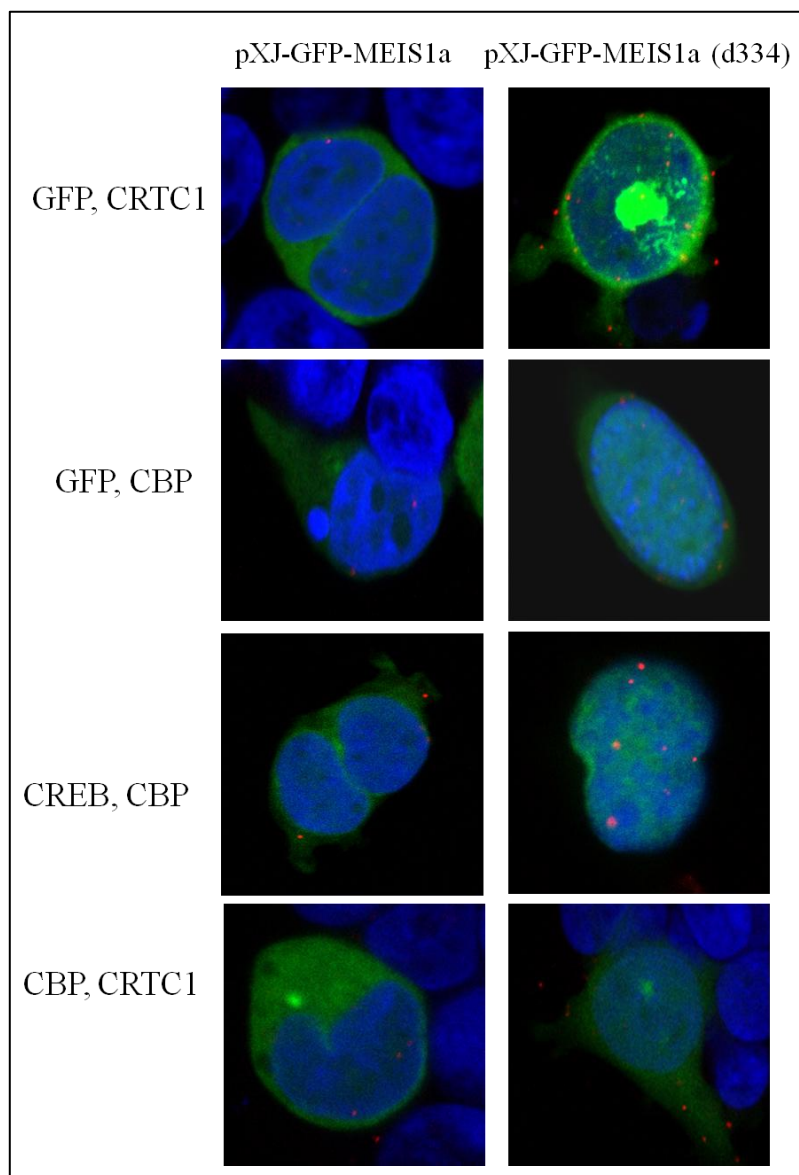
**Figure 14 Importance of the MEIS1A C-terminus for interaction with CRTC1**

A representation of interactions between MEIS1A and CRTC1, MEIS1A (Δ 334) and CRTC1 in HEK293T cells are shown.

Red signals indicate interactions; green signal indicates GFP-transfected hence MEIS transfected cells and DAPI staining served to visualize nuclear staining.

All images are taken at a magnification of 63X and enlarged digitally for a clearer view.

# 3   DISCUSSION

## 3.1   CRE reporter is modestly responsive to overexpressed CREB, but strongly responsive to PKA and CRTC1

In this chapter, I seek to further reveal the role of CREB in PKA- and CRTC-induced transcriptional activation by the MEIS1A C-terminus. Using dominant negative mutants of CREB: ACREB and ACREBR314A. The robust cAMP response element (CRE) luciferase reporter was first used to validate the effects of CREB, PKA and CRTC. As expected, a strong PKA response was detected at the CRE luciferase reporter (Figure1). Overexpressed CREB did not alter the activity at the CRE reporter in HEK293T cells in the presence or absence of PKA (Figure1). A possible explanation for that could be due to the low levels of endogenous PKA activity within HEK293T cells, leading to a moderately low activity seen upon overexpression of CREB, where the major portion of over-expressed CREB remains unphosphorylated.

Upon exogenous stimulus of PKA, high endogenous CREB within the HEK293T cells would be phosphorylated, bind to and saturate the binding sites, thus no significant level of increase in activity could be observed in the presence of exogenous CREB and PKA. Western blot with lysates from the various phosphorylated levels of endogenous and overexpressed CREB in the assays to determine their phosphorylated state at Serine133 (by PKA) could be used to verify this.

The CREB coactivator CRTC1 generated a strong response at the CRE-reporter when expressed alone (Figure 2). Thus, these tests validated the appropriate

responsiveness to PKA and CRTC1.

### 3.2 CREB mutants ACREB and ACREBR314A inhibit activation of a CRE reporter by PKA and CRTC1

Having validated the effect of CRE reporter at mediating the activities of CREB, PKA and CRTCs in my hands, I then moved on to determine the ability of the dominant negative constructs to inhibit these functions. Both dominant CREB mutants showed an inhibitory effect at the CRE reporter. ACREB was seen to have an inhibitory effect on the CRTC1 activation but not on PKA, while the ACREBR314A inhibited activation by both PKA and CRTC1 (Figure 2). Published data have indicated that ACREB heterodimerize with CREB in the nuclear extracts and prevented them from binding to the CRE probe. Thus, the presence of ACREB was expected to have an inhibitory effect for both PKA and CRTC at the CRE promoter. The inhibitory effect of ACREB in the presence of CRTC1 is evident; however the effect at PKA is not as distinct due to the large error bar in the lane for both ACREB and PKA. Another possibility why the inhibitory effect of ACREB is not as strong in the presence of PKA can be inferred from published studies, which have shown that heterodimerization of unphosphorylated CREB with ACREB triggers CREB protein degradation, whereas phosphorylation prevents CREB from such degradation. Thus, the presence of exogenous PKA act to stabilize phosphorylated CREB homodimer (though less as stable as the ACREB-CREB heterodimer).

Physical properties of the dominant negative constructs were confirmed using

co-immunoprecipitation assay, which demonstrated the expected physical interaction of both ACREB and ACREBR314A with wild type CREB (Figure 3).

### 3.3 CREB mediates PKA-responsiveness of the 5XUAS luciferase reporter

Results have suggested that CREB plays an important role in facilitating MEIS1A C-terminus transcriptional activity in response to PKA signaling. This prompted us to investigate the mechanism by which the MEIS1A C-terminus responds to PKA signaling via the 5XUAS luciferase reporter. Consistent to the CRE reporter activity, overexpression of wild type CREB341 only modestly increase the activity at the 5XUAS reporter in the absence or presence of PKA (Figure 4). A reason for this modest response of CREB observed at the CRE luciferase reporter is possibly due to an existing high level of endogenous CREB within the HEK293T cells (own observations).

### 3.4 ACREB and ACREBR314A strongly and non-specifically activated the 5XUAS luciferase reporter

Both the CREB mutants showed strong transcription activated at the MEIS1A C-terminus 5XUAS luciferase reporter (Figure 5). However, for both mutants strongly induction of was observed through a mutated MEIS1A C-terminus (GQWHYM) that is non-responsive to PKA (SL. Goh, data not shown) and CRTC1 (Huang et al, 2005) (Figure 5). Thus, no insights into the role of CREB can be obtained through the use of these dominant negative constructs.

159

### 3.5 Efficacy of CREB knockdown on CRE luciferase reporter

To confirm the contribution of CREB at MEIS1A transactivation, knockdown studies using shRNA vector against endogenous CREB were used. Having verified the efficacy of the knockdown, the effect of CREB depletion on CRE luciferase reporter was assessed (Figure 6). Functionality of CREB knockdown was validated on the CRE luciferase reporter, an impairment of PKA- and CRTC- mediated transcriptional activation was seen, though with modest effect (Figure 7). Such modest reduction in the activity at the CRE reporter upon CREB knockdown could be due to the functional compensatory effect of another CRE-binding protein the ATF family, which had been shown to compensate for CREB function *in vivo* (Hummler et al, 1994).

### 3.6 CREB knockdown impairs PKA-induced transcriptional activation through the MEIS1A C-terminus

The system was then tested using CREB-specific shRNA for the effect of endogenous CREB depletion on transcriptional activation through the MEIS1A C-terminus. The knockdown studies revealed that the transcriptional activation by the MEIS1A C-terminus in response to PKA is indeed dependent on CREB (Figure 8). However, the effect of CREB knockdown for CRTC1 at the MEIS1A C-terminus appeared weak (Figure 8).

This non-specificity could arise from an off-target effect or a specific effect on the expression of the GAL4/UAS reporter since this effect was not observed at the CRE luciferase reporter (Figure 7). Thus, the non-specific effect could possibly arise from the 5XUAS reporter. Indeed, various proteins have to

160

shown to interact with the GAL4 activation domain lending possibilities to the non-specific effect of the CREB mutants at the 5XUAS luciferase reporter (Traven et al, 2006).

## 3.7 No observable effect was observed for the serine/threonine kinase GSK3 at the MEIS1A C-terminus

No observable change in transcription at the MEIS1A C-terminus with GSK3 beta, a serine/threonine kinase which has been promoting the conditional association of CREB and its coactivators CRTC and CBP with MEIS1 despite attempts at verifying the identity and expression levels of all three proteins (Figure 9 Upper panel) (Wang et al, 2010b). A possible explanation for this discrepency which could arise form a difference in cell line tested: leukemia cell lines versus embryonic kidney cell line, an explanation to the different response.

## 3.8 Additional evidence of MEIS and CRTC interactions

The success in co-immunoprecipitation between MEIS1 and CRTC1 in the previous chapter prompted much testing on the effect of CRTC1 at the MEIS1 C-terminus. However, the depletion of CREB at the effect of CRTC1 at the MEIS1A C-terminus did not prove significant in this case. Despite this, further evidence of the cooperative activity between MEIS and CRTC was further supported by immunofluorescence staining, revealing a highly overlapping nuclear and cytoplasmic CRTC1 and MEIS1/2/3 (Figure 10).

### 3.9 **Validation of proximity ligation assay via CREB-CRTC interaction**

PLA was employed to test for novel protein interactions involving MEIS-CREB, MEIS-CRTC and MEIS-CBP. Controls were carefully verified prior to verifying the interactions (Figure 11). In addition to the positive and negative control, the known interaction between CREB-CRTC was also tested. As expected, interactions were observed between CREB and CRTC (Figure 11).

### 3.10 **Association of MEIS and CRTC using proximity ligation assay**

Interactions between MEIS and CRTC1, MEIS and CRTC2 were successfully detected by PLA as shown in a representative image in Figure 11 providing another independent support to the MEIS-CRTC interaction. However, no visible increase between endogenous MEIS1 and CRTC1 or CRTC2 interaction were observed in the nucleus upon stimulus treatment with forskolin in HEK293T cells. When tested on other cell lines for the effect of forskolin, endogenous nuclear CRTC1 was successfully detected in CAD neuroblastoma cells, validating the efficacy of the FSK used as well as the technique used (Figure 12).

### 3.11 **Association of MEIS and CBP using proximity ligation assay**

Previous observation has established that the the role of CREB in regulating MEIS1A C-terminus activity is dependent on its association with CRTC as well as CBP (Choe et al, 2009). Supporting this, PLA interactions have identified interaction between endogenous MEIS and CBP in HEK293T cells (Figure 11).

Consistent with its inability to promote nuclear CRTC in HEK293T in my hands, no noticeable increase in interactions were observed upon FSK stimulus (Figure 11).

### 3.12  Association of MEIS andCREB using proximity ligation assay

Previous observation by the lab using CREB point mutation mutants CREBR314A and CREBS133A established that the the role of CREB in regulating MEIS1A C-terminus activity (Screaton et al 2004, Huang et al 2005). Fortifying the role of CREB on MEIS1A activity, association between endogenous MEIS and CREB proteins by proximity ligation assay (PLA) was shown (Figure 11), coinciding with a recent paper demonstrating an interaction between endogenous MEIS and CREB (Wang et al, 2010b).

### 3.13  The role of MEIS1A C-terminus at mediating protein interactions

GFP-tagged constructs of full length MEIS1A and MEIS1A (Δ334) lacking its C-terminus were generated, GFP provided a simple and direct means to identify MEIS1A transfected cells (Figure 13). The role of MEIS1A and its C-terminus at mediating protein interactions between CREB, CRTC and CBP was then identified from the MEIS1A transfected GFP expressing cells (Figure 14). It would be of interest to determine if the C-terminus of MEIS1A is required to mediate CREB-CRTC, CREB-CBP and CRTC-CBP interaction.

Previous observations by the Featherstone's lab have shown that CREBS133A is able to hinder the MEIS1A C-terminus response to PKA signaling. When co-

expressed with CRTC1, CREBR314A has been shown to block transcription by the MEIS1A C-terminus, suggesting a crucial role of the CREB-CRTC interaction in stimulating transcriptional activity by the MEIS1A C-terminus. Phosphorylation of CREB at position 133 has been shown to promote association with its co-activator CBP (Chrivia et al 1993). In addition, without phosphorylation by PKA and thus unable to bind CBP, CREBS133A impeded the CRTC1 mediated MEIS1A C-terminal activity. These results suggest an involvement of MEIS1A C-terminus in mediating interactions between CREB, CRTC and CBP interactions.

However, no conclusion could be drawn with the C-terminal deleted MEIS1A (Δ334) mutant where interactions were seen in all GFP-tagged assays even in the absence of the C-terminus. Some explanations could involve the possibility of the CRTC1 interaction domain on MEIS1A, which was hinted to reside within the MEIS1A C-terminus and hence removed in the MEIS1A (Δ334) mutant, the interactions visualized could hence be mediated instead by the homodimer formation of MEIS1A mutant with the endogenous MEIS1A. Another possibly reason could suggest that the interacting region of MEIS1A with the examined proteins as well as heterodimerization ability require other domains apart from the C-terminus, thus explaining the interaction seen within the MEIS1A(Δ334) overexpressed cells.

# CHAPTER 5

# TO DETERMINE THE NATURE OF THE MEIS-CRTC-CREB COMPLEX AND THE STRUCTURAL CHANGES ACCOMPANYING THE INTERACTIONS

## 1 ABSTRACT

We attempted to purify MEIS1A in significant quantities in order to determine its structure and to ascertain whether interactions with CRTC and CREB are direct. Despite numerous attempts using bacterial and baculoviral systems, as well as a collaboration with a protein platform facilities, success was limited. Even truncated forms of the proteins showed proved largely intractable, with both the quantity and quality of pure proteins not meeting the requirements for structural studies.

The furthest progress was obtained with PREP2, a TALE class homeodomain-containing protein with homology at the N-terminal regions to MEIS1. Protein purification yielded high quantities of a soluble PREP2 (49-230) fragment consisting of the N-terminal regions homologous to MEIS. However, the presence of a heterogeneous population of conformers within the purified samples again thwarted structural determination.

## 2  RESULTS

To ascertain the direct interactions between MEIS1A, CRTC and CREB and to determine the structure of MEIS1A and CRTC, pure proteins are required. Bacterial overexpression of His-tagged proteins were induced and examined by SDS-PAGE as well as in western blot prior to purification via chromatographic techniques. Protein expression and purification was performed in collaboration with the Gruber lab, NTU. Generous expertise and advice were offered by Dr Gerhard Gruber as well as lab member Dr Conelia Hunke.

## 2.1   Expression of MEIS1A, CREB341 and Flag-CRTC1 proteins

Coding regions for MEIS1A, CRTC1 and CREB341 were amplified by PCR and cloned into the bacterial vector pET15b and pSUMO both carrying a 6XHis tag. Bacteria (BL21 strain) harboring these vector were grown in LB broth to OD 600 between 0.4-0.6 at 37 °C. IPTG was then used to induce expression. Figure 1 shows the expression profile of MEIS1A protein where control indicates the expression of MEIS1A at time zero versus an IPTG-induced MEIS1A culture after 2.5 h. Western blotting for MEIS1A detected a dominant band (arrow) alongside several weaker and non-specific bands (Figure 1).

Figures 2 and 3 show the expression profile of CREB and CRTC1 upon IPTG induction. A strong band of CREB protein was seen following SDS-PAGE which correlates with a clean specific band (arrow) in the western blot (Figure 2).  No obvious Flag-CRTC1 protein band could be detected in the SDS-PAGE and no distinct band was detected in the western blot probing for the Flag-tagged protein (Figure 3). Instead, a series of smaller bands were detected with

the Flag-tagged antibody (Figure 3, right panel).

The induced cell lysate consists of two fractions, the soluble proteins as well as the insoluble proteins. Following centrifugation, the insoluble fraction resides within the pellet while the supernatant contains the soluble fraction. Only the soluble fraction is suitable for further purification. As such, a solubility test was conducted where the solubility of the protein can be used to determine the maximal soluble protein possible under various buffer conditions. One fifth the amount of pellet was loaded for comparison with the supernatant. As visualized by SDS–PAGE, the proportion of protein within the supernatant, an indication of the soluble fraction of the expressed protein, was highest under buffer condition of 50 mM Tris, 200 mM NaCl at pH 8.5 (Figure 4).
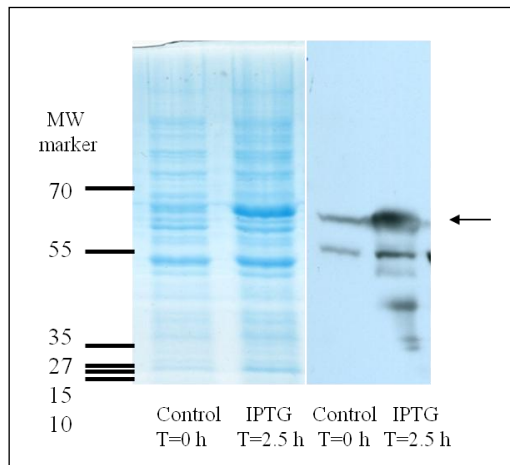


**Figure 1 Protein expression for MEIS1A protein**

The SDS-PAGE (left panel) and Western Blot (right panel) showed a visible induction of MEIS1A protein after 2.5 h of 1 mM IPTG induction. Protein identity and integrity were verified with anti-MEIS1/2/3 antibody.

Control   IPTG    Control  IPTG
T=0 h   T=2.5 h   T=0 h  T=2.5 h
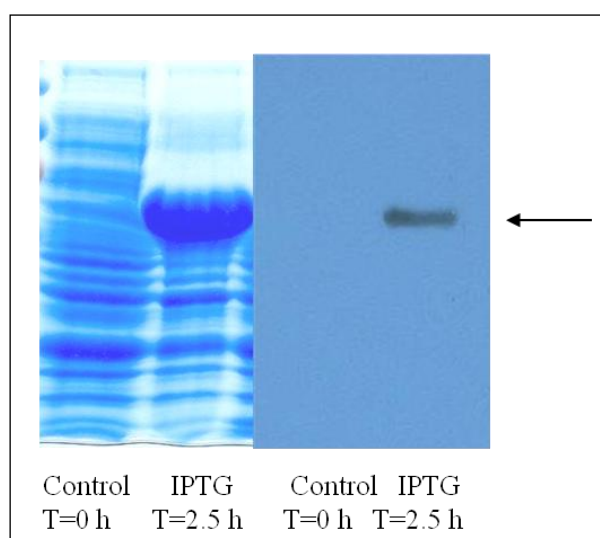
**Figure 2 Protein expression for CREB protein**

The SDS-PAGE (left panel) and Western Blot (right panel) showed a visible induction of CREB protein after 2.5 h of 1 mM IPTG induction. Protein identity and integrity were verified with anti-CREB antibody**.**
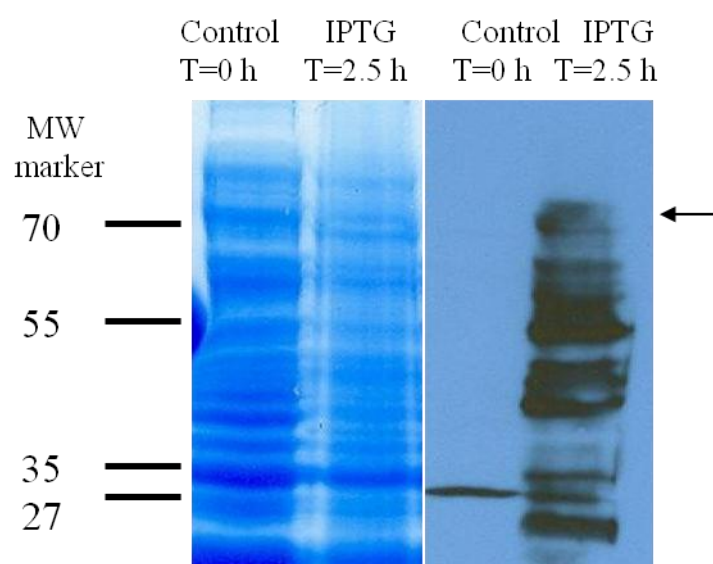


**Figure 3 Protein expression for Flag-CRTC1 protein**

The SDS-PAGE (left panel) and Western Blot (right panel) showed a visible induction of Flag-CRTC1 protein after 2.5 h of 1 mM IPTG induction. Protein identity and integrity were verified using an anti-Flag antibody.
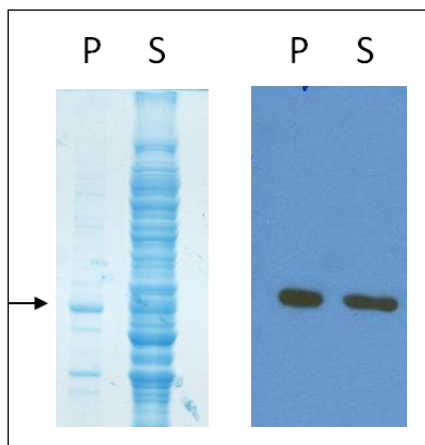
**Figure 4 Solubility test for MEIS1A protein**

SDS-PAGE (left panel) shows the solubility of MEIS1A protein at buffer conditions of 50 mM Tris, 200 mM NaCl, pH 8.5. Identity and integrity of the protein were verified in Western Blot using anti-MEIS1/2/3 antibody (right panel). The samples were re-suspended, P is a representation of 2 ul of the re-resuspended sample whilst S is a representation of 10 ul of the re-resuspended (P: Pellet; S: Supernatant).

## 2.2 Purification of MEIS1A protein

The recombinant pET15b-MEIS1A vector possessed six histidine-tags at the N-terminus of pET15b which binds to Nickel tagged resins, thereby allowing purification of the tagged protein. Lysates from the induced MEIS1A protein were incubated with Ni2+-NTA resin column, proteins were then purified using an Imidazole-gradient. Untagged proteins that do not specifically interact with nickel ion were first eluded with low concentration of Imidazole. Tagged-proteins of interest were usually eluted with 150 to 300 mM of Imidazole. Eluded samples were concentrated and loaded on a SDS-PAGE according to the eluded Imidazole concentration. Pellet represents the insoluble fraction of

MEIS1A protein and flow through indicates the proteins unbound to the Nickel resins. Optimal elution of soluble MEIS1A was found between 100-200 mM Imidazole (Figure 5). The purity and amount of protein can be assessed by SDS-PAGE and Western blotting. Imidazole concentration labeled blue were pooled and subjected to further purification steps.

### 2.3    Size exclusion chromatography purification of MEIS1A

Proteins from the elution (100-200 mM Imidazole) as detected from the SDS-PAGE in Figure 5 were pooled together and applied to either a size exclusion column (Superdex 75, 200) or ion exchange column for further purification *via* size or charge respectively. The Superdex columns were pre-packed glass columns for size fractionation and analysis of proteins to check for protein homogeneity. The optimum separation size for Superdex 75 is 3-70 kD while the Superdex 200 is 10-600 kD. With a protein size of 45 kD, both the columns were appropriate and hence used for MEIS1A purification. Chromatogram of MEIS1A proteins off the nickel column applied to Superdex 75 revealed 4 peaks, each with a different quantity of protein and with peak 1 containing most of the protein (Figure 6). However, the chromatogram from Superdex 200 revealed 4 peaks with a thin protein band detected only in peak 2 (Figure 7).
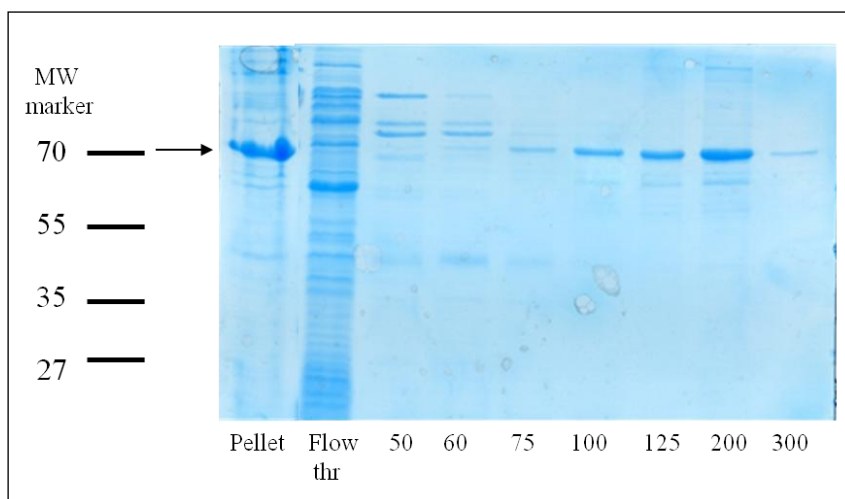
**Figure 5 Nickel-NTA purification of MEIS1A protein**

SDS-PAGE showed the elution pattern of MEIS1A protein at a gradient of increasing Imidazole. The insoluble fraction of MEIS1A protein and flow through indicated in the pellet lane represents the proteins unbound to the Nickel resins. Optimal elution of soluble MEIS1A was found between 100-200 mM Imidazole. Imidazole concentration labeled blue were pooled and subjected to further purification steps.
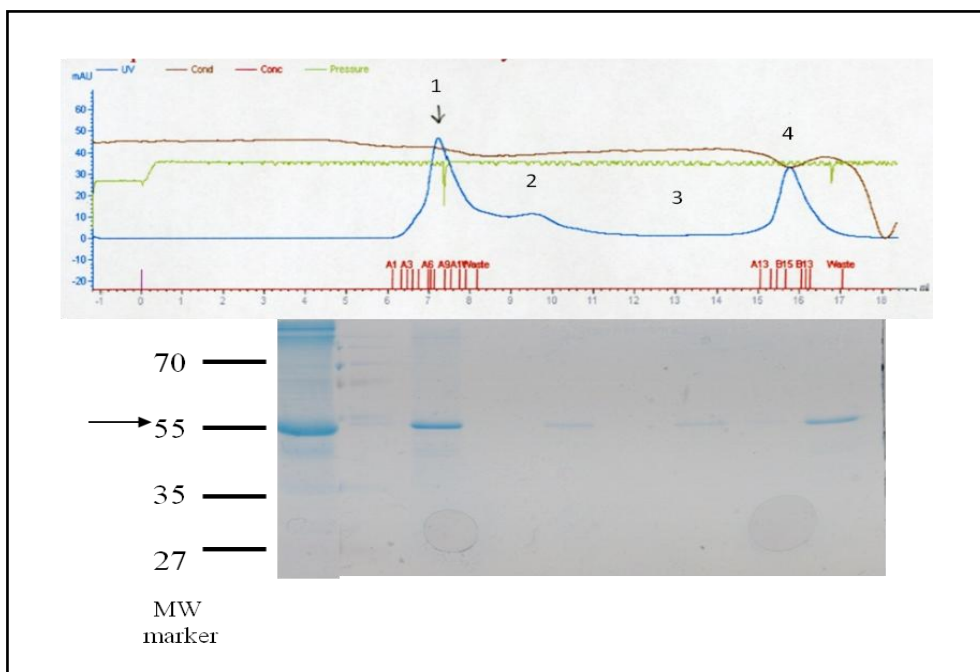
**Figure 6 Protein purification of MEIS1A via size exclusion column Superdex 75**

Chromatogram off the size exclusion column (Superdex 75) where the number 1 and 4 represent the 2 prominent elution peaks (Top panel). Samples from Peaks 1 to 4 were concentrated and ran on the SDS-PAGE (Lower panel). Sample represents the loading MEIS1A proteins off the Nickel column. Buffer conditions: 50 mM Tris, 200 mM NaCl, pH 8.5, 10 mM EDTA.
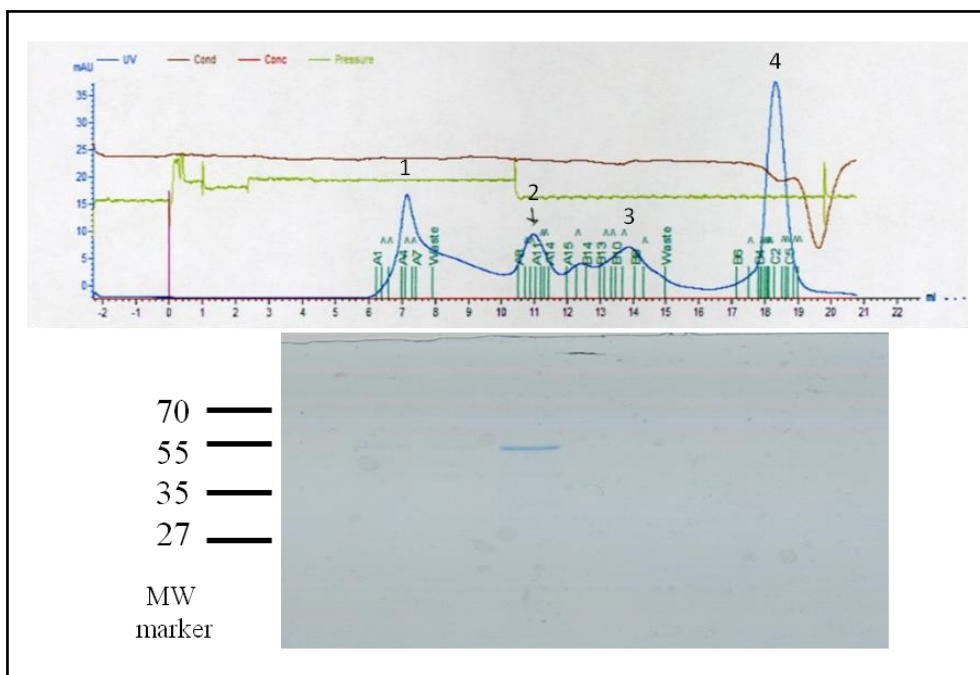
**Figure 7 Protein purification of MEIS1A via size exclusion column Superdex 200**

Chromatogram off the size exclusion column (Superdex 200) where the number 2 represent the prominent elution peak (Top panel). Samples from Peaks 1 to 4 were concentrated and ran on the SDS-PAGE (Lower panel). Sample represents the loading MEIS1A proteins off the Nickel column. Buffer conditions: 50 mM Tris, 200 mM NaCl, pH 8.5, 10 mM EDTA.

2.4 **Ion exchange chromatography purification of MEIS1A protein**

Concurrently, proteins eluded from Imidazole gradient were pooled and applied to an ion exchange chromatography column. Ion exchange chromatography is often use for the separation and purification of proteins on the basis of charge and it works on the basis that charged amino acids on the surface of a protein binds oppositely charged ligands of the ion exchanger. An anion exchanger (Resource Q) with a pH range from 6.5 to 9 was used where negatively charged

molecules were attracted to a positively charged solid support. When injected into the column, MEIS1A proteins with a pI of 6.13 were thus adsorbed on the support. A gradient of increasing salt concentration was then applied to elute the sample from the column. The elution profile can be divided into 4 peaks in which MEIS1A protein at salt concentrations of 19 % and 22.5 % was seen as thin bands by Coomassie staining (Figure 8).
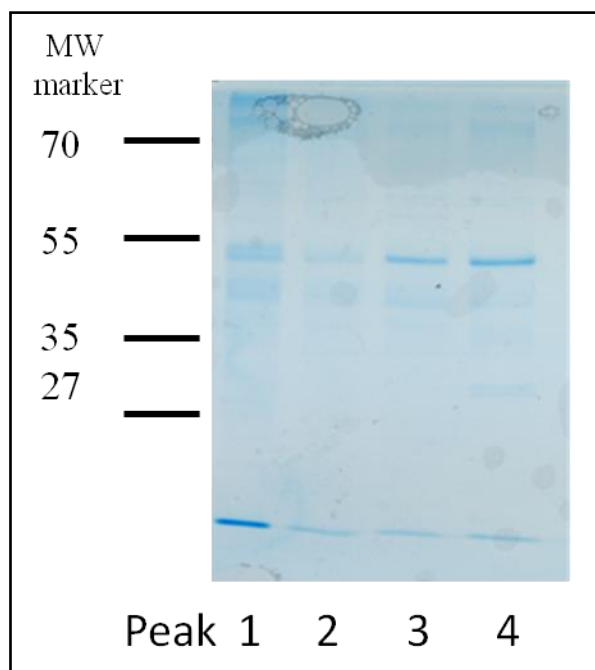


**Figure 8 Protein purification of MEIS1A via ion-exchanger**

SDS-PAGE showed the elution pattern of MEIS1A protein. Elution profile can be divided into 4 peaks, the quality and quantity of each elution peak can be seen in the SDS-PAGE. MEIS1A proteins were eluded at peak 3 and peak 4. Buffer conditions: 50 mM Tris, 200 mM NaCl, pH 8.5, 10 mM EDTA.

## 2.5 Baculovirus expression of Flag-CRTC1 protein

The baculovirus expression system has been shown to provide high levels of properly folded post-translationally modified, active and functional recombinant proteins. Given the poor protein expression of CRTC1 in bacterial culture, the baculovirus expression system was used to express CRTC1 protein for large scale expression of the recombinant protein of Flag-CRTC1. The expression of Flag-CRTC1 as recombinant baculovirus was verified in sf9 insect cells by immunoflorescence using anti-flag and anti-CRTC1 antibody. Figure 9 showed the successful expression of Flag-tagged CRTC1 in the otherwise CRTC null insect cells after six rounds of viral amplification. The identity of Flag-CRTC1 was also verified by Western blot (Figure 10). Given the low titer count of the baculovirus stock, insect cells were re-infected several more rounds. However, upon further amplification, SDS-page still failed to show any observable band of induction. SDS-PAGE and the Western blot showed Flag-tagged proteins of various smaller sizes suggestive of cleavage of the Flag-tagged protein at the C-terminus since the CRTC1 harbors an N-terminus Flag-tag (Figure 11).

## 2.6 Expression of Flag-CRTC1 (1-290) protein

Due to the lack of pure CRTC1 protein expressed at higher titer count in insect cells, truncated versions of the CRTC1 protein were constructed and expressed according to the binding studies with MEIS1A in Chapter 3. As seen in Figure 12, the CRTC1 (1-290) consisting of the N-terminal minimal region of CRTC1

was sufficient and able to retain binding to MEIS1A. Thus, a pSUMO-Flag-CRTC1 (1-290) vector, also harboring an N-terminus 6xHis-tag was generated and expressed in BL21 cells. However, the expression profile of the truncated version of CRTC1 revealed persistent problems with expression level and multiple cleavage products (Figure 13).
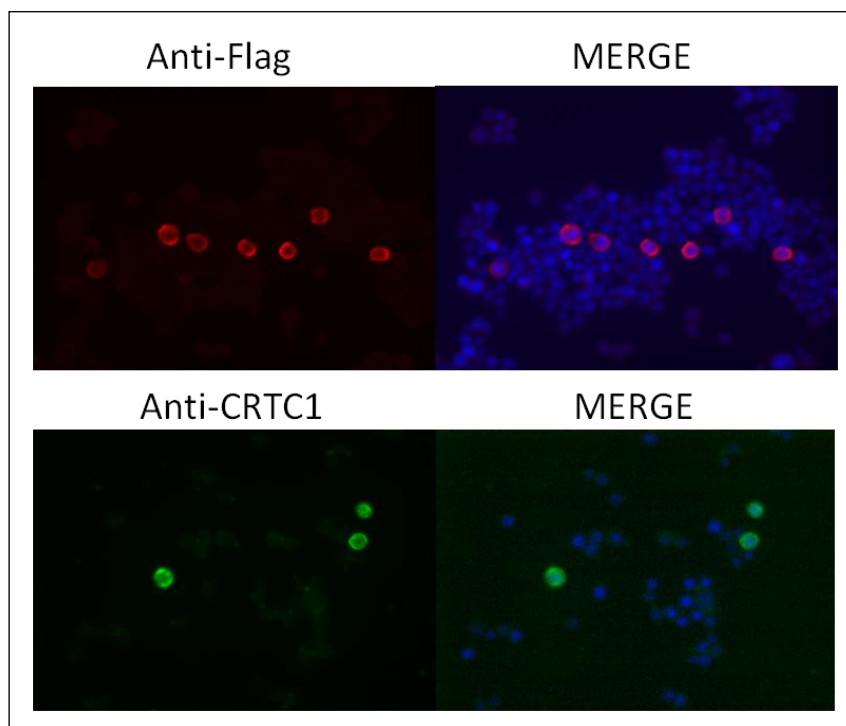


**Figure 9 Baculovirus expression of Flag-CRTC1 protein in sf9 cells**

Overexpressed Flag-tagged CRTC1 proteins in sf9 were shown after the 6th baculoviral infection. Red signals correspond to Flag-tagged CRTC1 expressing sf9 cells and MERGE corresponds to an overlay with the nuclear DAPI stain. Green signals correspond to CRTC1 expressing sf9 cells and MERGE corresponds to an overlay with the nuclear DAPI stain.
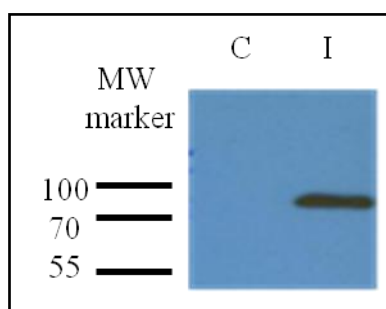
**Figure 10 Detection of Flag-CRTC1 protein identity and levels in induced sf9 cells after 6 rounds of infection**

Western Blotting using anti-Flag antibody detection of Flag-CRTC1 induced sf9 cells after six rounds of amplification C: control, I: Baculovirus induction of Flag-CRTC1 protein expression.
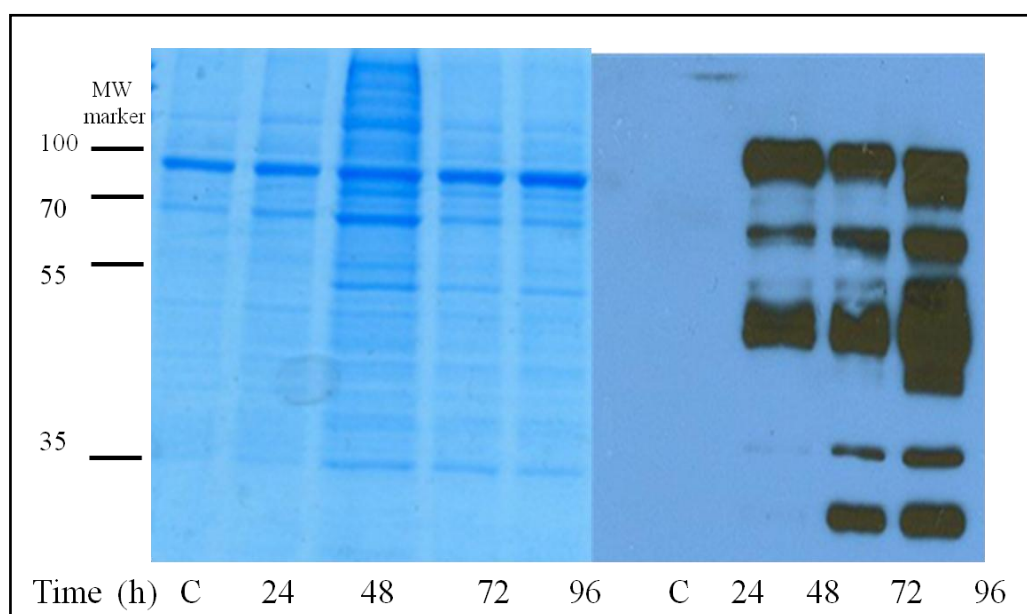


**Figure 11 Detection of Flag-CRTC1 protein identity and levels in induced sf9 cells after 12 rounds of infection**

SDS-PAGE and Western Blotting detection of Flag-CRTC1 induced sf9 cells after twelve rounds of amplification C: control, 24 h, 48 h, 72 h, 96 h: Detection of baculovirus induction timing post infection.

**Figure 12 Binding of MEIS1A with various truncations of Flag-CRTC1**

Schematic diagram of various CRTC1 mutants and their binding to MEIS1A. The plus and minus signs below Binding to MEIS1A correlate with the extent of binding of MEIS1A by the various CRTC1 mutants.
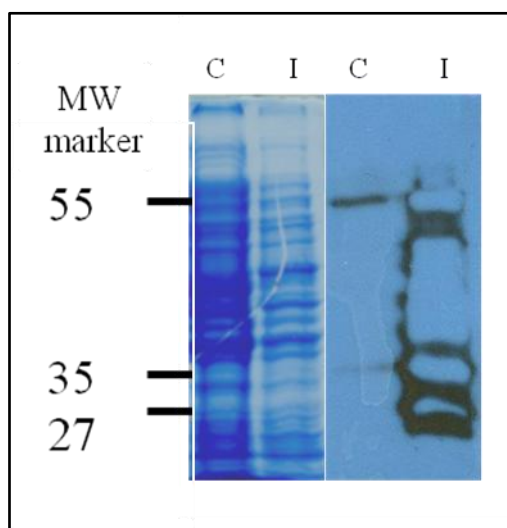


**Figure 13 Expression profile of Flag-CRTC1 (1-290) in HEK293T cells**

The SDS-PAGE (left panel) and Western Blot (right panel) showed a visible induction of Flag-CRTC1 (1-290) protein after 2.5 h of 1mM IPTG induction in LB broth. Protein levels were detected and verified with Anti-Flag antibody. C: control, I: IPTG induced.

# 3   DISCUSSION

## 3.1   Expression of MEIS1A, CREB341, Flag-CRTC1 proteins

To identify the mode of interaction between MEIS1A, CRTC and CREB proteins and to ultimately reveal their structures, proteins were expressed in bacterial strains (Figure 1, 2 and 3). Given that the structure of CREB has been known, effort was focused on MEIS1A and CRTC1 with no known structures (Chrivia et al, 1993; Gonzalez et al, 1991; Parker et al, 1996; Radhakrishnan et al, 1997). Protein expression level of MEIS1A appeared promising with a proportion of soluble proteins (Figure 1 and 4) while no distinct expression of Flag-CRTC1 could be detected (Figure 3).

## 3.2   Purification of MEIS1A protein

The histidine-tagged MEIS1A proteins were further purified using Nickle column (Figure 5) followed by size exclusion chromatography or Ion exchange chromatography to separate the pure MEIS1A proteins from other contaminating proteins. Following chromatography purification, the expressed MEIS1A proteins appeared to be significantly purer; however the quantity is too low for any structural determination (Figure 6 to 8).

## 3.3   Baculovirus expression of Flag-CRTC1 protein

The initial baculoviral expression of Flag-tagged CRTC1 appeared promising during infection in the insect cells (Figure 9 and 10). However, upon further re-infection, the same problem surfaced in that no significant nor pure CRTC1

band could be detected (Figure 11).

One alternative would be to attempt to recover the insoluble CRTC1 protein.

The multiple bands seen on SDS-PAGE and Western blot could arise from partial degradation of fusion proteins by proteases, or denaturation due to over-sonication. Since protease inhibitor has been added to the lysis solution, addition of lysozyme and a care to reduce frothing during sonication may also improve results. Increase flow rates may also reduce degradation and denaturation of protein. Another approach could be to try to tag the protein at the C-terminus instead to remove the possibility of degradation/cleavage occurring from the C-terminal end.

### 3.4 Expression of Flag-CRTC1 (1-290) protein

A third attempt was made at obtaining decent protein levels by using a truncated CRTC1 consisting of only the N-terminal 1-290 amino acids which have been shown in our binding studies (Figure 12) to be sufficient for binding to MEIS1A. However the same outcome persisted with various bands of truncated proteins products (Figure 13).

# CHAPTER 6

# GENERAL DISCUSSION

MEIS1, a cofactor of HOX and PBX proteins has been implicated in embryonic patterning and oncogenesis. Previous studies by our lab established that MEIS1A harbors a C-terminal transcriptional activation domain that responds to PKA signaling. Supporting studies have also mapped a conserved transcriptional function to the C-terminus of MEIS1A which is required for accelerating leukemogenesis, particularly in collaboration with HOXA9 (Mamo et al, 2006; Wang et al, 2005). In Chapter 3, I discussed how the mechanism behind the transcriptional activity of MEIS1A at the C-terminus involves the CREB coactivator CRTCs. Chapter 4 assessed the effects of CREB using CREB mutants, CBP and other protein interactions at the MEIS1A C-terminus. Finally in Chapter 5 I discussed my attempts to determine the nature of the MEIS-CRTC-CREB complex in order to reveal the structural changes accompanying their interactions. This thesis thus describes the mechanism by which the MEIS1A C-terminus achieves its transcriptional function as well as elucidates its interactions involving CREB and its co-activators CRTC. In this chapter, I present the role of phosphorylation in the regulation of CREB, CRTC and MEIS proteins. I also provide preliminary data for cooperative interaction of these proteins *in vivo* and *in vitro*. In addition, I propose experiments to further understand the role of MEIS1, CRTC and CREB in transcriptional regulation and in developmental and oncogenic processes.

# 1 Role of phosphorylation in the regulation of CREB, CRTC and MEIS proteins

Protein kinase A (PKA) has been known to phosphorylate CREB at Serine 133 and promote interaction with its coactivator CBP. Recently, a new role for PKA-mediated CREB activation has been identified (Bittinger et al, 2004; Conkright et al, 2003; Iourgenko et al, 2003; Screaton et al, 2004). In this pathway, the CRTC family has been shown to be directed from the cytoplasm into the nucleus via PKA or calcium signaling. Once in the nucleus, CRTC interacts with CREB in a phosphorylation-independent manner. This interaction between CRTC and CREB acts to stabilize CREB in at least two ways. Firstly, CRTC stabilizes the binding of phosphorylated CREB and CBP, an important role for the transcriptional activation by CREB in response to PKA (Ravnskjaer et al, 2007). Secondly, CRTC has also been shown to stabilize the DNA binding ability of CREB (Wang et al, 2010a).

In addition, deregulation of PKA and CREB have both been implicated in human and rodent cancers (Kinjo et al, 2005; Rosenberg et al, 2002; Shankar et al, 2005a; Shankar et al, 2005b; Shankar & Sakamoto, 2004). PKA phosphorylation sites within PBX have been shown to induce nuclear localization (Kilstrup-Nielsen et al, 2003). In addition, evidence of phosphorylation in various HOX functions have been reported (Bei et al, 2005; Eklund et al, 2000; Vijapurkar et al, 2004; Yaron et al, 2001). While no known PKA sites reside within MEIS, data by our lab showed a highly overlapped localization between MEIS and CRTC upon Forskolin treatment. In addition,

data from our lab showed a doublet in the western blot of MEIS1A further hinting at the possibility of a phosphorylated form of MEIS1A protein. Taken together with the collective phosphorylation information of its partners, MEIS may thus be the substrate of an intermediate kinase that is responsive to PKA which might be responsible for regulating nuclear localization of MEIS1. Phosphorylation is the most widespread form of post translational modification and usually occurs on serine, threonine and tyrosine residues in eukaryotic proteins (Ciesla et al, 2011). To validate the modification status of MEIS, phospho-specific (anti-phosphoserine, anti-phosphothreonine, anti-phosphotyrosine) antibodies can be tested on immunoprecipitated MEIS1 as well as forskolin stimulated MEIS1A. A kinase assay can also be used to determine the phosphorylation state on purified MES1A protein. Having identified the phosphorylation residues, PLA can be used once more to verify whether its nuclear localization activity of MEIS correlates with its phosphorylation state.

## 2   Examination of the nature of interactions between MEIS and CRTC in zebrafish development

Collective findings by my lab as well as in this thesis provided evidence of the physical and functional association between MEIS and CRTC. Using the zebrafish system, I have begun exploring the physiological as well as developmental impact of both MEIS and CRTC interactions. Using probes designed against zebrafish *crtc* (named: *crtc1* and *crtc1b*), which most closely

resemble human CRTC1 and mouse *Crtc1*; I seek to determine the expression of *crtc* during early zebrafish development. Zygotic transcription of *crtc1b* is low at 12 hpf, peaks at 24 hpf and maintains at 48 hpf (Appendix 8). What is significant is that the domains of expression of *crtc1b* overlap the known expression domain of *meis1.1*, an ortholog of the *Meis1* (Appendix 6 and data from zfin). The overlapping regions include the eye, the tectum and the hindbrain, all which are known to be patterned by *Meis* family members (Bessa et al, 2008; Choe et al, 2002; Heine et al, 2008; Hisa et al, 2004; Stedman et al, 2009; Vlachakis et al, 2001; Zhang et al, 2002). Preliminary evidence for coexpression of *crtc1b* and *meis1.1* is thus consistent for their concerted action in patterning of these tissues. Studies of *crtc* expression during early zebrafish development will be completed with a set of expression data ranging from as early as 20 min post fertilization up to 48 hpf, with complements and comparison to the expression of *meis1.1*.

In addition, the shared and concerted action of *crtc1* and *meis1.1* could be further tested using complementary knockdown approaches. Morpholinos (MO) directed against splice donors and acceptors could be designed to knock down the *crtc* and *meis* genes. First, levels of MO against *meis1.1* could be reduced down to a concentration at which no abnormalities could be detected. Using this concentration of *meis1.1* MO, *crtc1* MO would then be injected. By reducing the *meis1.1* expression to a minimum during development, it would not be able to tolerate any more reduction in its interacting proteins. Thus, should *meis* and *crtc* work together as partners in development, the knockdown of *crtc* at the

minimum required level of *meis* should lead to defects during the zebrafish development. Such defects could be detected visually or assessed using genetic markers against specific organs patterned by *meis* and *crtc* (eyes, hindbrain, tectum).

# 3 Examination the role of MEIS and CRTC proteins in oncogenic processes

Given the recent demonstration of high levels of *Meis* expression in neuroblastoma cell lines, as well as our physical and functional association of MEIS and CRTC, it would be of interest to investigate the contribution of MEIS and CRTC in the context of neuroblastoma. I have previously detected high levels of endogenous *Meis1*, *Crtc2* and *Crtc3* expression in the two neuroblastoma cell lines N2A and CAD (Appendix 9). Having established the expression of *Meis* and *Crtc2* and *Crtc3* in the two neuroblastoma cell lines, future work could head in the direction of determining a concerted action of MEIS and CRTC in neuroblastoma phenotype. Such work could involve dominant negative constructs which will then be used to screen for oncogenic phenotype such as migration assay, growth rate and tumor formation. In addition, RT-PCR could be used to assess the change in levels of certain key regulators, which have also been identified as MEIS1 targets.

# 4   The nature of interactions between MEIS, CRTC and CREB

We have had some successful in bringing to light the physical and functional interactions between MEIS and CRTC, however, no structures for MEIS beyond the homedomain and no structure for CRTC have been determined. As such, it is our goal to understand the molecular conformations that allow MEIS, CRTC and their partners to interact to perform their function in development as well as in cancer.

Several assays could be applied to verify the interactions between MEIS, CRTC, CREB and CBP complex. Crosslinking assays in combination with a pull-down assay could be applied to verify the transient protein interactions as well as to identify novel protein proteins. Other attempts to assess protein interactions could be the use of sucrose density gradients, in which the semi pure proteins were quantified in each fraction by mass spectrometry. Given that proteins in the same complex should generally co-sediment, physically associated proteins should contain correlated elution profiles. Gel staining and western blot detection could then be used to assess the identity as well as the ratio of each protein in the complex.

As terminal tags can become buried/blocked and inaccessible within the protein, future possible work could involve another collaboration with the SBS/NTU protein platform facility located at A*STAR, Biopolis to generate other batches of bacterially expressing vectors of different N- and C-terminal His-tag fusions with the aim to obtain highest overall yield. In addition, a series

of truncated proteins could be assessed in parallel in the hope of reduce the incidence of proteolytic cleavage as well as optimising the levels of protein expression for protein crystallization. Once the proteins are successfully purified and optimised, physical characterization of MEIS and CRTC would be assessed by using one-dimensional NMR and circular diochroism, which would provide the first information of the folded stated of the proteins. Small angle X-ray scattering (SAXS) in solution would also provide valuable information on the protein's propensity for homodimerization as well as formation of higher order oligomers. The crystal structure of MEIS and CRTC at high resolution would be useful to determine the structural basis for their interactions on and off DNA and help promote our understanding of transcriptional regulation, development and oncogenesis.

# 5   A word about the Appendix

The results chapters preceding the appendix have established a strong association for functional and physical interactions between MEIS1A, CREB and CRTCs. By means of *in-situ* hybridization and RT-PCR, part of the appendix seeks to further refine this association. Data obtained has further revealed multiple domains of overlapping expression between *zfcrtc1b* and *meis1.1* present at the same developmental stages. This is consistent with the suggestion of a shared role in patterning during embryogenesis, at least within the tissues identified. Endogenous MEIS, CRTC2 and CRTC3 proteins were detected in neuroblastoma cell lines which provided a promising future direction for their cooperative effect in oncogenesis. In addition data generated by the protein platform facility as well as my work on determining the homogeneity of PREP2 at the zetasizer can also serve to build upon future work.

## PERSPECTIVE

The broad and cross species expression of MEIS, CREB and CRTC family members and their control of HOX expression and function, combined with numerous roles for PKA in cellular and developmental processes suggest that the functions of these factors will converge in many such events. In conclusion, our work thus points towards both functional and physical evidence implicating the PKA-dependent complex formation of MEIS1A C-terminus with the cAMP-inducible transcription factor CREB and its coactivator CRTC. Refining the initial view in which MEIS1A mediates its oncogenicity by interaction of its C-terminus with CREB and CRTC.

## SCIENTIFIC CONTRIBUTION

During the course of this PhD program, a scientific publication was released by the Featherstone lab:

J Biol Chem. 2009 Jul 10;284(28):18904-12. Epub 2009 May 27.

**Transcriptional activation by MEIS1A in response to protein kinase A signaling requires the transducers of regulated CREB family of CREB co-activators**

Goh* SL, Looi* Y, Shen H, Fang J, Bodner C, Houle M, Ng AC, Screaton RA, Featherstone M.

*co-first authors

# APPENDIX

# 1   EXPRESSION OF CRTC IN ZEBRAFISH

### 1.1   Detection of zebrafish crtc1 and crtc1b in early embryogenesis in similar regions of the embryo which expresses *meis* genes

By means of *in situ* hybridization, expression of zebrafish *crtc1* and *crtc1b* were detected in embryogenesis as early as 8 to 24 hours of zebrafish development. Sequence analysis of the genome database demonstrates three *crtc* homologs in zebrafish. Since two of the three genes resemble the human and mouse CRTC1/crtc1, we designate them as *zfcrtc1* and *zfcrtc1b* (zfcrtc1 accession: XM_001331969, zfcrtc1b accession: NM_001077457 and zfcrtc3 accession: XM_693452). The sequence of *zfcrtc1* and *zfcrtc1b* bears higher similarities at the N and the C-terminus while *zfcrtc3* sequence differs from the other two *crtc* homologs. The probes for detecting regions within *zfcrtc1* and *zfcrtc1b* were successfully synthesized but not the sequence divergent *zfcrtc3*. Hence *in-situ* data in this appendix will only cover the expression of *zfcrtc1* and *zfcrtc1b*.

Zebrafish *in situ* staining for several stages of early embryogenesis were performed: blastula period extends from 2.25 to 5.25 hpf, gastrula period extends from 5.25-10 hpf and Prim-5 stage extends from 12-24 hpf. Zebrafish embryos from oblong stage (3 hpf) of the blastula period were used for detection and expression of *zfcrtc1* could be detected within the cell nuclei as seen in Appendix 1. The blastula stage observed is characterized by the migrating layer of multople rows of nuclei yolk syncytial layer (YSL) directed

along the animal-vegetal axis of the embryo. Zebrafish embryos from 80% epiboly (8 hpf) were used for detection of expression of *zfcrtc1*. Expression of *zfcrtc1* could be detected as seen in Appendix 2 where the embryonic shield along the animal-vegetal axis extends almost enveloping the yolk.

At 24 hpf (Prim-5 stage), expression of *zfcrtc1* can be distinctly detected in the eyes, midbrain tectum and hindbrain. Image taken at magnification of 49.5 times (Appendix 3). This expression pattern was highly indicative of known *meis1* expression in zebrafish, with common expression domain at the tectum, hindbrain and eyes (Kudoh et al, 2001; Minehata et al, 2008; Wu et al, 2006a). The specificity of the probes can be seen in the absence of expression was detected in the sense *crtc1* probe at the same embryonic stage (Appendix 4). An interesting observation of the zebrafish *crtc1* expression found in some 24 hpf embryos was observed in Appendix 5 whereby an expression at the pectoral fin bud is observed, a time coinciding with the developing of fin buds.

Expression of zebrafish *crtc1b* also bears similar expression to zebrafish *crtc1* as distinctively expression can be seen in 24 hpf developing embryos particularly in the eyes, midbrain tectum and hindbrain (Appendix 6). The specificity of the *crtc1b* probe can also be seen in the absence of expression was detected in the sense *crtc1b* probe at the same embryonic stage (Appendix 7). In addition to the *in vitro* data on the association between *meis* and *crtc*, these highly overlapping *in vivo* studies of *zfcrtc* and *meis* expression is thus an indicative of the collaborative effort involve in embryonic development.

Note: The sequence of the predicted *zfcrtc1* (accession: XM_001331969) has been removed from the database during the writing of the thesis.
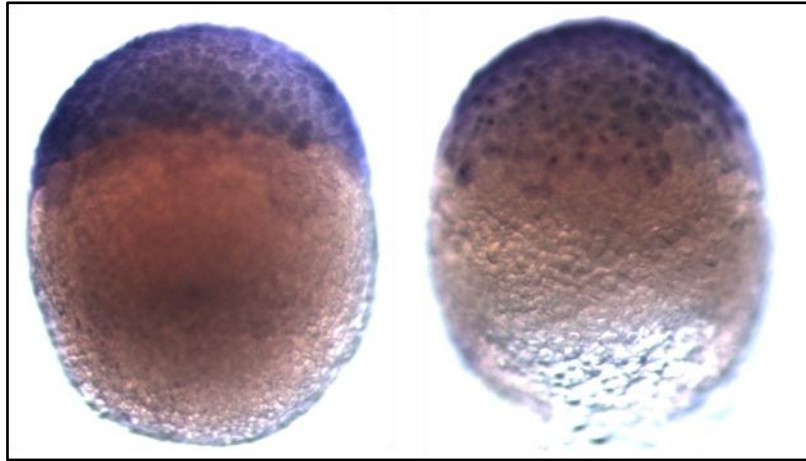


**Appendix 1 Expression pattern of antisense zebrafish *crtc1* probe at 3 hpf**

Expression pattern of *zfcrtc1* during blastula  period of zebrafish development. Nuclear staining were observed in embryos at 3 hpf during 1000 cells stage. Note the expression in expanding cells as well as the migrating nuclei of the YSL.
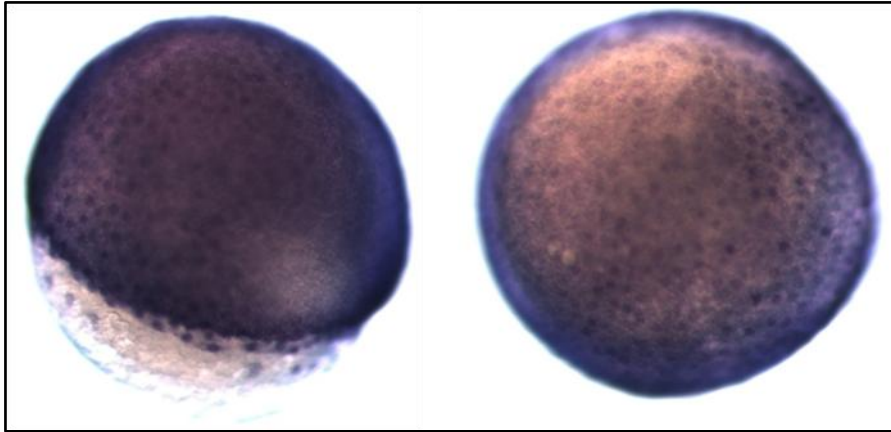
**Appendix 2 Expression pattern of antisense zebrafish *crtc1* probe at 8 hpf**

Expression pattern of *zfcrtc1* during gastrula period of zebrafish development. Nuclear staining were observed in embryos at 8 hpf during 80% epiboly. Note the embryonic shield along the animal-vegetal axis extends almost enveloping the yolk.
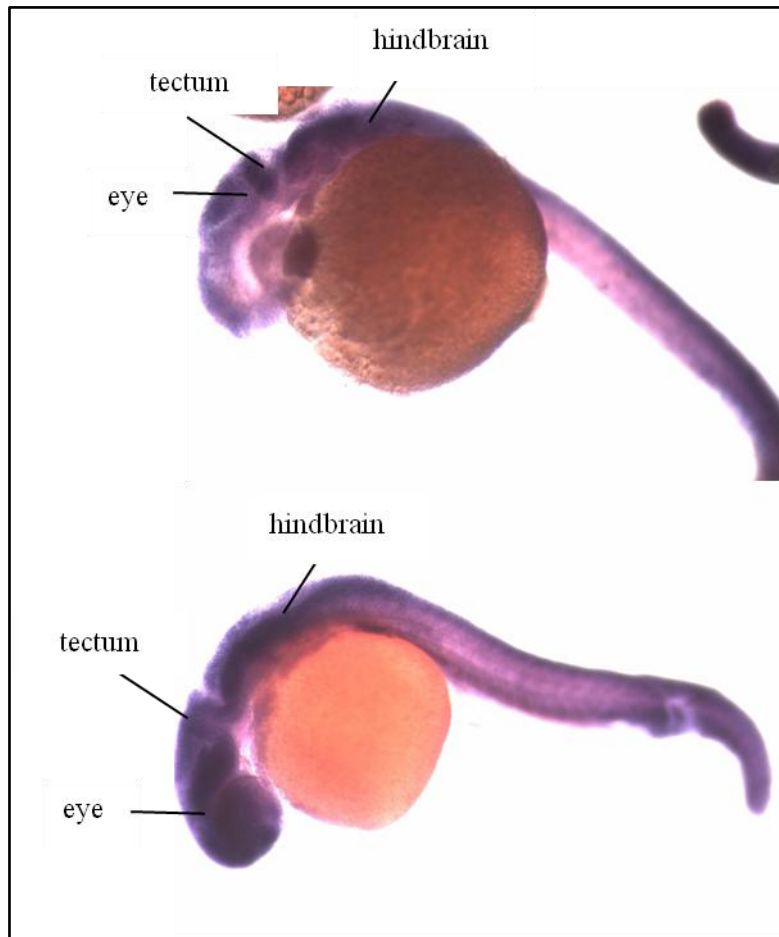
**Appendix 3 Expression pattern of antisense zebrafish *crtc1* probe at 24 hpf**
At 24 hpf (Prim-5 stage) with an antisense probe, expression of *zfcrtc1* can be distinctly detected in the eyes, midbrain tectum and hindbrain. Image taken at magnification of 49.5 times.
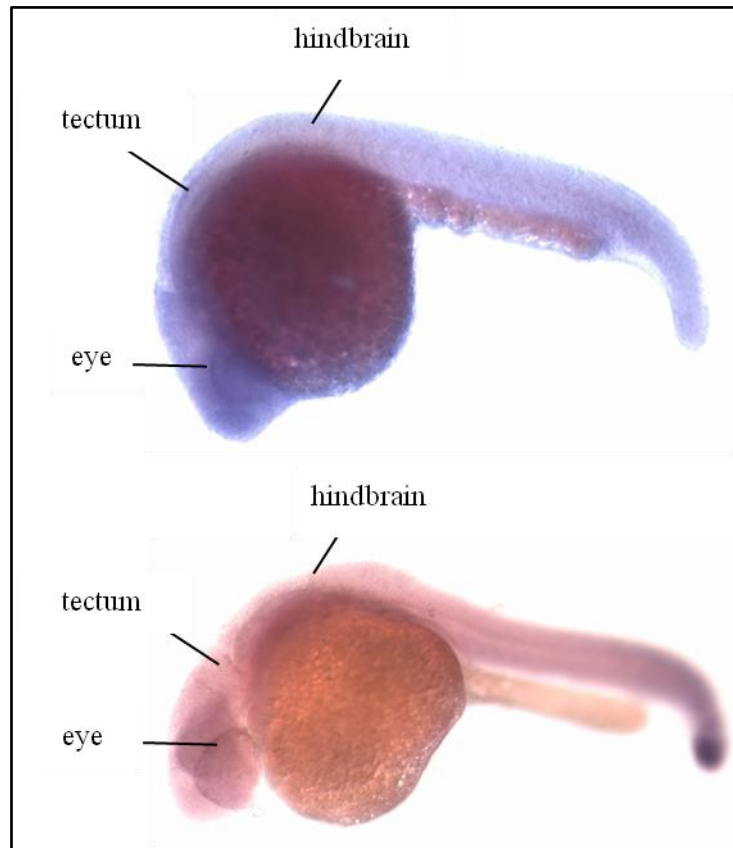
**Appendix 4 Expression pattern of sense zebrafish *crtc1* probe at 24 hpf**

At 24 hpf (Prim-5 stage) with a sense probe, no expression of *zfcrtc1* can be detected in the eyes, midbrain tectum and hindbrain. Image taken at magnification of 49.5 times.
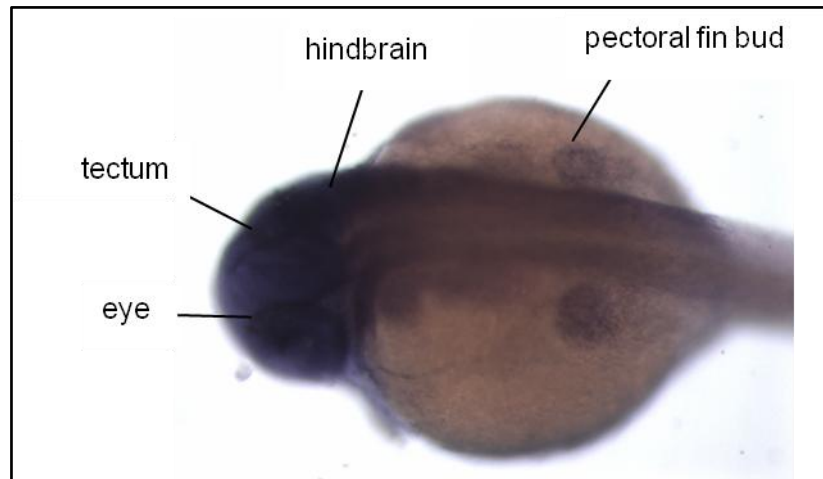
**Appendix 5 Expression pattern of antisense zebrafish *crtc1* probe at 24 hpf**

*In situ* staining for *zfcrtc1* antisense as probe at 24 hpf (dorsal view anterior to left). Image taken at magnification of 80 times.
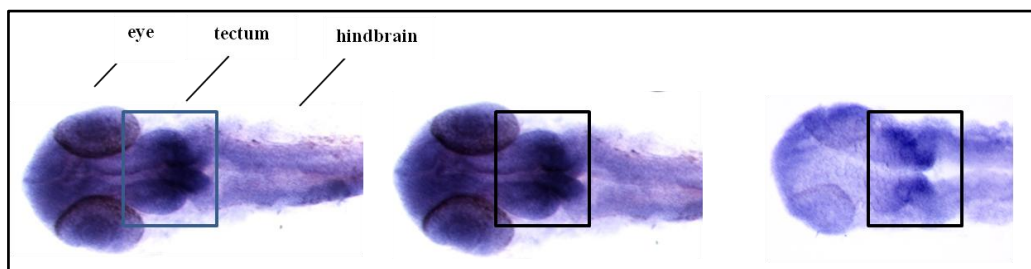


**Appendix 6 Expression pattern of antisense zebrafish *crtc1b* probe at 24 hpf**

Flat mount *In situ* staining for *crtc1b* antisense probe at 24 hpf (Prim-5 stage), (dorsal view anterior to left). Expression of *zfcrtc1b* can be distinctly detected in the eyes, midbrain tectum and hindbrain. Image taken at magnification of 80 times.
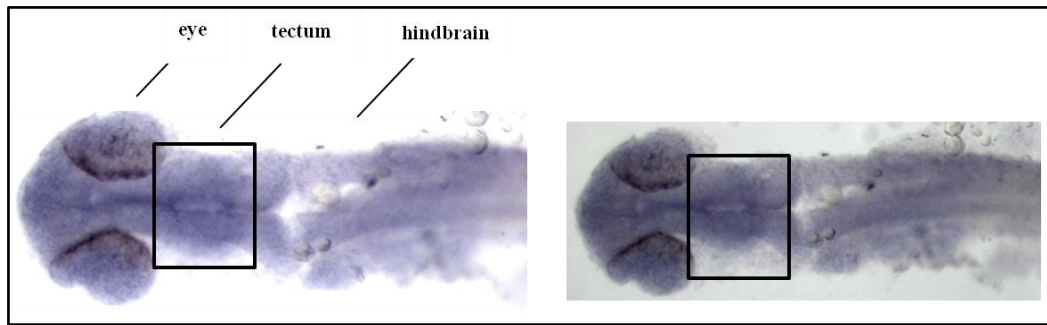
**Appendix 7 Expression pattern of sense zebrafish *crtc1b* probe at 24 hpf**

Flat mount *in situ* staining for *crtc1b* sense probe at 24 hpf (Prim-5 stage), (dorsal view anterior to left). No expression of *zfcrtc1b* can be detected in the eyes, midbrain tectum and hindbrain. Image taken at magnification of 80 times.

## 1.2    Expression of *meis1, crtc1, 1b* in zebrafish at oh, 12h and 24h

Relevant primers detecting various zebrafish *meis1.1, crtc1* and *1b* were designed for RT-PCR amplification. Transcript of *zfcrtc1b* within the zebrafish embryo is low at 12 hpf and increases with expression by 24 hpf, continuing through 48 hpf (Appendix 8). The transcript of *zfcrtc1* was hardly detected and alongside its removal from the database suggestive of it not being a true homolog (Appendix 8). Beta-actin was used alongside as an internal control (Appendix 8). Transcripts of *meis1.1* were also detected at various stages when the *zfcrtc1b* was expressed (Appendix 8).

Thus, the study on zebrafish in this appendix have by means of *in-situ* hybridization and RT-PCR further revealed multiple domains of overlapping expression between *zfcrtc1b* and *meis1.1* present at similar developmental stages.
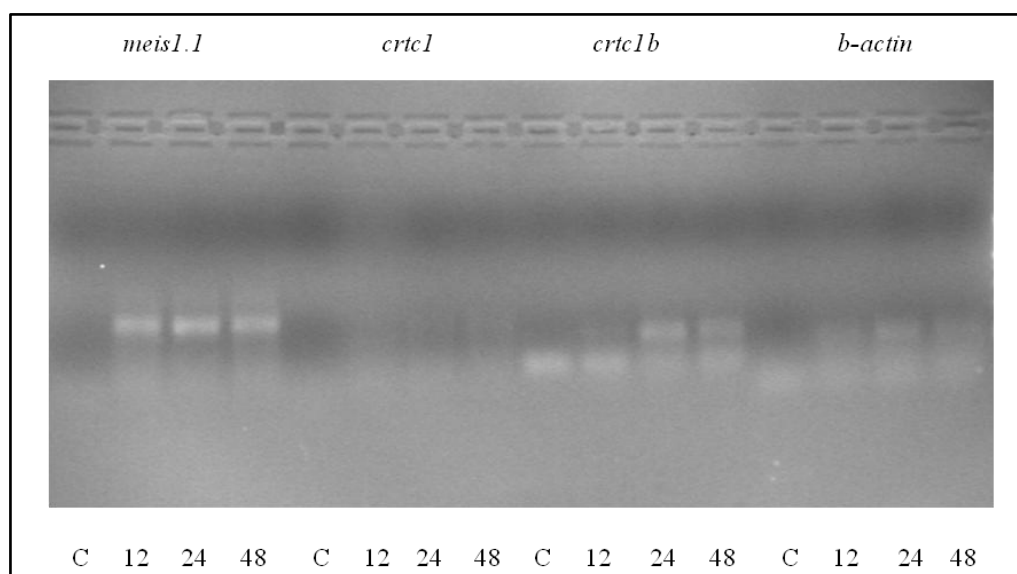
**Appendix 8 Expression of zebrafish *crtc1*, *crtc1b* and *meis1.1* by RT-PCR**
Primers were chosen to span an intron. Only spliced mRNA will give the expected sized band. Expected size for the amplicons: MEIS1 173 bp, CRTC1 166 bp, CRTC1b 119 bp, actin 124 bp. C is a control RT-PCR reaction with no template added. 12, 24, 48 correspond to the CDNA from embryos harvested at various hpf.

## 2 Expression of endogenous MEIS1/2/3, CRTC2 and CRTC3 in N2A and CAD cells
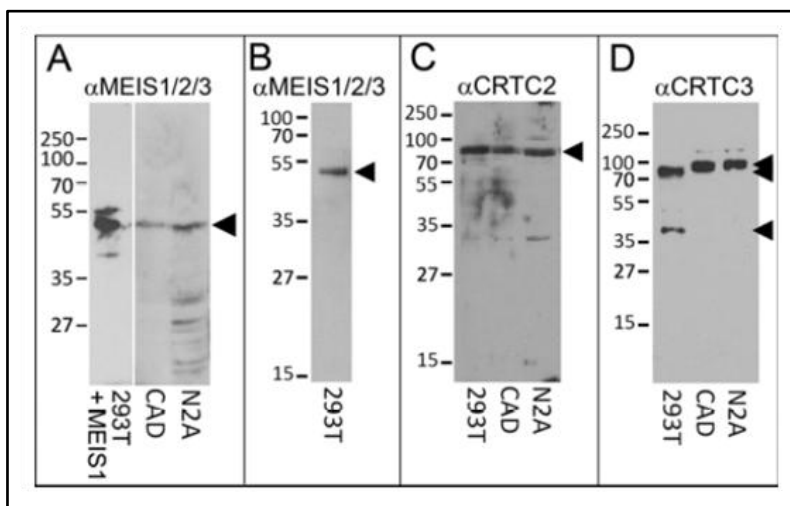


**Appendix 9 Levels of endogenous MEIS1/2/3, CRTC2 and CRTC3 in CAD and N2A neuroblastoma cell lines**

(A) Detection of endogenous MEIS1/2/3 in CAD and N2A cells. Overexpressed MEIS1A in HEk293T cells is shown alongside as comparison. (B) Endogenous MEIS1/2/3 in HEK293T cells. (C) Expression of CRTC2 cells in HEK293T, CAD and N2A cells. (D) Expression of CRTC3 cells in HEK293T, CAD and N2A cells.

## 3 Protein expressions of MEIS1A, PREP2 and CRTC1 *via* a protein production platform

In addition to the efforts at the Featherstone lab, protein expression and purification expertise by the protein production platform at A*STAR were also requested with the aim at structural determination of the proteins of interest using NMR. MEIS1A, PREP2 and CRTC1 proteins were submitted for expression and purification. Nine constructs each were selected for protein

expression.

## 3.1 Protein expressions of MEIS1A via the protein production platform

As there are no known protein structures of MEIS1A, nine constructs for mouse MEIS1A were selected based on the known interacting regions of MEIS1A which include, the homeodomain which mediates DNA binding, the PBX interacting motif (HR1 and HR2) and the MEIS1A C-terminus, which has been shown to mediate PKA as well as CRTC-responsiveness and confers transcriptional activity (Figure 10). Only proteins with high expression levels will be subjected to solubility test and further purified via gel filtration columns. Results from protein expression and purification column showed a MEIS1A construct MEIS1A (65-390) with a high expression level of 4 out of 5 and with a 3 out of 5 rating for soluble proteins (data not shown). Amino acid 64-390 contained the known regions of MEIS1A (the PBX-interacting domain, the homeodomain and the C-terminus). Chromatogram of MEIS1A (64-390) revealed broad inseparable peaks (Figure 11). The SDS-page profile of MEIS1A (64-390) revealed the presence of additional bands apart from the 36 kD MEIS1A (64-390) protein which also appeared larger on gel (Lane 8. Figure 12). The inseparable peaks along with the large additional protein bands are indicative of the presence of multimer within the purified protein.

## 3.2 Protein expressions of CRTC2 *via* the protein production platform

The known interaction domains of human CRTC1 include the coiled coil N-

terminus 56 amino acid which mediates CREB binding. The same N-terminus is also important in CRTC tetramer formation. The C-terminus of CRTC comprises a transactivation domain which is constitutively active (Figure 13). The SDS-PAGE profile of CRTC1 (2-315) and CRTC1 (4-295), CRTC1 (4-315) and CRTC1 (2-295) with an average protein size of 34 kD revealed a series of multiple inseparable bands (Figure 12 Lanes 3-7).

### 3.3    Protein expression of PREP2 *via* the protein production platform

The MEIS and PREP family proteins exhibit sequence similarities at the homeodomain and the PBX interacting motif. Based on the information, nine constructs for mouse PREP2 were selected based on the PBX-interacting motif (HR1 and HR2). Only proteins with high expression levels will be subjected to solubility test and further purified via gel filtration columns. Results from protein expression and purification column showed a PREP2 (49-230) construct with a high expression level of 3 out of 5 and with a 3 out of 5 rating for soluble proteins (data not shown). Chromatogram of PREP2 (49-230) showed a single asymmetrical peak (Figure 15). Its SDS-page profile revealed presence of weak bands of larger weight apart from the expected dominant band of 20kD PREP2 (49-230) protein which also appeared larger on gel (Lane 1. Figure 12). The broad peak along with the large additional protein bands present is suggestive of multimer formation (Figure 15). A second construct with a similar range as the first PREP2 construct with a relatively viable protein expression and solubility profile of 3 out of 5 for both rating was the PREP2 (43-230) construct

(Figure 16). Its chromatogram and SDS-page profile revealed a highly similar profile to PREP2 (49-230) (Lane 2. Figure 12).
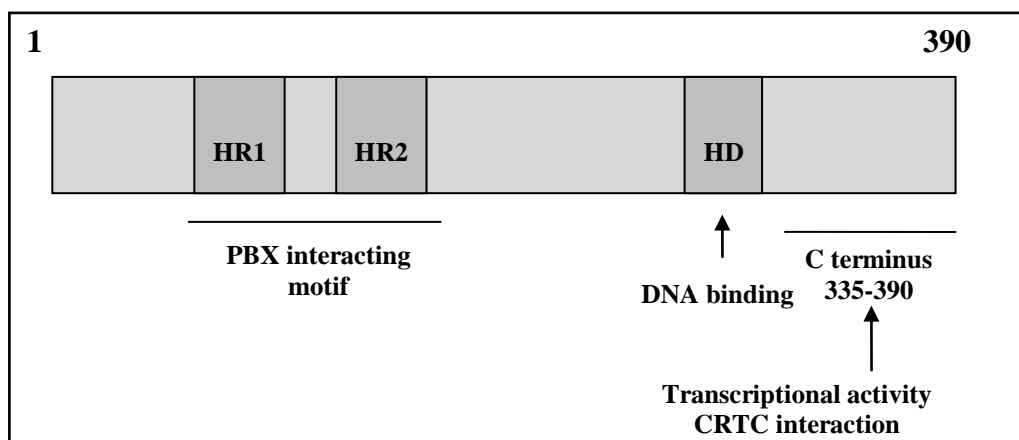


**Figure 10 Schematic diagram of the interaction domains of MEIS1A**

Schematic diagram of MEIS1A and its known interaction domains. HD represents the DNA binding homeodomain HR1 and HR2 indicate the conserved PBX-interacting motif of MEIS1. MEIS1A C-terminus contains a transcriptional activation domain (amino acid 335-390).
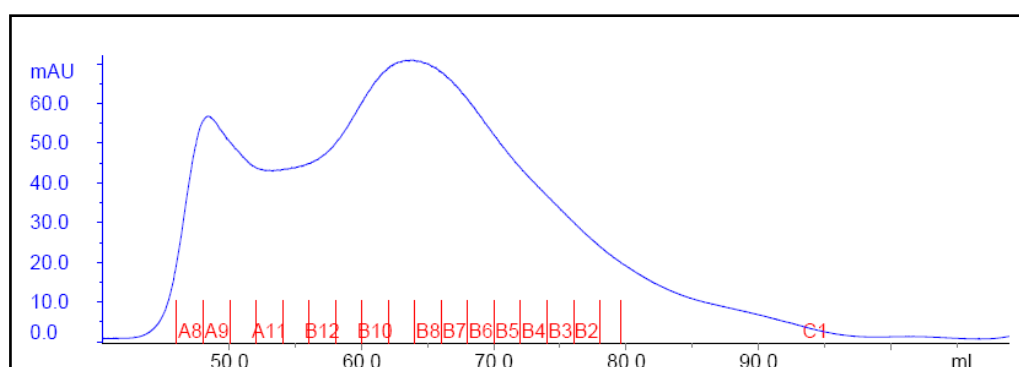


**Figure 11 Chromatogram profile of MEIS1A protein**

Chromatogram from gel filtration column for MEIS1A (64-390) protein revealed broad inseparable peaks, suggestive of the presence of multimers within the purified fractions.
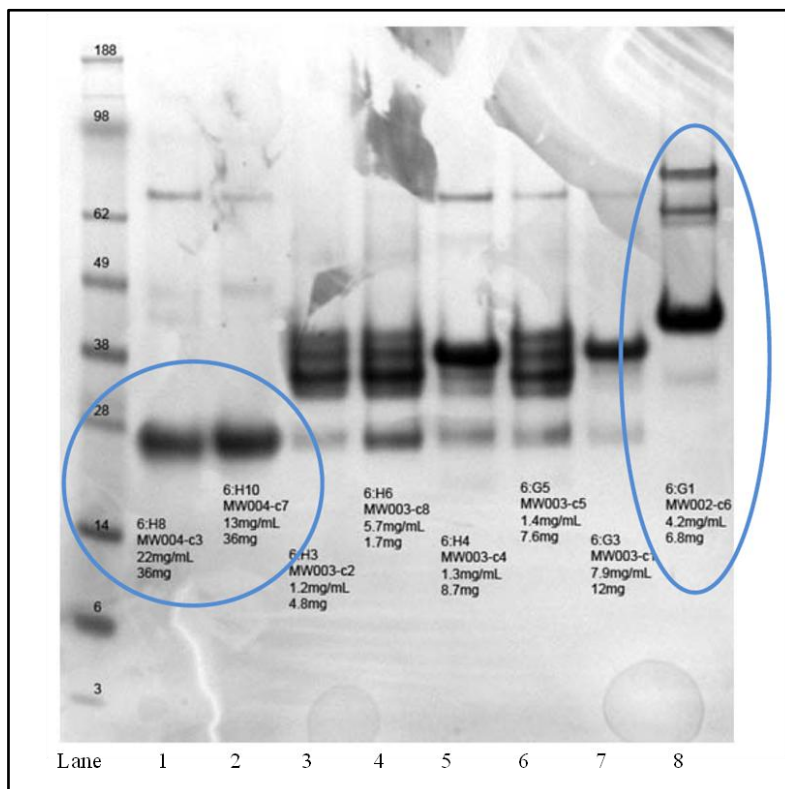
**Figure 12 Protein expression profile of MEIS1A, PREP2 and CRTC1**

SDS-page revealed the elution pattern of various proteins following gel filtration purification. Lane 1 and 2 represent PREP2 (49-230) and PREP2 (43-230) respectively. Lane 3 and 4 represent CRTC1 (2-315) as duplicates. Lane 5 and 6 represent CRTC1 (4-295) and CRTC1 (4-315) respectively. Lane 7 represents CRTC1 (2-295) and Lane 8 represents MEIS1A (64-390).
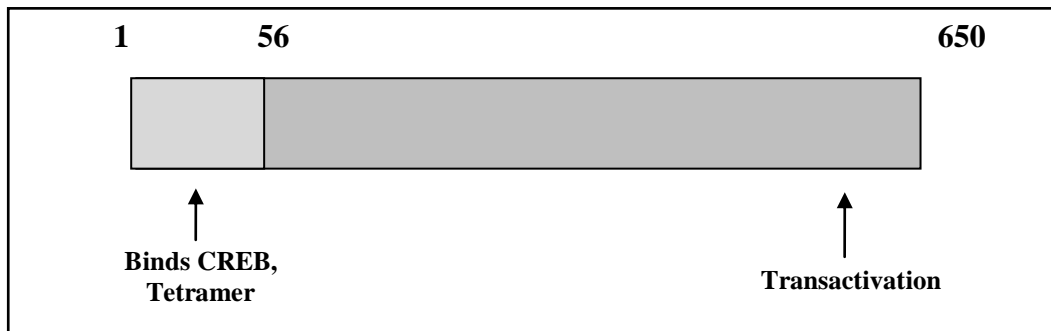
**Figure 13 Schematic diagram of the interaction domains of CRTC1**

Schematic diagram of CRTC1 and its known interaction domains. The N-terminus end mediates binding to CREB as well as CRCT1 tetramer (amino acid 1-56). The C-terminus of CRTC1 harbors a transactivation domain.
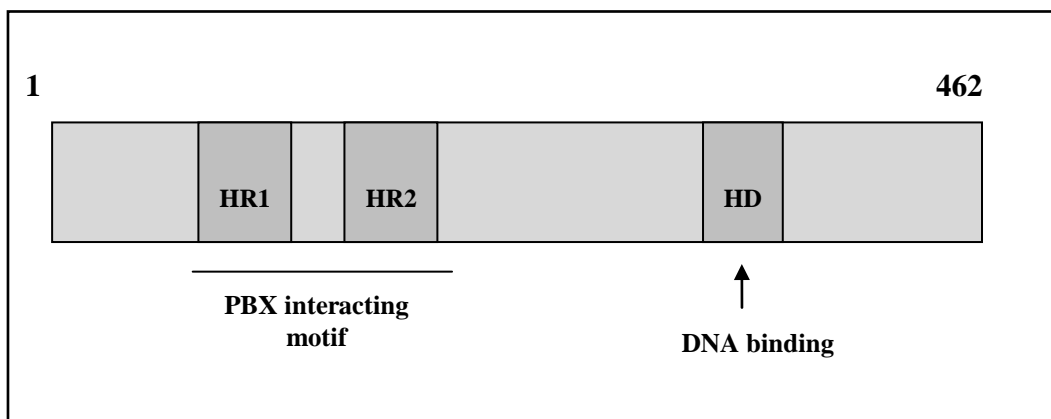


**Figure 14 Schematic diagram of the interaction domains of PREP2**

Schematic diagram of PREP2 and its known interaction domains. HD represents the DNA binding homeodomain. HR1 and HR2 indicate the conserved PBX-interacting motif of PREP2.

206
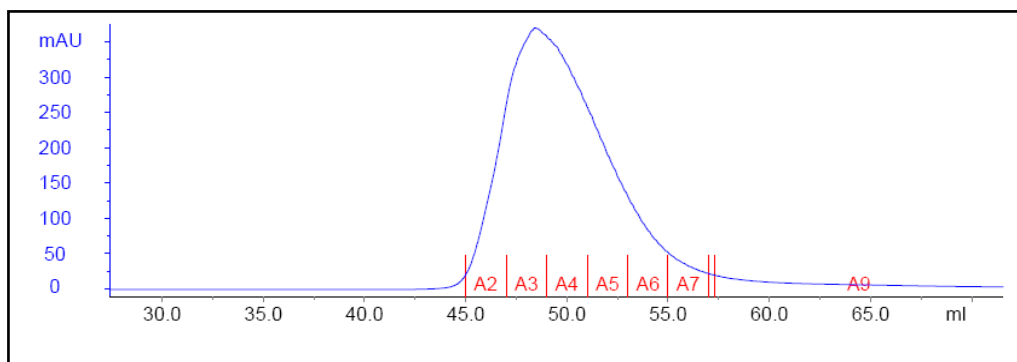
**Figure 15 Chromatogram profile of PREP2 (49-230) protein**

Chromatogram from gel filtration column for PREP2 (49-230) revealed a single asymmetrical peak, suggestive of the presence of tetramer within the purified complex.
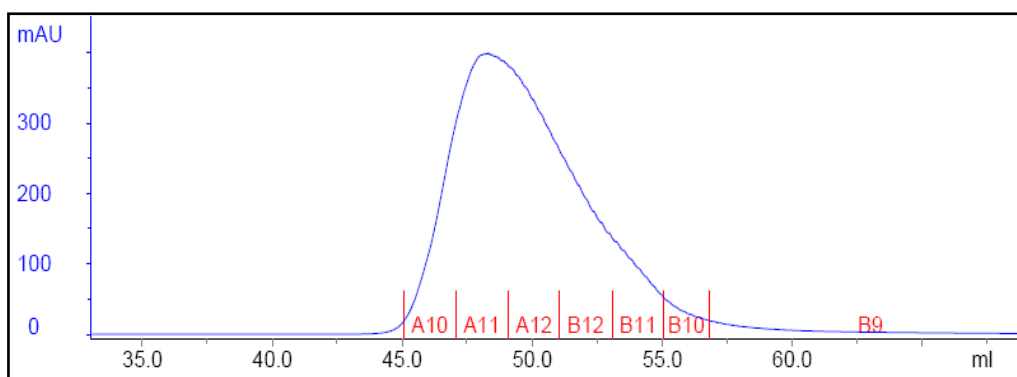


**Figure 16 Chromatogram profile of PREP2 (43-230) protein**

Chromatogram from gel filtration column for PREP2 (43-230) revealed a single asymmetrical peak, suggestive of the presence of tetramer within the purified complex.

3.4    **Characterization of gel filtration purified PREP2 (49-230)**

Amongst the three protein submitted for protein platform expression, PREP2 (49-230) and PREP2 (43-230) proteins appeared as single peak on the

chromatogram with a dominant band of PREP2 protein. The quality of each protein off the gel filtration column was also relative pure with a total protein quantity of 36 mg each.

Structural studies generally require protein samples to be mostly homogenous monodispersed ie: when a protein consists of only one molecular mass. A native gel and dynamic light scattering was thus applied to the PREP2 (49-230) proteins to distinguish the forms of proteins present within the purified samples. The PREP2 (49-230) was first ran on a native gel electrophoresis where the proteins were not denatured and thus separated base on their charge, size and shape (Lane 1 and 2 Figure 17). Migration pattern showed a larger non-dominant band of PREP2 (49-230) proteins (arrow) which appeared reduced upon addition of DTT, and could be indicative of multimer formation (Lane 3 and 4 Figure 17).

Given the favor for monodispersed proteins for structural studies, dynamic light scattering technique (DLS) was used to determine the distribution of the protein population. Dynamic light scattering technique measures time-dependent fluctuations in the intensity of scattered light which occurs due to the random Brownian motion of the protein, which are then converted into a size distribution. Figure 18 showed the intensity size distribution graph of PREP2 (49-230). In all DLS assays, samples were read three times with the second and third run indicated in the graphs. The wide bell curve hinted at various forms of PREP2 proteins which are unresolved and masked under the wide curve.

Lithium chloride (LiCl) treatment of 10 min was applied onto the sample to separate the protein complex. Observation from the data off the DLS detection showed that one of the bands appeared to separate upon treatment (Figure 19). DTT concentration of 0.05 M to 0.5 M appeared to continually promote distribution of the complexes (Figure 20-23). However, upon higher salt concentration of 0.8 to 1 M, the integrity of the complex started to fall and multiple bands were observed (Figure 24 and 25).

Given the suggested range of DTT on proteins were around 40-50 mM, separation of the protein complex would have happened at the earlier concentration of 0.05 M DTT. The data from DLS indicated a non-homogeneity of the purified complex which cannot be separated despite several attempts. Without homogeneity, structural studies such as 1D NMR would not be feasible.
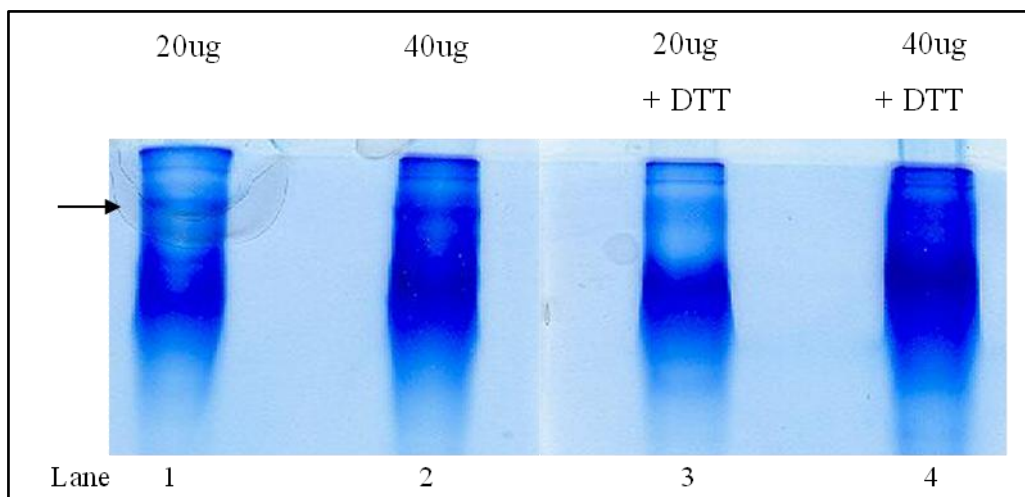
**Figure 17 Characterization of PREP2 (49-230) homogeneity on native gel**

A sample of 20 ug and 40 ug of PREP2 (49-230) protein was loaded onto a 15% native gel. Prior to loading, the samples were treated without/with 0.1 M of DTT for 10 mins at room temperature. Lane 1 and 2 revealed migration pattern of 20 ug and 40 ug of PREP2 (49-230) protein while Lane 3 and 4 indicated migration pattern of 20 ug and 40 ug of PREP2 (49-230) protein upon DTT treatment.
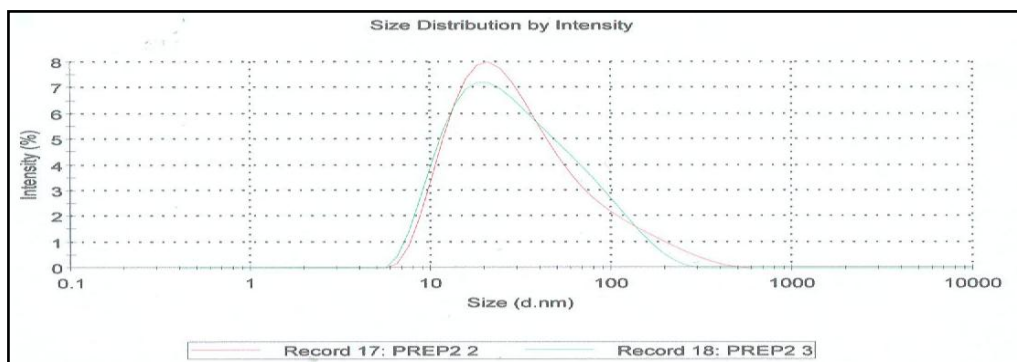
**Figure 18 Intensity size distribution of PREP2 (49-230)**

Intensity size distribution graph of PREP2 (49-230). The two lines on the graph represent two separate runs.
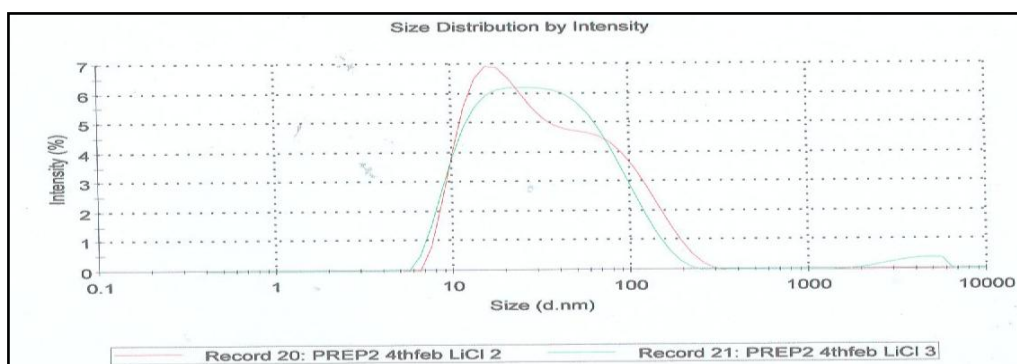


**Figure 19 Intensity size distribution of PREP2 (49-230) following LiCl treatment**

Intensity size distribution graph of PREP2 (49-230). The two lines on the graph represent two separate runs after 10 mins treatment with 20 mM LiCl.
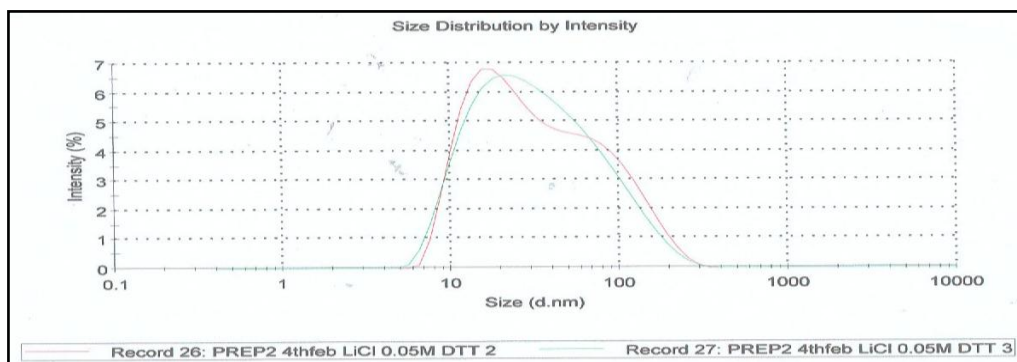
**Figure 20 Intensity size distribution of PREP2 (49-230) following LiCl and 0.05 M DTT treatment**

Intensity size distribution graph of PREP2 (49-230). The two lines on the graph represent two separate runs after 20 mins treatment with 20 mM LiCl and 0.05 M DTT.
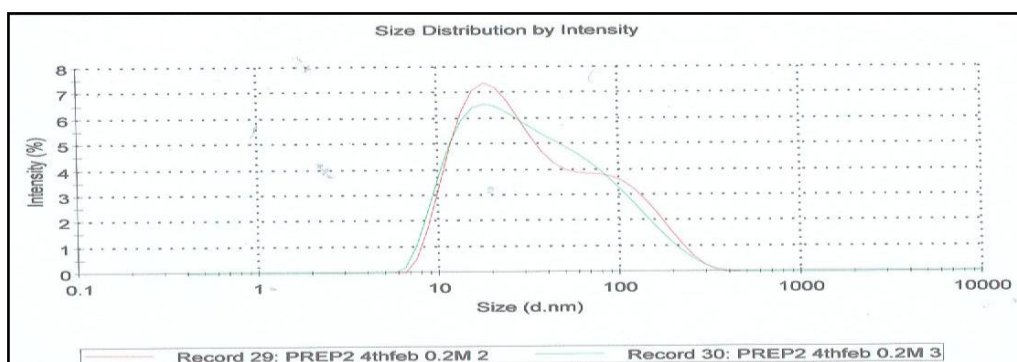


**Figure 21 Intensity size distribution of PREP2 (49-230) following LiCl and 0.2 M DTT treatment**

Intensity size distribution graph of PREP2 (49-230). The two lines on the graph represent two separate runs after 20 mins treatment with 20 mM LiCl and 0.2 M DTT.
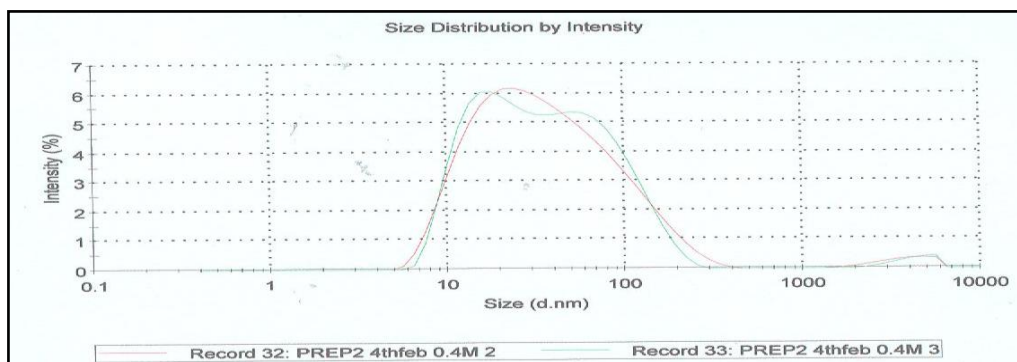
**Figure 22 Intensity size distribution of PREP2 (49-230) following LiCl and 0.4 M DTT treatment**

Intensity size distribution graph of PREP2 (49-230). The two lines on the graph representstwo separate runs after 20 mins treatment with 20 mM LiCl and 0.4 M DTT
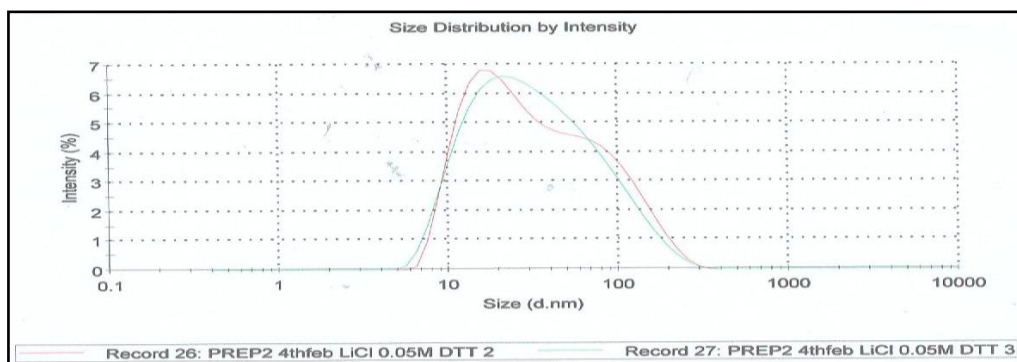


**Figure 23 Intensity size distribution of PREP2 (49-230) following LiCl and 0.5 M DTT treatment**

Intensity size distribution graph of PREP2 (49-230). The two lines on the graph represent two separate runs after 20 mins treatment with 20 mM LiCl and 0.5 M DTT.
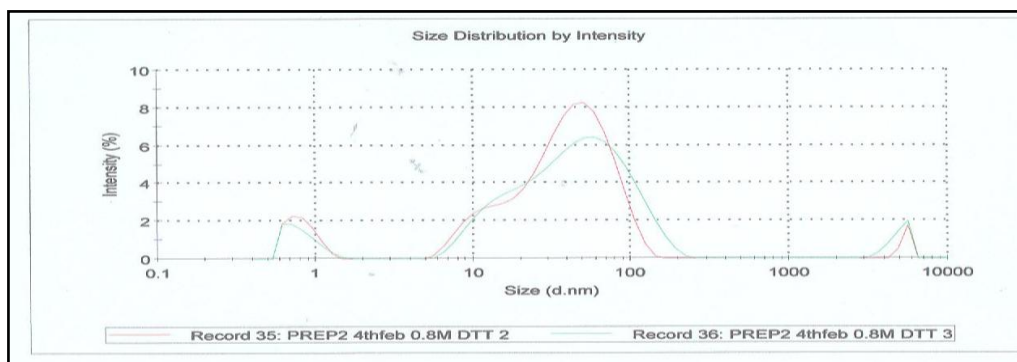
**Figure 24 Intensity size distribution of PREP2 (49-230) following LiCl and 0.8 M DTT treatment**

Intensity size distribution graph of PREP2 (49-230). The two lines on the graph representstwo separate runs after 20 mins treatment with 20 mM LiCl and 0.8 M DTT.
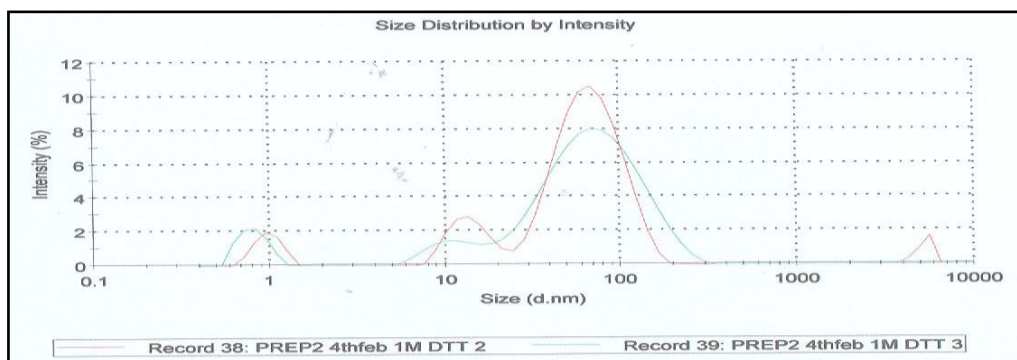


**Figure 25 Intensity size distribution of PREP2 (49-230) following LiCl and 1.0 M DTT treatment**

Intensity size distribution graph of PREP2 (49-230). The two lines on the graph represents two separate runs after 20 min treatment with 20 mM LiCl and 1.0 M DTT.

214

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