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RANBP17, A Novel Non-bHLH Binding Partner of bHLH Transcription Factor E12

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bHLH TRANSCRIPTION FACTOR E12

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DEDICATION

With my deepest love and gratitude, I dedicate this work to my parents, Jae-Wook Lee and Yoo-Za Hwang, and my younger brother, Seouk-Ho Lee. I also confess that God has always been with me throughout the long journey of my Ph.D. studies.
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INTRODUCTION

The basic helix-loop-helix (bHLH) family of transcription factors has been demonstrated to regulate gene transcription during cell differentiation, proliferation, lineage commitment, cell cycle regulation and neoplastic transformation in a variety of cell types (Kadesch 1992; Jan and Jan 1993; Duprey and Lesens 1994; Stewart, Zoidl et al. 1997; Dambly-Chaudiere and Vervoort 1998; Kako and Ishida 1998; Reya and Grosschedl 1998; Cepko 1999; Massari and Murre 2000; Perry and Soreq 2002; Desprez, Sumida et al. 2003). This family is defined by the bHLH signature, a highly conserved sequence motif near their C-termini consisting of two functionally distinct regions, the basic domain rich in positively charged amino acids that are necessary for DNA binding, and the HLH region responsible for interaction and dimerization with other bHLH family members. The canonical core DNA sequence motif recognized by bHLH proteins is a hexanucleotide consensus sequence known as an E-box (5'-CANNTG-3') element and it is present in a wide variety of tissue-specific enhancers (Murre, McCaw et al. 1989; Murre, McCaw et al. 1989; Deed, Armitage et al. 1996; Norton, Deed et al. 1998; Massari and Murre 2000; Hikima, Lennard et al. 2005).

E12 and E47 are alternative spliced transcripts of the E2A gene. They are classified as ubiquitously expressed class I bHLH transcription factors and founding members of mammalian E-proteins (Murre, McCaw et al. 1989; Murre, McCaw et al. 1989; Hu, Olson et al. 1992; Bain, Gruenwald et al. 1993; Sawada and Littman 1993; Zhuang, Barndt et al. 1998; Conway, Pin et al. 2004). They homodimerize or heterodimerize with other bHLH transcription factors including tissue-specific class II
bHLH factors (Braun, Rudnicki et al. 1992; Rudnicki, Braun et al. 1992; Hasty, Bradley et al. 1993; Nabeshima, Hanaoka et al. 1993; Rudnicki, Schnegelsberg et al. 1993; Rawls, Morris et al. 1995; Zhang, Behringer et al. 1995). The cell type selectivity of ubiquitously expressed class I bHLH proteins such as E12/E47 is conferred by various tissue-specific class II bHLH dimerization partner proteins (Massari and Murre 2000). This is exemplified by the myogenic regulatory factors (MRFs), such as MyoD, MRF-4, Myf-5 and myogenin in skeletal muscle development (French, Chow et al. 1991; Weintraub, Dwarki et al. 1991; Venuti and Cserjesi 1996), BETA2/NeuroD in neurogenesis, pancreatic development and insulin gene activation, and SCL/Tal1 in the development of hematopoietic cell lineages (Hsu, Huang et al. 1994; Hsu, Wadman et al. 1994; Gould and Bresnick 1998). The regulatory role of these bHLH interactions can be further controlled by ubiquitously expressed dominant negative regulators such as Id proteins, a distinct group of HLH proteins that lack basic domain (Benezra, Davis et al. 1990; Langlands, Yin et al. 1997). In addition, the physiological specificity of bHLH factors in transcriptional activation is thought to be not solely dependent on their intrinsic DNA binding specificities but also achieved through cooperative interactions with other components of transcriptional machinery such as coactivators or corepressors (Eckner, Yao et al. 1996; Massari and Murre 2000; Turner, Cureton et al. 2004).

While bHLH-bHLH protein interactions are perhaps the best studied, the interactions of E12/E47 proteins are not restricted to other bHLH family members or to the bHLH domain. A number of non-HLH interacting partners of E12/E47 have been described. These non-bHLH interactions of E12/E47 proteins appear to be involved in diverse biological settings. E12/E47 can interact with p300/ CBP and SAGA histone
acetyltransferase complex to regulate transcriptional activity of downstream target genes (Eckner, Yao et al. 1996; Massari, Grant et al. 1999; Bayly, Chuen et al. 2004). The MAPK-activated protein kinases 3pK and MK2 were identified as interaction partners of E47 and the expression of either kinase results in a repression of the transcriptional activity of E47 on an E-box containing promoter (Neufeld, Grosse-Wilde et al. 2000). The Ca\(^{2+}\) sensor protein calmodulin appears to directly bind to the basic region of E12/E47 proteins to inhibit their DNA binding (Saarikettu, Sveshnikova et al. 2004). A C-terminal 31-kDa fragment that is a caspase mediated cleavage product of p130\(^{casp}\) heterodimerizes with E12/E47 proteins and can inhibit E2A-mediated p21\(^{Waf1/Cip1}\) transcription (Kim, Kook et al. 2004). A GAP-related interacting protein to E12 (GRIPE) binds to the HLH region of E12 and is suggested to negatively regulate E12-dependent target gene transcription (Heng and Tan 2002). The rat homologue of the human polymyositis-scleroderma autoantigen (rPM-Scl) and the ubiquitin-conjugating enzyme UbcE2A (or mUbc9), specifically interact with amino acids 477-530 of E12/E47 proteins, a region that is distinct from the bHLH domain and deletion of this domain extends the half-life of E47 and expression of antisense UbcE2A delays E12 degradation (Kho, Huggins et al. 1997; Kho, Huggins et al. 1997; Huggins, Chin et al. 1999). Given the variety of protein-protein interactions noted for E12/E47, it appears that E12/E47 proteins may serve as an integration point for the various types of intracellular signals that likely reflect overall cellular status.

Proteins that function in the nucleus including transcription factors, must be regulated in precise temporal and cell type-specific manners. A number of studies indicate that nucleocytoplasmic shuttling of nuclear factors is a further mechanism by which their activities can be regulated and deregulation in this transport mechanism or
alterations in post-translational modification of nuclear factors including transcription factors have shown to be related to cancers (Flamini, Curigliano et al. 1996; Rayet and Gelinas 1999; Viglietto, Motti et al. 2002; Fabbro and Henderson 2003; Kau, Way et al. 2004; Nakamura 2005). After the first demonstration that identified the presence of a short stretch of basic residues in the SV40 Large T-antigen required for nuclear localization (Kalderon, Roberts et al. 1984), similar basic-rich nuclear localization sequences were identified in many other proteins including transcription factors (Boulikas 1993). The studies in transcription factors for example, Nuclear Factor kappa B (NF-κB), led to the realization that transcription factors are not necessarily constitutively present in the nucleus, and both nuclear import and export can be regulated at multiple levels via particular nuclear localization sequences (NLSs) and nuclear export sequences (NESs) (Fischer, Huber et al. 1995; Wen, Meinkoth et al. 1995; Lee and Hannink 2003).

The E12/E47 proteins contain potential nuclear localization sequences in their N-termini and the characteristics related to DNA binding specificity, transcriptional activity and protein degradation, can be regulated by post-translational modifications (Mitsui, Shirakata et al. 1993; Sloan, Shen et al. 1996; Huggins, Chin et al. 1999; Chu and Kohtz 2001). However, the specific details for the nuclear transport mechanism of E2A have not yet been illuminated and moreover, E2A as a transcription factor has not been demonstrated to have a functional connection with any member of importin-β superfamily in the nucleus as will be described herein. Our goal to identify novel binding partners of E12 stems from an interest in Id proteins (Ids) in cancer, particularly prostate cancer. Ids are implicated in various cancers including colon cancer, breast cancer as well as prostate cancer (Wilson, Deed et al. 2001; Ouyang, Wang et al. 2002; Desprez, Sumida et al. 2003; Fong, Itahana et al. 2003; Coppe, Itahana et al. 2004; Gautschi,
Tepper et al. 2008). Therefore, we postulated that a well-known binding partner of Id proteins, E12 and its intracellular protein interaction network may also impact Ids-mediated tumorigenesis. To search for novel interacting molecules of the bHLH transcription factor E12, a yeast two-hybrid screening was conducted and a non-bHLH protein named RANBP17 is identified as a candidate binding partner for E12. RANBP17 and its close relative, RANBP16, are the most distant members of the importin β-related receptor family (Mattaj and Englmeier 1998; Gorlich and Kutay 1999; Nakielny and Dreyfuss 1999; Macara 2001; Fried and Kutay 2003). The nucleocytoplasmic shuttling of most proteins largely relies on members of the importin-β superfamily, which is in turn governed by differential RanGTP gradient between nucleus and cytoplasm (Gorlich, Pante et al. 1996; Kutay, Bischoff et al. 1997). RANBP16 has been demonstrated to bind RAN and function as a nucleocytoplasmic transporter (Kutay, Hartmann et al. 2000; Mingot, Bohnsack et al. 2004) specifically as an exportin (Mingot, Bohnsack et al. 2004). To date, the function of RANBP17 is completely unknown. Here we carry out studies that indicate RANBP17 is a novel binding partner for E12 and RANBP17 functions to impact E12-mediated transcriptional activity.
Helix-Loop-Helix proteins

The helix-loop-helix (HLH) class of transcription factors has been shown to play an important role in the regulation of cell cycle, lineage commitment, cell differentiation and proliferation in a wide range of biological settings (Kadesch 1992; Jan and Jan 1993; Duprey and Lesens 1994; Stewart, Zoidl et al. 1997; Dambly-Chaudiere and Vervoort 1998; Kako and Ishida 1998; Reya and Grosschedl 1998; Cepko 1999; Massari and Murre 2000; Perry and Soreq 2002; Desprez, Sumida et al. 2003). To date, hundreds of HLH protein members have been identified in organisms ranging from the yeast *Saccharomyces cerevisiae* to human and they have been classified into several specific groups depending on the methodological tools and criteria applied for categorization. For example, M.E. Massari and C. Murre categorize HLH factors into seven classes based on tissue distribution, dimerization capability, and DNA-binding specificity (Massari and Murre 2000) while W.R. Atchley and W.M. Fitch have classified them into four groups mainly based on the phylogenetic conservation of amino acids at certain positions, DNA binding pattern and specificity, and the presence or absence of a leucine zipper motif (Atchley and Fitch 1997). Most HLH proteins are members of the basic helix-loop-helix (bHLH) family, a hallmark of which is the presence of a highly conserved bHLH motif that is typically at the C-terminal region. The bHLH motif is comprised of a basic region that is rich in positively charged basic residues and responsible for DNA binding, particularly making contact with the major groove of the DNA. The HLH region possesses two amphipathic $\alpha$-helices with stringent conservation...
of hydrophobic residues connected by a loop segment with various lengths and provides a protein-protein interaction interface and dimerization capability (Murre, McCaw et al. 1989; Voronova and Baltimore 1990; Ferre-D'Amare, Prendergast et al. 1993). The bHLH transcription factors act as transcriptional activators or inhibitors of various downstream target genes through direct binding to the consensus DNA binding sequence named the E-box element (CANNTG).

A distinctive member of this family includes Id (Inhibitor of differentiation or DNA binding) proteins which show ubiquitous tissue distribution (Benezra, Davis et al. 1990). There are four members of the Id gene family reported in the mammalian genome, Id1-Id4. Id proteins can interact with variety of bHLH transcriptional factors including E-proteins since they contain a HLH domain. However, they can not bind to DNA due to the lack of a basic domain, thus resulting in the formation of non-DNA binding complexes and the sequestration of bHLH proteins from bound DNA. Therefore, Id proteins function as dominant-negative regulators of other bHLH transcription factors and give rise to inhibition of transcriptional activities induced by other bHLH factors.

There are also other members of HLH transcription factors. For example, the bHLH-LZ factors contain a leucine zipper (LZ) motif characterized by heptad repeats of leucines. The bHLH-PAS factors have a bHLH domain located at their amino termini and have two well-conserved regions, PAS-A and PAS-B, separated by a poorly conserved spacer adjacent to the bHLH domain. As increasing numbers of new bHLH transcription factors were identified in the last decade, it has become more difficult to understand their relationship and classify them into definitive categories.
E-proteins

Based on the categorization from M.E. Massari and C. Murre, class I bHLH proteins are also known as E-proteins because their DNA binding is restricted to the E-box consensus sequence. It includes mammalian E-proteins such as E12, E47, HEB, E2-2 (also called ITF-2) and Daughterless, the Drosophila melanogaster homologue of mammalian E-proteins. As a subgroup of HLH family members, E-proteins also contain a bHLH motif thus, sharing the same essential characteristics with bHLH proteins in terms of structural features and DNA binding specificities. They are widely expressed and are known to play key regulatory roles in a variety of developmental processes. They also have the ability to form either homo- or heterodimers with other members of the HLH family but in most cases so far, they preferentially form heterodimers with class II bHLH proteins conferring tissue-specific characteristics (Murre, McCaw et al. 1989; Quong, Romanow et al. 2002). It is also well known that E-proteins can interact with Id proteins functioning as dominant negative inhibitors of E-proteins (Engel and Murre 2001; Yokota 2001).

It has been reported that in B- and T-lymphocyte development, the major functioning species of E-proteins are homodimers or heterodimers with other E-proteins. In muscle cells, E-proteins are also able to form heterodimers with muscle regulatory factors (MRFs) exerting their effects on myogenic differentiation. However, the physiologically relevant E-protein binding partner of each MRF during myogenic differentiation is still obscure partially due to the functional redundancy present in MRFs and E-protein members (Hu, Olson et al. 1992; Dias, Dilling et al. 1994; Rudnicki and Jaenisch 1995; Wang, Schnegelsberg et al. 1996; Zhuang, Barndt et al. 1998; Berkes and
The mammalian E-proteins commonly possess two transactivation domains (TADs), AD1 and AD2. “AD1 is located within the first 100 amino acids of the proteins and contains a putative \(\alpha\)-helix. AD2 is located between AD1 and the bHLH domain and possesses a loop-helix structure. These transactivation domains provide the potential to recruit coactivator or corepressor complexes and play an important role in transcriptional regulation as well as in cellular transformation in the context of chimeric oncoproteins such as E2A-RLF and E2A-Pbx1 (Aronheim, Shiran et al. 1993; Quong, Massari et al. 1993; Inaba, Shapiro et al. 1994; Lu, Wright et al. 1994; Yoshihara, Inaba et al. 1995; Massari, Jennings et al. 1996; Inukai, Inaba et al. 1998)” (Markus, Du et al. 2002).

**E2A proteins (E12 and E47)**

The E2A proteins are founding members of the E-protein family and they include E12 and E47, two major alternative spliced transcripts of the \(E2A\) (also referred to \(TCF3\)) gene, which is located in human chromosome 19p13.3. The E12 and E47 proteins only differ in the bHLH motif as the result of alternative splicing and are known to have different DNA binding specificities and affinities attributed to the different bHLH elements, which show 77% amino acid identity and 89% similarity between E12 and E47 proteins. Another alternative splicing transcript, E2-5 (ITF-1), has been also reported (Henthorn, Kiledjian et al. 1990). However, the actual existence and any detailed characteristic of this protein have not yet been firmly established. To date, studies for E2A gene products have almost exclusively focused on the major two splicing variants, E12 and E47. As such, the following details regarding the protein products of \(E2A\) gene
will be described only for E12 and E47 proteins.

(1) Transcript Expression and Protein Structure of E2A

Two cDNAs were initially isolated and characterized from the binding to the κE2 site of immunoglobulin light chain enhancer (Murre, McCaw et al. 1989) and were identified as E12 and E47 encoded by the \textit{E2A} (\textit{TCF3}) gene. E2A transcripts have been found to be expressed in various cell types (Murre, McCaw et al. 1989; Nelson, Shen et al. 1990; Walker, Park et al. 1990) and Northern blot analyses showed that a ~3 kb E2A transcript that was ubiquitously expressed in rat and mouse tissues (Nelson, Shen et al. 1990; Walker, Park et al. 1990). Human cell lines demonstrated a heterogeneous population of E2A mRNAs (Murre, McCaw et al. 1989; Kamps, Murre et al. 1990).

As mentioned, E12 and E47 are the alternatively spliced products of the \textit{E2A} gene and E12 and E47 proteins are founding members of E-protein subfamily. The nucleotide and amino acid sequences of these two proteins are identical except for the bHLH motif and the protein sequences are highly conserved across human, mouse, \textit{Xenopus} and chicken in both E12 and E47 (Conlon and Meyer 2004). They contain two transactivation domains (TADs) and a putative nuclear localization signal (NLS) in their N-termini and most importantly, the bHLH domain, a region of extensive identity to the \textit{Drosophila daughterless} gene and similarity to Myc, MyoD and members of the \textit{Drosophila achaete-scute} gene family located in the vicinity of the C-termini (Murre, McCaw et al. 1989). In addition, it is also reported that E12/E47 proteins have an ubiquitin ligase interaction domain, which is approximately mapped to 478-533 amino acids and this domain is involved in the degradation of E12/E47 proteins.
Figure 1. The Human E2A Gene and E12/E47 Protein Structure. (A) Schematic representation of human E2A gene structure. Human E2A gene consists of 19 exons and among those, exon 17 and exon 18 are differentially expressed by alternative splicing generating E12 and E47 proteins, respectively. Therefore, the coding sequence of E12 protein is generated from exons 1-17 and exon 19 while that of E47 is from exons 1-16 and exons 18-19 of E2A gene. The black boxes (or lines) represent assigned exons and the open box and the hatched box represent exon 17 or exon 18, respectively (Hikima, Lennard et al. 2005). The actual scale bar is presented below. (B) Schematic representation of human E12/E47 protein structures. Human E12/E47 proteins contain activation domain 1 (AD1) and loop-helix activation domain 2 (LH-AD2) at amino acids 1-99 and 325-479 respectively (hatched boxes). E12/E47 proteins also contain a putative nuclear localization signal at amino acids 170-175 (black bar) and bHLH domains which are defined at amino acids 547-607 for E12 and 544-604 for E47 (black boxes). However, the actual differences between E12 and E47 proteins exist in amino acids 530-607 for E12 and 530-604 for E47. The ubiquitin ligase interaction domain (478-533) is not shown in this diagram.
FIG. 1

A. Human E2A gene structure

5' UTR 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 3' UTR

E12 exons (1-16,17,19)
E47 exons (1-16,18,19)

Alternative splicing:

5 kb

Jun-ichi Hikima et al. (Physiol Genomics. 2005 Apr 14;21(2):144-51. Epub 2005 Feb 15)

B. E12/(E47) (full-length)

AD1  NLS  LH-AD2  bHLH

1  99  170-175  325  479  547  607  654 aa : E12
(544) (604) (651) aa : (E47)

(2) Binding Partners of E2A

The dimerization of HLH proteins is a prerequisite for DNA binding as well as for functioning as a transcription factor. Whereas some of the HLH proteins appear to form exclusively homodimers (e.g. upstream stimulatory factor (USF)) or heterodimers (e.g. SCL/TAL1, Myc and Mad) (Hsu, Huang et al. 1994; Littlewood and Evan 1995), HLH proteins are generally thought to be able to form either homo- or heterodimers. The E2A proteins can also form homo- or heterodimers with other HLH transcription factors as mentioned. However, in most other cases, E12/E47 proteins form heterodimers with other bHLH proteins and the function of E12/E47 homodimers are known to be restricted in B lymphocyte developmental processes. In addition, not only HLH binding partners, but also non-HLH binding proteins of E2A have been reported in different biological settings with implications of various functional roles of E2A proteins. The interaction of E2A with various binding partners may lead to diverse biological consequences depending on the characteristics of the respective E2A binding partners, in addition to its major role as a transcription factor.

(a) HLH Binding Partners of E2A

E2A proteins can interact with various members of HLH transcription factors and the tissue selectivity of E2A heterodimers is achieved by the cell type-specific bHLH binding partners of E2A including myogenic regulatory factors (MRFs) in skeletal muscle development that constitute MyoD, MRF-4, Myf-5, and myogenin (French, Chow
The E2A proteins play an important role in myogenesis by enhancing DNA binding specificity and affinity (Chakraborty, Brennan et al. 1991; Lassar, Davis et al. 1991; Shirakata, Friedman et al. 1993). In neurogenesis and pancreatic development as well as in transcriptional activation of the insulin gene, BETA2/NeuroD plays an important role as an E2A binding partner (Lee 1997; Naya, Huang et al. 1997; Glick, Leshkowitz et al. 2000). The interaction between E2A and SCL/Tal1 which is originally reported to be involved in T cell acute lymphoblastic leukemia (T-ALL) has also been shown to be essential for the development of hematopoietic cell lineages (Hsu, Huang et al. 1994; Hsu, Wadman et al. 1994; Voronova and Lee 1994; Gould and Bresnick 1998). The MASH genes, vertebrate homologues of *achaete-scute* in *Drosophila*, also interact with E2A and play a role in the generation of both autonomic and olfactory neurons (Johnson, Birren et al. 1992; Guillemot, Lo et al. 1993). ATOH1/MATH1, murine homolog of *atonal* in *Drosophila* that is essential for the generation of cerebellar granule neurons and inner ear hair cells (Ben-Arie, Bellen et al. 1997; Bermingham, Hassan et al. 1999; Uittenbogaard and Chiaramello 1999; Scheffer, Sage et al. 2007) can form heterodimers with E2A, as well (Uittenbogaard and Chiaramello 1999; Scheffer, Sage et al. 2007).

The regulatory role of these bHLH interactions with E2A proteins can further be controlled by ubiquitously expressed dominant negative regulators such as Id proteins, a distinct group of HLH proteins that lack a basic domain as previously mentioned (Benezra, Davis et al. 1990; Langlands, Yin et al. 1997). The interaction between Id1 and E2A proteins appear to be stronger than other E2A protein interactions such as the interaction between E12/E47 and MyoD, consistent with the notion that inhibition of
MyoD action occurs largely by the sequestration of E2A proteins by Id (Lingbeck, Trausch-Azar et al. 2008). Additionally, the E2A proteins can physiologically function as homodimers that are the major relevant DNA binding species in the regulation of B-cell development (Shen and Kadesch 1995). Therefore, as transcription factors, homodimerization or heterodimerization of E12/E47 proteins with other HLH proteins can exert their effects mainly on transcriptional regulation in a wide array of developmental processes.

(b) Non-HLH Binding Partners of E2A

The interactions of E2A proteins are not restricted among the (b)HLH family members. Non-HLH interacting partners of E2A proteins have also been described. The rat homologue of the human polymyositis-scleroderma autoantigen (rPM-Scl) which localizes to the granular layer of the nucleolus and distinct nucleocytoplasmic foci have been shown to specifically interact with a domain distinct from the bHLH motif of E12/E47 (Kho, Huggins et al. 1997). UbcE2A (mUbc9), a ubiquitin-conjugating enzyme has also been demonstrated to interact with the same domain of E12/E47 (477-530 amino acids) as does rPM-Scl and this domain is required for rapid degradation of E12/E47 proteins by the ubiquitin-proteasomal pathway. Deletion of the domain showed an extended half life of E47 and expression of antisense UbcE2A inhibited E12 degradation (Kho, Huggins et al. 1997; Huggins, Chin et al. 1999). More interestingly, by utilizing truncated E47 protein, it was demonstrated that phosphorylation of two serine residues (Ser-514, Ser-529) in the acidic region N-terminal to the E47 bHLH domain could abolish DNA binding of E47 homodimer in vitro and the two serine residues were
hypophosphorylated in B lymphocyte, which may also regulate B cell differentiation (Sloan, Shen et al. 1996). Based on these results, it was suggested that three regulatory mechanisms (degradation, phosphorylation, and possibly nuclear matrix attachment) can converge in the one E2A protein region (Kho, Huggins et al. 1997). Related to the phosphorylation of E12/E47 proteins, the MAPK-activated protein kinases 3pK and MK2 were identified as E47 kinases *in vitro* and the expression of either kinase results in a repression of the transcriptional activity of E47 on an E-box containing promoter (Neufeld, Grosse-Wilde et al. 2000).

The Ca\(^{2+}\) sensor protein, calmodulin, is another case of non-(b)HLH interaction with E12/E47 proteins. Overexpressed E12 and calmodulin demonstrated nuclear co-localization and by utilizing calmodulin inhibition-deficient E12 mutants, this interaction suggested inhibition of DNA binding of E12/E47 proteins through direct binding of Ca\(^{2+}\)/calmodulin to the basic region (Saarikettu, Sveshnikova et al. 2004). During apoptosis, the caspase-mediated cleavage product of p130\(^{\text{cas}}\), which is a C-terminal 31-kDa fragment with high sequence homology to Id proteins can heterodimerize with E12/E47 and translocate to the nucleus resulting in the inhibition of E2A-mediated p21\(^{\text{Waf1/Cip1}}\) transcription (Kim, Kook et al. 2004). A GAP-related interacting protein to E12 named as GRIPE has also been shown to bind to the HLH region of E12 and was suggested to be required for the nuclear import of E12 as well as the negative regulation of E12-dependent target gene transcription (Heng and Tan 2002). Regarding the mechanisms by which E2A proteins regulate transcription and recombination, a transactivation motif, LDFS, present in a subset of HLH proteins including E2A proteins where it is located in AD1 can directly interact with SAGA histone acetyltransferase.
(HAT) complex (Massari, Grant et al. 1999). In addition, interestingly, the coactivator p300, also containing HAT activity, has been shown to interact with E2A proteins when bound to DNA and this interaction is mapped to the bHLH region of E2A. These observations suggest that E-proteins can recruit enzymes involved in chromatin modification and the ability of bHLH proteins to activate transcriptional activity correlates with those transcriptional modulators as exemplified in SAGA and p300/CBP as E box-dependent DNA-binding complexes (Eckner, Yao et al. 1996; Massari, Grant et al. 1999; Massari and Murre 2000). Similar to the complexity among HLH interactions functioning as transcription factors, the non-HLH interactions of E2A as well as those of other HLH proteins such as Id proteins, which are also able to interact with non-bHLH proteins including pRb, Ets, Pax and MIDA-1 (Lasorella, Uo et al. 2001; Zebedee and Hara 2001), appear to have distinctive functional complexities in diverse regulatory stages of biological settings.

(3) DNA Binding Specificity and Transcriptional Regulation of E2A

The homo- or heterodimerization of E2A proteins is a prerequisite to achieve a transcriptional activity (Murre, Bain et al. 1994; Massari and Murre 2000). Most HLH proteins belong to the basic helix-loop-helix (bHLH) family and act as transcriptional activators or suppressors of various target genes by direct binding to canonical E-box element(s) in promoter regions. To date, the transcriptional regulatory functions of E2A have been exploited in many cell lineages, but have been most extensively studied as a critical regulator of B lymphocyte development and in myogenic differentiation. Although the detailed functional mechanism remains unclear, E2A proteins are also
known to have a role in regulation of gene rearrangement and class switch recombination of immunoglobulin genes. The homozygous E2A-null mice showed complete block of B cell development at an early stage and interestingly, the heterozygous E2A mutant mice embryos contain about half the number of B cells compared to wild-type embryos, suggesting the regulation of E2A expression level might translate into the number of B cells (Bain, Maandag et al. 1994; Zhuang, Soriano et al. 1994). E2A proteins were initially identified as binding to the κE2/μ5 sites of immunoglobulin gene enhancers (Murre, McCaw et al. 1989; Henthorn, Kiledjian et al. 1990). The unique sets of Immunoglobulin (Ig) and T cell receptors (TCRs) are generated during lymphocyte maturation through site-specific V(D)J recombination. Particularly, the activation of Igκ locus recombination has been extensively investigated and demonstrated to be regulated by various trans-acting DNA binding proteins including E2A transcription factors (Schlissel 2004). Ectopic expression of E2A and RAG recombinase enables the induction of a diverse repertoire of Igκ recombination in a nonlymphoid cell lines as well (Romanow, Langerak et al. 2000). This study also showed that the transactivation domains of E2A proteins are required to promote VJ recombination and germ line transcription. As such, E12/E47 proteins are known to activate or repress gene expression mainly by the AD1- and/or AD2-mediated transactivation activities (Aronheim, Shiran et al. 1993; Quong, Massari et al. 1993; Massari, Jennings et al. 1996).

As previously mentioned, it is noteworthy that the AD1 of E2A interacts with the SAGA complex, an assembly of proteins containing histone acetyltransferase (HAT) activity and amino acid substitution within AD1 abrogated transcriptional activation in vivo (Massari, Grant et al. 1999). The E2A proteins as well as MyoD have also shown to
interact with nuclear coactivator, p300/CBP, originally identified as nuclear proteins that bind E1A oncoprotein and cAMP-responsive transcription factor CREB, respectively (Eckner, Yao et al. 1996; Massari and Murre 2000; Bradney, Hjelmeland et al. 2003). Different from the interaction with SAGA complex, the region responsible for the interaction of E2A proteins with p300 has been mapped to the bHLH domain of E2A (Eckner, Yao et al. 1996). The involvement of bHLH domain in this interaction is somewhat exceptional since it has been recognized that the intrinsic transcriptional activity resides in the transactivation domains of E2A. Therefore, the transactivation domains as well as bHLH domain of E2A proteins can recruit transcriptional modulators, which contain the capability to modify chromatin structure such as HAT activity and subsequently can alter the transcriptional activity of downstream target genes.

It is recognized that E-proteins including E2A proteins are regulated by the relative intracellular concentrations of E-proteins, class II transcription factors and Id proteins (Zhuang, Cheng et al. 1996; Langlands, Yin et al. 1997). Additionally, it is likely that covalent modifications also affect their activities. For example, the homodimers of E47 are unable to bind DNA when two serines N terminal to the basic helix-loop-helix domain are phosphorylated (Sloan, Shen et al. 1996) and intermolecular disulfide cross-link formation is required for stable homodimerization of E-proteins and DNA binding (Benezra 1994; Markus and Benezra 1999). It was suggested that the homo- or heterodimerization preference is ascribed at least in part to disulfide bond formation regulated by protein disulfide isomerase (PDI) family members. The PDI proteins can reduce the disulfide bond present in E2A homodimeric complexes and render them competent to form heterodimers with other bHLH transcription factors. The E2A
homodimers can form intermolecular disulfide bonds in B cells but not in muscle cells where only heterodimers have been found. Therefore, PDI-mediated regulation is considered to play an important role in the dimerization status of E2A proteins and also in the development of the B lymphocyte lineage (Benezra 1994; Markus and Benezra 1999).

The homo- or heterodimers of E12 and E47 have different DNA binding specificities and affinities. E47 strongly binds to DNA as either a homodimer or a heterodimer with MyoD, while E12 and MyoD bind to DNA efficiently only as a heterodimer by measuring dimerization constants and binding strengths to the κE2 site or its near relative DNA sequences. Thus, E47 homodimers or MyoD heterodimers with E12 or E47 dimerize efficiently and bind to DNA avidly, but E12 homodimerize efficiently while bind to DNA poorly. In contrast, MyoD homodimerizes poorly but binds to DNA strongly (Sun and Baltimore 1991). It is also interesting that electrophoretic mobility shift assay (EMSA) and immuno-depletion experiments utilizing whole cell and nuclear extracts of differentiated C2C12 cells and B lymphocytes (BJAB cells) respectively, along with oligonucleotides that bear one or two adjacent E box elements and monoclonal antibodies for p300/CBP, suggested the interaction between p300/CBP and bHLH proteins such as E47 and MyoD. This experiment also demonstrated that retarded supershift bands were only detected when two E-box containing oligonucleotides were utilized (Eckner, Yao et al. 1996). This observation is correlated well with the notion that bHLH transcription factors to induce transcriptional activation requires at least two E-box binding sites (Weintraub, Davis et al. 1990; Ruezinsky, Beckmann et al. 1991). Therefore, this indicates the number of E-box elements present in
a specific promoter region may be another determinant for bHLH transcription factors to bind DNA and recruit transcriptional modulators to induce E-box dependent transcriptional activation.

It is notable that the presence of an inhibitory domain N-terminal to the basic region of E12 was found to prevent DNA binding in E12 homodimers but not in E12/MyoD heterodimers (Sun and Baltimore 1991). When the κE2 site in the intronic enhancer of immunoglobulin κ light chain gene was utilized as a probe, the intrinsic DNA binding activity of E12 homodimers and stability of E12-DNA complexes were increased by substitution of basic amino acids in the DNA binding domain of E12, indicating the repression effect elicited by the inhibitory domain was alleviated (Vitola, Wang et al. 1996). Therefore, it is also conceived that E12 preferentially forms heterodimers with tissue restricted class II bHLH factors while E12 binds poorly as a homodimer in non-B cells due to the presence of an inhibitory domain. Similar to the notion of an inhibitory domain, another autoregulatory inhibition domain termed the Rep domain has been reported to be a potent inhibitor of AD1 and AD2 transactivation activities (Markus, Du et al. 2002). The Rep domain is mapped between AD2 and the bHLH domain of E2A proteins similar to the inhibitory domain, but it is located more N-terminally than the inhibitory domain. This Rep domain region is found in all E proteins and prevents E-protein homodimers from binding to myogenic enhancers such as MCK enhancer. Thus, while MyoD-E protein heterodimers in immunoglobulin enhancers are transcriptionally inactive possibly by the presence of a cis-acting repression region on enhancer (Weintraub, Genetta et al. 1994), the inactivation of E protein homodimers in myogenic enhancers including MCK enhancer is considered to be due to the
autoregulatory action of the inhibitory Rep domain (Markus, Du et al. 2002). Since this conclusion is quite similar to that of inhibitory domain as described above, at least the region N-terminal to the bHLH motif appears to have a potential to control DNA binding specificity as well as modulate transcriptional activity of E-protein homo- and/or heterodimers.

E2A proteins are also able to modulate the transcriptional activity of other bHLH transcription factors when heterodimerized. The transactivation domains of myogenin, another muscle-specific basic helix-loop-helix (bHLH) transcription factor, were phosphorylated when myogenin was heterodimerized with E2A proteins. This dimerization-dependent phosphorylation of myogenin also resulted in a diminished transcriptional activity, suggesting that E2A proteins have a potential to modulate transcriptional activity by controlling the phosphorylation status of their dimerization partners without affecting DNA binding. It is also intriguing that the region responsible for the dimerization-dependent phosphorylation of myogenin is mapped to the bHLH domain of E2A proteins (Zhou and Olson 1994). In addition, co-expression of E2A proteins with MyoD or Id1 led to alterations in nuclear localization and turnover rates of MyoD and Id1 (Deed, Armitage et al. 1996; Lingbeck, Trausch-Azar et al. 2005) and Cdk2-dependent phosphorylation of Id2 correlates with the restoration of E12/E47 binding to DNA (Hara, Hall et al. 1997). Therefore, the protein degradation and post-translational modification of E12/E47 binding partners such as MyoD and Id proteins can provide another level of controlling mechanism for the transcriptional activity mediated by E2A proteins. It is suggested that to some extent, not only the E-box consensus sequence but also the surrounding sequences of E-box elements can influence the binding
specificity of bHLH complexes and therefore, combinations of dimerized proteins can have their own distinctive binding sequence preferences (Blackwell and Weintraub 1990). This notion is also evidenced by the presence of a cis-acting repression region in immunoglobulin heavy chain enhancer (Weintraub, Genetta et al. 1994).

(4) Post-translational Modification and Degradation of E2A

E2A proteins have shown to be regulated by post-translational mechanisms (Nie, Xu et al. 2003; Lluis, Ballestar et al. 2005). Phosphorylation at specific residue(s) of E2A proteins can influence their DNA binding affinity as well as modulate transcriptional activity. As mentioned, by employing N-terminally truncated E47, phosphorylation of two serines located N-terminal to the E47 bHLH domain was noted in many cell types while hypo-phosphorylation at these sites was found in B lymphocytes. In vitro studies demonstrated that this phosphorylation inhibits DNA binding of E47 homodimers but not of E47 heterodimers, indicating phosphorylation is another level of regulation to determine the DNA binding specificity in early B cell development (Sloan, Shen et al. 1996).

Dependence on substrate phosphorylation is considered to be a characteristic of ubiquitin-mediated protein degradation (Karin and Ben-Neriah 2000; Harper 2001). It is interesting there is evidence which can link phosphorylation and degradation of E2A. The phosphorylation of E2A proteins by p42/p44 MAP kinases is required for Notch-induced degradation of E2A proteins (Nie, Xu et al. 2003). The expression of the intracellular domain of Notch1 (N1-IC) or MEK1 promotes the interaction between E47 and the SCF^{skp2} E3 ubiquitin ligase and N1-IC stimulates proteasome-mediated degradation of
E47 (Nie, Xu et al. 2003). Therefore, although the molecular mechanism regarding the
downstream effectors of Notch signaling that is involved in Notch-induced degradation
of E47 still needs to be explored in more detail, it has been proposed Notch regulates
lymphocyte differentiation by controlling E2A protein turnover (Nie, Xu et al. 2003).

Certain transcription factors are degraded rapidly in vivo (Rogers, Wells et al.
1986) and the ubiquitin-proteasome pathway promotes rapid turn over (Treier,
Staszewski et al. 1994; Tsurumi, Ishida et al. 1995). The mUbc9 (UbcE2A), an E2
ubiquitin conjugating enzyme and mammalian homologue of Saccharomyces cerevisiae
Ubc9p, was identified as a binding partner of E12 from yeast two hybrid interaction
analysis (Kho, Huggins et al. 1997). The S. cerevisiae Ubc9p is a nuclear protein
necessary for cell viability and is likely to be involved in cell cycle regulation and protein
stability. The E2A proteins are shown to be degraded by the ubiquitin-proteasome
pathway since the half life of E2A proteins is extended in the presence of the proteasome
inhibitor, MG132, and mUbc9 antisense leads to a reduction in E12 protein degradation.
Removal of the domain responsible for interaction with mUbc9 (∆478-531) stabilizes the
E2A proteins from degradation (Huggins, Chin et al. 1999). Since the degradation
domain deletion mutant of E2A proteins maintains E-box mediated transcriptional
activation capacity, the degradation domain of E2A is considered to be functionally
separated from other transactivation domains (TADs) and the bHLH domain.

(5) Downstream Target Genes of E2A

The physiological functions of E12/E47 proteins are closely related to their
transcriptional activities. E2A is essential for B lymphocyte development and important
for muscle development as a binding partner of tissue specific muscle regulatory factors (MRFs). Several potential lineage-specific E2A target genes have been reported even though whether these target genes are directly regulated by E12/E47 still remains elusive. The regulatory functions of E2A proteins have been examined in a selected subset of genes crucial for B lymphocyte development. Immunoglobulin heavy and κ light chains, early B cell factor (EBF), surrogate light chains λ5 and VpreB, mb-1 (Igα) promoter, RAG-2 enhancer, the 5' regions of the B29 and TdT loci as well as the butyrophilin-like gene, NG9 (BTL-II), Nfil3 and FGFR2 are suspected to be direct target genes of E2A. It is also suggested that E2A proteins function as either positive or negative regulators in B lymphocyte development (Kee and Murre 1998; Romanow, Langerak et al. 2000; Sigvardsson 2000; Greenbaum and Zhuang 2002; Greenbaum, Lazorchak et al. 2004). The EBF transcription factor is able to form heterodimers with E47 when it binds to the λ5 promoter (Sigvardsson 2000). This is noteworthy since it has been considered that E2A proteins function as homodimers in B lymphocytes. Additionally, EBF can also bind to its own promoter indicating the presence of an auto-regulatory mechanism (Smith, Gisler et al. 2002).

Numerous studies have demonstrated that E12/E47 can inhibit cell cycle progression supporting the notion of E2A proteins as a tumor suppressor (Peverali, Ramqvist et al. 1994; Park, Nolan et al. 1999; Herblot, Aplan et al. 2002). E2A proteins induce p21, INK4A and INK4B that inhibit G1 cell cycle progression (Prabhu, Ignatova et al. 1997; Pagliuca, Gallo et al. 2000). However, although most studies support the tumor suppressive function of E2A proteins, other studies reported that E2A proteins do not inhibit but can even promote cell cycle progression (Yoshihara, Inaba et al. 1995;
Zhao, Vilardi et al. 2001; Song, Cooperman et al. 2004). The cyclin D3, cyclin D2 and cyclin A were identified as potential target genes of E2A proteins (Zhao, Vilardi et al. 2001) and the recruitment of E47 to the cyclin D3 promoter is considered to be independent of the DNA binding activity of E2A proteins (Song, Cooperman et al. 2004).

The tissue specificity of E2A is conferred by the heterodimerization with tissue-specific bHLH proteins such as muscle regulatory factors (MRFs) including MyoD (Weintraub 1993), and E2A proteins can activate MyoD-mediated transcriptional activity (Lassar, Davis et al. 1991). The E12 heterodimers with MyoD or myogenin bind to muscle creatine kinase (MCK) enhancer with high affinity and can exert their effect on myogenic differentiation (Murre, McCaw et al. 1989; Brennan and Olson 1990; Jen, Weintraub et al. 1992; Maleki, Royer et al. 2002). Several MyoD- and/or myogenin-regulated genes were also identified (Bergstrom, Penn et al. 2002; Cao, Kumar et al. 2006) and possibly some portions of those identified genes might be the potential targets of E2A proteins as well. However, the functional redundancy of E-protein dimerization with various muscle regulatory factors makes it difficult to precisely define the direct downstream target genes of E12/E47 in myogenesis.

(6) E2A-Fusion Proteins in Acute Lymphoblastic Leukemia (ALL)

The E2A gene on chromosome 19p13.3 has been demonstrated to be linked to acute lymphoblastic leukemia (ALL) through a chromosomal translocation event and the resulting fusion of 5’ E2A sequences with 3’ portions of other genes generates two well-known fusion proteins, E2A-PBX1 and E2A-HLF (Yoshihara, Inaba et al. 1995; LeBrun 2003). The truncated E2A gene fused to the PBX1 homeobox gene (Kamps, Murre et al.
and to the \textit{HLF} basic leucine zipper gene (Yoshihara, Inaba et al. 1995) are produced by translocations t(1;19)(q23;p13.3) and t(17;19)(q22;p13.3) respectively, and the occurring incidence of E2A-HLF has been reported to be relatively lower than E2A-PBX in pediatric ALL. The most prevailing idea regarding the oncogenic property of E2A-fusion proteins is that leukemogenesis can occur \textit{via} excessive transcriptional activity induced by the transcriptional activation domains of E2A exerting its effect on downstream target genes defined by DNA binding domains of PBX1 or HLF.

E2A has been implicated as a tumor suppressor (Massari and Murre 2000). The role of E2A as a tumor suppressor was first suggested by the observation that most E2A-deficient mice develop spontaneous thymic lymphomas (Yan, Young et al. 1997). Loss of E2A activity results in tumor progression and restoration of its activity gives rise to inhibition of cell growth and increased apoptosis in human leukemic T cells (Park, Nolan et al. 1999). The ectopic expression of E2A proteins in E2A-deficient lymphomas can promote apoptosis (Engel and Murre 1999) and interestingly, in NIH3T3 fibroblast, ectopic expression of E2A blocks the cell cycle in the G1 phase and growth-suppressive activity is counteracted by Id proteins (Peverali, Ramqvist et al. 1994). E2A-PBX1 transgenic mice demonstrated some features observed in E2A-null mice showing perturbation in T- and B-cell development (Dedera, Waller et al. 1993; Bain, Maandag et al. 1994; Bain, Engel et al. 1997). E2A-HLF stimulated foci and anchorage-independent growth in NIH 3T3 cells and the E2A-HLF transgenic mice also induced lymphoid malignancies (Yoshihara, Inaba et al. 1995; Inaba, Inukai et al. 1996; Honda, Inaba et al. 1999). Considering the tumorigenic property of E2A fusions and the anti-proliferative effects of E2A proteins, it was suggested that E2A fusion proteins may function as
dominant negative effectors in regard to wild-type E2A proteins or other related proteins including transcriptional modulators such as coactivators or corepressors, thus possibly impairing the tumor-suppressive function of wild-type E2A and/or downstream target gene regulation controlled by E2A transcriptional complexes (Bayly and LeBrun 2000; LeBrun 2003). Taken together, since the E2A portion of those fusions is required for the oncogenic transformation, E2A proteins appear to play a role in cell growth control.

Nucleocytoplasmic Transport

The compartmentalization of eukaryotic cells is vital for cell survival and normal cellular function. Particularly, the nucleus is one of the most defining features of eukaryotic cells and provides another intracellular controlling point of spatial regulation. The macromolecular movement of proteins and nucleic acids occurs through a complex structure spanning the nuclear envelope (NE) named the nuclear pore complex (NPC). It is a large proteinaceous structure composed of estimated 30-80 nucleoporins (NUPs) in mammalian cells (Kau, Way et al. 2004; Sebastian, Sreeja et al. 2004). The NPC allows both passive and active transport of molecules. However, as molecular weight approaches 20-40 kDa, the passive diffusion via the NPC becomes inefficient and increasingly restricted (Fried and Kutay 2003; Mingot, Bohnsack et al. 2004). In contrast, facilitated receptor-mediated active transport can accommodate molecules of several MDa and a diameter of ~40 nm (Mattaj and Englmeier 1998; Gorlich and Kutay 1999; Conti and Izaurrelde 2001; Mingot, Kostka et al. 2001; Weis 2003). The tight regulation of active nucleocytoplasmic transport is achieved in part by the selectivity of the NPC structure and the various carrier proteins, which recognize their selective cargos, shuttling back
Figure 2. Schematic Diagram of Nucleocytoplasmic Transport Mechanism via Importin-β Transport Receptors. The major function of importin-β superfamily members is the mediation of nucleocytoplasmic transport and they are classified as importins or exportins depending on the directionality where they transport their substrate cargos. The small GTPase Ran confers directionality to the transport reaction. While the concentration of RanGTP is high in the nucleus due to the action of chromatin-associated nucleotide exchange factor RCC1, the concentration of RanGDP is high in the cytoplasm by the concerted action of RANBP1 and RanGTPase-activating proteins (RanGAP) that triggers the conversion of RanGTP into RanGTP. Upon entry to the nucleus, the direct binding of RanGTP to importins causes the release of import cargos while binding of RanGTP to exportins enables them to stably associate with export cargos. Importins leave the nucleus as a complex with RanGTP devoid of cargos while exportins leave the nucleus as a complex with RanGTP and export cargos and then, release their export cargos upon hydrolysis of RanGTP in the cytoplasm. Thus, importins and exportins are regulated in an opposite manner.
and forth efficiently through the NPC (Damelin and Silver 2000; Fried and Kutay 2003). Unlike the unidirectional translocation across other organelle membranes, bidirectional transport through the NPC does not require unfolded protein structure. Instead, the carrier-cargo complex transiently interacts with FG-rich hydrophobic repeat motifs present in NUPs and seemingly dissolves the meshwork formed by the weak hydrophobic interactions inside the NPC channel, which functions as a molecular sieve. Therefore, while hydrophilic proteins remain excluded, the carrier-cargo complexes, that transiently interact with NUPs, rapidly cross the permeability barrier of NPC with little resistance and eventually transit NPC into the nucleus or cytoplasm allowing them to circulate constantly between the two compartments (Mingot, Kostka et al. 2001; Ribbeck and Gorlich 2001; Sebastian, Sreeja et al. 2004).

Nuclear transport receptors can be classified as importins and exportins depending on the directionality whereby they transport their cargos. Importins carry their cargos into the nucleus while exportins take cargos out of the nucleus. Accumulated evidence has revealed that the asymmetric distribution of RanGTP concentration is the major driving force for this active transport of nucleocytoplasmic shuttling (Gorlich, Pante et al. 1996; Izaurralde, Kutay et al. 1997; Kalab, Weis et al. 2002; Smith, Slepchenko et al. 2002). Members of importin β-related transport receptors can form importin-cargo complex independent of or with the help of importin α, which recognizes the nuclear localization signal (NLS) on the substrate cargo. Importin β-related receptors mediate interactions with the nuclear pore complex in a classical import mechanism (Adam and Gerace 1991; Gorlich, Kostka et al. 1995; Weis, Mattaj et al. 1995; Gorlich, Henklein et al. 1996; Gorlich, Pante et al. 1996). Inside the nucleus, high concentration of
GTP-bound Ran is maintained by the action of chromatin-associated nucleotide exchange factor, RanGEF (termed RCC1 in higher eukaryotes). When the importin complex enters the nucleus, the direct binding of RanGTP to the importin-cargo complex causes the release of substrate cargo from the complex (Rexach and Blobel 1995; Gorlich, Pante et al. 1996). In contrast, RanGTP binding to exportin enables cargo loading onto the RanGTP-exportin complex, leading to cytoplasmic translocation of RanGTP-exportin-cargo ternary complex. In the cytoplasm, the relative concentration of RanGDP is high due to the concerted actions of RanBP1 and RanGTPase activating protein (RanGAP) which mediates the conversion of RanGTP to RanGDP. Therefore, the RanGTP is released from the transported importin, enabling it to bind and reload the next substrate cargo for nuclear import. In contrast, the transported exportin-cargo complex is liberated from RanGTP, allowing the cargo to be displaced from the exportin. Thus, upon release of its cargo, the exportin can reenter the nucleus on its own to participate in the next round of the export cycle. Accordingly, importins and exportins behave in a diametrically opposed manner in response to nucleocytoplasmic RanGTP gradient (Gorlich, Pante et al. 1996; Bischoff and Gorlich 1997; Floer, Blobel et al. 1997; Kutay, Bischoff et al. 1997; Kutay, Hartmann et al. 2000).

**Importin-β superfamily (Karyopherin)**

The active transport of macromolecules through the NPC is largely mediated by nucleocytoplasmic carrier proteins of the importin-β superfamily of nuclear transport receptors (also referred to as karyopherins) (Kuersten, Ohno et al. 2001; Macara 2001; Fried and Kutay 2003; Mosammaparast and Pemberton 2004). As previously mentioned,
depending on the direction in which a cargo is transported, members of importin-β superfamily can be classified as importins or exportins. Most members of this family are known to carry their cargos exclusively either into the nucleus (importins) or out of nucleus (exportins) with the exception that yeast Kap142p/Msn5p and mammalian importin 13 transport cargos in both directions (Mingot, Kostka et al. 2001; Yoshida and Blobel 2001; Fried and Kutay 2003). There are many more cargos than known importin-β related transport receptors, suggesting each karyopherin can recognize and transport multiple cargos.

Representative karyopherins include importin β1 and Crm1. Importin β1 (also known as Kapβ1 or yeast Kap95) was the first identified transporter and is responsible for the nuclear import of proteins containing basic nuclear localization signals (NLSs) recognized by importin α (Macara 2001; Fried and Kutay 2003). The basic or classical NLSs, which contain one or two clusters of basic amino acids separated by a linker, were originally characterized in SV40 Large T antigen and nucleoplasmin (Dingwall, Sharnick et al. 1982; Kalderon, Roberts et al. 1984; Gorlich and Kutay 1999). Although the nuclear import of many proteins is considered to be mediated by such basic NLSs, import signals unrelated to the classical basic NLS also exist for example, in M9 sequence of hnRNPA1 (Siomi, Eder et al. 1997; Truant, Fridell et al. 1998). CRM1 (exportin 1) was the first identified export carrier and also possesses broad substrate specificities. It functions in the nuclear export of various substrate cargos that contain a leucine-rich nuclear export signal (NESs) (Kalderon, Roberts et al. 1984; Fornerod, Ohno et al. 1997; Fukuda, Asano et al. 1997; Ossareh-Nazari, Bachelerie et al. 1997; Stade, Ford et al. 1997). The NES recognized by CRM1 is a short motif rich in leucine or related hydrophobic residues as
found in protein kinase A inhibitor PKI or HIV protein Rev (Fischer, Huber et al. 1995; Fornerod, Ohno et al. 1997; Holaska and Paschal 1998; Henderson and Eleftheriou 2000). There are also efficient NESs that do not conform to the prototypical NESs such as occurs in the NFAT transcription factor (Klemm, Beals et al. 1997; Macara 2001).

The crystal structure of importin α (karyopherin α) bound to SV40 large T antigen NLS peptide provided the first insight regarding karyopherin-NLS interaction (Conti, Uy et al. 1998; Kobe 1999). Importin α forms a cylindrical superhelical structure consisting of 10 armadillo (ARM) repeats wherein each ARM repeat consists of approximately 40 amino acids arranged into three helices, and a series of ARM repeats create a binding pocket for the NLS (Conti, Uy et al. 1998; Kobe 1999). Similar to importin α, importin β1 (karyopherin β1) also contains a compact superhelical structure composed of 19 HEAT (Huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase Tor) repeats wherein each individual HEAT motif forms two antiparallel α helices linked by a turn that is reminiscent of an ARM repeat (Malik, Eickbush et al. 1997; Cingolani, Petosa et al. 1999; Vetter, Arndt et al. 1999). A structural model of human CRM1 employing X-ray crystallography, homology modeling, and electron microscopy provided the information that CRM1 resembles transportin1 (Trn1), which mediates the import of mRNA binding and ribosomal proteins, and contains 19 HEAT repeats with a large loop implicated in Ran binding (Petosa, Schoehn et al. 2004; Pemberton and Paschal 2005). Notably, “CRM1 shares sequence similarity with Impβ and other karyopherins over an N-terminal region termed the CRIME (Crm1) domain (Fornerod, van Deursen et al. 1997; Gorlich, Dabrowski et al. 1997). In Impβ and Trn1, this domain corresponds to HEAT repeat 1-3, which recognize RanGTP via its
switch II region (Chook and Blobel 1999; Vetter, Arndt et al. 1999). Consistent with a role in Ran binding, deletion of the CRIME domain eliminates the ability of CRM1 to form a ternary export complex (Ossareh-Nazari and Dargemont 1999). CRM1 possesses a highly conserved central region implicated in RanGTP-dependent NES recognition (Ossareh-Nazari and Dargemont 1999). A cysteine residue in this region is covalently modified by leptomycin B (Holmberg, Buchbinder et al.), a specific inhibitor of CRM1-mediated export (Nishi, Yoshida et al. 1994; Wolff, Sanglier et al. 1997; Kudo, Matsumori et al. 1999)” (Petosa, Schoehn et al. 2004). Considering the crystallography data, the structural flexibility and versatility residing in karyopherins appear to confer the capability to recruit multiple cargos.

The common features of importin β-related nuclear transport receptors are similar molecular weight range (90-145kDa), acidic isoelectric points (4.5-5.9) and most importantly, the presence of an N-terminal RanGTP binding domain (Koch, Bohlmann et al. 2000; Chook and Blobel 2001; Conti 2002). Based on the functional categorization defined by Migot et al. (Mingot, Bohnsack et al. 2004), the importin β-related nuclear transport receptors can also be classified into four major categories based on their functional role in active nucleocytoplasmic transport. First is mediating metabolic transport of constitutively produced macromolecules from one compartment to another such as import or export of tRNA, mRNA and ribosomal subunits (Mattaj and Englmeier 1998; Gorlich and Kutay 1999). The second is recycling reactions such as the intrinsic function of importin β receptors for the transport of their cargos or constitutive import of Ran from one compartment to another (Kutay, Bischoff et al. 1997; Ribbeck, Lipowsky et al. 1998). The third is called regulated transport. This category includes many
transcription factors, which are held in the cytoplasm until appropriate signals trigger their import into the nucleus to control respective downstream target genes (Kaffman and O'Shea 1999). Finally, nuclear export machinery that counteracts slow but steady leaking of materials back to the cytoplasm, such as exclusion of accumulated nuclear actins by exportin 6 or translation factors eIF2β, eIF2Bε, eIF5 and eRF1 by CRM1 (Bohnsack, Regener et al. 2002; Stuven, Hartmann et al. 2003).

**RANBP17 and RANBP16**

The RANBP17 gene was found in the process of cloning genes at the breakpoint of the recurring chromosomal translocation, t(5;14) (q34;q11) in acute lymphoblastic leukemia (ALL) (Whitlock, Raimondi et al. 1994; Koch, Bohlmann et al. 2000; Hansen-Hagge, Schafer et al. 2002). This novel gene is located at 5q34 and identified as a close homologue of RANBP16 (XPO7) that was mapped to 8p21 with 66% amino acid identity and 82% similarity to RANBP17 protein. RANBP16 was also identified by affinity chromatography studies employing immobilized RanGTP and HeLa cell extracts (Kutay, Hartmann et al. 2000). RANBP17 and RANBP16 appear to be conserved among higher eukaryotes and belong to phylogenetically distant members of the importin-β superfamily. Based on the presence of an importin β N-terminal domain, acidic isoelectric point and protein size of ~120 kDa, RANBP17 and RANBP16 are suggested to be novel members of the importin-β superfamily of nucleocytoplasmic transport receptors.

(1) Transcript Expression of RANBP17 and RANBP16
To date, various alternative splicing products of RANBP17 and RANBP16 have been reported. Full-length human RANBP17 protein contains 1088 amino acids and is encoded from a 4450 base mRNA transcript with a 3267 base open reading frame, and originally identified in a human testis cDNA library (Koch, Bohlmann et al. 2000). In addition, mRNA transcripts generated by the introduction of premature stop codon(s) in exon 14 producing four slightly different isoforms, whose protein product we refer to short form(s) of RANBP17 (sRANBP17), were also reported along with a low number of alternatively spliced transcripts that are missing exon 9 (Hansen-Hagge, Schafer et al. 2002). Three isoforms of human RANBP16 have been reported and designated as RANBP16 (a), (b) and (c), which generate 1096, 1087 and 1088 amino acid products, respectively. Compared to RANBP16 isoform (a), RANBP16 isoforms (b) and (c) use an alternate in-frame splice site in the 5’ coding region resulting in shorter proteins. Although there is a difference in their 5’ UTR regions, the protein coding region of RANBP16 isoform (b) and (c) shows only one amino acid difference.

While RANBP16 transcript shows a ubiquitous expression pattern in human tissues, the expression of RANBP17 transcript was reported to be tissue-specific by Northern blot analysis (Koch, Bohlmann et al. 2000). Human ~2.5 and 4.5 kb RANBP17 transcripts were detected in testis and a lower level of expression of 4.5, 7.5 and 10 kb transcripts were found in pancreas (Koch, Bohlmann et al. 2000). A second study reported somewhat different transcript expression, which demonstrated heart, kidney, liver and placenta expressed readily detectable levels of human RANBP17 transcript. However, testis and pancreas samples were not included in this study (Bernard, Busson-LeConiat et al. 2001). RANBP16 transcript expression with a major ~4.8 kb transcript is
Figure 3. Physiological Dendrogram of Importin-β Superfamily Members.

Phylogenetic analysis reveals RANBP17, RANBP16 and exportin 4 (EXP 4) form a separate subgroup distinctive from other members of importin-β superfamily. The members of importin-β superfamily from *Saccharomyces cervisiae* are indicated by the prefix *S.c.* (Kutay, Hartmann et al. 2000).
highest in testis, thyroid and bone marrow, while many other tissues have a moderate level of RANBP16 transcript expression. Two other RANBP16 transcripts with sizes of ~3.5 and 2.5 kb are readily detected in bone marrow (both transcripts) and testis (only the 3.5 kb transcript), while various other tissues show weak expression of RANBP16. Overall, compared to the ubiquitous and readily detectable expression of RANBP16 transcripts in human tissues, RANBP17 transcripts appear remarkably restricted as mentioned. The transcript expression patterns observed for human tissue samples are fairly consistent in murine tissues, though some differences in the details of RANBP16 transcript expression exist. RANBP17 transcript expression is still markedly enriched in murine testis and RNA in situ hybridization studies indicated that RANBP17 transcript was expressed in murine testis at stages IX-XII of primary spermatocytes. The physiological relevance for enriched expression of RANBP17 transcript in testis is currently unknown. The expression of RANBP17 in various cell lines has also been reported, albeit in a rather sporadic manner. RANBP17 expression appears prominent in cell lines of megakaryocytic origin such as MEG01 and MO7E while weaker expression is observed in the erythroid HEL cell line (Bernard, Busson-LeConiat et al. 2001; MacLeod, Nagel et al. 2003).

(2) Protein Structure and Expression of RANBP17 and RANBP16

RANBP17 and RANBP16 proteins are evolutionarily conserved for example, human RANBP17 and RANBP16 have overall 93% and 99% amino acid identity with their murine counterparts. A BLAST search for conserved domains showed that RANBP17 and RANBP16 contain a Crml domain at 8-167 and 29-305 amino acids
respectively, and an importin β N-terminal domain within the Crm1 domain at 30-95 and 30-96 amino acid sequences, respectively. When compared to other members of importin-β superfamily members, the closest homology of RANBP17 and RANBP16 is found in the CRM1 (exportin1) protein, but this homology is restricted to the N-terminal region including the Crm1 domain (Kutay, Hartmann et al. 2000). Crm1 domain includes importin-β N-terminal domain but the difference between Crm1 and importin-β N-terminal domain has not been clearly defined yet. Crm1 domain is important for the binding of Ran that contains the intrinsic GTPase activity. It was suggested that RANBP16 evolved from CRM1 after the divergence of higher and lower eukaryotes (Kutay, Hartmann et al. 2000).

Regarding the intracellular localization of RANBP17 protein, to date, only murine RANBP17 has been investigated. Ectopic expression of eGFP fusion of murine RANBP17 (mRANBP17) in HeLa cells, showed a faint cytoplasmic staining and strong nuclear signal. A speckled distribution of mRANBP17 with less intense nucleoli signal was also observed. However, the intracellular localization of human RANBP17 has not been investigated yet. The intracellular localization of human RANBP16 also demonstrated similar distribution to mRANBP17 in HeLa cells by indirect immunocytochemistry (Koch, Bohlmann et al. 2000). This result was confirmed by a study that showed interaction of RANBP16 with nucleoporins such as Nup153 and nuclear entry independent of energy or additional transport receptors (Kutay, Hartmann et al. 2000).

The biochemical properties of human RANBP16 were examined by the determining the dissociation constant for recombinant RANBP16 and RanGTP. The
result confirmed the direct interaction between RANBP16 and RanGTP and supported RANBP16 is a bona fide member of the importin-β superfamily. In contrast to a high affinity of importins for RanGTP with dissociation constants in the low nanomolar range, in the absence of substrate, exportins show a low affinity in the micromolar range (Kutay, Bischoff et al. 1997; Askjaer, Jensen et al. 1998; Kutay, Lipowsky et al. 1998; Askjaer, Bachi et al. 1999; Paraskeva, Izaurralde et al. 1999). Kutay et al. reported that RANBP16/RanGTP complex had an intermediate dissociation constant (~200 nM), thus making it unable to define the role of RANBP16 as an importin or exportin. They suggested that RANBP16 has a dual function for nuclear import and export (Kutay, Hartmann et al. 2000). However, work by Mingot et al. concluded that RANBP16 (Exp7) functions as an exportin with cargos such as p50RhoGAP, 14-3-3σ as well as possibly eIF1 and mammalian retromer (Mingot, Bohnsack et al. 2004). These proteins were identified to share no apparent specific region of significant homology and no rigid single consensus sequence as a RANBP16 binding motif. Instead, site-directed mutagenesis studies for eIF1 revealed that a short acidic region mainly composed of aspartic acids followed by an amphipathic helix where positively charged hydrophilic lysine residues are exposed to solvent, is important for the interaction of eIF1 with RANBP16. Several positively charged basic patches in p50RhoGAP appear to be critical for the recruitment of RANBP16. This study suggested RANBP16-mediated export differs from CRM1-dependent nuclear export in that substrate specificity that is critical for RANBP16 recruitment is not merely derived from single linear sequence such as the leucine-rich sequence (Nakamura, Largaespada et al.) recognized by CRM1. Instead, a folded structure of motifs where positively charged basic patches play an important role for
RANBP16 recruitment, is considered to be a genuine part of the signature (Mingot, Bohnsack et al. 2004).

(3) Truncated Isoforms and Chromosomal Translocation of RANBP17

As mentioned, it was in testis that alternative splicing at exon 14 of human RANBP17 gene produces four slightly different transcript variants. These are designated 14c, 14b/c, 14c/e and 14c/d/e (Hansen-Hagge, Schafer et al. 2002). Although the endogenous protein products of these transcripts have not been delineated, based on the existence of RANBP17 transcript isoforms, these transcripts can yield two short forms of RANBP17 (sRANBP17) proteins compared to full-length form by the introduction of an in-frame premature stop codon in exon 14 of the RANBP17 gene. While 14b/c can produce a short form of RANBP17 protein that contains 580 amino acids of full-length RANBP17, short form of RANBP17 generated by 14c, 14c/e or 14c/d/e transcripts can have 576 amino acids of full-length RANBP17. Furthermore, aside from the naturally occurring short forms of RANBP17, it is also of interest to note that the chromosomal translocation t(5;14)(q33-34;q11) wherein the chromosomal breakpoint exists at intron 24 or 3' downstream region of RANBP17 gene on chromosome 5 is found in acute lymphoblastic leukemia (ALL) involving T cell receptor (TCR) δ locus on chromosome 14 (Hansen-Hagge, Schafer et al. 2002). It was during the course of cloning of the breakpoint sequences from a leukemia patient with this translocation that Janssen and coworkers initially identified a truncated form of RANBP17 and subsequently cloned the full-length cDNA for RANBP17 (Koch, Bohlmann et al. 2000; Hansen-Hagge, Schafer et al. 2002). Recurrent chromosomal aberrations including chromosomal translocation and
inversion are recognized to be associated with lymphoid malignancy possibly by illegitimate V(D)J recombinase activity. Frequently, inappropriate protein expression of the translocation partner protein is elicited by the juxtaposition of regulatory elements of Ig and TCR genes and the chromosomal translocation t(5;14)(q33-34;q11) is reported to be the case. The aberrant TCR δ rearrangement was found in two ALL patients with t(5;14)(q33-34;q11) resulting in the juxtaposition of TCR δ enhancer element to the immediate 3’ vicinity of the RANBP17 gene on chromosome 5 or to the upstream region of Hox11L2 gene on chromosome 14 (Hansen-Hagge, Schafer et al. 2002). This head-to-tail orientation of TCR δ with RANBP17 and Hox11L2 is able to activate Hox11L2 gene expression and is predicted to generate an increased amount of C-terminally truncated RANBP17 and/or full-length RANBP17 protein possibly leading to activation in cell growth and eventually human leukemias. However, the quantitative and qualitative differences in RANBP17 gene expression between the samples from two ALL patients and the normal samples were not able to be further analyzed due to the limited amount of patient materials in this study (Hansen-Hagge, Schafer et al. 2002). A second type of recurrent chromosomal translocation t(5;14)(q35;q32) involving RANBP17 gene is also associated with T-ALL (Bernard, Busson-LeConiat et al. 2001). The breakpoint is located in intron 20 of RANBP17 gene and this translocation event is expected to generate C-terminally truncated 744 amino acids of sRANBP17. However, the premature termination of RANBP17 transcript, which ends in intron 20, was identified from one T-ALL patient sample, but this study could not find any fusion with chromosome 14 sequences in cloned RANBP17 cDNA fragments (Bernard, Busson-LeConiat et al. 2001).
Figure 4. The Alternatively Spliced Isoforms of RANBP17 and Chromosomal Translocation Events of t(5;14)-Positive Patients. (A) Schematic representation of alternatively spliced RNA products of human RANBP17 cDNA. The largest human RANBP17 cDNA is depicted with relative size and position of the various exons indicated by vertical lines. The grey boxes represent the open reading frame (ORF) and white regions mark 5’ and 3’ untranslated sequences. The three polyadenylation sites and the breakpoint in the t(5;14)-positive patient, are indicated. The alternatively spliced RNAs with their respective ORFs are also indicated below. (B) Schematic representation of the translocation events of t(5;14)-positive patients. The structural map of RANBP17 and Hox11L2 genes on chromosome 5q34-35 and chromosomal translocation events at the breakpoints of the two t(5;14)(q34;q11)-positive patients are depicted. The TCR δ locus with its various V, D and J elements, constant region (Cδ) and transcription enhancer element (δenh) are also depicted with the location of the breakpoints of the two t(5;14)(q34;q11)-positive patients. The bottom of the figure shows the resulting derivative chromosomes in the cases with t(5;14)(q34;q11) (Hansen-Hagge, Schafer et al. 2002).
FIG. 4

Hansen-Hagge et al., JWG Janssen
(Leukemia. 2002 Nov. Vol 16, Number 11, 2205-2212)
**Alterations in nucleocytoplasmic machineries and Cancer**

Nucleocytoplasmic transport *via* NPC is a defining feature of eukaryotic cells. This active transport mechanism requires several intracellular components that include nucleoporins in the NPC, a RanGTP concentration gradient between nucleus and cytoplasm, and transport receptors (karyopherins) for the nucleocytoplasmic transport of various macromolecular cargos. Deregulations in post-translational modifications of cargo substrates or related molecules, nucleocytoplasmic transport machineries and variations in NPC itself are associated with cancers (Kau, Way et al. 2004). Thus, alterations in the nucleocytoplasmic transport mechanism are considered to induce tumorigenesis, allowing it as a novel target for a therapeutic intervention.

Related to the alterations in post-translational modification, various nuclear factors including transcription factors are mislocalized in cancers. NFκB is normally localized in cytoplasm bound to IκB, but when IκB is phosphorylated by IKK and degraded by the 26S proteasome, NFκB can enter the nucleus as a result of unmasking the NLS of NFκB (Beg, Ruben et al. 1992; Ganchi, Sun et al. 1992; Henkel, Zabel et al. 1992). Alterations in IκB phosphorylation status can elicit nuclear mislocalization of NFκB. In addition, the action of p300/CBP and HDAC3 can alter the acetylation status of NFκB and impact the intracellular localization of NFκB (Chen and Greene 2003). In many types of cancers, nuclear distribution of NFκB was found and this nuclear mislocalization is possibly attributed to alterations in post-transcriptional modifications in IκB and NFκB as well as defective activity of related factors including upstream kinases (Rayet and Gelines 1999; Chen and Greene 2003). The cytoplasmic mislocalization of inactivated transcription factor FOXO1a has also been found in PTEN-null cancer cells.
as well as colon and renal cancer cells that do not express PTEN and the cytoplasmic mislocalization of FOXO1a transcription factor is considered to be due to the alterations in phosphorylation status of FOXO1a related to PI3K/AKT signaling pathway (Ramaswamy, Nakamura et al. 1999; Nakamura, Ramaswamy et al. 2000). Similarly, cytoplasmic mislocalization of other factors such as the cell cycle inhibitor p27, and the tumor suppressor p53, were also found in many different types of cancer cells (Moll, Riou et al. 1992; Flamini, Curigliano et al. 1996; Blain and Massague 2002; Liang, Zubovitz et al. 2002; Shin, Yakes et al. 2002; Viglietto, Motti et al. 2002). Therefore, deregulated post-translational modifications of nuclear factors including transcription factors are thought to induce cancer development.

Regarding the alterations in nucleocytoplasmic transport machineries including transport receptors, the presence of a truncated form of karyopherin-α, which is devoid of NLS was identified in a breast cancer cell line (Kim, Kim et al. 2000). The overexpression of the karyopherin-α export receptor, CAS/CSE1 was also detected in various cancers (Brinkmann, Gallo et al. 1996; Behrens, Brinkmann et al. 2001; Wellmann, Flemming et al. 2001). Alterations in karyopherin-α by itself or CAS, which mediates the nuclear export of karyopherin-α may induce alterations in the level of many nuclear factors including p53, BRCA1 and RB as cargos of karyopherin-α leading to cell proliferation (Behrens, Brinkmann et al. 2003). Indeed, the nuclear localization of p53, which is a cargo of karyopherin-α was hampered in breast cancer cells (Kim, Kim et al. 2000). Taken together, these observations support a link between alterations in nuclear machineries and tumorigenesis.

Changes in nucleoporin structure are also associated with cancer. Chromosomal
aberrations in nucleoporin genes can generate several oncogenic fusion proteins such as various NUP98-HOX fusions and a CAN (NUP214)-DEK fusion protein that is involved in acute myeloid leukemia (AML) (Nakamura, Largaespada et al. 1996; Kasper, Brindle et al. 1999; Kau, Way et al. 2004; Nakamura 2005; Palmqvist, Pineault et al. 2007; Ageberg, Drott et al. 2008). Various NUP98 fusion proteins are reported in patients with acute and chronic myelogenous leukemia (AML & CML), myelodysplastic syndrome (MDS), and T cell acute lymphoblastic leukemia (T-ALL) (Lam and Aplan 2001). The resulting chimeric fusions of NUP98 are composed of N-terminal FG repeats juxtaposed to C-terminal fusion proteins. Since FG repeats in nucleoporins are a docking site for karyopherins and also interact with p300/CBP, which can elicit transcriptional activation, the FG repeats in NUP98 fusion proteins may act as a transcription activator by allowing those fusion proteins to interact with other nuclear factors including transcription factors (Kau, Way et al. 2004).

Although currently existing evidence is very limited, the physiological connection between abnormalities in nucleocytoplasmic transport mechanisms and tumorigenesis is feasible and attractive in terms of the development of therapeutic intervention. Since each karyophererin transports its own cargo(s), identification of molecules controlling the nucleocytoplasmic transport of each specific karyophererin as exemplified in leptomycin B for CRM1, might provide a useful tool to precisely control tumorigenesis. Therefore, it is very useful, significant and important to understand the characteristics of karyopherins and related protein-protein interactions as well as their abnormalities in various human diseases including cancer.
MATERIALS AND METHODS

Cell Culture

Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1x non-essential amino acid (Gibco-BRL) at 37°C under a humidified atmosphere of 5% CO₂. To induce myogenic differentiation of murine C2C12 myoblasts, cell culture growth medium were changed at 70-80% confluency to DMEM supplemented with 2% horse serum and 10 µg/ml insulin (differentiation medium) and maintained until 6 days after switching the medium. The differentiation medium was replenished at least every 2 days. For LNCaP cells, 1 mM sodium pyruvate and 10 mM HEPES were added to standard cell culture growth medium.

Yeast Two-Hybrid Screening and Mating Assays

Yeast two-hybrid screening was carried out using the Matchmaker System 3 Kit (Clontech Corp.). A DU145 prostate cancer cell two-hybrid library was generated in the pGADT7-Rec vector using the Clontech Matchmaker 3 Library Construction Kit (Clontech), following manufacturer's instructions using 10 µg of total RNA for cDNA synthesis. The human E12 bait used in yeast two hybrid screening contained amino acids 508-654 subcloned into the EcoRI and BamHI sites of the pAS2 vector (Clontech). Approximately 1 x 10⁶ clones of the amplified library were subject to screening by yeast mating. The yeast mating mixture was grown on Leu⁻, Trp⁻, His⁻ nutrient selective media for 10 days followed by processing for the standard filter-lift β-galactosidase assay.
Resultant clones were subject to isolation of yeast plasmid DNA and transformation into *E. coli* DH5α, followed by preparation of *E. coli* DNA. The library clone DNA was retransformed into *S. cerevisiae* strain AH109 and tested for bait specificity by mating with *S. cerevisiae* strain Y187 that harbored the indicated E12 bait construct, lamin negative control construct, or pGBTK7 empty vector. Mating mixtures were plated on the either double (Leu−, Trp−) dropout (DDO) or triple (His−, Leu−, Trp−) dropout (TDO) nutrient selective media and bait specificity of the interaction scored by appropriate growth pattern on selective media and β-galactosidase and/or X-α gal activity. E12 bait-specific library clones were subject to DNA sequencing analysis.

**Mammalian Two-Hybrid Assay**

For mammalian two hybrid assay, the same fragments of E12 bait in pAS2 and RANBP17 library clone in the pGADT7-Rec yeast two-hybrid vectors that generate 508-654 amino acids of E12 and 1-252 amino acids of RANBP17, were transferred into the EcoRI and BamHI sites of the mammalian two-hybrid expression vectors, pVP16 and pM (Clontech), such that they were in reading frame with VP16 transactivating and the GAL4-DNA binding proteins, respectively. A firefly luciferase construct, pGAL4-Luc, was kindly provided by Dr. B. Rowan (Tulane University, New Orleans, LA) and utilized as a reporter gene for protein-protein interaction. pGAL4-Luc contains a GAL4 DNA binding site upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene. Subconfluent HeLa cells (4x10^4 per well in a 24 well plate) were plated and transfected the following next day with Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA). Indicated combinations of DNA constructs (0.2 µg for each) were co-
transfected with the pGAL4-Luc luciferase reporter and pRLTK vector plasmids (0.4 µg and 0.2 µg per well respectively) and 48 hrs after transfection, cells were lysed with Passive lysis buffer and 20 µl of sample was used to measure dual luciferase activity according to manufacturer’s protocol (Dual-Luciferase Reporter Assay System, Promega corp, Madison, WI). The Renilla luciferase activity for pRLTK vector was measured to normalize variation in transfection efficiencies. Experiments were performed at least in triplicate and the relative luciferase activity was calculated by the ratio of firefly and Renilla luciferase activity.

**Semi-quantitative End-Point RT-PCR and Northern Blot Analysis**

RNA was isolated from cultured cells using TriZol Reagent (Invitrogen Corp.), according to manufacturer’s instruction. For semi-quantitative end-point RT-PCR, cDNA was prepared with 1.5 µg of total RNA by using SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen Corp.) and subsequently, PCR was performed with ¼ diluted amount of prepared cDNA by utilizing the RT² PCR Primer Set for Human RANBP17 (SuperArray Bioscience Corp.) that detects both the full-length and short forms of the RANBP17 transcripts generating a 193 bp amplicon. The signal was visualized by ethidium bromide staining on a 1.5% agarose gel and human GAPDH was used for normalization. For Northern blots, 5 µg of total RNA was fractionated in 1% agarose-formaldehyde gels in MOPS buffer and transferred to Hybond-N membrane (GE Healthcare, Piscataway, NJ). Blots were hybridized in ExpressHyb solution (BD Biosciences Clontech, Palo Alto, CA) with a ³²P-labeled random-primed fragment of 561 base pairs of RANBP17 cDNA that is positioned at 2379 - 2939 bps of the NM_022897
sequence for human RANBP17. After washing, membranes were exposed at -80°C to Kodak Biomax film with a Kodak Biomax intensifying screen and also subjected to phosphorimager analyses using Typhoon 8600 PhosphorImager and ImageQuant software (GE Healthcare).

Quantitative Real-Time PCR

First-strand cDNA was synthesized using 5 µg of DNase I-treated total RNA. The cDNA levels of target genes were analyzed by SYBR Green-based real-time PCR in 25 µl reactions containing 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM each forward and reverse primers, and 10 ng of cDNA. Real-time PCR was conducted with an ABI 7500 Real-Time PCR System. PCR was carried out over 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 40 s, with an initial cycle of 50°C for 2 min and 95°C for 10 min to activate AmpliTaq Gold DNA polymerase; a dissociation curve was generated over the range of 60–95°C. Expression of each gene was normalized against GAPDH transcript level. The cycle threshold value was generated using ABI Prism 7500 SDS software, version 1.2, and then exported to a Microsoft Excel spreadsheet. Fold changes in relative gene expression values were determined by the \( \Delta\Delta C_T \) method and are shown as means ± SD in triplicate. Among the samples tested, the lowest transcript expression level was set at a value of 1. The gene-specific primer pairs utilized in this analysis were as follows: \( RANBP17 \) forward 5’-TTGATAAACAGCCAGCCC-3’, reverse 5’-CCTCTGCCACATCTCTTCTG-3’; \( RANBP16 \) (\( XPO7 \)) forward 5’-TTCCGAGAAACCAGCAAGATG-3’, reverse 5’-TAACTCCCACTGAGAGCAGC-3’; \( E12 \) (\( TCF3 \)) forward 5’-AGAAGCCCCAGACCA-3’.
AACTG-3’ reverse 5’-TCTCCAACCACACCTGACAC-3’; p21 forward 5’-CCAGCCTGACAGATTTCTATC-3’, reverse 5’-AAGACACACAGAGTGAGGG-3’; MyoD1 forward 5’-ATCCGCTACATCGAAGGTC-3’, reverse 5’-CCGCTGTAATCCATCATGC-3; MYH3 forward 5’-AGATTGACGACCTCTCCAGC-3’, reverse 5’-TCTCCTCATTCTTGCCCCTG-3’; MCK forward 5’-TTCTCACCCTGCTTCGCTTTCTG-3’, reverse 5’-AGCTGCACCTGTTCGACTTC-3’.

E-box Dependent Functional Assay

An E-box containing firefly luciferase reporter construct, termed E-box pGL3, was generated via insertion of three copies of the consensus E-box element, CAGGTG to the upstream of an SV40 minimal promoter, followed by a firefly luciferase gene in pGL3-Promoter vector (Promega Corp.). Full-length E12 (E121-654) was subcloned into pcDNA3.1 vector (+) (Invitrogen Corp.) at the HindIII and BamHI restriction sites. The short form of human RANBP17 (RANBP171-576) was obtained from American Type Culture Collection (ATCC) and full-length human E47 from Open Biosystems as I.M.A.G.E. cDNA clones with GenBank accession numbers BI824159, BC110579 respectively. RANBP171-576 was present in pCMV-SPORT6 vector and full-length E47 clone was subcloned into the pcDNA3.1 (+) vector at the EcoRV and XbaI restriction sites. A mammalian expression construct for full-length human RANBP16 and RANBP17 contain three copies of an HA tag at their N-termini. RANBP16-pMT2SM and RANBP17-pMT2SM were a kind gift from Dr. W.G. Janssen (University of Heidelberg, Germany). The Crm1 domain of RANBP17 (RANBP172-167) was constructed in the pCMV-HA vector utilizing PCR primer sets with an introduction of EcoRI and
KpnI restriction sites as follows: \textit{RANBP17}^{2-167} forward 5’-GCCCGAATTCTGGCGCTG CACTTCCAGAGTTTG-3’, reverse 5’-GGCGGTACCTCATGTTCTCCAAGTTGTTG GAATCTG-3’. The mass of DNA plasmids transfected per well of a 24 well culture plate were E12-1-654–pcDNA3.1 (0.004 µg), MyoD-pcDNA3.1 (0.1 µg), 3X-HA tagged full-length RANBP17 (RANBP17^{1-1088}-pMT2SM, 0.02 µg) or 3X-HA tagged full-length RANBP16 (RANBP16-pMT2SM, 0.02 µg), a short form of RANBP17 (RANBP17^{1-576}-pCMV-SPORT6, 0.02 µg), Crm1 domain of RANBP17 (RANBP17^{2-167}, 0.02 µg), full-length E47-pcDNA3.1 (0.004 µg) and Id1-pcDNA3.1 (0.5 µg) together with internal control plasmid pRLTK (0.1 µg) and either E-box pGL3 (0.2 µg) or pGL3 luciferase plasmid (0.2 µg). As needed, the total combined mass of DNA per well of a 24 well plate was equalized to 1 µg or 1.2 µg (the latter when Id1-pcDNA3.1 was utilized) with the addition of empty pcDNA3.1 vector. Cotransfections were carried out in HeLa cells using Lipofectamine 2000 (LP 2000) reagent (Invitrogen Corp). At 48 hrs post-transfection, cell lysates were prepared as directed by manufacturer’s instructions (Promega Corp., Madison, WI). However, since we noted that expression from the same internal control pRLTK construct was markedly affected by combinations of plasmid constructs used, luciferase activity was ultimately determined by normalization to total protein content using a standard protein assay (Bio-Rad). Each sample was prepared at least in triplicate and measured using a Turner Systems dual luminometer in accordance with the manufacturer’s instruction.

**Immunocytochemistry and Intracellular Localization Studies**

The DNA plasmid constructs utilized for immunocytochemistry and co-
immunoprecipitation experiments are the same as those used in E-box dependent functional assays. In addition, the truncated form of E12 (E12$^{502-654}$) was initially constructed in the pGBK7 vector utilizing PCR primer sets with an introduction of EcoRI and SalI restriction sites as follows: $E12^{502-654}$ forward 5’-GCCGAATTCAAGGAGGAGGACGAGGAGGACGAGGAGGACCGAGGAGAAGACG-3’, reverse 5’-GGCGTCGACTCACATGTGCCGGCGGGGT-3’. Then the $E12^{502-654}$ fragment was transferred into EcoRI and SalI sites of pEGFPC2 vector and finally resubcloned in BglII and KpnI sites of pCMV-Myc vector. For immunostaining studies, COS cells were plated on coverslips in 6 well plates and transfected with total of 4 µg of DNA plasmids for full-length E12-pcDNA3.1 (E12$^{1-654}$, 0.8 µg), Myc-tagged truncated form of E12-pCMVMyc, which contains bHLH domain of E12 (E12$^{502-654}$, 0.8 µg) and 3xHA tagged full-length RANBP17-pMT2SM (RANBP17$^{1-1088}$, 3.2 µg) either separately or in the indicated combinations using Lipofectamine 2000 reagent (Invitrogen). Empty vector (pcDNA3.1) was used to make up total mass of 4 µg of DNA. 48 hrs after transfection, cells were washed twice with PBS and fixed with 100% ice-cold methanol for 10 min., washed again in PBS, followed by incubation in a blocking solution comprised of 0.1% BSA in PBS for 30 min. at room temperature. After blocking, cells were incubated with polyclonal E2A/E12 (1:100, Santa Cruz Biotech.) and/or monoclonal HA (1:100, Covance Corp.) primary antibodies added to 0.1% BSA in PBS for 1.5 hrs. Cells were then washed with 0.1% BSA in PBS three times and incubated with Alexafluor 568-conjugated goat anti-mouse secondary antibody (1:800, Invitrogen Corp.) and/or FITC-conjugated rabbit IgG (1:200, Bio-Rad) secondary antibodies, as indicated. In some instances, following immunostaining, cells were washed with dH2O and incubated with 10 µM DAPI (Invitrogen Corp.) for 10 min to stain nuclei.
Coverslips were mounted on glass slides and cells were observed at 400 X or 600 X magnifications using a Nikon Eclipse E800 fluorescence microscope equipped with a digital camera. Image acquisition and merging was performed with Image-Pro Plus software (Media Cybernetics, Carlsbad, CA). For quantitative assessment of intracellular localization of immunostaining signals, cells were scored for the cellular localization of E12<sup>1-654</sup>, E12<sup>502-654</sup> and RANBP17<sup>1-1088</sup> as predominately cytoplasmic, predominantly nuclear or equally distributed between the nucleus and the cytoplasm, similar to a previously published methodology (Yagita, Tamanini et al. 2002; Lingbeck, Trausch-Azar et al. 2005). For each determination, 50-100 cells were scored from three independent transfections.

**Subcellular Fractionation, Co-immunoprecipitation and Immunoblot Analyses**

For protein expression, HeLa cells were grown in 6 well plates and transfected with a total of 4 µg of empty vector (pcDNA3.1) or 3xHA-tagged full-length RANBP17-pMT2SM (RANBP17<sup>1-1088</sup>) DNA per well, using Lipofectamine 2000 reagent (Invitrogen Corp.). Cells were harvested at 48 hrs post-transfection by lysis in TNN<sup>+</sup> buffer (10 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA) supplemented with a protease inhibitor cocktail. Cell lysates were incubated on ice for 30 min. with intermittent vortexing and after centrifugation at 20,800 x g for 15 min, supernatant was collected and protein content determined (Bio-Rad, Hercules, CA). For subcellular fractionation, cell lysates were prepared according to instructions supplied with the Nuclear Extract Kit (Active Motif, Carlsbad, CA). For immunoblot analysis, 30 µg of protein extract was loaded on 10% SDS-PAGE gels, followed by electroblotting onto
PVDF membrane with 0.025 M Tris/0.192 M glycine transfer buffer supplemented with 20% methanol. Membranes were blocked for 1 hr in 5% non-fat milk in PBS containing 0.5% Tween 20 (PBS-T) followed by 1 hr incubation at room temperature with mouse monoclonal HA (1:2000, Covance Research Products, Berkeley, CA), monoclonal lamin A/C (1:200, Affinity BioReagents) or rabbit polyclonal tubulin-β antibodies (1:10,000, COVANCE). Secondary antibody was HRP-conjugated goat anti-mouse (1: 2000, Santa Cruz Biotech.) or anti-rabbit (1: 2000, Bio-Rad) antibody and the signal was detected by ECL Plus enhanced chemiluminescence (GE Healthcare).

For co-immunoprecipitation, COS cells were transfected separately or in combination with the indicated expression constructs of 3xHA tagged RANBP171-1088-pMT2SM, RANBP172-167-pCMVHA, full-length E121-654-pcDNA3.1 or truncated E12502-654-pCMVMyc plasmids. Total of 4 µg of DNA plasmids for E12 (0.8 µg) and RANBP17 (3.2 µg) constructs were used for transient transfection. Empty vector (pcDNA3.1) was utilized to make up total mass of 4 µg of DNA when necessary. At 48 hrs post-transfection, cell lysates were prepared by sonication in TNN(+) lysis buffer (10 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA supplemented with a protease inhibitor cocktail) and 30 or 50 µg of cell lysate was incubated with E2A/E12 primary antibody by rotation overnight at 4°C followed by incubation for 3-5 hrs with 20-30 µl of 50% (v/v) suspension of protein A-agarose beads (Santa Cruz Biotech, Inc.). After centrifugation at 10,800 x g for 30 seconds, pelleted beads were washed three times with cell lysis buffer and resuspended in 100 µl of 1 x SDS-PAGE gel loading buffer followed by boiling at 95-100°C for 10 min. After recentrifugation, 30-50 µl of supernatant was subjected to immunoblot analysis.
RESULTS

Two Hybrid Analyses for Identification of a Novel Interaction Partner of E12

The yeast two-hybrid system was employed to search for novel binding partners of E12, using a form of E12 that includes the bHLH domain in its C-terminus (E12<sup>508-654</sup>) fused to the GAL4 DNA-binding domain as bait. Within the bHLH domain located at amino acids 547-607 of E12, it is the basic region that confers DNA binding and the HLH region that provides a protein-protein interaction interface for homo- or heterodimerization with other bHLH transcription factor family members. Furthermore, since full-length E12 also contains two intrinsic transactivation domains (AD1 and AD2) in its N-terminus, which are found to autonomously induce transcriptional activation of the yeast-two hybrid reporter genes, E12<sup>508-654</sup> was chosen as the bait in our yeast two-hybrid assay. Following screening of 2 x 10<sup>6</sup> clones of a DU145 prostate cancer cell yeast two-hybrid library, one library clone was consistently detected in our assay and identified as an N-terminal coding region of an importin-β superfamily member, named RANBP17. It encompasses the amino acids 1-252 of full-length RANBP17 (RANBP17<sup>1-252</sup>) and maintains reading frame with the GAL4 activation domain.

Figure 5A shows the schematic illustration of the full-length E12 protein consisting of 654 amino acids and also depicts the region of E12 used as bait that encompasses the bHLH domain of full-length E12 (E12<sup>508-654</sup>). Also shown in Figure 5A is a schematic representation of full-length RANBP17 of 1088 amino acids and the RANBP17<sup>1-252</sup> library clone identified in yeast two-hybrid screening. RANBP17 is a little-studied member of the importin-β superfamily and phylogenetic analysis has
revealed that RANBP17 and its closely related protein, RANBP16, are the least conserved members of the importin-β superfamily (Kutay, Hartmann et al. 2000).

To confirm the bait specific interaction of the yeast two-hybrid E12 bait (E12\(^{508-654}\)) with the RANBP17 library clone (RANBP1\(^\text{1-252}\)), these fusion plasmids were separately transformed into either Y187 or AH109 strains of \(S.\ cervisiae\), and diploids were produced by yeast mating. The diploid yeast harboring both bait and the library vectors can grow on tryptophan- and leucine- deficient double dropout (DDO) agar media plates. The left panel of Figure 5B indicates successful mating of each of the indicated pairwise combinations of DNA binding and DNA activation domain plasmids. The middle panel of Figure 5B shows mated yeasts grown on tryptophan-, leucine- and histidine-deficient triple dropout (TDO) agar media plates; here the interaction was assessed by the ability of yeast to grow in the absence of histidine (His\(^-\)). Since Id1 is a well known binding partner of E12, the interaction of E12 and Id1 was utilized as a positive control, and empty vector mating as a negative control. In regard to RANBP1\(^\text{1-252}\) interaction with E12\(^{508-654}\), the result showed it to be bait-dependent in that only the yeast that contains both E12\(^{508-654}\) and RANBP1\(^\text{1-252}\) constructs evidenced growth on TDO media (Figure. 5B middle panel). No growth was found for TDO media with RANBP1\(^\text{1-252}\) in combination with empty bait vector or with an unrelated bait, lamin C.

The colorimetric detection of X-α-galactosidase activity, an additional reporter gene for protein-protein interaction in this system was shown in the right panel of Figure 5B and confirmed the bait -specific interaction of RANBP1\(^\text{1-252}\) and E12\(^{508-654}\). Next, since the protein-protein interaction in yeast two-hybrid analysis may sometimes show false positive results due to, for example, lack of post-translational modification or
Figure 5. Yeast Two-Hybrid Analysis Identifies RANBP17^1-252 As a Binding Partner of E12^{508-654} protein. The interaction of E12^{508-654} and RANBP17^{1-252} in yeast two-hybrid assay. (A) Schematic representation of full-length E12 (E12^{1-654}) and full-length RANBP17 (RANBP17^{1-1088}) as well as E12 bait (E12^{508-654}) and RANBP17 library clone (RANBP17^{1-252}) used in yeast two-hybrid assay. (B) The E12^{508-654} and RANBP17^{1-252} expressing vectors (E12^{508-654}-pAS2 or E12^{502-654}-pGBKT7 and RANBP17^{1-252}-pGADT7) as well as empty vector (pGBKT7 or pGADT7) were transformed into two different mating types of yeast strains, Y187 and AH109 respectively. After transformation, growing yeasts were mated in various combinations as indicated and streaked on tryptophan and leucine deficient double dropout (DDO) and tryptophan, leucine and histidine deficient triple dropout (TDO) media plates. Lamin C was used as a negative control and mating of E12^{508-654} and full-length Id1 was used as a positive control. Note that only the mating of E12^{508-654} and RANBP17^{1-252} transformed yeasts and the positive control (E12-Id1) demonstrated positive growth on TDO media plates. Interaction was also confirmed by the blue colony growth of yeasts on medium agar plates containing X-\alpha-gal (20 mg/ml).
FIG. 5

A.

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654

508

1088

252

B.

- PGADT7
- RANBP17
- Id1

- DDO (Trp⁺, Leu⁻)
- TDO (Trp⁻, Leu⁺, His⁻)
- X-α-Gal

pGBK7
E12
Lamin C
Figure 6. Mammalian Two-Hybrid Analysis Confirms the Interaction Between E12<sup>508-654</sup> and RANBP17<sup>1-252</sup> In Mammalian Cells. The interaction of E12<sup>508-654</sup> and RANBP17<sup>1-252</sup> in HeLa cells. (A) Schematic illustration of mammalian two-hybrid assay. The specifics are tailored for use in mammalian cells. Instead of GAL4-AD fusion protein and nutritional selection marker HIS gene utilized in yeast two-hybrid analysis, VP16 fusion protein and luciferase gene to measure luciferase activity were utilized in mammalian two-hybrid analysis (asterisks). (B) The validity of mammalian two-hybrid assay system was evaluated by using two well-known interactors of MyoD and Id1. (C) The interaction of E12<sup>508-654</sup> and RANBP17<sup>1-252</sup> fusion proteins was tested in HeLa cells. A total 1µg of DNA was used for each transfection. Along with pRLTK (0.2 µg) and pGAL4-Luc plasmids (0.4 µg), combinations of tested plasmids (0.2 µg for each) as indicated were co-transfected into HeLa cells (4x10<sup>4</sup> cells/well plated in a 24 well plate on the day before transfection). 48 hrs after co-transfection, HeLa cell lysates were analyzed to measure the relative luciferase activity. Data represent the mean ± S.D. from a minimum of three independent transfections.
FIG. 6

A. GAL4 Luc. Reporter Construct

B. Relative Luciferase Activity (A.U.)

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C. Relative Luciferase Activity (A.U.)

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<td>+</td>
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* p < 0.01
mislocalization of the protein of interest, mammalian two-hybrid analysis was also employed to assess the protein-protein interaction between E12 and RANBP17. The same cDNA inserts for E12 and RANBP17 utilized in the yeast two-hybrid experiments were transferred into the activation and binding domain vectors, pVP16 and pM for mammalian two-hybrid analysis (Figure. 6A). These plasmid constructs along with the pGAL4-Luc reporter and a pRLTK internal control construct were co-transfected into HeLa cells and luciferase activity was assessed at 48 hrs post-transfection. Before testing the interaction of E12\textsuperscript{508-654} and RANBP17\textsuperscript{1-252}, we tested the mammalian two-hybrid system using two well-known interactors, MyoD and Id1 as a positive control and a significant increase in luciferase activity validates this assay (Figure. 6B). Figure 6C shows that, in comparison to empty vector transfectants, a 5.7 fold increase in relative luciferase activity was observed in cell lysates transfected with E12 and RANBP17 mammalian two-hybrid fusion constructs. Compared to other negative controls, more than a 5 fold increase in relative luciferase activity was also observed. The significance of $p < 0.01$ was obtained from the comparison of cell lysates co-transfected with E12\textsuperscript{508-654} and RANBP17\textsuperscript{1-252} with all negative controls.

**Protein Expression and Subcellular Fractionation of Full-length RANBP17**

An interaction between E12\textsuperscript{508-654} and RANBP17\textsuperscript{1-252} was observed by yeast two-hybrid and mammalian two-hybrid analyses. However, these two assays employed fusion proteins namely DNA binding and activation domain proteins. As such, it is important to assess the interaction between full-length E12 and full-length RANBP17. Compared to the well-studied details for E12 protein, to date characteristics of the
RANBP17 protein is minimal. Therefore, we first examined the protein expression and intracellular localization of RANBP17 using a HA-tagged RANBP17 plasmid construct. Expression of the exogenous protein by transient transfection was employed in our studies for RANBP17 since no RANBP17 primary antibody with a reasonable quality is currently available based on our previous testing results (data not shown).

The N-terminally 3x HA-tagged full-length RANBP17-pMT2SM (RANBP17$^{1-1088}$) was a kind gift from Dr. W.G. Janssen (University of Heidelberg, Germany). However, there was a point mutation at amino acid 67 of full-length RANBP17 (reference sequence; NM_022897), which substitutes leucine with proline and to date, no polymorphism at this amino acid was reported. Therefore, we fixed it utilizing restriction enzymes with RANBP17$^{1-252}$-pGADT7 used in the yeast two-hybrid analysis, which has a correct RANBP17 sequence. Transfection of HeLa cells with the corrected plasmid construct of 3xHA-tagged RANBP17$^{1-1088}$ yielded a single species of protein with a molecular weight of $\sim$120 kDa consistent with a previous report (Hansen-Hagge, Schafer et al. 2002) (Figure. 7A). To assess the intracellular localization of full-length RANBP17, HeLa cell lysates that had been transfected with the 3xHA-tagged full-length RANBP17$^{1-1088}$ expression construct were separated into nuclear and cytoplasmic fractions and assessed for subcellular protein expression by immunoblotting with a HA primary antibody. The results shown in Figure 7B revealed the presence of full-length RANBP17 protein in both nuclear and cytoplasmic fractions. In this Figure, immunoblot analysis of both cell fractions with tubulin and lamin A/C primary antibodies indicated efficient fractionation of cytoplasmic and nuclear protein fractions, respectively.
Figure 7. Nuclear and Cytoplasmic Subcellular Fractionation Demonstrates the Full-length RANBP17 Localizes in both Nucleus and Cytoplasm. (A) Protein expression of RANBP17\textsubscript{1-1088} by immunoblot analysis. The empty vector (pcDNA3.1) or N-terminally 3xHA-tagged full-length RANBP17 expression construct (RANBP17\textsubscript{1-1088}) was transfected into HeLa cells using LP2000 transfection reagent. 48 hrs post-transfection, whole cell lysates were subjected to immunoblot analysis using monoclonal HA primary antibody (1:2000). Tubulin was used as a loading control and detected with a polyclonal antibody to tubulin-β (1:10,000). (B). Nuclear and cytoplasmic subcellular fractionation. Cell lysates were subjected to nuclear and cytoplasmic fractionation. The HeLa cell lysates transfected with empty vector (pcDNA3.1) or 3xHA-tagged RANBP17\textsubscript{1-1088} expression construct were prepared according to a manufacturer’s instruction (Active Motif) and a total of 30 µg protein extracts from either nuclear or cytoplasmic fraction were subjected to immunoblot analysis. Tubulin and lamin A/C were used as positive markers for the cytoplasmic and nuclear fraction. Protein expression was detected by monoclonal lamin A/C (1:200) and polyclonal tubulin-β (1:10,000) primary antibodies, respectively (M = molecular mass, marker in kDa).
FIG. 7

A.

Ab:

HA

Tub β

B.

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Immunostaining for Intracellular Localization of Full-length RANBP17

To further confirm the intracellular localization of full-length RANBP17, immunocytochemical assessment was carried out utilizing COS cells transfected with 3xHA-tagged RANBP17$^{1-1088}$ plasmid construct. While a previous report using a RANBP17-GFP fusion protein indicated that mouse RANBP17 is primarily nuclear with a faint signal in cytoplasm, our immunostaining data in Figure 8 demonstrated 3xHA-tagged human RANBP17 is localized primarily in both nucleus and cytoplasm. The immunocytochemical signal of RANBP17 also demonstrated distinctive intracellular localization patterns such as predominantly cytoplasmic, predominantly nuclear or equal distribution in both nucleus and cytoplasm whose numerical assessment is described more in detail in Figure 16. A prominent nuclear rim staining suggested its association with NPC. Intense nuclear speckles, possibly through its association with nuclear factors involved in cellular proliferation, DNA synthesis or DNA repair mechanisms, were also observed in a subset of population of cells similar to a previous report (Koch, Bohlmann et al. 2000). Under the same immunostaining conditions, the intracellular localization of full-length E12 showed an almost exclusively nuclear distribution. In the absence of HA primary antibody or in an empty vector transfection (pCDNA3.1), no significant positive immunostaining signal was detected.

Transcript Expression of RANBP17 by Northern Blot and Semiquantitative End-Point Reverse Transcription PCR

Characterization of human RANBP17 to date is limited mostly to demonstration that multiple forms of the transcript exist in human and mouse tissues and that testis is
Figure 8. Indirect Immunocytochemistry Demonstrates Various Patterns of Intracellular Localization of Full-length RANBP17. Indirect immunocytochemistry for RANBP171-1088 and E121-654. To visualize the subcellular localization of 3xHA tagged full-length RANBP17 (RANBP171-1088), COS cells were transfected with RANBP171-1088 and 48 hrs post-transfection, subjected to indirect immunostaining with monoclonal HA primary antibody (1:100) followed by incubation with Alexafluor 568-conjugated goat anti-mouse secondary antibody (1:800) for visualization (upper panel). Under the same conditions, COS cells were also transfected with full-length E12 (E121-164) and incubated with polyclonal E2A/E12 primary antibody (1:100) followed by incubation with FITC-conjugated rabbit IgG (1:200) for comparison (middle panel). No signal was detected when empty vector (pCDNA3.1) or when either no HA primary antibody or no E12/E2A primary antibody was utilized (bottom panel).
where RANBP17 evidences the highest transcript expression. Studies of RANBP17 transcript expression in a number of human tissues indicates that testis appears to be the most predominant site of RANBP17 expression with two major transcripts (~2.5 and ~4.5 kb) and moderate expression of RANBP17 is observed in pancreas with ~4.5, 7.5 and 10 kb transcripts (Koch, Bohlmann et al. 2000). Its transcript expression in cell lines has not been as thoroughly examined. However, expression of RANBP17 was reported prominent in those of megakaryocytic origin, namely MEG01 and MO7E while weak RANBP17 transcript expression was also observed for the erythroid HEL cell line (Bernard, Busson-LeConiat et al. 2001; MacLeod, Nagel et al. 2003). In an effort to further elucidate the cell type-specific expression of RANBP17, Northern blot analysis was conducted on a panel of human cancer cell lines. As is shown in Figure 9A, all the cell lines tested showed a similar level of RANBP17 transcript expression. The expression level among these cell lines was considerably less than that detected in the human testis sample included as a positive hybridization control.

Given that the yeast two-hybrid library we screened was generated from DU145 prostate cancer cells, we asked whether RANBP17 transcript might show a differential level of expression in prostate cancer cell lines that represent distinct stages of malignancy. Three human prostate cancer cell lines were compared. LNCaP cells are androgen-dependent and do not evidence anchorage-independent growth, two features found in less aggressive prostate cancer. DU145 and PC3 cells are androgen-independent and exhibit anchorage-independent growth, indicative of a more aggressive prostate cancer phenotype. Semi-quantitative reverse transcription PCR was carried to assess the level of RANBP17 transcript with GAPDH used as an internal control. Data showed that
Figure 9. Northern Blot and Semi-Quantitative End-Point RT-PCR Analyses Shows the Presence of RANBP17 Transcript in Various Human Cancer Cell Lines. (A) Northern blot analysis. 5 µg of total RNA obtained from various cancer cell lines were subjected to Northern blot analysis. The blot was hybridized with $^{32}$P-labeled random-primed 561 bp RANBP17 probe (position 2379 to 2939 of NM_022897 for human RANBP17). (B) Semi-quantitative end-point RT-PCR. 1.5 µg of total RNAs from LNCaP, DU145 and PC3 prostate cancer cell lines were utilized for cDNA synthesis and 1/4 diluted amount of prepared cDNA were subjected to semi-quantitative end-point RT-PCR according to manufacturer’s instruction (SuperArray Bioscience). The resulting 193 bp amplicon was visualized by ethidium bromide staining on 1.5% agarose gel. Human GAPDH was used for normalization.
the three prostate cancer cell lines expressed comparable levels of RANBP17 transcript (Figure. 9B).

**Transcript Expression of RANBP17, RANBP16 and E12 by Quantitative Real-Time PCR**

To confirm the relative expression of RANBP17 transcript in various human cancer cell lines, quantitative PCR was performed by SYBR Green-based real-time PCR. The human cancer cell lines tested were categorized as those originated from breast (MCF7 and ZR75), prostate (LNCaP, PC3 and DU145), bone (SAOS2, U2OS2 and MG63), skin (A431), liver (HepG2), connective tissue (HT1080), cervix (HeLa) and blood (HL60, THP-1, MEG01) cancers and compared to normal human testis tissue where RANBP17 expression is known to be readily detectable. Overall, RANBP17 mRNA expression in various human cancer cell lines showed significantly lower expression level than the testis positive control \( (p < 0.001) \). When cancer cell lines of same origin were individually compared, RANBP17 mRNA expression in MCF7, LNCaP and THP-1 cells was considerably higher than other cell lines of same tissue origin. Particularly, in contrast to the comparable level of RANBP17 transcript expression among prostate cancer cell lines observed in semiquantitative RT-PCR, quantitative real-time PCR result showed RANBP17 transcript expression in LNCaP cells is approximately 6.3 and 2.1 times higher than PC3 and DU145 cells, respectively (Figure. 10).

As a novel binding partner of RANBP17, E12 transcript expression was also investigated by real-time PCR. Interestingly, among various cancer cell lines, MCF7 and
Figure 10. Quantitative Real-Time PCR Analysis Demonstrates RANBP17 Transcript Expression in Various Human Cancer Cell Lines. Quantitative real-time PCR for RANBP17. Full-length human RANBP17 mRNA expression in various human cancer cell lines was evaluated by quantitative real-time PCR. 10 ng of input cDNA was used for SYBR Green-based real-time PCR. The relative expression of RANBP17 transcript in each sample was measured and normalized against GAPDH expression level. Among the samples, the lowest expression level of RANBP17 transcript in HeLa cells was set at a value of 1. The fold change was calculated and shown as means ± SD in independent triplicate samples. Note that compared to all human cancer cells, normal testis sample demonstrated significantly higher expression of RANBP17 transcript ($p < 0.001$).
FIG. 10

![Graph showing RANBP17 mRNA Expression (Fold Change) for different tissues and cell lines.](image)

* $p < 0.001$
Figure 11. Quantitative Real-Time PCR Analysis Demonstrates E12 Transcript Expression in Various Human Cancer Cell Lines. Quantitative real-time PCR for E12. Full-length human E12 mRNA expression in various human cancer cell lines was evaluated by quantitative real-time PCR. 10 ng of input cDNA was used for SYBR Green-based real-time PCR. The relative expression of E12 transcript in each sample was measured and normalized against GAPDH expression level. Among the samples, the lowest expression level of E12 transcript in MEG01 cells was set at a value of 1. The fold change was calculated and shown as means ± SD in independent triplicate samples. Note that compared to all human cancer cells, normal testis sample demonstrated significantly higher expression of E12 transcript ($p < 0.001$).
FIG. 11

![Bar graph showing E12 mRNA Expression (Fold Change) for different cell lines and tissues.](image)

- * $p < 0.001$

**Cell Lines:**
- MCF1
- ZRT75
- LNCaP
- PC3
- DU145
- SAOS2
- U2OS
- MEG01
- A431
- HepG2
- HT1080
- HeLa
- HL60
- THP-1
- MEG01
- Testis

**Tissues:**
- breast
- prostate
- bone
- skin
- liver
- connective tissue
- cervix
- blood
THP-1 also demonstrated a significant increase in E12 mRNA expression compared to other cancer cell lines similar to that observed for RANBP17 transcript expression but LNCaP did not in this case (Figure. 11). In addition, as a close human homologue of RANBP17, the transcript expression of RANBP16 was also examined in these cancer cell lines and the result is presented later in Figure 22. It seems that the human acute monocytic leukemia cell line, THP-1 is the human cancer cell line, which expresses readily detectable levels for each of RANBP17, RANBP16 and E12 transcripts and thus, might be a useful cancer cell model for studying the details of those genes.

**Co-immunoprecipitation of Full-length E12 and Full-length RANBP17**

Previously, the interaction between E12 and RANBP17 was confirmed from yeast two-hybrid and mammalian two-hybrid analyses. However, since E12 and RANBP17 utilized in these assays were truncated fusion proteins, the interaction of full-length E12 (E12\(^{1-654}\)) and 3xHA-tagged full-length RANBP17 (RANBP17\(^{1-1088}\)) requires additional analysis. As the protein expression of E12\(^{1-654}\) and RANBP17\(^{1-1088}\) were verified in COS and HeLa cells with expected sized bands detected in immunoblot analysis (Figure. 7A), the physical interaction of E12\(^{1-654}\) and RANBP17\(^{1-1088}\) was investigated by ectopic expression of these two proteins in COS cells. COS cell lysates expressing E12\(^{1-654}\) and/or RANBP17\(^{1-1088}\) separately or in combinations were subjected to co-immunoprecipitation (CO-IP) with E2A/E12 polyclonal primary antibody (Santa Cruz Biotech.), which can recognize the last C-terminal 19 amino acids of E12 and E47 proteins as an epitope. COS cell lysates transfected with an empty vector (pcDNA3.1) or either E12\(^{1-654}\) or RANBP17\(^{1-1088}\) alone were set to be negative controls. After pulling
Figure 12. Full-length E12\textsuperscript{1-654} Co-immunoprecipitates with Full-length RANBP17\textsuperscript{1-1088}. Co-immunoprecipitation of E12\textsuperscript{1-654} and RANBP17\textsuperscript{1-1088}. (A) Schematic representation of full-length E12\textsuperscript{1-654} and 3xHA-tagged full-length RANBP17\textsuperscript{1-1088} used in co-immunoprecipitation. (B) Co-immunoprecipitation of full-length E12\textsuperscript{1-654} and full-length RANBP17\textsuperscript{1-1088} was assessed by transient transfection in COS cells. HA-tagged RANBP17\textsuperscript{1-1088} and E12\textsuperscript{1-654} proteins were expressed separately or in combination. 50 µg of each cell lysate was subjected to co-immunoprecipitation with polyclonal E2A/E12 antibody (1:100) and blotted with monoclonal HA (1:2000) or polyclonal E2A/E12 (1:2000) primary antibodies. 1/20 of total protein (2.5 µg) was loaded as input and equal loading of protein was verified by Coomassie blue staining.
### FIG. 12

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<td>E2A</td>
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<td>Coomassie</td>
</tr>
</tbody>
</table>

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82
down by immunoprecipitation with E2A/E12 antibody and immunoblotting with monoclonal HA antibody for each immunoprecipitated cell lysate, only the COS cell lysate co-expressing E12\(^{1-654}\) and RANBP17\(^{1-1088}\) showed a positive interaction in this analysis (Figure. 12B). This interaction is specific since when an unrelated HA-tagged protein with a similar protein expression level to that of RANBP17\(^{1-1088}\) was co-immunoprecipitated with E12\(^{1-654}\), no interaction was detected (data not shown). Equivalent amounts of co-immunoprecipitated E12 protein and each protein expression as an input utilized for co-immunoprecipitation were confirmed along with the same amount of sample loading by Coomassie staining. Since the physical interaction of E12\(^{1-654}\) and RANBP17\(^{1-1088}\) was confirmed by co-immunoprecipitation, a myc-tagged C-terminal region of E12, which contains bHLH domain (E12\(^{502-654}\)), was tested for the physical interaction with RANBP17\(^{1-1088}\). The result for this co-immunoprecipitation also showed a positive interaction between E12\(^{502-654}\) and RANBP17\(^{1-1088}\) (Figure. 13B). The Crm1 domain, which also contains importin-β N-terminal domain in its structure, is important for small GTPase Ran binding and possibly for the interaction with nuclear pore complexes (NPCs). Therefore, E12\(^{502-654}\) and an N-terminal region of RANBP17 containing the Crm1 domain at 8-167 amino acids of RANBP17 (RANBP17\(^{2-167}\)) were assessed for their interaction. Similar to the positive interaction between E12\(^{508-654}\) and RANBP17\(^{1-252}\) observed in yeast-two hybrid and mammalian two-hybrid analyses, the physical interaction between E12\(^{502-654}\) and RANBP17\(^{2-167}\) turned out to be positive by immunoblotting (Figure. 14B). Therefore, this result enables us further to make a conclusion that it is the Crm1 domain inside the N-terminal region of RANBP17 that is responsible for the interaction with C-terminal bHLH region of E12. However, it is also
Figure 13. Truncated E12$^{502-654}$ Co-immunoprecipitates with Full-length RANBP17$^{1-1088}$. Co-immunoprecipitation of E12$^{502-654}$ and RANBP17$^{1-1088}$. (A) Schematic representation of N-terminally truncated E12$^{502-654}$ and 3xHA-tagged full-length RANBP17$^{1-1088}$ used in co-immunoprecipitation is depicted. (B) Co-immunoprecipitation of truncated E12$^{502-654}$ and full-length RANBP17$^{1-1088}$ was assessed by transient transfection in COS cells. HA-tagged RANBP17$^{1-1088}$ and E12$^{502-654}$ proteins were expressed separately or in combination. 50 µg of each cell lysate was subjected to co-immunoprecipitation with polyclonal E2A/E12 antibody (1:100) and blotted with monoclonal HA (1:2000) or polyclonal E2A/E12 (1:2000) primary antibodies. 1/20 of total protein (2.5 µg) was loaded as input and an equal loading of protein was verified by Coomassie blue staining.
FIG. 13

A.

E12 (bHLH) :

\[ \text{bHLH} \]

\[ \text{502 - 654} \]

Crm

RANBP17 (full) :

\[ \text{1 - 1078} \]

B.

\[ \text{E12}^{502-654} : \quad - \quad + \quad - \quad + \]

\[ \text{HA-RANBP17} : \quad - \quad - \quad + \quad + \]

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<td>Coomassie</td>
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Figure 14. Truncated E12<sup>502-654</sup> Co-immunoprecipitates with Crm1 Domain of RANBP17. Co-immunoprecipitation of E12<sup>502-654</sup> and RANBP17<sup>2-167</sup>. (A) Schematic representation of N-terminally truncated E12<sup>502-654</sup> and HA-tagged Crm1 domain of RANBP17<sup>(RANBP17<sup>2-167</sup>)</sup> used in co-immunoprecipitation. (B) Co-immunoprecipitation of truncated E12<sup>502-654</sup> and RANBP17<sup>2-167</sup> was assessed by transient transfection in COS cells. HA-tagged RANBP17<sup>2-167</sup> and E12<sup>502-654</sup> proteins were expressed separately or in combination. 50 μg of each cell lysate was subjected to co-immunoprecipitation with polyclonal E2A/E12 antibody (1:100) and blotted with monoclonal HA (1:2000) or polyclonal E2A/E12 (1:2000) primary antibodies. 1/20 of total protein (2.5 μg) was loaded as input and an equal loading of protein was verified by Coomassie blue staining.
FIG. 14

A.

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B.

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necessary to mention that RANBP17\textsuperscript{2-167} was subjected to co-immunoprecipitation with full-length E12\textsuperscript{1-654} under the same conditions but we were unable to detect a positive interaction for reasons that are currently unknown.

**Immunocytochemical Co-localization Studies of E12 and RANBP17**

Next, it was asked whether co-expression of RANBP17 and E12 would alter their intracellular localization and if so, which protein exerts a dominant effect on their respective intracellular distribution. To test this, HA-tagged full-length RANBP17\textsuperscript{1-1088} and either full-length E12\textsuperscript{1-654} or N-terminal truncation of E12, E12\textsuperscript{502-654} which contains bHLH region of E12 similar to the E12 construct (E12\textsuperscript{508-654}) used in yeast two-hybrid and mammalian two-hybrid assessments as previously mentioned, were expressed in COS cells by transient transfection. Although the nuclear localization signal (NLS) for E12 is not clearly delineated, it appears a putative NLS is located in an N-terminal region of E12, particularly at amino acids 170-175. Prior to conducting co-localization studies of RANBP17 with E12, the intracellular localization of E12\textsuperscript{1-654} and E12\textsuperscript{502-654} was ascertained. As expected, E12\textsuperscript{1-654} exhibited exclusive nuclear localization while E12\textsuperscript{502-654} demonstrated exclusive cytoplasmic localization. This was quantitatively assessed in the left two graphs in Figure 15A. A microscopic representation is shown in Figure 15B in the absence or presence of nuclear DAPI staining where typical nuclear localization of E12\textsuperscript{1-654} (upper panel) and nuclear exclusion of E12\textsuperscript{502-654} with exclusively cytoplasmic staining (lower panel), were contrasted. Thus, it was next determined whether co-expression of RANBP17 would alter the subcellular localization of E12 or vice versa employing indirect double immunostaining. In the right two graphs in Figure 15A, cells
that exhibit predominantly cytoplasmic, predominantly nuclear or equal distribution in both compartments were quantitated after the co-expression of E12\textsuperscript{1-654} or E12\textsuperscript{502-654} with full-length RANBP17 (RANBP17\textsuperscript{1-1088}). The results showed that no significant change in intracellular localization of E12\textsuperscript{1-654} and E12\textsuperscript{502-654} is induced by co-expression of full-length RANBP17\textsuperscript{1-1088}. This observation is somewhat different from our initial expectation since we postulated that E12 might be a cargo candidate of RANBP17 with the assumption that RANBP17 functions as a nucleocytoplasmic transporter. As previously shown in Figure 12B and 13B, both E12\textsuperscript{1-654} and E12\textsuperscript{502-654} co-immunoprecipitated with RANBP17\textsuperscript{1-1088}. Thus, if E12 is a substrate cargo of RANBP17 and RANBP17 is an exportin, the nuclear localization of E12\textsuperscript{1-654} might be anticipated to be decreased. On the other hand, if RANBP17 functions as an importin, the cytoplasmic localization of E12\textsuperscript{502-654} is likely to be decreased. However, the result showed no change in nuclear and cytoplasmic localization of E12\textsuperscript{1-654} and E12\textsuperscript{502-654} regardless of co-expression of RANBP17. Therefore, the expression of RANBP17 did not influence the intracellular localization of E12 proteins.

Next, the intracellular localization of RANBP17\textsuperscript{1-1088} co-expressed with E12\textsuperscript{1-654} was examined. The E2A proteins appear to play a dominant role for nuclear translocation of other proteins such as MyoD, Id1 and Id3 as suggested by previous studies, which point to a nuclear chaperoning mechanism for E2A proteins (Deed, Armitage et al. 1996; Lingbeck, Trausch-Azar et al. 2005). Therefore, it is also probable that E12 can exert a dominant function in regard to localization of RANBP17. Figure 16A shows quantification of cells that exhibit predominantly cytoplasmic, predominantly nuclear or both cytoplasmic and nuclear localization of RANBP17\textsuperscript{1-1088} with or without co-
Figure 15. The Intracellular Localization of E12 Is Not Affected by RANBP17 Expression. (A) Analysis of predominant intracellular localization of E12\(^{1-654}\) and E12\(^{502-654}\) in the absence or presence of full-length RANBP17 expression. The localization of E12\(^{1-654}\) and E12\(^{502-654}\) was individually evaluated as predominantly cytoplasmic (indicated as C), nuclear (indicated as N), and distributed equally between nucleus and cytoplasm (indicated as B) shown as percentage of total cells observed. Each of 50-100 COS cells expressing either E12\(^{1-654}\) or E12\(^{502-654}\) or cells co-expressing either E12\(^{1-654}\) or E12\(^{502-654}\) with full-length RANBP17 were counted. The data represent the mean ± S.D. from three independent transfections and the significance value (\(p < 0.001\)) was obtained from individual comparisons within the same transfection (C vs. N and B or N vs. C and B). (B) Immunocytochemistry for subcellular localization of E12\(^{1-654}\) and E12\(^{502-654}\). The full-length E12 (E12\(^{1-654}\)) and N-terminally truncated form of E12 (E12\(^{502-654}\)) were expressed in COS cells by transient tranfection. E2A/E12 polyclonal primary antibody (1:100) was used for immunostaining of E12\(^{1-654}\) and E12\(^{502-654}\) followed by 10 µM DAPI staining. Representative images were presented from more than five separate transfections.
FIG. 15

A.

B.
Figure 16. Nuclear Localization of Full-length RANBP17 Is Significantly Increased by Co-transfection of Full-length E12 Expression. (A) Analysis of predominant intracellular localization of full-length RANBP17 in the absence or presence of full-length E12\textsuperscript{1-654} expression. The localization of full-length RANBP17 protein was individually evaluated as predominantly cytoplasmic (indicated as C) nuclear (indicated as N), and distributed equally between nucleus and cytoplasm (indicated as B) shown as percentage of total cells observed. 50-100 COS cells expressing either full-length RANBP17 alone or COS cells co-expressing full-length RANBP17 along with full-length E12 (E12\textsuperscript{1-654}) were enumerated. Data represent the mean ± S.D. from three independent transfections and the significance value ($p < 0.01$) was obtained from the comparison between each respective signal (C, N, B) in the two transfections (RANBP17 transfection vs. E12 and RANBP17 co-transfection). (B) Immunocytochemistry for subcellular co-localization of RANBP17 and E12\textsuperscript{1-654}. Full-length E12\textsuperscript{1-654} and 3xHA tagged full-length RANBP17 were co-expressed in COS cells. HA monoclonal primary antibody (1:100) and E2A/E12 polyclonal primary antibody (1:100) were used for immunostaining of full-length RANBP17 and E12\textsuperscript{1-654} followed by incubation with Alexafluor 568-conjugated goat anti-mouse secondary antibody (1:800) and FITC-conjugated rabbit IgG (1:200 Bio-Rad) secondary antibodies respectively. Representative images were presented from more than five separate transfections.
FIG. 16

A.

![Bar graph showing the number of cells (C, N, B) for RANBP17 and RANBP17 + E121-654.](image)

B.

![Images showing FITC, Alexafluor568, and Merged.]
expression with full-length E12\textsuperscript{1-654}. This data indicated that in cells transfected with RANBP17\textsuperscript{1-1088} alone, the signal was often found in both cytoplasmic and nuclear compartments however, \(\sim 25\%\) of cells showed predominantly nuclear and \(\sim 18\%\) predominantly cytoplasmic staining (Figure 15A, left). Upon combined expression of RANBP17\textsuperscript{1-1088} with E12\textsuperscript{1-654}, \(\sim 59\%\) of cells evidenced a predominantly nuclear localization for RANBP17\textsuperscript{1-1088} while \(\sim 5\%\) and \(\sim 34\%\) of cells showed predominantly nuclear or equal distribution in both compartments respectively as demonstrated in the right graph in Figure 16A. This shift in RANBP17 localization signal to predominantly nuclear distribution was evident and up to a 34\% increase in predominant nuclear signal of RANBP17 was noted with concomitant decreases in the two other RANBP17 signals. Therefore, this indicates that when E12\textsuperscript{1-654} is present with RANBP17\textsuperscript{1-1088}, RANBP17 protein is redistributed to some extent from cytoplasmic regions to the nucleus, presumably \textit{via} the dominant function of E12\textsuperscript{1-654} in nuclear translocation of RANBP17\textsuperscript{1-1088} while strictly nuclear localization of E12\textsuperscript{1-654} is not affected by the co-expression of RANBP17\textsuperscript{1-1088} as previously assessed in Figure 15A. This observation supported that predominantly nuclear shift of RANBP17\textsuperscript{1-1088} induced by E12\textsuperscript{1-654} expression resulted from the interaction of RANBP17\textsuperscript{1-1088} with E12\textsuperscript{1-654}. Figure 16B showed a representative example of typical cells showing nuclear co-localization of E12\textsuperscript{1-654} and RANBP17\textsuperscript{1-1088}. Taken together, this result provides evidence supporting a consistent interaction of E12 and RANBP17 that occurs in a cellular context and that is in line with all our previous observations for this protein-protein interaction. Additionally, however, when E12 was artificially localized to the cytoplasm by employing E12\textsuperscript{502-654}, which lacks an NLS and shows an exclusive cytoplasmic localization, although a slight decrease in nuclear
localization of RANBP17 is noticed, the overall intracellular localization of RANBP17\textsuperscript{1-1088} signal did not seem to be remarkably affected by E12\textsuperscript{502-654} expression (data not shown).

The E-box Dependent and E12/MyoD-mediated Transcriptional Activation of RANBP17

We next addressed if RANBP17 exerts a functional effect on E12, namely alteration of E12 transcriptional activity \textit{via} E-box enhancer elements. For this, we designed an E-box dependent functional assay by generating an E-box responsive luciferase reporter construct, termed E-box pGL3 that contains three copies of E-box consensus enhancer element (CAGGTG) inserted in the pGL3 minimal promoter construct (Promega Corp.) such that E-box regulatory sequences are upstream of an SV40 minimal promoter and a firefly luciferase gene. In general, E12 binds to its E-box consensus sequence weakly as a homodimer but avidly as a heterodimer. In addition, under most physiological conditions, E12 exerts its action as a heterodimer with other bHLH transcription factors. Therefore, MyoD, a well-characterized myogenic bHLH transcription factor as well as a well-known binding partner of E12, was employed as a heterodimerization partner of E12 in this assay. The co-transfection of the E-box pGL3 reporter construct along with expression constructs of E12\textsuperscript{1-654} and full-length MyoD, as anticipated, led to a 3-4 fold increase in luciferase activity as shown in columns 1 and 2 of Figure 17B. Moreover, co-transfection of E12 and MyoD failed to alter the luciferase activity when the minimal pGL3 reporter construct wherein no E-box consensus element is present, was employed in this E-box dependent functional assay (Columns 5 and 6 in
Figure 17. Full-length RANBP17 Significantly Increases E12/MyoD-mediated Transcriptional Activity in an E-box Dependent Manner. The effect of full-length RANBP17 protein expression on E-box dependent transcriptional activity. (A) Schematic diagram of luciferase reporter constructs used in the E-box dependent functional assay. The 3x E-box element (CAGGTG) was inserted in front of SV40 minimal promoter followed by firefly luciferase gene. Note that the E-box pGL3 promoter construct contains three E-box elements while the pGL3 promoter does not. (B) The indicated combinations of plasmids including E12, MyoD and full-length RANBP17 and either E-box pGL3 or pGL3 promoter constructs were utilized for co-transfection into HeLa cells to test E-box dependent and E12/MyoD-mediated transcriptional activity. 48 hrs after co-transfection, cell lysates were analyzed to measure luciferase activity and values were normalized by protein content. The empty vector (pcDNA3.1) and E-box pGL3 transfection was set at a value of 1 (column 1). Data represent the mean ± S.D. from a minimum of independent triplicate samples. Note that E12/MyoD-mediated transcriptional activity was significantly increased compared to that of empty vector transfection (p < 0.01) and co-expression of RANBP17 with E12 and MyoD further enhanced the E12/MyoD-mediated transactivation (p < 0.01).
FIG. 17

A.

B. 

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<th>E-box pGL3</th>
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* $p < 0.01$
Figure 17B). This indicated that the transcriptional action of the E12/MyoD heterodimer was mediated by the E-box consensus enhancer element. Next we tested the effect of co-transfection of full-length RANBP17\textsuperscript{1-1088} on the ability of E12/MyoD heterodimer to transactivate the E-box pGL3 reporter gene. As shown in column 2 and 3 of Figure 17B, the presence of RANBP17\textsuperscript{1-1088} led to a 2.7-fold enhancement (p < 0.01) in E-box dependent and E12/Myo-mediated transcriptional activity. In contrast, no significant transactivation effect was noted either for RANBP17 alone on the E-box-pGL3 activity or with co-transfection of RANBP17, E12, and MyoD on the pGL3 promoter activity (Fourth column and the last four columns of Figure 17B respectively). This result also strongly supports the notion that RANBP17 interacts with E12 in a functionally important manner that enhances the transactivation activity of E12 heterodimers and that the transactivation we observed is dependent on E-box mediated signals.

The yeast two-hybrid and mammalian two-hybrid analyses utilized the RANBP17\textsuperscript{1-252} fusion proteins to test the interaction of E12 and RANBP17 and their positive interaction was evidenced as previously shown in Figures 5 and 6. Thus, the N-terminal region of RANBP17, which includes Crm1 domain, was suspected to be a region responsible for the interaction of E12. The physiologically relevant short form of RANBP17 protein, which also contains the Crm1 domain in its N-terminus, was tested in the E-box dependent functional assay to determine if it activates E12/MyoD-mediated transcriptional activity. The major short form of RANBP17, which is identical to the possible protein products of the 14c, 14 c/e or 14 c/d/e RANBP17 transcripts, was tested in our assay and briefly, it is termed herein as short RANBP17 (sRANBP17) or RANBP17\textsuperscript{1-576}. Figure 18B shows that a 2.1-fold increase (p < 0.05) in E-box dependent
Figure 18. The C-terminally Truncated Short Form of RANBP17 Results in a Significant Increase in E12/MyoD-mediated Transcriptional Activation in an E-box Dependent Functional Assay. The effect of sRANBP17 protein expression on E-box dependent transcriptional activity. (A) Schematic diagram of sRANBP17 used in E-box dependent functional assay. (B) The indicated combinations of E12, MyoD, sRANBP17 and E-box pGL3 plasmids were utilized for co-transfection into HeLa cells to test E-box dependent and E12/MyoD-mediated transcriptional activity. 48 hrs after co-transfection, cell lysates were analyzed to measure luciferase activity and values were normalized by protein content. The empty vector (pcDNA3.1) and E-box pGL3 transfection was set at a value of 1 (column 1). Data represent the mean ± S.D. from a minimum of independent triplicate samples. Note that E12/MyoD-mediated transcriptional activity was significantly increased compared to that of empty vector transfection ($p < 0.05$) and co-expression of short form of RANBP17 with E12 and MyoD further enhanced the E12/MyoD-mediated transactivation ($p < 0.01$).
FIG. 18

A.

[Diagram showing Crm1 and sRANBP17 with a ratio of 576:1]

B.

[Bar chart showing luciferase activity (fold change) with E12, MyoD, and sRANBP17 conditions. The chart includes error bars and significance markers: * p < 0.05, ** p < 0.01.]

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<th>E12</th>
<th>MyoD</th>
<th>sRANBP17</th>
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<tr>
<td>Luciferase Activity (Fold Change)</td>
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Significance markers:
* p < 0.05
** p < 0.01
Figure 19. The Crm1 Domain of RANBP17 is Sufficient to Induce E12/MyoD-mediated Transcriptional Activation in an E-box Dependent Functional Assay. The effect of the Crm1 domain of RANBP17 on E-box dependent transcriptional activity. (A) Schematic diagram of N-terminal Crm1 domain of RANBP17 used in E-box dependent functional assay. (B) The indicated combinations of each plasmid including E12, MyoD, the N-terminal Crm1 domain of RANBP17 (RANBP17\textsuperscript{2-167}) and E-box pGL3 were utilized for co-transfection into HeLa cells to test for E-box dependent and E12/MyoD-mediated transcriptional activity. 48 hrs after co-transfection, cell lysates were analyzed to measure luciferase activity and values were normalized by protein content. The empty vector (pcDNA3.1) and E-box pGL3 transfection was set at a value of 1 (column 1). Data represent the mean ± S.D. from a minimum of independent triplicate samples. Note that E12/MyoD-mediated transcriptional activity was significantly increased compared to that of empty vector transfection ($p < 0.01$) and co-expression of the Crm1 domain of RANBP17 with E12 and MyoD further enhanced the E12/MyoD-mediated transactivation ($p < 0.01$).
FIG. 19

A.

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Luciferase Activity (Fold Change)

* $p < 0.01$
and E12/MyoD-mediated transcriptional activity was found in the presence of sRANBP17. More specifically, the Crm1 domain of RANBP17 is suspected to be responsible for the interaction with E12 based on the positive co-immunoprecipitation result for RANBP17^{2-167} and E12^{502-654}. RANBP17^{2-167}, which includes mostly the Crm1 domain of RANBP17, was also tested to E-box dependent functional assay. The result showed a 2.5-fold increase in E12/MyoD-mediated transcriptional activity (Figure. 19B). This fold change in transcriptional activation is comparable to that previously observed when full-length RANBP17^{1-1088} was utilized in this assay. Therefore, the Crm1 domain by itself appears sufficient to induce E12/MyoD-mediated transcriptional activity and strongly supports the conclusion that the Crm1 domain is responsible for the interaction of RANBP17 with E12.

The Effect of RANBP17 on the Inhibitory Action of Id1 in E12/MyoD-mediated Transcriptional Activation.

Given that RANBP17 interacts with E12 resulting in augmentation of E-box dependent and E12/MyoD-mediated transactivation, next it was investigated whether RANBP17 can exert its transactivation effects to offset Id1’s dominant negative action for E12/MyoD-mediated transcriptional activity. Id1 is a well-characterized inhibitory member of the HLH protein family. Id1 has a HLH protein interaction interface, but lacks a basic DNA binding domain. As such it functions in a dominant negative manner via sequestration of E12 and other bHLH factors into non-functional heterodimers and thus can diminish E12-mediated transactivation of E-box regulated genes (Benezra, Davis et al. 1990; Ellis, Spann et al. 1990; Garrell and Modolell 1990). As previously noted, co-
Figure 20. RANBP17 Can Partially Rescue E12/MyoD-mediated Transactivation from the Action of Id1. The effect of RANBP17 on the inhibitory action of Id1 in E12/MyoD-mediated transcriptional activity. The indicated combinations of each plasmid including Id1 with or without full-length RANBP17 as indicated were employed for co-transfection into HeLa cells to test the effect of RANBP17 on the inhibitory action of Id1. 48 hrs after co-transfection, cell lysates were analyzed to measure luciferase activity and values were normalized by protein content. The empty vector (pcDNA3.1) and E-box pGL3 transfection was set at a value of 1 (column 1). Data represent the mean ± S.D. from a minimum of independent triplicate samples. Note that E12/MyoD-mediated transcriptional activity was significantly increased compared to that of empty vector transfection ($p < 0.01$) and co-expression of RANBP17 with E12 and MyoD further enhanced the E12/MyoD-mediated transactivation ($p < 0.01$). Co-expression of RANBP17 and Id1 along with E12 and MyoD rescued E12/MyoD-mediated transactivation from the inhibitory action of Id1 ($p < 0.01$). It was also significantly different from RANBP17 transfection alone ($p < 0.05$).
FIG. 20

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* p < 0.05
** p < 0.01
transfection of E12 and MyoD resulted in increased E-box regulated transcriptional activity that was further enhanced by approximately 3-fold when full-length RANBP17\textsuperscript{1-1088} was utilized. This is depicted in columns 1, 2, and 3 of Figure 20. Upon co-expression of Id1 with E12 and MyoD as shown in column 4, the luciferase activity is similar to that of the empty vector transfection in column 1, indicating that co-expression of Id1 with E12 and MyoD led to a complete inhibition of E12/MyoD-mediated transcriptional activity. When RANBP17\textsuperscript{1-1088} was co-expressed with E12, MyoD and Id1 as in column 5, the dominant negative effect of Id1 was alleviated and the transcriptional activity was partially restored to the level of E12/MyoD-mediated transcriptional activation in column 2. This result showed a 2.9-fold increase in transcription activity compared to the co-trasfection of E12, MyoD and Id1 in column 4 and also showed a significant difference from the luciferase activity induced by only RANBP17\textsuperscript{1-1088} transfection in column 6. Id1 alone did not show any significant change in luciferase activity as shown in column 7 of Figure 20.

E-box Dependent Transcriptional Activation by RANBP16 and E47

RANBP16 is a recently identified close homologue of RANBP17 and RANBP16 as well as RANBP17 are evolutionarily distant members of the importin β superfamily. Since RANBP16 also contains a Crm1 domain in its N-terminus, which shows approximately 68% identity and 86% similarity to the Crm1 domain of RANBP17, RANBP16 was tested in our E-box dependent functional assay to determine whether RANBP16 can also activate E12/MyoD-mediated transcription. As predicted, this result demonstrated RANBP16 induces a 2.2-fold increase ($p < 0.01$) in E12/MyoD-mediated
transcriptional activity (Figure. 21B). This result also implicated the possible interaction of RANBP16 with E12. Considering all the proteins that contain Crm1 domains in their structure such as full-length RANBP17\textsuperscript{1-1088}, RANBP17\textsuperscript{1-576} (sRANBP17), RANBP17\textsuperscript{2-167}, and full-length RANBP16 are able to demonstrate E12/MyoD-mediated transcriptional activation, the Crm1 domain is considered to be the domain responsible for the E-box dependent and E12 heterodimer-mediated transcriptional activation. Regarding RANBP16 transcript expression in Figure 22, the overall expression levels of RANBP16 transcript in various human cancer cells were still significantly lower than the testis positive control ($p < 0.001$) except the peripheral blood cancer cell line, THP-1.

E12 and E47 are alternate products of the \textit{E2A} gene. Although their respective bHLH sequences differ as a result of alternate splicing, in many cases, E12 and E47 are considered to have similar binding to other bHLH partner proteins and to some degree, they can functionally substitute for each other or other E-proteins (Zhuang, Barndt et al. 1998; Massari and Murre 2000). Since both E12 and E47 contain a bHLH motif wherein their amino acids evidence 77% identity and 89% similarity, we thus hypothesized that E47 and RANBP17 might interact. As it was previously confirmed, the interaction of E12 and RANBP17 was also employed for comparison as well as a positive control. The result showed E47 also co-immunoprecipitated with RANBP17 (Figure. 23B). However, since E2A/E12 primary antibody recognizes both E12 and E47 with the same epitope that is mapped at C-terminal ends outside their bHLH motif, the relatively weaker signal of E47 as shown in column 4 than that of E12 as in column 6 is possibly translated as a weaker binding of E47 with RANBP17 than that of E12 with RANBP17. If it is the case, it can also indirectly support that bHLH is responsible for the interaction of E12 and
Figure 21. Full-length RANBP16 Increases E12/MyoD-mediated Transcriptional Activation in an E-box Dependent Functional Assay. The effect of full-length RANBP16 on E-box dependent transcriptional activity. (A) Schematic diagram of full-length RANBP16 used in E-box dependent functional assay. (B) The indicated combinations of each plasmid including E12, MyoD and full-length RANBP16 with E-box pGL3 were utilized for co-transfection into HeLa cells to test E12/MyoD-mediated and E-box dependent transcriptional activity. 48 hrs after co-transfection, cell lysates were analyzed to measure luciferase activity and values were normalized by protein content. The empty vector (pcDNA3.1) and E-box pGL3 transfection was set at a value of 1 (column 1). Data represent the mean ± S.D. from a minimum of independent triplicate samples. Note that E12/MyoD-mediated transcriptional activity was significantly increased compared to that of empty vector transfection ($p < 0.01$) and co-expression of RANBP16 with E12 and MyoD further enhanced the E12/MyoD-mediated transactivation ($p < 0.01$).
FIG. 21

A.

1
Crm1
RANBP16
1087

B.

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<th></th>
<th>E12</th>
<th>MyoD</th>
<th>RANBP16</th>
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<tr>
<td>Luciferase Activity (A.U.)</td>
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* * p < 0.01
**Figure 22. Quantitative Real-time PCR Analysis Demonstrates RANBP16 Transcript Expression in Various Human Cancer Cell Lines.** Quantitative real-time PCR for RANBP16. Full-length human RANBP16 mRNA expression in various human cancer cell lines was evaluated by quantitative real-time PCR. 10 ng of input cDNA was used for SYBR Green-based real-time PCR. The relative expression of RANBP16 transcript in each sample was measured and normalized against GAPDH expression level. Among the samples, the lowest expression level of RANBP16 transcript in SAOS2 cells was set at a value of 1. The fold change was calculated and shown as means ± SD in independent triplicate samples. Note that compared to all human cancer cells except THP-1, normal testis sample demonstrated significantly higher expression of RANBP16 transcript ($p < 0.001$).
FIG. 22

![Graph showing RANBP16 mRNA Expression (Fold Change) for various cell lines and tissues. The graph includes data points for cell lines such as MCF7, ZR75, LNCaP, PC3, DU145, SAOS2, U2OS, MG63, A431, HepG2, HT1080, HeLa, HL60, THP-1, MEG01, and Testis. The x-axis represents different tissues and cell lines, while the y-axis indicates the fold change in mRNA expression. Significant differences are indicated by asterisks (* p < 0.001).]
RANBP17 since the sole difference between E12 and E47 is only the bHLH motif, which might be able to control their binding strength to RANBP17.

Next, we asked if the effect of RANBP17 on the transactivation of E12 would extend to E47. Figure 23C demonstrates the ability of RANBP17 to transactivate E-box regulated gene transcription generated by employing E47 and MyoD. Here, we found that co-transfection of E47 with MyoD resulted in a 3.4-fold increase ($p < 0.01$) in E-box dependent transcriptional activity and that the inclusion of RANBP17$^{1-1088}$ further augmented this response by 2.2-fold ($p < 0.05$). Given that it is the C-terminal region of E12 containing the bHLH domain that showed a positive interaction with RANBP17 in yeast two-hybrid, mammalian two-hybrid and co-immunoprecipitation assessments in addition to the evidence of positive interaction between E47 and RANBP17, the interaction interface of E12 for RANBP17 is considered to involve the bHLH domain of E12.

The Physiological Relevance of RANBP17 in Myogenic Differentiation

So far, the interaction between E12 and RANBP17 has been investigated, and the positive interaction of RANBP17 with E12 was confirmed throughout various experimental approaches. Now, we asked whether RANBP17 indeed exerts its effect under physiological conditions involving E12 activity. To address this question, primarily the physiological conditions where E12 plays its role were investigated. It has long been known that ubiquitously expressed E12/E47 exerts its function by heterodimerization with tissue-restricted bHLH transcription factors to confer tissue specificity, and among those factors, muscle regulatory factors (MRFs), which include MyoD, MRF-4, Myf-5
Figure 23. E47 Can Co-Immunoprecipitate Full-length RANBP17 and Increase E-box Dependent Transcriptional Activation. Co-immunoprecipitation and E-box dependent transcriptional activity of E47 and RANBP17. (A) Schematic diagram of full-length E47 used in E-box dependent functional assay. (B) Co-immunoprecipitation of full-length E47 and 3xHA-tagged full-length RANBP17 was assessed by transient transfection in COS cells. The E47 and RANBP17 proteins were expressed separately or in combination. Co-immunoprecipitation of E12 and RANBP17 was used as a positive interaction control in this analysis. 50 µg of each cell lysate was subjected to co-immunoprecipitation with polyclonal E2A/E12 antibody (1:100) and blotted with monoclonal HA (1:2000) or polyclonal E2A/E12 (1:2000) primary antibodies. 1/20 of total protein (2.5 µg) was loaded as input and an equal amount of loading was verified by Coomassie blue staining. (C) The indicated combinations of each plasmid including E47, MyoD, full-length RANBP17, and E-box pGL3 were used for co-transfection into HeLa cells to test E12/MyoD-mediated and E-box dependent transcriptional activity. 48 hrs after co-transfection, cell lysates were analyzed to measure luciferase activity and values were normalized by protein content. The empty vector (pcDNA3.1) and E-box pGL3 transfection was set at a value of 1 (column 1). Data represent the mean ± S.D. from a minimum of independent triplicate samples. Note that E47/MyoD-mediated transcriptional activity was significantly increased compared to that of empty vector transfection ($p < 0.01$) and co-expression of RANBP17 with E47 and MyoD further enhanced the E47/MyoD-mediated transactivation ($p < 0.01$).
and myogenin, are well-studied examples related to the action of E12/E47 in muscle development (French, Chow et al. 1991; Funk, Ouellette et al. 1991; Rashbass, Taylor et al. 1992; Becker, Dorman et al. 2001). The E12 protein forms heterodimers with muscle regulatory factors including MyoD and can regulate the transcriptional activity of myogenic downstream target genes in myogenesis (Lassar, Davis et al. 1991). Therefore, it is worthwhile to ask whether RANBP17 can exert its effect on E12/MRFs-mediated transactivation of myogenic downstream target genes to address the physiological relevance of RANBP17 expression associated with the function of E12. Thus, the mouse myoblast cell line, C2C12 was employed as a model to address this question. C2C12 cells were infected with an empty vector (pLNCX2) or a retroviral construct of 3xHA-tagged full-length RANBP17 (RANBP17\(^{1-1088}\)-pLNCX2) and a myogenic differentiation protocol was applied. Triplicate cultures of infected C2C12 cells were prepared and after RANBP17 protein expression was confirmed by utilizing monoclonal HA antibody in immunoblot analysis (Figure 24A), total RNA was prepared to generate cDNA for quantitative real-time PCR analysis. To assess the RANBP17 effect on myogenic downstream genes in C2C12 cell differentiation, several potential downstream target genes including \( p21 \), myosin heavy chain (\( MYH \)) and muscle creatine kinase (\( MCK \)) genes were investigated (Murre, McCaw et al. 1989; Brennan and Olson 1990; Jen, Weintraub et al. 1992; Halevy, Novitch et al. 1995; Ojamaa, Samarel et al. 1995; Prabhu, Ignatova et al. 1997; Talmadge 2000; Yam, Chan et al. 2000; Bergstrom, Penn et al. 2002; Maleki, Royer et al. 2002; LaFramboise, Guthrie et al. 2003; Cao, Kumar et al. 2006). Independent triplicate infection samples from empty vector or RANBP17 infections were prepared and again, each triplicate from every sample was subjected to
quantitative real-time PCR analysis. As expected, compared to the C2C12 cells at day 0 indicating cells before differentiation, the transcript expression of \( p21 \), \( MYH \) and \( MCK \) genes from each independent triplicate of C2C12 cells infected with empty vector or RANBP17 retroviral constructs were significantly increased at day 6 after C2C12 myogenic differentiation while that of MyoD was decreased as previously reported (Miller 1990; Nishiyama, Kii et al. 2004; Sun, Trausch-Azar et al. 2005) (Figure. 24B, Figure 25 and Figure 26).

Next, all independent triplicates of either empty vector or RANBP17 retroviral infections were compared to determine whether RANBP17 expression affects the transcript expression of these genes during C2C12 myogenesis. We defined it to be significantly different only when every independent triplicate in one group of infection shows a statistical difference to every triplicate of another group of infection (pLNCX2 vs. RANBP17-1-1088-pLNCX2 retroviral infections). Although individual variation can be detected in some cases, when each triplicate sample in two infection groups were compared, according to our definition, no significant differences in \( p21 \) and \( MYH \) gene expressions between the two infection groups were noticed. Therefore, it appears that RANBP17 expression did not influence transcript expression of \( p21 \) and \( MYH \) genes. The transcript expression of other potential downstream target genes such as \( desmin \) and \( CDH15 \) were also investigated and did not show significantly differences in these two infection groups before and after C2C12 cell differentiation (data not shown). However, when the transcript expression of the \( MCK \) gene was examined, a significant difference was found for all independent triplicate infection samples between the empty vector and RANBP17 retroviral infections at day 6 after C2C12 differentiation. Compared to the
Figure 24. The Protein Expression of RANBP17 in C2C12 Retroviral Infection is Confirmed and C2C12 Myogenic Differentiation is Validated by p21 Transcript Expression. Immunoblot analysis for RANBP17 and Quantitative Real-time PCR for p21 gene in C2C12 stable cell pools. (A) Three independent triplicates of C2C12 cells were infected with either empty retroviral vector (pCLNCX2) or 3xHA tagged full-length RANBP17 retroviral vector (RANBP17-pLNCX2) and subjected to immunoblot analysis for protein expression of RANBP17. 50 µg of each cell lysate was used and signal was detected by monoclonal HA primary antibody (1:2000). (B) Empty vector- or RANBP17-infected C2C12 cells were differentiated under low serum conditions and 5 µg of total RNA was used to generate cDNA from the either C2C12 cells before or 6 days after myogenic differentiation. The relative transcript expression levels of the p21 gene in each sample were evaluated by SYBR Green-based quantitative real-time PCR and determined through normalization against GAPDH gene expression level. Fold changes were calculated and are shown as means ± SD.
FIG. 24

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Figure 25. The C2C12 Myogenic Differentiation is Confirmed by MyoD and MYH Transcript Expressions. Quantitative real-time PCR for MyoD and MYH genes in C2C12 stable cell pools. The empty vector- or RANBP17-infected C2C12 cells were differentiated under low serum conditions and 5 µg of total RNA was used to generate cDNA from either C2C12 cells before or 6 days after myogenic differentiation. The relative transcript expression levels of the MyoD and MYH genes in each sample were evaluated by SYBR Green-based quantitative real-time PCR and determined through normalization against GAPDH gene expression level. Fold changes were calculated and are shown as means ± SD.
FIG. 25

A.

MyoD

B.

MYH (Myosin Heavy Chain)
Figure 26. The RANBP17 Can Regulate MCK Gene Expression During C2C12 Myogenesis. Quantitative Real-time PCR for MCK gene. The empty vector- or RANBP17-infected C2C12 cells were differentiated under low serum conditions and 5 µg of total RNA was used to generate cDNA from either C2C12 cells before or 6 days after myogenic differentiation. The relative transcript expression levels of the MCK gene in each sample were evaluated by SYBR Green-based quantitative real-time PCR and determined through normalization against GAPDH gene expression level. Fold changes were calculated and are shown as means ± SD. The significance value (p < 0.01) was obtained from the individual comparison between each sample in two infection groups. The significance value was assigned only when all triplicate samples in two groups show significant differences when individually compared. Among the samples, the lowest transcript expression level was set at a value of 1.
FIG. 26

MCK (Muscle Creatine Kinase)

MCK mRNA Expression (Fold Change)

* * * p < 0.01

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The graph shows the MCK mRNA expression levels in different conditions (Vector and RANBP17) at two time points (D0 and D6). The asterisks indicate statistically significant differences.
triplicate retroviral infections of empty vector, all the RANBP17 infections demonstrated a significant decrease ($p < 0.01$) in $MCK$ gene expression at day 6 after C2C12 differentiation with a range of 1.6- to 3-fold differences in those two groups. Therefore, the expression of RANBP17 altered $MCK$ transcript level but not other genes tested at day 6 after C2C12 myogenic differentiation. It is also noted that the $MCK$ gene expression is most prominently upregulated among the genes in our test during C2C12 myogenic differentiation. Under physiological conditions, gene expression is controlled and regulated not solely by transcription factors but also by the cooperative actions of other transcriptional modulators such as coactivators or corepressors generating distinctive transcriptional complexes in a specific promoter region for each gene. Therefore, in a physiological context, RANBP17 is considered to exert its effect on E12/MRFs-mediated transcriptional regulation of myogenic downstream target genes in a promoter specific manner.
DISCUSSION

By utilizing a yeast two hybrid screening approach, a largely uncharacterized member of the importin-β superfamily, RANBP17 was identified as a novel non-bHLH binding partner of E12. RANBP17 interacted with E12 in a manner that confers enhanced transcriptional activity when examined in mammalian yeast two-hybrid analysis. These results revealed that the N-terminal region of RANBP17 (RANBP17^{1-252}) and the C-terminal bHLH domain of E12 (E12^{508-654} or E12^{502-654}) are responsible for this interaction. Furthermore, studies on the physical interaction between E12 and RANBP17 tested by co-immunoprecipitation verified that the Crm1 domain within the N-terminal region of RANBP17 (RANBP17^{2-167}) accounts for the interaction with E12. This result is also supported by the finding that the Crm1 domain in RANBP17^{2-167} is sufficient to activate E12/MyoD-mediated transcription comparable to the level observed when full-length RANBP17^{1-1088} is tested in an E-box dependent functional assay. Therefore, the bHLH domain of E12 and the Crm1 domain of RANBP17 are concluded to be responsible for this interaction. However, since the bHLH domain is mapped to amino acids 547-607 within the E12^{508-654} or E12^{502-654} construct and the Crm1 domain exists at amino acids 8-167 within the RANBP17^{2-167} construct, the possibility that the amino acids outside the bHLH and Crm1 domain may also participate in this interaction can not be completely dismissed.

Migot et al. have previously reported that positively charged basic residues are critical for the recruitment of RANBP16, a close homologue of RANBP17. Their observations also provided suggestive evidence that it is the bHLH domain of E12 that is
important for the interaction with RANBP17. The motif important for the interaction with RANBP16 contains a short acidic extended region followed by an amphipathic helix where the hydrophilic residues are lysines in a folded structure, but with no rigid short linear consensus sequence. RANBP16 also appears to have an ability to make multiple interactions with bipartite or tripartite positively charged basic patches where an unfavorable residue in one position can be compensated somewhere else in the molecule (Mingot, Bohnsack et al. 2004). These observations are somewhat interesting since E12 also has a similar feature in the vicinity of the basic motif in the bHLH domain. A sequence located N-terminal to the basic region termed the A region or inhibitory domain, was reported to be responsible for the interference of E12 homodimer for DNA binding possibly due to the steric hindrance effect induced by negatively charged amino acids in the inhibitory domain. This domain possesses an intrinsic acidic property composed of four glutamic acids and four aspartic acids within 19 amino acid residues of the defined inhibitory domain which was mapped at amino acids 530-548 of E12 protein (Sun and Baltimore 1991) and this inhibitory domain is immediately followed by the basic domain of E12/E47 proteins. Considering the positive but seemingly weaker interaction of E47 with RANBP17 compared to that of E12 with RANBP17 from co-immunoprecipitation assessment, the specific involvement of the bHLH domain in the interaction of E12 and RANBP17 is strongly supported because E12 and E47 are known to differ only in the bHLH motif of their primary amino acid structures. However, in more details for the differences between E12 and E47, only the bHLH region of E12 contains this inhibitory domain. Since the difference of E12 from E47 protein occurs at amino acids 530-607 of E12 protein while actual bHLH domain of E12 is defined at
amino acids 547-607. The inhibitory domain is reported to be approximately at 530-548 amino acid positions. Therefore, it is conceivable that the inhibitory domain may also participate in the interaction with RANBP17 along with the actual bHLH motif of E12.

Previously, we introduced mutations in the basic domain that is mapped at 547-563 amino acids of E12 where an arginine residue is the major basic amino acid conferring a positively charged basic property instead of lysine. All of the five arginine amino acid residues in the basic region of E12 were substituted with alanine residues (mutE12508-654) and subjected to mammalian two-hybrid assay. The result showed this mutation induced more than seven times stronger transcriptional activation than wild type (E12508-654) in our analysis (data not shown). This result appears to indicate the basic domain is not necessary for the interaction with RANBP17. However, the involvement of the basic motif with the interaction of RANBP17 can not be completely excluded since the RANBP16 has demonstrated possible multiple interactions and some redundancy for the interaction with its binding partners where unfavorable residue in one position can be compensated for another amino acid residue(s) at different position of the molecule. Therefore, mutations in a basic motif can still be compensated by interaction with another region(s) of E12 protein and it is also possible that other amino acids instead of arginine residues in the basic domain of E12 may also interact with RANBP17. Considering the prominent increase in transcriptional activity induced by the mutations in the basic region of E12, the involvement of the inhibitory domain needs to be considered. It was previously proposed that in the absence of DNA binding, the conformation of the basic domain may allow the inhibitory domain to directly block the HLH domain (Shirakata and Paterson 1995). Thus, mutations in the basic domain may also possibly reduce the
blocking effect present in the inhibitory domain and HLH motif, thereby either the inhibitory or HLH domain is more readily accessible to participate in the interaction with RANBP17. In addition, the possible release from the blocking effect may be more prominent in this truncated E12 fusion protein (mutE12^{508-654}), which was utilized for mammalian two-hybrid analysis than full-length E12, allowing RANBP17 fusion protein more easy access to the interaction interface(s) of E12 leading to prominently enhanced transcriptional activation. Based on the multiple characteristic of RANBP16, not only the basic and/or HLH motif but also inhibitory domain may possibly involve the interaction with RANBP17. Additionally, as mentioned in the previous Literature section, the existence of the Rep domain, which is also mapped between AD2 and the bHLH domain of E2A and slightly N-terminal to the inhibitory domain showed quite similar features to the inhibitory domain. This may allow a model regarding the action of inhibitory domain, to be extended to the Rep domain as well since some part of the Rep domain is also included in the truncated E12 construct utilized in our various analyses.

Regarding the involvement of the Crm1 domain of RANBP17, since the RANBP17^{2-167} utilized in co-immunoprecipitation and E-box dependent functional analyses includes only six amino acids that are not formally a part of the Crm1 domain, we concluded that it is the Crm1 domain that is responsible for the interaction with E12. Although the other C-terminal regions outside the Crm1 domain of RANBP17 was not investigated, our data demonstrated the Crm1 domain of RANBP17 is sufficient to interact with E12. Taken together, it was relatively complicated to precisely define the domain(s) of E12 responsible for the interaction with RANBP17. It is apparent however, that at least the Crm1 domain of RANBP17 and basic and/or HLH region possibly along
with the N-terminal region of the basic motif (inhibitory domain or Rep domain) are the regions responsible for the interaction of E12 and RANBP17.

It was demonstrated that RANBP17 is able to enhance the ability of E12/MyoD heterodimers to activate transcriptional responses in an E-box dependent functional assay. RANBP17 is a member of the importin-β family, whose major function is known to the mediation of nucleocytoplasmic transport. If RANBP17 functions as an importin for E12 as we initially hypothesized, the increased transcriptional activity could be partially ascribed to increased nuclear localization of E12 imported by the action of RANBP17. However, our immunocytochemical study revealed that E12 exerts a dominant effect on the nuclear localization of RANBP17 and that RANBP17 can not alter the intracellular localization of E12. In addition, based on our Northern blot analysis, since the expression level of RANBP17 transcript appears to be relatively lower than other genes, a small change in RANBP17 expression may have much stronger effect than other genes. Therefore, the prominent increase in nuclear localization of RANBP17 induced by the dominant role of E12 may exert an indirect effect on E12/MyoD-mediated transactivation. The question remains as to how increased RANBP17 induces E12/MyoD-mediated transactivation once RANBP17 is transported into nucleus partially by the dominant role of E12. One of the possibilities is an alteration in the half-life of E12. The E2A proteins can acquire an extended half life via the interaction with MyoD or Id1 (Lingbeck, Trausch-Azar et al. 2005). The interaction of E12 with RANBP17 may also confer extended half-life to E12 and this enhanced E12 protein stability would, in turn, lead to enhanced transcriptional activity. In addition, the rapid degradation of E2A proteins is mediated by the ubiquitin-proteasome system and the ubiquitin ligase
interaction domain has been mapped at amino acids 478-533 of E12 (Kho, Huggins et al. 1997; Huggins, Chin et al. 1999; Conlon and Meyer 2004). This domain partially overlaps with the region of E12 protein utilized in our yeast and mammalian two-hybrid analyses as well as in co-immunoprecipitation. Although it is located N-terminally outside of the bHLH motif, the binding of RANBP17 with E12 in close proximity to this domain may restrain the accessibility of ubiquitin enzymes such as mammalian Ubc9 (UbcE2A) to E12. Therefore, it is possible that the interaction of RANBP17 with E12 delays the ubiquitin-proteasomal degradation of E12.

Related to these posttranslational events, there is another possibility. It has been reported that not only the transactivation domains, but also the bHLH domains of E2A proteins possesses the ability to modulate transcriptional activity. The dimerization of E2A proteins with myogenin, one of the muscle regulatory bHLH transcription factors, can potentiate the phosphorylation of myogenin, possibly by unmasking the phosphorylation sites and/or recruitment of the kinase(s) necessary for the phosphorylation of myogenin (Zhou and Olson 1994). Furthermore, this dimerization-dependent phosphorylation was suggested to induce diminished myogenin transcriptional activity since mutations in dimerization-dependent phosphorylation sites of myogenin demonstrated enhanced transcriptional activity (Zhou and Olson 1994). Similarly, MyoD is a phosphoprotein in myogenic cell nuclei and deletion mutants of MyoD lacking potential dimerization-dependent phosphorylation sites demonstrated increased myogenic activity (Tapscott, Davis et al. 1988). The association with E47 also resulted in the enhanced phosphorylation of MyoD (Lassar, Davis et al. 1991). In the E-box dependent functional assay we utilized, since MyoD is employed as a heterodimerization partner of
E2A, it is also possible that the interaction of E12 with RANBP17 may exert an impact on its binding partner, MyoD. If this is the case, the interaction of RANBP17 with E12 might inhibit the dimerization-dependent MyoD phosphorylation. Based on previous observations, this would result in enhanced transcriptional activity. However, this is paradoxical in that the interaction of E12 with myogenin or MyoD induces diminished transcriptional activities. Regarding this issue, based on the existence of the fast- and slow-migrating forms of myogenin in differentiated muscle cells, which is attributable to the phosphorylation status at a specific serine site, it was also suggested that only a fraction of myogenin might participate in dimerization-dependent phosphorylation, and large fractions of myogenin heterodimers still remain as unphosphorylated form of myogenin which is considered to be only transcriptionally active in myogenic differentiation. In addition, this phosphorylation process may be a normal process to inactivate excessive myogenin molecules to restrict its possible indiscriminate activity (Zhou and Olson 1994). Thus, major portions of E2A-myogenin heterodimers still can induce transactivation as a transcriptionally active form while transcriptionally inactive form of E2A-myogenin heterodimers also exist to control transcriptional activity with a partial suppressive action and explains why the mutations in dimerization-dependent phosphorylation sites of myogenin can generate enhanced transcriptional activity. In addition, it is also suggested E2A-induced phosphorylation of myogenin is caused possibly by E2A’s recruitment of growth factor-inducible kinase(s) that can suppress myogenin’s activity as well (Zhou and Olson 1994). Although more detailed mechanisms how the dimerization-dependent phosphorylation can be partially achieved in a selective manner and how myogenin phosphorylation and related kinases can modulate
transcriptional activities need to be explored, taken together with this point of view, it seems E12 functions as a regulatory nodal point that controls the degree of transcriptional activation of muscle regulatory downstream genes as previously suggested (Lassar, Davis et al. 1991). If this event is also able to be extended to MyoD, the interaction of E12 and RANBP17 will apparently affect the dimerization-dependent phosphorylation of MyoD. The interaction of RANBP17 with E12 may reduce the phosphorylation of MyoD to diminish the transcriptional activity and the resulting transcriptional activity will be increased. In addition, instead of the indirect contribution of E2A for the phosphorylation of its dimerization partner(s) and its transcriptional activity, the transactivation directly induced by phosphorylation of E2A is also possible. The E47 protein is demonstrated to be phosphorylated by p38 MAPK and this phosphorylation can enhance E47/MyoD dimerization as well as activate muscle-specific transcriptional activity (Lluis, Ballestar et al. 2005). Taken together, it seems the phosphorylation and transcriptional activity are closely connected each other, and the interaction of RANBP17 with E12 may interfere with possible association of E12 with other kinase(s) such as p38 MAPK leading to changes in phosphorylation of E12 by itself and/or its binding partner(s) and also generating alterations in their transcriptional activities.

Based on the observation that RANBP17 can partially release E12/MyoD-mediated transcriptional activity from the inhibitory action of Id1, it is also possible that RANBP17 can convert the functionally inactive E12 bound to ubiquitously expressing endogenous Id proteins into functionally active forms of E12 leading to enhanced transactivation. However, it is also suspicious how many portions of ectopically expressed E12 are functionally inactive by the interaction with endogenous Id proteins.
Due to the largely unknown characteristics of RANBP17, it was also questioned whether RANBP17 has an intrinsic ability to regulate transcriptional activity. However, based on our observations in E-box dependent functional assay, RANBP17 alone did not significantly induce transcriptional activation. Thus, it concluded that RANBP17 does not appear to possess intrinsic transctactivation ability. However, the question persists whether when RANBP17 interacts with E12, it can function as a transcriptional modulator forming a large transcriptional complex with E12 and generating a synergistic transcriptional activity in this experimental setting. Since E12/MyoD heterodimers already occupy their bHLH domains for dimerization, if the bHLH domain of E12 is the sole interaction interface for interaction with RANBP17, how RANBP17 achieves both its interaction with E12 and an effect on E12/MyoD-mediated transactivation is another unanswered question. Although it has not been tested, if RANBP17 can also interact with Id1 and the dimerization capability and strength of RANBP17 with Id1 is stronger than that with E12, then RANBP17 may be simply able to sequester the Id1 proteins from E12/MyoD heterodimers, thus leading to the enhanced transcriptional activation. However, this may not be such a simple case and as mentioned, it is also considered possible for RANBP17 to share the bHLH domain of E12 with another bHLH binding partners forming a large transcriptional complex. It might be necessary to narrow down their interaction domain or pinpoint the major amino acid(s) participated in these interactions and also test the potential interaction of RANBP17 with Id1 to help understanding this question more clearly. In conclusion, the enhanced transcriptional activity of E12 heterodimer in an E-box dependent functional assay may not come from a sole factor but may result from the combined effects of the consequences as suggested.
In contrast, when RANBP17 was expressed in C2C12 cells employing retroviral infection, diminished transcriptional activity was also observed in the transcript expression of MCK gene among the genes we tested after C2C12 cell differentiation. This observation appears opposite to the result expected based on the result of the E-box dependent functional assay. Considering this observation, the previous proposed mechanisms apparently are not sufficient to explain the regulation of transcriptional activity controlled by RANBP17. Moreover, the previous myogenin studies adopt CAT (chloroamphenicol acetyltransferase) reporter construct that contains basal promoter and muscle-specific enhancer from the mouse muscle creatine kinase (MCK) gene and transiently transfected in COS or 10T1/2 cells to test transcriptional activity (Zhou and Olson 1994). In our retroviral C2C12 infection result demonstrated the increased MCK gene expression was detected in C2C12 cells infected with empty retroviral vector after differentiation as expected, but RANBP17 expression has demonstrated to induce diminished MCK gene expression compared to the empty vector infection after C2C12 cell differentiation and no significant difference in MCK gene expression was noticed between before and after RANBP17-pLNCX2 infected C2C12 cell differentiation. The diminished effect in MCK transcript expression is MCK gene-specific since other genes tested did not show any significant difference when RANBP17 infections were compared with the empty vector infections.

As mentioned earlier, the expression of RANBP17 appears unlikely to generate only one definitive consequence in regard to effects on transcriptional activity. Instead, depending on the cell type where it is expressed, on which gene promoter region it exerts its action, as well as what other factors it interacts with or recruits, the resulting
consequences of RANBP17 expression are apparently different. It is also likely associated with the other binding partners of E12 connected to gene transcription since the bHLH domain or transactivation domains (TAD) can recruit other factors involved in gene transcription such as coactivator(s) and/or corepressor(s) (Eckner, Yao et al. 1996; Massari, Grant et al. 1999; Bradney, Hjelmeland et al. 2003). In the latter case, it is considered highly possible that RANBP17 expression can impair the capability of E12 to recruit such factors under certain physiological conditions and thereby give rise to different consequences in transcriptional activity. Indeed, the coactivator p300/CBP interacts with E12 and E47 as well as other myogenic factors including MyoD when bound to DNA and the bHLH motif of those bHLH transcription factors is sufficient for the recruitment of p300 while AD1 and AD2, the two transctivation domains of E2A appear to be involved in the interaction with CBP (Eckner, Yao et al. 1996; Massari and Murre 2000; Bradney, Hjelmeland et al. 2003). p300/CBP also has been shown to possess histone acetyltransferase (HAT) activity (Bannister and Kouzarides 1996; Ogryzko, Schiltz et al. 1996). In addition, the LDFS motif in AD1 of E2A can recruit another HAT complex, SAGA, leading to gene transactivation as well (Massari, Grant et al. 1999). Currently whether RANBP17 can interact with MyoD and HAT factors such as p300/CBP or SAGA complex is not known, but based on previous observations, at least the interaction of E12 and p300/CBP is considered to be closely linked to E-box mediated transcriptional regulation. As the responsible region for the interaction of E2A and p300 was mapped at the bHLH domain (Eckner, Yao et al. 1996; Massari and Murre 2000), the interaction of RANBP17 with E12 may restrict the interaction between E2A and p300 and moreover, since other muscle regulatory factors including MyoD is also able to
interact with p300, the impact of the interaction of RANBP17 and E12 can be expanded. In addition, it is completely unknown whether RANBP17 possesses an intrinsic repressive characteristic functioning as a transcription repressor associated with distinctive sets of nuclear factors in a specific promoter region, whose environment may only be affordable under physiological settings.

Compared to the enhanced transcriptional activity induced by ectopic expression of RANBP17 in an artificial reporter assay system such as the E-box dependent functional assay in HeLa cells, the effect of retroviral RANBP17 expression in C2C12 cells where the transcriptional activity of MCK gene is selectively diminished after C2C12 myogenesis, appears more physiologically relevant. However the two systematic approaches are very different in regard to, for example, expression level; relatively higher levels of expression would occur in the transient transfection vs. retroviral infection. Also, the cell types utilized are different. While the Hela cells utilized in the E-box dependent functional assay contains only ectopically expressed MyoD, the C2C12 cells express various endogenous muscle regulatory factors including MyoD, that are E12 binding partners. Therefore, E12 may heterodimerize with other myogenic factors influencing C2C12 myogenic differentiation. Similarly MyoD can also interact with other E-proteins such as E47, HEB and E2-2, which may participate in the myogenic differentiation of C2C12 cells. Thus, there is a limitation as to what extent one can precisely define the major species of the MRFs and E-proteins that function in C2C12 differentiation. Since we have not tested the interaction of RANBP17 with other various MRFs or E-proteins, it is also possible the effect of RANBP17 expression in C2C12 cells can be extended to these factors if they interact with RANBP17. Although we utilized
C2C12 myoblasts to assess the effect of RANBP17 expression, we have not yet ascertained the endogenous expression level of RANBP17 protein in C2C12 myoblasts or myotubes. Since we could not obtain a reasonable quality of RANBP17 primary antibody, we were unable to compare the endogenous expression levels of RANBP17 protein in HeLa cells and C2C12 cells. Instead, it may be necessary to examine the RANBP17 transcript expression in muscle cells including C2C12 cells. The E-box pGL3 utilized in E-box dependent functional assay is an artificial reporter construct to measure the transcriptional activity. This cell type specificity is also previously exemplified in E1A oncoprotein which initially identified its interaction with p300. The E1A has been shown to repress IgH enhancer-induced transcriptional activity in B cells (Borrelli, Hen et al. 1984; Hen, Borrelli et al. 1985) while it can activate IgH enhancer-induced transcriptional activity in mouse fibroblast L cell lines (Borrelli, Hen et al. 1986) suggesting p300/CBP has a dual role and maybe contains a monitoring mechanism that assures whether correct transcription factors are involved in a given promoter (Eckner, Yao et al. 1996).

Collectively, regarding the effect of RANBP17, the seemingly opposite two consequences we observed, do not necessarily have to be contradictory. These observations may provide another clue to understand E12/MyoD- or E12/MRFs-mediated gene transcriptional mechanism. Based on the selective repression of MCK gene by RANBP17 in C2C12 cells, it is considered that unknown MCK gene promoter-specific factor(s) which may or may not interact with RANBP17 is present probably in association with p300/CBP or SAGA nearby promoter region and possibly three dimensional overhead access to the E-box bound E12 heterodimers allow to form a
Figure 27. The Proposed Model for the Functional Effect of RANBP17 on E12-mediated Transcriptional Activity. After the translocation of RANBP17 into the nucleus induced by its intrinsic and E12’s dominant actions, the interaction of E12 and RANBP17 may enhance their protein stabilities inside the nucleus by delaying the proteasomal degradation or forming transcriptional complexes possibly with concomitant promoter-specific recruitment of various nuclear coactivators or corepressors under certain physiological conditions such as C2C12 myogenic differentiation. The interaction of RANBP17 with E12 may also interrupt the E12’s recruitment of coactivators required for E12-mediated transactivation in a specific promoter region such as *MCK* gene promoter.
distinctive promoter-specific transcriptional complex eventually exerting its effect to modulate relevant chromatin structures. Therefore, the possible mechanism whereby RANBP17 exerts its effect on selective transcriptional activation in a promoter-specific manner needs to be deeply investigated in future studies including our proposed model. The transactivation effects induced by other proteins related to E12 and RANBP17 proteins such as E47, the short form of RANBP17(RANBP17_{1-576}), RANBP16 and Crm1 domain of RANBP17 (RANBP17_{2-167}) in the E-box dependent functional assay are currently understood in the same context with full-length E12 and full-length RANBP17 since they all possess the same bHLH motif (or similar for E47) or Crm1 domain in their structures. In addition, our study regarding the interaction of E12 and RANBP17 not only simply showed the functional consequence of their interaction but also can provide a new perspective for the possible functional connection between transcription factors including bHLH transcription factor and the nucleocytoplasmic transporters including importin-β related one.

In the initial assessment of RANBP17 gene expression and protein localization reported that while RANBP16 showed rather widespread tissue expression, RANBP17 evidenced particular enrichment in testis (Koch, Bohlmann et al. 2000). Our work here also demonstrated that the transcript expression level of RANBP17 in human cancer cells was apparently less than that we found in normal testis and this expression pattern was also resembled in the transcript expression of E12 and RANBP16 even there are sample-to-sample variations in gene expression exist. As previously mentioned, however, among the various human cancer cell lines, a human acute monocytic leukemia cell line THP-1 demonstrated a significant increase in transcript expression for RANBP17, E12 and
RANBP16 providing a possibility that THP-1 cell line is a useful model to study the intracellular relationship among those genes along with normal testis tissue and some megakaryocytic cell lines as reported. It is speculated that cells, which need to maintain themselves as high proliferation and hypermutation rates may require higher expression of E12 based on the abundance of E2A in rapidly proliferating cells and sub-regions such as germinal center dark zone (Rutherford and LeBrun 1998). Additionally, even no significant difference in the transcript expression of RANBP17 was noticed from Northern blot and semi-quantitative RT-PCR analyses, quantitative real-time PCR result showed androgen dependent LNCaP cells appear to express relatively higher level of RANBP17 transcript expression than the other two androgen independent cell lines, DU145 and PC3 cells and thus, its physiological relevance in prostate cancer development may need to be further addressed in the future studies.

Preciously, Koch et al. reported largely nuclear localization of RANBP17 employing a GFP fusion of murine RANBP17 protein. However our data using indirect immunostaining for human RANBP17 showed that its subcellular expression exhibits distinct cytoplasmic and nuclear patterns on a cell-to-cell basis. When it was co-expressed with E12 in a subset of cells, the prominent increase in nuclear localization of RANBP17 protein was observed. This supports the notion RANBP17 and E12 may functionally interact in the nucleus with the consequence of affecting gene transcription events. Surprisingly little is known of the machinery specifically involved in E12 transport. However given its molecular mass of ~70 kD, it exceeds the estimated 20 to 40 kDa upper range to enter the nucleus by diffusion, and thus is likely to depend on receptor-mediated translocation. The transport between nucleus and cytoplasm through
the nuclear pore complex is largely mediated by importin β-related factors which represent the largest class of nuclear transport receptors (Kutay, Hartmann et al. 2000; Mingot, Bohnsack et al. 2004) and it requires the energy source of RanGTP hydrolysis to accumulate cargo against gradients. The common features of importin β-related transport receptors include their similar molecular weight ranging from ~90 to ~145 kD and the presence of an importin β-like conserved region in their N-termini which can mediate RanGTP binding (Fornerod, Ohno et al. 1997; Gorlich, Dabrowski et al. 1997; Koch, Bohlmann et al. 2000; Kutay, Hartmann et al. 2000). RANBP16 is a member of the importin-β superfamily and a close human homologue of RANBP17. They show overall 67% amino acid identity and phylogenetic analysis defines these two proteins along with exportin 4 as the most evolutionarily distant members of the importin-β superfamily (Kutay, Hartmann et al. 2000). While the function of RANBP17 in nuclear transport machinery has yet to be addressed, that of RANBP16 has been reported. Mingot et al. showed that RANBP16 is an exportin suggesting broad substrate specificity while Kutay et al. could not define its specific function as importin or exportin based on an intermediate dissociation constant of RanGTP hydrolysis analysis; They suggested that RANBP16 may be a novel type of transporter that functions in bidirectional cargo transport.

As observed in some importin-β superfamily members including CRM1 exportin (Kudo, Kholobin et al. 1997; Schlenstedt, Smirnova et al. 1997; Siomi, Eder et al. 1997), we have demonstrated that the intracellular localization of human RANBP17 occurs both in nucleus and cytoplasm; a strong nuclear rim staining is found in a subset of cells by indirect immunostaining studies. This observation may indicate that
RANBP17 is a member of importin-β superfamily, which is able to function as a nucleocytoplasmic transporter shuttling between two compartments through the NPC to carry its cargo substrates. Full-length E12 (E12\textsuperscript{1-654}) or N-terminally truncated E12 (E12\textsuperscript{502-654}) was co-expressed with full-length RANBP17 (RANBP17\textsuperscript{1-1088}), and initially, the intracellular E12 signal was investigated employing indirect immunocytochemistry. It was anticipated that if RANBP17 functions as an exportin of E12, a degree of decrease in nuclear E12\textsuperscript{1-654} would be expected, while on the other hand, if it functions as importin of E12, a degree of decrease in cytoplasmic E12\textsuperscript{502-654} signal would be observed. However, the exclusively nuclear or exclusively cytoplasmic signal of E12\textsuperscript{1-654} or E12\textsuperscript{502-654} respectively, was not altered by the expression of RANBP17. From these observations, RANBP17 only does not appear to function as a nucleocytoplasmic transporter of E12 but moreover E12 is unlikely to be a transport cargo of RANBP17. If RANBP17 indeed functions as an importin of E12, then it may be possible that the nuclear import of E12 requires the presence of an NLS, which can be recognized directly by RANBP17 or importin α, which in many cases, cooperate with importin-β transporters to import their cargos and this might explain why the overexpression of full-length RANBP17 could not change the exclusively cytoplasmic localization of E12\textsuperscript{502-654}. Next, the intracellular RANBP17 signal was examined when it was co-expressed with E12. In this case, surprisingly, the full-length E12\textsuperscript{1-654} exerted a dominant effect on nuclear localization of RANBP17. However, co-expression with E12\textsuperscript{502-654} did not change the intracellular localization of RANBP17 (data not shown). As previously reported, E12 may exert its dominant effect on nuclear localization of RANBP17 by its putative intrinsic chaperoning property (Deed, Armitage et al. 1996; Lingbeck, Trausch-Azar et al. 2005). Since E12\textsuperscript{502-}
which showed an exclusively cytoplasmic localization, did not induce an increase in cytoplasmic localization of RANBP17 even they co-immunoprecipitated, thus the reason is speculated such that the endogenous full-length E12 may be dominant and sufficient for the predominant nuclear shift in the intracellular localization of overexpressed RANBP17. In addition, E12<sup>502-654</sup> does not appear to possess the ability to reverse the nuclear RANBP17 to cytoplasm. Since certain proteins including transcription factors and transcriptional modulators can be accumulated and mislocalized in the nucleus due to the disrupted nuclear export mechanism caused by alteration in posttranslational modification or some environmental insults such as oxidative stress (Kuge, Toda et al. 1998; Kudo, Taoka et al. 1999; Rayet and Gelinas 1999; Hoshino, Kobayashi et al. 2000; Huang, Kudo et al. 2000; Yashiroma and Yoshida 2003; Kau, Way et al. 2004), it is also possible that ectopically expressed RANBP17 in the nucleus might experience similar alterations yielding its nuclear accumulation presumably by concomitant indirect effects of ectopically expressed nuclear E12, which may lead to aberration in nuclear export of RANBP17 where RANBP17 is also suspected to function as a transcription repressor. So far, many proteins including transcription factors (Biggs, Meisenhelder et al. 1999; Rayet and Gelinas 1999; Brownawell, Kops et al. 2001), tumor suppressors (Moll, Riou et al. 1992; Harris and Hollstein 1993; Kong, Suzuki et al. 1997; Kondo, Yao et al. 2001), cell cycle regulators (Blain and Massague 2002; Shin, Yakes et al. 2002) have shown evidence of intracellular mislocalization, which may in turn lead to tumorigenesis. Previous studies have implicated that E2A can function as a tumor suppressor (Peverali, Ramqvist et al. 1994; Park, Nolan et al. 1999; Massari and Murre 2000; Herblot, Aplan et al. 2002). However, whether mislocalization of E12 can elicit
tumorigenesis is unknown and in addition, the role of RANBP17 in disease including tumorigenesis remains to be determined.

It was of interest to note that aberrant TCR delta rearrangements in two acute lymphoblastic leukemia patients with T-ALL have been described to involve t(5;14) translocations of the TCR locus with RANBP17 and HOX11L2 gene where the breakpoints are localized within and immediately 3' of the RANBP17 gene respectively upstream of the HOX11L2 gene on chromosome 5. This results in a head-to-tail juxtaposition of RANBP17 with the TCR δ enhancer elements in the immediate vicinity of the RANBP17 gene, and has been predicted to result in an inappropriate activation of RANBP17 that would give rise to expression of a C-terminally truncated or full RANBP17 protein in these patients. To date, the molecular etiology of these leukemias have nearly exclusively focused on the role of HOX11L2 activation and deregulation as a result of the translocation of HOX11L2 gene just distal to TCR δ element and/or loss of putative 5’ regulatory sequence of Hox11L2 gene as well as juxtaposition with BCL11B/CTIP2 gene (Bernard, Busson-LeConiat et al. 2001; Hansen-Hagge, Schafer et al. 2002; MacLeod, Nagel et al. 2003; Pan, Li et al. 2005; Su, Della-Valle et al. 2006; Baak, Gokbuget et al. 2008). It was also mentioned that inappropriate activation of RANBP17 was predicted to occur due to the juxtaposition of TCR δ enhancer elements and the presumable increase in RANBP17 expression may activate cell growth and contribute to leukemogenesis (Hansen-Hagge, Schafer et al. 2002). However, while preliminary analysis indicated an increase of RANBP17 transcript level in these patients, constraints of sample size limited a thorough assessment.

Considering numerous evidence that showed aberrations in protein expression,
intracellular localization, and transcriptional activity of many transcription factors and nucleocytoplasmic machineries are involved in diseases, particularly cancers including leukemogenesis, it is also possible that the importin-β superfamily member, RANBP17 plays a role in the development of disease possibly via alterations in E12-mediated transcriptional activities. In addition, our studies also suggest the possibility for functional involvement of importin-β superfamily members in gene regulation inside the nucleus in addition to their major role in mediation of nucleocytoplasmic transport.
CONCLUSIONS

1. The interaction of E12 and RANBP17 is identified in a yeast-two hybrid analysis and this interaction is bait-specific.

2. The interaction of E12 and RANBP17 is confirmed in mammalian HeLa cells by mammalian two-hybrid analysis.

3. The ~120 kD size of full-length RANBP17 protein is demonstrated to be localized in both nucleus and cytoplasm from subcellular fractionation studies.

4. The immunostaining for the intracellular localization of full-length RANBP17 shows distinct patterns of intracellular distribution of predominantly nuclear or predominantly cytoplasmic distribution as well as both nuclear and cytoplasmic equal distribution while full-length E12 shows an exclusive nuclear distribution under the same conditions.

5. The major 4.5 kb-sized transcript of RANBP17 is detected in various human cancer cell lines by Northern blot analysis and the relative transcript expression of RANBP17 gene in less and more malignant prostate cancer cell lines is not significantly different by semi-quantitative reverse transcription PCR analysis.

6. Overall, compared to normal human testis tissue, relatively low expression of RANBP17, RANBP16 and E12 transcripts are observed in various human cancer cells from quantitative real-time PCR analysis.

7. The various physical interactions between E12 and RANBP17 are demonstrated by co-immunoprecipitation and these results support that the bHLH region of E12 and the Crm1 domain of RANBP17 are responsible for their interaction.
8. The co-expression of E12 and RANBP17 does not alter the intracellular localization of E12, but it increases nuclear localization of RANBP17, suggested that E12 has a dominant function in regard to nuclear localization of RANBP17 and also supporting the interaction of E12 and RANBP17.

9. The E12/MyoD-mediated transcriptional activity is enhanced by RANBP17 in an E-box dependent manner.

10. The Crm1 domain of RANBP17 is sufficient to increase E-box dependent and E12/MyoD-mediated transcriptional activation, also supporting the evidence that the Crm1 domain of RANBP17 is responsible for the interaction of E12.

11. The short form of RANBP17 and full-length RANBP16 that contain Crm1 domain in their N-termini also demonstrate transactivation of E12/MyoD-mediated transcriptional activity.

12. The co-expression of Id1 and full-length RANBP17 demonstrates that RANBP17 is able to rescue E12/MyoD-mediated transactivation from the inhibitory effect of Id1.

13. E47 also physically interacts with full-length RANBP17 and increases E12/MyoD-mediated transcriptional activity.

14. RANBP17 can differentially regulate myogenic downstream target genes during C2C12 cell differentiation and under physiological conditions, is considered to exert its effect on transcriptional regulation in a promoter-specific manner.
SUMMARY

The interaction of E12 and RANBP17 was initially identified from our yeast-two hybrid screening and mating analyses utilizing E12^{508-654} and RANBP17^{1-252}. The interaction of E12 with RANBP17 was bait-specific evidenced by the observation that empty vector or an unrelated protein bait was unable to interact with RANBP17. This positive interaction between E12 and RANBP17 was also examined in mammalian cells. When the E12^{508-654} and RANBP17^{1-252} constructs used in yeast-two hybrid analysis, were subcloned into mammalian expression vectors and expressed in HeLa cells, greater than 5 times increase in relative luciferase activity was detected by mammalian two-hybrid analysis, thus indicated a positive interaction of E12 and RANBP17 in mammalian cells and confirmed the previous yeast-two hybrid based interaction. Since fusion proteins were utilized in two types of two-hybrid analyses, we extended these observations by examining the interaction for the physiologically relevant full-length forms of E12 and RANBP17 in mammalian cells. First, full-length human RANBP17 was characterized for its transcript and protein expression as well as intracellular localization. As a binding partner of E12, RANBP17 clone was initially identified from a DU145 prostate cancer library in the yeast-two hybrid screening. Therefore, the transcript of \textit{RANBP17} gene in various human cancer cell lines was investigated including prostate cancer cell lines. A \textasciitilde4.5 kb transcript was detected as the sole transcript in all the human cancer cell lines by Northern blot analysis. However, transcript expression levels were relatively much lower than human testis tissue positive control. Transcript expression in prostate cancer lines was also examined by semi-quantitative end-point reverse transcription PCR, but no
obvious difference was observed among three prostate cancer cell lines. The quantitative real-time PCR analysis showed same distinctions in expression level in various human cancer cell lines, and also the overall transcript expression levels were considerably lower than that in the human testis tissue. After ~120 kD sized protein expression of 3xHA-tagged full-length RANBP17 was confirmed by immunoblot analysis, the intracellular localization of full-length RANBP17 was investigated by subcellular fractionation and immunocytochemistry. The results obtained from these two experiments were consistent in that the expression of RANBP17 protein is detected in both nucleus and cytoplasm. The immunostaining for RANBP17 revealed various patterns of intracellular localization such as either predominantly nuclear or predominantly cytoplasmic distribution as well as cells with an equal distribution of RANBP17 protein signal in both compartments. Quantitative assessment of this result reveals the majority of cells expressing RANBP17 show equal distribution in both nucleus and cytoplasm. In a subset of cells, intense nuclear speckles and nuclear rim staining were also observed. Next, the physical interaction of E12 and RANBP17 is examined by co-immunoprecipitation utilizing E12\(^{654}\) and 3xHA-tagged RANBP17\(^{1-1088}\). The result demonstrated that full-length E12 and full-length RANBP17 indeed interact, evidenced by detection of a ~120 kD sized band in immunoblot analysis with monoclonal HA primary antibody after co-immunoprecipitation with E2A/E12 primary antibody. Furthermore, the interaction between E12\(^{502-654}\) and RANBP17\(^{2-167}\) was also shown by co-immunoprecipitation followed by immunoblot analyses demonstrating that the bHLH domain of E12 and the Crm1 domain of RANBP17 is the region responsible for this interaction, similar to the results from yeast two-hybrid and mammalian two-hybrid analyses. When RANBP17 is
co-expressed with E12, immunostaining results revealed that the exclusively nuclear localization of E12 is not changed, however, the intracellular localization of RANBP17 was shifted from a predominantly equal distribution in both nuclear and cytoplasmic compartments to a predominantly nuclear localization. This revealed the dominant role of E12 in the intracellular localization of RANBP17 and provided evidence that supports the interaction of E12 with RANBP17. To find additional evidence for this interaction and to determine if this interaction has a functional relevance in regard to transcriptional regulation, E-box mediated transcriptional activity was examined by utilizing expression of E12/MyoD heterodimer and an E-box containing luciferase reporter construct. When E12 and MyoD expression constructs were co-transfected with luciferase reporter construct that contains E-box enhancer element, the E12/MyoD-mediated and E-box dependent transcriptional activity was considerably increased compared to empty vector transfection. After co-expression of full-length RANBP17 with E12/MyoD heterodimer, the E-box dependent transcriptional activity was further significantly enhanced. When RANBP172-167, which is mostly comprised of the Crm1 domain of RANBP17, was tested, a comparable level of transactivation to full-length RANBP17 was observed. This demonstrates that Crm1 domain is sufficient to induce E-box dependent and E12/MyoD-mediated transcriptional activation and also that the Crm1 domain is responsible for this interaction. Other RANBP17-related proteins such as short form of RANBP17 or RANBP16 could induce E-box dependent transcriptional activation, seemingly by virtue of possession of a Crm1 domain in their protein structure. Another alternative splicing product of the E2A gene, E47, which contains a bHLH domain distinct from that in E12, demonstrated its interaction with RANBP17 by co-immunoprecipitation and also by
E12/MyoD-mediated transactivation in the E-box dependent functional reporter assay. Taken together, the C-terminal region, particularly the bHLH domain of E12 and the Crm1 domain of RANBP17 are concluded to be responsible for the interaction of E12 and RANBP17. To pursue the physiological relevance of RANBP17 expression related to the function of E12, the effect of RANBP17 on the expression of genes involved in myogenesis such as p21, myosin heavy chain and muscle creatine kinase was investigated. The mouse myoblast cell line, C2C12, were infected with empty retroviral vector or RANBP17 retroviral construct and each triplicate of infected C2C12 myoblasts or myotubes after differentiation were prepared for analysis of transcript expression by quantitative real-time PCR. The result demonstrated that while genes such as p21 and myosin heavy chain did not show differences in their transcript expression level between empty vector and RANBP17 retroviral infections. However, significant differences in those two sets of infections were found for expression of muscle creatine kinase gene at 6 days after C2C12 cell differentiation. RANBP17 infectioned cells showed a significant decrease in muscle creatine kinase transcript expression level. This result is somewhat unexpected from the previous observations wherein RANBP17 was demonstrated to induce an increase in E-box dependent and E12/MyoD-mediated transcriptional activity. The underlying mechanism whereby RANBP17 exerts its effect on transcriptional activity on a specific subset of E12-regulated genes is currently unclear. Considering the result obtained from our retroviral infection studies in C2C12 cells, it is suggested that RANBP17 can regulate the transcriptional activity of a subset of genes such as muscle creatine kinase gene among various myogenic downstream target genes and likely exerts its effect in a promoter specific manner under physiological conditions.


Huang, T. T., N. Kudo, et al. (2000). "A nuclear export signal in the N-terminal regulatory domain of IkappaBalpba controls cytoplasmic localization of
inactive NF-kappaB/IkappaBalpha complexes."


ABSTRACT

E12 is a ubiquitously expressed basic helix-loop-helix (bHLH) transcription factor regulating various developmental processes via homo- or heterodimerization with other bHLH family members and it also has a number of non-bHLH binding partners in diverse biological settings. To search for novel binding partners of E12, the bHLH domain of E12 was utilized as bait in yeast two-hybrid screening of a DU145 prostate cancer cell library. As a consequence, RANBP17, a member of the importin-β superfamily was identified as a binding partner of E12. The interaction of E12 and RANBP17 was also confirmed in mammalian two hybrid analysis evidenced by significantly enhanced relative luciferase activity in HeLa cells. Semiquantitative RT-PCR, quantitative real-time PCR and Northern blot analyses revealed that RANBP17 is present as a major ~4.5 kb transcript showing somewhat ubiquitous expression across a panel of human cancer cell lines. Immunostaining and subcellular fractionation of nuclear and cytoplasmic cell extracts demonstrated that RANBP17 is localized to both cytoplasm and nucleus. Upon co-expression with E12, the intracellular localization of RANBP17 showed a shift to a predominant nuclear localization implicating the dominant role of E12 in nuclear localization of RANBP17 and also supporting the interaction of E12 with RANBP17. The interaction of E12 and RANBP17 is further verified by co-immunoprecipitation and E-box dependent functional assay wherein co-expression of RANBP17 with E12 and MyoD revealed that RANBP17 can induce E12/MyoD-mediated transcriptional activation. Several proteins related to E12 and RANBP17 such as E47, short form of RANBP17 and RANBP16 also showed similar E12/MyoD-
mediated transactivation in this assay and taken together, these observations substantiate the specific interaction of E12 and RANBP17 and support the conclusion that the bHLH domain of E12 and the Crm1 domain of RANBP17 are responsible for this interaction. Based on our retroviral infection studies in C2C12 cells, it is considered that RANBP17 exerts its effect on transcriptional regulation of a subset of E12-regulated genes in a promoter-specific manner under physiological conditions.