ZNF451 is a Novel Binding Partner of the bHLH Transcription Factor E12

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In partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences

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Date of Defense: August 19, 2008
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By
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To my parents
Without your love and guidance I would not be who I am today

To my husband
Your love and support have been unconditional and unending
   I Thank You
   And
   I Love You

To my dear little son, Daniel
You are my sunshine and I love you
   With all my heart
Acknowledgements

I sincerely appreciate Dr. Cynthia M. Smas, my major advisor for her valuable guidance and encouragement throughout my research. As my advisor, she has taught me to think critically, to experimentally query the unknown, and to write about science. Her passion for research has deeply affected my way of thinking of science. Also, I appreciate all of my advisory committee members: Dr. Ivana de la Serna, Dr. KV Chin, Dr. Ronald Mellgren, and Dr. Xiaodong Wang for their advice and support. My appreciation is extended to the help from all the incredible past and present members of Dr. Smas’ lab for their great help in my experiments.

Thanks go to my parents for their unending and unconditional love, for their guidance and support throughout my life. Also, thanks go to my husband and my son for their understanding and support. In particular, I thank my son for all the after-hours and weekends he spends with me in the lab.

I truly thank you all.
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Introduction

Prostate cancer continues to be the most common lethal malignancy diagnosed in American men and the second leading cause of male cancer mortality (Jemal et al., 2005). Approximately 1 in 5 men will be diagnosed with prostate cancer during his lifetime, and 1 in 33 men will die of this disease (Pienta et al., 2006). At the beginning, almost all metastatic prostate cancers require testosterone for growth, and androgen deprivation becomes a first-line therapy for metastatic prostate cancer (Huggins CB, 1967). However, recurrent tumors often arise within several years, and few therapies are available to manage recurrent prostate cancers, which is an incurable stage of the disease (Balk and Knudsen, 2008). The primary mechanism by which the androgen-dependent prostate cancer progresses to the androgen-independent stage is not yet clearly elucidated; identifying the major causal factors during this progression is useful to the development of effective treatment strategies. Among suspected molecular targets, Ids (Inhibitor of DNA binding) have been suggested to be a primary causal factor in various cancers, such as breast cancer, cervical cancer, and prostate cancer (Desprez et al., 2003; Schoppmann et al., 2003; Schindl et al., 2001; Ouyang et al., 2002) and to be a potential diagnostic marker in prostate malignancy progression (Coppe et al., 2004). Aberrant expression of Id-1 has been reported in over 20 types of cancer (Wong et al., 2004). Its well-known interacting partner E12, a bHLH transcription factor, has been highlighted in B- and T- lymphomagenesis (Xin et al., 1993; Bain et al., 1994; Yan et al., 1997; Park et al., 1999; Engel and Murre, 1999). The interaction between Ids and E12 may play key roles in prostate tumor malignancy as well as in other cancer development.
The basic-helix-loop-helix (bHLH) family of transcription factors can recognize and bind to E-box “CANNTG” sequences and has been shown to play a key role in the differentiation of a number of cell lineages, including B- and T-lymphocytes, muscle cells, pancreatic β cells, neurons and osteoblasts (Jan and Jan, 1993; Weintraub, 1993; Olson and Klein, 1994; Massari and Murre, 2000; Zebedee and Hara, 2001). This family of proteins is characterized by a helix-loop-helix (HLH) dimerization domain, consisting of highly conserved amphipathic helices separated by a loop of variable length and sequence, and an adjacent DNA-binding domain that is rich in basic amino acids (Murre et al., 1989a; Davis et al., 1990; Ellenberger et al., 1994; Ma et al., 1994). The class I bHLH proteins, also known as the E proteins, are exemplified by the differentially spliced transcripts of the E2A gene (E12, E47 and E2-5/ITF1 proteins), E2-2/ITF2 and HEB/HTF4. These genes are ubiquitously expressed (Murre et al., 1989b; Henthorn et al., 1990; Zhang et al., 1991; Hu et al., 1992; Skerjanc et al., 1996; Massari and Murre, 2000). Class II bHLH proteins include MyoD, myogenin, NeuroD/BETA2, MASH, HAND and TAL and show a tissue-restricted expression pattern.

Id proteins also contain an HLH domain but are distinct from the bHLH transcription factors in that they lack the basic domain necessary for DNA binding. As such form, Ids form heterodimers with bHLH proteins and act as dominant negative regulators of bHLH transcription factors. There are four members of this family in mammalian cells, Id1-Id4 (Benezra et al., 1990; Christy et al., 1991; Sun et al., 1991; Biggs et al., 1992; Ellmeier et al., 1992; Deed et al., 1993; Hara et al., 1994; Riechmann et al., 1994; Pagliuca et al., 1995). Of the Id proteins, Id1 is the best studied. Id1 binds to bHLH transcription factors and inhibits the transactivation function and consequent
biological responses, such as cell differentiation (Benezra et al., 1990). Id1 has also been implicated as an oncogene which may play role in the tumorigenesis of a wide range of cancers (Ling et al., 2006). However, compared to the studies on the function of Id proteins in cancer, there are limited reports on the role of E12, a frequent binding partner of Id proteins in cancer development.

Studies in our lab have demonstrated that the bHLH transcription factor E12, a spliced form of E2A gene, is overexpressed in aggressive prostate cancer cell lines, such as DU145 and androgen independent LNCaP (LN-AI) derivatives, versus less aggressive LNCaP cells. Quong et al. also reported that E2A is involved in a significant percentage of childhood pro-B and pre-B cell leukemias (Quong et al., 2002). Together these data suggest that the Id and E12 interaction network may be involved in the action of Id-mediated tumorigenesis. To further examine the mechanisms and pathways for E12 action, we utilized yeast two-hybrid screening to identify novel protein binding partner(s) for E12, and a zinc finger protein, ZNF451, was identified as a candidate binding partner for E12. Zinc finger proteins are a class of regulatory proteins that participate in a variety of cellular activities such as development, differentiation, and tumor suppression (Iuchi, 2001). In our project, studies were carried out to address the interaction of ZNF451 with E12 and illustrate the characteristics of this protein molecule.
Helix-Loop-Helix transcription family

The helix-loop-helix (HLH) proteins form a large family of transcriptional regulators and play important roles in a wide range of biological processes, such as the regulation of cell cycle, cell differentiation and proliferation as well as organogenesis and sex determination (Kadesch, 1992; Jan and Jan, 1993; Duprey and Lesens, 1994; Stewart 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 et al., 2003). To date, over 240 HLH proteins have been identified in organisms ranging from the yeast *Saccharomyces cerevisiae* to humans (Atchley and Fitch, 1997). Since a large number of HLH proteins have been described, a classification scheme was developed based on tissue distribution, dimerization capabilities, and DNA-binding specificities (Massari and Murre, 2000), which divides the superfamily into seven classes (Figure 1).

Members of the bHLH family have two highly conserved and functionally distinct domains, which together make up a region of approximately 60 amino-acid residues (Jones S, 2004). At the amino-terminal end of this region is the basic domain, which binds the transcription factor to DNA at a consensus hexanucleotide sequence, CANNTG, known as the E box. At the carboxy-terminal end of this region is the HLH domain, which facilitates interactions with other protein subunits to form homo- and hetero-dimeric complexes (Murre et al., 1989b).
Ids (Inhibitors of DNA binding) belong to class V HLH factors. They are a distinctive group from other HLH family proteins and show ubiquitous tissue distribution, have a HLH region, but lack a basic region (Benezra et al., 1990). They often interact with variety of bHLH transcriptional factors to form inactive heterodimers and act as negative regulators of class I and class II bHLH proteins. There are four members of this family in mammalian cells, Id1-Id4. Except the common HLH domain that mediates Id dimerization, each Id protein has unique feature that may regulate specific functional roles. For example, Id2, Id3 and Id4 contain a consensus cdk2 phosphorylation site in their N-terminus and are phosphorylated by cyclin-cdk2 complexes, but Id1 does not have this feature (Benezra et al., 1990; Christy et al., 1991; Sun et al., 1991; Biggs et al., 1992; Ellmeier et al., 1992; Deed et al., 1993; Hara et al., 1994; Riechmann et al., 1994; Pagliuca et al., 1995). Id proteins have been implicated in regulating various cellular processes, including cellular growth, senescence, differentiation, apoptosis, angiogenesis and neoplastic transformation (Sikder et al., 2003), and been implicated as oncogenes which may play roles in the tumorigenesis of a wide range of cancers (Ling et al., 2006). For example, in prostate cancer studies, the high expression level of Id1 was associated with the hyperplasia of prostate cancer in a Noble rat model (Ouyang et al., 2001). Also, the expression level of Id1 increased as the Gleason scores of the tumors increased (Ouyang et al., 2002), suggesting that over-expression of Id-1 may play a role in prostate cancer progression.

There are also other members of HLH transcription factors, for example, c-Myc and E2F belong to the class III HLH proteins, which have a leucine zipper adjacent to the HLH motif; Mad and Max are the class IV HLH factors; and the bHLH-PAS factors,
which have a bHLH domain located at their amino termini and two well-conserved regions, PAS-A and PAS-B, separated by a poorly conserved spacer adjacent to the bHLH domain (Jones S, 2004). Since more and more numbers of new bHLH transcription factors are identified continuously, it has become more difficult to understand their relationship and classify them into definitive categories.

**E Proteins**

The class I bHLH proteins are also known as E proteins because their DNA binding is restricted to the E box (CANNTG) element (Massari and Murre, 2000). They include E12, E47, HEB and E2-2 (also called ITF-2) (Figure 2A). As a subgroup of HLH family members, E-proteins contain a bHLH motif and share the essential characteristics with other bHLH proteins in terms of structural features and DNA binding specificities. They have the ability to form either homo- or heterodimers with other members of the HLH family. But in most instances, they prefer to form heterodimers with class II bHLH proteins (Murre and McCaw, 1989b; Quong and Romanow, 2002). It is also well known that E-proteins prefer to interact with their dominant negative inhibitors, Id proteins (Engel and Murre 2001; Yokota 2001) (Figure 2B). E proteins are widely expressed and are known to play key regulatory roles in a variety of developmental processes (Engel and Murre, 2001). For example, in B- and T-lymphocyte development, one E-protein can form homodimers or heterodimers with other E-proteins family members (Greenbaum and Zhuang, 2002; Xin, et al, 1993; Bain G et al, 1994, 1997); in muscle cells, E-proteins are also able to form heterodimers with muscle regulatory factors (MRFs) to exert their effects on myogenic differentiation (Berkes and Tapscott, 2005). In addition to bHLH domain, the mammalian E-proteins also contain two transactivation domains, AD1 and
AD2. AD1 is located at N-terminal of the proteins and contains a putative α-helix, and AD2 is located between AD1 and the bHLH domain and possesses a loop-helix structure (Massari et al., 1996; Inukai et al., 1998). These transactivation domains provide the potential to recruit coactivator or corepressor complexes and play an important role in transcriptional regulation (Markus et al., 2002).

**Figure 1**

*Figure 1*: Classification of representative members of the HLH family of transcription factors.

Figure 2: A: Schematic depiction of the E- proteins, with the AD1- and LH (AD2)-activation domains and the basic (b) and helix–loop–helix (HLH) DNA-binding. B: Illustration of E-protein dimers binding to E-box sites in promoters and enhancers, and activating transcription of target genes. Id–E-protein dimers are unable to bind promoters and activate transcription due to the lacking of basic domain required for DNA binding.

(Engel and Murre, Nature Review, Immuno., Dec, 2001)

The best-studied main members of E proteins are the E2A proteins. E12 and E47 are two major alternative spliced transcripts of the E2A (also referred to TCF3) gene, which is located at human chromosome 19p13.3. They were originally identified as binding proteins to the E2/E5 sequence elements located in the enhancers of immunoglobulin genes (Murre, 1989a). The nucleotide and amino acid sequences of E12 and E47 are identical except for the bHLH motif, and the protein sequences of them are highly conserved across human, mouse, Xenopus and chicken (Conlon and Meyer, 2004). Due to the different sequence in bHLH motif of E12 and E47, these two proteins have different specificities and affinities when they binding to DNA. While E47 strongly binds to DNA
as either a homodimer or a heterodimer with MyoD, E12 and MyoD bind to DNA efficiently only as a heterodimer (Sun and Baltimore, 1991). In additional to MyoD, E2A proteins can interact with other members of HLH transcription factors and play an important role in neurogenesis and pancreatic development, as well as in transcriptional activation of the insulin gene (Lee, 1997; Naya and Huang, 1997; Glick, Leshkowitz et al., 2000). As mentioned previously, the regulatory role of E2A proteins can be affected by ubiquitously expressed dominant negative regulators Id proteins (Benezra et al., 1990; Langlands 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 (Lingbeck, 2008).

E2A proteins not only can interact with bHLH family member, but also can interact with non-HLH partners. For example, the rat homologue of the human polymyositis-scleroderma autoantigen (rPM-Scl) localized 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 et al., 1997b); UbcE2A (mUbc9), a ubiquitin-conjugating enzyme has also been demonstrated to interact with E12/E47 as does rPM-Scl and the interact domain of E12/E47 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 et al., 1997a; Huggins et al., 1999). Other interaction partners of E2A protein are the MAPK-activated protein kinases 3pK and MK2, which were identified as E47 kinases in vitro and the expression of either one will result in a
repression of the transcriptional activity of E47 on E-box containing promoter (Neufeld et al., 2000).

The homo- or heterodimerization of E2A proteins is required to achieve a transcriptional regulation by direct binding to canonical E-box element(s) in promoter regions (Bain et al., 1994; Massari and Murre, 2000). To date, the transcriptional regulatory functions of E2A have been focused on the regulation of the development of B- and T-lymphocytes, and tumorigenesis of B- and T-cell lymphomas (Bain et al., 1999; Barndt et al., 1999, 2000; Bergqvist et al., 2000; Engel and Murre, 1999; Engel and Murre, 2001). E2A proteins are highly expressed in lymphoid progenitor populations and identified as essential regulators of gene expression during lymphocyte development (Greenbaum and Zhuang, 2002; Bain et al., 1994; Zhuang et al., 1994). E2A-deficient mice show a complete and persistent block of early B-cell development prior to initiation of immunoglobulin heavy chain rearrangement (Greenbaum and Zhuang, 2002), this result supports that the E2A gene is required for B-cell development. The E2A proteins are also required for proper T-cell development (Engel and Murre, 1999, 2001). Loss of E2A results in a partial block at the earliest stage of T-lineage development and E2A is essential for properly coordinated temporal regulation of V(D)J rearrangements within the T-cell receptor (TCR) gamma and delta loci (Bain et al., 1997; 1999). In addition to the regulatory function in the development of B- and T-cells, E2A also can regulate myogenic differentiation. The transactivation domains of myogenin, a muscle-specific basic helix-loop-helix (bHLH) transcription factor, are phosphorylated when it heterodimerizes with E2A proteins, and this phosphorylation of myogenin will 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 (Zhou and Olson, 1994). In addition, co-expression of E2A proteins with MyoD or Id1 led to alterations in nuclear localization. Turn over rates of MyoD and Id1 (Deed and Armitage, 1996; Lingbeck et al., 2005) and Cdk2-dependent phosphorylation of Id2 correlates with the restoration of E12/E47 binding to DNA (Hara et al., 1997). Therefore, the protein modification of E12/E47 binding partners such as MyoD and Id proteins can provide another mechanism for the transcriptional activity mediated by E2A proteins.

E2A is not only an important transcription regulator, but also a tumor suppressor. The role of E2A as a tumor suppressor was first demonstrated by the observation that most E2A-deficient mice develop spontaneous thymic lymphomas and that nearly half of the surviving E2A-null mice develop acute T-cell lymphoma (Yan et al., 1997). Furthermore, E2A inactivation is a common feature of a wide variety of human T-cell proliferative disorders, which result in T-cell malignancies (Engel and Murre, 2002). The loss of E2A results in tumor progression and restoration of E2A can result in inhibition of cell growth and apoptosis of human leukemic T cell. Enforced expression of E2A in E2A-deficient lymphomas can also lead to programmed cell death in these lymphomas (Park et al., 1999; Engel and Murre, 1999). The function of E2A as a tumor suppressor is also demonstrated by cell cycle progression studies. For example, E2A can block cell growth at the G1 to S phase transition in NIH3T3 cells (Hara et al., 1994); E2A proteins can induce p21, INK4A and INK4B that inhibit G1 cell cycle progression (Prabhu et al., 1997; Pagliuca et al., 2000), overexpression of E2A can transcriptionally activate the p21 gene, which encodes an inhibitor of the cyclin-dependent kinases (CDK) (Harper et al.,
1993), also, overexpression of E2A in 293T cells activates expression of the endogenous p21 gene at mRNA and protein level (Prabhu et al., 1997). Some studies reported that E47 protein binds to the E-box-containing region in p16INK4a, regulating the activity of p16INK4a and this regulation is silenced by Id1 (Zheng et al., 2004). Further more, Rothschild et al. reported that p57Kip2 is a target gene of E47, and p57Kip2 is a functionally relevant target recruited by bHLH transcription factors to induce cell cycle arrest in developing neuroblasts (Rothschild et al., 2006). Collectively, these numerous studies have demonstrated that E12/E47 can inhibit cell cycle progression, supporting their tumor suppressor function. However, while most of studies support the suppressive function of E2A, some others showed that E2A does not inhibit but even promote cell cycle progression (Zhao et al., 2001; Song et al., 2004). So the role of E2A can be distinctive depending on its temporal interacting molecular networks, which may be continuously changing in response to various cellular contexts or developmental stages.

**Zinc finger proteins**

Zinc finger proteins are small protein domains in which zinc plays a structural role to stabilize the domain. The first identified zinc finger protein is transcription factor IIIA (TFIIIA) from Xenopus, and it can bind to the internal control region of 5S RNA gene to regulate its transcription (Miller et al., 1985), since then numbers of zinc finger proteins have been identified and the DNA-binding properties of zinc finger has been explored in extreme details (Wolfe et al., 2000). Zinc finger proteins, which are extremely abundant in higher eukaryotes, typically function as interaction modules and bind to nucleic acids, proteins and small molecules (Krishna et al., 2003). According to the structure of the
zinc stabilizing amino acids, zinc finger proteins are categorized into about 20 types, such as C2H2, C2HC, C2C2, C2Hc C2C2, and C2C2 C2C2 (Krishna et al., 2003; Matthews and Sunde, 2002). The most common type is the C2H2 type, or “classical” zinc finger, which is often described as CX_{2,4}CX_{12}HX_{2,6}H (Wolfe et al., 2000). The finger structure contains two to three β strands in its N-terminal and one α helix in the C-terminal. This structure can self-fold to form ββα structure and coordinated binding of a zinc ion by the two conserved cysteine and histidine residues (Frankel et al., 1987; Parraga G et al., 1988; Pavletich and Pabl, 1991). The primary role of C2H2 fingers is to bind to DNA segments and control transcription of target genes together with other factors (Iuchi, 2001). They are not only ubiquitous, but also the most common proteins within eukaryotic proteomes (Brayer and Segal, 2008). It is estimated that as much as 1% of total mammalian proteins are C2H2 zinc finger proteins, and to date, about one hundred thirty-three species of C2H2 type zinc finger cDNA have been identified in human brain alone (Klug, 1999; Becker et al., 1995; Berg and Shi, 1996). Based on the distribution of zinc finger structures, C2H2 zinc finger proteins can be divided into three groups: triple-fingered, multi-adjacent-fingered and separated-paired-fingered (Iuchi, 2001). Triple-fingered protein, such as Zif268, contains three C2H2 fingers, each of which forms two β strands and one α helix, and also contains linkers between the fingers; multiple-adjacent-C2H2 zinc finger proteins have four or more fingers located close to one another at similar intervals, these proteins often have more than one binding activity. TFIIIA, the first identified zinc finger protein belongs to this group; the third group, separated-paired-C2H2 zinc fingers proteins contain one or more pairs C2H2 structures, such as Tramtrack (TTK) and Basonuclin (Iuchi, 2001).
In addition to their DNA-binding capability, C2H2 zinc finger proteins have been shown to interact with RNA and proteins (Mackay and Crossley, 1998; Brown, 2005; Hall, 2005), and to be involved in RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding (Laity et al., 2001). In recent years, some novel C2H2 zinc finger proteins have been designed to bind unique sequences of the human genome, and been proved to be valuable for human gene therapy (Iuchi, 2001). However, the other functional significance of zinc finger proteins is partially or completely unknown. In order to detect the new significance of zinc finger proteins, to detect the potential roles of zinc finger proteins to their target genes or target interacted proteins is necessary.
Materials and Methods

Cell Culture

293T, HeLa and COS cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine and 1% non-essential amino acidS (Gibco-BRL) at 37°C under a humidified atmosphere of 5% CO2.

Generation of ZNF451-expression Construct

DKFZp686M03226 (BX648081) cDNA clone (imaGenes, Germany, Berlin), which contains the whole coding region of human ZNF451 except a mutation site at 2145 (amino acid R is changed into C) was used as a template for PCR to generate a human full-length ZNF451-HA cDNA. BaeI site was chosen as a start point of an 1166bp PCR product to correct the mutate site. Using primer pairs: 5’-GCCACAAGTTTCATAGATACAGCTGTGCTCACTGCAGAAAGCCT-3’ and 5’-GGCCTCGAGTTAAGCGTAATCTGGAACATCGTATGGGTACATCATTTCCTCAA GAC-3’, a PCR fragment spanning from BaeI to XhoI site of the template clone was amplified to correct the mutation site and HA tag at 3’ end of the clone on the same time was added. Then the whole coding region of human ZNF451 with HA tag at 3’ end was cloned into pcDNA3.1 using KpnI and XhoI to generate human full-length ZNF451-HA construct.

Yeast Two-Hybrid Screening Assay
An LNCaP prostate cancer cell two-hybrid cDNA library was generated in the pGADT7-Rec vector with the Clontech Matchmaker 3 Library Construction Kit (Clontech). Following manufacturer’s instructions, 10 µg of total RNA is used for synthesis of cDNA. 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 1.3x10^6 clones of the amplified library were subject to screening by yeast mating. The yeast mating mixture was grown on Leu-, Trp-, His- and Ade- nutrient selective media for 7 days followed by processing for standard filter-lift β-galactosidase assay. Clones positive for β-galactosidase, visualized by the development of blue color, were subject to isolation of yeast DNA. Isolated yeast DNA was transformed into *E. coli* DH5α to prepare *E. coli* plasmid DNA. The plasmid DNA were sent to be sequenced. Inserts in the correct reading frame were tested in a bait specificity assay. For this, DNA for each cDNA library clone was co-transformed into *S. cerevisiae* strain AH109 with E12 bait construct or Lamin C construct or pGBTK7 empty bait vector, which were used as negative controls. Following co-transformation, yeasts were plated on either double (Leu-, Trp-) dropout (DDO) or quadruple (His-, Leu-, Trp-, Ade-) dropout (QDO) nutrient selective media and bait specificity of the interaction scored by appropriate growth pattern on selective media and by β-galactosidase activity.

**Immunoblot Analysis**

For immunoblot analysis of ZNF451 protein expression, 293T, HeLa and COS cells were cultured in 35mm dishes and transfected with a total of 4 µg of empty vector (pcDNA3.1) or ZNF451-HA using Lipofectamine 2000 reagent (Invitrogen Corp.). Cells
were harvested at 24 and 48 hrs post-transfection by lysis in TNN (+) 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. and centrifuged at 14,000 rpm for 15 min. Supernatant was collected and protein concentrations were determined. 20 µg of protein sample was loaded per lane of 10% SDS-PAGE gels, followed by overnight electroblotting onto PVDF membrane with 0.025 M Tris/0.192 M glycine transfer buffer supplemented with 20% methanol. The next day, membranes were blocked for 1 hr in 5% non-fat milk in 1X PBS containing 0.5% Tween 20 (PBS-T) and incubated for 1 hr at room temperature with mouse monoclonal HA primary antibody (1:1000, Covance Research Products, Berkeley, CA) or tubulin-β antibody (1:10,000). Secondary antibody was HRP-conjugated goat anti-mouse (1: 2000, Santa Cruz Biotech.). Following washing 3 times (10 minutes per time) in 1X PBS-T, signal was detected by ECL Plus enhanced chemiluminescence (GE Healthcare).

**Immunocytochemical Analysis of Protein Localization**

HeLa cells were plated on coverslips placed in 6-well plates (1.5x10^5 cells/well). After 24 hours cells were transfected with total of 4 µg of full-length E12-pcDNA3.1 and/or ZNF451-HA using Lipofectamine 2000 reagent (Invitrogen). As needed, empty vector pcDNA3.1 plasmid was added such that total mass of transfected DNA was 4 µg. After 22 hours, cells were washed twice with 1X PBS and fixed with 100% ice-cold methanol for 10 min., followed by incubation in 0.1% BSA in PBS (blocking solution) for 30 min. After washing with 1X PBS at room temperature, cells were incubated for 1.5 hours in blocking solution containing polyclonal E2A/E12 antibody (1:100, Santa Cruz
Biotech.) and/or monoclonal HA (1:100, Covance Corp.). 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. Cells were then washed with dH2O and incubated with 10 µM DAPI (Invitrogen Corp.) for 10 min to stain nuclei. Coverslips were mounted on glass slides and observed at 200X magnification 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).

Co-Immunoprecipitation Assay

For co-immunoprecipitation, 293T cells were transfected separately or with the indicated combination of expression constructs for ZNF451-HA and/or full-length E12-pcDNA3.1. A total of 4 µg of DNA was used for transient transfection and empty vector (pcDNA3.1) was added as needed to maintain a total mass of 4 µg of DNA per transfection. At 48 hours 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). 100 µg of cell lysate was added to TNN(+)-buffer lysis buffer to 300 µl and incubated with 3 µl E2A/E12 primary antibody (1:100) by rotation overnight at 4°C, followed by incubation for 3-5 hrs with 50 µl of 50% (v/v) suspension of protein A-agarose beads (Santa Cruz Biotech, Inc.). After centrifuging 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. After
heating at 95-100°C for 10 min, 40 µl of supernatant was subjected to western blot analysis.

**RNA Purification and Transcript Analysis**

RNA was isolated from cultured cells using TriZol Reagent (Invitrogen Corp.) following the manufacturer’s instruction, and purified with an RNeasy RNA purification kit with DNase I treatment (Qiagen, Valencia, CA). 4 µg of RNA was used for first-strand cDNA synthesis with SuperScript II RNase H-RT (Invitrogen Corp.) and an oligo(dT)-22 primer. Semiquantitative PCR for human ZNF451 was performed with 10 ng of cDNA by utilizing the primer pair: 5’-ACATTGCTTCTTACAGAAACCC-3’, and 5’-GCTCCAGAAACCTTGATCTC-3’ to detect the ZNF451 transcript. This primer pair will generate a 600 bp amplicon from transcript isoform 1 or a 456 bp amplicon from isoform 2. Products at 30 cycles were run on a 1.2% agarose gel and signal visualized by ethidium bromide staining. Human GAPDH (5’-GTTGCCATCAATGACCCCTTCATTG-3’ and 5’-GCTTCACCACCTTCTTGATGTCATC-3’) was used as internal control.

**Quantitative Real-Time PCR**

For quantitative real time PCR, transcript levels were analyzed using SYBR Green-based real-time PCR in 25-ul 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
34 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. Gene expression 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 ΔΔCT method and are shown as means ±SD in triplicate. The gene specific primer pairs utilized in this analysis were as follows:

Human ZNF451: 5’- GGAGAAAGGAGTTGAGAATGAC-3’ and 5’-ACTACAGCGAGGATGAGG-3’; Mouse ZNF451: 5’-TTGTTTACCCAAGGATGAGGAG-3’ and 5’- CAGAGAGACCACCTTCCCTTTGCTTC-3’;

Human GAPDH: 5’- GTTGCCATCAATGACCCCTTCATTG-3’, 5’- GCTTCACCACCTTCTTGTGGATGTCATC-3’; Mouse GAPDH: 5’-ACAGCCGCATCTTTCTTGTGGCAGTG-3’ and 5’-GCCTTGACTGTGCAGTGGAATTTT-3’.

**E-box 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 just upstream of an SV40 minimal promoter linked to a firefly luciferase gene in the pGL3-promoter vector (Promega Corp.). 293T cells (10^5 cells/well) were plated into 24-well culture plates. The mass of DNA plasmids transfected per well were E12-pcDNA3.1 (0.004 μg), MyoD-pcDNA3.1 (0.1 μg), ZNF451-HA (0.02 μg). All transfections include the internal control plasmid pRLTK (0.1 μg) and the E-box pGL3
(0.2 μg) luciferase reporter plasmid (0.2 μg). The total combined mass of DNA per well of a 24 well plate was maintained to 1 μg with the addition of empty pcDNA3.1 vector as needed. Cotransfections were carried out in 293T cells using Lipofectamine 2000 (LP 2000) reagent (Invitrogen Corp). At 48 hours post-transfection, cell lysates were prepared by lysis in passive lysis buffer as manufacturer’s instructions (Promega Corp., Madison, WI). Samples were prepared at least in quadruplicate and luciferase activities were measured using a Turner Systems dual luminometer (Promega Corp.).
Results

Yeast Two Hybrid Screening Identify ZNF451 as an E12 Partner

The bHLH domain of E12 is located at amino acids 547-607 of E12, which includes 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 (Murre et al., 1989a). Since full-length E12 contains two intrinsic transactivation domains (AD1 and AD2) in its N-terminus that can autonomously induce transcriptional activation of yeast-two hybrid reporter genes, an E12 bait containing amino acids 508-654 was utilized for yeast two hybrid screening. Following screening of 1.3X10^6 clones of an LNCaP prostate cancer cell yeast two-hybrid library, we identified a cDNA clone that contained an N-terminal portion of the coding region of a zinc finger protein family member named ZNF451. This yeast two hybrid cDNA clone contained amino acids 1-96 of human ZNF451 and maintained reading frame with the GAL4 activation domain present in the pAS2 bait vector.

Figure 3A shows a schematic illustration of the full-length E12 protein, the region of E12 used as bait, full-length ZNF451 (1-1061) and the ZNF451 (1-96) library clone identified in yeast two-hybrid screening. To confirm bait-specific interaction of the E12 bait (E12 (508-654)) with ZNF451 library clone, plasmids were transformed into AH109 strains of *S. cerevisiae*. Id1, a well-known binding partner of E12, was used as a positive control, and empty vectors are used as a negative control. Only the yeast harboring both bait and the library constructs are able to grow on tryptophan- and leucine- deficient double dropout (DDO) agar media plates. The left panel of Figure 3B indicates successful co-transformation of each of the indicated combinations of DNA GAL4 binding and
DNA GAL4 activation domain plasmids. The middle panel of Figure 3B shows that yeast containing both the E12 (508-654) and the ZNF451 (1-96) or Id1 constructs are able to grow on tryptophan-, leucine-, histidine- and adenine- deficient dropout (QDO) agar media plates. No growth was found in QDO media with ZNF451 (1-96) in combination with either empty bait vector or with the unrelated bait, lamin C. The colorimetric detection of X-α-galactosidase activity, an additional reporter gene for protein-protein interaction in this system, is shown in the right panel of Figure 3B and confirms bait-specific interaction of ZNF451(1-96) with E12(508-654).

The human ZNF451 gene is located at 6p12.1 and its mRNA transcript contains 5241 bases with a 3186 base open reading frame. Full-length human ZNF451 protein contains 1061 amino acids with a calculated 121 KD molecule weight. Its protein sequence is dominated by 11 C2H2 zinc finger structures, and it has a C-terminal ubiquitin interaction motif (UIM) (Figure 4). There are two alternative splicing products of ZNF451 gene, isoform 1 and isoform 2. Isoform 2 lacks amino acids 870-917, which are encoded by exon 11. Current knowledge is very limited regarding the function of ZNF451, therefore, the interaction between ZNF451 and E12 is attractive in terms of detecting the role of E12 in the development of prostate cancer. It might be a useful molecular tool to illustrate a mechanism of prostate tumorigenesis. Also, it may be important to better understand the characteristics of ZNF451 and its related protein-protein interactions, as well as its expression and regulation in various human diseases including cancer.
Figure 3: Yeast two-hybrid analysis identifies ZNF451 as a binding partner for E12 (508-654) protein. A: Schematic representation of full-length E12 (E12(1-654)) and full-length ZNF451 as well as E12 bait (E12(508-654)) and ZNF451 library clone identified in yeast two-hybrid assay. B: The E12(508-654) and ZNF451(1-96) expressing vectors as well as empty vector (pGBKT7 or pGADT7) were co-transformed into AH109 in the indicated combinations. Lamin C was used as a negative control and co-transformation of E12(508-654) and full-length Id1 was used as a positive control. Interaction was also assessed by the blue colony growth of yeasts on medium agar plates containing X-α-gal (20 mg/ml).
**Figure 4:**

A: ZNF451 transcript and amino acids sequence. The double-underlined amino acids indicates the sequence of ZNF451 (1-96) present in the yeast two-hybrid cDNA clone for ZNF451. The single underlined sequences are the 11 zinc fingers. Sequence labeled with dashed line is the region missing in isoform 2; the sequence labeled at C terminal is ubiquitin interaction motif (UIM). The bolded and italic amino acids are products between two exons.

B: The alignment of 11 C2H2 zinc finger sequences, the Cys and His residues are all boxed, amino acid positions of zinc finger are listed at right.
**Protein Expression of Full-length ZNF451**

An interaction of E12(508-654) with ZNF451(1-96) was observed by yeast two-hybrid analysis. However, compared to the well-studied details for E12 protein, to date the report about characteristics of the ZNF451 protein is limited. Therefore, we first examined the size of the protein encoded by an expression construct for ZNF451 with a C’ HA epitope tag, ZNF451-HA. 293T, HeLa and COS cells were transfected with ZNF451-HA or pcDNA3.1, and then cells were harvested at 24 and 48 hours post-transfection. Whole cell lysates were subjected to immunoblot analysis using monoclonal HA primary antibody. The western blot in Figure 5A shows that the expression level of ZNF451 is various in three cell lines, the protein level is higher in 293T cells than that in HeLa cells, however, no protein expression was detected in COS cell. A protein band with a molecular weight in accord with the calculated mass of ZNF451 primary translation product was detected (Figure 5A).

**Immunostaining for Intracellular Localization of Full-length ZNF451**

We next conducted studies to detect the intracellular localization of ZNF451. Immunostainning was carried out using HeLa cells transfected with either the ZNF451-HA construct or an empty vector negative control. At 22 hours post-transfection, cells were fixed and incubated with HA primary antibody and Alexafluor 568-conjugated goat anti-mouse secondary antibody. The data demonstrate that human ZNF451 is localized primarily in the nucleus (Figure 5B).
Figure 5

A

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<th></th>
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B

Alexafluor568  DAPI  Merged
Empty Vector No Primary Antibody

Negative Control
**Figure 5:** Immunobloting and immunocytochemical analysis of ZNF451. A: Protein expression of ZNF451 by immunoblot analysis. The empty vector (pcDNA3.1) or ZNF451-HA expression construct was transfected into 293T cells, COS cells and HeLa cells. At 24 and 48 hrs posttransfection, whole cell lysates were subjected to immunoblot analysis using monoclonal HA primary antibody. Tubulin was used as a loading control. B: Immunocytochemistry for ZNF451-HA. HeLa cells were transfected with ZNF451-HA and at 22 hrs post-transfection, subjected to immunostaining with monoclonal HA primary antibody, followed by incubation with Alexafluor 568-conjugated goat anti-mouse secondary antibody (left panel). DAPI was used to stain nuclei (middle panel). No signal was detected when empty vector (pCDNA3.1) or when no HA primary antibody was utilized (bottom panel). Representative images for three individual studies are shown. Images with 200x magnification are used.

**Co-immunoprecipitation of E12 and ZNF451**

An interaction of E12 with ZNF451 was detected from yeast two-hybrid analysis. However, since E12 and ZNF451 utilized in this assay were truncated fusion proteins, the interaction between full length E12 and full-length ZNF451 was confirmed by co-immunoprecipitation. 293T cells were transfected with expression construct for E12 and ZNF451-HA either separately or in combination with empty vector, pcDNA3.1 was set up as a negative control. Following immunoprecipitation with E2A/E12 antibody and immunoblotting with monoclonal HA antibody, only the 293T cell lysate co-expressing E12 and ZNF451-HA showed a positive interaction in this analysis (Figure 6). The
detected interaction is specific since when an unrelated antibody was added to perform coimmunoprecipitation, no interaction was detected (data not shown).

**Figure 6**

| pcDNA3.1: | + | + | + | - |
| E12: | - | - | + | + |
| ZNF451-HA: | - | + | - | + |

**Figure 6**: E12 Co-immunoprecipitates with ZNF451-HA. Co-immunoprecipitation of E12 and ZNF451-HA was assessed by transient transfection in 293T cells. ZNF451-HA and E12 proteins were expressed separately or in combination. 100 μg of each cell lysate was subjected to co-immunoprecipitation with polyclonal E2A/E12 antibody and immunoblot for monoclonal HA (first Panel) or polyclonal E2A/E12 primary antibodies (second Panel). 20 μg of total protein was loaded as input and tubulin was used as loading control (third, fourth and fifth panels).
**Immunocytochemical Co-localization Studies of E12 and ZNF451**

After we confirmed the interaction between E12 and ZNF451 with coimmunoprecipitation, we carried out immunostaining to test their respective intracellular distribution in cells co-expressing both of these proteins. ZNF451-HA and full-length E12 were expressed in HeLa cells by transient transfection and the intracellular localization of ZNF451-HA co-expressed with E12 was examined. From the images in Figure 7, we found that the signal for ZNF451 was primarily in the nucleus, appearing as random punctuated distribution. E12 signal distributes in the whole nucleus. However, in cells that co-expressed ZNF451 and E12, the ZNF451 signal appeared as a large aggregated structure, which obviously colocalized with E12. Taken together, this result provides evidence that after co-expression with E12, the distribution of ZNF451 is changed and it also supports a consistent interaction of E12 and ZNF451 that occurs in a cellular context.
Figure 7

A

ZNF451-HA

Alexafluor568  DAPI  Merged

B

E12

FITC  DAPI  Merged

C

ZNF451-HA and E12

Alexafluor568  FITC  Merged  DAPI

D

Empty Vector  No Primary Antibody

Negative Control
Figure 7: Immunocytochemistry demonstrates that ZNF451 colocalizes with E12 in the nucleus. Panel A shows HeLa cells transfected with ZNF451-HA alone, and panel B shows cells transfected with E12 alone. Panel C shows cells that cotransfected with ZNF451-HA and E12. Panel D is negative control.

Quantitative PCR Analysis of Expression of ZNF451 Transcript in Various Cancer Cell Lines and Tissues

To gain further information on the expression pattern of ZNF451 transcript, we used real-time PCR to analyze various human and mouse tissues, and various human cancer cell lines, including those from breast (MCF7 and ZR75), prostate (LNCaP), bone (U2OS2 and MG63), skin (A431), liver (HepG2), connective tissue (HT1080), cervix (HeLa) and blood (HL60, MEG01). Figure 8A indicates that the ZNF451 transcript level is enriched in HepG2, MEG01 and LNCaP cells compared to that in the other cell lines, however, it is still less abundant than that in normal human testis tissue, which is used as positive control tissue. Since the study from our lab showed that the expression level of E12 is lower in LNCaP androgen-dependent prostate cancer cell than that in LNCaP androgen-independent derivatives LN95, LN96, LN97, LN98, we question whether the ZNF451 transcript level in these cell lines have similar distribution pattern. Figure 8B illustrates that compared to the ZNF451 transcript levels in LN95, LN96, LN97 and LN98, the level of it in LNCaP is low, which is consistent with the result of E12 expression. However, the ZNF451 transcript level in LNCaP cell is higher than that in PC3 and DU145, which does not show the same trend as that in LNCaP cell and its derivatives (Figure 8C). Next we detected ZNF451 transcript in various human and mouse tissues that are available currently in our lab (Figure 8D and E). Mouse ZNF451
(zfp451) contains 1056 amino acids, 12 zinc finger structures and 79.8% identification with human ZNF451. From the result of human tissues, the ZNF451 transcript shows random distribution, however, it shows significant testis specific expression in mouse tissues, suggesting that ZNF451 might ubiquitously expressed in adult human tissues.

**Figure 8**

A

![Graph A](image1)

B

![Graph B](image2)
**Figure 8:** Quantitative real-time PCR analysis demonstrates ZNF451 transcript expression in various human cancer cell lines, LNCaP cell and its derivatives, PC3 and DU145 cells, human tissues and mouse tissues. 10 ng of input cDNA was used for real-time PCR. The relative expression of ZNF451 transcript in each sample was measured and normalized against GAPDH expression level. The lowest expression level of ZNF451 transcript was set at a value of 1 in ZR75, LNCaP, human small intestine and mouse stomach. The fold change was calculated and shown as means ± SD in independent triplicate samples. A: ZNF451 transcript level in different human cancer cell lines; B: ZNF451 transcript level in LNCaP cells and its androgen-independent derivatives; C: ZNF451 transcript level in LNCaP, PC3 and DU145 cell lines; D: ZNF451 transcript level in various human tissues; E: Mouse ZNF451 transcript level in various mouse tissues.
Using Semiquantitative End-Point Reverse Transcription PCR to Detect the Distribution of ZNF451 Transcripts in Cells and Tissues

From NCBI database, we know there are two isoforms of this protein. The difference between isoform 1 and isoform 2 is that isoform 2 lacks an exon that encodes 870-917 amino acids. To detect the distribution of two similar isoforms, isoform 1 and isoform 2 in various human cell lines, semi-quantitative PCR was carried out. With a primer pair that is predicted to generate 600 bp product from isoform 1, and 456 bp product from isoform 2. Figure 9 illustrated that only 600 bp amplicon was found, and no product from isoform 2 is obvious, suggesting that isoform 1 is the main transcript exist naturally, and isoform 2 might be an artificial result from inappropriate splicing.

Figure 9
**Figure 9:** Semi-quantitative RT-PCR showing the distribution of ZNF451 isoform 1 and isoform 2 in various human tissues and cancer cell lines. A: Schematic illustration of the strategy to detect the distribution of isoform 1 and isoform 2 transcripts of ZNF451. B: Semi-quantitative end-point RT-PCR. Human GAPDH was used as internal control.

**The E-box Dependent and E12/MyoD-mediated Transcriptional Activation Assay**

We next addressed if ZNF451 could affect E12 transcriptional activity via E-box enhancer elements, for this, we designed an E-box dependent functional assay using an E-box responsive luciferase reporter construct, termed E-box pGL3. This 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; 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 and a well-known binding partner of E12, was employed as a heterodimerization partner for E12 in this assay. As expected, co-transfection of the E-box pGL3 reporter construct and expression constructs of E12 and MyoD led to a ~3.5 fold (P) increase in luciferase activity (Figure 10, columns 1&2). However, as shown in column 2 vs. 3 of Figure 10, the presence of ZNF451 does not have significant transactivation effect (P>0.5) on the E-box-pGL3 activity with co-transfection of ZNF451, E12, and MyoD.
Figure 10: ZNF451 does not affect E12/MyoD-mediated transcriptional activity in E-box function assay. The indicated combinations of plasmids including E12, MyoD and ZNF451 and E-box pGL3 constructs were used for co-transfection into 293T cells to test E-box dependent and E12/MyoD-mediated transcriptional activity. 48 hrs after cotransfection, cell lysates were analyzed to measure luciferase activity. *P<0.01 for column 2 vs. column 1; #P>0.05 for column 3 vs. column 2.
Discussion

Androgens are primary regulators of normal prostate as well as prostate cancer cell growth and proliferation. In androgen-dependent stage, prostate cancer cells depend on the androgen receptor as the primary mediator of growth and survival (Feldman and Feldman, 2001; Nelson et al., 2003; Debes and Inkdall, 2004). During androgen-independent progression, prostate cancer develops in various cellular pathways to survive and grow in an androgen-depleted environment (Feldman and Feldman, 2001). The mechanisms postulated in the process from androgen-dependent stage to androgen-independent stage include androgen receptor (AR) gene amplification, AR gene mutations, activation of coregulators, ligand-independent activation of the androgen receptor, and the tumor stem cells (Shah et al., 2004; Feldman and Feldman, 2001; Nelson et al., 2003; Debes and Inkdall, 2004; Tindall et al., 2004). Androgen receptor (AR) is a critical effector of prostate cancer development and progression. Recently, Karvonen et al. reported that interaction of ZNF451 with PIAS1 (protein inhibitor of activated STAT 1) results in disintegration of ZNF451 nuclear domains and recruitment of ZNF451 to androgen receptor (AR) speckles, and that ablation of endogenous ZNF451 in LNCaP cells significantly decreases expression of several AR target genes, PSA, ELK4 and TMPRSS2, which suggests ZNF451 is a regulatory protein in androgen signaling (Karvonen et al., 2008).

By utilizing a yeast two hybrid screening approach, Zinc Finger Protein 451 was identified as a novel non-bHLH binding partner of E12 in our study. Furthermore, co-immunoprecipitation study result verified the interaction between E12 and ZNF451. The function of E12 has been demonstrated in details in the development of B- and
T-cells, and E12 is very important during lymphopoiesis (Bain and Murre, 1998). Yan et al. also reported that E2A is a tumor suppressor from the study that most E2A-deficient mice develop spontaneous thymic lymphomas and that nearly half of the surviving E2A-null mice develop acute T-cell lymphoma (Yan et al., 1997). Id1, the well known binding partner of E12, has been indicated in promotion of cell survival and proliferation, and it is a key regulator of oncogenic transformation and the growth of malignancies (Alani et al., 1999; Nickoloff BJ et al., 2000). Based on the importance of E12 and Id1 during tumourigenesis and the report from Karmonen’s study, the interaction of ZNF451 and E12 may implicate the potential role of ZNF451 during the development of prostate cancer. ZNF451 might be a bridge molecule between AR and E12, Id1 network. The previous study in our lab showed that E12 expression level is lower in LNCaP androgen-dependent prostate cancer cell than that in its androgen-independent derivatives. The quantitative real-time PCR analysis also demonstrated that the ZNF451 transcript level in LNCaP cell is lower than that in its derivatives, which is consistent with the expression level of E12. So the interaction between ZNF451 and E12 might be involved in the transformation of prostate cancer from androgen-dependent stage to androgen-independent stage.

ZNF451 is a protein that contains 1061 amino acids with 11 C2H2 structures, and belongs to C2H2 zinc finger protein family. C2H2 zinc finger domains were originally identified as DNA-binding domains and function as transcriptional regulators (Brown, 2005). From our study, ZNF451 is identified to interact with E12, however, from our E-box function assay results, ZNF451 does not affect the transcription activity of E12 through E-box element. This result suggests that ZNF451 may link E12 to the other
pathway and the regulation between them may involve in various molecular activities. In 1997, Kho et al. once reported that UbcE2A, which is highly homologous to and functionally complements the yeast ubiquitin-conjugating enzyme UBC9, can specifically interact with E2A protein and that E2A protein is degraded by the ubiquitin-proteasome pathway. On the other hand, ZNF451 can be associated with promyelocytic leukemia (PML) bodies, interact with SUMO E2 conjugase Ubc9 and SUMOs, and be sumolated by SUMOs (Karvonen et al., 2008). Therefore, both E12 and ZNF451 are all involved in ubiquitin-proteasome pathway, and the interaction between them might regulate the degradation of proteins.

An aggresome is formed by aggregated abnormal polypeptides that fail to be degraded in ubiquitin-proteasome system (UPS) (Corboy et al., 2005). It is related to cell death in many degenerative diseases, such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and alcoholic liver disease (Tran, et al., 1999). The formation of aggresome is a protective cellular response that the aggregated proteins are stored and degraded by autophagy (Zaarur, 2008). Kopito has reported that aggresomes are inclusion bodies formed by aggregated protein on microtubules (Kopito, 2000). From our immunostaining study results, we found that ZNF451 aggregates into an aggresome-like structure after co-expression with E12. This result suggests that the interaction between ZNF451 and E12 might affect the structure and distribution of ZNF451 and this changing might improve cell death. The mechanism of the regulation between E12 and ZNF451 may be similar as that of the formation of aggresome. Thus the interaction of E12 and ZNF451 might have potential role(s) during different cellular activities and this is also a good direction in our future study.
Collectively, our present results demonstrated that ZNF451 is a novel binding partner of E12, and the interaction of them may change the structure and distribution of ZNF451 and improve cell death. ZNF451 might be a potential molecule with important roles and the interaction between it and E12 might be a new clue to detect its functional involvement in gene regulation and the development of diseases.
1. The interaction of E12 and ZNF451 is identified in a yeast-two hybrid analysis and the interaction is bait-specific.

2. The interaction between E12 and ZNF451 is confirmed by co-immunoprecipitation study.

3. ZNF451 protein is demonstrated to be localized in nucleus from immunocytochemistry study.

4. The co-expression of E12 and ZNF451 does not alter the intracellular localization of E12, but ZNF451 looks like to accumulate after cotransfected with E12, which suggested that E12 might have a function in regard to the structure of ZNF451 and also supported the interaction of E12 and ZNF451.

5. Compared to ZNF451 transcript level in LNCaP androgen-dependent prostate cancer cell, relatively high expression of ZNF451 transcripts are observed in LNCaP androgen-independent derivatives from quantitative real-time PCR analysis.

6. The ZNF451 transcript level in different human cancer cell line varied, and had higher level in HepG2, and LNCaP cell lines.

7. Compared to the testis specific distribution in mouse tissues, the ZNF451 transcript does not show testis specificity in human tissues.

8. The isoform2 transcript of ZNF451 is not detected in various human cancer cell lines by semi-quantitative reverse transcription PCR analysis.

9. From E-box function assay, ZNF451 does not affect the E12/MyoD-mediated transcriptional activity.
Bibliography


Abstract

E12 is a member of the class I basic helix-loop-helix (bHLH) proteins, which contains a basic DNA binding domain and a helix-loop-helix (HLH) protein interaction domain. E12 is a multifunctional transcription factor that is involved in a large variety of developmental processes. It plays an important role in embryonic patterning, cell fate determination, cell differentiation and proliferation. In human prostate cancer cells, E12 interacts with Id1 (DNA binding inhibitor 1), a dominant negative member of the HLH family of transcriptional regulators. This complex may alter transcriptional activity, promote malignancy and facilitate transition to androgen independence. Using the bHLH domain of E12 as bait in a yeast two hybrid screen of an LNCaP prostate cancer cell cDNA library, we identified an interaction between E12 and Zinc Finger Protein 451 (ZNF451) and the interaction of E12 and ZNF451 is further verified by coimmunoprecipitation. While the DNA-binding properties of some zinc finger proteins have been explored in detail, the function of ZNF451 is currently unknown. Real time PCR detection showed expression of ZNF451 transcript in multiple human cancer cell lines, including HepG2 (human hepatocellular carcinoma cells) and MEG-01 (human megakaryoblast cells). Furthermore, we found increased ZNF451 transcript level in LNCaP androgen independent prostate cancer sublines compared to parental androgen dependent LNCaP cells. Also, from the quantitative PCR analysis, ZNF451 transcript level shows testis specific in mouse tissues compared to that in human tissues. ZNF451 is comprised of 1,061 amino acids, and contains 11 C2H2-type zinc fingers and an ubiquitin interaction motif at C terminal. Immunostaining demonstrated that ZNF451 is localized
to nucleus as tiny speckles. Upon co-expression with E12, ZNF451 accumulated and colocalized with E12 in nuclear and also supporting the interaction of E12 with ZNF451. These results demonstrate for the first time the interaction of E12 with ZNF451 and indicate a possible novel mechanism by which E12 is linked to the development of disease including cancer.