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Research paper

# Inhibitor of differentiation 4 (ID4) acts as an inhibitor of ID-1, -2 and -3 and promotes basic helix loop helix (bHLH) E47 DNA binding and transcriptional activity

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ABSTRACT

The four known ID proteins (ID1-4, Inhibitor of Differentiation) share a homologous helix loop helix (HLH) domain and act as dominant negative regulators of basic-HLH transcription factors. ID proteins also interact with many non-bHLH proteins in complex networks. The expression of ID proteins is increasingly observed in many cancers. Whereas ID-1, ID-2 and ID-3, are generally considered as tumor promoters, ID4 on the contrary has emerged as a tumor suppressor. In this study we demonstrate that ID4 heterodimerizes with ID-1, -2 and -3 and promote bHLH DNA binding, essentially acting as an inhibitor of inhibitors of differentiation proteins. Interaction of ID4 was observed with ID1, ID2 and ID3 that was dependent on intact HLH domain of ID4. Interaction with bHLH protein E47 required almost 3 fold higher concentration of ID4 as compared to ID1. Furthermore, inhibition of E47 DNA binding by ID1 was restored by ID4 in an EMSA binding assay. ID4 and ID1 were also colocalized in prostate cancer cell line LNCaP. The alpha helix forming alanine stretch N-terminal, unique to HLH ID4 domain was required for optimum interaction. Ectopic expression of ID4 in DU145 prostate cancer line promoted E47 dependent expression of CDKNI p21. Thus counteracting the biological activities of ID-1, -2 and -3 by forming inactive heterodimers appears to be a novel mechanism of action of ID4. These results could have far reaching consequences in developing strategies to target ID proteins for cancer therapy and understanding biologically relevant ID-interactions.



## 1. Introduction

The basic helix-loop-helix (bHLH) family of transcription factors are critical cell type determinants that play important roles in cellular differentiation [1]. The highly conserved bHLH domain (reviewed in Refs. [2,3] consists of two amphipathic helices separated by a loop that mediates homo and hetero-dimerization adjacent to a DNA-binding region rich in basic amino acids [4]. The bHLH dimers bind to an E-Box (CANNTG) DNA consensus sequence present in a wide variety of tissue-specific promoters [5]. The transcriptionally active bHLH dimer usually consists of heterodimers between class I proteins E2–2 [6], HEB [7], and E12 and E47 (i.e. differentially spliced products of the E2A gene [8]) and tissue restricted class II proteins such as MyoD [9] and NeuroD [10].

The members of class V, the ID (inhibitor of differentiation/DNA binding) family regulate the transcriptional activity of class I and II bHLH heterodimers. The four known ID proteins (ID1, ID2, ID3, and ID4) share a homologous HLH domain, but lack the basic DNA binding region [11]. Thus, the ID proteins sequester bHLH transcription factors by forming inactive heterodimers and prevent binding of bHLH proteins to the E-box responsive elements [12,13]. Therefore, ID proteins are largely considered as dominant negative regulators of differentiation pathways but positive regulators of cellular proliferation [13–15]. Apart from bHLH proteins, the ID proteins also interact with many non-bHLH proteins with different affinities [16–19] in complex transcriptional and non-transcriptional networks.

As key regulators of cell cycle and differentiation, the expression of ID proteins is increasingly observed in many cancers and in most cases associated with aggressiveness of the disease including poor prognosis [20–23], metastasis [24] and angiogenesis [25]. ID1, ID2 and ID3, are thus generally considered as tumor promoters/

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supporting oncogenes. On the contrary ID4 has emerged as a tumor suppressor [26–33] based on the evidence that it is epigenetically silenced in many cancers [28,29,34,35]. In few cancers ID4 also acts as an oncogene such as in Ovarian Cancer [36,37], Malignant rhabdoid tumors [38] and Glioblastoma [39].

The associated molecular pathways and unique expression profile during development suggests that ID4 may have functions distinct from other ID family members [40–43]. In vitro studies have also demonstrated that ectopic ID4 expression in cancer cells inhibits proliferation, promotes senescence, apoptosis and sensitivity to chemotherapeutic drugs [44]. Thus the biological effect of ID4 appears to be almost completely opposite to those observed by ID-1, -2 and -3 suggesting that the core function of ID proteins as dominant negative bHLH transcriptional regulators may be just a fraction of their overall activity. The majority of ID functions could involve unknown and perhaps yet undefined interactions with sequence specific bHLH or non-bHLH proteins resulting in non-overlapping biological endpoints.

Of all the four ID proteins, the expression of ID1 and ID2 in cancer and the underlying molecular mechanism is relatively well known [45–48]. As compared to ID1, ID2 and ID3, the mechanism of action of ID4 remains largely unexplored. Based on the observations that ectopic expression of ID4 in cancer cell lines attenuates the biological pathways promoted by ID-1, -2 and -3, we conceptualized a highly simplistic model in which ID4 could form a heterodimeric complex with ID-1, -2 or -3 and essentially neutralize their dominant negative bHLH activity. In this study we demonstrate that ID4 in fact has the unique ability to heterodimerize with ID-1, -2 and -3 and promote bHLH DNA binding. These results could have far reaching consequences in developing strategies to target ID proteins for cancer therapy and understanding the ID-bHLH and ID-non-bHLH interactions in cell growth and development.

## 2. Materials and methods

### 2.1. Cell line and cell culture

Human prostate cancer cell lines LNCaP, DU145 and PC3 were obtained from American Type Culture Collection (ATCC, Rockville, MD). LNCaP cells were cultured in RPMI-1640 supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta GA) and antibiotics (pen/strep, Normocin and Gentamycin). DU145 and PC3 cell were cultured in Ham's F12 (Meditech, VA) supplemented with 5% bovine calf serum (Atlanta Biologicals) and antibiotics (pen/strep, Normocin and Gentamycin). All cells were cultured at 37 °C and 5% CO2. DU145 cells over-expressing full length human ID4 (DU145 + ID4) is described elsewhere [49,50].

### 2.2. Recombinant plasmids

The pReceiver-B04 with GST tagged ID4 (OmicsLink Expression Clone) was obtained from GeneCopeia Inc. The pET28a-His-Id1 and pET30a-His-E47plasmids were kindly provided by Dr. Robert Benezra (Memorial Sloan Kettering Medical Center). The GST-ID2 plasmid was kindly provided by Dr. Chuanju Liu (New York University School of Medicine).

### 2.3. Recombinant protein expression and purification

The above mentioned bacterial expression plasmids containing affinity tagged ID-1, -2 and -4 were transformed into E. Coli host strain BL21 (DE3). Optimum recombinant protein expression was obtained with 1 mM IPTG followed by incubation at 30 C for 3–4 h. Cells were subsequently harvested by centrifugation at 3000 g for 15 min at 4 °C. The cell pellet was resuspended in lysis buffer (B-PER, Thermo Scientific) supplemented with 1 mg/ml lysozyme, DNase and complete protease inhibitor cocktail (Invitrogen). The lysate was then clarified by centrifugation at 14,000 rpm for 15 min. The supernatant was loaded on pre-equilibrated affinity column (Glutathione for GST and Ni-NTA for His tagged proteins) and eluted according to manufacturer's protocol (Thermo scientific). On-column cleavage of GST tag from ID4 and ID2 was performed by enterokinase (Gene Script) and thrombin (NEB) respectively. The eluted recombinant proteins were dialyzed against 50 mM Tris (PH7.2) (Tube-O-Dialyzer Medi, 8kMWCO, G-Biosciences).

### 2.4. Western blot

Prostate cancer cell lines were lysed using M-PER (Thermo-Scientific). Twenty five microgram of total protein was electrophoretically separated on 12% SDS-PAGE gel and blotted onto nitrocellulose membranes (Millipore). Western blotting was performed according to standard procedures. After incubation with primary and secondary antibodies, the membranes were developed using an ECL kit (GE Life Sciences, Piscataway, NJ) as described earlier [51]. The ID-1, -2, -3, -4 and E47 antibodies used in this study have been validated as described in our previous studies [51–53].

### 2.5. Immunofluorescence

The cells were grown in six well plates with cover slips. The cells were fixed and permeabilized in methanol for 20 min and then rehydrated with PBS. Before immunostaining with the protein specific antibodies, cells were blocked in 1%BSA in PBS with 0.5% Tween 20. Mouse polyclonal ID4 (Novus), Rabbit polyclonal E47 (Santa Cruz) and Rabbit monoclonal ID1 (BioCheck) antibodies were used for colocalization studies. The primary antibodies were detected by either goat anti-rabbit DyLight 594 or goat anti-mouse Dylight 488 fluorophores (Thermo Scientific). Cells were analyzed using Carl Zeiss AxioVision 4 microscope equipped with AxioCam digital camera and software.

### 2.6. Coimmunoprecipitation assay

To detect the protein–protein interactions, coimmunoprecipitation was performed using protein A coupled to magnetic beads (Protein A Mag beads, GenScript) as per manufacturer's instructions. Briefly, protein specific IgG (anti-ID1 or–ID4) was first immobilized to Protein A Mag Beads by incubating overnight at 4 °C. To minimize the co-elution of IgG following immunoprecipitation, the immobilized IgG on protein A mag beads was cross-linked in the presence of 20 mM dimethyl pimelimidate dihydrochloride (DMP) in 0.2 M triethanolamine, pH8.2, washed twice in Tris (50 mM Tris pH7.5) and PBS followed by final re-suspension and storage in PBS. The cross-linked protein specific IgG-protein A-Mag beads were incubated overnight (4 C) with freshly extracted total cellular proteins (500 μg/ml). The complex was then eluted with 0.1 M Glycine (pH 2–3) after appropriate washing with PBS and neutralized by adding neutralization buffer (1 M Tris, pH 8.5) per 100 μl of elution buffer.

### 2.7. Affinity column based pull down assay

To find out the possible ID4 protein interactions, the bacterial cell lysate of BL21 (DE3) expressing the protein of interest was allowed to bind to the respective affinity column. For In vitro pull down assay, the protein bound affinity columns were used as bait. The purified recombinant proteins (GST cleaved or His-tagged) were used as prey proteins separately. Alternatively, total cell protein was used as prey protein. The column was washed with at