European Journal of Cell Biology 94 (2015) 214–222

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

ING3 protein expression profiling in normal human tissues suggest its role in cellular growth and self-renewal☆

Arash Nabbi[a,b], Amal Almami[a,b], Satbir Thakur[a,b], Keiko Suzuki[a,b], Donna Boland[a,b], Tarek A. Bismar[a,b,c], Karl Riabowol[a,b,*]

[a] Department of Biochemistry & Molecular Biology, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada
[b] Department of Oncology, Southern Alberta Cancer Research Institute, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada
[c] Department of Pathology & Laboratory Medicine, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada

ARTICLE INFO

Article history:
Received 17 January 2015
Received in revised form 3 March 2015
Accepted 3 March 2015

Keywords:
Tumor suppressor
PHD protein
Epigenetic regulator
Monoclonal antibody
Tissue expression
Histone code

ABSTRACT

Members of the INhibitor of Growth (ING) family of proteins act as readers of the epigenetic code through specific recognition of the trimethylated form of lysine 4 of histone H3 (H3K4Me3) by their plant homeodomains. The founding member of the family, ING1, was initially identified as a tumor suppressor with altered regulation in a variety of cancer types. While alterations in ING1 and ING4 levels have been reported in a variety of cancer types, little is known regarding ING3 protein levels in normal or transformed cells due to a lack of reliable immunological tools. In this study we present the characterization of a new monoclonal antibody we have developed against ING3 that specifically recognizes human and mouse ING3. The antibody works in western blots, immunofluorescence, immunoprecipitation and immunohistochemistry. Using this antibody we show that ING3 is most highly expressed in small intestine, bone marrow and epidermis, tissues in which cells undergo rapid proliferation and renewal. Consistent with this observation, we show that ING3 is expressed at significantly higher levels in proliferating versus quiescent epithelial cells. These data suggest that ING3 levels may serve as a surrogate for growth rate, and suggest possible roles for ING3 in growth and self renewal and related diseases such as cancer.

Introduction

The first member of the INhibitor of Growth (ING) family of proteins was identified using subtractive hybridization between normal and transformed mammary epithelial cells followed by an in vivo screen to detect regulators of growth and tumorigenesis (Garkavtsev et al., 1996). Since then, four other members of this family have been identified through sequence homology and bioinformatic analyses (Satpathy et al., 2013; Nagashima et al., 2001, 2003; Feng et al., 2002; Tallen and Riabowol, 2014). The ING proteins are well conserved evolutionarily (He et al., 2005) and both yeast and human ING proteins associate with histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity (Loewith et al., 2000; Vieyra et al., 2002; Doyon et al., 2004). INGs affect HAT and HDAC activity through serving as stoichiometric members of the mSin3A HDAC (ING1 and ING2), Tip60 (ING3), HBO1 (ING4 and ING5) or Moz/Morf (ING5) HAT complexes (Doyon et al., 2006). The most highly conserved feature of the ING family, their plant homeodomain form of zinc finger (He et al., 2005) specifically recognizes the H3K4Me3 histone mark of active transcription (Pena et al., 2006; Shi et al., 2006; Martin et al., 2006), targeting different HAT and HDAC complexes to subsequently alter chromatin structure. In addition to this canonical role in chromatin regulation, several biological functions have been ascribed to the ING proteins, some of which do not appear to be chromatin dependent such as a role in apoptosis through the mitochondria (Bose et al., 2013), roles in DNA damage/repair (Scott et al., 2001; Cheung et al., 2001; Pena et al., 2008; Bua et al., 2013; Larrieu et al., 2009) and modulation of cell motility (Shen et al., 2007; Thakur et al., 2014a). ING3 is the most evolutionarily conserved of this family (He et al., 2005), and was first described as a p53-dependent cell cycle and apoptosis regulator in RKO human colorectal cancer cells (Nagashima et al., 2003). Subsequently many studies have reported ING3 to be down-regulated in different types of cancer including head and neck carcinoma (Gunduz et al., 2002, 2008), melanoma (Wang et al.,

Abbreviations: ING, INhibitor of Growth; HAT, histone acetyltransferase; HDAC, histone deacetylase; TMA, tissue microarray; ELISA, enzyme-linked immunoabsorbent assay; IHC, immunohistochemistry.

☆ Grant number and sources of support: Canadian Institutes of Health Research #MOP-86607; Alberta Innovates Health Solutions #20110276.

* Corresponding author at: 3330 Hospital Drive, NW, Calgary, AB, Canada T2N 4N1. Tel.: +1 403 220 8865.
E-mail address: karl@ucalgary.ca (K. Riabowol).

http://dx.doi.org/10.1016/j.ejcb.2015.03.002
0171-9335/

2007), ameloblastoma (Borkosky et al., 2010), hepatocellular carcinoma (Yang et al., 2012; Lu et al., 2012) and colorectal cancer (Gou et al., 2014). To date, other than one northern blot analysis of ING3 RNA levels in human tissues (Nagashima et al., 2003), ING3 expression in human normal tissues has not been reported and neither protein levels nor localization have been addressed.

Our initial attempts to examine ING3 levels using a commercial antibody were problematic as noted below, and indeed many studies have reported on the ineffectiveness of numerous antibodies and lack the proper controls to detect the protein or proteins that the antibody is believed to recognize (Voskuil, 2014; Yu and Hill, 2013; Talmont and Mouledous, 2014; Herrera et al., 2013). While this is less of a problem when immunoblotting since protein size can be confirmed, when an antibody recognizes proteins other than its target, interpretation of immunofluorescence, immunohistochemistry and immunoprecipitation results can be very misleading (Herrera et al., 2013; Egelhofer et al., 2011). In our experience, available commercial antibodies against ING3 have been ineffective in molecular biology experiments. We have been previously successful in generating well characterized monoclonal antibodies (CAbs1-9) against INGs (Garkavtsev et al., 1997; Boland et al., 2000; Suzuki et al., 2011), which have been used extensively in numerous applications such as western blotting, immunoprecipitation and tissue microarray (TMA) staining (Rajarajacholan et al., 2013; Thakur et al., 2014b). These antibodies are commercially available for other investigators. In this study we have generated a new monoclonal antibody against ING3, and show that it detects specifically ING3 and can be used in various molecular biology techniques such as western blotting, indirect immunofluorescence, immunohistochemistry and immunoprecipitation. Using this antibody, we present the first analysis of ING3 staining in normal human tissues and find that expression of ING3 strongly correlates with rapid cell growth.

Materials and methods

Cell culture, plasmids and transfection

HEK293, RWPE-1 and hTERT-HME human cells and BALB-3T3 and NIH-3T3 mouse cells were obtained from ATCC. 3×10^5 or 2×10^6 HEK293 cells were seeded in 6-well plates or in 10 cm dishes (Fisher Scientific), respectively, and were transfected with 0.5 or 5 μg of plasmid using Lipofectamine 2000 (Invitrogen) in serum free media as per the manufacturer's instructions. The media was changed to DMEM supplemented with 10% FBS after 6 h. A GFP expression vector was used at a 10% molar ratio as a control for transfection efficiency. Whole ING3 protein was subcloned into the bacterial expression vector pET (GE Healthcare) and expressed in *Escherichia coli* for use as an antigen.

Generation of a hING3 monoclonal antibody

Four female BALB/c mice were each injected intraperitoneally with 10 μg of bacterially expressed ING3-GST mixed with Complete Freund's Adjuvant (CFA) initially, and with two subsequent injections at two week intervals using the antigen mixed with incomplete Freund's Adjuvant (IFA). Three to four days after the third injection, one or two mice were sacrificed, and their spleen cells were fused with the myeloma cell line Sp2/mIL6 using polyethylene glycol 1500. The fused cells were plated into 96-well culture plates and when colonies formed, the supernatants were screened by enzyme-linked immunoabsorbent assay (ELISA) to detect positive clones. Selected clones were then subcloned by limiting dilution, as previously described (Zhang, 2012).

Enzyme-linked immunoabsorbent assay (ELISA)

96-well plates (Nunc-Immuno, Thermo Scientific) were coated with either 1 μg ING3-GST/ml in carbonate buffer, pH 9.2 or 1 μg GST/ml carbonate buffer, pH 9.2 and incubated at 37 °C for 2 h or at 4 °C overnight. Plates were then washed three times and non-specific binding sites were blocked with 1% BSA/PBS, pH 7.4 at 37 °C for 30 min. Supernatants from candidate clones were added to plates previously washed, and were then incubated at 37 °C for 1 h. Goat anti-mouse IgG-horseradish peroxidase (HRP) (Jackson Laboratories) was added as secondary antibody at a 1:1000 dilution and plates were incubated at 37 °C for 30 min. Plates were washed three times, ABTS-peroxidase substrate (Mandel scientific, Inc) was added to wells and incubated at 37 °C for 30 min, and then the absorbance was read using a plate reader at 405 nm.

SDS-PAGE and western blotting

After washing twice with ice-cold PBS, cells were lysed (50 mM TRIS pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 supplemented with protease inhibitor cocktail (Roche). After centrifugation and addition of Laemmli's sample buffer, samples were boiled for 5 min and electrophoresed. Nitrocellulose membranes (Pall Inc.) were used for transfer and membranes were blotted with in-house mouse ING3 2A2 monoclonal antibody, mouse anti-beta-actin (Santa Cruz), rabbit anti-HA tag (Santa Cruz) or rabbit anti-GAPDH (Cell Signaling), as indicated in the figures. As secondary antibody, horse radish peroxidase-conjugated secondary antibodies for mouse or rabbit (Millipore) were used. ECL reagent (Millipore) was used to visualize protein bands on X-ray film (Kodak).

Immunoprecipitation (IP)

Cell pellets were lysed under denaturing conditions (0.5% SDS) and sonicated briefly. IP samples were incubated with mouse anti-HA antibody (Covance) or different dilutions of 2A2 antibody, as indicated in the figure for 2 h at 4°. Protein G beads (GE Healthcare) were added to antibody-antigen complex and were incubated for 2 h at 4° with mixing. After centrifugation, post-IP samples were taken and beads were washed three times with IP buffer. Laemmli's sample buffer was added to washed pellets that were then boiled for 10 min and electrophoresis was subsequently performed.

Immunofluorescence (IF)

Cells were washed with PBS, fixed using 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were then blocked with 5% BSA in PBS for 1 h at room temperature (RT), incubated with 2A2 anti-ING3 antibody (1:100 dilution) in 5% BSA in PBS for 1 h. As secondary antibody, Alexa Fluor 568 (Invitrogen) in 1X PBS was used. Nuclei were then stained using 0.1% Hoechst dye (Sigma–Aldrich) in PBS for 10 min at RT. Images were taken using Zeiss Axiovert 200 M inverted microscope. For the blocking experiment, 5×10^7 HEK293 cells transfected with 50 μg of either GFP or ING3 expression constructs were rinsed with PBS, fixed with 4% paraformaldehyde for 15 min and collected into tubes. The cell pellets were permeabilized with 0.2% Triton X-100 in PBS for 10 min and washed with PBS twice. The ING3 antibody was diluted using 5% BSA in PBS and then incubated with the GFP-transfected or ING3-transfected cell pellets at room temperature for 30 min and were centrifuged at 13,000 rpm for 1 h at 4°. The supernatants were used to stain HEK293 cells transfected with ING3 and GFP constructs. For knock down experiment, cells were transfected with 20 pmol of