Histochemistry and Cell Biology (2025) 163:56
https://doi.org/10.1007/s00418-025-02385-2

SHORT COMMUNICATION

Tissue-specific localization of the ING4 targeting subunit of the HBO1 histone acetyltransferase in the cytoplasm and nucleus of secretory cells

Arthur Dantas[1,2,4] · Buthaina Al Shueili[1,2,5] · Jeongah Park[1,2,6] · Suleyman Abdullah[1,2,7] · Jessica Bertschmann[1,2,3] · Hokan Krowicki[1,2] · Mahbod Djamshidi[1,2,3] · Yang Yang[1,2] · Karen Blote[1,2] · Subhash Thalappilly[1,8] · Karl Riabowol[1,2,3]

Accepted: 1 May 2025
© The Author(s) 2025

Abstract

Members of the INhibitor of Growth protein family (ING1-5) function as epigenetic regulators by targeting different histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes to the H3K4Me3 mark of active transcription. The INGs recognize H3K4Me3 by specific interaction with their well-conserved plant homeodomains, and affinity can be increased by interactions between DNA and disordered regions within the ING proteins. They are classified as type II tumor suppressors since they are downregulated in numerous cancer types and knockout of ING family members results in tumorigenesis. ING4 targets the HBO1 HAT complex, which is known to affect acetylation of the H4 core nucleosomal histone, to affect local chromatin structure and knockout results in deficient innate immunity. Reports indicating roles in cell cycle regulation, tumor suppression, and apoptosis suggest that ING4 may be a promising target for cancer treatment by targeting pathways of innate immunity. Given the relatedness between ING4 and the closely related ING5 proteins, we have developed and characterized two mouse monoclonal antibodies to specifically recognize human and mouse ING4, but not ING5, to more accurately characterize ING4 levels by western, immunofluorescence and immunohistochemical assays. Using them, we show that ING4 differentially partitions between the nucleus and cytoplasm in different tissues and localizes largely to the cytoplasm of cells having a secretory role in different tissue types.

Keywords ING4 · Antibody · Immunofluorescence · Immunohistochemistry · Innate immunity · Hematopoiesis · HBO1

Introduction

Antibodies are produced by B-lymphocytes and usually have very high specificity and selectivity for their target molecules, making them useful for numerous applications. Although monoclonal antibodies generally recognize a single epitope, it has become evident that a significant proportion of commercially available antibodies frequently do not recognize the expected epitope (Couchman 2009; Kalyuzhny 2009). In fact, even monoclonal antibodies that do recognize an epitope of interest can recognize the same epitope in different antigens/proteins, and some monoclonals can express additional functional variable regions and so recognize numerous epitopes (Bradbury et al. 2018). We have found that a proportion of antibodies advertised to recognize members of a family of epigenetic regulatory proteins called the INhibitors of Growth (INGs) do not actually recognize the protein of interest (Nabbi et al. 2015). This can result in artifactual results, especially in protocols where antibody specificity needs to be particularly specific, such as in ChIP-seq (Krebs et al. 2014) and DNA-immunoprecipitation-based genomic profiling (Lentini et al. 2018). In some extreme cases, numerous antibodies against the same protein have been shown, upon proper characterization, to be non-specific (Jensen et al. 2009). To make our analyses of the INGs more accurate, we have produced multiple monoclonals to different ING family members and have characterized them extensively before use (Boland et al. 2000; Suzuki et al. 2011; Nabbi et al. 2015). Here, we present the characterization and use of monoclonal antibodies to determine the expression patterns and subcellular localization of the ING4 epigenetic regulator, which affects chromatin structure to

Extended author information available on the last page of the article

Published online: 22 May 2025

modify innate immunity (Mathema and Koh 2012; Dantas et al. 2019).

Our group discovered the first member of the INhibitor of Growth (ING) family of type II tumor suppressors using a combination of PCR-mediated subtractive hybridization between normal and cancerous breast epithelial cells followed by screening of a senescent mesenchymal expression library (Garkavtsev et al. 1996). ING2-5s were later identified through sequence homology and found in all vertebrates examined (He et al. 2005; Jacquet and Binda 2021). All ING family members contain a highly conserved plant homeodomain (PHD) that interacts with trimethylated lysine residue 4 in core histone H3 (H3K4Me3) (Pena et al. 2006), which targets different histone acetyltransferase (HAT) or histone deacetylase (HDAC) complexes to alter local chromatin acetylation levels. These domains and the proteins and HAT complexes with which they directly interact are shown for the related ING4 and ING5 proteins in Fig. 1. While all ING proteins contain nuclear localization signals including nucleolar targeting sequences (Scott et al. 2001; He et al. 2005), various types of cancer cells frequently show decreased levels of ING proteins and relocation of INGs from the nucleus to cytoplasm (Toyama et al. 1999; Nouman et al. 2002), and an allele of ING1 (p.*Pro319Leu*) has been identified as predisposing to breast cancer (Kuligina et al. 2020). ING1 has been reported to act together with p53 to activate or repress specific DNA damage response pathways, and p33ING1b, p33ING2, p29ING4, and p28ING5 promote induction of G1-phase cell cycle arrest or apoptosis after DNA damage in a p53-dependent manner (Thalappilly et al. 2011; Archambeau et al. 2019). The major isoform of ING1, called p33ING1b, was also reported to direct the Growth Arrest after DNA Damage 45 (GADD45) protein to the H3K4Me3 mark, thus affecting DNA demethylation in addition to histone acetylation (Schäfer et al. 2013).

Like other ING proteins, ING4 binds the H3K4Me3 epigenetic mark and shares the ability to target HBO1 HAT activity with ING5 (Doyon et al. 2006). Like ING5, which has been shown to promote stemness in normal (Mulder et al. 2012) and cancer (Wang et al. 2018) stem cells, ING4 also regulates normal hematopoietic stem cell homeostasis (Thompson et al. 2024) and cancer cell stemness. By binding liprin-α1, which is necessary for focal adhesion at lamellipodia in the cytoplasm, ING4 also plays an extranuclear role affecting cell migration, potentially inhibiting cancer cell invasion and metastasis (Shen et al. 2007).

Knockout revealed that ING4 regulates innate immunity by regulating NF-κB (Nozell et al. 2008; Coles et al. 2010), and in vitro studies indicate it suppresses hypoxia-inducible

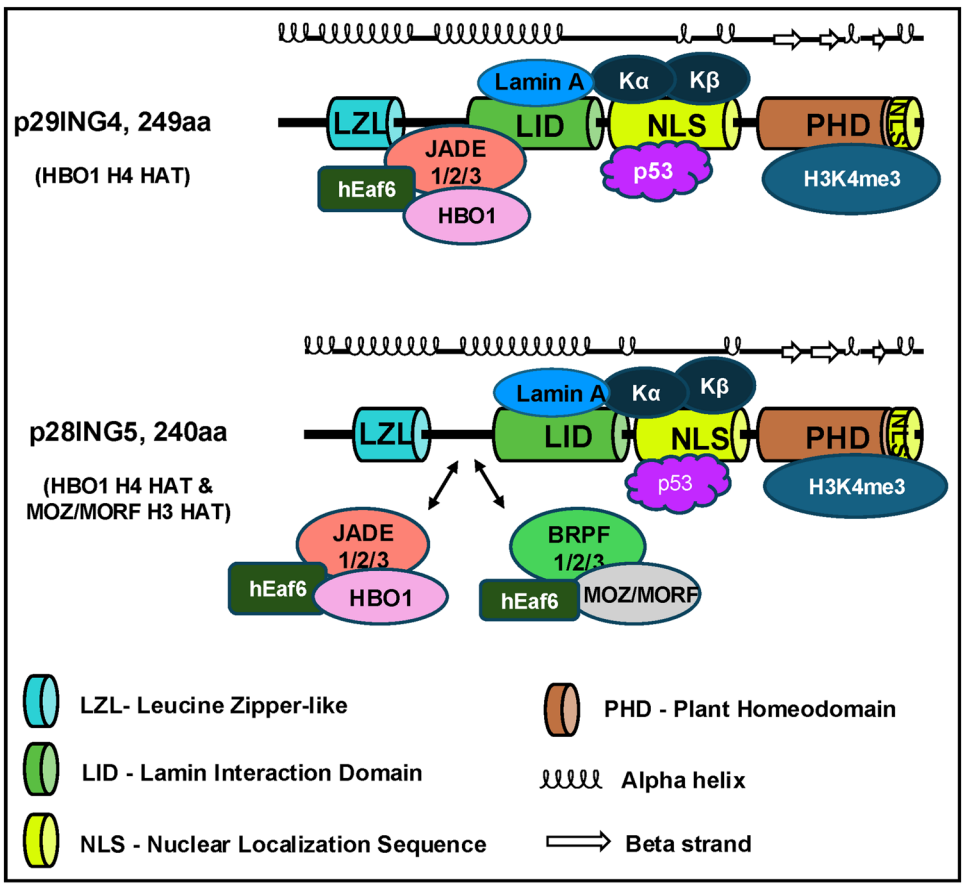

Fig. 1 Domains of the ING4 and related ING5 proteins. ING4 and ING5 contain similarly located domains, bind the H3K4Me3 histone mark through their plant homeodomain (PHD) form of zinc finger, bind p53 in their unstructured nuclear localization sequences (NLS) and bind lamin A through the lamin interaction domain (LID), a sequence unique to the ING proteins in the human proteome. They are transported into the nucleus by the alpha and beta karyopherins (Kα and Kβ) and encode leucine zipper-like (LZL) regions through which they form homo- and hetero-dimers. ING4 targets the HBO1 histone H4 HAT complex to H3K4Me3, while ING5 can target either HB01 or the MOZ/MORF histone H3 HAT complex to H3K4Me3

factor-1 (HIF-1α) (Ozer et al. 2005; Colla et al. 2007) and regulates angiogenesis (Garkavtsev et al. 2004). NF-κB is a transcription factor with major roles in apoptosis, angiogenesis and innate and adaptive immune responses. Consistent with these observations, ING4 knockout mice showed higher levels of the RelA-p50 protein in macrophages, resulting in increased NF-κB activity and higher levels of cytokines (Coles et al. 2010). The suppressive nature of ING1 on certain κB promoters helps to explain the increase in NF-κB activity in tumor cells and the role of ING4 as a tumor suppressor. To gain a better understanding of the roles undertaken by ING4 in different cell types under different conditions of growth and metabolism, we generated and characterized ING4 monoclonal antibodies with which to unambiguously determine the subcellular localization of endogenous ING4.

Materials and methods

Monoclonal antibody production

ING4 antigen production and purification from bacteria, mouse immunization and screening for titer, hybridoma fusion and screening of clones by enzyme-linked immunosorbent assay (ELISA) were done as described (Boland et al. 2000). In brief, a dilution series of purified bacterial recombinant ING4 (1–1000 ng/ml) was bound to 96-well enzyme-linked immunosorbent assay (ELISA) plates (catalog no. 77589-184, VWR, Radnor, PA, USA) at pH 9.0 for 2 h at 37 °C. After non-specific binding was blocked by incubation with 1% BSA in t-TBS (catalog no. 10791-790, VWR), ING4-coated plates were incubated with each candidate monoclonal IgG at a concentration of 10 µg/ml for 1 h at 37 °C. The plates were then washed with t-TBS followed by incubation with goat anti-mouse IgG-HRP (catalog no. AP124P, MilliporeSigma, Oakville, ON, Canada) for 20 min at 37 °C. After washing, ABTS peroxidase substrate system (catalog no. 11684302001, MilliporeSigma) was used for substrate development and OD 405 was measured. Three individual wells for each condition were measured each time, and the experiments were repeated two to four times for each of the candidate ING4 monoclonals. For production of hybridoma supernatants, cell lines were maintained in either serum-free medium (catalog no. 12045084, Thermo Fisher Scientific Canada, Edmonton, Canada) or Dulbecco's modified Eagle's medium (Thermo Fisher Scientific Canada) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (catalog no. 15140122, Gibco, Thermo Fisher Scientific Canada, Edmonton, Canada), and 8% fetal bovine serum (catalog no. 12483020, Gibco, Thermo Fisher Scientific Canada) in a Steri-Cycle C100 CO_2 incubator (Thermo Fisher Scientific Canada) at 37 °C and 5% CO_2. The serum-free culture media were collected every 3–4 days and prepared for antibody purification. Antibodies from each hybridoma were purified using HiTrap Protein G HP columns (catalog no. 45-000-053, Cytiva, Thermo Fisher Scientific Canada, Edmonton, Canada) following the company's protocol. The culture media containing fetal bovine serum were also collected every 3–4 days and directly used for western blot analysis.

Expression construct subcloning

ING1-5 and FLAG-tagged versions of ING4 were subcloned into pCI (Clonetech, 1290 Terra Bella Avenue, Mountain View, CA, USA). Tagged ING4 deletions were generated by one-step-PCR. Transient transfections of plasmids were performed using a modified calcium phosphate method for HEK293 cells (CRL-1573, ATCC, Manassas, VA, USA) growing in log phase.

ING4 knockdown

ING4 was knocked down in growing HEK293 cells by transfection of a SMARTpool siRNA mixture (Dharmacon™ ON-TARGETplus™ SMARTpool siRNA, Horizon Discovery Biosciences Limited, Cambridge, UK) following the manufacturer's suggestions.

Cell culture and cell transfection

HEK293 cells were grown in Dulbecco's modified Eagles medium (DMEM, D6429-500ML, Sigma-Aldrich, Burlington, MA, USA) containing 10% FBS plus 100 U/ml of penicillin and 100 mg/ml of streptomycin. Cells were maintained at 37 °C in 95% air and 5% CO_2. Cells were tested weekly for mycoplasma, and all tested negative.

Bacterial protein expression

Production of full-length GST-ING4 protein was cloned in the pGEX4 T3 expression vector (Clonetech, Mountain View, CA, USA), expressed and purified from bacteria as described (Suzuki et al. 2011).

Western blotting

The samples obtained from either *Escherichia coli* or HEK293 cells overexpressing different fragments of ING4 or different whole ING family proteins were run on 10% sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose or nylon membranes (catalog no. GE10600007, Millipore-Sigma, Oakville, ON, Canada). After non-specific binding was blocked by incubating in a solution containing 3% BSA and 0.1%

Tween-20 in 20 mM Tris-buffered saline (t-TBS, pH 8.0) for 1 h at room temperature (RT), the membranes were incubated with ING4 hybridoma culture supernatant diluted 1:9 (tenfold) in PBS containing 0.5% BSA. Rabbit anti-GST, -ING1, -ING2, -ING3, -ING4 and -ING5 (all produced by SACRI Antibody Services, University of Calgary, Calgary, Alberta, Canada) were used to visualize all potential target proteins to confirm expression levels in sample lysates (data not shown). After washing membranes with t-TBS, membranes were incubated with HRP-conjugated goat α-mouse IgG (catalog no. AP124P, Millipore-Sigma) for 20 min at RT and then washed with t-TBS three times for 10 min. Signals were subsequently detected using ECL reagent (Clarity Western ECL Substrate, #1705061, Bio-Rad, Hercules, CA, USA) on X-Omat x-ray films (Kodak, Rochester, NY, USA).

Indirect immunofluorescence

HEK293 cells were seeded on acid-washed glass coverslips and 24 h later were transfected with ING4 plasmids using Lipofectamine 2000 (Invitrogen™, catalog no. 11668027, Thermo Fisher Scientific Canada). After another 24 h, cells were washed with ice-cold PBS and then fixed using 3.7% paraformaldehyde in PBS and permeabilized with 0.1% Triton-X100 in PBS. Cells were incubated in PBS containing 5% BSA to block non-specific binding, rinsed in PBS and then incubated at 37 °C for 1 h with purified 4F4, 6C6 or anti-FLAG antibody diluted 1:50 or 1:100 with PBS containing 1% BSA in a humidified chamber. After incubation and three washes in PBS containing 0.1% Triton X-100, coverslips were incubated with goat a-mouse Alexa 568 or goat a-mouse Alexa Fluor 488 secondary antibodies (catalog nos. ab175473 and ab150113, Abcam, Waltham, MA, USA). Images were captured using a Zeiss Axiovert 200 using Axioset software or an Axio Observer.Z1 using an EC Plan-Neofluar 40x/0.72 M27 objective with a working distance of 0.71 mm (Zeiss Canada, Toronto, Canada). Image acquisition was carried out using a Zeiss AxioCam MRm (1388 × 1040 pixels) using Zen blue 2.6 software. Scaling per pixel was 0.161 mM × 0.161 mM with a scaled image size of 223.82 mM × 167.7 mM with a 12-bit bit depth.

Immunohistochemistry

For mouse samples, tissues were harvested from C57BL/6 mice (Health Sciences Animal Resource Centre, University of Calgary, Alberta, Canada) and snap frozen in Clear Frozen Section Compound (catalog no. 95057–838, VWR, Radnor, PA, USA). Frozen tissue blocks were sectioned at 5 µm thickness and were loaded onto Superfrost plus slides [catalog no. CA48311-703, Avantor (VWR), Radnor, PA, USA] and stored at −80 °C. Slides were fixed in ice cold acetone prior to staining. For human samples, multiple tissue microarray (TMA) slides were used (catalog no. BN1002a, US Biomax, Inc., Derwood, MD, USA). The TMA samples were initially formalin fixed and paraffin embedded and were subsequently deparaffinized and rehydrated. The slides were heated in a 1000-Watt microwave (GE®, catalog no. JE635 WW, GE Appliances, Louisville, KY, USA) for 2 min at 100% power followed by 20 min at 20% power using Rodent Decloaker AR buffer (catalog no. 50–832-71, Biocare Medical, LLC, Fisher Scientific, Edmonton, Canada) for antigen retrieval. Slides were treated with peroxidase block and then incubated for 15 min with 1% BSA in TBST wash buffer at room temperature. Samples were then incubated with 1:50 dilutions of concentrated 4F4 ING4 monoclonal antibody in Signal Stain® antibody diluent [catalog no. 8112L, NEB Canada (CST), Ontario, Canada] overnight at 4 °C. Analysis of ING4 expression was made by Labeled Polymer-HRP anti-mouse reagent for 1 h at RT and a 5-min incubation with DAB + substrate and chromogen (catalog no. K346711-2, Agilent Technologies, Santa Clara, CA, USA). Lastly, slides were counterstained with Mayer's hematoxylin (catalog no. S330930-2, Agilent Technologies, Santa Clara, CA, USA).

Results and discussion

Two mouse monoclonal antibodies (4F4, 6C6) that recognize human and mouse ING4 were identified by ELISA and were tested to determine the region of the ING4 protein that they recognized. As shown in Fig. 2a, constructs encoding fragments of ING4 encompassing increasing lengths of the ING4 protein tagged with the FLAG epitope were transfected into HEK293 cells. Lysates from cells harvested 24 h after transfection were electrophoresed and probed with the ING4 monoclonals (Fig. 2b) and, after stripping, sequentially with a commercial α-FLAG antibody (Fig. 2c) to confirm expression. Both monoclonals recognized fragments containing the nuclear localization signal (NLS) but not one missing it, although all fragments were expressed at similar levels (Fig. 2c). While the monoclonals robustly recognized fragments of overexpressed ING4, we next asked whether they could specifically recognize endogenous ING4 protein. Lysates from untransfected HEK293 cells or from HEK293 s transfected with 50 or 100 pico-moles of ING4-specific siRNA were harvested 48 h after transfection, and lysates were electrophoresed, blotted and probed with both the 4F4 and 6C6 monoclonals. As seen in Fig. 2d, two endogenous isoforms of ING4 (p28 and p29) (Unoki et al. 2006) are recognized by both monoclonals, and these are knocked down by the ING4 siRNA, confirming that the monoclonals recognize ING4. We also asked whether the 4F4 monoclonal

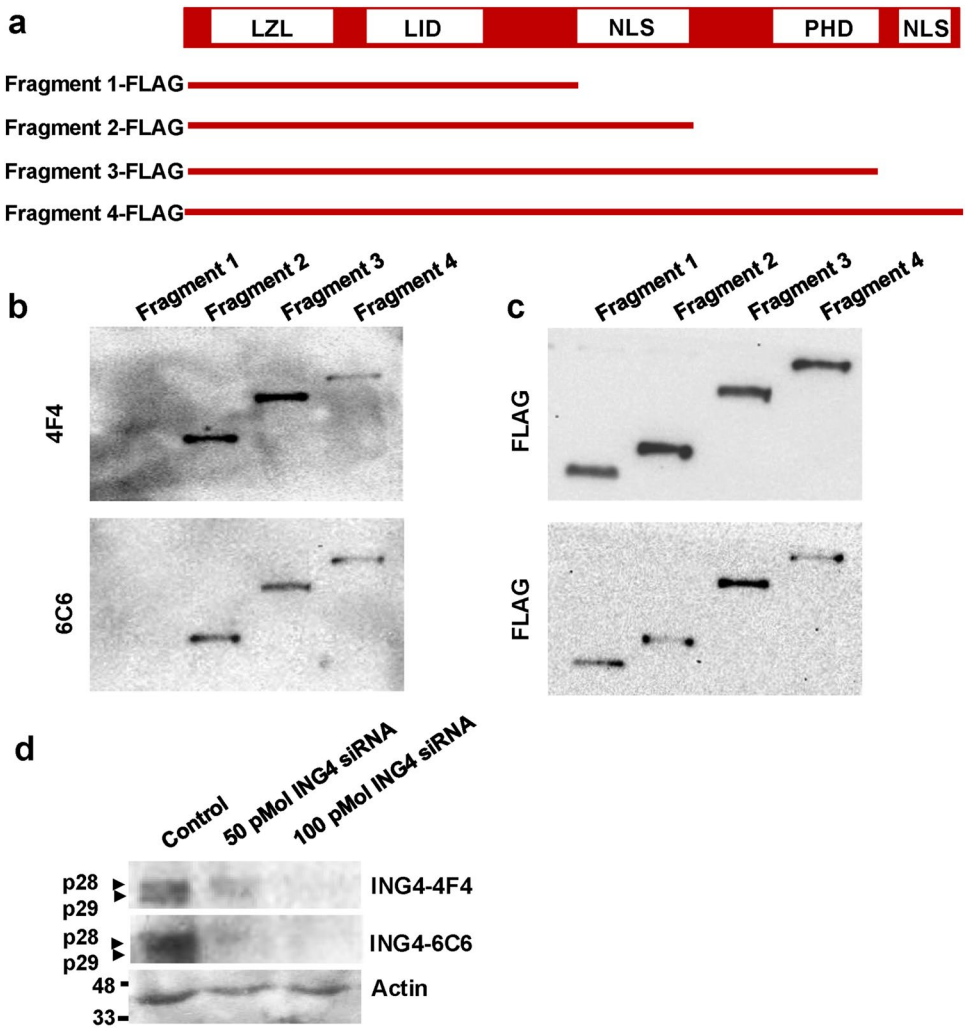

Fig. 2 Domain recognition and specificity of 4F4 and 6C6 monoclonal antibodies. **a** FLAG-tagged ING4 fragments generated by PCR-based sub-cloning. **b** Plasmids expressing the ING4 fragments shown in **a** were transfected into HEK293 cells, and cell lysates were prepared after 24 h. Western blots using the 4F4 and 6C6 antibodies identify fragments detected by these antibodies. **c** Blots were stripped and re-probed with anti-FLAG antibody to confirm expression of FLAG-tagged ING4 fragment. **d** The specificity of anti-ING antibodies for endogenous ING4 was tested using untransfected (control) HEK cell lysates and lysates from cells in which ING4 levels were knocked down for 48 h using ING4-specific Smartpool siRNAs at the amount indicated. The arrowheads indicate the two major isoforms (p28 and p29) of ING4 and actin was used as a loading control

would recognize ING4 in immunoprecipitation experiments. As shown in Supplementary Fig. 1, although recognizing ING4 robustly in western blot loading controls, it did not immunoprecipitate ING4.

Since all the ING proteins contain several well-conserved domains, we next asked whether the ING4 monoclonals would cross-react with other ING proteins. Constructs encoding all ING proteins were individually transfected into HEK293 cells, and lysates from cells were electrophoresed and probed with different ING4 monoclonals. As seen in Fig. 3a, the monoclonals all recognized overexpressed ING4. Given the much greater similarity between ING4 and ING5 compared to the rest of the ING proteins (He et al. 2005), it was important to confirm robust expression of ING5. Therefore, in the rightmost panel of Fig. 3a, the blot shows that ING5 was indeed expressed and recognized by a commercial ING5 antibody, but no signal was evident when probing only with the ING4 monoclonals.

We next asked whether the ING4 monoclonals would recognize the largely native form of ING4 in immunofluorescence assays. HEK293 cells were transfected with a pCI-FLAG-ING4 construct and fixed in formalin 24 h later. Immunofluorescence was carried out using 4F4 and 6C6 antibodies in two dilutions (1:50 and 1:100), and staining using an α-FLAG antibody was included as a positive control. As shown in Fig. 4a, both monoclonals detected overexpressed ING4 with intensities approaching that seen for the α-FLAG antibody. As anticipated for a chromatin reader that recognizes the H3K4Me3 mark of active transcription (Pena et al. 2006), staining was largely nuclear in HEK293 cells, although some signal was seen in the cytoplasm of transfected cells. This is likely due to alterations in karyopherin-induced shuttling between the cytoplasm and nucleus as described for the related ING1 protein (Russell et al. 2008). We next tested whether the 4F4 monoclonal was able to visualize endogenous levels of ING4. As seen in Fig. 4b in fields exposed tenfold longer than cells in panel a, transfected cells showed intense staining, and it is possible to also detect weak, nuclear and cytoplasmic staining in untransfected cells. Since yellow-green cell autofluorescence

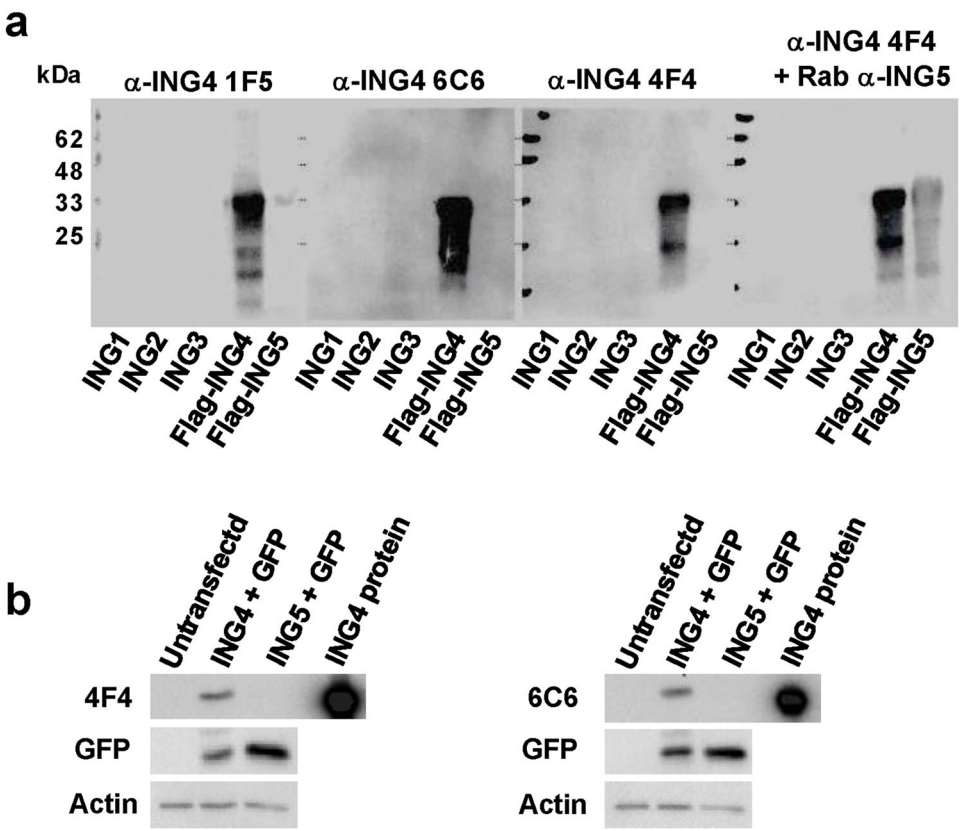

Fig. 3 ING4 mococlonal antibodies recognize ING4 but not other ING proteins. **a** Expression constructs encoding INGS 1–3 and FLAG-tagged ING4 and ING5 were expressed in HEK293 cells that were lysed 24 h after transfection, and cell lysates were electrophoresed, transferred to nylon membranes and probed in western blots with the indicated monoclonal antibodies. The rightmost panel was also probed with a commercial rabbit ING5 polyclonal to confirm expression of ING5. **b** The 4F4 and 6C6 antibodies were further tested for recognition of ING4 but not ING5. Cells were co-transfected with expression constructs containing GFP as transfection controls and bacterially expressed ING4 protein as an additional positive control and blotted for actin as a loading control

due to the accumulation of lipofuscins occurs in aging and stressed cells and tissues (Jung et al. 2010) and secondary antibodies can sometimes produce spurious signals due to nonspecific binding, we omitted the primary antibody in the lowermost panels of Fig. 4b and noted that no staining was evident, confirming that the monoclonals were specifically recognizing ING4.

Antibodies that work well in blotting, where proteins are denatured, and in immunofluorescence after fixation with mild bifunctional crosslinkers like formalin, where proteins remain largely nondenatured, frequently also work well in immunohistochemistry. To test our monoclonals, we first fixed, sectioned and stained different mouse tissues. As shown in Fig. 5a–f, the 4F4 monoclonal stained ependymal cells surrounding the lateral ventricles of the brain (Fig. 5a, b), the interior of villi in the small intestine (Fig. 5d) and follicle-like regions of the spleen (Fig. 5e), all of which act as secretory cells, while the cerebellum (Fig. 5c) and liver (Fig. 5f) showed very infrequent and low levels of staining. Magnification of images of the brain (Fig. 5g) and intestine (Fig. 5h) indicated both nuclear and cytoplasmic localization of ING4. Examination of a commercial panel of human tissue samples showed similar results in which pancreatic acinar cells (Fig. 5i) and stomach parietal and/or chief cells (Fig. 5j) also showed significant levels of staining. However, in contrast to the largely nuclear staining seen in HEK293 cells by immunofluorescence, secretory cells of various types showed cytoplasmic staining. Low levels of diffuse cytoplasmic staining were also seen in human liver hepatocytes (Fig. 5k). The reason for the cytoplasmic localization in fixed tissue versus the largely nuclear localization seen in cultured HEK293 cells is unknown, but one possibility could be related to whether cells are actively growing since HEK293 cells in culture would all be actively traversing the cell cycle whereas most cells in the tissue sections would be expected to be quiescent and in the G_0 phase of the cell cycle despite being metabolically active. Another potential explanation may be related to the ability of ING4 to interact with liprin-α1 at lamellipodia (Shen et al. 2007), suggesting a cytoplasmic, membrane-associated role, perhaps contributing to cell secretion. Finally, it should be noted that mouse tissues were snap frozen and fixed with ice-cold acetone that precipitates proteins, whereas human tissues were formalin-fixed, so nuclear ING4 could be cross-linked in the HBO1 HAT complex, possibly blocking recognition by the antibodies. A small subset (1–2%) of cells in the uterus (Fig. 5l), breast (Fig. 5m) and prostate (Fig. 5n) tissue displayed intense staining that appeared both cytoplasmic and nuclear, but in these cases, staining was likely non-specific because of the presence of mast cells (Schiltz et al. 1993). Staining of all human tissue samples was done using the

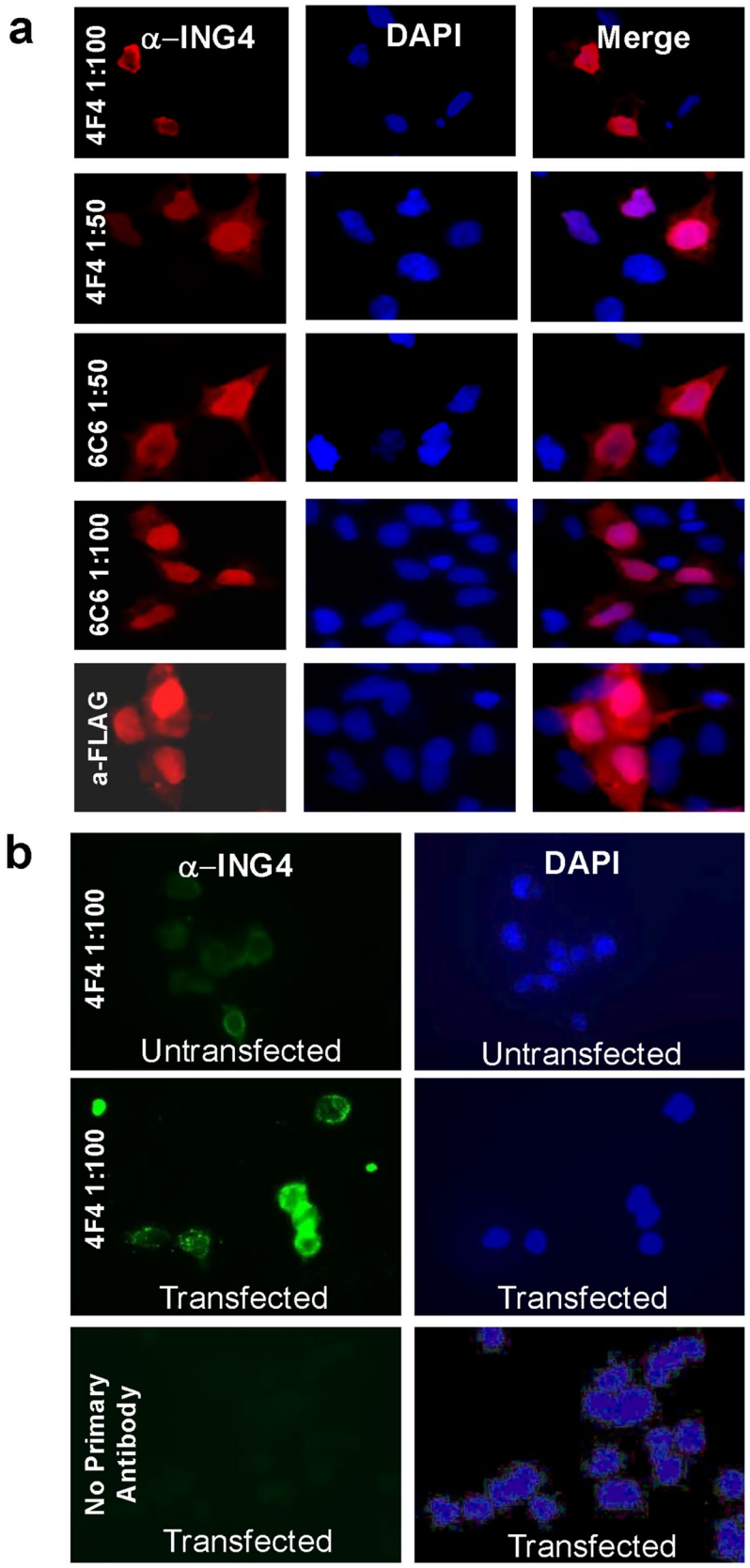

Fig. 4 Detection of ING4 expression by immunofluorescence. **a** HEK293 cells were transfected with pCl-FLAG-ING4 construct and fixed 24 h later, and immunofluorescence was carried out using 4F4 and 6C6 antibodies at the dilutions of hybridoma supernatant noted followed by a Texas Red-conjugated secondary antibody. Immunofluorescence using anti-FLAG antibody serves as a positive control. **b** Untransfected HEK293 cells and cells transfected with pCl-ING4 with no tag were stained with 4F4 monoclonal followed by Alexa 488-conjugated secondary antibody. Cells were exposed for tenfold longer than in panel **a**, and cells stained with secondary antibody only (no primary antibody) served as a control for endogenous fluorescence. Most ING4 staining is seen in the nuclei, except in the case of cells stained with α-FLAG

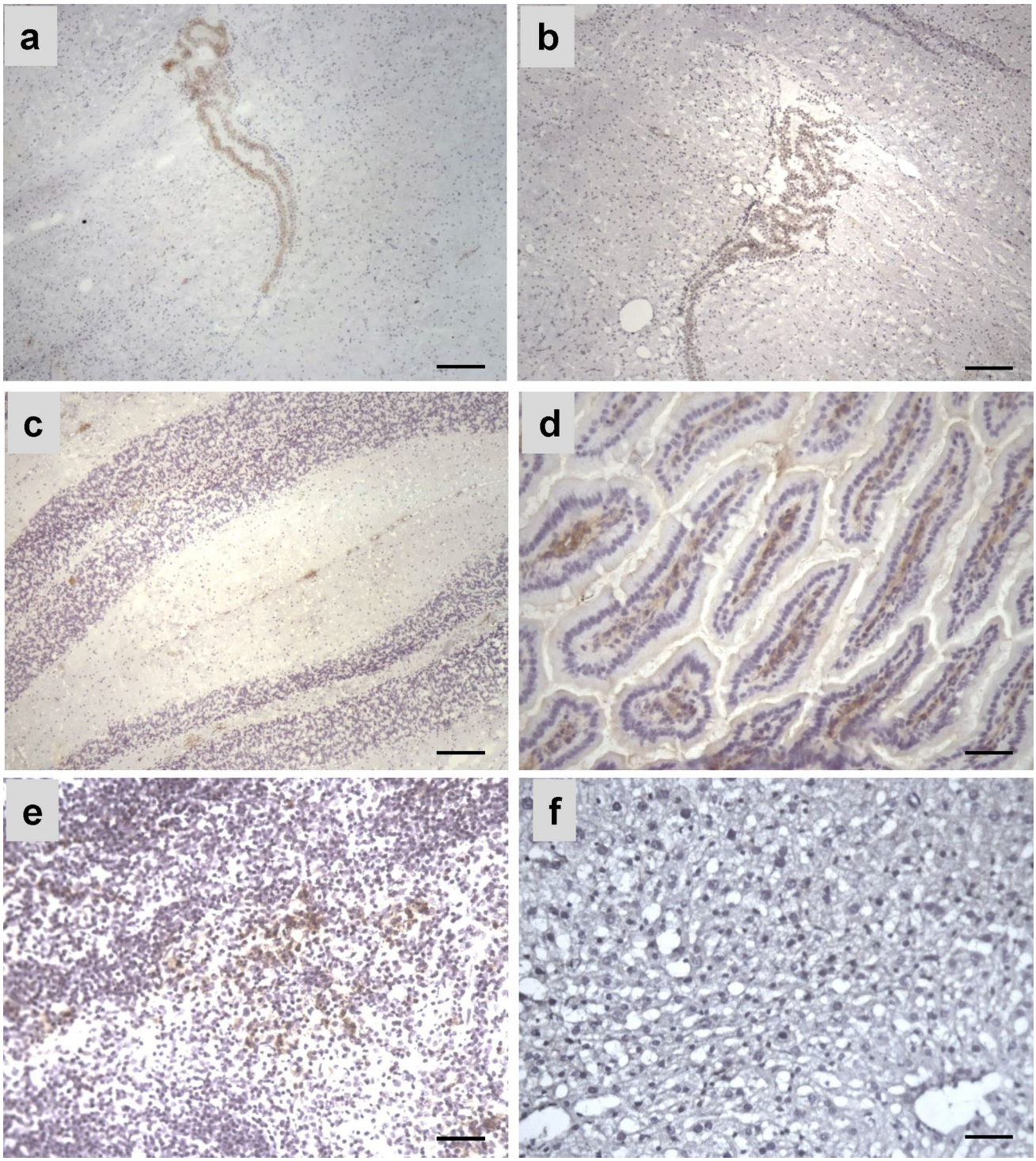

Fig. 5 ING4 staining in murine tissues by immunohistochemistry using a mixture of 4F4 and 6C6 ING4 monoclonals. A tissue array containing the samples noted was stained using a 1:100 dilution of 4F4 hybridoma supernatant followed by horseradish peroxidase-conjugated rabbit α-mouse IgG. Strong staining was seen in the ependymal cells of the lateral ventricles of the brain (**a**, **b**) responsible for production of cerebrospinal fluid as well as in the interior of villi of the small intestine (**d**) and follicle-like regions of the spleen (**e**), but very few cells of the cerebellum (**c**) or liver (**f**) showed staining. Scale bar = 100 µM. Higher magnification of **g** the right brain verticle and **h** the small intestine stained using a 1:100 dilution of 4F4 hybridoma supernatant followed by horseradish peroxidase-conjugated rabbit α-mouse IgG. Scale bar = 100 µM. Strong staining was seen in the cytoplasm of pancreatic acinar cells (**i**) and in stomach parietal and/or chief cells of the lamina propria (**j**). A subset of hepatocytes weakly stained in the liver (**k**). Occasional staining was seen in uterine (**l**), breast (**m**) and prostate (**n**) samples and probably represents non-specific staining of mast cells. Staining patterns suggest that cell types with strong secretory functions appear to express higher levels of ING4. Scale = 100 µM

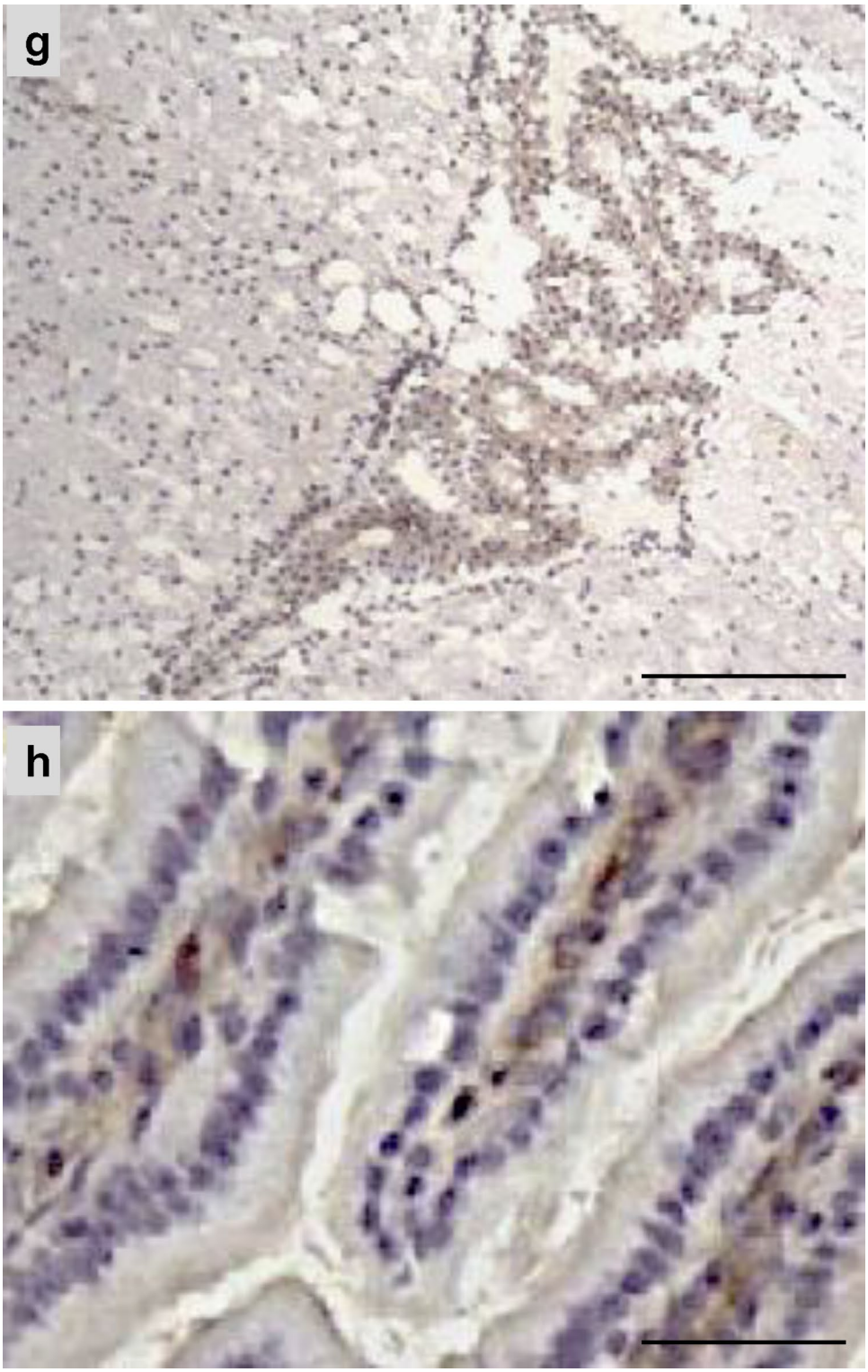

Fig. 5 (continued)

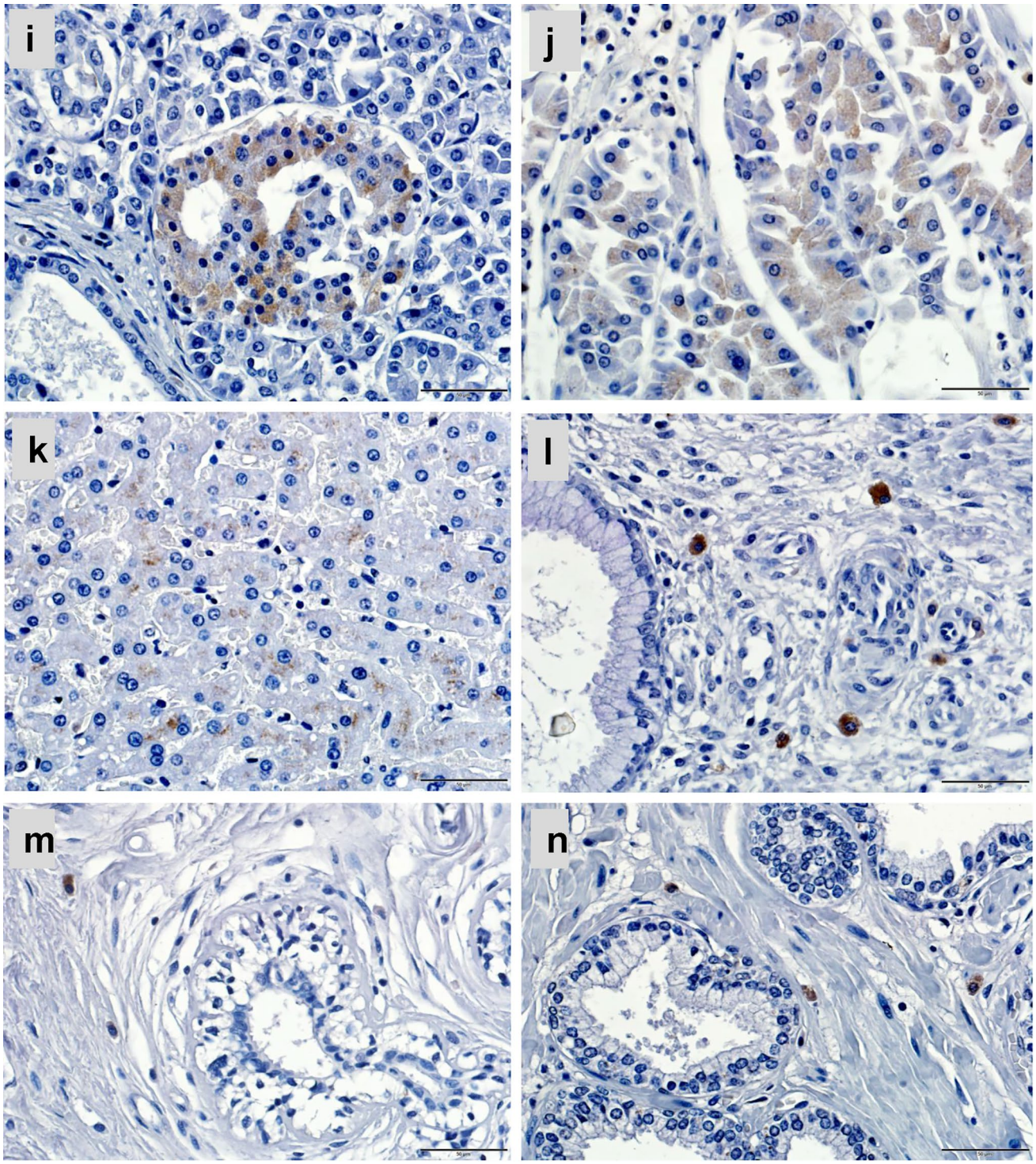

Fig. 5 (continued)

same experimental method on a single membrane so relative levels of expression are shown.

In summary, production and rigorous quality control and specificity testing allowed us to identify two mouse monoclonal antibodies that recognize the relatively unstructured nuclear localization sequence (NLS) of ING4 but not any of the related NLS regions of other ING proteins, including the closely related ING5. The monoclonals recognize two similarly sized splicing variants of ING4 that are 28 and 29 kDa but not several others that have been

reported to be produced from the *ING4* locus (Raho et al. 2007). This is likely due to expression of only these isoforms in the cells examined since, if other variants were expressed, the majority should also encode the NLS that is recognized by the monoclonals. Knockdown of ING4 using a Smartpool mixture of siRNA further demonstrated that the antibodies recognized the two ING4 isoforms, while both endogenous and overexpressed ING4 was detected by the monoclonals in HEK293 cells by immunofluorescence. Using the monoclonals to stain various mouse and human tissues by immunohistochemistry indicated that only a relatively small subset of cells expressed ING4 and that cells associated with high levels of secretion, such as ependymal cells of the brain ventricles, cells within intestinal villi, follicular cells of the spleen, pancreatic acinar cells and stomach parietal and/or chief cells all express significant levels of ING4 that appears to largely or exclusively localize to the cytoplasm. This suggests that ING4 may play a function that is distinct from affecting chromatin structure through histone modification by the HBO1 HAT complex, which has been proposed as the major function of ING4 in regulating transcription. This may be related to regulating cell secretion since ING proteins were previously shown to alter the cell secretory phenotype, in particular the secretion of cytokines and metalloproteases (Thakur et al. 2015). There are some examples of ING proteins having cellular effects that do not involve chromatin modification for epigenetic tuning of transcription. For example, ING1 and ING2 have both been implicated in regulating the activity of p53 by regulating its acetylation (Nagashima et al. 2001; Kataoka et al. 2003; Pedeux et al. 2005). For ING1, this occurs by physical interaction with hSIR2 to induce acetylation of p53 residues Lys373 and Lys382 (Kataoka et al. 2003). ING2 also appears to promote p300-dependent acetylation of p53, and ING1 can stabilize p53 by blocking the polyubiquitination of p53 on the six lysine residues known to affect p53 turnover by the proteosome (Thalappilly et al. 2011). Furthermore, ING3 has been shown to also act independently of chromatin through its ability to target the TIP60 HAT complex directly to the cytoplasmic androgen receptor, promoting nuclear localization and androgen receptor activation as a transcription factor (Nabbi et al. 2017). It will be interesting to further examine non-nuclear roles of the ING proteins, including those associated with mitochondria as reported for both ING1 (Bose et al. 2013) and ING2 (Ma et al. 2020; Ricordel et al. 2021), possibly in association with the p15 PCNA-associated factor p15-PAF (Simpson et al. 2006), which associates with ING1 and localizes to both the mitochondria and nucleus. For ING4, production of these specific monoclonals will allow further examination of ING4 in the cytoplasm, where it appears to be expressed selectively in cells with potential roles in secretion.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00418-025-02385-2.

Acknowledgements We thank Donna Boland of SACRI Antibody Services for expert help in generating ING4 monoclonal antibodies. This work was supported by grants to KR from the Canadian Institutes of Health Research (MOP-133646; PJT-178099) and the National Sciences and Engineering Research Council (NSERC) of Canada.

Author contributions AD curated data. AD, BAS, JP, SA, HK, MD and JB undertook cell culture, western and immunofluorescence experiments. KB and ST supplied resources. YY and KB supervised students. AD, ST and KR wrote the original draft. KR conceptualized the study, secured funding, reviewed and edited the manuscript.

Funding This work was supported by grants to KR from the Canadian Institutes of Health Research (MOP-133646; PJT-178099) and the National Sciences and Engineering Research Council (NSERC) of Canada.

Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval All experiments undertaken using animals were reviewed by the University of Calgary Animal Ethics Committee.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

References

Archambeau J, Blondel A, Pedeux R (2019) Focus-ING on DNA integrity: implication of ING proteins in cell cycle regulation and DNA repair modulation. Cancers (Basel) 12(1):58. https://doi.org/10.3390/cancers12010058

Boland D, Olineck V, Bonnefin P, Vieyra D, Parr E, Riabowol K (2000) A panel of CAb antibodies recognize endogenous and ectopically expressed ING1 protein. Hybridoma 19(2):161–165. https://doi.org/10.1089/02724570050031202

Bose P, Thakur S, Thalappilly S, Ahn BY, Satpathy S, Feng X, Suzuki K, Kim SW, Riabowol K (2013) ING1 induces apoptosis through direct effects at the mitochondria. Cell Death Dis 4(9):e788. https://doi.org/10.1038/cddis.2013.321

Bradbury ARM, Trinklein ND, Thie H, Wilkinson IC, Tandon AK, Anderson S, Bladen CL, Jones B, Aldred SF, Bestagno M, Burrone O, Maynard J, Ferrara F, Trimmer JS, Görnemann J, Glanville J, Wolf P, Frenzel A, Wong J, Koh XY, Eng HY, Lane D, Lefranc MP, Clark M, Dübel S (2018) When monoclonal

antibodies are not monospecific: hybridomas frequently express additional functional variable regions. Mabs 10(4):539–546. https://doi.org/10.1080/19420862.2018.1445456

Coles A, Gannon H, Cerny A, Kurt-Jones E, Jones S (2010) Inhibitor of growth-4 promotes IκB promoter activation to suppress NF-κB signaling and innate immunity. Proc Natl Acad Sci USA 107(25):11423–11428. https://doi.org/10.1073/pnas.0912116107

Colla S, Tagliaferri S, Morandi F, Lunghi P, Donofrio G, Martorana D, Mancini C, Lazzaretti M, Mazzera L, Ravanetti L, Bonomini S, Ferrari L, Miranda C, Ladetto M, Neri TM, Neri A, Greco A, Mangoni M, Bonati A, Rizzoli V, Giuliani N (2007) The new tumor-suppressor gene inhibitor of growth family member 4 (ING4) regulates the production of proangiogenic molecules by myeloma cells and suppresses hypoxia-inducible factor-1 alpha (HIF-1alpha) activity: involvement in myeloma-induced angiogenesis. Blood 110(13):4464–4475. https://doi.org/10.1182/blood-2007-02-074617

Couchman JR (2009) Commercial antibodies: the good, bad, and really ugly. J Histochem Cytochem 57(1):7–8. https://doi.org/10.1369/jhc.2008.952820

Dantas A, Al Shueili B, Yang Y, Nabbi A, Fink D, Riabowol K (2019) Biological functions of the ING proteins. Cancers (Basel) 11(11):1817. https://doi.org/10.3390/cancers11111817

Doyon Y, Cayrou C, Ullah M, Landry AJ, Côté V, Selleck W, Lane WS, Tan S, Yang XJ, Côté J (2006) ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. Mol Cell 21(1):51–64. https://doi.org/10.1016/j.molcel.2005.12.007

Garkavtsev I, Kazarov A, Gudkov A, Riabowol K (1996) Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. Nat Genet 14(4):415–420. https://doi.org/10.1038/ng1296-415

Garkavtsev I, Kozin S, Chervova O, Xu L, Winkler F, Brown E, Jain R (2004) The candidate tumour suppressor protein ING4 regulates brain tumour growth and angiogenesis. Nature 428(6980):328–332. https://doi.org/10.1038/nature02329

He GH, Wagner M, Helbing CC, Sensen CW, Riabowol K (2005) Phylogenetic analysis of the ING family of PHD finger proteins. Mol Biol Evol 22(1):104–116. https://doi.org/10.1093/molbev/msh256

Jacquet K, Binda O (2021) ING proteins: tumour suppressors or oncoproteins. Cancers (Basel) 13(9):2110–2122. https://doi.org/10.3390/cancers13092110

Jensen BC, Swigart PM, Simpson PC (2009) Ten commercial antibodies for alpha-1-adrenergic receptor subtypes are nonspecific. Naunyn Schmiedebergs Arch Pharmacol 379(4):409–412. https://doi.org/10.1007/s00210-008-0368-6

Jung T, Höhn A, Grune T (2010) Lipofuscin: detection and quantification by microscopic techniques. Methods Mol Biol 594:173–193. https://doi.org/10.1007/978-1-60761-411-1_13

Kalyuzhny AE (2009) The dark side of the immunohistochemical moon: industry. J Histochem Cytochem 57(12):1099–1101. https://doi.org/10.1369/jhc.2009.954867

Kataoka H, Bonnefin P, Vieyra D, Feng X, Hara Y, Miura Y, Joh T, Nakabayashi H, Vaziri H, Harris CC, Riabowol K (2003) ING1 represses transcription by direct DNA binding and through effects on p53. Cancer Res 63(18):5785–5792

Krebs W, Schmidt SV, Goren A, De Nardo D, Labzin L, Bovier A, Ulas T, Theis H, Kraut M, Latz E, Beyer M, Schultze JL (2014) Optimization of transcription factor binding map accuracy utilizing knockout-mouse models. Nucleic Acids Res 42(21):13051–13060. https://doi.org/10.1093/nar/gku1078

Kuligina ES, Sokolenko AP, Bizin IV, Romanko AA, Zagorodnev KA, Anisimova MO, Krylova DD, Anisimova EI, Mantseva MA, Varma AK, Hasan SK, Ni VI, Koloskov AV, Suspitsin EN, Venina AR, Aleksakhina SN, Sokolova TN, Milanović AM, Schürmann P, Prokofyeva DS, Bermisheva MA, Khusnutdinova EK, Bogdanova N, Dörk T, Imyanitov EN (2020) Exome sequencing study of Russian breast cancer patients suggests a predisposing role for USP39. Breast Cancer Res Treat 179(3):731–742. https://doi.org/10.1007/s10549-019-05492-6

Lentini A, Lagerwall C, Vikingsson S, Mjoseng HK, Douvlataniotis K, Vogt H, Green H, Meehan RR, Benson M, Nestor CE (2018) A reassessment of DNA-immunoprecipitation-based genomic profiling. Nat Methods 15(7):499–504. https://doi.org/10.1038/s41592-018-0038-7

Ma Y, Yan R, Wan Q, Lv B, Yang Y, Lv T, Xin W (2020) Inhibitor of growth 2 regulates the high glucose-induced cell cycle arrest and epithelial-to-mesenchymal transition in renal proximal tubular cells. J Physiol Biochem 76(3):373–382. https://doi.org/10.1007/s13105-020-00743-3

Mathema V, Koh Y (2012) Inhibitor of growth-4 mediates chromatin modification and has a suppressive effect on tumorigenesis and innate immunity. Tumour Biol 33(1):1–7. https://doi.org/10.1007/s13277-011-0249-3

Mulder KW, Wang X, Escriu C, Ito Y, Schwarz RF, Gillis J, Sirokmány G, Donati G, Uribe-Lewis S, Pavlidis P, Murrell A, Markowetz F, Watt FM (2012) Diverse epigenetic strategies interact to control epidermal differentiation. Nat Cell Biol 14(7):753–763. https://doi.org/10.1038/ncb2520

Nabbi A, Almami A, Thakur S, Suzuki K, Boland D, Bismar T, Riabowol K (2015) ING3 protein expression profiling in normal human tissues suggests its role in cellular growth and self-renewal. Eur J Cell Biol 94(5):214–222. https://doi.org/10.1016/j.ejcb.2015.03.002

Nabbi A, McClurg UL, Thalappilly S, Almami A, Mobahat M, Bismar TA, Binda O, Riabowol KT (2017) ING3 promotes prostate cancer growth by activating the androgen receptor. BMC Med 15(1):103. https://doi.org/10.1186/s12916-017-0854-0

Nagashima M, Shiseki M, Miura K, Hagiwara K, Linke SP, Pedeux R, Wang XW, Yokota J, Riabowol K, Harris CC (2001) DNA damage-inducible gene p33ING2 negatively regulates cell proliferation through acetylation of p53. Proc Natl Acad Sci USA 98(17):9671–9676. https://doi.org/10.1073/pnas.161151798

Nouman G, Anderson J, Mathers M, Leonard N, Crosier S, Lunec J, Angus B (2002) Nuclear to cytoplasmic compartment shift of the p33ING1b tumour suppressor protein is associated with malignancy in melanocytic lesions. Histopathology 40(4):360–366. https://doi.org/10.1046/j.1365-2559.2002.01369.x

Nozell S, Laver T, Mosele D, Nowoslawski De Vos M, Atkinson GE (2008) The ING4 tumor suppressor attenuates NF-κB activity at the promoters of target genes. Mol Cell Biol 28(21):6632–6645. https://doi.org/10.1128/MCB.00697-08

Ozer A, Wu L, Bruick R (2005) The candidate tumor suppressor ING4 represses activation of the hypoxia inducible factor (HIF). Proc Natl Acad Sci USA 102(21):7481–7486. https://doi.org/10.1073/pnas.0502716102

Pedeux R, Sengupta S, Shen JC, Demidov ON, Saito S, Onogi H, Kumamoto K, Wincovitch S, Garfield SH, McMenamin M, Nagashima M, Grossman SR, Appella E, Harris CC (2005) ING2 regulates the onset of replicative senescence by induction of p300-dependent p53 acetylation. Mol Cell Biol 25(15):6639–6648. https://doi.org/10.1128/MCB.25.15.6639-6648.2005

Peña PV, Davrazou F, Shi X, Walter KL, Verkhusha VV, Gozani O, Zhao R, Kutateladze TG (2006) Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. Nature 442(7098):100–103. https://doi.org/10.1038/nature04814

Raho G, Miranda C, Tamborini E, Pierotti MA, Greco A (2007) Detection of novel mRNA splice variants of human ING4 tumor suppressor gene. Oncogene 26(36):5247–5257. https://doi.org/10.1038/sj.onc.1210335

Ricordel C, Chaillot L, Blondel A, Archambeau J, Jouan F, Mouche A, Tiercin M, Burel A, Lena H, Desrues B, Guillaudeux T, Pedeux R (2021) ING2 tumor suppressive protein translocates

into mitochondria and is involved in cellular metabolism homeostasis. Oncogene 40(24):4111–4123. https://doi.org/10.1038/s41388-021-01832-3

Russell MW, Soliman MA, Schriemer D, Riabowol K (2008) ING1 protein targeting to the nucleus by karyopherins is necessary for activation of p21. Biochem Biophys Res Commun 374(3):490–495. https://doi.org/10.1016/j.bbrc.2008.07.076

Schäfer A, Karaulanov E, Stapf U, Döderlein G, Niehrs C (2013) Ing1 functions in DNA demethylation by directing Gadd45a to H3K4me3. Genes Dev 27(3):261–273. https://doi.org/10.1101/gad.186916.112

Schiltz PM, Lieber J, Giorno RC, Claman HN (1993) Mast cell immunohistochemistry: non-immunological immunostaining mediated by non-specific F(ab′)$_2$-mast cell secretory granule interaction. Histochem J 25(9):642–647. https://doi.org/10.1007/BF00157878

Scott M, Boisvert FM, Vieyra D, Johnston RN, Bazett-Jones DP, Riabowol K (2001) UV induces nucleolar translocation of ING1 through two distinct nucleolar targeting sequences. Nucleic Acids Res 29(10):2052–2058. https://doi.org/10.1093/nar/29.10.2052

Shen JC, Unoki M, Ythier D, Duperray A, Varticovski L, Kumamoto K, Pedeux R, Harris CC (2007) Inhibitor of growth 4 suppresses cell spreading and cell migration by interacting with a novel binding partner, liprin alpha1. Cancer Res 67(6):2552–2558. https://doi.org/10.1158/0008-5472.CAN-06-3870

Simpson F, Lammerts van Bueren K, Butterfield N, Bennetts JS, Bowles J, Adolphe C, Simms LA, Young J, Walsh MD, Leggett B, Fowles LF, Wicking C (2006) The PCNA-associated factor KIAA0101/p15(PAF) binds the potential tumor suppressor product p33ING1b. Exp Cell Res 312(1):73–85. https://doi.org/10.1016/j.yexcr.2005.09.020

Suzuki K, Boland D, Gong W, Riabowol K (2011) Domain recognition of the ING1 tumor suppressor by a panel of monoclonal antibodies. Hybridoma (Larchmt) 30(3):239–245. https://doi.org/10.1089/hyb.2010.0124

Thakur S, Nabbi A, Klimowicz A, Riabowol K (2015) Stromal ING1 expression induces a secretory phenotype and correlates with breast cancer patient survival. Mol Cancer 14:164. https://doi.org/10.1186/s12943-015-0434-x

Thalappilly S, Feng X, Pastyryeva S, Suzuki K, Muruve D, Larocque D, Richard S, Truss M, von Deimling A, Riabowol K, Tallen G (2011) The p53 tumor suppressor is stabilized by inhibitor of growth 1 (ING1) by blocking polyubiquitination. PLoS ONE 6(6):e21065. https://doi.org/10.1371/journal.pone.0021065

Thompson Z, Anderson GA, Hernandez M, Alfaro Quinde C, Marchione A, Rodriguez M, Gabriel S, Binder V, Taylor AM, Kathrein KL (2024) Ing4-deficiency promotes a quiescent yet transcriptionally poised state in hematopoietic stem cells. iScience 27(8):110521. https://doi.org/10.1016/j.isci.2024.110521

Toyama T, Iwase H, Watson P, Muzik H, Saettler E, Magliocco A, DiFrancesco L, Forsyth P, Garkavtsev I, Kobayashi S, Riabowol K (1999) Suppression of ING1 expression in sporadic breast cancer. Oncogene 18(37):5187–5193. https://doi.org/10.1038/sj.onc.1202905

Unoki M, Shen JC, Zheng ZM, Harris CC (2006) Novel splice variants of ING4 and their possible roles in the regulation of cell growth and motility. J Biol Chem 281(45):34677–34686. https://doi.org/10.1074/jbc.M606296200

Wang F, Wang AY, Chesnelong C, Yang Y, Nabbi A, Thalappilly S, Alekseev V, Riabowol K (2018) ING5 activity in self-renewal of glioblastoma stem cells via calcium and follicle stimulating hormone pathways. Oncogene 37(3):286–301. https://doi.org/10.1038/onc.2017.324

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Authors and Affiliations

Arthur Dantas[1,2,4] · Buthaina Al Shueili[1,2,5] · Jeongah Park[1,2,6] · Suleyman Abdullah[1,2,7] · Jessica Bertschmann[1,2,3] · Hokan Krowicki[1,2] · Mahbod Djamshidi[1,2,3] · Yang Yang[1,2] · Karen Blote[1,2] · Subhash Thalappilly[1,8] · Karl Riabowol[1,2,3]

✉ Karl Riabowol
karl@ucalgary.ca

Arthur Dantas
adantas@uottawa.ca

Buthaina Al Shueili
buthainas@squ.edu.om

Jeongah Park
jeongah@ualberta.ca

Suleyman Abdullah
sabdul45@uwo.ca

Jessica Bertschmann
Jessica.bentschmann@ucalgary.ca

Hokan Krowicki
hokan.krowicki@ucalgary.ca

Mahbod Djamshidi
mahbod.djamshidi@ucalgary.ca

Yang Yang
yang4@ucalgary.ca

Karen Blote
blote@ucalgary.ca

Subhash Thalappilly
subhash.t@gmail.com

1 Robson DNA Sciences Centre, Calgary, Canada

2 Arnie Charbonneau Cancer Institute, Calgary, Canada

3 Departments of Biochemistry and Molecular Biology, Alberta Children's Hospital Research Institute, University of Calgary, Calgary, AB T2N 4N1, Canada

4 Present Address: Faculty of Health Sciences, School of Human Kinetics, University of Ottawa, Ottawa, ON, Canada

5 Present Address: Department of Biology, College of Science, Sultan Qaboos University, Muscat, Oman

6 Present Address: University of Alberta, Edmonton, AB, Canada

7 Present Address: University of Western Ontario, London, ON, Canada

8 Present Address: National University of Singapore, Queenstown, Singapore