Reliable hexokinase 3 protein detection in human cell lines and primary tissue

Yasmeen H. Mady,[1,2,3]* Carmen G. Kalbermatter,[1,2]* Maarij Khan,[1] Anna M. Schläfli,[1] Rina Mehmeti,[1,2] Inti Zlobec,[1] Lucine Christe,[1] Mario P. Tschan[1,2]

[1]Institute of Tissue Medicine and Pathology, University of Bern, Switzerland
[2]Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland
[3]Department of Clinical Pathology, South Egypt Cancer Institute, Assiut University, Assiut, Egypt

*These authors contributed equally to this work.

ABSTRACT

Accurate differentiation of homologous proteins that share high sequence identity remains a significant challenge in biomedical research, as conventional antibodies often lack sufficient specificity, leading to potential misinterpretations. This issue is particularly evident in the study of hexokinases, a family of isoenzymes that catalyze the first step of glycolysis by phosphorylating glucose. Beyond their canonical metabolic roles, hexokinases play critical non-glycolytic functions, especially in cancer biology. However, their unique tissue distributions and context-dependent roles are often obscured by the overlapping specificities of commercially available antibodies, which can produce misleading results. In this study, we rigorously evaluated a panel of antibodies targeting hexokinase isoenzyme 3 (HK3), highlighting the widespread issue of cross-reactivity and insufficient validation. Through this process, we identified and validated a highly specific antibody for HK3, demonstrating its reliability in Western blot and immunohistochemistry applications. Using this validated tool, we reveal the distinct localization of HK3 in myeloid cell populations, providing new insights into its potential functional roles in these cells. This work addresses a critical gap in antibody specificity and establishes HK3 as a uniquely expressed gene in myeloid and immune cells and is absent in other cell types under basal conditions, providing a foundation for future investigations into its context-dependent functions

Key words: hexokinase; HK3; immunohistochemistry; leukemia; myeloid cells; antibody.

Correspondence: Mario P. Tschan, Institute of Tissue Medicine and Pathology, University of Bern, Murtenstr. 31, 3008 Bern, Switzerland. E-mail: mario.tschan@unibe.ch

Contributions: YHM, CGK, conducted experiments and drafted the manuscript; MK, analyzed publicly available datasets; RM, LC, analyzed and scored immunohistochemistry data; AMS, IZ, MPT, conceptualized and designed the study, revised the manuscript. All the authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors declare no conflict of interests.

Availability of data and materials: the data used to support the findings of this study are available from the corresponding author upon reasonable request.

Funding: this study was supported by grants from the Swiss National Science Foundation (310030_197786 to IZ and MPT;31003A_173219 to MPT). YHM is supported by a scholarship provided by the Ministry of Higher Education and Scientific Research of the Arabic Republic of Egypt. RM is supported by a Swiss Government Excellence Scholarship (ESKAS, nr. 2021.0019 / Kosovo / OP).

Introduction

Hexokinases (HKs) catalyse the first and rate limiting step of glycolysis by converting glucose into glucose-6-phosphate (G6P). The 100kDa enzymes HK1-3 are thought to have arisen from a common 50kDa precursor by gene duplication and tandem ligation. The fourth 50kDa isoform HK4 with its high degree in sequence similarity supports this hypothesis.[1] HK1 and 3 show catalytic activity at the C-terminal half and glucose binding properties at the N-terminal half, whereas in HK2 both the N-terminal and C-terminal halves are catalytically active.[2] All HKs show a cytoplasmic localization whereas HK1 and HK2 can bind to the outer mitochondrial membrane *via* a 21 hydrophobic amino acid sequence at their N-terminus. HK3 however, does not contain the conserved mitochondrial binding region.[3] On the subcellular level the HK2 isoform has also been shown to localize to the nucleus[4] and HK3 may have a perinuclear localization.[5] Although the primary function of HKs is linked to glycolysis, these enzymes have been shown to play additional regulatory roles within cells. Depending on factors such as cell type, subcellular localization, and the cell's metabolic state, different isoforms of HK are involved in diverse processes such as autophagy, cell death, DNA damage, and reactive oxygen species (ROS) production.[5] For instance, mitochondrial HK1 and HK2 inhibit apoptosis by selectively translocating pro-apoptotic Bcl-2 family members such as tBid, Bax, and Bak away from the mitochondria.[6] Nuclear HK2, on the other hand, maintains stem cell fate, modulates chromatin accessibility, and enhances the DNA damage response.[4] Among the HK family, HK3 has gained particular attention for its pro-survival role in hematological malignancies, specifically acute promyelocytic leukemia (APL)[7] and acute myeloid leukemia (AML).[5] Notably, HK3 is a transcriptional target of PU.1[7] a critical regulator of myeloid differentiation,[8-10] highlighting its potential importance in both AML and myeloid cells in general. However, research into HK3 has been hindered by the lack of a highly specific antibody. This has made it difficult to study HK3's expression and function using traditional methods such as Western blotting. As a result, researchers have had to rely on alternative techniques, such as PCR or endogenous tagging, which are more cumbersome and semi-artificial techniques. Moreover, the absence of a specific antibody may have contributed to some preliminary misinterpretations or incomplete conclusions regarding HK3's role, hindering a more comprehensive understanding of its functions.

Materials and Methods

Cell lines, culture and treatment conditions

HEK 293T cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS), 1% HEPES and were kept in 7.5% CO_2 -92.5 % air humified atmosphere at 37°C.

The human AML cell lines HL60, THP1 and OCI-AML2 were maintained in RPMI-1640 with 10% FBS in 5% CO_2-95% air humified atmosphere at 37°C. HL60 cells were differentiated into neutrophils with 1 µM all-trans retinoic acid (ATRA) (DMSO, Merck KGaA Darmstadt, Germany) over 4 days. Macrophage differentiation of HL60 and THP1 cells was induced with 1 nM phorbol-12-myristate-13-acetate (PMA) (DMSO) for 48 h or 65 nM PMA for 24 h respectively.

The solid cancer cell lines A549 and HCT116 were maintained in DMEM/F12 with 10%FBS and DMEM with 10%FBS and 1% L-Glutamine, respectively. Cells were kept in 5% CO_2-95% air humified atmosphere at 37°C.

Generation of knockout cell lines

HK3 knockout cell lines were generated using the lentiCRISPRv2 vector containing the Cas9 endonuclease gene, gRNA (HK3: GGATGCTGCCTACATACGTG) and a puromycin selection marker. Lentiviral vectors for CRISPR knockouts were generated by transient transfection of the corresponding plasmid and third-generation packaging plasmids pMD2.G (VSV-G), pMDLg/pRRE (gag and pol), and pRSV-Rev (rev) into HEK 293 T cells. Briefly, cells were transfected using calcium phosphate, and the supernatant was collected 72 h post transfection. The media was filtered through a 0.45 µm nitrocellulose membrane. About $4x10^5$ THP1 cells were incubated with 400 µL virus and 8 µg/mL polybrene; after 4 h a second transduction was performed. After 2 days cells were selected with 1.5 µg/mL puromycin for 4 days followed by 3 days of 0.5 µg/mL. Following selection cells were sorted using HANA Cell Sorter and Single Cell Dispenser (Biotechne, Minneapolis, MN, USA) and single cells grown in the 96-well format.

Endogenous HiBit-tagging of hexokinase 3

Endogenous tagging of HK3 with the HiBiT sequence was performed following the published protocol[11] using the Neon Electroporation Kit (Invitrogen, Carlsbad, CA, USA). Alt-R HiFi Cas9, ssODN, sgRNA, and HDR enhancer were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA). Briefly, the RNP complex was assembled by incubating sgRNA with Alt-R HiFi Cas9 in a 2.5:1 ratio in Buffer R for 15 min at room temperature (RT). The ssODN was then added to the RNP mix, which was subsequently incubated at 4°C. Concurrently, cRPMI was preincubated with HDR enhancer (1 µM), while HL60 cells were washed with PBS and resuspended at a concentration of 2×10^7 cells/mL in Buffer R. The RNP and ssODN mix was then added to the cells, which were electroporated using a 10 µL tip with the following settings: 1 pulse at 1700 V for 20 ms. After 24 h, the HDR-containing medium was replaced with fresh medium containing 20% FBS. A HiBiT lytic assay was performed 96 h post-electroporation to detect luminescence.

HiBiT lytic detection

Luminescent detection of HiBit was performed using Nano-Glo® HiBit Lytic detection System according to manufacturer's instructions (Promega, Madison, WI, USA). Briefly, adding the cells (1:1) to the reaction buffer (prepared by adding 1:50 Substrate and 1:100 LgBit in assay buffer) in 96 well flat white plates and read after 10 min using Tecan Reader with 1000 ms integration time. The values were normalized to cell number using CellTiter-Glo® luminescent cell viability assay (Promega).

Transient transfection

HEK 293T cells were transiently transfected with lentiviral plasmids containing Flag-HK1, Flag-HK2 or Flag-HK3, using the calcium-phosphate method. In total 10 µg of plasmid was used and after the transfection cells were cultured in 5% CO_2-95% humified atmosphere at 37°C for 48 h.

Western blotting

Whole cell extracts were prepared using RIPA buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X lysis buffer) with addition of protease inhibitor (Roche, Basel, Switzerland). Protein concentration was measured by Bradford Assay (BioRad, Hercules, CA, USA) and 0.4-25 µg total protein was loaded onto 12% TGX stain free, fast cast acrylamide gels (BioRad). Blots were incubated with the primary antibodies in TBS 0.1% Tween-20/ 5% non-fat milk or 5% BSA overnight at 4°C plus 2 h at RT, then incubated with HRP coupled secondary

anti-rabbit or anti-mouse antibody at 1:3000-1:10000 for 1-3 h at RT. For re-incubation membranes were stripped using 1.5% Glycine (w/v), 0.1% SDS (w/v), 1% Tween-20 (v/v) in dH_2O, pH 2.2. Successful stripping was verified by incubation with the according secondary antibody and visualized with ECL solution (BioRad). Primary antibodies used are listed in Table 1. Uncropped Western blots and stripped membranes are shown in the supplement.

Embedding of cells for immunohistochemical Labeling

Cells were pelleted by centrifugation before 3-4 drops of plasma (Blood Transfusion Service, Bern, Switzerland) and 1-2 drops of thrombin (Diagnotec, Liestal, Switzerland) were added to initiate coagulation. After 2 min of incubation, the cell pellet was transferred into an embedding cassette. The sample was then fixed in 4% paraformaldehyde overnight at RT. After fixation, the cells were embedded in paraffin following standard procedures in the routine pathology laboratory.

Immunohistochemical Labeling

Before immunohistochemical Labeling paraffin-embedded samples were pre-processed, which included de-paraffination, rehydration, and antigen retrieval. IHC Labeling was performed using an automated immunostainer (Bond RX; Leica Biosystems, Heerbrugg, Switzerland). Antibodies used were HK3 (Merck KGaA; HPA056743 dilution 1:2000) and the CD68 antibody (Dako Omnis, Agilent, Santa Clara, CA, USA; clone PG-M1; dilution 1:200). Visualization was performed using the Bond Polymer Refine Detection kit (Leica Biosystems) as instructed by the manufacturer. The ratio of HK3/CD68 positive cells was determined by counting the positive cells in an area of 2 mm^2 of sequential cuts.

Results

Hexokinase 3: a 'white blood cell hexokinase' with expression predominantly associated with myeloid cells

Upon analyzing the publicly available datasets on the human protein atlas (HPA), an enrichment of *HK3* gene expression in bone marrow and lymphoid tissues becomes evident (Figure 1A). A closer examination at the single-cell level reveals that the highest *HK3* mRNA expression occurs in phagocytic cells, including macrophages, monocytes, and tissue-resident macrophages like Hofbauer cells in the placenta and Kupffer cells in the liver (Figure 1B). We then focused on *HK3* mRNA expression across various immune cell types and found it to be predominantly associated with cells of myeloid origin (Figure 1C). This observation prompted us to explore HK3 expression during myeloid differentiation, where we noted a 2-fold increase in *HK3* transcript levels as differentiation progressed (Figure 1D). If we then examined HK3 levels in cancer cell lines published on the HPA, it becomes apparent that *HK3* mRNA is confined to myeloid leukemia cell lines, with no transcripts detected in solid cancer cell lines (Figure 1E).

Testing several commercially available HK3 antibodies for their specificity by Western blotting

The HK family consists of isoenzymes that, despite being located on different chromosomes, exhibit up to 70% sequence and structural identity. The three HKs, HK1, HK2 and HK3 are comprised of two highly analogous domains: an N-terminal domain and a C-terminal domain. HK3, however, lacks significant similarity to other HK isoforms at its N-terminus due to the absence of the mitochondrial binding domain. In terms of the small and large subdomains of HKs, the shared sequence identity of HK3 with other isoforms ranges between 44% and 54%. At the C-terminal region, the sequence similarity among the HK isoforms is higher, ranging from 61% to 70% (Figure 2A). Due to the pronounced sequence similarity among HKs, identifying a truly specific antibody for HK3 is challenging. We evaluated several commercially available antibodies for HK3, some of which have been used in other studies exploring the role of HK3 in solid cancers. The immunogen sequence used to produce the respective antibodies are depicted in Figure 2B. To assess specificity, these antibodies were tested on 293T cells, which have no detectable basal expression of *HK3* mRNA according to the HPA (Figure 1E). The cells were transiently transfected to overexpress Flag-HK1, Flag-HK2, or Flag-HK3, and serial dilutions of protein were loaded to evaluate antibody sensitivity (Figure 2C). We used THP1, an AML cell line with moderate HK3 expression according to the HPA, as a positive control. THP1 cells depleted of HK3 using CRISPRCas9 technology (validated by TIDE analysis; *Supplementary Figure S1 A,B*) served as negative control. Our results indicate that most tested antibodies for HK3 also cross-react with HK1 and HK2, often detecting them more effectively than HK3 (Figure 2C). Lastly, we verified that HK1 and HK2 are indeed expressed at endogenous levels in THP1 HK3 knockout (KO) cells as well as in 293T cells that were used for transient transfections of the different HK expression plasmids (Figure 2D). The notable exception not showing cross-reactivity was the Prestige Antibody (HPA056743), which specifically recognized HK3. This specificity can be attributed to its recognition site at the N-terminus, where there is no significant sequence similarity with HK1 or HK2 (Figure 2B).

Table 1. Primary antibodies used for Western blotting.

Name	Company	Cat. number	Blocking	Species	Work conc.	2nd AB and detection	Immunogen
HK3 (C1C3-2)	GeneTex (Irvine, CA, USA)	GTX111409	5% BSA	Anti rabbit	1:1000	1:10000	No info, discontinued
HK3 Rabbit pAB	Prestige Antibodies (Merck KGaA, Darmstadt, DE)	HPA056743	5% Milk	Anti rabbit	1:500	1:3000	1-61 aa
HK3 Rabbit pAB	Thermofisher (Invitrogen, Carlsbad, CA, USA)	PA5-29304	5% BSA	Anti rabbit	1:500	1:10000	587-826 aa
HK3 Monoclonal antibody	Proteintech (Rosemont, IL, USA)	67803-1-Ig	5% Milk	Anti-mouse	1:1000	1:10000	622-923 aa

HK3, Hexokinase 3; pAB, polyclonal antibody.

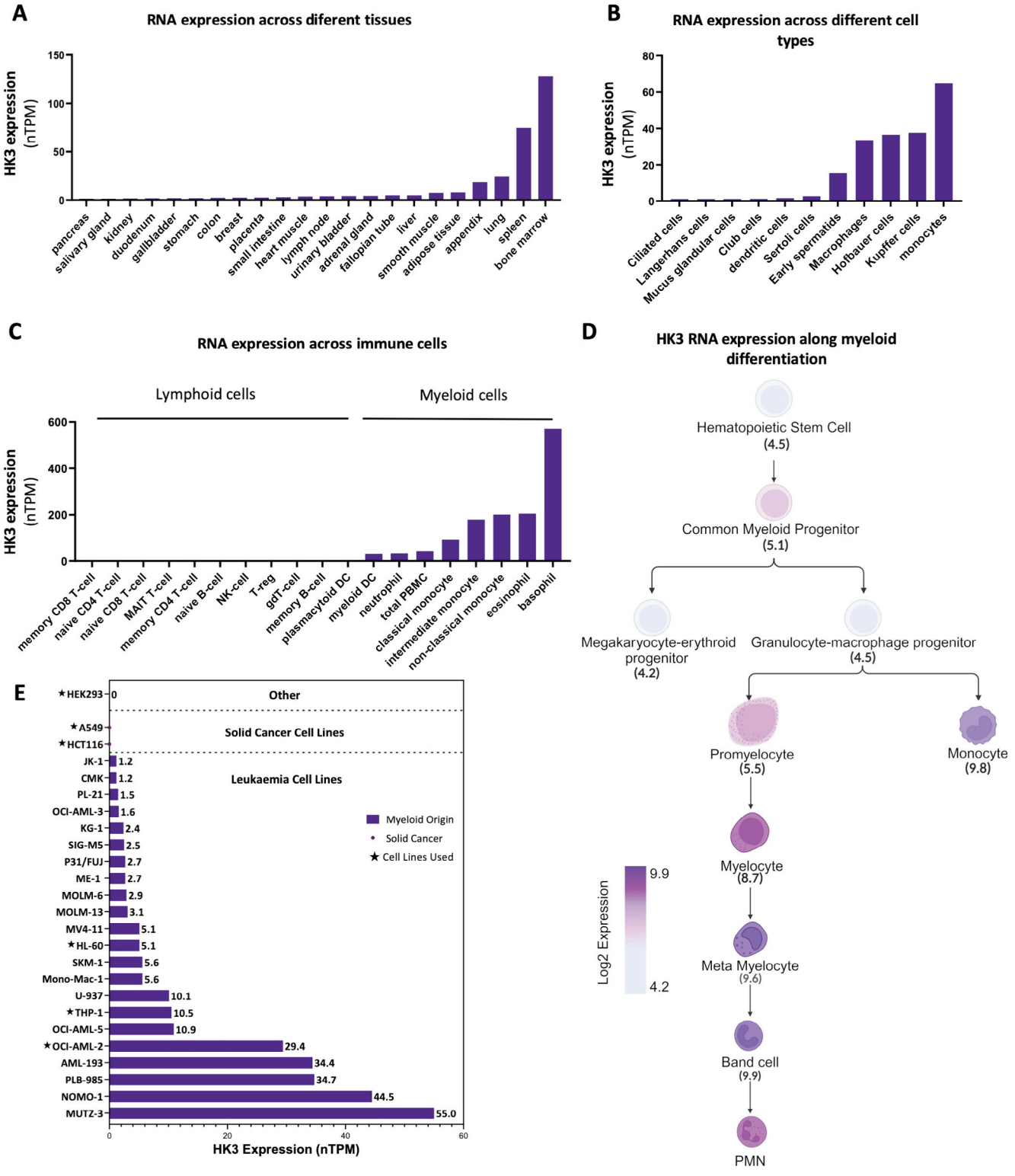

Figure 1. Hexokinase 3 (HK3) expression is highly associated with myeloid cells. **A**) RNA-seq data from the consensus dataset of the human protein atlas (HPA) database showing HK3 expression across various tissue types. **B**) Single-cell RNA-seq data obtained from the consensus dataset of the HPA depicting HK3 expression across different cell types. **C**) Single-cell RNA-seq data obtained from the HPA dataset focusing on HK3 expression within immune cell types. **D**) HK3 expression profiles in different stages of myeloid differentiation, obtained from Blood Spot samples from normal hematopoiesis with AMLs dataset, demonstrating expression changes during differentiation. **E**) HK3 expression levels in various cancer cell lines, as reported by the HPA (nTPM, normalized transcript per million).

Expression of HK3 is absent in solid cancer cells but induced upon myeloid differentiation

According to mRNA transcript data from the HPA, solid cancer cell lines do not express *HK3* mRNA (Figure 1E). To validate this, we determined HK3 protein levels in the lung adenocarcinoma cell line (A549) and the colorectal cancer cell line (HCT116). By comparing the results from different antibodies, we observed that only the HK3 specific Prestige antibody (HPA056743) confirmed the absence of the HK3 protein in these cell lines. In contrast, the other two antibodies, Thermo Fisher (PA5-29304) and Proteintech (67803-1-IG), detected non-specific bands corresponding very likely to other HKs (Figure 3A; *Supplementary Figure S2A*).

We further investigated whether HK3 expression levels change during myeloid differentiation. Therefore, we utilized the AML cell line HL60, which can be induced to differentiate into neutrophils in response to ATRA or into macrophages using PMA. We also included the THP1 AML cell line, which differentiates into macrophages

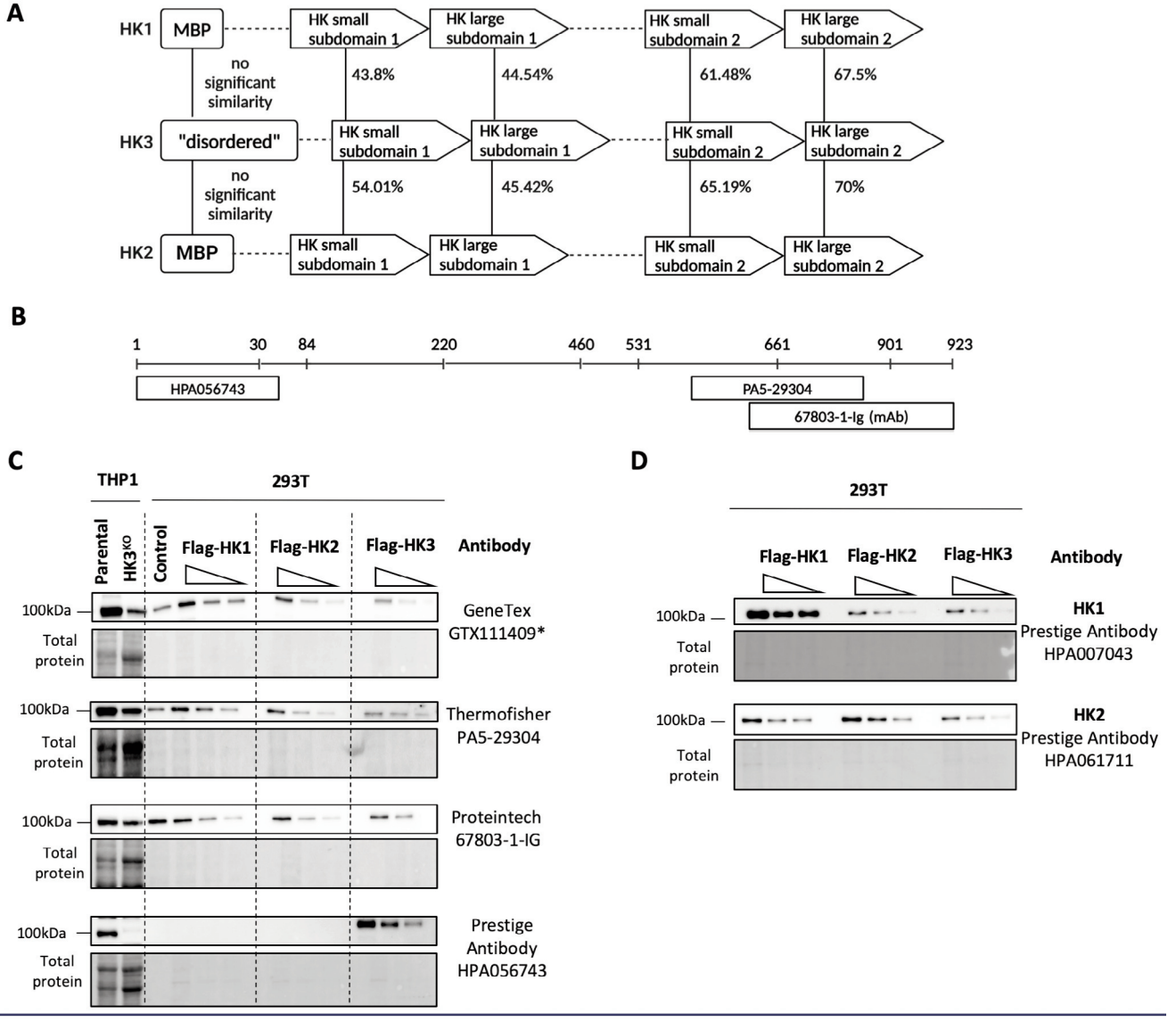

Figure 2. Hexokinase isoenzyme similarity complicates antibody specificity. **A**) Schematic representation illustrating the structural domains of human hexokinases, entailing the percentage of amino acid sequence similarity between hexokinase (HK) 3 and other HK1 and HK2. **B**) Diagram showing the amino acid regions used as immunogens for various antibodies tested: Prestige antibody (HPA056743): immunogen region spanning amino acids 1-61. Thermo Fisher antibody (PA5-29304): immunogen region spanning amino acids 587-826. Proteintech antibodies (67803-1-Ig): immunogen region spanning amino acids 622-923.*GeneTex antibody (GTX111409): immunogen region could not be identified as the product has been discontinued by the company. **C**) Western blot analysis demonstrating the specificity of different HK3 antibodies. **D**) Protein lysates from HK1, HK2, and HK3 overexpression in HEK 293T cells are shown. Serial dilutions of 2.5 µg, 1 µg, and 0.4 µg of each lysate were loaded, with THP1 parental cells as a positive control, and THP1 HK3 knockout (KO) and HEK 293T parental cells as negative controls. Total protein was used as loading control.

upon PMA treatment. In both cell lines, HK3 expression levels increased after differentiation (Figure 3A; Supplementary Figure S2B). In addition to the THP1 HK3 KO cells we also used HL60 HK3 KO cells (confirmed by MS analysis[5]) as negative controls. To confirm these findings, we employed a highly sensitive HK3-HiBiT-endogenous tagging system in the HL60 cell line.[11] Following endogenous tagging of HK3 with the HiBiT tag, we assessed HK3 expression using a lytic luminescence assay, which further demonstrated the elevated expression of HK3 upon neutrophil and macrophage differentiation in HL60 cells (Figure 3B).

Anti-HK3 antibody from Prestige antibodies specifically stains macrophages in tissue IHC

Immunohistochemistry (IHC) is a valuable tool in translational research, particularly for detecting protein expression in patient samples. To ensure the Prestige antibody's effectiveness and specificity in IHC, we validated it using two AML cell lines (OCI AML2 and THP1) known to express HK3. Both parental and HK3 knockout cell lines were labeled and the results showed HK3 detection in the parental cell lines, with no expression in the KO variants (Figure 4A). Next, we sought to identify the cell types expressing HK3 in lung and colon tissues. To achieve this, we analyzed published single-cell RNA sequencing datasets, including the Human Lung Cell Atlas (HLCA) and the Human CD Atlas on the Single Cell Portal, alongside the HPA dataset. In the lung, both HLCA and HPA datasets demonstrated that HK3 expression is restricted to macrophage and monocyte clusters (Figure 4B). These findings are consistent with our IHC labeling, which revealed robust HK3 labeling in large mononuclear cells characterized by round, oval, or kidney-shaped nuclei and abundant granular cytoplasm-a morphology consistent with macrophages. Notably, epithelial and alveolar cells, as well as all other cell types, lacked HK3 labeling (Figure 4C). In the colon, HK3 expression was similarly confined to macrophage and monocyte immune subsets (Figure 5A). Among all cell types in the colon, the highest enrichment of HK3 was observed in macrophages and neutrophils (Figure 5B). This was corroborated by CD68 labeling of macrophages in the same colon tissues, which revealed a similar distribution pattern of HK3 expression in these cells (Figure 5C). Sequential sections of normal colon tissue demonstrated a ratio of HK3-positive to CD68-positive cells of 132:490. These results align with single-cell RNA sequencing data from colon immune tissues, which reported that only a subset of macrophages exhibits HK3 positivity. Together, these findings highlight the selective expression of HK3 in macrophages and monocytes across lung and colon tissues, emphasizing its potential role in immune cell-specific functions.

Discussion

The structurally and on a sequence level very similar HK isoforms 1-3 show distinct tissue expression. HK1 is ubiquitously expressed, whereas HK2 is limited to insulin sensitive tissue such as skeletal muscle and adipose tissue.[12] Lately researchers have focused on the role of HK3 in solid cancers and concluded it to have an effect on glycolysis in intestinal microbial-induced metastasis of colorectal cancer (CRC),[13] that it's overexpression promotes orthotopic tumor growth[14] and that HK3 supported malignant biologic behaviour in renal cell carcinoma cell lines,[15] and it's inhibition promotes invasion of hepatocellular carcinoma cells (HCC).[16] In our study and in publicly available databases, however, we found that HK3 is almost exclusively expressed in cells of myeloid origin and that no *HK3* mRNA could be detected in solid cancer cell lines under basal conditions. Our previously published paper, Seiler *et al.*[5] demonstrates an upregulation of HK3 during myeloid differentiation, consistent with the analysis of publicly available datasets and the experimental outcomes presented in this paper. These findings confirm increased HK3 levels in more differ-

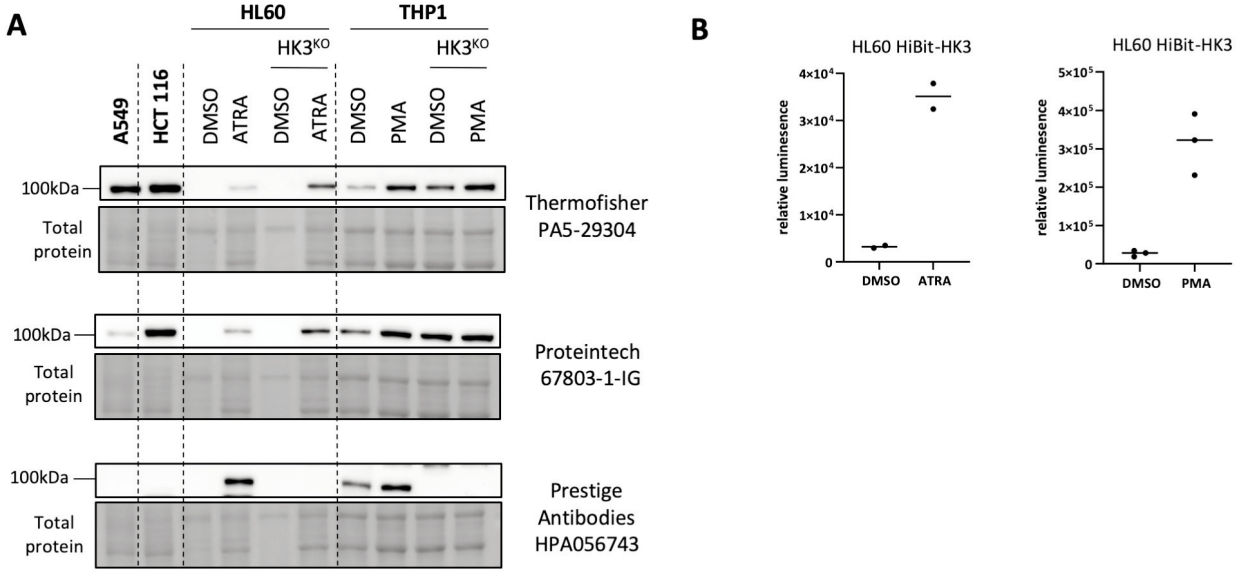

Figure 3. HK3 protein expression is upregulated during myeloid differentiation. **A**) Western blot analysis of HK3 levels in solid cancer cell lines, HL60 control and HK3 KO cell clones ± 4 days of ATRA treatment, THP1 control and HK3 KO cell clones ± 24 h PMA treatment with the indicated antibodies. **B**) Relative protein expression of HK3 in HL60 ± 4 days of ATRA treatment or ± 48 h PMA treatment measured by luminescent quantification of HiBiT-tag expression, normalized to viability as determined by CellTiter-Glo® Luminescent Cell Viability Assay.

entiated myeloid cells. Despite this tissue specific expression, the relatively high degree of sequence similarity between the HK isoforms can lead to cross reactivity of antibodies and potentially inaccurate interpretations. In our hands only 1/4 commercially available antibodies tested showed specific binding to HK3 on Western blotting and IHC.

Antibody validation and characterization of specificity is not a new concern; this issue has been recognized and discussed extensively with the challenges nicely laid out here.[17] Several reports, including those from our group[5,11] and others[18] have highlighted the challenges posed by poorly validated antibodies. The so-called "antibody characterization crisis" has been thoroughly reviewed.[18] Over the years, various solutions have been proposed to address these issues, offering valuable suggestions for both companies[19] and researchers.[20]

We believe that reporting problems with antibody specificities are critical for the scientific community for correct interpretation of results. Our work with HK3 serves as a clear example: many studies have drawn misleading conclusions about HK3 expression that may be due to antibody cross-reactivity. While these findings are not entirely erroneous, the data become clearer when combined with RNA-seq data from bulk sequencing. Our interpretation is that the observed HK3 expression is largely attributable to immune cells present in the tumor microenvironment of solid cancers as we could only detect HK3 expression in the macrophages of tissues and it was nicely demonstrated by Liang *et al*. naming it a 'poten-

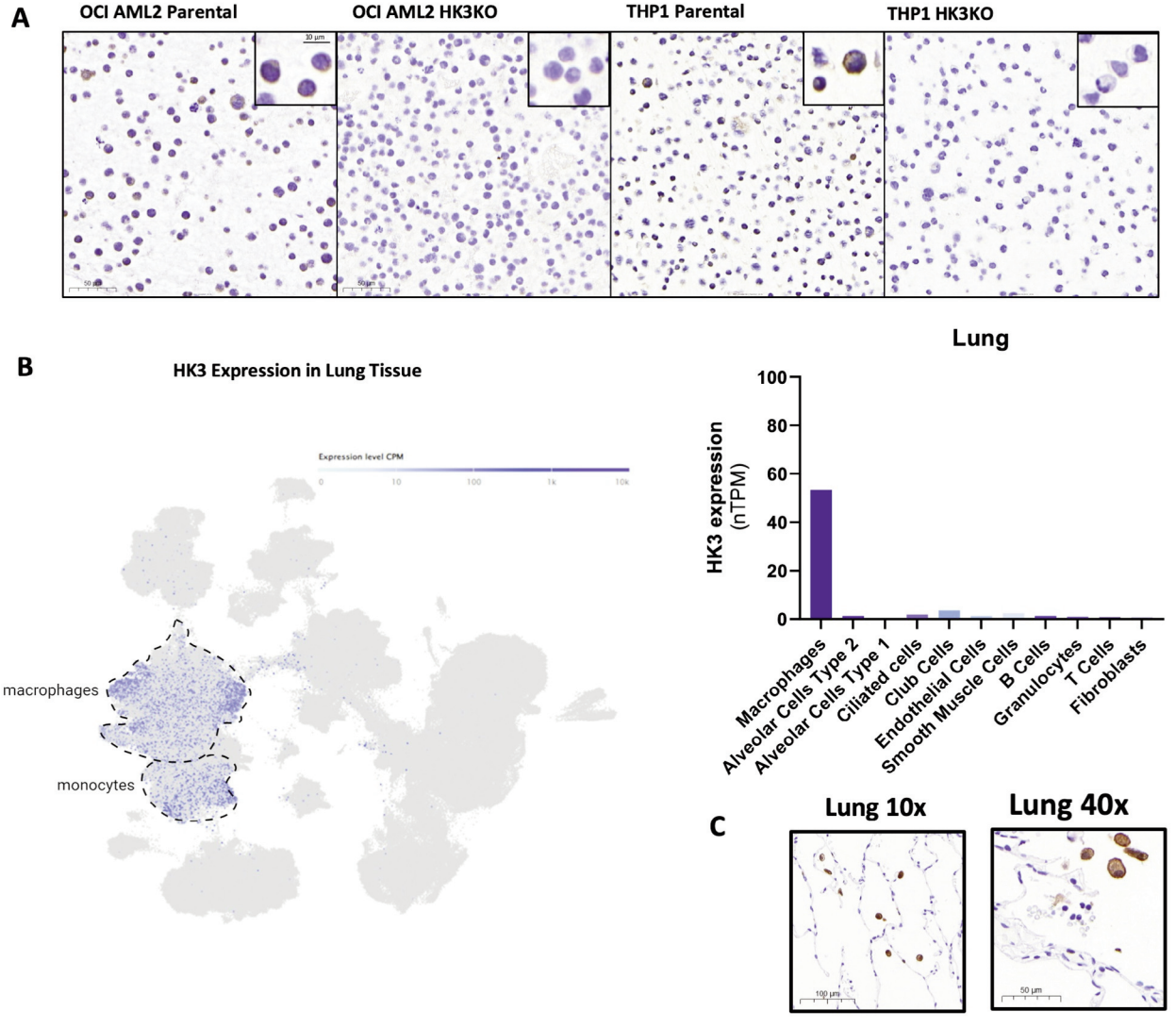

Figure 4. Reliable detection of HK3 in cell and lung tissue using immunohistochemistry. **A**) IHC HK3 labeling with anti-HK3 antibody from Prestige antibodies HPA056743 in OCI-AML2 parental cell lines, OCI AML2 HK3 KO cells, THP1 parental and THP1 HK3 KO cells (from left to right); positive immunolabeling is indicated by brown color. **B**) Single cell RNA seq mRNA of different cell types of the lung from Human Lung Cell Atlas and Single Cell Expression Atlas, European Bioinformatics Institute (EMBL-EBI), accessed October 22, 2024, Available at https://www.ebi.ac.uk/gxa/sc/home (on the left) and from the HPA dataset (on the right). **C**) IHC labeling of HK3 in lung tissue.

tial immune-related biomarker'.[23] The precise role of HK3 in these contexts, however, remains an open question that requires further investigation. Our results have shown that HK3 expression is predominantly found in myeloid cells, though it can be induced even in cells with low basal levels of HK3 under certain stimuli. Whether this holds true in non-hematopoietic cells is still unresolved. With the development of proper tools and more rigorous validation methods, we hope to have laid the groundwork for future studies on HK3, enabling researchers to explore its role with greater accuracy and confidence.

Acknowledgments

The Translational Research Unit (TRU), Institute of Tissue Medicine and Pathology, and Deborah Shan-Krauer are gratefully acknowledged for excellent technical support.

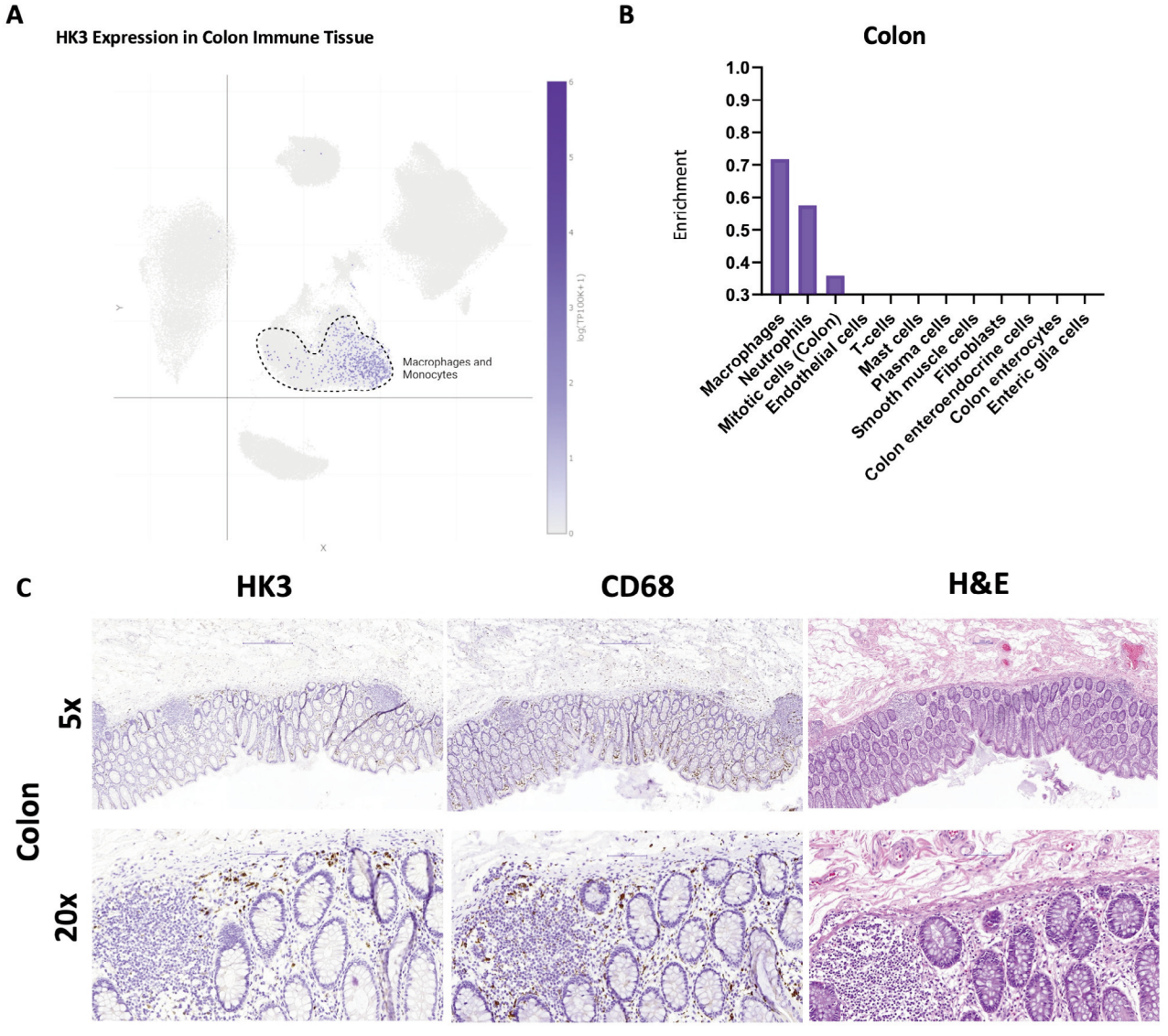

Figure 5. Reliable detection of HK3 colon tissue using immunohistochemistry. **A**) single cell RNAseq of colon immune tissue from the human CD atlas study between colon and terminal ileum on Single Cell Portal (accessed October 22, 2024). **B**) Enrichment score of HK3 in colon tissue from human protein atlas v23. **C**) IHC labeling of HK3 in colon tissue with anti-HK3 form Prestige antibodies HPA056743, CD68 antibody and H&E staining (left to right).

References

1. Ardehali H, Yano Y, Printz RL, Koch S, Whitesell RR, May JM, et al. Functional organization of mammalian hexokinase II. J Biol Chem 1996;271:1849-52.
2. Robey RB, Hay N. Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt. Oncogene 2006;25:4683-96.
3. Wyatt E, Wu R, Rabeh W, Park HW, Ghanefar M, Ardehali H. Regulation and cytoprotective role of hexokinase III. Wölfl S, editor. PLoS One 2010;5:e13823.
4. Thomas GE, Egan G, García-Prat L, Botham A, Voisin V, Patel PS, et al. The metabolic enzyme hexokinase 2 localizes to the nucleus in AML and normal haematopoietic stem and progenitor cells to maintain stemness. Nat Cell Biol 2022;24:872-84.
5. Seiler K, Humbert M, Minder P, Mashimo I, Schläfli AM, Krauer D, et al. Hexokinase 3 enhances myeloid cell survival via non-glycolytic functions. Cell Death Dis 2022;13:448.
6. Lauterwasser J, Fimm-Todt F, Oelgeklaus A, Schreiner A, Funk K, Falquez-Medina H, et al. Hexokinases inhibit death receptor-dependent apoptosis on the mitochondria. Proc Natl Acad Sci USA 2021;118:e2021175118.
7. Federzoni EA, Valk PJM, Torbett BE, Haferlach T, Löwenberg B, Fey MF, et al. PU.1 is linking the glycolytic enzyme HK3 in neutrophil differentiation and survival of APL cells. Blood 2012;119:4963-70.
8. Kueh HY, Champhekar A, Nutt SL, Elowitz MB, Rothenberg E V. Positive feedback between PU.1 and the cell cycle controls myeloid differentiation. Science 2013;341:670-3.
9. McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, et al. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J 1996;15:5647-58.
10. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 1994;265:1573-7.
11. Seiler K, Rafiq S, Tschan MP. Isoform-specific, semi-quantitative determination of highly homologous protein levels via CRISPR-Cas9-mediated HiBiT tagging. Bio Protoc 2023;13:e4777.
12. Wilson JE. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. J Exp Biol 2003;206:2049–57.
13. Wu X, Qian S, Zhang J, Feng J, Luo K, Sun L, et al. Lipopolysaccharide promotes metastasis via acceleration of glycolysis by the nuclear factor-κB/snail/hexokinase3 signaling axis in colorectal cancer. Cancer Metab 2021;9:23.
14. Li S, Li Z, Wang X, Zhong J, Yu D, Chen H, et al. HK3 stimulates immune cell infiltration to promote glioma deterioration. Cancer Cell Int 2023;23:227.
15. Xu W, Liu WR, Xu Y, Tian X, Anwaier A, Su JQ, et al. Hexokinase 3 dysfunction promotes tumorigenesis and immune escape by upregulating monocyte/macrophage infiltration into the clear cell renal cell carcinoma microenvironment. Int J Biol Sci 2021;17:2205-22.
16. Du Y, Zhang H, liu J, Duan X, Chen S, Jiang W. HK3: A potential prognostic biomarker with metastasis inhibition capabilities in hepatocellular carcinoma. Biochem Biophys Res Commun 2024;741.
17. Voskuil JLA. The challenges with the validation of research antibodies. F1000Res 2017;6:161.
18. Kahn RA, Virk H, Laflamme C, Houston DW, Polinski NK, Meijers R, et al. Antibody characterization is critical to enhance reproducibility in biomedical research. Elife 2024:13:e100211.
19. Uhlen M, Bandrowski A, Carr S, Edwards A, Ellenberg J, Lundberg E, et al. A proposal for validation of antibodies. Nat Methods 2016;13:823-7.
20. Laflamme C, Edwards AM, Bandrowski AE, McPherson PS. Opinion: Independent third-party entities as a model for validation of commercial antibodies. N Biotechnol 2021;65:1-8.
21. Liang T, Zhou X, Wang Y, Ma W. Glioma hexokinase 3 positively correlates with malignancy and macrophage infiltration. Metab Brain Dis 2024;39:719-29.

Online supplementary material

Supplementary Figure S1. TIDE analysis of HK3 KO clones.

Supplementary Figure S2. HK3 upregulation upon myeloid differentiation of myeloid leukemic cells.

Uncropped Western blots.

Received: 20 December 2024. Accepted: 27 February 2025.

European Journal of Histochemistry 2025; 69:4175
doi:10.4081/ejh.2025.4175