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Review

If this is true, what does it imply? How end-user antibody validation facilitates insights into biology and disease

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Abstract Antibodies are employed ubiquitously in biomedical sciences, including for diagnostics and therapeutics. One of the most important uses is for immunohistochemical (IHC) staining, a process that has been improving and evolving over decades. IHC is useful when properly employed, yet misuse of the method is widespread and contributes to the "reproducibility crisis" in science. We report some of the common problems encountered with IHC assays, and direct readers to a wealth of literature documenting and providing some solutions to this problem. We also describe a series of vignettes that include our approach to analytical validation of antibodies and IHC assays that have facilitated a number of biological insights into prostate cancer and the refutation of a controversial association of a viral etiology in gliomas. We postulate that a great deal of the problem with lack of accuracy in IHC assays stems from the lack of awareness by researchers for the critical necessity for end-users to validate IHC antibodies and assays in their laboratories, regardless of manufacturer claims or past publications. We suggest that one reason for the pervasive lack of end-user validation for research antibodies is that researchers fail to realize that there are two general classes of antibodies employed in IHC. First, there are antibodies that are "clinical grade" reagents used by pathologists to help render diagnoses that influence patient treatment. Such diagnostic antibodies, which tend to be highly validated prior to clinical implementation, are in the vast minority (*e.g.* < 500). The other main class of antibodies are "research grade" antibodies (now

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numbering >3 800 000), which are often not extensively validated prior to commercialization. Given increased awareness of the problem, both the United States, National Institutes of Health and some journals are requiring investigators to provide evidence of specificity of their antibody-based assays.

1. Introduction

Immunohistochemical (IHC) staining is a widely employed method for single cell level localization of protein/antigen expression in tissue samples. IHC staining assays, which have produced unprecedented insights into gene function and disease states, are employed extensively as biomarkers of diagnosis, prognosis, prediction of drug response, and pharmacodynamic sensors. The techniques for IHC are widely employed, evolving, and increasing in use. For example, methods for multiplexing from 2 to 7 antibodies using multispectral approaches [1,2], or up to 12 antibodies using iterative staining, stripping and scanning on a single glass slide [3], are gaining in popularity. Furthermore, newer technologies in which dozens of antibodies are mass-tagged and evaluated simultaneously are generating even more capabilities in this field [4–7].

The increased use of IHC across thousands of laboratories is being fueled by a large increase in commercial antibody production and marketing that accelerated after the completion of the human genome project. Goodman [8] recently discussed the rapidly increasing numbers of available commercial antibodies, which have expanded over the last 15 years from approximately 10 000 to over 3.8 million, which is on par or faster than Moore's law for transistor number doubling in integrated circuits every 18 months. The usefulness of IHC in clinical practice and research is undeniable, yet it is now well recognized that many research IHC assays are poorly implemented. While there are a number of reasons for this, one of the most glaring problems is lack of rigorous research antibody validation by the commercial vendors that develop and market them [8]. Of course, poor antibody validation contributes significantly to the larger overall problem of lack of reproducibility in science in general [9–12]. The lack of proper antibody validation is clearly widespread. The magnitude of the problem is unknown, but our anecdotal experience suggests that more than 50% of all IHC staining shown in manuscripts we have reviewed (as journal reviewers or editors), or papers from the extant literature that we read, contain either overtly incorrect IHC staining or staining results that cannot be reliably determined to be correct, given the lack of demonstrated analytical validation of the assays employed. One might infer that this problem is poorly documented in the scientific literature. However, this is distinctly not the case as there are a number of published articles that directly deal with problems of antibody specificity for many types of assays, including those that focus on problems with IHC [8,11–24]. In addition, a number of organizations, including the United State National Institutes of Health (NIH) have been focusing on the problem of antibody validation and the NIH now requires a section in grant applications that describes efforts to authenticate antibodies [12]. Furthermore, it has been argued that vendors should be held to higher standards when selling antibodies [12] that are marketed to be employed in specific "fit-for-purpose" assays. If vendors were held to such standards, it is likely that the reliability of many IHC assays reported in the literature would greatly improve. Along these lines, the Global Biological Standards Institute has deployed a working group and is testing a novel antibody scoring system that they hope will help to establish guidelines and standards for a number of applications including IHC (see https://www.gbsi.org/news/novel-antibody-scoring-system-enters-alpha-tests/). Interestingly, when antibodies are marketed as companion diagnostics, usually as *In Vitro* Diagnostics (IVD) kits, the antibody assay itself is regulated by the Food and Drug Administration (FDA) and must be shown to be highly analytically validated in relevant clinical samples.

When flawed IHC studies are published, this leads to biased results that can confound or "pollute" the literature [8,13,24]. This results in potential wasted time and research dollars for those performing these studies, as well as for future researchers, and can even have implications for pharmaceutical research and patient care (see below section on cytomegalovirus [CMV] and gliomas). While there has been a clear increase in vigilance by some commercial vendors regarding pre-market antibody validation [12], the continued rapid increase in growth of commercial antibody companies and offerings is stunning. In some cases, the release of such a large number of antibodies for sale has been so immense that it is difficult to fathom how all of the reagents could possibly have been appropriately validated prior to marketing. For example, the highly commendable Human Protein Atlas Project [25–28], whose website of gene expression can be highly useful, introduced approximately 20 000 antibodies at one time through a commercial vendor. While this was a monumental endeavor that included some reasonably strong antibody validation at the time which is still ongoing, in our opinion it still appears that many of the IHC results posted on the website show assays that cannot be interpreted with high confidence. In this manuscript we agree with the College of American Pathologists that the "aim of analytic validation is to determine a test's ability to accurately and reliably detect the antigen or marker of interest in specimens consistent with those to be tested in clinical practice" [29]. In other words, the antibody performs well in tissue in a "fit-for-purpose" assay. We define IHC assay validation here as follows: "objective evidence that the staining observed in

an optimized IHC assay in the tissue in question is the result of specific binding by the antibody to the purported target".

Although controls for antibodies can be nearly infinite (see our current approach to working up new research antibodies below), and there is no perfect assay (*e.g.* one size does not fit all in antibody validation), in our opinion the most important controls are "true negative" controls. By this we mean cell lines known to be negative by real time quantitative PCR (qPCR) for the mRNA encoding the protein of interest, cells that have undergone bi-allelic gene knockout (using CRISPR-cas9 or other means), or tissues from biallelic gene knockout mice, that are fixed in formalin and processed into paraffin blocks. Using and developing these types of controls in our laboratories, there have been a number of occasions by which technicians and/or trainees at the graduate and postgraduate level have spent upwards of 1—2 years to ensure the validity of IHC assays to individual protein targets (*e.g.* Refs. [30,31]). Others have also indicated how difficult and time consuming developing a working IHC assay can be [23,32].

In terms of other routine controls, an important one is to leave out the primary antibody. The reason for this is that most modern commercial IHC kits used in clinical and research labs employ a primary antibody of a given species followed by a labeled secondary antibody (*e.g.* with an enzyme such as horseradish peroxidase) raised against the immunoglobulins of the correct type from the first species. This omission of the primary antibody in your tissue of interest ensures that the staining seen is not due to the binding of the secondary antibody non-specifically, or to endogenous peroxidase activity for example. Another important control that is often used for antibodies raised against synthetic peptides is a peptide competition experiment whereby an affinity purified, or other antibody, being used is first preincubated (preabsorbed) with an excess of specific peptide comprised of the amino acid sequence used for the original antibody production. This can represent an important control such that if the staining reaction is not prevented or markedly decreased using the specific peptide, this calls into serious question whether the reaction you are visualizing is specific. However, we would also point out, as indicated by Holmseth et al. [19], "antigen preadsorption blocks all binding of the affinity purified antibodies, regardless of whether this binding is to the proteins under study or to cross-reacting epitopes". In other words, while this reaction should block the staining observed, what it really tells the investigator is that whatever binding is taking place in a tissue sample by IHC is most likely occurring via the antigen combining domain of the antibody, as opposed to the Fc or other region for example, but it does not preclude that the staining is occurring because of binding to another epitope and not the purported target.

All of the problems of poorly implemented IHC assays cannot, however, be blamed on commercial vendors' lack of extensive validation of antibody specificity. For example, it is quite possible to incorrectly implement an IHC assay using a highly specific antibody, which can lead to false positive results ([33]; see below section describing CMV and v-akt murine thymoma viral oncogene homolog 1 (AKT1) IHC. The extent of this problem is unknown.

2. Why is antibody usage in IHC assays such a problem?

One of the potential problems in the field with the lack of high quality IHC assays is that, although there is no official designation by a governing body, there are two major classes of antibodies used in IHC. In our experience, however, this dual nature is either unknown or underappreciated by the majority of investigators. One class of IHC antibodies are "clinical-grade" diagnostic antibodies (CGDA), which are employed in routine practice by surgical pathologists. These antibodies, which likely number less than 500, are generally highly validated prior to implementation in clinical labs [29]. This validation is certainly necessary since pathologists using IHC-stained slides from clinical laboratories render diagnoses that often are critical in determining patient care decisions. The other class of antibodies are referred to as "research antibodies" or by Goodman [8] as "commercial research-tool" antibodies. A third class may be considered therapeutic antibodies used as drugs manufactured by pharmaceutical companies that are generally very highly validated as highly specific, high-affinity binders to their targets [8]. We classify research antibodies as those available from commercial vendors (or specific researchers) that are not used in diagnostic surgical pathology settings for clinical use. It has recently been documented that there are approximate 3.8 million research antibodies available currently through commercial vendors [8].

We postulate that there is a lack of knowledge regarding this key difference in antibody classes by many laboratory-based scientists and pathologists who do not run their own research or IHC laboratories. Many researchers are accustomed to performing Western blots and can follow their protein of interest by viewing the blot in the region of the correctly predicted molecular weight. In the case in which additional bands of the incorrect molecular weight are present, in many instances these can be ignored as off-target binding. However, if there are multiple bands on a Western blot from a given tissue sample or cell line, one usually cannot know if the IHC staining in that cell line or tissue corresponds to the correct target being visualized in the tissue; this precise dilema was clearly shown by Bordeaux et al. [18] using commercial antibodies and various cell lines.

Many of the reviews and manuscripts cited above regarding antibody and IHC specificity problems provide details regarding methods to mitigate and prevent incorrect IHC assay deployment, and these should serve as a resource for all researchers interested in implementing a new IHC assay. Rather than cover these in detail in this review, we will present a number of published and unpublished examples where we have validated IHC assays. These assays have allowed us to make novel biological insights into normal and diseased tissues, to provide evidence disputing prior "high profile" findings in the literature, or both. As we introduce the examples, we will point out tips and approaches we used to mitigate these pitfalls. At the end, we provide our current overall approach to working up new IHC antibodies, which actually must to be tailored to each specific case, since one size does not always fit all in

developing "fit for purpose" assays, and, often extensive experiments must be performed prior to acceptance of assay validity. This is also the case even for antibodies that have previously been validated by others; each antibody-based assay needs to be revalidated in the laboratory performing the work [8].

3. The importance of orthogonal studies to increase confidence in IHC results

Given the fact that there are no perfect scientific experiments that can "prove" that a certain result is true, one of our most important tools for antibody validation is to use orthogonal (*e.g.* multiple different) means to help support a given IHC-based finding. For example, if a certain pattern of expression (*e.g.* overexpressed in cancer *vs.* benign) is observed by IHC using one antibody against one epitope, the extent of certainty regarding this staining is greatly enhanced if another antibody against another epitope produces a highly similar staining pattern. In fact, this type of validation is inherently present in assays used for proximity ligation reactions (see below). However, validation with a second complementary antibody is not always possible because in many cases there are no two different antibodies available to known proteins or targets. In this instance, one can perform Western blotting from the same tissue that is being used for the IHC, assuming the IHC-based antibody also works in Western blots. In the majority of cases we attempt to utilize this approach and recommend antibodies that work both by Western blots and IHC, whenever possible. Also, one can attempt to corroborate the findings by providing, for example, correlation with mRNA levels using *in situ* hybridization and/or real time qPCR. Of course, at times mRNA and protein do not correlate since there are many methods of regulating protein levels that can be uncoupled from mRNA levels. However, while it is not a perfect validation, in the great majority of cases one should not expect protein to be present if the corresponding mRNA is not detectable. Further, when mRNA and protein expression do correlate, for example in specific cells types within a heterogeneous tissue, this can provide increased confidence for the validity of both measures.

4. Examples of IHC assays and insights they have facilitated

4.1. V-myc avian myelocytomatosis viral oncogene homolog (MYC) in prostate cancer

A region on chromosome 8q24 encompassing the *MYC* locus is amplified in a subset (~30%) of primary prostate cancers. This somatic alteration occurs mostly in high grade cases and is more frequently found in metastatic castrate resistant disease than in primary tumors, suggesting that *MYC* alterations at 8q24 are associated with disease progression [34]. By contrast, after the publication of initial RNA expression microarray studies, it became clear that *MYC* mRNA was elevated in the great majority of primary prostate cancers, even those of relatively low stage and grade (*e.g.* Gleason score 6), suggesting that MYC overexpression is much more common than 8q24 amplification and that it may play a role in disease initiation in addition to progression [35]. However, since mRNA expression does not necessarily mean that the protein is present, it was important to determine whether MYC protein was elevated in human prostate cancer, and if so, at what stage of the disease this elevation occurs [35]. Prior studies using IHC in primary prostate cancer and the major precursor, high-grade prostatic intraepithelial neoplasia (PIN), were performed but were most likely confounded by lack of rigorously validated IHC-suitable antibodies. For example, a few studies localized MYC either exclusively or predominantly to the cytoplasm in human formalin-fixed and paraffin-embedded (FFPE) tissues, which did not coincide with the known literature. Also, in the single study that had reported nuclear localization of MYC, there was essentially no increased expression in neoplastic cells as compared to benign epithelium [35]. Attempts in our laboratory using IHC were performed using the mouse monoclonal antibody, clone 9E10, which recognizes the epitope from MYC that is often used to "tag" recombinant proteins (so called MYC-tag). As a positive control, we used prostate tissues from transgenic mice that overexpress human MYC ("Lo-MYC" mice) in the mouse prostate [36,37]. However, we were unable to obtain specific nuclear staining using the 9E10 antibody. We then turned to a newly introduced (at the time) rabbit monoclonal antibody raised against N-terminus of the human MYC protein (from Epitomics, now Abcam, clone Y69), which was reported by the manufacturer to perform well in IHC and Western blotting. Using a positive control for Western blotting from a cell line with a tetracycline-regulated promoter ("Tet-off" system) driving MYC, we found a doxycycline-repressible single band migrating near the predicted molecular weight. In addition, lysates from prostate carcinoma cell lines (LNCaP and CWR22Rv1) each showed a single band at the same apparent molecular weight. To test the specificity of this antibody for IHC, we stained prostate tissue sections from the Lo-MYC mouse. Although there was very little or no staining in the prostates from the wild-type (FVB) mice, there was intense staining in the nuclei of the epithelial cells of the MYC-expressing transgenic animals in their ventral prostates. As another control we found doxycycline repressible positive nuclear staining of liver tissue from an additional transgenic mouse line that was programed to express MYC in a liver-specific repressible fashion. Taken together, the results established the specificity of this antibody for MYC for IHC on archival tissue specimens.

4.1.1. Biological insights and ongoing studies

Using this newly validated assay we were able to establish that nuclear overexpression of MYC protein occurred frequently in luminal cells of high grade PIN as well as in most primary carcinomas and metastatic disease, as compared with normal appearing prostatic luminal epithelial cells. MYC protein did not correlate with somatic amplification or gain of chromosome 8q24, suggesting alternative mechanisms for MYC overexpression. These results indicated that upregulation of nuclear MYC protein expression is a highly prevalent and early change

in prostate cancer (approximately 80% of cases showed clear overexpression in high grade PIN and cancer). Taken together with already known data that overexpression of MYC in mouse prostate leads to high-grade PIN and early carcinoma lesions that highly resemble the corresponding human lesions [36], the results supported the hypothesis that increased nuclear MYC may be a critical oncogenic event driving human prostate cancer initiation and progression. In subsequent work [37,38], we found that overexpression of MYC controls a nucleolar program of gene expression that correlates with increased nucleolar size (and at times increased numbers) and nucleolar activity in human and mouse PIN and adenocarcinoma of the prostate, providing a compelling molecular explanation for the long-standing and well-known diagnostic feature of enlarged and increased numbers of nucleoli in neoplastic prostate epithelial cells. In both human and transgenic mice overexpressing MYC, the overexpression occurred only in the luminal compartment, which fit nicely with prior work that De Marzo et al. showing decreased p27Kip1 found in the luminal compartment of high grade PIN; this finding is in part what prompted us to postulate that stem cell features, normally found in basal cells, were "shifted" to the luminal compartment in PIN [39–41].

4.1.2. IHC assays can evolve, providing new information over time

Those who knew or worked with Don Coffey often heard him say "don't tell me what you did, but tell me how you did it". In the study by Gurel et al. [35] showing MYC overexpression in human PIN and carcinoma, the IHC assay used at the time was state-of-the-art. Also, we used FFPE specimens only. More recently, we have improved our assay for MYC IHC protein staining by using what is now (10 years later) state-of-the-art IHC, using Ventana/Roche Optiview technology, on an automated IHC system. Using this system we have recently achieved even greater signal and less noise for MYC IHC, and, coupled with using frozen sections, we found that more consistent results were obtained for MYC overexpression in both normal and neoplastic prostate tissues [42]. For example, our original estimates for normal basal cell staining in the prostate were 0.75% of cells from Gurel et al. [35], and our updated estimates show a marked increase in the fraction of normal basal cells staining, with a median of 20%, and approximately 75% of samples ranging from 10% to 40%. This fits nicely with a recent paper describing a prominent MYC and ribosome expression signature in prostate basal cells [43]. Also, using this updated improved assay, the fraction of cases of high-grade PIN and carcinoma with clear overexpression is increased from approximately 80% to 90%–95%. Overall this shows that even when using the same antibody, further improvements in signal-to-noise ratio can occur over time because of improvements in IHC technology, which can further enhance our ability to confidently estimate expression. The results with the new IHC assay were highly correlated with overexpression of MYC mRNA using an ACD RNA scope *in situ* hybridization assay [42], providing further support for the accuracy of the new assay.

4.2. Phosphatase and tension homolog (PTEN) in prostate cancer

Over a decade ago we sought to perform IHC for PTEN in prostate cancer and we started with a polyclonal antibody from Zymed (now part of Invitrogen). We used PC3 prostate cancer cells that have a known homozygous deletion in *PTEN* as a negative control, and used PC3 cells that were transfected with a plasmid encoding human *PTEN* cDNA as a positive control. The staining appeared as expected using FFPE cell plugs from these cell lines. Next, we performed studies on tissue microarrays containing prostate cancer and other normal tissues, and during these studies we found that in normal tissues there was only strong staining in the prostate, endometrium, and brain. This was surprising since what was known at the time suggested that PTEN was expressed rather ubiquitously throughout the body. This produced a lack of confidence in the findings in our tissue studies and we stopped using this antibody and did not publish using it.

Next, we tried an existing mouse monoclonal antibody that others had used in breast and endometrial cancer (6H2.1) in which they performed similar genetically controlled cell line experiments and showed good specificity of staining [44,45]. While this antibody reacted appropriately with our positive and negative control cell lines, the staining in tissues was generally rather weak and the appearance resembled that of nonspecific staining, and our confidence in the staining was again diminished. Although there is no specific pattern of staining that can be definitively scored as background/non-specific staining, many times when working up antibodies the pattern of staining one obtains in cells known to be negative for the protein is that of relatively weak and diffuse signals across all cells and all cellular compartments. In this case, however, this concern that much of the staining was nonspecific turned out to be incorrect. Later, a rabbit monoclonal antibody (clone D4.3) became available from Cell Signaling Technologies Inc. (while we are not endorsing or denigrating any specific companies in this article, in our experience, some companies, such as Cell Signaling Inc., and increasingly Abcam, have generally been very diligent at performing analytical validation of their antibodies in terms of fit-for-purpose assays, often with evidence posted online that could serve as models for other companies). This antibody gave the same pattern of expected staining in positive and negative controls and also a similar pattern to the 6H2.1 antibody in tissues, although the signal-to-noise was improved compared to the 6H2.1 antibody. Given the somewhat concerning pattern of staining that resembled nonspecific background staining, we performed a number of additional controls consisting of three other cell lines in which other investigators had targeted both endogenous *PTEN* alleles for disruption (using older and much more difficult technology than present day CRISPR-cas9), as well as a mouse embryo fibroblast cell line from *Pten*$^{-/-}$ knockout mice [30]. The D4.3 antibody showed the expected staining in these cell lines, which were also transfected with a human *PTEN* cDNA clone as a positive control. We then examined additional cell lines with known genomic alterations in *PTEN*, consisting of the NCI-60 (59 cell lines),

as well as other cell lines we often used in the laboratory. For each of these cell lines we fixed them in formalin and submitted them for FFPE and performed IHC and found that our IHC assay was 100% sensitive, and 95% specific for detecting genomic alterations in *PTEN*, whether it be deletion or point mutation, *etc*.

Given this extensive level of validation we moved forward with IHC studies with human prostate cancer and found a significant fraction of cases with complete or near complete loss of PTEN signal in tumor cells. With the D4.3 antibody nearly all cells throughout all areas of the body that we have tested stain positively for PTEN, such that in nearly all cases there are "built in" positive control non-neoplastic cells. In fact, such built-in internal controls are crucial to have when one is evaluating the loss of expression of a given protein; for example if non-tumor cells in the region of cancer do not stain robustly in a given tissue sample for PTEN, we do not evaluate that case. And, if it is a clinical case, we attempt to resolve the question of PTEN status by sending for fluorescence *in situ* hybridization (FISH). In addition to showing that PTEN loss by protein staining generally correlated with genomic alterations in PTEN [46], one of the other important early findings we had using IHC for PTEN was that more often than not, when a tumor had PTEN loss, only a subfraction of tumor cells in a given primary tumor focus showed the loss. In addition, PTEN loss correlated strongly with increased grade and stage of prostate cancer [30]. We were later able to show that PTEN loss was generally subsequent to ETS-related gene (ERG) rearrangement in prostate cancer, providing novel insights into the order of events of molecular alterations in primary prostate cancer [47]. Others have found similar results using FISH and other approaches such as next generation sequencing [48,49]. We also used this assay in a study tracking the starting point within the prostate of a lethal clone found in widespread metastatic disease at autopsy [50], and, to help determine whether a presumptive precursor lesion, high grade PIN, could at times actually represent pre-existing invasive carcinoma masquerading as high grade PIN [51].

This initial assay for PTEN IHC that we employed was improved upon over time using the same antibody, and additional studies were performed to show that loss of PTEN by IHC correlated tightly with genomic alterations in prostate cancer as well as with outcome including death from prostate cancer (reviewed by Ref. [46]). We are currently employing PTEN IHC as an inexpensive and highly accurate clinical test for determining *PTEN* status in human prostate cancer biopsies at our institution that are graded as Gleason score 6, since *PTEN* status can help urologists with decision-making regarding additional biopsies and/or enrollment in active surveillance programs or not. While PTEN loss is not common in Gleason score 6 tumors, its loss in such tumors is associated with upgrading at prostatectomy [52], and studies are ongoing in higher grade lesions [53]. In addition, Guedes et al. [54] recently reported in a study related to pre-analytical variables that this assay is robust to wide variations in tissue fixation and processing.

Interestingly, while we did most of our studies using the Cell Signaling D4.3 rabbit monoclonal antibody, we did find that the staining we had observed using the 6H2.1 antibody was also specific. We learned from this not to trust this "gut feeling" of whether staining appears specific since in this case this subjective impression was incorrect. The Don "Coffey-ism" that best describes this is that "we do not see with our eyes, we see with our minds".

4.3. Xenotropic murine leukemia virus-related virus (XMRV) in prostate cancer

A novel gammaretrovirus with high similarity to murine leukemia viruses, designated XMRV, was discovered in 2006 in prostate cancer tissues [55]. Localization of the viral DNA sequences was found in approximately 1% of stromal cells in prostate cancer tissues using FISH [55]. In addition, localization of a viral protein by IHC using a monoclonal antibody against a Moloney leukemia virus (MLV) protein gave a similar pattern. A number of other groups subsequently found evidence for infection in some cases of prostate cancer [56], mostly by PCR. In 2009, a paper was published in the *PNAS* using IHC staining and PCR in which IHC showed the presence of XMRV in prostate cancer epithelial cells in 23% of cases and in ~4% of controls, which consisted of benign prostatic hyperplasia (BPH) tissues [57]. The staining pattern was cytoplasmic, which was similar to XMRV infected cells in culture, and was mostly in the epithelial cells and was focal within the tumor. Unrelated to prostate cancer, in an article in *Science*, XMRV was detected in the blood of patients with chronic fatigue syndrome in approximately 70% of cases, whereas less than 5% of controls showed this virus [58].

Our studies in prostate cancer by both PCR ($n = 200$ and $n = 161$) and IHC staining ($n = 596$) were entirely negative for XMRV [59,60]. After a great deal of additional effort, money spent, and time involving studies in chronic fatigue syndrome and prostate cancer, a subsequent paper in *Science* found that the virus was almost certainly generated by recombination of two endogenous murine proviruses when a prostate cancer cell xenograft was passaged through nude mice [61]. A prostate cell line derived from this xenograft, CWR22-RV1, was widely used in the prostate cancer research community and was shown with high confidence to be the likely source of contamination that resulted in the incorrect association of this virus with human disease. PCR reactions that were positive from nucleic acids isolated from patient samples were either from contamination of DNA from this cell line, or, from mouse DNA that was contaminating PCR reagents. Thus, since this virus most certainly does not circulate in the wild, all positive studies in prostate-derived and other types of specimens that showed positive signals by IHC or other means, were incorrect. The original paper on chronic fatigue syndrome was ultimately retracted, at first in part by some of the authors and then fully by the editors of *Science* [62,63]. The manuscript published in *PNAS* in 2009 was retracted in 2014, in which the authors indicated.

> "the detection of XMRV DNA in various human tissues by PCR has been attributed to contamination of commercially available reagents with mouse DNA. This explanation is the most likely for the PCR findings were reported. The IHC staining with anti-XMRV antiserum that we reported in our *PANS* publication was most likely due to cross-reactivity of our antiserum with a protein

present almost exclusively in malignant prostatic epithelial cells. We are in the process of identifying this cross-reacted protein" [64].

While the paper by Schlaberg et al. [57] did perform a number of control experiments for their IHC assay, there was one key aspect of the paper that in retrospect should have raised significant red flags. There were a number of cases in which samples were positive by IHC but negative by PCR. While it is possible that the focal nature of the infection could have explained these results, the disconnect between the two assays in a relatively large number of cases was quite striking. In many of our studies using IHC, we employ complementary methods, such as the detection of mRNA in the tissues, or the use of another antibody against another epitope, and/or the use of Western blots showing bands of the correct size from that tissue. If two methods do not show similar or compatible findings, we generally will not publish those results, at least not until we determine a scientifically valid explanation.

In our study we employed two different antibodies raised against two different MLV proteins and found that they cross reacted well by Western blotting and IHC with corresponding proteins from XMRV [59]. We analytically validated IHC staining for both antibodies using Western blotting and IHC from uninfected (negative controls) and infected cell lines and found the expected staining patterns. We confirmed the negative nature of our negative control cell lines by a number of assays, including PCR, and in developing and optimizing the signal-to-noise for the staining, we always verified that the negative cell lines were completely negative by IHC staining. We cannot overestimate the need for true negative controls when validating IHC assays.

Using this IHC assay we were able to find that some cell lines that were not previously known to be infected with murine retroviruses, were in fact infected, and, at times very high levels of viral proteins were present in the cell culture media in which the cells were grown [65]. The IHC assays were quite robust and can be used to screen cell lines for known murine retroviral infection, which is relatively common in human cell lines that have been passaged through immunocompromised mouse hosts [65].

4.4. IL-6 in prostate cancer

IL-6 has been shown to be a critical immunological mediator important in a number of different diseases including cancer. A number of studies have previously implicated IL-6 as an important factor for the progression of prostate cancer [31]. Further, using IHC, a number of prior studies had shown that prostate cancer epithelial cells often overexpressed the protein and that several different prostate cancer cell lines showed expression as well. In our own studies we had difficulty obtaining positive IHC staining for IL-6 in cell lines expressing endogenous IL-6 mRNA. For negative controls, we chose cell lines that were negative by qPCR, and, as positive controls we used some of the same negative cell lines that were transfected with a human IL-6 encoding cDNA clone. Interestingly, while we did obtain specific staining by IHC in the transfected cell lines, other cell lines that were positive by real-time qPCR for endogenous levels of IL-6 mRNA were negative using the same IHC assay. Using this IHC assay on tissue sections showed a lack of any convincing positive IHC staining in a series of prostate cancer tissues. Therefore, we next employed a highly sensitive and specific *in situ* hybridization assay for IL-6 mRNA. For this assay, we used the same positive and negative cell lines expressing endogenous IL-6 mRNA, as well as the transfected controls. The *in situ* hybridization on FFPE versions of the cell lines showed excellent concordance with the mRNA levels by qPCR. When we applied this *in situ* hybridization assay to tissue samples, we found hybridization signals in prostate cancer tissues. Interestingly, while we did find variable amounts of staining in stromal cells, inflammatory cells within the stromal compartment, and endothelial cells, in no cases did we find hybridization signals in cancer or normal prostate epithelial cells, other than occasional positive staining in atrophic benign cells [31]. Furthermore, in castrate resistant metastatic cancers, we found striking expression exclusively in endothelial cells from bone metastases, but no expression in endothelial cells from soft tissue metastases.

Overall this work allowed us to present a refined view for IL-6 in prostate cancer. While it is still possible that IL-6 is involved in prostate cancer progression, our results indicate that it does not act in an autocrine fashion, since prostate cancer cells virtually never express IL-6 mRNA. In attempts to determine why the IHC did not work in the cell lines with endogenous levels of IL-6 mRNA, we found that if we employed a pharmacological block of Golgi protein transport (using Golgi-stop containing monensin), a well-known method often used by immunologists employing flow cytometry to detect expression of cytokines in leukocytes, we could detect signals by IHC and by Western blotting in whole cell extracts in the cell lines that endogenously express IL-6 mRNA [31]. This implies that some cytokines that are produced in cells may be processed and secreted so rapidly that it is nearly impossible to detect protein inside the cells, unless protein trafficking is exogenously blocked; a method currently is not feasible for with IHC in tissues samples from humans.

4.5. Glutathione S-transferase-pi gene (*GSTP1*) in prostate

GSTP1 encodes the pi class of glutathione S transferases and has been shown in many studies to undergo frequent inactivation in prostate cancer by hypermethylation of sequences within its upstream regulatory CpG island [66—68]. Also, hypermethylation of the *GSTP1* CpG island occurs in approximately 70% of high grade PIN [69,70]. The functional significance of *GSTP1* silencing in prostate cancer is still under study, but one example is that it may be involved in protection against cytotoxicity and DNA adduct formation occurring in the setting of exposure to charred meat carcinogens [71], and others suggest a role as a tumor suppressor after carcinogen exposure in skin and lung cancer models [72,73]. We have preliminary evidence for a role of *GSTP1* as a bone fide tumor suppressor in prostate cancer [74], and, using a newly updated IHC assay have provided strong evidence that normal prostatic luminal epithelial

cells in human express GSTP1, albeit at much lower levels than normal basal cells or atrophic cells [42]. Since the target cell for prostate cancer initiation appears to be a luminal epithelial cell, these new results provide additional insights into the earliest steps in human prostate cancer formation. The development of the improved IHC assay for GSTP1 was facilitated by the use of *Gstp1/2* knockout mice and human cells lines with *GSTP1* methylation as negative controls, and by the release of a new monoclonal antibody combined with using state-of-the-art IHC reagents [42].

4.6. Cyclooxygenase 2 (COX2) in prostate

Cyclooxygenase (COX) enzymes are the rate limiting step in prostaglandin synthesis, and are known to be important inflammatory mediators [75]. Two major COX enzymes are known, a constitutive isoform named COX1, and an inducible isoform, named COX2 [76]. These enzymes are the major known targets of the non-steroidal anti-inflammatory drugs (NSAIDs), including non-selective inhibitors aspirin and sulindac, and COX2-selective inhibitors celecoxib, and rofecoxib, all of which have been tested in multiple cancer prevention and therapy settings. Numerous early studies used IHC methods for characterization of COX2 in many human tissues and associated cancers, including prostate. In the early reports in prostate cancer, the general findings from these studies suggested that the expression of COX2 was low to weak in normal prostate tissues and significantly elevated in prostate cancer [77—81], generating enthusiasm around targeting COX2 for reversal of prostate cancer. However, the early studies were largely predicated on IHC assays with poor assay validation. Application of a robust and validated IHC protocol for evaluation of prostate tissues revealed that COX2 indeed had low to absent expression in normal prostate glands, but also had absent expression in the majority of PIN and adenocarcinoma lesions [82,83]. Interestingly, like GSTP1, COX2 was significantly induced and elevated in proliferative inflammatory atrophy, both in luminal epithelial cells as well as in infiltrating macrophages [82,83]. These findings were supported by robust assay validation, including use of positive and negative control cell line systems, Western blots validations, and corroboration by qPCR. These validations also showed that multiple antibodies used in the prior studies likely had non-specific staining. In a subsequent study, the promoter of the *PTGS2* gene, which encodes COX2, was found to be hypermethylated in the majority of prostate cancers (~80%—90%), providing additional evidence that the gene was likely to be epigenetically repressed in prostate cancers [84].

4.7. Retinoblastoma-associated protein 1 (RB1) in prostate

The retinoblastoma protein (referred to as Rb), encoded by *RB1*, was the first tumor suppressor to be cloned [85]. It encodes a protein that regulates the cell cycle and is inactivated in human retinoblastoma and in several other human tumor types. We have tried over the years to stain for Rb by IHC antibodies and were never satisfied with the staining results because we found often that many cells were negative for RB, when the expected pattern is that most cells throughout the body should have detectable levels in their nuclei. Relatively recently we obtained a monoclonal antibody where we were able to show high-level IHC signals restricted to nuclei in a vast majority of cells in multiple tissues. Using this antibody on a large series of cell lines with known *RB1* gene status, we were able to show that it could specifically and sensitively detect Rb protein levels in the nuclei [86] and this detectability correlated well with underlying *RB1* gene status. Using this antibody we performed IHC staining on a series of primary and metastatic castrate resistant prostate carcinomas and found that the vast majority of primary conventional acinar adenocarcinomas retained at least some Rb protein expression. Furthermore, only ~15% of castrate resistant metastatic human prostate carcinomas showed complete Rb protein loss. This was in distinct contrast to small cell neuroendocrine carcinomas of the prostate, either primary or metastatic, in which 80%—90% showed complete Rb protein loss [86]. For these studies we also only evaluated a case for Rb if there were internal non-tumoral cells staining positive (*e.g.* internal positive control cells) in the vicinity of the negatively staining tumor cells. These data provide evidence that Rb loss is a critical event in the development of small cell carcinomas of the prostate and IHC for Rb protein may be a useful diagnostic tool in the setting of neuroendocrine differentiation in castration resistant or primary untreated prostate cancer.

4.8. Tumor protein 53 (TP53) in prostate cancer

A large number of studies starting over 2 decades ago showed that missense mutations in the *TP53* gene lead to the abnormal accumulation of p53 protein due to enhanced protein stability that is detectable by IHC. This was seen in many cancer types including ovarian, lung, bladder, squamous head and neck cancers and prostate. However, over time it became clear that positive staining for p53 protein did not always strictly correlate with *TP53* mutations [87]. Moreover, studies in prostate cancer showed that overexpression of p53 in tumor cells was predictive of poor outcome [88], but there were very few studies analyzing in detail with automated clinical grade IHC assays whether positive IHC staining for p53 in prostate carcinoma correlated with *TP53* mutation. Recently, using a series of cell lines with known *TP53* status we worked up a previously existing IHC antibody that was being used in a clinical laboratory and found that it was highly specific for *TP53* missense mutations in prostate cancer [89]. Using this assay on human prostate samples we reported that p53 overexpression was approximately 85% sensitive for detecting a *TP53* missense mutation. In addition, using this assay Maughan et al. [90], found that p53 overexpression is correlated with poor outcome and poor response to hormonal therapies. These findings show that even older antibodies can be re-examined using more modern technologies to validate their assay properties. Also, as is the case for PTEN, the p53 IHC assay was robust to pre-analytic fixation conditions and could be useful in

identifying heterogeneous, subclonal *TP53* alterations in the setting of prostate biopsies that may be difficult to detect without next generation sequencing approaches [89]. The ability to interrogate p53 status by a simple IHC assay in primary and metastatic prostate tumors could be highly useful for studies of disease pathogenesis and for developing predictive biomarkers.

4.9. Programmed cell death 1 ligand 1 (PD-L1) in prostate cancer

PD-L1 is an important T-cell inhibitory molecule that is inducible in a number of different types of cell types. It has been shown in many studies to be predictive of response to anti-PD-L1 or anti-PD-1 immunotherapies in different tumors including melanoma, non-small cell lung carcinoma, and bladder cancers. In prostate cancer it was relatively contentious in terms of what fraction of cases stained positive. The rate of positivity ranged from 0% to 95%. Notably, a small study from our group showed that PD-L1 immunoreactivity was quite infrequent in primary tumors and only patchy positivity was observed in a subset of tumor cells [91]. This finding was in strong contrast to another study showing that the majority of aggressive prostate tumors overexpressed the PD-L1 in the vast majority of tumor cells [92]. Recently, we evaluated a number of monoclonal antibodies using positive and negative cell lines, as well as, Western blotting and various human tissues known to express or not express PD-L1 and reported this assay to be highly specific for determining PD-L1 expression in prostate cancer and benign tissues. Using this approach, we found a very small minority of primary tumors were positive for any PD-L1 staining, and when there was positive staining, it was usually a small fraction of cells tumor cells (1%–5%), with only a very small subset expressing it in 20% or more of tumor cells [93]. By contrast, there was at least some expression in tumor cells in castrate resistant metastatic prostate cancers in 33% of cases [93]. While studies are ongoing to determine the efficacy of PD-1/PD-L1 targeted checkpoint inhibitors in prostate cancer, and what subsets of patients may benefit, this assay can provide important information regarding the correlation with response. Interestingly, the one manuscript that reported PD-L1 was highly overexpressed in the large majority prostate cancer cells [92] used an antibody that was subsequently removed from the manufacturer's website.

4.10. CMV in glioma

CMV is known to infect more than half of the human population. It is a major cause of disease in newborns, and in the immunosuppressed. In 2002, human CMV (HCMV) was described as commonly infecting human gliomas [94]. Multiple gene products from HCMV were detected and it was postulated that HCMV may play an active role in the pathogenesis of gliomas. In a number of additional studies, the prevalence of CMV was reported to be very high, with some studies showing low magnification images of IHC in which virtually all cells (tumor and non-tumor cells) in the area were positive for viral proteins [95]. However, not all studies have been positive for HCMV in gliomas and the presence of this virus became quite controversial [96,97]. Given that there were a number of both positive and negative studies in the literature, we sought to definitively address this question using three orthogonal approaches, including real-time qPCR for CMV DNA against two different genes, *in situ* hybridization for CMV DNA against two different gene targets, and IHC against two different protein targets [33]. For IHC, human foreskin fibroblasts were used as negative controls, which are routinely employed by one of the co-authors on the study by Holdhoff et al.[33], and continually tested as negative for human CMV by multiple different assays. As positive controls, these cells were infected with various multiplicity of infections (MOIs) of an HCMV isolate. Using this titration approach, along with other positive and negative controls, we found very high sensitivity and specificity for all six assays. Using these assays on a series of fresh frozen and FFPE brain tumors, including high-grade gliomas and pediatric gliomas, we found no evidence of CMV in any of the cases tested.

Given that the study by Holdhoff et al. [33] is not the only one with completely negative findings, there appear to be essentially two mutually exclusive sets of results in published studies regarding the presence of CMV in gliomas. The first are those that find that CMV is present, which is usually in a very high percentage of cases (at times nearly 100%), and another set of studies that virtually never observes it. That is in part why Holdhoff et al. [33] employed six different validated assays to assess this question. It should also be noted that the negative findings have also been corroborated to a certain extent by studies doing whole genome DNA and RNA sequencing from gliomas [96,97]. Given the serious nature of this discrepancy, with implications for patient care since some studies have treated glioma patients with valganciclovir and clinical trials are underway using anti-CMV vaccines, it is likely that additional studies will need to be performed before the matter is settled. For example, despite a number of negative studies in both prostate cancer and chronic fatigue syndrome in the case of XMRV, a number of studies were undertaken in which investigators not previously involved were the stewards of samples for blinded studies with known positive and negative controls sent to various laboratories [98,99]. Along with the paper showing XMRV was most likely the result of viral recombination in human cells passaged in mice, these studies helped to firmly solidify that XMRV could not be a cause of disease in humans.

In one part of our study on HCMV we realized that in one of the monoclonal antibodies we validated and used for IHC was also used by others who had reported positive staining with the same antibody [33]. Although the protocols used were different in these studies, we found that if we simply changed the dilution of our primary antibody to a somewhat more concentrated form, we could obtain false-positive IHC staining in cells known to be negative for HCMV. Using the same approach, when we applied this antibody using IHC with this increased antibody concentration on the glioma specimens, we detected abundent false-positive IHC signals in most of the cases in most of the cells [33].

5. Methods for enhancing IHC validity

5.1. It is not difficult to develop an IHC staining protocol that leads to a false-positive result — an example of AKT1

The results with the anti-CMV protein antibodies in which it was relatively simple to obtain non-specific, false-positive IHC staining, even with an otherwise excellent antibody, imply that virtually any IHC staining assay can be mishandled to provide false-positive results. Fig. 1 shows an experiment in which we obtained HCT116 colorectal cancer cells that had targeted disruption of both alleles of AKT1 and AKT2 (double knockout cells) [100] in which we subjected cells to FFPE followed by IHC staining with an AKT-specific antibody. Using this antibody for Western blotting, we found a single band at the correct molecular weight for AKT1 in wild-type HCT116 cells and in double knockout cells that were transfected with a cDNA expression vector encoding human AKT1 (not shown), and an absence of bands in the untransfected double knockout cells. By IHC, the double knockout cells were negative at a dilution of 1:6400 of the primary antibody, and there were reasonably strong signals in the transfected cells in a subset of cells at this dilution (note that only a subset of cells staining positive is expected in these transient transfection assays). However, when we diluted the antibody to 1:800, we found strong signals in all of the knockout cells, which by definition must be non-specific, false-positive binding (Fig. 1).

5.2. General approach and practical tips for validating new antibodies in IHC assays

In our opinion, the most important part of working up a new or existing non-clinical grade antibody against a target protein is the use of appropriate negative controls. In the best case, and increasingly in most cases, one can use online literature searches to identify a cell line (or cell lines)

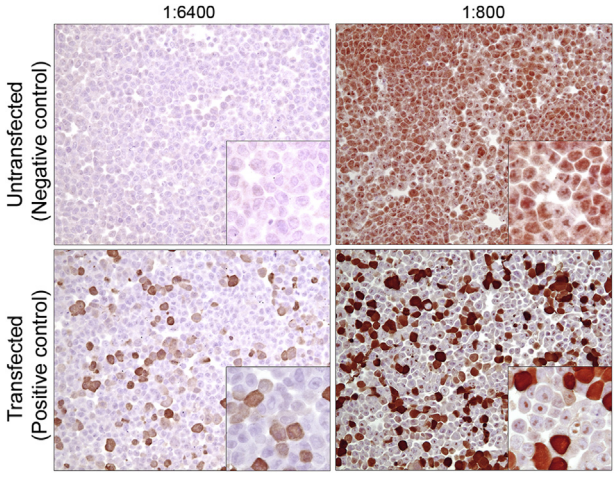

Figure 1 False-positive AKT1 staining in double knockout cells. Original magnification ×100, and the insets are original magnification ×400.

with a known homozygous deletion or targeted disruption of both alleles encoding the epitope of interest. In the latter cases, it is also useful to obtain the parental wild-type cells. If such knockout cells are not readily available, we usually next turn to a search of the NCI-60 series of cell lines to try to find cells with very low expression according to online databases of mRNA expression, performed on these either using microarrays (https://discover.nci.nih.gov/cellminer/) [101] or RNA-seq (https://portals.broadinstitute.org/ccle) [102]. At the same time one can examine these sites for potentially high expressor cell lines. Cell lines of interest can often be obtained from the American Type Culture Collection or other sources (https://www.atcc.org/). Cells expected to be negative or low for the encoding RNA can be tested by either in-house developed or commercial based real time qPCR assays. Also, we have found that robust detection of a number of protein markers using IHC can be confounded by issues related to formalin fixation, with the most common problem we find being related to under fixation [103]. Thus, for our cell line controls we fix the cells in formalin for 16—24 h and for our rodent or human research tissues we fix routinely for 48 h. We have found this to be useful for many dozens of antibodies as well as dozens of probe sets for *in situ* hybridization using ACD RNAscope assays.

The important aspect of having "true negative" cell lines is that when attempts are being made in the laboratory to maximize signal-to-noise, one must maintain negative staining in the true negative cell lines because any adjustments to enhance signals cannot be accepted if the negative lines become positive. As indicated above, it is quite easy to have true negative cells appear positive by IHC (Fig. 1). We also employ knockout mice at times where appropriate because if they are known not to express the epitope of interest, they can also be used to help with enhancing signal-to-noise.

Methods that we often employ to increase signals are to vary antigen retrieval buffers, times, and temperatures, to try different staining kits with different types of secondary antibodies, or to employ tyramide amplification. Moreover, we have found that the best signal-to-noise is generally now obtained using commercial reagents sold with IHC auto-stainers (we currently use Ventana reagents on a Ventana Discovery Ultra auto-stainer), often employing any "amplification" steps that the manufacturers offer. While this does not allow one full control and knowledge about all reagents, we are interested in maximizing signal-to-noise and ultimately having some assays be readily portable to clinical laboratories. Regardless of whether one has access to auto-stainers, the most important aspect of working up new and existing commercial research grade antibodies is having robust negative controls, and including those in each antibody run.

For cells validated to be negative for mRNA by qPCR, investigators can generate or purchase cDNA expression vectors for use in transfections of the proven negative cells, which can be used as an isogenic positive controls. If no good negative controls can be identified, one should consider siRNA knockdown [18] and/or CRISPR-cas9 knockouts, both of which are now facilitated by commercial companies that sell siRNA or sgRNAs to virtually all known open reading frames, and which are being increasingly

employed by some commercial vendors to help better validate their antibodies prior to marketing (*e.g.* Abcam now sells "knockout validated" antibodies [104]). In addition to cell lines, we often prefer to test antibodies on multiple human tissues. For positive and negative control tissues, one can search the literature or examine the Gtex database to get some ideas of mRNA expression (https://www.gtexportal.org/home/), as well as the Human Protein Atlas (https://www.proteinatlas.org/) for RNA and at times protein validation (see above caveats regarding the Human Protein Atlas).

Once high quality specific staining is achieved using appropriate controls, it is time to use on your tissue of interest. We also would like to stress that no cell line or tissue can serve as a surrogate for your tissue of interest, because since no antibody is a perfect binder to only one epitope [105], it is possible that your tissue will have epitopes that cross react with your antibody even if your cell lines or other control tissues do not (see example above on retracted *PNAS* paper on XMRV). It is wise, therefore, that if one is taking a serious attempt at localizing and quantifying a given protein in a specific tissue, to also perform Western blotting if possible on that tissue with that antibody. If multiple bands are present at the incorrect molecular weight and you cannot show they either relate to the peptide of interest or are not recognized by IHC, then one should not use that antibody for IHC [18]. As with all "rules" in this field there can be exceptions. For example, there is a recent study using an androgen receptor variant 7 antibody that recognizes a band in PC3 prostate cancer cells by Western blotting at a specific molecular weight, but there is no staining by IHC in the same PC3 cells with the same antibody [106]. Nevertheless, when a better antibody becomes available that does not have this issue, it is recommended to use that. Another applicable finding is that if a given target molecule is already known to have a specific cellular or subcellular localization from other studies, the internal controls where one observes the correct pattern of staining (*e.g.* epithelial specific or blood vessel specific) can be very helpful.

Fig. 2 shows an example of data from the cancer cell line encyclopedia on *ERG* mRNA levels as well as IHC staining with an anti-ERG antibody [47] in a number of cells lines, showing an excellent and robust example of how to find good positive and negative controls.

6. Additional approaches and considerations

6.1. *In situ* hybridization

Over the last several years a number of new technologies based on "z-pair" probe binding, requiring two separate oligonucleotide probes to hybridize in close proximity, and

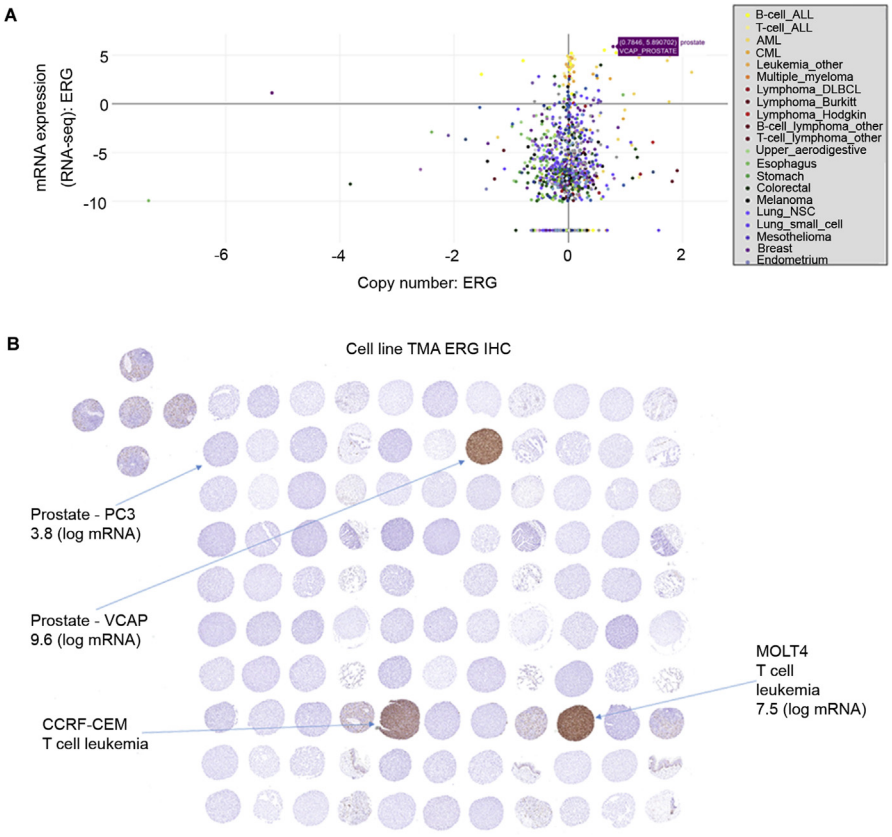

Figure 2 Example of using online database search to find positive and negative control cells for ERG mRNA. (A) Cancer cell line encyclopedia RNA-seq results for ERG mRNA showing prostate cancer VCaP cells to be strongly positive; (B) IHC for ERG protein using a tissue microarray with a number of cell lines including VCaP in which there is a good correlation between mRNA and protein by IHC. ERG, ETS-related gene; IHC, immunohistochemical.

branched DNA amplification have been introduced that have greatly enhanced the sensitivity, specificity, and ease of implementation for research and clinical laboratories to perform *in situ* hybridization for RNA and some DNA species in cells and tissue sections [107,108]. In addition to localizing virtually any coding and non-coding RNA at the single cell level with up to single molecule detection, as mentioned above, these technologies can also be used as orthogonal approaches to help provide increased evidence for the validity of IHC findings. In our experience of using more than 50 probe sets on both high and low expressing genes, we have found this technology to be invaluable in many of our studies, including in helping to validate IHC [31,33,109—112]. One caveat that we found, however, is that using this technology there can be relatively rapid loss of *in situ* hybridization signals from FFPE tissue blocks stored at room temperature, but that these signals can be preserved by freezing unstained, unbaked slides or FFPE blocks at -20°C [110].

6.2. Proximity ligation assays (PLAs)

The PLA is a recent addition to the toolbox of molecular pathology. It is based on a two-component detection system, which requires two molecular targets to be in close spatial proximity. Similar to the z-pair based probe hybridization indicated above, a signal is only generated when two oligonucleotides that are conjugated to detection antibodies come in close enough proximity (<100 nm apart) to form the template for a rolling circle amplification [113,114] which generates a single stranded DNA molecule that can be detected and visualized by standard bright field or fluorescence microscopy. Essentially, this assay generates a positive signal if two targets are in close proximity and therefore is perfectly suited to detect macromolecular binding events *in situ*. The specificity of the assay is ensured by its two-component nature; the very high sensitivity is the result of enzymatic signal amplification that allows robust single molecule detection [113,114]. This assay was first developed to detect protein—protein interactions and represents an important bridging technology in translational research [113,114]. The widespread use of PLA in recent years is partly due to the availability of commercial kits which enhances ease of use for establishing PLA in any laboratory with limited molecular biology expertise [115]. Numerous studies have established the great versatility of this assay in visualizing protein—protein interactions in preclinical models and clinical specimens [113,114,116,117]. Due to the modular nature of this assay, this approach can also be used to study post-translational modifications of proteins as well as protein—RNA and protein—DNA interactions [118—120]. Furthermore, given the fact that the PLA relies on the binding of two affinity reagents in close proximity, this assay can help to increase the specificity of antibody-based detections assays. As recently shown by Zieba et al. [115], using a dual labeling approach, PLA can be used to greatly improve the specificity of polyclonal antibodies or antisera. We are certainly hopeful that PLA will become more widely available and used in the near future to help bolster the validity of IHC results.

Author contributions

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Final approval of manuscript: Angelo De Marzo, Karen Sfanos.

Conflicts of interest

The authors declare no conflict of interest.

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This reviewed is dedicated to the memory of Donald S. Coffey. In fact, anyone who knows Don would know the first part of the title is a direct quote from him: "If this is true, what does it imply?". Don was a mentor and friend to each of us. For me (AMD), other than my parents and my wife Sandy, I can easily say that no one person had more influence over my life, including the realms of the personal, spiritual and scientific. Don was truly one-of-a-kind. I believe that the way he approached life, as a deeply caring individual to all people he came in contact with, and the way he approached science as a critical and unique thinker in many ways, exemplify the best of these characteristics that human beings have to offer.

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Note: While the manuscript was in production we also learned that the Journal of Histochemistry and Cytochemistry has several publications about standards of practice for immunohistochemistry and validation. Those publications and the current editor's perspective on this

(by Steven Hewitt) can be found at: https://journals.sagepub.com/doi/abs/10.1369/0022155416636547.

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