BIOCHEMISTRY

The Myc tag monoclonal antibody 9E10 displays highly variable epitope recognition dependent on neighboring sequence context

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Epitope tags are short, linear antibody recognition sequences that enable detection of tagged fusion proteins by antibodies. Epitope tag position and neighboring sequences potentially affect its recognition by antibodies, and such context-dependent differences in tag binding may have a wide-ranging effect on data interpretation. We tested by Western blotting six antibodies that recognize the c-Myc epitope tag, including monoclonal antibodies 9E10, 4A6, 9B11, and 71D10 and polyclonal antibodies 9106 and A-14. All displayed context-dependent differences in their ability to detect N- or C-terminal Myc-tagged proteins. In particular, clone 9E10, the most cited Myc-tag antibody, displayed high context-dependent detection variability, whereas others, notably 4A6 and 9B11, showed much less context sensitivity in their detection of Myc-tagged proteins. The very high context sensitivity of 9E10 was further substantiated by peptide microarray analyses. We conclude that recently developed, purpose-made monoclonal antibodies specific for Myc have much more uniform reactivity in diverse assays and are much less context sensitive than is the legacy antibody 9E10.

INTRODUCTION

Antibodies bind to conformational or linear epitopes in proteins. The average size of a linear epitope sequence is between six and nine amino acids (*1*). Fusing a linear antibody epitope to recombinant proteins by genetic engineering (epitope tagging) offers the possibility to detect, isolate, and characterize newly discovered or otherwise undetectable proteins for which no specific antibodies are available. Protein tags—for example, the substance P tag (*2*), the FLAG tag (*3*), and the 6xHistidine (His) tag (*4*)—were first developed to facilitate protein detection and purification. Today, researchers can choose from a large repertoire of epitope tag–antibody pairs to suit their particular experimental needs, including immunoblotting, immunoprecipitation and chromatin immunoprecipitation, immunofluorescence microscopy, or affinity purification [reviewed in (*5*)]. Popular tags include the FLAG tag, the hemagglutinin (HA) tag derived from the human influenza virus HA surface protein (*6*–*8*), and the Myc tag derived from the human c-Myc oncoprotein (*9*). Small tags have the advantage that in many cases they can be fused to the protein of interest without affecting its structure, function, subcellular localization, or stability. Therefore, tagging of proteins has become an indispensable technique in molecular biology, biochemistry, and cell biology, and consequently, a large number of antibodies are available for the detection of epitope tags. Most of these tag-specific antibodies were developed against short linear peptides that had to be coupled to carrier molecules to elicit an immune response toward these peptide haptens.

Although the minimum size of a proteinaceous linear epitope is considered to be only about six amino acids, most epitopes are part of larger three-dimensional structures on the surface of proteins, which are in direct or indirect contact with the antibody surface (*10*). Fusion of the tag sequence to the termini of proteins extends the linear epitope sequence at the N or C terminus by additional sequences. Hence, the binding of epitope tag–specific antibodies to their targets is most probably influenced by the surrounding protein environment. It is, therefore, likely that the affinity of antibodies for epitope tags can vary depending on the sequence context, with profound consequences for detectability and the quantitative assessment of different tagged proteins. Because epitope tags can be fused to the N or the C terminus of a protein and can be even inserted at internal positions (*11*), this may be of particular importance when differently tagged proteins are compared to each other. Here, we have tested by Western blot analysis six different commercially available Myc tag–specific antibodies for their ability to quantitatively detect the Myc epitope tag in different sequence contexts. Our results show that the sequence preceding or after the Myc epitope has a strong influence on its recognition by some of the antibodies and in particular by the most cited Myc tag antibody, clone 9E10.

RESULTS

The context of the Myc epitope sequence influences detection by the antibody 9E10

The Myc tag sequence, EQKLISEEDL, is derived from the loosely defined epitope of the monoclonal antibody 9E10 (*9*). 9E10 was generated against a larger peptide immunogen spanning amino acids 408 to 439 of human p62Myc (Fig. 1A). Within this larger sequence, the N-terminal half was thought to be the 9E10 epitope because 9E10 detects human but not chicken or mouse Myc in Western blot analyses, and only the N-terminal sequence of this peptide differs between these species (*9*). Subsequent epitope mapping experiments identified LISE within the N-terminal immunogen sequence EQKLISEEDL as the key binding residues for 9E10, and KLISEEDL as the shortest high-affinity peptide recognized by 9E10 (*12*). A substitutional array of N- and C-terminally extended peptides did not identify additional key positions and confirmed EQKLISEEDL as the 9E10 epitope sequence. A variant of the 9E10 epitope with an additional C-terminal asparagine residue originating from the expression vector (*13*) is used for tagging as well.

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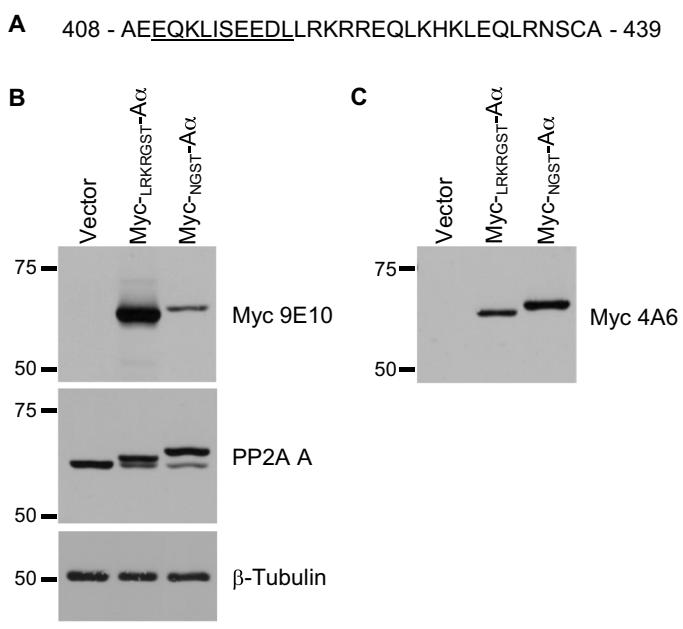

Fig. 1. Recognition of Myc-tagged PP2A Aα subunit by clone 9E10 is strongly influenced by the epitope sequence context. (**A**) Immunogen sequence corresponding to amino acids 408 to 439 of the human c-Myc used for generating clone 9E10. Clone 4A6 was raised against amino acids 410 to 419 (underlined). (**B**) Immunoblotting of lysates from NIH3T3 mouse fibroblasts either transfected with empty vector or expressing the indicated versions of Myc-tagged PP2A Aα subunit using the Myc-specific antibody 9E10 and a PP2A A–specific polyclonal antibody. One membrane was immunoblotted for β-tubulin as loading control. (**C**) Immunoblotting of lysates from NIH3T3 mouse fibroblasts either transfected with empty vector or expressing the indicated versions of Myc-tagged PP2A Aα subunit using antibody 4A6. The same amounts of the identical lysates that were used in (B) were loaded for this blot. Blots are representative of $n = 4$ independent experiments.

We found that the context of the Myc sequence had a marked effect on detection in Western blotting. In an experiment comparing two different versions of N-terminally Myc-tagged protein phosphatase 2A (PP2A) regulatory Aα subunit (PPP2R1A) derived from two different cloning strategies, we observed differences in the amounts of the proteins detected with the most commonly used and most often cited (www.citeab.com/antibodies/search?q=Myc) Myc tag–specific antibody, clone 9E10 (Fig. 1B). 9E10 detected much higher amounts of the Myc–PP2A Aα protein, in which the Myc tag was followed by a series of positively charged amino acids (LRKR), than of the Myc-Aα protein, in which the Myc tag was followed by polar and rather small and neutral amino acids (NGST). This marked difference in abundance was not observed when the blot was probed with a polyclonal antibody specific for the PP2A A subunit (Fig. 1B), suggesting that the context of the Myc epitope might be affecting its recognizability in the immunoblot.

We next examined in more detail the context of the epitope. In the original immunogen, the 9E10 epitope sequence EQKLISEEDL is followed by the basic amino acids LRKRR (Fig. 1A) (*9*, *13*, *14*). Four of these amino acids (LRKR) are also immediately adjacent to the Myc tag in the Myc–PP2A Aα version that was preferentially detected by the 9E10 antibody. This finding suggested a sequence bias of the 9E10 antibody and prompted us to generate a new Myc tag–specific monoclonal antibody without a C-terminal sequence bias. We used a shorter peptide encompassing only amino acids 410 to 419 of the human c-Myc for the immunization of mice and generated a monoclonal antibody, termed clone 4A6 (see Materials and Methods for details) (*15*). To increase the chances for a highly specific yet largely context-independent immune response, we included a spacer consisting of three nonproteinogenic β-alanine residues between the Myc epitope sequence and the C-terminal cysteine, resulting in the following immunogen sequence: MEQKLISEEDL(βA)(βA)(βA)C. We then tested this antibody for its ability to detect the two different Myc–PP2A Aα proteins. The relative amounts of Myc–PP2A Aα detected by clone 4A6 were similar to those detected by the PP2A A–specific antibody (Fig. 1C). Although clone 4A6 showed a slight preference for the NGST cloning context, the sequence bias was far less pronounced than the one observed for clone 9E10 for the LRKR context. Myc epitope recognition by clone 9E10 was hampered by the NGST context not only in Western blot but also in immunoprecipitation, whereas clone 4A6 immunoprecipitated the Myc-tagged PP2A A subunit in both sequence contexts equally well and more efficiently than did 9E10 (fig. S1).

Peptide microarray analysis highlights the context dependence of Myc epitope binding by antibody 9E10

To better understand the influence of the sequence context on Myc tag detection, we analyzed both antibodies on PEPperCHIP Custom Peptide Microarrays, in which 15–amino acid peptides corresponding to the Myc epitope tag in the two cloning contexts described above (MEQKLISEEDLLRKR and MEQKLISEEDLNGST) were permuted in a single-substitution scan to all 20 proteinogenic amino acids at each of the four positions C terminal to the epitope tag. This single-substitution scan demonstrated that 9E10 was much more sensitive to sequence context than was 4A6. The variability of the 9E10 signals was profoundly larger compared with the 4A6 signals at all four positions C terminal to the Myc epitope not only in the NGST variants but also in this antibody's preferred LRKR sequence context (table S1). The ratio of the highest to lowest signal of the position +1 permutations in the NGST context was only 2.6-fold for 4A6 but 28.7-fold for 9E10 (excluding one context with 0 signal). Likewise, for the LRKR context, this was 6.6-fold for 4A6 but 44-fold for 9E10 (again excluding one context with 0 signal). Five of the 154 peptides were not detected by clone 9E10, and a further 19 displayed only very low signals (less than 10% of the average signal). Of these 24 very low- or nonbinding peptides, only two contained the basic context. Asparagine at position +1 in combination with glycine at position +2 had a negative impact on antibody binding in many of the peptides, and proline at position +1 completely abolished tag recognition by 9E10 in both sequence contexts.

In agreement with the Western blot data, 9E10 showed a preference for the LRKR context, but this preference did not extend to all similar peptides. Several peptides in the NGST permutation series, in particular those with hydrophobic residues at position +1 (for example, IGST, LGST, or VGST), were much better detected by 9E10 than the one in the LRKR context. In contrast to 9E10, clone 4A6 recognized all 154 sequence variants with much less variability and a signal strength higher than 10% of the average signal. To identify the best and worst C-terminal epitope context, we carried out a double-substitution scan at positions +1 and +2 of the Myc tag peptides MEQKLISEEDLXXKR and MEQKLISEEDLXXST (Fig. 2 and table S2). Again, clone 9E10 showed much greater signal variability than did clone 4A6 at the same antibody concentration, with a variation coefficient (CV) of 135%. Twenty peptides were not detected

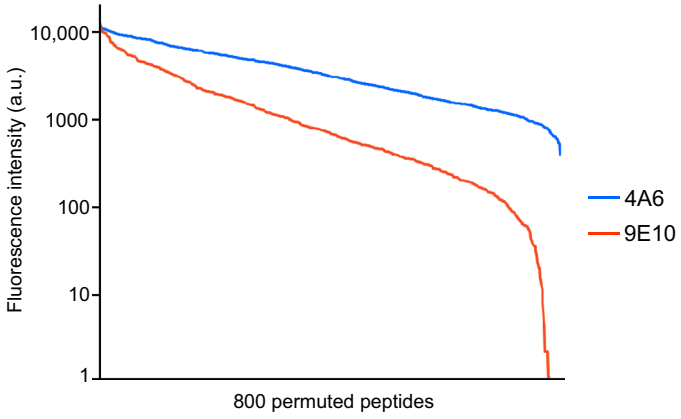

Fig. 2. Clone 9E10 shows much higher signal variability than clone 4A6 on peptide microarray. Peptides corresponding to the Myc epitope tag in the contexts MEQKLISEEDLXXKR or MEQKLISEEDLXXST, in which the XX positions were permuted to all 20 amino acids, were incubated with the indicated antibody on PEPperCHIP Custom Peptide Microarrays. Fluorescence intensity values (*y* axis, log scale) are plotted for 800 double-substitution permuted peptides (*x* axis). Twenty peptides were not detected by clone 9E10 and are therefore not represented in the log diagram. Primary data are shown in table S2. a.u., arbitrary units.

by clone 9E10, and an additional 122 peptides were detected at less than 10% of the average signal. In contrast, clone 4A6 showed not only a 2.5-fold higher average signal compared with 9E10 but also a more narrow signal distribution (CV = 70%), with only a single peptide yielding a signal just below 10% of the average signal and none without any signal. Nevertheless, it is important to note that also for clone 4A6, the ratio between the weakest (-RKKR) and the strongest signal (-FEST) was 35-fold in this peptide microarray format. For both antibodies, the top 10 peptides contained the XXST context, with XX most often being small, hydrophobic amino acids (table S3). At the same time, 19 of the 20 nonbinding peptides of the 9E10 antibody also contained the XXST context (most often with P, W, or N at position +1, and H, W, or C at position +2), which highlights the big impact of the first two residues following the Myc epitope on its recognition by 9E10. Together, the peptide microarray analysis revealed a large variability of Myc peptide recognition dependent on the adjacent sequence environment.

Antibody 9E10 exhibits greater binding variability than antibody 4A6

Next, we wanted to know whether the results with the peptide microarrays could be recapitulated in Western blot analysis. Therefore, we fused the Myc epitope sequence in six different sequence contexts to the N terminus of a 147–amino acid C-terminal fragment of rat protein phosphatase 2A B55α regulatory subunit (PPP2R2A). The sequence contexts included NGST and LRKR, representing the initial sequence contexts; IAST and IGST, the microarray high-scoring permutations of the NGST context; TRKR, the high-scoring permutation of the LRKR context; and $L(GS)_3$, an often used "unstructured" linker motif (Table 1). The resulting Myc tag fusion proteins were expressed in *Escherichia coli* and detected by SDS–polyacrylamide gel electrophoresis (PAGE) followed by Western blot analysis with 9E10 and 4A6 (Fig. 3, A and B). As an internal control, we used a mouse monoclonal antibody, clone 2G9, which was raised against the B55α-specific peptide 398-CASGKRKKDEISVD-411 (Fig. 3A). This internal peptide is located far away from both termini, and its

Table 1. Protein sequences of six N-terminal Myc-tagged PP2A Bα subunit fragments. The core Myc tag epitope is underlined, and the different sequence contexts are shown in red. The epitope of clone 2G9 is shown in bold.

Myc-NGST-B55α 301 to 447
MEQKLISEEDL**NGST**MTRDYLSVKVWDLNMENRPVETYQVHEYLRSKLCSLYE NDCIFDKFECCWNGSDSVVMTGSYNNFFRMFDRNTKRDITLEASRENNKPR TVLKPRKV**CASGKRKKDEISVD**SLDFNKKILHTAWHPKENIIAVATTNNLYI FQDKVN
Myc-IGST-B55α 301 to 447
MEQKLISEEDL**IGST**MTRDYLSVKVWDLNMENRPVETYQVHEYLRSKLCSLYE NDCIFDKFECCWNGSDSVVMTGSYNNFFRMFDRNTKRDITLEASRENNKPR TVLKPRKV**CASGKRKKDEISVD**SLDFNKKILHTAWHPKENIIAVATTNNLYI FQDKVN
Myc-IAST-B55α 301 to 447
MEQKLISEEDL**IAST**MTRDYLSVKVWDLNMENRPVETYQVHEYLRSKLCSLYE NDCIFDKFECCWNGSDSVVMTGSYNNFFRMFDRNTKRDITLEASRENNKPR TVLKPRKV**CASGKRKKDEISVD**SLDFNKKILHTAWHPKENIIAVATTNNLYI FQDKVN
Myc-LRKR-B55α 301 to 447
MEQKLISEEDL**LRKR**MTRDYLSVKVWDLNMENRPVETYQVHEYLRSKLCSLYE NDCIFDKFECCWNGSDSVVMTGSYNNFFRMFDRNTKRDITLEASRENNKPR TVLKPRKV**CASGKRKKDEISVD**SLDFNKKILHTAWHPKENIIAVATTNNLYI FQDKVN
Myc-TRKR-B55α 301 to 447
MEQKLISEEDL**TRKR**MTRDYLSVKVWDLNMENRPVETYQVHEYLRSKLCSLYE NDCIFDKFECCWNGSDSVVMTGSYNNFFRMFDRNTKRDITLEASRENNKPR TVLKPRKV**CASGKRKKDEISVD**SLDFNKKILHTAWHPKENIIAVATTNNLYI FQDKVN
Myc-L$(GS)_3$-B55α 301 to 447
MEQKLISEEDL**LGSGSGS**MTRDYLSVKVWDLNMENRPVETYQVHEYLRSKLCS LYENDCIFDKFECCWNGSDSVVMTGSYNNFFRMFDRNTKRDITLEASRENN KPRTVLKPRKV**CASGKRKKDEISVD**SLDFNKKILHTAWHPKENIIAVATTNN LYIFQDKVN

recognition by clone 2G9 should be unaffected by any N- or C-terminal tagging, reflecting the "true" amounts of the Myc-tagged B55α fragments. The signals of eight Western blot experiments were quantified with the LI-COR Odyssey fluorescence imaging system and are shown relative to the "true" protein amount as determined with clone 2G9 (Fig. 3B). Our analysis revealed substantial differences in the recognition patterns of the six sequence contexts, in particular by 9E10. 4A6 showed relatively consistent binding across all six contexts. In agreement with our initial observation with Myc–PP2A Aα, the basic contexts reduced recognition by 4A6 by half compared with the more neutral contexts. However, the microarray high-scoring permutations of NGST (IAST and IGST) and LRKR (TRKR) did not improve detectability by 4A6, indicating that the signal strength measured in the peptide array cannot be translated quantitatively (1:1) into Western blot signal strength.

In stark contrast to 4A6, 9E10 displayed great sequence bias: Whereas the NGST context strongly obstructed tag binding, the "original" basic c-Myc context, LRKR, was about 20-fold better detected, again confirming our initial findings with Myc–PP2A Aα. Impaired recognition was—as the array data indicated—due to an asparagine at position +1 in combination with glycine at position +2, because replacing asparagine or asparagine-glycine with isoleucine

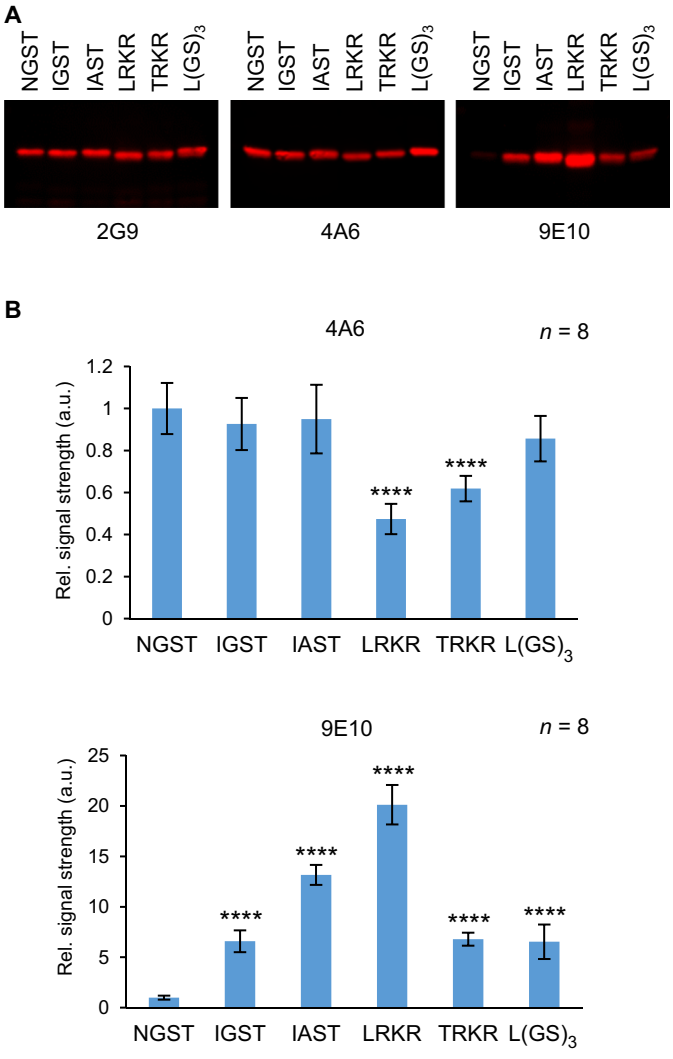

Fig. 3. Amino acid content C terminal to the Myc tag strongly influences the detection of tagged proteins by clone 9E10 in Western blotting. (**A**) Immunoblotting of lysates from bacteria expressing six versions of an N-terminal Myc-tagged PP2A B55α subunit fragment in different C-terminal sequence contexts [NGST, IGST, IAST, LRKR, TRKR, and L(GS)$_3$] using the PP2A B55α–specific monoclonal antibody 2G9 as reference antibody and the Myc tag–specific antibodies, clone 9E10 and clone 4A6. Representative images of $n = 8$ independent LI-COR Western blot analyses are shown. (**B**) Quantification of fluorescence signal intensities adjusted to the 2G9 signals, which represent the amounts of Myc-tagged PP2A B55α subunit fragment, shown as average ± SD from eight Western blot replicates ($n = 8$). The average NGST context signal was arbitrarily set to 1, and values are shown relative to NGST. Statistical significance was calculated with a one-way ANOVA + post hoc Tukey's HSD test. *P* values relative to NGST are depicted as ****$P \leq 0.001$.

or isoleucine-alanine, respectively, increased detectability substantially. However, changing leucine to tryptophan in the LRKR context, a replacement that increased signals in the microarray assay, rather impaired recognition of the Myc epitope in the Western blot analysis. Hence, although the Western blot data did not fit quantitatively well to the PEPperCHIP microarray data (thereby highlighting the importance to validate antibodies in the particular technique of choice), this analysis confirmed the generally much larger binding variability of clone 9E10 compared with 4A6.

Researchers often use epitope repeats—consisting of, for example, six (or more) serial Myc tags—to increase detection sensitivity. Given that the presence of such a multiple epitope tag may influence the context bias of Myc tag–directed antibodies, we also investigated the binding of clones 9E10 and 4A6 to three different versions of a 6xMyc-tagged B55α fragment (fig. S2, A and B). Two versions contained a 6xMyc tag that is found in several popular mammalian expression vectors in the Addgene database (for example, plasmids 17439, 17457, 13627, or 13460), with the 6xMyc tag followed by NGST in one version and by LRKR in the other. In the third version, we also substituted the internal NEM linkers between the individual Myc peptides by LRK linkers (fig. S2A). Clone 4A6 recognized all three versions with similar strength in Western blot–LI-COR analyses. In contrast, although the variability was reduced compared to the single Myc–tagged versions, clone 9E10 still showed a 3.5-fold preference for the LRKR context (fig. S2, B and C).

Most Myc tag–specific antibodies display highly variable, context-dependent epitope tag recognition

These findings raised our concerns about a potential variability of Myc peptide recognition by different Myc tag–specific antibodies. Such a variability may have severe consequences for the estimation of the relative abundance of different Myc-tagged proteins and consequently for the interpretation of data obtained with such antibodies. We therefore extended our analysis to test the influence of the immediate surroundings of the Myc epitope tag on the antigen recognition by six different Myc tag–specific antibodies. These antibodies were chosen on the basis of the numbers of citations [www.citeab.com/search?q=Myc; (*16*)], the host animal, and the suppliers to include some of the most cited Myc tag antibodies from different species and sold by a variety of commercial providers. Our panel included the following antibodies: mouse monoclonal clone 9E10 (Millipore), mouse monoclonal clone 4A6 (Millipore), mouse monoclonal clone 9B11 (Cell Signaling Technology), rabbit monoclonal clone 71D10 (Cell Signaling Technology), rabbit polyclonal ab9106 (Abcam), and rabbit polyclonal A-14 (Santa Cruz Biotechnology, sc-789; discontinued in 2017). We chose to scrutinize the six antibodies again by Western blotting because this is one of the most common applications for the detection of proteins by tag-specific antibodies. To test their sensitivity to different sequence contexts, the Myc epitope sequence was fused in four different contexts either to the N or the C terminus of the 147–amino acid C-terminal fragment of rat PP2A B55α (Table 2). The chosen sequence contexts (highlighted in red) represent seven amino acids immediately up- or downstream of the Myc epitope tag sequence that are encoded in popular mammalian expression vectors such as pcDNA3.1/Myc-His B (Invitrogen), pCMV-Myc-N (Clontech), pCMV-Myc-C (Clontech), and pCruz Myc (reading frame A, Santa Cruz Biotechnology). For the ease of the analysis, the resulting Myc tag fusion proteins were expressed in *E. coli* and detected by Western blot with the different Myc tag–specific antibodies (Fig. 4). As internal control, we again used the mouse monoclonal antibody, clone 2G9 (Fig. 4A). As additional control, we also probed for Myc-B55α with a polyclonal rabbit serum (Δ237) that was raised against amino acids 238 to 447 of rat B55α and gave almost identical results as clone 2G9 (Fig. 4A). The signals of eight Western blots were quantified using the LI-COR Odyssey fluorescence imaging system and are shown relative to the "true" protein amount as determined with clone 2G9 (Fig. 4B).

Table 2. Protein sequences of four Myc-tagged PP2A Bα subunit fragments. The core Myc tag epitope is underlined, and the different sequence contexts are shown in red. The epitope of clone 2G9 is shown in bold.

pCMV-Myc-N-B55α 301 to 447
MEQKLISEEDL**LMAMEAR**MTRDYLSVKVWDLNMENRPVETYQVHEYLRSKL CSLYENDCIFDKFECCWNGSDSVVMTGSYNNFFRMFDRNTKRDITLEASR ENNKPRTVLKPRKV**CASGKRKKDEISVD**SLDFNKKILHTAWHPKENIIAVA TTNNLYIFQDKVN
pCMV-Myc-C-B55α 301 to 447
MTRDYLSVKVWDLNMENRPVETYQVHEYLRSKLCSLYENDCIFDKFECC WNGSDSVVMTGSYNNFFRMFDRNTKRDITLEASRENNKPRTVLKPRKV **CASGKRKKDEISVD**SLDFNKKILHTAWHPKENIIAVATTNNLYIFQDKVN **EISRGTG**EQKLISEEDL
pCruz-Myc-B55α 301 to 447
MGSEEQKLISEEDL**EFSTAAA**MTRDYLSVKVWDLNMENRPVETYQVHEY LRSKLCSLYENDCIFDKFECCWNGSDSVVMTGSYNNFFRMFDRNTKRD ITLEASRENNKPRTVLKPRKV**CASGKRKKDEISVD**SLDFNKKILHTAWH PKENIIAVATTNNLYIFQDKVN
pcDNA3.1/Myc-His B-B55α 301 to 447
MTRDYLSVKVWDLNMENRPVETYQVHEYLRSKLCSLYENDCIFDKFECC WNGSDSVVMTGSYNNFFRMFDRNTKRDITLEASRENNKPRTVLKPRKV **CASGKRKKDEISVD**SLDFNKKILHTAWHPKENIIAVATTNNLYIFQDKVN **SLEGPRF**EQKLISEEDL

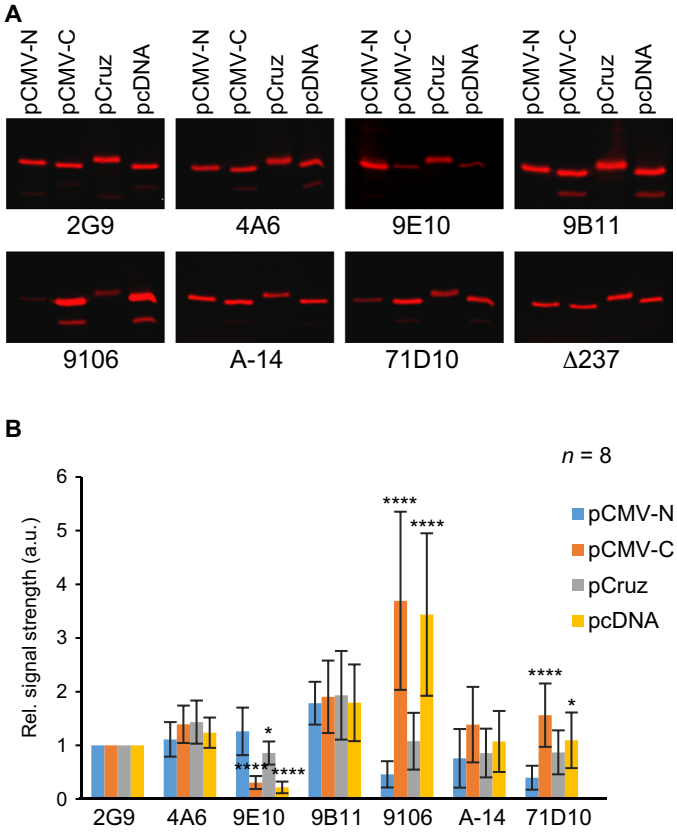

Fig. 4. Antibody binding to Myc tag on Western blots depends on tag position and sequence context. (**A**) Immunoblotting of lysates from bacteria expressing the four indicated different context versions of an Myc-tagged PP2A B55α subunit fragment using the indicated antibodies. Representative images of $n = 8$ independent LI-COR Western blot analyses are shown. Two antibodies recognizing PP2A B55α (clone 2G9 and rabbit polyclonal Δ237) were used as reference antibodies. (**B**) Quantification of fluorescence signal intensities adjusted to the 2G9 signals, which represent the amounts of Myc-tagged PP2A B55α subunit fragment, shown as average ± SD from eight Western blot replicates ($n = 8$). Statistical significance of signal differences between contexts was calculated separately for each antibody with a one-way ANOVA + post hoc Tukey's HSD test. *P* values are shown relative to the pCMV-N value for the respective antibody: $^{*}P \leq 0.05$, $^{****}P \leq 0.001$.

Our analysis revealed that only two of the tested antibodies—mouse clones 4A6 and 9B11—detected the four different Myc-B55α proteins with comparable signal strengths and gave a very similar pattern compared with clone 2G9. In contrast, some of the other antibodies displayed considerable differences in their ability to detect the four tagged proteins. All three rabbit antibodies showed a preference for the Myc epitope tag in a C-terminal position (in the contexts of vectors pCMV-Myc C and pcDNA3.1/Myc). In particular, antibody 9106 (Abcam) displayed a strong bias for the C-terminal tag position, with the pCMV-Myc C context protein about eight times better detected than the pCMV-Myc N counterpart. Furthermore, antibody 9106 showed some preference for the pCruz Myc N-terminal context compared with the pCMV-Myc N context, which may be due to the fact that in the pCruz context the Myc tag is not located at the far N terminus but rather in a more internal position with several amino acids preceding it. Last, clone 9E10 again exhibited a substantial sequence bias. In contrast to the rabbit antibodies, it preferentially recognized the N-terminally tagged B55α versions and showed a strongly reduced ability to detect the two C-terminally tagged versions, with the pCMV-Myc N version about six times better recognized than the pcDNA3.1/Myc version. These results corroborated that the ability of 9E10 to detect the Myc epitope sequence varies very much with its N- or C-terminal position and the sequence composition of residues neighboring the epitope.

DISCUSSION

Antibody specificity and selectivity are critical for data reproducibility. However, a large number of scientific findings have been difficult or impossible to replicate due to inconsistent and nonspecific antibodies (*17*, *18*). Consequently, during the last years, several groups have called for comprehensive and (more) vigorous antibody validation [see, for example, (*19*–*21*)], and the establishment of the proper common practices for validating antibodies has emerged as a prime agenda both in basic life science research as well as in clinical applications. Mostly, antibody validation focuses on antibodies against particular (protein) targets. In the course of our own research, we have observed substantial performance differences of antibodies that are assumed to be widely applicable in many different assay formats, namely, antibodies specific for short epitope tags. Here, we showed that four of six Myc epitope tag antibodies were very much influenced by the sequence context in which the peptide tag was embedded. This variability was observed for both monoclonal and polyclonal antibodies, indicating that the nature of the immunogen (length and exact sequence) and how it is presented are probably more important for the performance of the resulting antibody than is its clonality.

Our analysis included one of the most frequently applied antibodies in basic research, namely, mouse monoclonal antibody 9E10. Clone 9E10 is by far the most often used Myc tag–specific antibody

with more than 6500 citations in the CiteAb database (www.citeab.com/antibodies/search?q=Myc), and its binding mode to the Myc tag peptide has been studied in detail. Mutational analysis of the Myc tag peptide and random peptide library screening identified the core peptide stretch of LISE as crucial for the interaction with 9E10 (*12*, *22*), but the impact of amino acid changes adjacent to the Myc tag sequence on antibody binding has never been reported in detail. X-ray crystallography of the 9E10 antigen-binding fragment (Fab) in complex with the epitope peptide EQKLISEEDLN has revealed an unusual structure in which two antibody fragments bind asymmetrically to one peptide (*23*). Further truncation of the peptide to KLISEEDL results in only a moderate loss of affinity, but isothermal titration calorimetry and gel filtration reveal an altered 1:1 stoichiometry of Fab and truncated peptide (*23*), indicating that amino acid changes outside the core epitope sequence may have profound effects on binding by 9E10. The Western blot data presented here show that this antibody is particularly sensitive to the identity of the amino acids located in the vicinity of the tag.

A comparison of clones 9E10 and 4A6 by peptide microarrays also revealed a much larger signal variability for the 9E10 antibody. A preference of 9E10 for the LRKR cloning context, which is found in the genuine human c-Myc sequence that was used to generate this antibody, was only observed in our Western blot analyses. In the peptide array format, the 10 highest-scoring peptides were based on the XXST context. Clone 4A6 showed a preference for the XXST context in both the Western blot and the microarray analyses; however, this bias was much more pronounced in the peptide array. These differences are likely due to different epitope presentation: In a Western blot, the Myc epitope is presented to the antibody in the context of—at least partially—a denatured protein disorderedly immobilized on a membrane. In comparison, in the peptide array, short peptides are immobilized in a given orientation through their C terminus on a coated glass slide surface. This macroscopic, static surface most likely affects antibody binding in a different way than does the additional downstream sequence present in a fusion protein. For 9E10, permutation of the amino acid in closest proximity to the glass surface (the fourth amino acid position downstream of the Myc epitope sequence) showed the highest relative signal variability (table S1), indicating that this amino acid had an above-average influence on peptide accessibility and antibody binding. Fan *et al.* reported that two of four N-terminally Myc-tagged proteins could not be detected by Western blotting with clone 9E10 (*24*). At the same time, however, all four proteins could be—with varying efficiency—immunoprecipitated with clone 9E10 and could also be detected by immunofluorescence microscopy, corroborating that the tertiary protein structure also has a strong influence on tag accessibility and/or binding affinity. In the case of our Myc–PP2A Aα proteins, clone 9E10 was hampered by the NGST context not only in Western blotting but also in immunoprecipitation. One of the proteins that were undetectable by Western blotting in the paper by Fan *et al.* contained the amino acids PLKR immediately adjacent to the Myc epitope (*24*). This peptide context was also included in our peptide microarray analysis, where it scored very low (table S2). Another approach in which the context sensitivity of 9E10 may have influenced the results is the protein truncation test (PTT), a diagnostic test to identify truncating (frameshift and nonsense) mutations in cancer susceptibility genes (*25*). For protein detection, this assay used a Myc epitope sequence with an asparagine at position +1, which—according to our array data—is one of the least favorable amino acids at this position. Depending on the sequence C-terminal to the asparagine, some of the analyzed exon sequences may have escaped detection by 9E10. Along these lines, a recent reassessment by next-generation sequencing of 190 patients with breast cancer who had tested negative for *BRCA* mutations in the PTT analysis revealed 17 pathogenic mutations in breast cancer susceptibility genes, 6 of them in *BRCA1* or *BRCA2*, including a frameshift deletion in *BRCA2* exon 10 that had been missed by PTT for unknown reasons (*26*).

We have shown that sequence context–dependent epitope tag recognition is a widespread phenomenon among Myc tag–specific antibodies. Although the binding variability will be different for other antibodies and for other short epitope tags and depends on the technique and most likely the particular experimental conditions used, it is conceivable that such artifacts are not restricted to Myc tag antibodies. For instance, the FLAG tag antibody clone M2 is known to detect the FLAG tag independent of its position at the N or C terminus or even in the interior of the target protein, whereas FLAG tag antibody clone M1 is strongly influenced by the tag position and requires Ca^{2+} as cofactor for high-affinity binding (*3*). Similar to 9E10, the HA tag–specific monoclonal antibody, 12CA5, was also raised against a larger peptide from HA (*6*) and might, therefore, also be influenced by the surrounding sequence. Last, detection variability has also been reported for a series of poly-His–specific antibodies (*27*).

Because of their high specificity, ease of use, and broad applicability, tag antibodies are among the most widely used reagents in basic and applied biomedical research. Our results show that even these seemingly unsuspicious antibodies should be used with great care and should be individually validated in the particular technique of choice before drawing any quantitative conclusions. On the basis of our findings, we recommend using identical sequence contexts for the epitope tagging of proteins, for example, a 3× glycine-serine flexible linker, and an antibody that has been validated for the least context sensitivity for epitope detection.

MATERIALS AND METHODS

Generation of Myc tag clone 4A6

A synthetic peptide corresponding to amino acids 410 to 419 of the human c-Myc and containing an N-terminal methionine, three β-alanine residues, and a C-terminal cysteine [MEQKLISEEDL-(βA)(βA)(βA)C] was coupled to keyhole limpet hemocyanine (KLH) as carrier protein via the C-terminal cysteine using succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) as a conjugating agent (provided by PiChem GmbH, Austria). Balb/c mice were immunized with the KLH-peptide conjugate, and splenocytes were fused with X63-Ag8.653 myeloma cells using polyethylene glycol. Hybridoma supernatants were screened 7 days after fusion for the presence of Myc epitope tag–specific antibodies using enzyme-linked immunosorbent assay against the Myc peptide.

Cloning and expression of Myc tag clone 4A6 scFv

cDNA was prepared from clone 4A6 using the RNeasy Mini Kit (QIAGEN) and AccuScript 1st Strand cDNA Synthesis Kit (Agilent) according to the manufacturers' manuals. The variable parts of the heavy and light chains, respectively, were amplified with polymerase chain reaction (PCR) primer mix sets from Sigma-Aldrich Merck (*28*), cloned into the pJET1.2 cloning vector, and sequenced. The obtained sequences for the heavy and light variable fragments were analyzed with the V-Quest tool on the International Immunogenetics

Information System (IMGT) homepage (www.imgt.org), and allele-specific forward primers corresponding to the first 20 base pairs of the best-matching heavy-chain V and light-chain V region alleles were designed. The correct variable regions were cloned as scFv fragment together with an N-terminal signal peptide and connected by an 18–amino acid glycine-serine–rich linker into the pcDNA3.1 expression plasmid, and the 4A6 scFv fragment was expressed in transiently transfected human embryonic kidney (HEK) 293T cells. Briefly, cells were seeded in 60-mm petri dishes and transfected with TurboFectin transfection reagent according to the manufacturer's instruction. Four days after transfection, the growth medium was harvested and used to detect Myc-tagged proteins by Western blotting to ensure that the correct DNA sequences had been recombined. The sequence of clone 4A6 can be obtained upon request and provision of a material transfer agreement (MTA) of the Medical University of Vienna.

Cloning of expression constructs

Ectopic Myc-tagged PP2A Aα subunit was expressed from pLPC-N MYC (Addgene, plasmid # 12540). For the LRKRGST sequence context, the PP2A Aα cDNA was amplified by reverse transcription PCR from HeLa cells and inserted via the Bam HI/Xho I sites. For the NGST sequence context, the complete Myc–PP2A Aα cassette was excised from a different plasmid (pBABE-Puro-Aα) by Hind III/Xho I and inserted into pLPC-N MYC via the Hind III/Xho I sites. Codon-optimized cDNAs of the rat Myc-PP2A B55α fragments corresponding to six different Myc tag sequence contexts [NGST, IGST, IAST, LRKR, TRKR, and L(GS)$_3$; Table 1], three different 6xMyc tag sequence contexts (fig. S2), or four different Myc tag sequence contexts (designated as pCMV-Myc-N, pCMV-Myc-C, pCruz-Myc, and pcDNA3.1-Myc B; Table 2) were synthesized and cloned into the pET23a(+) bacterial expression vector via the Nde I/Xho I restriction sites by GenScript Inc. All four latter versions contain an in-frame stop codon immediately upstream of the Xho I restriction site to avoid read through into the 6xHis tag sequence of the vector. The exact DNA sequences can be obtained upon request.

Bacterial expression of recombinant Myc-PP2A B55α proteins

Recombinant Myc-PP2A B55α proteins were expressed in the bacterial strain Tuner(DE3)pLysS (Novagen). Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3.5 hours at 37°C. The bacterial cells were lysed by sonication on ice for 3× 30 s with 20-s breaks between the sonication steps in 25 mM tris (pH 8.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and aprotinin (0.03 U/ml). After centrifugation, the cleared lysates were mixed with Laemmli buffer and boiled for 5 min at 95°C.

Mammalian tissue culture and lysates

NIH3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM GlutaMAX, and 1% penicillin-streptomycin at 37°C in an atmosphere containing 7.5% CO_2. Cells regularly tested negative for mycoplasma infection. Cells were infected with pLPC-N Myc–LRKRGST–PP2A Aα or pLPC-N Myc–NGST–PP2A Aα retroviral supernatant as described (*29*), and stable expressing mix clones were established by selection with puromycin (5 μg/ml) for 2 weeks. Whole-cell lysates were prepared from exponentially growing cells by incubation for 20 min at 4°C in Nonidet P-40 (NP-40) lysis buffer [20 mM tris (pH 8.0), 135 mM NaCl, 1% NP-40, 10% (v/v) glycerol, 1 mM PMSF, aprotinin (0.03 U/ml), and Roche cOmplete Protease Inhibitor Mix], followed by centrifugation at 14,000 rpm in a tabletop centrifuge for 15 min at 4°C. Before loading on SDS-PAGE, cleared lysates were mixed with Laemmli buffer and boiled for 5 min at 95°C.

Western blotting and data analysis

Proteins were separated by tris-glycine SDS-PAGE, followed by blotting onto nitrocellulose membrane (0.2 μm; Amersham Protran, GE Healthcare). For horseradish peroxidase (HRP)/enhanced chemiluminescence (ECL) detection, the membranes were blocked with 3% non-fat dry milk (NFDM) in phosphate-buffered saline (PBS)–Tween 20 (0.05%; PBST) for 1 hour at room temperature (RT) and incubated with primary antibody diluted in 0.5% NFDM/PBST overnight at 4°C. Incubation with secondary peroxidase–conjugated antibody [1:10,000 in 0.5% NFDM/PBST; AffiniPure Goat Anti-Mouse immunoglobulin G (IgG) (Fcγ fragment) or AffiniPure Goat Anti-Rabbit IgG (Fcγ fragment)–specific antibody, Jackson ImmunoResearch] was performed for 1 hour at RT, followed by incubation with ECL Western Blotting reagent (GE Healthcare) as suggested by the manufacturer. The signals were detected by Super HR-HA 30 film (Fuji). HRP/ECL-generated signals were quantified using ImageQuant TL 8.1 software (1D gel analysis, rolling ball background correction) and ImageJ software (https://imagej.nih.gov/ij/). For detection by fluorescence, the membranes were blocked with 2% bovine serum albumin (BSA) in PBS for 1 hour at RT and incubated with primary antibody diluted in 2% BSA in PBS overnight at 4°C. Incubation with secondary IRDye 680LT goat anti-mouse or anti-rabbit antibody (1:20,000; LI-COR) was done for 1 hour at RT in the dark. After washing three times with PBST and once with PBS, images were captured with the Odyssey system (LI-COR). LI-COR/fluorescence-generated signals were quantified using Image Studio Lite 4.0 software. All Western blot experiments were performed at least three times (n), and the values are presented as means ± SD; n in the figure panels and figure legends indicates the number of independent experiments. For quantifications shown in Figs. 3 and 4 and fig. S2, signals were adjusted relative to the respective 2G9 signals. For Fig. 3 and fig. S2, the average of the adjusted NGST context signals was arbitrarily set to 1, and all other contexts are shown relative to NGST. In Fig. 4, all signals are shown relative to the 2G9 signals, which were arbitrarily set to 1. Statistical significance of immunoblotting data was assessed using a one-way analysis of variance (ANOVA) followed by Tukey's honestly significantly different (HSD) post hoc test for multifold comparisons. In all cases, P values <0.05 were considered to be significant and are indicated with $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.001$. Statistical analysis was performed in Excel using the data analysis and real statistics add-ins.

The following primary antibodies against the Myc epitope tag were used: anti–Myc tag mouse clone 4A6 (Millipore and Ogris laboratory), anti–Myc tag mouse clone 9E10 (Millipore and Ogris laboratory; also available from Santa Cruz Biotechnology/Abcam/Invitrogen/Novus/Origene/Genetex/Enzo Life Sciences/Bio-Rad/Developmental Studies Hybridoma Bank), anti–Myc tag mouse clone 9B11 (Cell Signaling Technology), anti–Myc tag rabbit clone 71D10 (Cell Signaling Technology), anti–Myc tag rabbit polyclonal A-14 (Santa Cruz Biotechnology; discontinued), and anti–Myc tag rabbit polyclonal 9106 (Abcam). The following primary antibodies against other proteins were used: anti–PP2A Aα rabbit polyclonal (Millipore, catalog no. 07-250), anti–PP2A B55α mouse clone 2G9 (Abcam, Cell Signaling Technology, Millipore, Santa Cruz Biotechnology, and Sigma), anti–PP2A B55α rabbit polyclonal Δ237 (Ogris laboratory), and anti–β-tubulin (Millipore, catalog no. 05-661).

Immunoprecipitation

Monoclonal antibodies 4A6 and 9E10 were cross-linked to protein A–Sepharose beads (GE Healthcare) with dimethyl pimelimidate in Na-borate (pH 9.0) (*30*). Myc–PP2A Aα was immunoprecipitated for 2 hours at 4°C with either clone 4A6 or clone 9E10 from whole-cell lysates of NIH3T3 cells stably expressing the respective tagged protein. Immunoprecipitated proteins were analyzed by SDS-PAGE and Western blotting.

PEPperCHIP custom peptide microarrays

Fifteen–amino acid–long linear peptides corresponding to either MEQKLISEEDLLRKR or MEQKLISEEDLNGST and permuted at each of the underlined positions, as well as 15–amino acid–long linear peptides corresponding to either MEQKLISEEDLXXKR or MEQKLISEEDLXXST and permuted at each of the X positions, were immobilized via the C terminus on a coated glass-slide surface (www.pepperprint.com/technology/glass-slide-coatings). The resulting peptide microarray contained 952 different 15–amino acid peptides printed in duplicate on different places of the array (that is not adjacent to each other; a total of 1904 peptide spots). These spots were further framed by additional HA control peptides (YPYDVPDYAG, 98 spots), which served as internal controls for uniform incubation/signal strength over the whole peptide array. Arrays were incubated in Rockland blocking buffer MB-070 for 30 min and incubated with antibody (clone 4A6 or 9E10, both from Millipore) at 1 μg/ml in incubation buffer (PBS/0.05% Tween-20 + 10% blocking buffer) for 16 hours at 4°C. After washing three times for 1 min in PBST, arrays were incubated with goat anti-mouse IgG (H + L) DyLight 680 antibody in incubation buffer for 45 min at RT. After washing three times for 1 min in PBST, arrays were incubated with DyLight 800–coupled anti–HA tag antibody (12CA5) in incubation buffer for 45 min at RT. Images were obtained with the LI-COR Odyssey imaging system. All incubations and scans were done by PEPperPRINT GmbH.

Ethics statement

The maintenance of mice and rabbits and experimental procedures have been conducted according to the Austrian Animal Experiments Act and have been approved by the Austrian Federal Ministry of Science and Research GZ 66.009/40-Pr/4/2002 and GZ 66.009/34-BrGT/2004 and the animal experiments ethics committee of the Medical University of Vienna.

SUPPLEMENTARY MATERIALS

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Fig. S1. Clone 9E10 is greatly diminished in immunoprecipitation of Myc–NGST–PP2A Aα.

Fig. S2. Clone 9E10 shows sequence context bias toward 6xMyc-tagged proteins.

Table S1. 9E10 shows a larger signal variability than 4A6 in a single-substitution scan of Myc peptide contexts NGST or LRKR.

Table S2. 9E10 shows a larger signal variability than 4A6 in a double-substitution scan of the first two amino acid positions C terminal to Myc peptide.

Table S3. Highest- and lowest-scoring Myc context peptides in a double-substitution scan for clones 4A6 and 9E10.

View/request a protocol for this paper from *Bio-protocol*.

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The Myc tag monoclonal antibody 9E10 displays highly variable epitope recognition dependent on neighboring sequence context

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Know thy antibodies

Knowing whether a given antibody is specific for its intended target or is sensitive to posttranslational modifications of that target is critical for interpreting experimental data generated with antibody reagents. Two Research Resources from the Ogris group highlight the importance of knowing the capabilities and limitations of antibody reagents. Frohner *et al.* found that various antibodies raised against the catalytic (C) subunit of protein phosphatase 2A (PP2A) were sensitive to methylation and phosphorylation of PP2A C, cross-reacted with related phosphatases, and failed to immunoprecipitate major subsets of trimeric holoenzymes. This implies that findings using a common commercial phosphatase assay kit that relies on one of these antibodies should be reevaluated. Schüchner *et al.* found that the recognition of Myc-tagged proteins by a widely used Myc-specific antibody varied depending on sequences adjacent to the tag. A Focus by Janes highlights the importance of systemic validation of research antibodies.