Comparison of human Nrf2 antibodies: A tale of two proteins

Zachary A. Kemmerer[1,*], **Nicholas R. Ader**[1,*], **Sarah Mulroy**[1], and **Aimee L. Eggler**[1,2,*]

[1]Department of Chemistry, Villanova University, Villanova, PA

Abstract

The Nrf2 transcription factor is a master regulator of the cellular defense against oxidative and electrophilic stress. An increase in Nrf2 protein levels and an accumulation of Nrf2 in the nucleus are key parts of the Nrf2 activation mechanism. The western blot technique remains the most widely used method to assess these changes. A well-characterized, specific antibody that is commercially available would greatly enhance these studies in the field. Here, an apparently highly specific Nrf2 monoclonal antibody, EP1808Y from Abcam, is compared with the most widely used Nrf2 antibodies, H-300 and C-20, both from Santa Cruz Biotechnology, in a panel of human cell lines. In addition to detecting Nrf2, EP1808Y avidly detects another protein present in two of the three cell lines tested. This protein can be mistaken for Nrf2 as it co-migrates with verified Nrf2 on two different polyacrylamide gel types. However, unlike Nrf2, its levels and cytoplasmic localization are unaffected by treatment with Nrf2 activators. The possibility that this band corresponds to a form of Nrf2 was excluded by siRNA and immunodepletion experiments. Finally, the monoclonal antibody D1Z9C from Cell Signaling was found to detect Nrf2 with the highest specificity of these four antibodies.

1. Introduction

The cytoprotective response mediated by the Nrf2 transcription factor is one of the primary cellular defenses against toxic stresses, protecting the major organ systems against heavy metal exposure, electrophiles, nephrotoxins, and a variety of other toxins (Baird and Dinkova-Kostova, 2011; Jennings et al., 2013). The crucial role of Nrf2 in many other areas of cell health is becoming increasingly apparent (Hayes and Dinkova-Kostova, 2014). There is a diverse body of literature on Nrf2 involvement in fields ranging from antioxidant defense (Ma, 2013), metabolism (Vomhof-Dekrey and Picklo, 2012), and neurological disorders (Gao et al., 2014) to cancer (Namani et al., 2014). The number of publications in the National Center for Biotechnology Information database in which Nrf2 appears in the title or abstract is at nearly 5000 at the time of submission of this work. A substantial amount of this literature characterizes Nrf2 activity via western blot analysis, using a variety of commercially available and in-house generated primary antibodies.

It was noted early in the characterization of Nrf2 that the protein displays a reduced mobility on Tris-glycine gels, migrating at a rate of just over 100 kDa despite its molecular weight of

[2]Corresponding author. aimee.eggler@villanova.edu; 800 East Lancaster Avenue, Villanova PA, 19085.
[*]These authors contributed equally to this work.

~66 kDa (Moi et al., 1994). This aberrant mobility has muddled the field of Nrf2 research and has remained a topic of discussion (Lee et al., 2001; Venugopal and Jaiswal, 1998). Recently, Lau *et al.* reaffirmed the original conclusion of Moi *et al.* and conclusively demonstrated that the Nrf2 band has a mobility corresponding to approximately 100 kDa on Tris-glycine gels, often appearing as two bands close together (Lau et al., 2013). The identities of Nrf2 bands were established in that work by treating cells with the canonical Nrf2 activators sulforaphane (SFN) and tert-butyl hydroquinone (tBHQ), which allow Nrf2 to escape from ubiquitination and degradation, leading to accumulation of Nrf2 protein levels. The slower migrating band of the two appears to be due to phosphorylation (Apopa et al., 2008; Pi et al., 2007). The anomalously high migration and the occurrence of two bands are both resolved to a large extent by utilizing Bis-Tris gradient gels (Lau et al., 2013).

Three of the four commercially available antibodies used in Lau *et al.* have rather low specificity to Nrf2, with a number of non-specific bands detected on a blot, and one antibody has high specificity. The low-specificity antibodies include the two that are most widely used, the H-300 and C-20 polyclonal antibodies from Santa Cruz Biotechnology, which are cited in hundreds of papers. This lack of specificity can make detection of Nrf2 in whole cell lysates difficult, depending on the extent to which Nrf2 protein levels increase in response to treatment. It is likely for this reason that many publications examine only nuclear accumulation of Nrf2, as nuclear lysates have fewer proteins making these less prone to provide a non-specific result. Thus, information on total cellular accumulation of Nrf2 is often lacking. However, the fourth commercially available antibody examined in that work, EP1808Y from Abcam, has high specificity (Lau et al., 2013). A faint double band at the correct migration for Nrf2 in the lysates of HEK293 cells greatly increases in intensity upon treatment with SFN, indicating it is indeed Nrf2, and it is by far the brightest band on the full-size blot after SFN treatment. Interestingly, the Abcam product literature for the EP1808Y antibody shows highly specific detection of a band in untreated HepG2 cell lysates at approximately the correct migration for Nrf2, in the form of a single bright band on a full-sized blot[i]. Based on current understanding, Nrf2 is present at a low level in HepG2 cells prior to treatment with tBHQ or SFN, whereupon the Nrf2 protein amount significantly accumulates (Nguyen et al., 2005; Niture et al., 2009; Yueh and Tukey, 2007). Thus, detection of a bright band in the lysates of untreated cells may indicate EP1808Y is a highly avid Nrf2 antibody, or it may indicate that Nrf2 levels are unexpectedly high in that cell line.

A well-characterized antibody with high specificity to Nrf2 would be valuable to the field. Several labs are using privately made antibodies that are specific (Furusawa et al., 2014; Ludtmann et al., 2014; McMahon et al., 2003; Nguyen et al., 2005), but these are not available to the general scientific public. We sought to characterize the EP1808Y antibody, which has been cited in at least 20 publications, and compare it across a panel of cell lines with the widely used H-300 and C-20 polyclonal antibodies. Results in HepG2, a tumor-derived human liver endothelial cell line (Knowles et al., 1980), were compared to those in the HaCaT cell line, a spontaneously transformed human normal keratinocyte cell line (Boukamp et al., 1988), and the THLE-2 cell line, which is derived from normal human liver

---

[i]http://www.abcam.com/nrf2-antibody-ep1808y-ab62352.html Page last accessed on June 11, 2015.

epithelial cells and was transformed with SV40 large T antigen (Pfeifer et al., 1993). The HepG2 cell line in particular is widely used in both toxicological studies and studies of Nrf2.

2. Materials and Methods

2.1 Chemicals and reagents

All compounds were purchased from Sigma-Aldrich unless otherwise indicated. R,S-Sulforaphane (LKT Laboratories, Inc. # S8044) was dissolved in anhydrous DMSO (99.9%) to a stock concentration of 50 mM. Single-use aliquots of stock compound solutions were placed under argon and stored at −80°C. Further dilutions for experiments were done in DMSO.

Cell culture reagents, transfection reagents and M-PER lysis buffer (78501) were purchased from Life Technologies, except bronchial epithelial cell basal medium (BEBM) and BEGM SingleQuots (Lonza), and L-glutamine and fetal bovine serum (FBS) (Atlanta Biologicals). Mouse anti-β-tubulin and lamin B antibodies were purchased from Life Technologies. The anti-Nrf2 antibodies used were the polyclonals H-300 (sc-13032) and C-20 (sc-722) (Santa Cruz Biotechnologies), the EP1808Y monoclonal (Abcam ab62352) and the D1Z9C monoclonal (Cell Signaling Technology, #12721), all sourced from rabbit. The three lots tested for C-20 were H2412, E0416, and E0514. Lot H2412 was used for the blots shown. Peroxidase AffiniPure goat anti-rabbit IgG and peroxidase AffiniPure goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories.

All siRNA reagents were purchased from Santa Cruz Biotechnologies, and the siRNAs used were control siRNA A (sc-37007), Nrf2 siRNA mix (sc-37030), and individual Nrf2 siRNAs (sc-37030 A–C). The Active Motif – Nuclear Extract Kit and Pierce Anti-HA Magnetic beads were purchased from Fisher Scientific. Problock Gold protease inhibitor cocktail and human EGF were purchased from Gold Biotechnology.

2.2 Cell culture

HepG2 (ATCC #HB-8065), HaCaT (Cell Line Service; L#300493-4212), and THLE-2 (ATCC #CRL-2706 F2) cell lines were maintained at 37°C in 5% CO_2. HepG2 and HaCaT cells were grown in phenol red-free and sodium pyruvate-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, containing 2 and 4 mM L-glutamine, respectively. THLE-2 cells were grown in BEBM supplemented with BEGM SingleQuots, 70 ng/mL phosphoethanolamine, and 5 ng/mL human epidermal growth factor. All three media were supplemented with 15 mM HEPES pH 7.2. All cells were maintained between 20–80% confluency to preserve cellular integrity. Unless otherwise indicated, cells were seeded in 6-well plates at various densities (HaCaT – 5.5×10^5; HepG2 – 1.0×10^6; THLE-2 – 6.0×10^5 cells/well) in 2 mL total volume medium. Once seeded plates reached 80% confluency, cells were subjected to vehicle and activator treatment for specified times. Cells were lysed in 100 μL of lysis buffer (M-PER with final concentrations of 1 mM sodium orthovanadate and 1% protease inhibitor cocktail).

2.3 Western blot analysis

Prepared samples were loaded on a 10% Tris-glycine polyacrylamide gel (freshly prepared). Where indicated, commercially available gels were used for Bis-Tris 4–12% gradient gel analysis (Bolt, Life Technologies). Precision Plus Protein All Blue Standards (Bio-Rad) marker was included in at least two lanes on all gels. After separation by electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were blocked in 5% dry milk blocking buffer (prepared in Tris-buffered saline with 1% Tween 20). Antibody dilutions were as follows: H-300 (1:300); EP1808Y (1:1000); C-20 (1:300); D1Z9C (1:1000); anti-rabbit secondary (1:1000); β-tubulin monoclonal mouse (1:4000); lamin B1 monoclonal mouse (1:200); and anti-mouse secondary (1:1000). These dilutions were determined empirically by testing concentrations near those recommended by the manufacturer. Blots were probed using a Snap ID apparatus (Millipore). Probed blots were treated with West Dura Substrate (Thermo Fisher) to detect horseradish peroxidase on the immunoblots. Bands were visualized using a Fluorchem Q imaging system (Alpha Innotech). Markers were visualized by fluorescence and overlaid above sample bands at 40% opacity using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2015). Stripping of blots was performed by a 10 min incubation in Re-Blot Plus Mild Antibody Stripping Solution (Millipore). The order of probing of a given blot with Nrf2 antibodies was as follows: either H-300, C-20, EP1808Y, or H-300, D1Z9C.

2.4 Cellular fractionation

HepG2 cells were plated in 60 mm dishes at 2.21×10^6 cells/dish in 3 mL total volume medium. After 24 h of growth, cells were treated with vehicle or activator for 2 h. Cells were then scraped from each dish and fractionated using the Active Motif – Nuclear Extract Kit with the provided protocol. Cytoplasmic and nuclear samples were prepared and loaded for western blot analysis.

2.5 Antibody Immunodepletion

Cells treated with activator as specified were lysed, and 20 μL was mixed with either M-PER (control) or antibody (2 μL H-300, 2 μL Abcam (3 μL for HepG2 lysates), or 6 μL C-20) and M-PER, up to 26 μL final volume for each. The lysate-antibody mixture was incubated for 1 h at 4°C while rotating. Next 7.5 μL Red Affinity beads (Sigma-Aldrich) that had been washed with M-PER were added to each solution, including the control without antibody. Samples were incubated for 1 h at 4°C while rotating. The mixture was then centrifuged at 5,200 ×g for 30 sec to separate the bead immunoprecipitation complex from the supernatant, and the supernatant was then analyzed by western blotting.

2.6 Reverse transfection for Nrf2 silencing

Transfection mixes (40 μL) were prepared for six different conditions (no siRNA, control siRNA, Nrf2 siRNA mix, and three individual Nrf2 transcripts) by mixing siRNA (45 nM total Nrf2/control siRNA or 15 nM individual Nrf2 siRNAs) with OptiMEM and then adding 8 μL Lipofectamine 3000 for a final volume of 40 μL. After a 5 min incubation, mixes were added to a 24-well plate. Immediately following, 460 μL of HepG2 cells in

DMEM (0.8×10^5 cells/well) were added to each well. The plate was rocked to mix and incubated for 24 h. After incubation, transfection mix was replaced with 2 mL fresh medium, followed by immediate treatment by vehicle or activator for 2 h. Cells were harvested with 25 µL lysis buffer and prepared for western blot analysis.

3. Results

3.1. EP1808Y detects a band in HepG2 that is independent of SFN treatment

The specificity and accuracy of the EP1808Y (monoclonal) and H-300 and C-20 (both polyclonal) antibodies were compared in three human cell lines. Lysates of cells either treated with vehicle alone (herein referred to as untreated) or treated with SFN for two hours were analyzed on 10% Tris-glycine SDS polyacrylamide gels followed by western blotting. As shown in Fig. 1A, a band was detected by EP1808Y in both untreated and treated HepG2 cell lysates just above 100 kDa, the expected migration of Nrf2 (Lau et al., 2013). This migration rate for Nrf2 was confirmed using *in vitro* translated full-length Nrf2 (Fig. S1A). Unexpectedly, this band in HepG2 lysates is remarkably brighter than the protein bands detected at a similar molecular weight by EP1808Y in the other two cell lines despite equal amounts of total protein present, as indicated by the β–tubulin control. Examination of the same blot, at full-length, (Fig. 1B, center) shows this band is significantly more intense than any other band in HepG2 lysates. The brightness in Fig. 1B has been adjusted to allow visualization of bands in both HaCaT and THLE-2 lanes. Importantly, for both HaCaT and THLE-2 lysates, band intensity detected by EP1808Y in the region just above 100 kDa increases after SFN treatment. This SFN-dependent increase suggests these bands indeed correspond to Nrf2 as previously demonstrated (Lau et al., 2013). However, while both bands increase in HaCaT lysates, only the upper band at ~120 kDa in THLE-2 increases, and the lower band at ~105 kDa remains unchanged (Fig. 1B, center). The intensity of the band detected at ~105 kDa in HepG2 cell lysates remains unchanged in response to SFN treatment (Fig. 1A), raising the question of whether this band is indeed Nrf2. H-300 antibody detection of the blot (Fig. 1B) displays expected increases in visual intensity for protein bands at 105 and 120 kDa in all cell lines when treated with SFN. C-20 antibody detection of this same blot (Fig. 1B, right) displays no increase in protein bands intensity for treated lysates across all cell lines, indicating the bands detected may not correspond to Nrf2.

3.2. The band detected by EP1808Y in HepG2 cell lysates co-migrates with Nrf2 on Bis-Tris gels

One explanation for detection of this band in HepG2 and THLE-2 cell lysates by the EP1808Y antibody may be that a different, co-migrating protein is also detected by this antibody. As the Nrf2 protein migrates at a much faster rate on Bis-Tris gradient gels, we attempted to separate this band from the confirmed Nrf2 band on a Bis-Tris gel. However, as shown in Fig. 2, the bright band detected in untreated HepG2 lysates by the EP1808Y antibody migrates at the same rate on the gradient gels as Nrf2 (~80 kDa). The migration rate of Nrf2 on this gel type, as for the Tris-glycine gel type, is established by the increased band intensity upon SFN treatment, detected in HaCaT lysates by the EP1808Y antibody (Fig. 2, center) and by the H-300 antibody in all three cell lines (Fig. 2, left). A similar result

was obtained for the THLE-2 cell line as for the HepG2 cell line, in that a band at the correct migration rate for Nrf2 is detected by EP1808Y in untreated cell lysates at a similar intensity to the band detected in SFN-treated cell lysates (Fig. 2, center). In contrast, the primary bands detected by the C-20 antibody do not co-migrate with Nrf2, and there is no increase in the band at ~80 kDa in response to SFN treatment (Fig. 2, right).

Given the avid detection of a band in HepG2 cell lysates by EP1808Y, with few other non-specific bands, and the co-migration of this band with verified Nrf2 on two gel types, the data suggest that the band may be a form of Nrf2 that is not detected by H-300.

3.3. The protein detected by EP1808Y in HepG2 cells is located solely in the cytoplasm

The canonical mechanism of Nrf2 activation results in nuclear accumulation of Nrf2. To determine if the band detected by the EP1808Y antibody in HepG2 lysates translocates into the nucleus upon treatment with Nrf2 activators, nuclear and cytoplasmic extracts were prepared from cells treated with tBHQ or SFN. These extracts, along with whole cell lysates, were analyzed by Tris-glycine SDS PAGE followed by western blotting (Fig. 3). As expected, Nrf2 bands are detected by H-300 only weakly in the cytoplasmic extract compared to nuclear extracts, and these bands increase in intensity upon treatment with tBHQ or SFN. Notably, the bright band detected by EP1808Y does not appear in the nuclear fractions even after treatment with Nrf2 activators. Interestingly, within the nucleus, EP1808Y detects Nrf2 in a manner practically identical to the H-300 antibody. This finding corresponds with the ability of these antibodies to detect recombinant or overexpressed Nrf2 protein. When used to detect purified *in vitro* translated full-length Nrf2, or HA-Nrf2 overexpressed and purified from HepG2 cells, H-300 and EP1808Y detect Nrf2 with similar avidity (Fig. S1A and S1B). Fig. S1B also illustrates the close co-migration of the band detected by EP1808Y in HepG2 lysates with the lower Nrf2 band. The lack of nuclear translocation by the bright band suggests that if this band is a form of Nrf2, it is localized specifically to the cytoplasm and does not respond to Nrf2 activators.

3.4. Immunodepletion and siRNA silencing strongly suggest the protein detected by EP1808Y in HepG2 cells is not Nrf2

The above results indicate there may be a cytoplasmic form of Nrf2 highly abundant in HepG2 cells that is not detected by H-300. Possible reasons why this form of Nrf2 would not be detected by H-300 are diagrammed in Fig. 4A. The immunogen used to raise the H-300 antibody in rabbits covers a major portion of the N-terminus of human Nrf2 (residues 37–336)[ii], and the possibilities would require that a smaller region contains the actual major epitope(s) for this polyclonal antibody. One possibility is that the putative Nrf2 protein in HepG2 has a high number of post-translational modifications (PTMs) in the H-300 epitope(s), which block H-300 binding, whereas the C-terminal epitope for EP1808Y would remain relatively free of PTMs, allowing EP1808Y to detect the highly modified Nrf2 protein. Alternatively, there are three known splice variants of Nrf2. Nrf2 isoforms 1 and 2 both contain the complete H-300 immunogen sequence, whereas isoform 3 is missing residues 135–141 of the immunogen (numbering corresponds to isoform 1) (Fig. 4A, black).

[ii]http://www.scbt.com/datasheet-13032-nrf2-h-300-antibody.html Page last accessed on June 11, 2015.

It is possible that the major point of recognition for polyclonal H-300 involves this missing segment of isoform 3, thus limiting its detection by H-300. Therefore, an alternative explanation for the prominent band detected by EP1808Y in HepG2 is an abundance of Nrf2 isoform 3. In order to determine if the protein is an Nrf2 isoform or the result of nonspecific binding, we took two approaches: immunodepletion of Nrf2 with each of the three antibodies and treatment of cells with siRNA to assess Nrf2 knockdown.

For the immunodepletion experiments, the C-20 epitope is reported by the manufacturer to map to the C-terminus of the protein[iii] but is otherwise undefined. Thus, if the C-20 antibody is proficient at immunodepletion, it will likely deplete the band detected by EP1808Y if this band corresponds to an isoform of Nrf2 that is either spliced or post-translationally modified in the region of the H-300 epitope. Nrf2 levels were maintained at stimulated levels in each of the three cell lines by treatment with either SFN or MG132, a proteasome inhibitor that blocks Nrf2 degradation. In HaCaT lysates (Fig. 4B, left) each of the three antibodies successfully immunodepleted the bands corresponding to Nrf2 as detected on a western blot by both H-300 and EP1808Y, whether stabilized by SFN or by MG132. Thus, C-20 and the other two antibodies were efficient at immunodepletion. (The band detected by C-20 at ~120 kDa is confirmed to be non-specific, as it is not depleted by any of the antibodies including C-20.) Notably, in the HepG2 cell line, only the EP1808Y antibody was able to deplete the bright band detected by EP1808Y, whereas the bands detected by H-300 could be immunodepleted by all three antibodies, as in HaCaT. A similar result was observed in THLE-2 cells. The inability of C-20 or H-300 to immunodeplete this band suggests that this band is not a form of Nrf2.

To further explore whether the band strongly detected by EP1808Y in HepG2 cells is Nrf2 or another protein, we employed three siRNAs that collectively flank the segment of Nrf2 mRNA that encodes the EP1808Y epitope (Fig. 4A), based on the antigen peptide CVFLVPKSKKPDVKKN listed on the product website[i]. It follows that silencing via recognition of these three sites on Nrf2 mRNA would target any Nrf2 protein containing the EP1808Y epitope. This configuration should thus allow any unknown alternatively spliced isoforms of Nrf2 that may be detected by EP1808Y to be knocked down, as well as Nrf2 that may be significantly post-translationally modified. As shown in Fig. 4C, all of the siRNAs, alone or in combination, were able to knock down Nrf2 as detected by the H-300 antibody. However, none of the siRNAs, alone nor in combination, were able to knock down the EP1808Y band. These data together with those from the immunodepletion experiments provide strong evidence that the bright band detected by the monoclonal EP1808Y antibody is not a form of Nrf2. We note that similar results were obtained with the Nrf2 S40-phospho specific antibody from Abcam, EP1809Y (Supplementary Results and Supplementary Fig. S2).

3.5. Evaluation of a fourth Nrf2 antibody from Cell Signaling Technology

The original goal of this work was to characterize an antibody (EP1808Y) that could potentially provide highly specific detection of human Nrf2 by western blot. We found that

---

[iii]http://www.scbt.com/datasheet-722-nrf2-c-20-antibody.html Page last accessed on June 11, 2015.

while EP1808Y does specifically detect human Nrf2, it also avidly detects another protein that co-migrates with Nrf2, present in some but not all human cell lines. Towards the original goal, we evaluated a relatively new commercial monoclonal Nrf2 antibody from Cell Signaling Technology, D1Z9C, against our panel of human cell lines. The immunogen for the D1Z9C antibody is a peptide from human Nrf2 containing Ala275[iv]. This antibody has been evaluated in *Nrf2*^{+/+} or *Nrf2*^{−/−} mouse astrocytes treated with MG132, tBHQ, or SFN, and it shows high specificity to mouse Nrf2 (Deighton et al., 2014). The product literature indicates the antibody is also specific to human Nrf2 in U-2 OS cells using MG132 treatment[iv]. Further, evaluating a fourth Nrf2 antibody in our panel of cell lines provides a third way to confirm that the band detected strongly by EP1808Y in HepG2 cells is not the Nrf2 protein.

We compared the D1Z9C antibody to the H-300 antibody in each of the three cell lines, both untreated and treated with SFN. As shown in Fig. 5, H-300 and D1Z9C both detect bands at 105 and 120 kDa whose intensity greatly increases in response to SFN treatment in all three cell lysates. This result further substantiates that a protein other than Nrf2 is strongly detected by EP1808Y in HepG2 cells. These bands detected by D1Z9C were confirmed to be Nrf2, as they were knocked down by treatment with either of two individual Nrf2 siRNAs (Fig. S3). D1Z9C detects Nrf2 with similar intensity when compared to H-300, but shows fewer nonspecific bands overall (Fig. 5). Thus, D1Z9C accurately detects Nrf2 with high specificity, and would serve as the best option amongst the four antibodies evaluated.

4. Discussion

As for many proteins, the cellular mechanisms of Nrf2 activation have been characterized in large part through western blot analysis. The field has been lacking a well-characterized, commercially available, and specific antibody with which to detect Nrf2. We sought to characterize the detection of Nrf2 by the monoclonal antibody EP1808Y from Abcam and compare it with the H-300 and C-20 antibodies from Santa Cruz Biotechnology. The HaCaT, HepG2, and THLE-2 lines provide an array of cell types and transformative stages in which to compare these antibodies. We find that while EP1808Y is a competent antibody in the HaCaT cell line, a bright band that electrophoretically imitates Nrf2 is present in the whole cell lysates of HepG2 cells, and to a lesser extent in THLE-2 cell lysates as well. Although this band migrates at the same molecular weight as Nrf2 on two types of polyacrylamide gels, treatment with Nrf2 activators, immunodepletion, and siRNA silencing experiments all indicate that this bright band is not Nrf2 protein or an alternatively spliced form of Nrf2. However, EP1808Y does both immunodeplete Nrf2 and accurately detect Nrf2 in nuclear extracts. An alternative monoclonal antibody for use in whole cell lysates, D1Z9C from Cell Signaling, accurately detects Nrf2 in all three cell lines with higher specificity than H-300.

The C-20 antibody has been used for approximately 15 years, and it is cited in more than one hundred references (listed on the product's website [iii]), many in the context of western blotting. Initially the strong detection of a band in HepG2 lysates near the correct migration

---

[iv]http://www.cellsignal.com/product/productDetail.jsp?productId=12721 Page last accessed June 11, 2015.

rate for Nrf2 by C-20, as well as EP1808Y, suggested that a form of Nrf2 was present in HepG2 that did not increase in response to SFN. However, this relatively bright band detected by C-20 does not co-migrate with Nrf2 on Bis-Tris gradient gels and is not knocked down by treatment with Nrf2 siRNA. Further, examination of the gradient gel (Fig. 2) indicates no increase in intensity of a band at the correct rate of migration for Nrf2, ~80 kDa. The inability of C-20 to detect Nrf2 in treated cell lysates was unexpected. Using the C-20 antibody, Lau *et al.* saw an increase in intensity of a band at the correct migration rate for Nrf2 upon SFN treatment of HEK293 cells. Similarly, Zucker *et al.* showed correct detection of Nrf2 in NIH3T3 cells by C-20, validated with SFN and Nrf2 siRNA treatments (Zucker et al., 2014). We note that C-20 is capable of detecting recombinant Nrf2 protein by western blot, but with a greatly reduced affinity compared to H-300 and EP1808Y (Fig. S1). Three lots of C-20 were evaluated using HepG2 cell lysates and 10% Tris-glycine gels, and each lot showed no increase in intensity of a band in the region from 105–120 kDa (data not shown). Thus the inability of C-20 to correctly detect Nrf2 in the cell lysates we evaluated is likely due to weak affinity by western blot, and possibly lot to lot antibody variability or lower induced levels of Nrf2 in the cell lines tested here. We also note that the C-20 antibody is nonetheless proficient at immunodepleting Nrf2.

In the literature, it is often difficult to discern how Nrf2 western blot results were obtained, due to incomplete Nrf2 antibody procedural annotation. Methods regularly state the company an antibody is from without specifying the product number, or two Nrf2 antibodies are listed, such as H-300 and C-20, without reporting which was used in a given experiment. Given the wide-ranging abilities of Nrf2 antibodies to accurately detect Nrf2, and the possibility for the most intense band on a full blot to masquerade as Nrf2, we advocate for the specification of an Nrf2 antibody product number for each experiment. In addition, it seems judicious to validate Nrf2 antibodies in a given cell lysate by treatment with SFN, tBHQ and/or Nrf2 knockdown, showing full-length blots with markers, to demonstrate their unambiguous identification of Nrf2. Various groups have adopted these practices (*e.g.,* (Deighton et al., 2014; Lau et al., 2013; Nguyen et al., 2005)). (MG132 treatment is less ideal due to its effect of increasing levels of any protein degraded by the proteasome.) These practices reflect a recent broader call in the life sciences to improve reproducibility of results by validating antibodies in each assay of interest and by reporting antibody usage details (Baker, 2015).

Finally, although the bright band detected by EP1808Y in HepG2 cell lysates is not Nrf2, it is intriguing that this monoclonal antibody would detect such a bright band that co-migrates with Nrf2 across gel types. A BLAST search of the antigen peptide, CVFLVPKSKKPDVKKN, resulted only in the three alternatively spliced Nrf2 transcripts. As this bright band appears to be most abundant in HepG2, the most fully transformed of these three cell lines, this protein may be overexpressed in the course of transformation. Interestingly, the intensity of the band in THLE-2 cell lysates was observed to increase when cells were grown to overconfluency prior to harvesting (data not shown). Efforts to identify this protein band avidly detected by EP1808Y in HepG2 lysates are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

BEBM	bronchial epithelial cell basal medium
FBS	fetal bovine serum
PVDF	polyvinylidene difluoride
PTM	post-translational modifications
SFN	sulforaphane
tBHQ	tert-butyl hydroquinone

References

Apopa PL, He X, Ma Q. Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. J Biochem Mol Toxicol. 2008; 22:63–76.10.1002/jbt.20212 [PubMed: 18273910]

Baird L, Dinkova-Kostova A. The cytoprotective role of the Keap1–Nrf2 pathway. Arch Toxicol. 2011; 85:241–272.10.1007/s00204-011-0674-5 [PubMed: 21365312]

Baker M. Reproducibility crisis: Blame it on the antibodies. Nature. 2015; 521:274–276.10.1038/521274a [PubMed: 25993940]

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. The Journal of Cell Biology. 1988; 106:761–771.10.1083/jcb.106.3.761 [PubMed: 2450098]

Deighton RF, Markus NM, Al-Mubarak B, Bell KF, Papadia S, Meakin PJ, Chowdhry S, Hayes JD, Hardingham GE. Nrf2 target genes can be controlled by neuronal activity in the absence of Nrf2 and astrocytes. Proc Natl Acad Sci U S A. 2014; 111:E1818–1820.10.1073/pnas.1402097111 [PubMed: 24753562]

Furusawa Y, Uruno A, Yagishita Y, Higashi C, Yamamoto M. Nrf2 induces fibroblast growth factor 21 in diabetic mice. Genes Cells. 2014; 19:864–878.10.1111/gtc.12186 [PubMed: 25270507]

Gao B, Doan A, Hybertson BM. The clinical potential of influencing Nrf2 signaling in degenerative and immunological disorders. Clin Pharmacol. 2014; 6:19–34.10.2147/cpaa.s35078 [PubMed: 24520207]

Hayes JD, Dinkova-Kostova AT. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. Trends Biochem Sci. 2014; 39:199–218.10.1016/j.tibs.2014.02.002 [PubMed: 24647116]

Jennings P, Limonciel A, Felice L, Leonard M. An overview of transcriptional regulation in response to toxicological insult. Arch Toxicol. 2013; 87:49–72.10.1007/s00204-012-0919-y [PubMed: 22926699]

Knowles BB, Howe CC, Aden DP. Human Hepatocellular Carcinoma Cell Lines Secrete the Major Plasma Proteins and Hepatitis B Surface Antigen. Science. 1980; 209:497–499.10.2307/1685123 [PubMed: 6248960]

Lau A, Tian W, Whitman SA, Zhang DD. The predicted molecular weight of Nrf2: it is what it is not. Antioxid Redox Signal. 2013; 18:91–93.10.1089/ars.2012.4754 [PubMed: 22703241]

Lee JM, Moehlenkamp JD, Hanson JM, Johnson JA. Nrf2-dependent activation of the antioxidant responsive element by tert-butylhydroquinone is independent of oxidative stress in IMR-32 human neuroblastoma cells. Biochem Biophys Res Commun. 2001; 280:286–292. [PubMed: 11162512]

Ludtmann MH, Angelova PR, Zhang Y, Abramov AY, Dinkova-Kostova AT. Nrf2 affects the efficiency of mitochondrial fatty acid oxidation. Biochem J. 2014; 457:415–424.10.1042/bj20130863 [PubMed: 24206218]

Ma Q. Role of Nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol. 2013; 53:401–426.10.1146/annurev-pharmtox-011112-140320 [PubMed: 23294312]

McMahon M, Itoh K, Yamamoto M, Hayes JD. Keap1-dependent Proteasomal Degradation of Transcription Factor Nrf2 Contributes to the Negative Regulation of Antioxidant Response Element-driven Gene Expression. J Biol Chem. 2003; 278:21592–21600.10.1074/jbc.M300931200 [PubMed: 12682069]

Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proceedings of the National Academy of Sciences. 1994; 91:9926–9930.

Namani A, Li Y, Wang XJ, Tang X. Modulation of NRF2 signaling pathway by nuclear receptors: implications for cancer. Biochim Biophys Acta. 2014; 1843:1875–1885.10.1016/j.bbamcr. 2014.05.003 [PubMed: 24851839]

Nguyen T, Sherratt PJ, Nioi P, Yang CS, Pickett CB. Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1. J Biol Chem. 2005; 280:32485–32492.10.1074/jbc.M503074200 [PubMed: 16000310]

Niture SK, Jain AK, Jaiswal AK. Antioxidant-induced modification of INrf2 cysteine 151 and PKC-delta-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance. J Cell Sci. 2009; 122:4452–4464. [PubMed: 19920073]

Pfeifer AM, Cole KE, Smoot DT, Weston A, Groopman JD, Shields PG, Vignaud JM, Juillerat M, Lipsky MM, Trump BF. Simian virus 40 large tumor antigen-immortalized normal human liver epithelial cells express hepatocyte characteristics and metabolize chemical carcinogens. Proceedings of the National Academy of Sciences. 1993; 90:5123–5127.

Pi J, Bai Y, Reece JM, Williams J, Liu D, Freeman ML, Fahl WE, Shugar D, Liu J, Qu W, Collins S, Waalkes MP. Molecular mechanism of human Nrf2 activation and degradation: Role of sequential phosphorylation by protein kinase CK2. Free Radic Biol Med. 2007; 42:1797–1806. [PubMed: 17512459]

Venugopal R, Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. Oncogene. 1998; 17:3145–3156. [PubMed: 9872330]

Vomhof-Dekrey EE, Picklo MJ Sr. The Nrf2-antioxidant response element pathway: a target for regulating energy metabolism. J Nutr Biochem. 2012; 23:1201–1206.10.1016/j.jnutbio. 2012.03.005 [PubMed: 22819548]

Yueh MF, Tukey RH. Nrf2-Keap1 signaling pathway regulates human UGT1A1 expression in vitro and in transgenic UGT1 mice. J Biol Chem. 2007; 282:8749–8758.10.1074/jbc.M610790200 [PubMed: 17259171]

Zucker SN, Fink EE, Bagati A, Mannava S, Bianchi-Smiraglia A, Bogner PN, Wawrzyniak JA, Foley C, Leonova KI, Grimm MJ, Moparthy K, Ionov Y, Wang J, Liu S, Sexton S, Kandel ES, Bakin AV, Zhang Y, Kaminski N, Segal BH, Nikiforov MA. Nrf2 amplifies oxidative stress via induction of Klf9. Mol Cell. 2014; 53:916–928.10.1016/j.molcel.2014.01.033 [PubMed: 24613345]
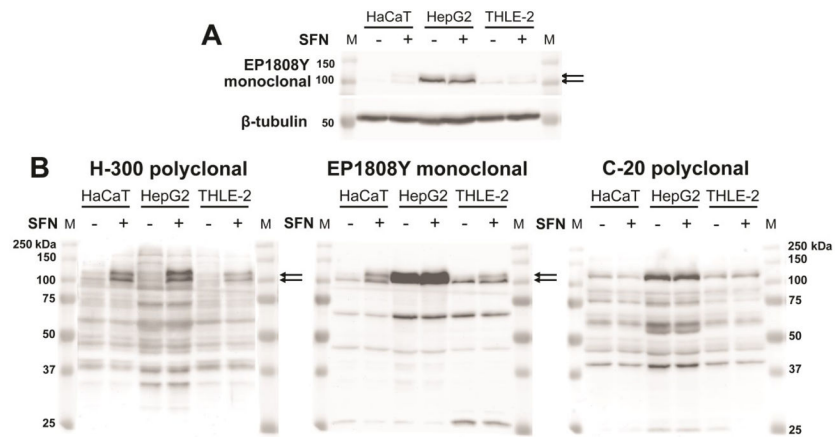
Fig. 1. Comparison of EP1808Y monoclonal antibody with two commonly used Nrf2 polyclonal antibodies

Cells were treated with 5 μM SFN for 2 h, and whole cell lysates were analyzed by 10% Tris-glycine SDS PAGE and western blotting. (A) Detection using the EP1808Y antibody at a brightness level that illustrates a single band is detected in HepG2 cell lysates and unchanged by SFN treatment. (B) The truncated blot in *A* is shown at full length (center), and brightness is increased to show detection in HaCaT and THLE-2 lysates. The same blot was probed with H-300 (left) and C-20 (right) antibodies. Arrows indicate the positions at which Nrf2 migrates. M, marker. Blots are representative of n = 3 biological replicates.
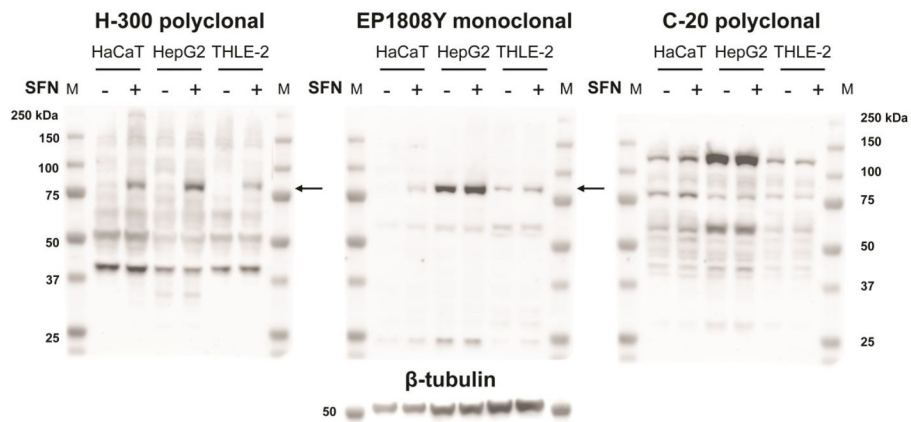
Fig. 2. Nrf2 band migration rate on Bis-Tris gradient gels

Cells were treated with 5 μM SFN for 2 h, and whole cell lysates were analyzed by 4–12% Bis-Tris SDS PAGE and western blotting. The same blot was probed with H-300 (left), EP1808Y (center), and C-20 (right) antibodies. Arrows indicate the positions at which Nrf2 migrates. Blots are representative of n = 2 biological replicates.
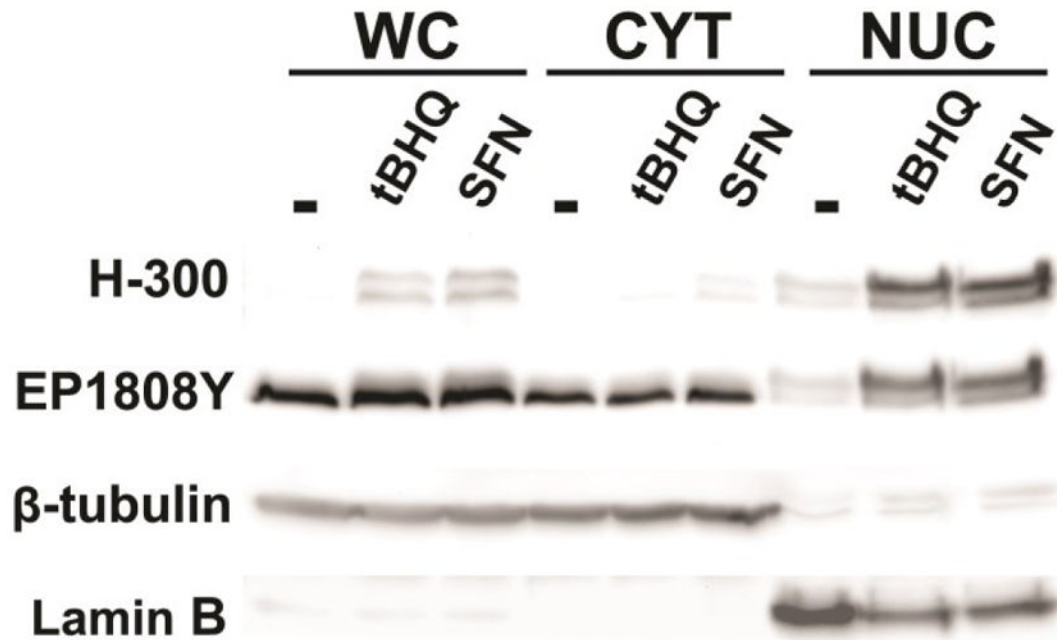
Fig. 3. Cellular localization of protein detected by EP1808Y monoclonal antibody in HepG2 cells
Cells were treated with 50 µM tBHQ or 5 µM SFN for 2 h, and whole cell lysates were subjected to nuclear/cytoplasmic fractionation. Whole cell lysates and fractions were analyzed by 10% Tris-glycine SDS PAGE and western blotting. The same blot was probed with H-300 and EP1808Y antibodies. Blots are representative of n = 3 biological replicates.
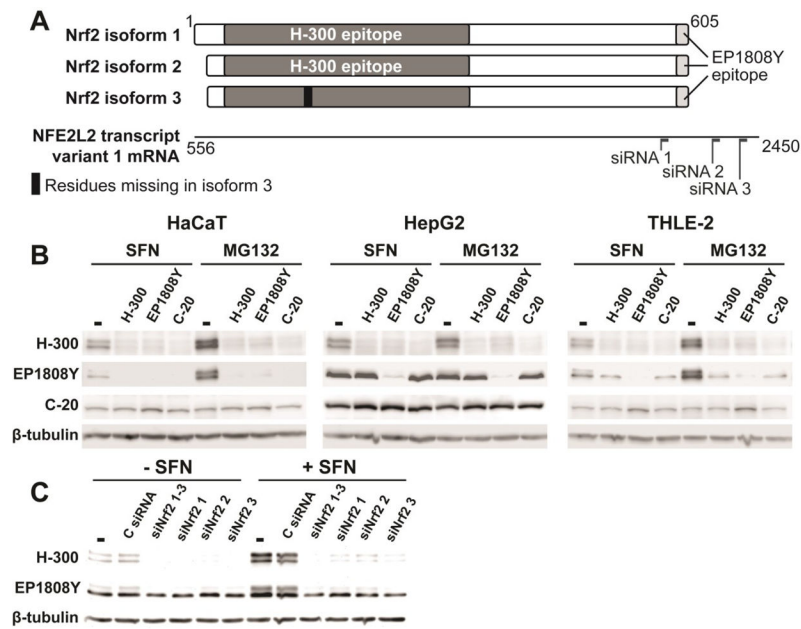
Fig. 4. Immunodepletion and Nrf2 knockdown used to assess the primary band detected in HepG2 cells by EP1808Y

(A) Schematic of the three Nrf2 isoforms from the three known alternatively-spliced transcripts with respect to H-300 and EP1808Y antibody epitopes. The transcript numbering aligns with that of the protein isoforms. Three commercially available siRNAs for Nrf2 mRNA target the indicated sites on the transcript. (B) HepG2 cells were treated with 5 μM SFN or 5 μM MG132 for 2 h, and whole cell lysates were immunodepleted with indicated antibodies, or beads alone (–). Depleted lysates were then analyzed by 10% Tris-glycine SDS PAGE and western blotting. The same blot was probed with H-300 and EP1808Y antibodies. Blots are representative of n = 3 biological replicates. (C) HepG2 cells were reverse transfected with siRNA (10 μM individual, 30 μM combined) for 24 h and then were treated with 5 μM SFN for 2 h. Whole cell lysates were analyzed by 10% Tris-glycine SDS PAGE and western blotting. The same blot was probed with H-300 and EP1808Y antibodies. C siRNA, control siRNA. Blot is representative of n = 2 biological replicates.
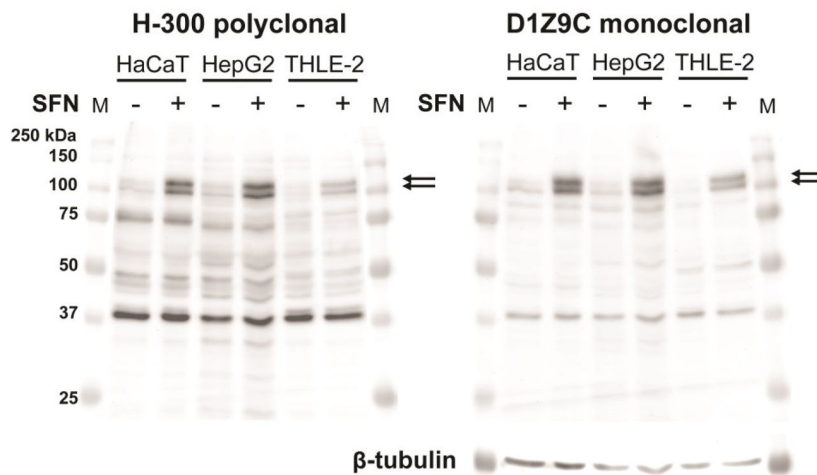
Fig. 5. Comparison of D1Z9C monoclonal antibody with H-300 in the panel of cell lines
Cells were treated with SFN, as indicated for 2 h, and whole cell lysates were analyzed by 10% Tris-glycine SDS PAGE and western blotting. Arrows indicate the location of bands that correspond to Nrf2 as indicated by their increase in response to SFN. Blots are representative of n = 2 biological replicates.