LETTER TO THE EDITOR

The Predicted Molecular Weight of Nrf2: It Is What It Is Not

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Dear Editor:

The transcription factor NF-E2-related factor 2 (Nrf2) is rapidly being recognized as a critical regulator of the cellular stress response. Explosions of publications in the field are investigating the role of Nrf2 in disease prevention and progression; however, this rapid expansion is coming at a cost. As new investigators break into the emerging field of Nrf2 research, confusion regarding the correct migratory pattern of Nrf2 is causing doubts about the accuracy and reproducibility of published results. This letter provides solid evidence that the biologically relevant molecular weight of Nrf2 is ~95–110 kilodalton (kDa) and not the predicted ~55–65 kDa based on its 2-kb open reading frame. The data discussed and presented here will hopefully lead to a uniform acceptance that future experiments and publications should be designed around detecting Nrf2 at the apparent molecular weight of ~95–110 kDa.

Since its discovery over a decade ago, Nrf2 has emerged as one of the cardinal transcription factors for the adaptive stress response. The Nrf2 pathway reaches broadly across many systems of biology and is involved in the prevention and pathogenesis of multiple complex human diseases such as cancer, diabetes, and cardiovascular and neurodegenerative diseases. The field of Nrf2 biology has exponentially grown over the past decade. Subsequently, the accurate reporting of Nrf2 molecular weight is a major issue that has arisen with the influx of researchers entering the Nrf2 field from other disciplines.

The common misconception relating to Nrf2 is that it migrates at a predicted molecular weight of ~55–65 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). This prediction is made according to Nrf2's open reading frame size of ~2.2-kb. In this letter, we provide evidence through chemical activation, vector driven mammalian expression, and recombinant protein expression that in fact, the biologically relevant species of Nrf2 migrates between ~95 and 110 kDa.

As a reviewer for journals and grants, it is worrisome to see that many researchers are reporting an incorrect migratory species of Nrf2. Furthermore, I receive weekly inquiries from investigators both nationally and internationally regarding the “abnormal” migration of Nrf2 from its predicted size. To remove the current confusion in the field, we want to make this information publically available in a highly credible, broadly

impactful journal. This report is crucial to allow investigators to report accurate and reliable data and to progress the field.

Nrf2 is a member of the cap ‘n’ collar subfamily of basic-region leucine zipper transcription factors that was first identified, cloned, and characterized in 1994 (5). Nrf2 knockout mice appear to be normal and fertile, indicating that Nrf2 is not essential for the normal development of mice (1).

Over the past decade, much of the molecular mechanisms regulating the Nrf2 pathway have been broadly elucidated. The substrate adaptor protein Kelch-like ECH-associated protein 1 (Keap1) forms an E3 ubiquitin ligase complex with Cullin 3 (Cul3) and RING-box protein 1 (Rbx1) to negatively regulate Nrf2 protein levels. Under unstressed conditions, the Keap1-Cul3-Rbx1 E3 ubiquitin ligase complex conjugates ubiquitin onto Nrf2, leading to its degradation by the 26S proteasome to maintain low basal levels of Nrf2. Upon exposure to electrophiles or oxidative stress, essential cysteine residues in Keap1 act as sensors and are modified, resulting in stabilization of Nrf2. Subsequently, Nrf2 enters the nucleus and heterodimerizes with a small Maf protein to activate transcription of targets bearing an antioxidant response element in the promoter. These cytoprotective genes encode for phase II detoxifying enzymes, intracellular redox-balancing proteins, and transporters (4).

Nrf2 is known to be the master regulator of a major cellular defense mechanism due to its ability to eliminate toxicants or carcinogens and reinstate cellular homeostasis. Therefore, activation of Nrf2 by small molecules and natural products has been shown to protect against a variety of human diseases. The beneficial role of Nrf2 inducers in preventing or alleviating pathological alterations by harmful substances has been demonstrated using various murine disease models. More importantly, several clinical trials have yielded promising results using broccoli sprouts to activate Nrf2 to prevent aflatoxin-induced liver cancer (3).

It was not until 2008, when the “dark” side of Nrf2 was revealed (7). Somatic mutations in Nrf2 or Keap1, and loss of Keap1 expression, were found in different types of tumors that allow Nrf2 to escape Keap1-mediated degradation, thus causing constitutive activation of Nrf2. Furthermore, high basal levels of Nrf2 have been proven to contribute to both intrinsic and acquired chemoresistance in cancer.

Nrf2 is widely expressed at low basal levels in all tissues. Its transcript is 2.2-kb, which is predicted to be a ~66-kDa protein.

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However, the very first *in vitro* transcribed and translated protein from full-length Nrf2 cDNA showed not only a band at ~66 kDa, but also at ~96 kDa (5). In the following year, Yamamoto's group made an antibody against the basic region leucine-zipper domain of Nrf2 (ECH) (2). This antibody detected bands at ~63 and ~97 kDa when the cDNA for the ECH domain was expressed *in vitro* (2). The first use of a commercial antibody from Santa Cruz Biotechnology detected two bands at 66 and 110 kDa (6). Now, mounting evidence supports the biologically relevant size Nrf2 to be ~95–110 kDa under reduced and denatured conditions. The nature of the aberrant migration pattern of Nrf2 is still uncertain. However, the abundance of

acidic residues in Nrf2 may offer an explanation for the unpredicted migration of Nrf2.

The dual role of Nrf2 in cancer and other diseases has piqued the interest of an interdisciplinary group of researchers causing the field to exponentially grow over the last few years. However, this aberrant migration of Nrf2 has caused major controversy and confusion in the field. Most commercial antibody sources indicate Nrf2 protein to be at its predicted molecular weight of ~55–65 kDa, which has caused investigators to litter the literature with somewhat misleading data and snowballing this molecular weight mystery. Therefore, the intention of this commentary is to make clear that the

FIG. 1. NF-E2-related factor 2 (Nrf2) migrates at ~95–110 kDa. (a) Vector alone (V) or hemagglutinin (HA)-tagged Nrf2 was transfected into human kidney epithelial (HEK293) cells. Cells were either not treated (N) or treated with 25 μ M tert-butylhydroquinone (tBHQ) (T) for 4 h. An antibody specific for HA was used to detect HA-Nrf2. (b) GST-tagged Nrf2 purified from *Escherichia coli* and GST-cleaved Nrf2 was run on SDS-PAGE gels and either Coomassie stained or analyzed by Western blot using the Nrf2 H300 antibody from Santa Cruz. (c) MDA-MB-231, HEK293, HeLa, and COS1 cells were left untreated (N) or treated with either 5 μ M sulforaphane (S) or 25 μ M tBHQ (T) for 4 h. Nrf2 was detected using the Nrf2 H300 antibody. (d) Mouse embryonic fibroblast cells were isolated from Nrf2^{+/+}, Nrf2^{+/-}, and Nrf2^{-/-} mice and either left untreated or treated with 5 μ M sulforaphane (S) for 4 h. (e) HEK293 cells either untreated (N) or treated with sulforaphane (S) for 4 h were subjected to immunoblot analysis using the indicated commercial antibodies. Asterisk (*) denotes Nrf2 protein band.

molecular weight of Nrf2 ranges from ~95 to 110 kDa depending upon composition of the SDS-PAGE gel used.

Here, we provide strong evidence that Nrf2 indeed migrates at ~95–110 kDa. Immunoblot analysis with overexpressed hemagglutinin-tagged Nrf2 shows a prominent protein band at ~110 kDa and 95 kDa on a 7.5% SDS-PAGE and a commercial 4%–12% gradient gel, respectively (Fig. 1a). The well-known Nrf2 activator, tert-butylhydroquinone (tBHQ), increased these ~110 and 95 kDa band intensities, further confirming that the ~95–110 kDa signal is indeed Nrf2 (Fig. 1a). A band of similar size was also detected when Nrf2 protein was produced and purified from *Escherichia coli*. Purified GST-Nrf2 or GST-cleaved Nrf2 was detected at ~80–130 kDa, by either Coomassie blue staining or immunoblot analysis using an Nrf2 antibody (Santa Cruz [SC]-H300) (Fig. 1b). Endogenous Nrf2 in a variety of cell lines was also detected at ~95–110 kDa (Fig. 1c). Moreover, endogenous Nrf2 displays induction by well-established activators, sulforaphane or tBHQ. This induction only occurs at the ~95–110 kDa range with no alterations in levels of other nonspecific bands or any migratory species of ~55–65 kDa (Fig. 1c). Mouse Nrf2 also migrates at ~110 kDa since only this band significantly increased by SF treatment in Nrf2^{+/+} and Nrf2^{+/-} mouse embryonic fibroblast (MEF) cells, whereas no ~110 kDa band was detected in Nrf2^{-/-} MEFs regardless of treatment (Fig. 1d). More importantly, no prominent bands were detected at the predicted molecular weight of 55–65 kDa in any of the experiments. Notably, human Nrf2 frequently appears as a doublet in low percentage SDS-PAGE gels (<7.5%), however, the reasons for the apparent doublet have not yet been scientifically postulated. Lastly, we compared commercially available antibodies from three different sources in addition to SC-H300 and were able to only detect a band that could be induced by sulforaphane at 110 kDa (Fig. 1e).

Due to the misconception that Nrf2 runs at ~55–65 kDa, companies that commercialize antibodies may actually discard more specific Nrf2 antibodies that only detect the relevant species of ~95–110 kDa and instead, commercialize antibodies that show inherently nonspecific bands. Quite often the ~95–110 kDa band is labeled as “nonspecific” on the antibody data sheet due to a lack of knowledge of an apparent Nrf2 molecular weight of ~95–110 kDa. The reality that Nrf2 in fact migrates at ~95–110 kDa has created a huge negative impact on Nrf2 research. A vast amount of the published literature refers to a change in the ~55–65 kDa band as modulation of Nrf2 protein expression by certain compounds or physiological conditions. As the field begins to move into the translational phase of research by targeting Nrf2 for disease prevention and intervention, proper recognition of the apparent Nrf2 molecular weight and generation of specific Nrf2 antibodies are essential to avoid misinterpretation of results.

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Abbreviations Used

Cul3 = Cullin 3

HA = hemagglutinin

kDa = kilodalton

Keap1 = Kelch-like ECH-associated protein 1

MEF = mouse embryonic fibroblast

Nrf2 = NF-E2-related factor 2

Rbx1 = RING-box protein 1

SC = Santa Cruz

SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis

tBHQ = tert-butylhydroquinone