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# Considerations for antibody-based detection of NRF2 in human cells

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ABSTRACT

Based on the knockdown and overexpression experiments, it is accepted that in Tris-glycine SDS-PAGE human NRF2 migrates above 100 kDa, depending on the percentage of the gel. In 8 % Tris-glycine gel, monoclonal anti-NRF2 antibodies detect NRF2 signal as three bands migrating between 100 and 130 kDa. Here we used mass spectrometry to identify proteins immunoprecipitated by anti-NRF2 antibodies migrating in this range under steady state, upon NRF2 activator tert-BHQ and after translation inhibition with emetine. Our results show that three commercial monoclonal antibodies with epitopes in the center and in the C-terminus of NRF2 also bind calmegin, an ER-residing chaperone, that co-migrates with NRF2 in SDS-PAGE and gives stronger signal in western blot than NRF2. Calmegin has a much longer half life than NRF2 and resides in the cytoplasm, which differentiates it from NRF2. The most specific anti-NRF2 antibody in western blot, Cell Signaling Technology clone E5F1 is also specific in staining nuclear NRF2 in immunofluorescence. Other antibodies, that recognize calmegin in western blot, still can be specific for nuclear NRF2 in immunofluorescence, but require prior validation with NRF2 knockdown or knockout. These results appeal for caution and consideration when analyzing and interpreting results from antibody-based NRF2 detection.

## 1. Introduction

NRF2 is a stress-induced transcription factor, considered the main defense factor in a cell and a major regulator of cell survival. NRF2 levels are kept low under homeostatic conditions mainly via the KEAP1-cullin3 E3 ubiquitin ligase complex, which mediates constitutive ubiquitination and degradation of the KEAP1-bound NRF2 [1–4]. KEAP1 is the adaptor protein of the cullin3 (CUL3) ubiquitin ligase that anchors NRF2 in the complex and at the same time it is a stress sensor which translates stress stimuli to cellular responses via mediating degradation of NRF2 [5–8] and other proteins [9]. Oxidative or electrophilic stress activates NRF2 via de-repression - it modifies distinct cysteine residues in KEAP1 resulting in conformational changes in KEAP1-NRF2 binding which impair NRF2 ubiquitination [10–13]. In consequence, freshly synthesized NRF2 accumulates in the nucleus and induces expression of a battery of cytoprotective genes, including phase II detoxification enzymes, anti-oxidative stress enzymes, or enzymes involved in glucose metabolism [14–18].

Due to the broad spectrum of NRF2 actions as a pro-survival and protective factor, molecules that stabilize NRF2 in cells and promote its transcriptional activity have been intensively searched for in various diseases [19–21]. The physiological context in which NRF2 activation is especially beneficial is when oxidative stress and inflammation underlie disease pathogenesis such as in lung, liver, eye, gastrointestinal, metabolic, neurodegenerative, and autoimmune diseases [20].

Studying NRF2 biology or druggability of its pathway involves monitoring NRF2 levels, localization and activity and requires specific and sensitive antibodies. Sadly to say, the anti-NRF2 antibodies available on the market are the Achilles' heel of the NRF2 research [22,23]. They have low specificity and sensitivity, which, along with a low abundance of NRF2 in cells under steady state, and an aberrant migration of NRF2 in SDS-PAGE, makes detection of NRF2 in western blot really tricky [24]. It is known since the discovery of NRF2, that in vitro translated NRF2 does not migrate in the Tris-glycine SDS-PAGE according to the predicted molecular weight calculated for its 605 amino acids (~66 kDa), but at around 100 kDa level [25]. So, it was known where to look for endogenous NRF2 in immunoblotting, but an issue of specificity and sensitivity of antibodies still remained [22]. An interesting study by Kemmerer et al. [24] reported that monoclonal anti-NRF2 antibody (Abcam EP1808Y) detected an unspecific band

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co-migrating with NRF2. Methods were proposed to differentiate NRF2 signal from this unspecific target, basing on the siRNA knockdown or immunodepletion. NRF2 knockdown or activation as a validation for NRF2 signal specificity were also strongly advised by Kopacz et al. in a recent and rich update on NRF2 knowledge and detection tools [23]. Our previous results were contrary to those from Kemmerer et al. as we observed downregulation of all NRF2 bands from 100 to 130 kDa range in both stable and siRNA-mediated NRF2 knockdown, detected with Abcam EP1808Y and Cell Signaling D1Z9C antibodies [26]. We decided to pursue this issue further and solve this inconsistency with a direct analysis of proteins bound by anti-NRF2 antibodies.

In this study, with the use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), we confirm the existence of a masquerading protein, bound by all monoclonal anti-NRF2 antibodies tested, and reveal what it actually is – a chaperon named calmegin. Only one anti-NRF2 antibody, whose epitope, contrary to others, is in the N-terminus of NRF2, binds little or no calmegin, and gives the most specific NRF2 signal in western blot and immunofluorescence.

## 2. Results

NRF2 was detected in three non-small cell lung cancer cell lines H1299, RERF and A549 at steady state and upon treatment with a classical electrophilic NRF2 activator, tert-butylhydroquinone (tert-BHQ) with monoclonal anti-NRF2 antibody, clone EP1808Y from Abcam (Fig. 1A). Three distinct bands migrating in 100–130 kDa range were observed in 8 % Tris-glycine SDS-PAGE and the top one accumulated in response to tert-BHQ treatment. Upon NRF2 knockdown with a pool of NRF2-targeting siRNAs, top and middle bands were undetectable, confirming they are NRF2 species, but results regarding the fastest migrating ~105 kDa band were inconclusive. Its expression was reduced, though incompletely, in RERF cells, but unaffected in H1299 cells, thus it was unclear if this band represents true NRF2 signal (Fig. 1B). Utilization of lambda phosphatase allowed to observe that the top NRF2 band was a phosphorylated NRF2, which upon dephosphorylation was reduced in mass to the middle band – dephosphorylated NRF2. This pattern was detected by two anti-NRF2 monoclonal antibodies recognizing different NRF2 epitopes (EP1808Y from Abcam and D1Z9C from Cell Signaling) (Fig. 1C and D). The bottom band was not affected by phosphatase treatment. Interestingly, this ~105 kDa protein also did not translocate to the nucleus upon tert-BHQ treatment, contrary to the phosphorylated and non-phosphorylated NRF2 species (Fig. 1E). Moreover the ~105 kDa protein detected by anti-NRF2 antibodies was very stable, unlike NRF2. While phosphorylated and unphosphorylated NRF2 forms were degraded within minutes after translation inhibition and undetectable after 2 h, the 105 kDa protein was still present in cells even after 4 h of emetine treatment (Fig. 1F).

We utilized this difference in stability to verify the identity of this 105 kDa band with liquid chromatography coupled to tandem mass spectrometry in RERF cells, which express high levels of it (Fig. 2). NRF2 was immunoprecipitated from: 1) control cells, 2) cells in which translation was inhibited for 2 h and from 3) cells treated with NRF2 activator tert-BHQ for 5 h. Precipitates were separated in 8 % Tris-glycine SDS-PAGE followed by Flamingo staining and NRF2 detection by western blot, detecting precipitated NRF2. Gel pieces with proteins migrating in 100–130 kDa range, corresponding to NRF2 signal in western blot, were

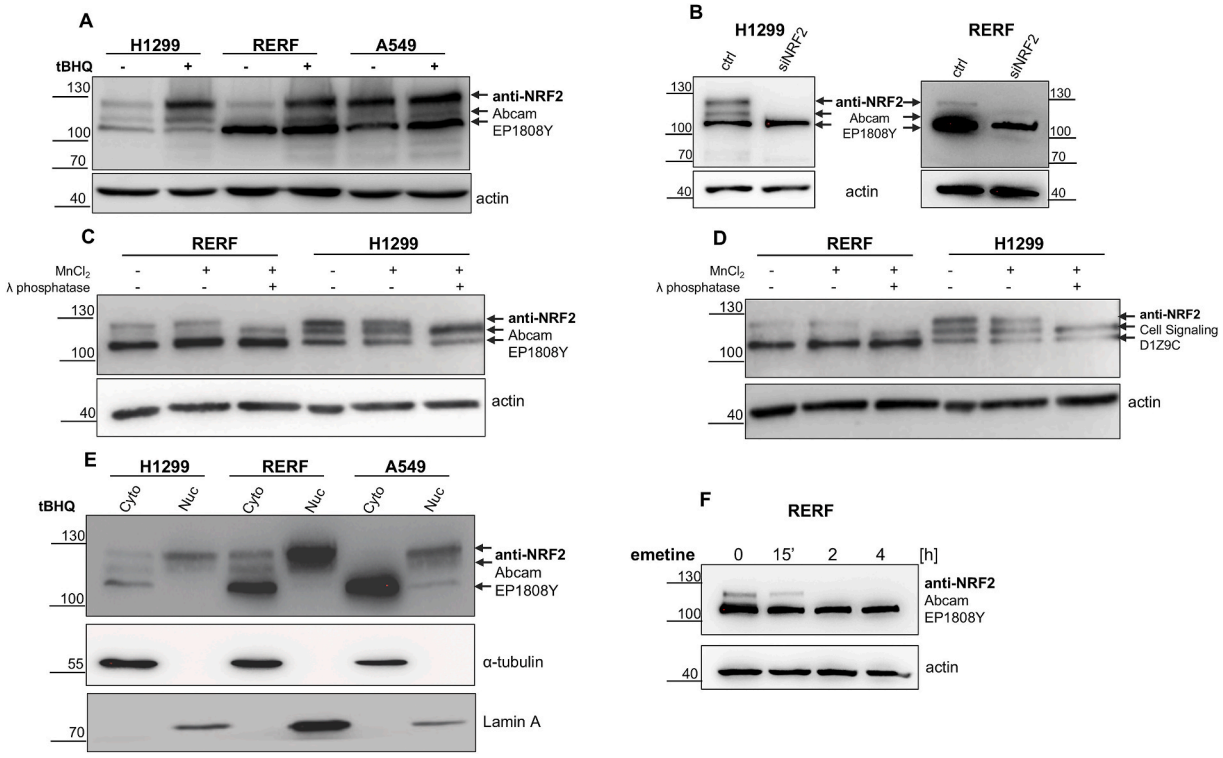


**Fig. 1.** NRF2 antibodies targeting different epitopes detect 3 bands migrating between 100 and 130 kDa in 8% Tris-glycine SDS-PAGE.
(A) NRF2 migration in Tris-glycine 8 % SDS-PAGE in H1299, RERF and A549 cells at steady state and upon treatment with tert-butylhydroquinone (t-BHQ). (B) Knockdown of NRF2 gene (*NFE2L2*) with a pool of siRNA targeting NFE2L2 (siNRF2) compared to transfection with control unspecific short RNAs (ctrl) in H1299 and RERF cells. (C,D) NRF2 dephosphorylation with λ phosphatase in RERF and H1299 cell lysates. Lysates were incubated with or without λ phosphatase for 30 min in 30 °C in the presence of $MnCl_2$ and NRF2 was detected by western blot. Arrows indicate bands detected by anti-NRF2 antibodies from Abcam [EP1808Y] (C) and Cell Signaling [D1Z9C] (D). (E) Detection of NRF2 in cytoplasmic and nuclear fractions upon treatment with tert-BHQ in H1299, RERF and A549 cells. Arrows indicate signal detected by anti-NRF2 antibodies from Abcam [EP1808Y]. Alfa-tubulin is a marker of cytoplasmic fraction and lamin is a marker of nuclear fraction. (F) Pulse-chase experiment upon translation inhibition with emetine in RERF cells. Cells were treated with emetine for indicated time and NRF2 levels were detected with Abcam [EP1808Y] antibodies. Molecular Weight is depicted at each western blot.

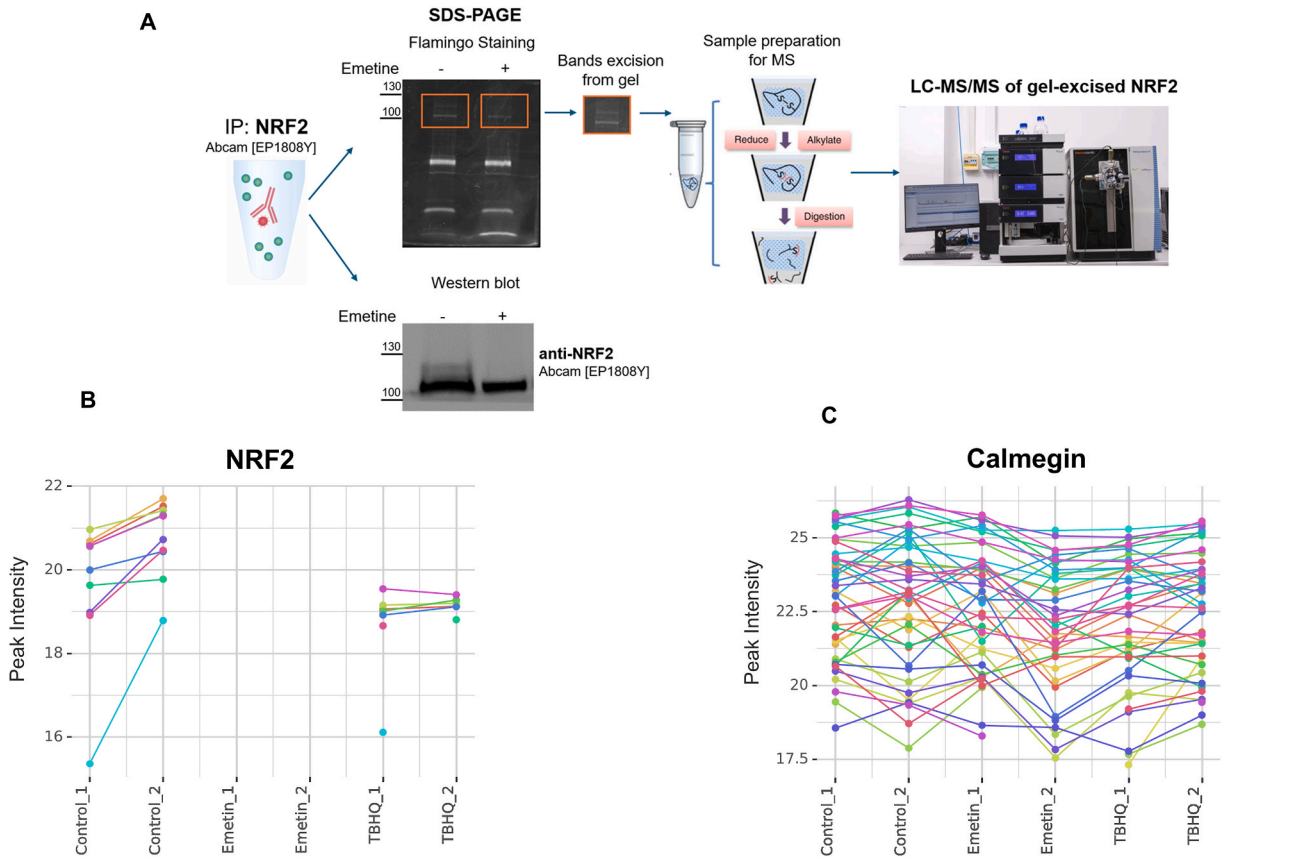


**Fig. 2.** Calmegin is precipitated by anti-NRF2 antibodies and co-migrates with NRF2 in SDS-PAGE.
(A) A scheme representing the workflow of identification of proteins precipitated by anti-NRF2 antibodies in RERF cells were treated with translation inhibitor emetine for 2 h cells or with tert-BHQ for 5 h. NRF2 was precipitated with Abcam EP1808Y antibodies. Precipitates were resolved in 8 % Tris-glycine SDS-PAGE and stained with Flamingo Fluorescent Gel Stain. Gel pieces with proteins migrating in 100–130 kDa range, representing NRF2 signal in western blot, were excised, followed by in-gel tryptic digestion of proteins. Diagrams show peptides of NRF2 (A) and calmegin (B) detected with LC-MS/MS in each sample (in duplicates). No NRF2 peptides were detected upon translation inhibition with emetine.

excised from the Flamingo-stained gel and, after tryptic digestion in gel, peptides were analyzed by LC-MS/MS (Fig. 2A). The analysis detected presence of NRF2 peptides in untreated cells and cells treated with tert-BHQ, but not in cells with inhibited translation, in which the 105 kDa protein recognized by anti-NRF2 antibodies remained present (Fig. 2A). To confirm the reliability of peptide detection and quantification, chromatograms of fragments of representative NRF2 peptides were extracted using Skyline and manually checked. Example results are presented in Sup. Fig. 1. These data clearly show that the 105 kDa protein is not NRF2. Interestingly, all samples showed high levels of calmegin, as observed by a high number of detected calmegin peptides (Fig. 2C) covering majority of calmegin sequence (Sup. Fig. 2).

Calmegin (encoded by *CLGN* gene) is an endoplasmic reticulum (ER)-residing $Ca^{2+}$-dependent chaperone, homologous to calnexin, which was previously described as a testis-specific factor required for sperm fertility [27,28]. To check if calmegin might be recognized by anti-NRF2 antibodies, we knocked down calmegin expression with specific *CLGN* siRNAs in two cell lines and probed lysates with four commercial anti-NRF2 antibodies (Fig. 3A,B,C). Calmegin migrates at 105 kDa - the same molecular weight (MW) as the protein recognized by anti-NRF2 antibodies. Upon calmegin knockdown in RERF and H1299 cells, three commercial anti-NRF2 antibodies showed decreased signal of the 105 kDa band, indicating that this band indeed represents calmegin and is recognized by all three anti-NRF2 antibodies binding different NRF2 epitopes (Fig. 4). Anti-calmegin antibodies used in this study do not bind to NRF2 (Fig. 3). There is no significant sequence similarity between NRF2 and calmegin, indicating that it is not the linear epitope in calmegin that is recognized by various anti-NRF2 antibodies. Only one monoclonal anti-NRF2 antibody that we analyzed, Cell Signaling clone E5F1A, did not bind to calmegin in H1299 cells or showed little binding in RERF cells (Fig. 3C). When we tested this antibody in RERF cells upon NRF2 knockdown and tert-BHQ treatment, we still could see the calmegin signal as the third band present upon NRF2 knockdown (Fig. 3D), but it was not that strong compared to the 'true' NRF2 bands, as with other antibodies. Therefore, we concluded that Cell Signaling E5F1A antibody gives the most reliable NRF2 signal in western blot among all antibodies tested.

Finally, we tested specificity of selected anti-NRF2 antibodies in immunofluorescence (IF) with NRF2 knockdown and tert-BHQ treatment in H1299 cells (Fig. 5). Cell Signaling E5F1A antibody turned out to be highly specific also in IF, but showed only nuclear signal – very weak upon no stimulation and strong nuclear NRF2 accumulation upon 5 h of tert-BHQ treatment (Fig. 5A). NRF2 knockdown abrogated nuclear signal accumulation upon tert-BHQ treatment indicating the specificity of this antibody in IF, same as in western blot under these conditions (Fig. 5B). IF-based detection of calmegin with monoclonal antibody recommended for IF (Thermo Scientific, clone 9C8G10) surprisingly showed its localization both in the cytoplasm and in the nucleus (Fig. 5C). This is at odds with fractionation results showing clearly that calmegin is not present in the nucleus (small nuclear fraction of calmegin was detected only in A549 cells) (Fig. 1E). Due to its function as a chaperon we would expect to see calmegin in the ER that surrounds the nucleus, but not inside it, whereas 3D view of IF staining clearly shows that signal from this antibody is present inside nuclei. Thus, we

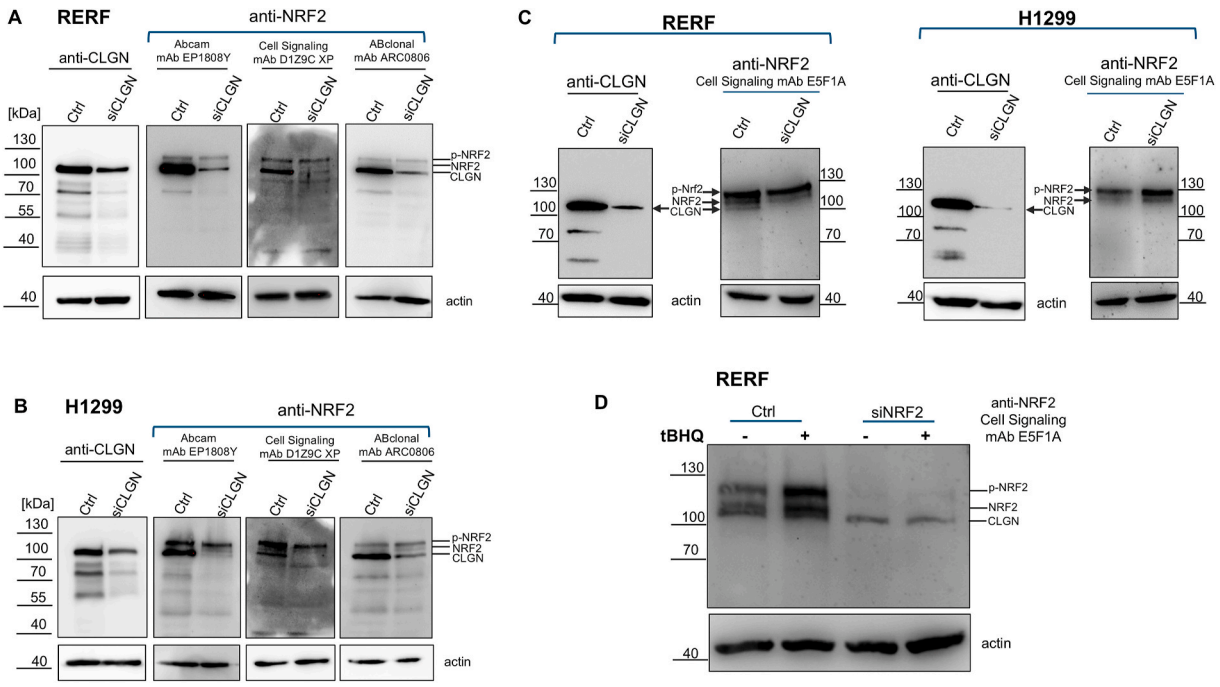


**Fig. 3.** Various commercial monoclonal anti-NRF2 antibodies bind calmegin.
Upon calmegin (CLGN) knockdown in RERF (A) and H1299 (B) cells, NRF2 was detected with three different monoclonal anti-NRF2 antibodies: Abcam EP1808Y, Cell Signaling D1Z9C and ABclonal ARC0806. All these three antibodies bind to calmegin. (C) Anti-NRF2 antibody from Cell Signaling (E5F1A) binds little calmegin in RERF or no detectable calmegin in H1299 cells. (D) RERF cells were knocked down for NRF2 expression (siNRF2) or transfected with control short RNA (Ctrl) for 48 h and treated with NRF2 activator tert-BHQ (tBHQ). NRF2 was detected with anti-NRF2 antibodies from Cell Signaling (E5F1A).

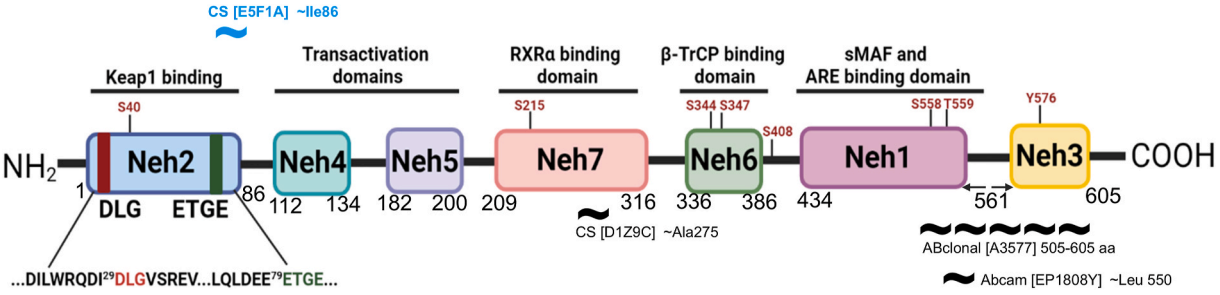


**Fig. 4.** NRF2 domain structure with marked localization of immunogens used for the production of monoclonal antibodies **used** in this study, marked by a wave (~), based on the information provided by a manufacturer. Antibodies which bind NRF2 with the highest specificity (Cell Signaling E5F1A) are marked with blue. Cell Signaling E5F1A - immunogen is a synthetic peptide corresponding to residues surrounding **Ile86**; Cell Signaling D1Z9C - immunogen is a synthetic peptide corresponding to residues surrounding **Ala275**; Abcam EP1808Y - immunogen is a synthetic peptide surrounding **Leu550**; ABclonal A3577 - immunogen is a synthetic peptide corresponding to a sequence within amino acids 505–605.

are not convinced if the anti-CLGN antibody used here is truly specific for calmegin in immunofluorescence.

We tested also two more antibodies – ABclonal ARC0806 (Fig. 5D) and Abcam EP1808Y (Fig. 5D and E) to see if, despite binding calmegin in western blot, they can still recognize nuclear NRF2 with specificity in IF (assuming that calmegin is not in the nucleus). Interestingly, ABclonal antibody was utterly unspecific in IF, showing only cytoplasmic signal, unaffected by stimulation with tert-BHQ or NRF2 knockdown. On the contrary, antibody from Abcam, showed cytoplasmic and nuclear signal and some nuclear accumulation upon tert-BHQ treatment abrogated by NRF2 knockdown, indicating it still can be used in IF for monitoring NRF2 accumulation in the nucleus, despite the overall signal was weak.

## 3. Discussion

Antibodies are essential tools in protein research. In classical workflow of monoclonal antibodies production, clones are screened and selected based on the antigen specificity and characterized functionally to confirm, validate, and specify their antigen binding capacity. But if the antigen used to construct the antibody bears similarity with a part of another protein, either in a linear sequence of amino acids or in a conformation – or both, the produced antibody will recognize and bind more proteins. One could assume that protein conformation is utterly lost upon denaturation at 95°C and reduction of disulfide bridges, that accompany SDS-PAGE, thus conformational antibodies should not work in western blot, but it is not always the case. Some proteins retain their conformation during SDS-PAGE, even partially, and can be bound by antibody recognizing conformational epitopes. Especially chaperones, such as calmegin, which evolved to retain their structure under high temperatures, might bear epitopes resistant to denaturation. This situation most probably underlies recognition of calmegin by anti-NRF2 antibodies, which precipitate calmegin both in its native state (Fig. 2) and upon denaturation and reduction in western blot (Fig. 3). Since there is no significant similarity in linear amino acid sequence between

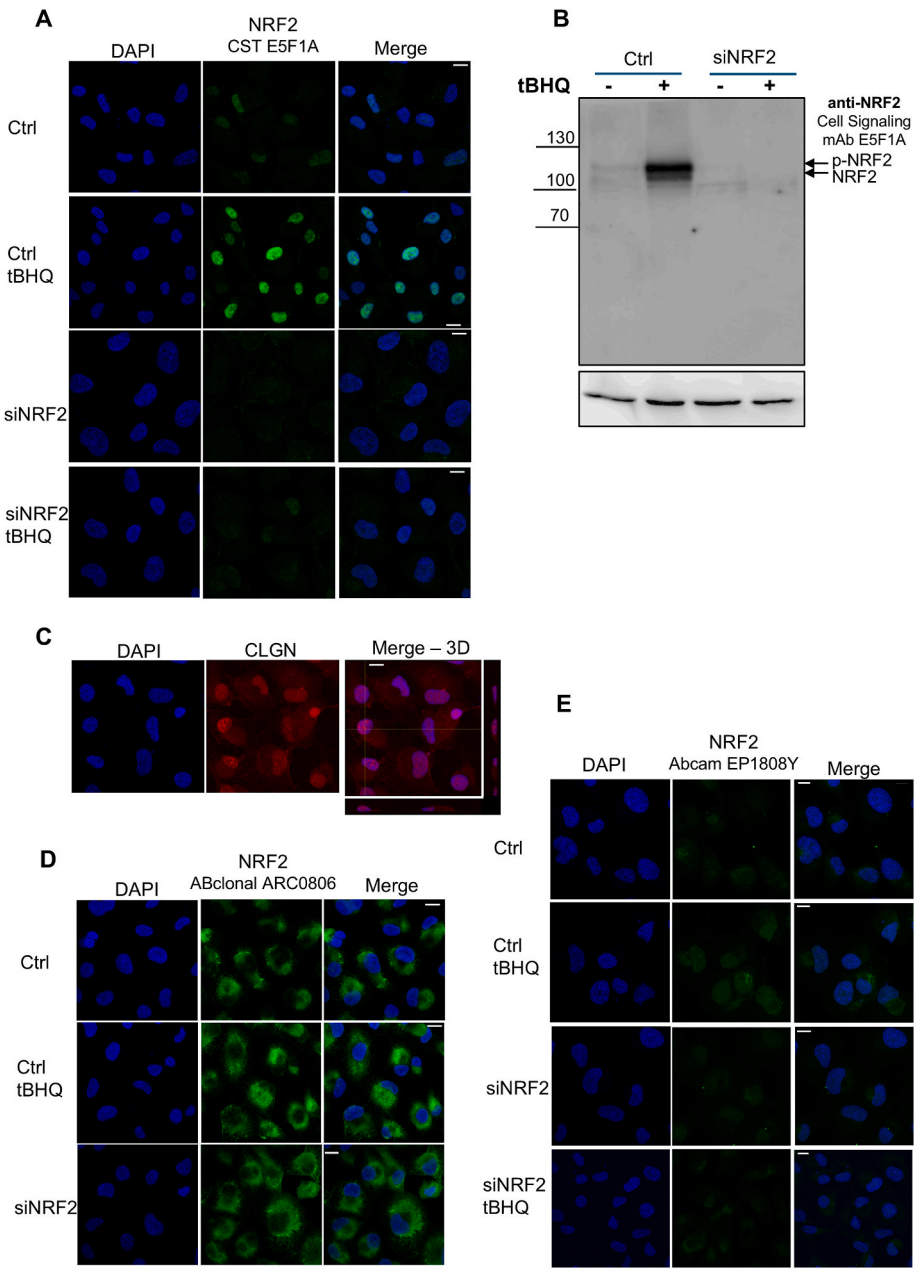


**Fig. 5.** Analysis of selected monoclonal anti-NRF2 antibodies specificity in immunofluorescence in H1299 cells. Cells were knocked down for NRF2 expression (siNRF2) or transfected with control short RNA (Ctrl) for 48 h and, where indicated, treated with tert-BHQ (tBHQ) for 5 h. After that cells were fixed with 4 % paraformaldehyde and permabilized with 0.2 % Triton X-100, followed by staining with various anti-NRF2 antibodies (A,D,E) or analyzed for NRF2 levels in western blot (B). Alternatively, non-transfected cells were stained with anti-calmegin (CLGN) antibodies (C). Nuclei were stained with DAPI. All experiments were performed in at least three independent biological repeats with two technical repeats for each condition.

calmegin and NRF2, it indicates that native conformational epitopes of calmegin 'survive' harsh SDS-PAGE conditions and are bound by anti-NRF2 antibodies.

Calmegin, a homolog of calnexin, is a 610 amino acid lectin chaperone, assisting in folding of other proteins in the ER [29]. Whereas amino acid sequences of calmegin and calnexin are quite similar, a difference in secondary structures was indicated by circular dichroism (CD) spectrum [29]. Calmegin also exhibited higher surface hydrophobicity than calnexin [29]. So far, calmegin has been considered a testis-specific chaperone, which is required for sperm fertility [27,28,30], but transcriptomics data show its elevated expression also in the heart [31–33]. Here we provide evidence that calmegin is expressed in various lung cancer cells. We have also identified it in cells from different origin (eg. U2-OS osteosarcoma cells), primary lung tumors as well as in normal lung fibroblasts (data not shown here). As calmegin is detected by various anti-NRF2 antibodies, it seems that many studies might have accidently 'revealed' its presence in various cell types.

Similarly to NRF2, calmegin migrates in SDS-PAGE not according to its molecular weight. As a 610 aa protein, its predicted molecular weight is 70 kDa, but in western blot the majority of the protein signal is detected around 105 kDa. The reason lies, most probably, similarly to NRF2, in the acidity of calmegin (theoretical pI = 4.57) that impedes SDS binding [34].

NRF2 is phosphorylated in cells by various protein kinases [35–37]. With all anti-NRF2 antibodies tested here we could see two NRF2 bands representing phosphorylated and unphosphorylated NRF2 (Fig. 3), as proved with dephosphorylation assay (Fig. 1C,D). This is in line with previous reports carefully examining endogenous NRF2

phosphorylation by casein kinase II (CK2) [36,37]. It is thus important to remember that NRF2 signal in western blot is represented by, at least, two bands. Why at least? Not only due to possible various levels of NRF2 phosphorylation [37] or other post-translational modifications (reviewed in Ref. [23]), but also due to the expression of alternative NRF2 isoforms. Splicing variants of *NFE2L2* gene with exon 2 deletion or exon 2 and 3 deletions have been identified in 2.1 % and 1.2 % lung squamous cell carcinoma patients respectively, but such NRF2 variants are much smaller than the full-length form and migrate below 100 kDa [38]. Interestingly, NRF2 can be expressed from alternative P2 promoter and these transcripts lack part of the open reading frame (ORF) from exon 1, encoding 16 amino acids (aa). Recently, the long-read RNA sequencing (Iso-seq) identified the NRF2 transcript originating from P2 as the second highest expressed NRF2 transcript under homeostatic and stress-induced conditions [39], but the presence of an encoded protein has not been reported so far. The 16 aa difference gives only ~1,77 kDa difference in mass between these isoforms which is highly challenging to detect in the one-dimensional Tris-glycine SDS-PAGE. Therefore, it is plausible that two NRF2 bands that we observed in western blot represent not one, but two NRF2 isoforms in their phosphorylated and not phosphorylated states. This could be further investigated, for example, with isoelectric point separation approaches.

It is unfortunate that the non-specific protein recognized by three different monoclonal anti-NRF2 antibodies, that we identified here as a calmegin, migrates in the Tris-glycine SDS-PAGE at the almost same level as NRF2. Due to much higher amount of calmegin in cells [40,41], it gives stronger signal in western blot than NRF2, and if lysates are not carefully resolved in low percentage (6–8%) or gradient gels, calmegin and NRF2 signals can merge into one, creating an artifact. Especially the fact that calmegin levels might be affected by NRF2 knockdown calls for caution and attention when interpreting western blot results. Contrary to NRF2, calmegin is a stable protein with half-life measured in hours (Fig. 1F) and it does not translocate to the nucleus, as observed in western blot upon fractionation (Fig. 1E). By these features, the true NRF2 signal can be differentiated from a signal derived from calmegin.

Only one anti-NRF2 antibody tested, Cell Signaling E5F1A, showed no or little binding to calmegin when compared with other antibodies. This antibody has already been reported by Kopacz et al. to detect NRF2 with high specificity [23]. However, whether this antibody recognizes calmegin or not seems to depend on the amount of calmegin expressed in a particular cell type. For example, RERF cells express more calmegin than H1299 (Fig. 1A) and Cell Signaling E5F1A detects calmegin in RERF but not in H1299 cells (Fig. 3C,D). The epitope recognized by Cell Signaling E5F1A is located within the N-terminus of NRF2 – around Ile86, according to the manufacturer (Fig. 4). All other anti-NRF2 antibodies, that strongly bind calmegin, have their epitopes in the middle of the protein (Cell Signaling D1Z9C) or at the C-terminus (Abcam EP1808Y and ABclonal ARC0806), indicating that N-terminus of NRF2 could provide the most reliable epitope for the anti-NRF2 monoclonal antibodies production.

In the last part of this study, we evaluated specificity of selected antibodies in immunofluorescence (Fig. 5). We wanted to know if the most specific antibody in western blot, Cell Signaling E5F1A, is also specific in the IF and if antibodies that recognize calmegin in western blot can still be used in immunofluorescence for detection of nuclear NRF2. Indeed, Cell Signaling E5F1A demonstrated high specificity for nuclear NRF2 (Fig. 5A). However, tyramide-based signal enhancement was required to obtain a detectable signal, as the signal was too weak with standard IF procedure. Interestingly, Abcam EP1808Y antibody also demonstrated specific nuclear NRF2 accumulation upon tert-BHQ, abrogated by NRF2 knockdown. This suggested that it can be used in IF to detect nuclear NRF2 accumulation. Thus we conclude that although majority of anti-NRF2 antibodies bind to calmegin, which resides in the cytoplasm (based on our fractionation and literature data), they may still be used in immunofluorescence, but require proper validation with NRF2 knockout or knockdown.

## 4. Materials and methods

### 4.1. Cell lines

Non-small cell lung cancer cell lines A549, RERF-LC-AI and H1299 were purchased from RIKEN BRC Cell Bank (Tsukuba, Ibaraki, Japan). They were cultured in Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific), with 8 % of Fetal Bovine Serum (Gibco, Thermo Fisher Scientific) and 1 % of Penicillin-Streptomycin (10,000 U/mL, Gibco, Thermo Fisher Scientific) and maintained at 37 °C under humidified conditions with 5 % CO2.

### 4.2. Lipid-mediated reverse transfection

Cells were seeded in 6-well plates, 100,000 cells/well and transfected with 25 nM siRNA for silencing NRF2 (NFE2L2) or CLGN (ON-TARGET plus SMARTpool, Dharmacon) or with 25 nM control siRNA-A (ON-TARGET plus Control Pool, Dharmacon) and 4 μl/well of Lipofectamine 3000 reagent (Invitrogen, Thermo Fisher Scientific), according to manufacturer's instructions. Western blot was performed 48 h after transfection.

### 4.3. Western blot analysis

Western blot analysis was performed as described previously [26]. The following primary antibodies were used: anti-NRF2 [EP1808Y] – ChIP Grade (cat. no. ab62352; Abcam), anti-NRF2 (D1Z9C) XP (cat. no. 12721; Cell Signaling Technology), anti-NRF2 (E5F1A) (cat.no 20733; Cell Signaling Technology), anti-NRF2 (ARC0806) (cat.no. A3577; ABclonal), anti-Calmegin (ARC2214) (cat.no. A19648, ABclonal), anti-tubulin (DM1A, Cell Signaling Technology), anti-lamin A (C-3, sc-518013; Santa Cruz Biotechnology) and anti-β-actin (cat. no. A2228; Sigma-Aldrich).

### 4.4. Flamingo staining of proteins

Proteins were electrophoretically separated in 8 % Tris-glycine SDS-PAGE, followed by gel fixation in 40 % ethanol, 10 % acetic acid and 50 % deionized water in a clean glass beaker for 4 h and staining overnight in 10 % Flamingo dye in dH2O (BIO-RAD, Hercules, CA, United States). Proteins were visualized using standard ultraviolet (UV) transilluminator and Chemidoc equipment (Biorad).

### 4.5. Dephosphorylation with lambda protein phosphatase (λPP)

800,000 cells were lysed in 250 μl of RIPA lysis buffer, sonicated for 15 min on ice and briefly centrifuged at 13,000×*g*. For dephosphorylation, 40 μl of cell lysate was incubated with 400 U of λPP (New England Biolabs) in the dedicated buffer and in the presence of manganese ions at 30 °C for 30 min. Control samples underwent the same treatment, but without the enzyme. The NRF2 phosphorylation was analyzed by western blot.

### 4.6. Cellular fractionation

Separation of nuclei from cytoplasm was performed according to the REAP method described by Suzuki et al. [42] Briefly, 4 × $10^6$ cells were resuspended in 400 μl of ice-cold 0.1 % NP-40 in PBS by gentle pipetting and centrifuged at 500 g for 10 s. Supernatant (cytosolic fraction) was collected and 5x Laemmli buffer was added to final concentration 1x. Pellets containing nuclei were washed with 300 μL of 0.1 % NP-40 in PBS, centrifuged at 500 g for 10 s, resuspended in 200 μl 1x Laemmli buffer and sonicated 15 min. Samples were boiled for 10 min and analyzed by western blot.

### 4.7. Identification of NRF2 with LC/MS/MS

Identification of NRF2 peptides upon NRF2 immunoprecipitation was performed in RERF cells under three conditions ($10 \times 10^6$ cells/condition): in control cells, upon inhibition of translation with emetine (20 μM) for 2 h and upon treatment with tert-BHQ (50 μM) for 4 h. Cells were lysed in IP buffer (25 mM Tris pH 7.5, 150 mM NaCl, 0.5 % TritonX-100), sonicated and centrifuged. 100 μg of total protein lysate was precleared with beads and NRF2 was immunoprecipitated overnight with anti-NRF2 antibodies Abcam [clone EP1808Y] ChIP Grade (ab62352), 2 μg/sample. 50 μl of protein G magnetic beads (Thermo Fisher Scientific) were used to pull down precipitated NRF2. After washing with IP buffer and PBS, elution was performed in 2 x Lamelli buffer 50 °C 10 min. Eluates were separated in 8 % SDS-PAGE. The gel was fixed and stained with Flamingo stain overnight (10 % in $dH_2O$). Gel pieces containing proteins migrating in 100–130 kDa range were cut from gel, fragmented to 3 mm fragments and rinsed with buffers A (25 % Acetonitrile/75 % 50 mM $NH_4HCO_3$) and B (50 % Acetonitrile/75 % 50 mM $NH_4HCO_3$) in the following order: B, A, B followed by reduction with 20 mM DTT in 50 mM $NH_4HCO_3$ for 30 min and alkylation in 60 mM iodoacetamide for 60 min in dark. In-gel digestion was performed with 10 ng/μl trypsin in 50 mM $NH_4HCO_3$ overnight. Peptides were extracted by addition of 1/20 V/V of 5 % TFA followed by sonication and second extraction with pure acetonitrile. Extracted peptides were evaporated to dryness under vacuum.

Before analysis, peptide samples were resuspended in 25 μl of 2,5 % acetonitrile and 0.08 % TFA in water by vortexing for 30 min at RT, sonication for 10 min at RT and vortexing for 10 min at RT.

LC/MS analysis was conducted with Thermo Scientific RSLC3000nano LC system (Thermo Scientific, MA, USA) liquid chromatograph coupled to Thermo Scientific Orbitrap Exploris 480 mass spectrometer (Thermo Scientific, MA, USA). Liquid chromatography was performed by concentrating peptides on (0.3 mm × 5 mm, 5 μm particle size) C18 PepMap trap column (Thermo Scientific, MA, USA) at flow of 5 μl/min for 10 min and analytical separation on (0.075 mm × 250 mm, 2 μm particle size) C18 PepMap RSLC column (Thermo Scientific, MA, USA). Peptides were separated by 60 min linear 2–36 % gradient of acetonitrile with 0.1 % formic acid.

DDA runs used full scan with 120,000 resolution and acquired spectra in profile mode. Ions from +2 to +6 charge state were selected for fragmentation. MS/MS scan used resolution of 60000 and normalized HCD energy to 30 %. Cycle time was set to 3 s. Fragmentation scans were acquired in centroided mode. For DIA data acquisition, full scan was performed at 120000 resolution and 350–1050 Th mass range. DIA scans used 12 Th wide windows with 1 Th overlaps, covering a mass range from 350 to 1020 Th. Resolution was set to 15000. Raw data files were converted to mzMLformat using MSConvert. Peptide identification and spectra library creation was done with FragPipe v. 20.0 using *Homo sapiens* UniProt sequence database (canonical sequences + isoforms). Briefly, precursor mass tolerance was set at 8 ppm, and fully tryptic peptides with maximum 2 missed cleavages of length between 7 and 45 amino acids were considered for search. Methionine oxidation and N-terminal peptide acetylation were set as variable modifications, while cysteine carbamidomethylation was set as static modification. Fragment mass tolerance was automatically optimized, and mass recalibration was performed before searching. Spectra library was prepared with easypqp python package within FragPipe workflow. DIA data analysis was performed with DIA-NN v. 1.8.2 beta 22 using double-pass analysis and composite high precision quantification mode.

### 4.8. Immunofluorescence

H1299 cells were seeded in 12-well plates on the 15 mm glass coverslips, 180,000/well. On the next day, the NRF2 knockdown was performed, where indicated, with 20 pmol/well of siNRF2 or ctrl siRNA (both from Dharmacon) and 3 μl/well of Lipofectamine (Invitrogen, Thermo Scientific) for 48 h. For the last 5 h, indicated cells were treated with tert-BHQ. Cells were fixed with ice-cold 4 % para-formaldehyde (Thermo Scientific) for 10 min, rinsed 3 times with cold PBS, permeabilized with 0.2 % Triton X-100 for 5 min and rinsed 3 times with PBS. Cells were blocked with 5 % FBS or 10 % goat serum when tyramide signal amplification was applied. Cells were incubated with primary antibodies overnight at 4 °C: NRF2 Cell Signaling Technology E5F1A (1:100), NRF2 ABclonal ARC0806 (1:100), NRF2 Abcam 1808Y (1:500) and anti-Calmegin 9C8G10 (#MA5-31704, Thermo Scientific) (1:200) in blocking buffer and washed 3 times with 1 % FBS in PBS. Subsequently, only cells incubated with NRF2 Abcam 1808Y were stained with secondary goat anti-rabbit antibody Alexa Fluor 488 (Thermo Scientific) in blocking buffer at 1:3000 dilution, in the dark at room temperature for 1 h. For the rest of samples, the tyramide signal amplification was applied according to the protocol (Tyramide SuperBoost kit GAR 488 and GAM 555, Thermo Scientific) with 7 min incubation with tyramide reagent. Cells were washed 3 times with 1 % FBS in PBS. Nuclei were stained with 300 nM of DAPI (Thermo Scientific), rinsed 3 x with PBS and 1 x with $dH_2O$ and samples were mounted using ProLong Diamond Antifade Mountant (Thermo Scientific). Specimens were imaged using a confocal laser scanning microscope (FluoView 3000, Olympus) with a 60x oil immersion lens. For each staining z-stacks were collected.

## CRediT authorship contribution statement

**Alicja Dziadosz-Brzezińska:** Writing – original draft, Methodology, Investigation. **Sara Kusiński:** Methodology, Investigation. **Artur Piróg:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Zuzanna Urban-Wójciuk:** Writing – review & editing, Investigation. **Monikaben Padariya:** Visualization, Software, Methodology, Investigation. **Umesh Kalathiya:** Visualization, Software, Methodology, Investigation. **Sachin Kote:** Resources, Funding acquisition. **Alicja Sznarkowska:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

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## Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2025.103549.

## Data availability

Data will be made available on request.

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