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Invited Review Article

Overlooked and valuable facts to know in the NRF2/KEAP1 field

Aleksandra Kopacz [a], Ana I. Rojo [b,c,d,e], Chinmai Patibandla [f], Diego Lastra-Martínez [b,c,d,e], Aleksandra Piechota-Polanczyk [a], Damian Kloska [a,1], Alicja Jozkowicz [a], Calum Sutherland [f], Antonio Cuadrado [b,c,d,e,**], Anna Grochot-Przeczek [a,*]

[a] Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland
[b] Department of Biochemistry, Medical College, Autonomous University of Madrid (UAM), Madrid, Spain
[c] Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC/UAM), Madrid, Spain
[d] Instituto de Investigación Sanitaria La Paz (IdiPaz), Madrid, Spain
[e] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain
[f] Division of Cellular and Systems Medicine, School of Medicine, University of Dundee, Ninewells Hospital and Medical School, James Arrott Drive, Dundee, United Kingdom

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1. Introduction

NRF2 (nuclear factor erythroid 2-related factor 2) is a critical regulator of the cellular stress response. It is a transcription factor that controls the expression of hundreds of genes. We will soon celebrate the thirtieth anniversary of its discovery [1]. Over the years, NRF2 and its repressor KEAP1 have become the subject of intensive research, ranging from the detailed molecular mechanisms of their activity to clinical perspectives and generating over a dozen thousand publications. This review intends to draw the attention of researchers to essential issues in the NRF2/KEAP1 field, highlighting the overlooked facts and clarifying potential misconceptions.

1.1. Fact 1: NRF2 migrates at ~95–110 kDa in Tris-Glycine SDS-PAGE

NRF2 is encoded by the *NFE2L2* gene. National Center for Biotechnology Information (NCBI) reports that human NRF2 mRNA has 8 transcript variants coding 6 different NRF2 isoforms, 505 to 605 amino acids long (Table 1). According to the Ensembl database, there are 14 human NRF2 transcript variants, coding 12 NRF2 isoforms. Both NCBI and Ensembl databases show that murine NRF2 has two transcript variants, which encode two NRF2 isoforms (Table 1). However, not all transcript variants seem to really exist in cells. Full-length transcriptome analysis in human lymphoblastoid cells revealed that among the 13 human *NFE2L2* isoforms identified in the GENCODE database, only 5 were confirmed by Iso-seq sequencing [2]. Two of them dominate in the level of expression over the others, both in basal and stress conditions. The identified ENST00000397062 and ENST00000397063 variants correspond to the NM_006164.5 and NM_001145412.3 sequences in the NCBI database. Interestingly, these variants use alternative first exons [2]. Up to date, no reports confirm the presence of murine short variant.

The molecular weight of human NRF2, calculated based on the amino acid sequence, ranges from 56.1 kDa to 67.8 kDa for the shortest isoform 6 to the longest isoform 1, respectively. Quite similar, the longer mouse NRF2 isoform is 66.9 kDa, whereas the shorter one is around 5 kDa (Table 1). However, it is critical to know that neither human nor murine NRF2 runs in Tris-Glycine SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) at this predicted molecular

* Corresponding author.
** Corresponding author. Department of Biochemistry, Medical College, Autonomous University of Madrid (UAM), Madrid, Spain.
E-mail addresses: antonio.cuadrado@uam.es (A. Cuadrado), anna.grochot-przeczek@uj.edu.pl (A. Grochot-Przeczek).
[1] Current affiliation: Malopolska Centre of Biotechnology, Jagiellonian University, Krakow, Poland.

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weight. The anomalous migration of NRF2 was reported already in the first paper describing this protein [1] and became the subject of a comprehensive commentary by Donna Zhang's group [3]. Yet, even now, many articles and antibody datasheets refer to the incorrect band, which migrates at ~55–70 kDa. It is a common misconception, carrying a significant risk of data misinterpretation.

The experiments based on the recombinant tagged-NRF2 and NRF2 inducing compounds performed by Donna Zhang's group showed that the relevant NRF2 band, detected with various anti-NRF2 antibodies, runs at ~95–110 kDa in SDS-PAGE [3]. Also, our research carried out in human cells transfected with siRNA against *NFE2L2* or transduced with adenoviral vectors for the overexpression of NRF2 demonstrated that a specific band for NRF2 migrates at ~100–110 kDa [4–6]. Furthermore, the only band that disappears in lysates from sh*NFE2L2*-transduced U373MG human glioblastoma astrocytoma cells is the one at 100–110 kDa, which has been shown with one homemade and four different commercially available antibodies (Fig. 1A). Also, NRF2 detection with A-10 anti-NRF2 antibody in the siRNA-controlled experiment on primary human aortic endothelial cells shows that the specific band can be found around 100 kDa (Fig. 1B). In like manner, wildtype (WT) but not NRF2 knockout (KO) mice NRF2 gives a band at ~100–110 kDa that in the brain is inducible by sulforaphane [7]. The knockout mouse NRF2 was built by inserting the coding sequence of β-galactosidase (LacZ) in a frame with the N-terminal region of *Nfe2l2,* and therefore, a band of around 180 kDa is detected in these mice as a fusion of NRF2 N-terminal domains with LacZ [4,8]. Accordingly, only the bands ~100 kDa and ~180 kDa for WT and KO cells, respectively, are significantly induced by a well-established NRF2 activator, sulforaphane (Fig. 1C).

Well-controlled experimental data shows the aberrant mobility of NRF2 in SDS-PAGE indisputably. However, this brings up an essential question of why it happens. The discoverers of NRF2 have already suggested that the overrepresentation of acidic amino acids might cause a discrepancy between the predicted and empirical NRF2 molecular weight [1]. Indeed, NRF2 is an acidic protein with an isoelectric point (pI) of 4.7. The acidic residues (D and E) content in NRF2 is one and a half times higher than the protein average (Fig. 2A). Therefore, at pH > 8.3, appropriate for Tris-Glycine SDS-PAGE, negatively charged residues of NRF2 repel anionic SDS, resulting in insufficient SDS binding. Hence, it may explain the lower electrophoretic mobility of NRF2 protein, similar as in the case of membrane proteins [9].

To elucidate which domains of NRF2 account for the aberrant migration in SDS-PAGE, we have generated five constructs with truncated NRF2. The recombinant proteins are tagged with His-tag, enhanced yellow fluorescent protein (EYFP) and V5 (Fig. 2B). Analysis of a Ponceau S-stained membrane revealed that the anomalous mobility of NRF2 is maintained in Neh2 mutant (Δ1) and still observed, but to a much lower extent, in Neh2, Neh4, Neh5, Neh7 (Δ2 and Δ3) mutants (Fig. 2C and D). The proteins lacking Neh2, Neh4, Neh5, Neh7, and Neh6 domains (Δ4), as well as containing only the domains Neh1 and Neh3 (Δ5), migrated according to their expected molecular mass (Fig. 2C and D). The distribution of charged amino acids is uneven between the individual fragments of NRF2. The net charge of the investigated mutant proteins is high for Δ1, and moderate for Δ2 and Δ3, whereas Δ4 and Δ5 mutants have a low net charge (Fig. 2E.) Thus, there is a significant correlation between mutated protein mobility delay with the net charge of the mutants ($R^2 = 0.9818$, $p = 0.0011$). It corresponds well with the full-length wildtype NRF2 migration delay (~40 kDa) and its high net charge (~−50 at pH 8.6) (Fig. 2E).

The electrophoretic shift of negatively charged proteins can be reversed by the amidation of acidic residue carboxyl groups [10]. Our experiments show that NRF2 mobility might be significantly improved (NRF2 detection at approx. 80 kDa) by using the NuPAGE Bis-Tris system with MOPS running buffer instead of Tris-Glycine SDS-PAGE (Fig. 3A and B). In accordance, pH of the precast NuPAGE Bis-Tris gel running buffer MOPS is 7.2, at which NRF2 net charge is ~ −40, which may explain a smaller shift in NRF2 migration in NuPAGE gel compared to standard Tris-Glycine SDS-PAGE electrophoresis (Fig. 3A and B).

The high net charge and anomalous mobility in SDS-PAGE are characteristic features of intrinsically disordered proteins (IDPs) [11]. The apparent molecular weight of IDPs is usually overestimated based on SDS-PAGE by 1.2–1.8 times compared to the real one, calculated from the sequence or determined by direct amino-acid mass measurement using, for example, mass spectrometry [11]. Indeed, in the case of NRF2, the observed molecular weight is ~1.6 times higher than the real one. Both *in silico* and experimental data confirmed that NRF2 contains unstructured regions (Fig. 4) [12,13]. The Neh2 domain of NRF2 is partially unstructured, except for 33-residue α-helix between DLG and ETGE, KEAP1 binding, motifs and anti-parallel short β-sheet [13]. The partial disorder also applies to Neh7 and Neh1 domains, whereas Neh6 and Neh3 are predicted to be mainly unstructured. On the other hand, transactivating domains Neh4 and Neh5 possess structured elements [12]. Interestingly, many disordered proteins belong to the class of proteins regulating transcription. More than half of eukaryotic transcription factors are estimated to possess unstructured domains [14], and many present abnormal mobility in SDS-PAGE [15].

1.2. Fact 2: Anti-NRF2 antibody validation is a must

Interpretation of experimental data on NRF2 obtained with antibody-based techniques, such as western blotting (WB), immunofluorescence (IF), and flow cytometry (FC), carries the risk of data misinterpretation due to the issue of anti-NRF2 antibody specificity. Many anti-NRF2 antibodies produce a ladder of bands in WB instead of one specific band. Optimisation of the protocol sometimes helps to limit or eliminate the non-specific pattern. We highly recommend using appropriate positive and negative controls for all techniques based on anti-NRF2 antibodies to minimise data misjudgement. Negative control samples such as NRF2 knockdown or knockout cells (siRNA, shRNA, CRISPR/Cas9) are best for *in vitro* experiments. Alike, we recommend using samples from NRF2 knockout mice as a negative control for murine tissues. It is, however, crucial to pay attention to the construction of the KO mice and the antibody used - which residues of NRF2 it

Table 1
Comparison of human and mouse NRF2 isoforms based on NCBI. NM RefSeq – mRNA reference sequence, NP – protein reference sequence, aa – number of amino acids. Based on https://www.ncbi.nlm.nih.gov.

	NM RefSeq	NP RefSeq	transcript variant	isoform	aa	predicted molecular weight [kDa]
human NRF2	NM_006164.5	NP_006155.2	1	1	605	67.8
	NM_001145412.3	NP_001138884.1	2	2	589	66.1
	NM_001145413.3	NP_001138885.1	3	3	582	65.3
	NM_001313900.1	NP_001300829.1	4	2	589	66.1
	NM_001313901.1	NP_001300830.1	5	2	589	66.1
	NM_001313902.2	NP_001300831.1	6	4	575	64.4
	NM_001313903.2	NP_001300832.1	7	5	532	59.1
	NM_001313904.1	NP_001300833.1	8	6	505	56.1
mouse NRF2	NM_010902.4	NP_035032.1	1	1	597	66.9
	NM_001399226.1	NP_001386155.1	2	2	45	5.1

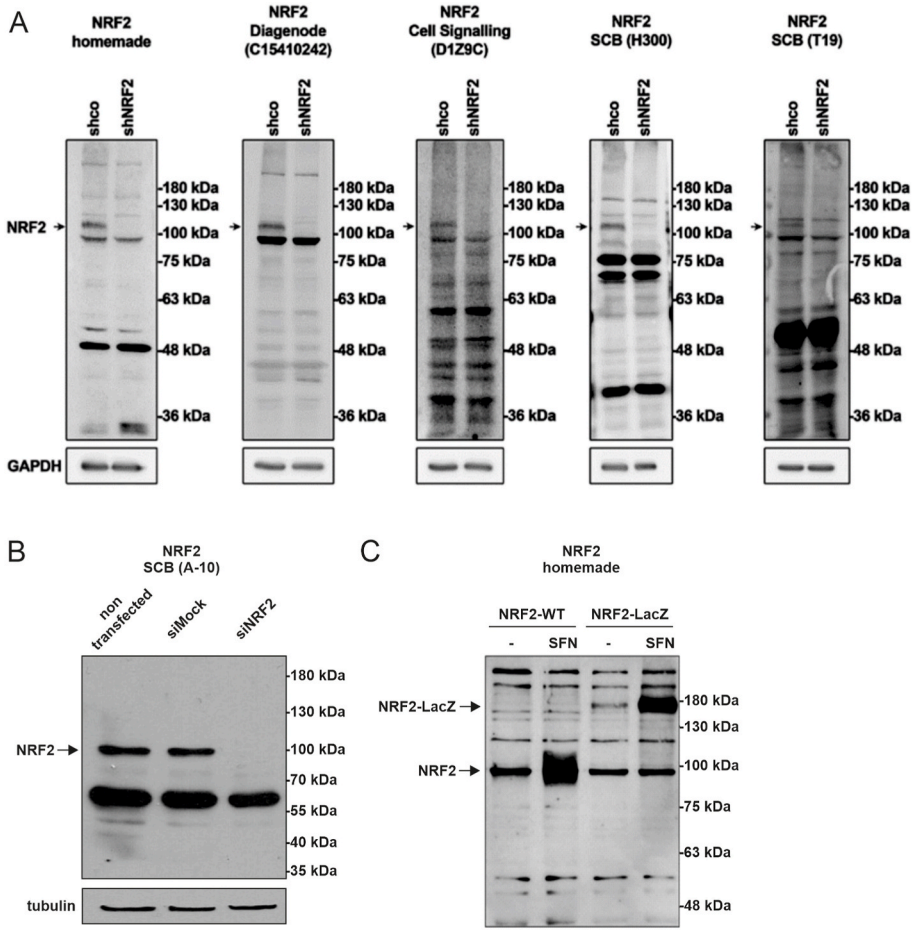


Fig. 1. Immunodetection of NRF2 by SDS-PAGE. **(A)** Comparison of five antibodies against NRF2. 20 μg of protein lysate from control (shco) or NRF2-knockdown (shNRF2) U373 MG cells were resolved in 10% SDS-PAGE and blotted with the indicated antibodies. Blocking was done in 5% non-fat milk in TBST. Antibodies were used as follows: homemade at Cuadrado lab against full-length recombinant protein (0.5% non-fat milk in TBST, 1:5000), Diagenode, Cell Signaling (D1Z9C) and SCB (H300) (0.4% BSA in TBST, 1:1000), and SCB (T19) (0.4% BSA in TBST, 1:500). The blot with the D1Z9C antibody using 5% non-fat dry milk, according to vendor's recommendation, provided the same results. The black arrow points specific NRF2 band. **(B)** Detection of NRF2 in human aortic endothelial cells. 15 μg of protein lysate from control (non-transfected or siMock) or NRF2-knockdown (siNRF2) primary human aortic endothelial cells were resolved in 10% SDS-PAGE and blotted with the SCB (A-10) antibody (1:500). Blocking was done in 5% non-fat milk in TBST. The black arrow points specific NRF2 band (A-10 antibody datasheet refers to the incorrect band ~60 kDa). Representative examples of 3 experiments. **(C)** Detection of NRF2 in NRF2 KO hepatocytes. 20 μg of protein lysate from untreated or SFN (10 μM, 6 h) immortalized hepatocytes derived from wildtype or NRF2 KO mice were resolved in 8% SDS-PAGE. Blocking was done in 5% non-fat milk in TBST and homemade anti-NRF2 antibody from Cuadrado lab was used (0.5% non-fat milk in TBST, 1:5000). Black arrows point specific NRF2 and NRF2-LacZ bands. Representative examples of 3 experiments.

recognizes (Fig. 5). N-terminal domains of NRF2 (up to 301 aa) are present in NRF2 KO mice generated by the Yamamoto group [4,8], and they are also present in NRF2 floxed mice generated by the Biswal group [16] (Fig. 5). The usage of antibodies recognizing N-terminal domains is pointless in this case. Antibodies produced with immunogens located in the proximity of the 300th amino acid could either give a positive signal or not, depending on the exact position of the epitope, sequence coverage between species and spatial structure of NRF2 protein in this region. Therefore, to distinguish between WT and KO, it is essential and best to use the antibodies that recognise C-terminal domains of murine NRF2, especially for methods other than those utilising molecular mass distinction, such as WB. For positive control samples, we recommend using NRF2 overexpressing cells or the sample treated with well-known NRF2 activators such as sulforaphane, prostaglandin J_2 or tert-butylhydroquinone (tBHQ).

1.3. Fact 3: KEAP1 sequestration by NRF2 implies NRF2 KO mice experimental limitations

KEAP1-dependent degradation of NRF2 and consequent limitation of NRF2 transcriptional activity [17,18] is a cornerstone of research in this field and textbook knowledge. However, recent papers reported that NRF2 activity might reach beyond gene transactivation. Occupancy and sequestration of KEAP1 by NRF2 are critical for angiogenesis, retrograde mitochondrial trafficking and proteostasis [4–6,19,20]. This entails the conclusion that NRF2 and KEAP1 mutually repress each other. In this view, the presence of N-terminal NRF2 domains in NRF2 KO mice [4,8], including Neh2, which is responsible for KEAP1 binding, implies some experimental limitations. These mice are KO in transcriptional activity but might retain transcription-independent NRF2 functions such as KEAP1 inhibition. The NRF2-LacZ fusion protein is not detectable in NRF2 KO mice in unstressed conditions, but it accumulates in response to diethylmaleate (DEM, a Keap1-modifying electrophile), or MG-132 (a proteasome inhibitor) treatment [8]. A quick turnover of NRF2-LacZ indicates that KEAP1 interacts with the Neh2 domain of NRF2 in these mice. Thus, the sequestration of KEAP1 by NRF2 is preserved. For further reading on the KEAP1 repression and the significance of NRF2/KEAP1 balance, please refer to Ref. [19].

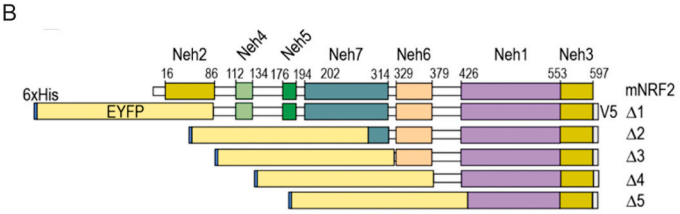
1.4. Fact 4: De novo translated NRF2, not released from KEAP1, translocates to the nucleus

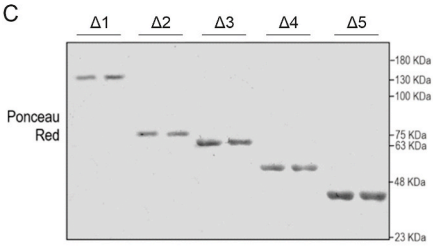
It is widely accepted that KEAP1 sequesters and represses NRF2 in the cytoplasm, thus impeding its translocation to the nucleus. Furthermore, the stress sensing capacity of KEAP1 allows the change of the interaction between these two proteins; as a result, NRF2 can accumulate in the nucleus. The regulation of KEAP1/NRF2 interaction has several nuances, which, when overlooked, can precipitate an oversimplification of the model.

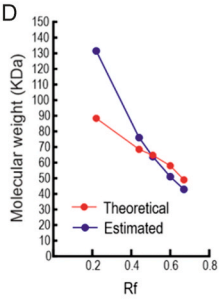
The stoichiometry of the KEAP1:NRF2 interaction is 2:1, as revealed by the isothermal calorimetry. Neh2 domain of NRF2 interacts with two KEAP1 molecules via two motifs differing in their affinity to KEAP1 of approximately two hundred times [21]: a stronger (ETGE) and a weaker one (DLG) [22]. Such stoichiometry is probably forced because the amino acid sequences of binding motifs in KEAP1 overlap, as shown by NMR titration [13]. This discovery fostered the development of the field significantly. It permitted the introduction of the "hinge and latch" model. According to this hypothesis, more substantial, ETGE-mediated interaction occurs first and is unchangeable (a hinge). Whereas the weaker DLG-driven interaction may occur or not (latch), permitting the

A

			mouse NRF2		human NRF2		average protein
			number	count [%]	number	count [%]	count [%]
Aspartic Acid	Asp (D)	Acidic	46	7.71	45	7.44	5.1
Glutamic Acid	Glu (E)	Acidic	57	9.55	58	9.59	6.2
Arginine	Arg (R)	Basic	22	3.69	17	2.81	6.2
Histidine	His (H)	Basic	18	3.02	17	2.81	2.2
Lysine	Lys (K)	Basic	36	6.03	39	6.45	5.3
Asparagine	Asn (N)	Hydrophilic	22	3.69	29	4.79	3.9
Cysteine	Cys (C)	Hydrophilic	7	1.17	6	0.99	1.5
Glutamine	Gln (Q)	Hydrophilic	40	6.7	39	6.45	3.9
Glycine	Gly (G)	Hydrophilic	26	4.36	21	3.47	6.8
Serine	Ser (S)	Hydrophilic	66	11.06	67	11.07	7.0
Threonine	Thr (T)	Hydrophilic	26	4.36	25	4.13	5.3
Alanine	Ala (A)	Hydrophobic	37	6.2	30	4.96	8.7
Isoleucine	Ile (I)	Hydrophobic	22	3.69	24	3.97	5.7
Leucine	Leu (L)	Hydrophobic	60	10.05	62	10.25	9.8
Methionine	Met (M)	Hydrophobic	14	2.35	12	1.98	2.4
Phenylalanine	Phe (F)	Hydrophobic	27	4.52	25	4.13	4.0
Proline	Pro (P)	Hydrophobic	39	6.53	42	6.94	5.0
Tryptophan	Trp (W)	Hydrophobic	2	0.34	2	0.33	1.3
Tyrosine	Tyr (Y)	Hydrophobic	10	1.68	10	1.65	2.9
Valine	Val (V)	Hydrophobic	20	3.35	35	5.79	6.5







E

	aa from mNRF2	delay [kDa]	net charge pH 8.6
mNRF2	1-597	41	-51.3
Δ1	98-597	49	-50.3
Δ2	280-597	13	-20.4
Δ3	316-597	5	-16.4
Δ4	381-597	-1	-5.1
Δ5	464-597	0	-6.3

Fig. 2. Analysis of the electrophoretic mobility of NRF2 N-terminal deletion mutants by SDS-PAGE. **(A)** Comparison of the amino acid composition of mouse NRF2, human NRF2 and average protein. **(B)** A diagram showing the general organization of the Neh domains in murine NRF2 according to Ref. [18] and the 6xHis-EYFP-mNRF2-V5 chimeras with N-terminal deletions of mNRF2. Each chimera carries the following residues from mNRF2: Δ1, 98 to 597; Δ2, 280 to 597; Δ3, 316 to 597; Δ4, 381 to 597; Δ5, 464 to 597. **(C)** Mobility of NRF2 N-terminal deletion mutants. 2 μg of recombinant EYFP-mNRF2-V5 proteins were resolved in 10% SDS-PAGE, transferred, and stained with Ponceau S. **(D)** A graph depicting the theoretical and estimated molecular weight for NRF2 chimeras from (C) vs Rf (migration distance of the protein/migration distance of the dye front). **(E)** Comparison of migratory delay and net charge of the NRF2 N-terminal domain mutants.

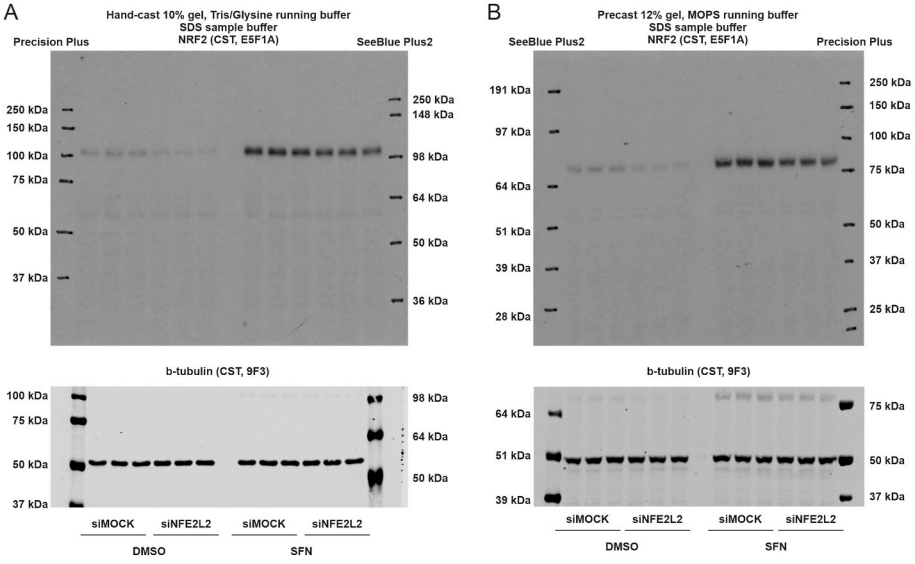


Fig. 3. Analysis of the electrophoretic mobility of NRF2 by SDS-PAGE: hand-cast gel, Tris-Glycine buffer vs precast gel, MOPS buffer. Mock or *NFE2L2* siRNA-transfected INS1 cells were treated for 3 h with DMSO or sulforaphane (SFN, 5 μM). 15 μg of total protein suspended in SDS sample buffer was resolved in **(A)** 10% SDS-PAGE hand-cast gel, Tris-Glycine running buffer, or **(B)** 4–12% NuPAGE Bis-Tris precast gel, MOPS running buffer. Blocking was done in 5% fatty acid-free BSA in TBST. The membranes were probed with anti-NRF2 antibodies (CST, E5F1A, 1:1000 in blocking buffer) or anti-β-tubulin antibody (CST, 9F3, 1:1000 in blocking buffer). Two different molecular mass marker ladders were used on all gels, Biorad Precision Plus Protein standards and SeeBlue Plus2 Pre-Stained Standard.

fluctuation of KEAP1/NRF2 complex between the open and closed conformation, respectively [23,24]. Furthermore, in the open conformation (based only on the ETGE interface), NRF2 ubiquitination and subsequential degradation are abolished. While, in the closed conformation (based on the interaction with both motifs), lysines targeted for ubiquitination are well-positioned, and NRF2 undergoes degradation, which releases KEAP1 for another cycle of interaction [24,25]. In particular, although NRF2 inducers cause significant conformational changes in KEAP1, NRF2 remains trapped due to the blockage of the complex in a close conformation. The ubiquitination machinery cannot dissociate but remains misaligned with NRF2 lysine residues, and the ubiquitination is not feasible [26,27]. Therefore, only *de novo*

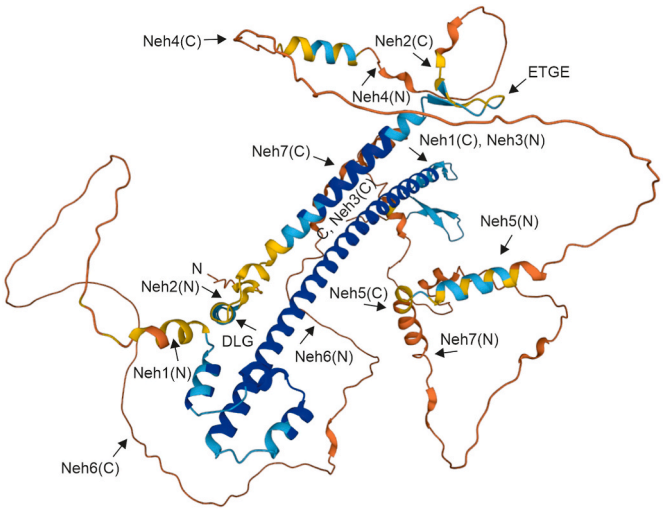


Fig. 4. 3D representation of human NRF2 protein. Representative positions of NRF2, such as Neh domains, ETGE and DLG motifs, are pointed out. Created with AlphaFold [130,131].

synthesised NRF2 can enter the nucleus [25,28] (see also detailed reviews [21,29]). These conclusions are supported by mathematical modelling of the regulation of nuclear translocation, introducing the allegory of two water tanks: bigger (cytoplasm), higher and smaller (nucleus) [30]. The smaller tank can start to be filled up only when the bigger one is full (for a detailed description of methods related to many NRF2 inducers, please refer to Ref. [30]). These findings suggest a close interplay between available KEAP1 and NRF2 protein pools and their particular dynamics. Regulation of KEAP1 levels (expression/degradation) should modify the availability of NRF2, and indeed this is the case with SQSTM-driven autophagic degradation of KEAP1, which leads to an increase in NRF2 levels [31]. For a comprehensive discussion of the current KEAP1/NRF2 interaction models, see Ref. [23].

1.5. *Fact 5: nuclear NRF2 may remain transcriptionally inactive*

A general notion is that if NRF2 translocates to the nucleus, its level increases and triggers an antioxidant response element (ARE)-mediated response. Therefore, the increased NRF2 protein level or its presence in the nuclear fraction is often identified as augmented transcriptional activity of NRF2. On the other hand, some factors promote NRF2 translocation to the nucleus without subsequent ARE-driven transcription [4]. To address this discrepancy, first and foremost, we should recall the complexity of transcription initiation. It relies on well-orchestrated recruitment of adaptors, mediators, histone-modifying enzymes, and RNA polymerase II, in addition to the transcription factor itself [32]. However, assuming the proper functioning of the whole transcription machinery, the primary determinant is the good context binding of NRF2 to its consensus sequence ARE. The main factors governing NRF2 nuclear presence and transcriptional activity are: accessibility of nuclear localisation sequence (NLS) and inaccessibility of nuclear export sequence (NES), the exact nuclear localisation, post-translational modifications, accessibility of ARE sequence, and interactors regulating ARE

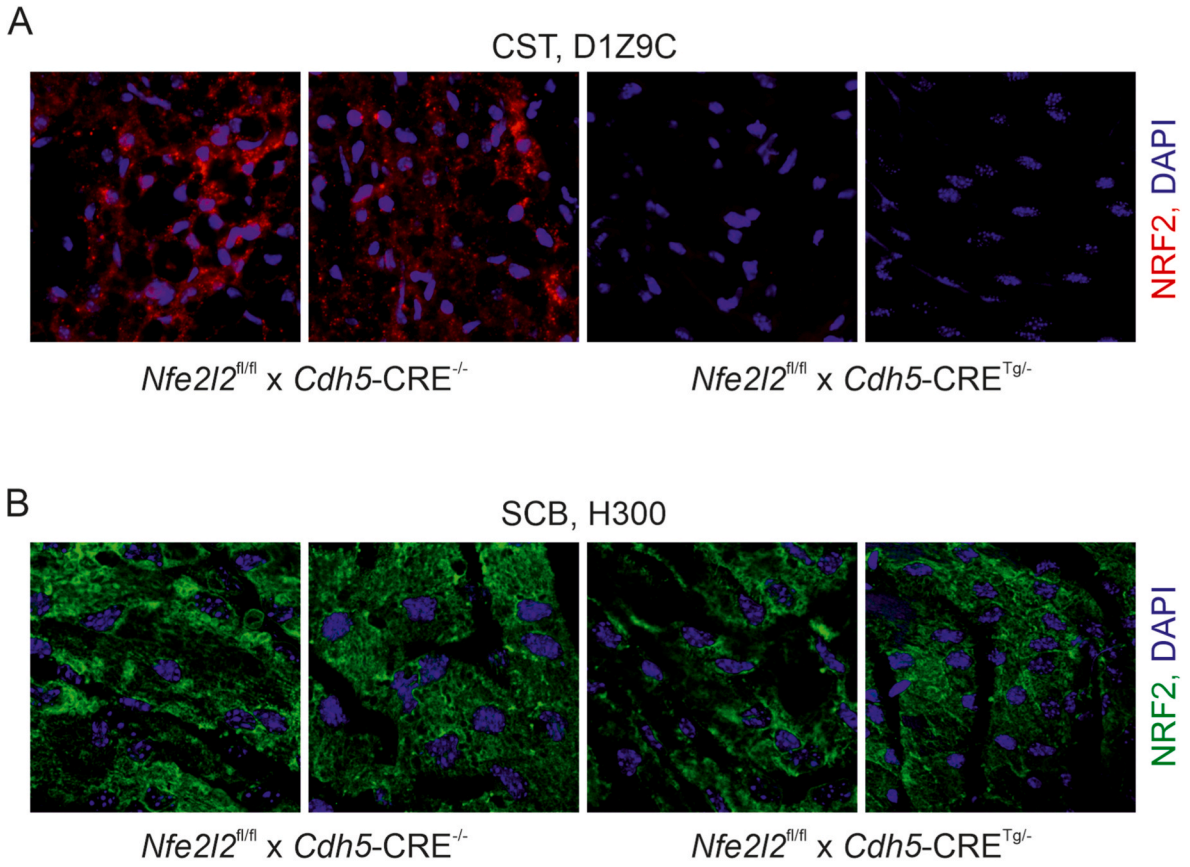


Fig. 5. Immunofluorescent detection of NRF2 in *en face* staining of the murine aorta. NRF2 was detected in mouse aortic endothelial cells with **(A)** D1Z9C, CST (1:100 in blocking buffer; D1Z9C is a monoclonal antibody obtained by immunizing rabbits with a synthetic peptide corresponding to residues surrounding 275 amino acid of human NRF2 protein) or **(B)** H300, SCB (1:100 in blocking buffer; H-300 is a polyclonal antibody with the epitope mapping to amino acids 37–336 of human NRF2) antibodies, according to the protocol described in Ref. [5]. *Nfe2l2*$^{fl/fl}$ x *Cdh5*-CRE$^{-/-}$ (control) and *Nfe2l2*$^{fl/fl}$ x *Cdh5*-CRE$^{Tg/-}$ (NRF2 deleted in endothelial cells) were used. Representative pictures.

binding (for more details, please refer to Ref. [33]) (Fig. 6). The newly translated NRF2 can enter the nucleus through the importin α/β complex owing to three NLS located in Neh1, Neh2 and Neh3 domains [34]. The uncovering of NES resulting in a transport of NRF2 from the nucleus can be triggered by either GSK3β-mediated phosphorylation of NRF2 [35] or by the reduction of cysteine 183 in redox-sensitive NES [34]. NRF2 can also be escorted out of the nucleus by KEAP1 [36,37].

Despite nuclear localisation of NRF2, the transcriptional activity is abolished when NRF2 interacts with components of nuclear membrane lamin A/C [38] or, when mutated, progerin [39]. Besides, NRF2 traffics and localises in PML bodies (one of the nuclear structures) [40]. There, it is post-translationally modified (PTM) by SUMOylation, which depending on the enzyme and targeted lysine residues, can either cause its degradation [41] or stabilisation [42].

NRF2 PTMs play a crucial role in the regulation of NRF2 transcriptional activity. To avidly bind to ARE, NRF2 should be acetylated, phosphorylated and not glycated. In more detail, an essential step in the induction of NRF2 transcriptional activity is the recruitment of co-activator molecule CBP by the synergy of Neh4 and Neh5 [43], which acetylates NRF2, hence stabilising it, promoting nuclear localisation [44] and enhancing its activity [45]. Consequently, histone deacetylases (HDACs), SIRT1 and SIRT2, decrease NRF2 transcriptional activity [46,47]. Furthermore, while NRF2 phosphorylation at serine 40 by MAPK kinases promotes its nuclear localisation [48], the CK2-driven phosphorylation in transactivation domains (Neh4 and Neh5) is crucial for ARE binding and NRF2 transcriptional activity [49]. Finally, deglycation by fructosamine-3-kinase (FN3K) fosters NRF2 stability, binding to transcriptional activators and consensus sequence [50] (Fig. 7).

The final regulatory step governing NRF2 transcriptional activity is binding to ARE and transactivation. NRF2 heterodimerization is a crucial step in switching the transcriptional activity on. Noteworthy, there are heterodimers capable of ARE activation independently of NRF2 [51–53]. Among all possible NRF2 interactors, only a tiny portion has an activatory role on ARE, including sMAF [54] and ATF4 [55]. Subsequently, many repressor proteins attenuate NRF2-driven transcriptional activity by preventing the binding to ARE or by the impediment of transactivation (Table 2) [56–63]. However, they are inhibited or exported from the nucleus in response to the need for NRF2

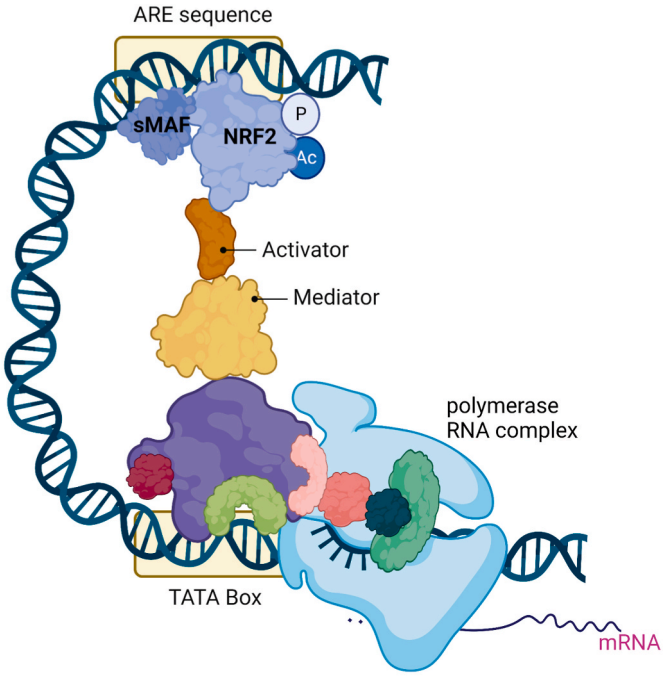


Fig. 6. The complexity of transcription initiation. ARE – antioxidant response element. Created with BioRender.com.

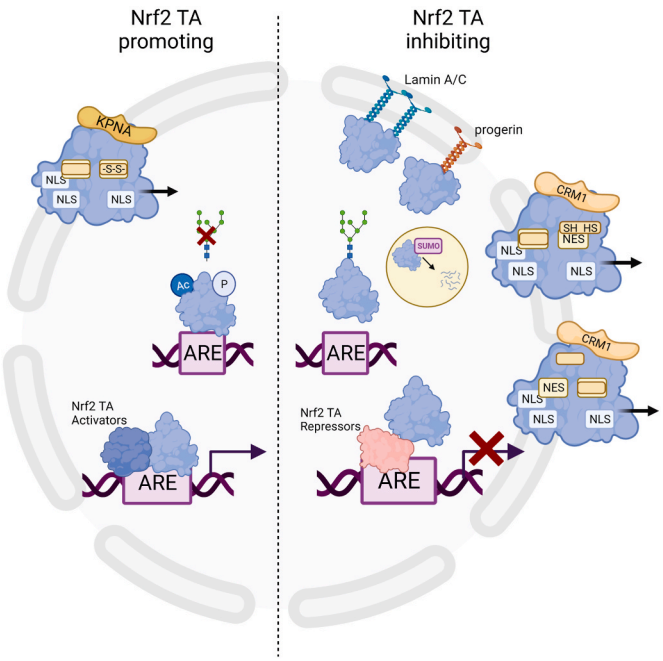


Fig. 7. Modulation of NRF2 transcriptional activity. TA – transcriptional activity, NLS – nuclear localisation signal, NES – nuclear export signal. Created with BioRender.com.

Table 2
Repression of NRF2 transcriptional activity – the mechanisms.

Repressor mechanisms – NRF2-driven transcriptional activity	
Mode of action	Examples
Heterodimers activating ARE, independently of NRF2	sMAF-c-FOS [52] NRF1-sMAF [51] NRF3-sMAF [53]
Homodimers or heterodimers occupying ARE	BACH1/2-sMAF [59] sMAF-sMAF [58]
Interaction with NRF2, sequestration from ARE	RXR [60] ATF3 [57]
Interaction with NRF2, inhibition of transactivation	ERα [56] ERRβ [62] RARα [61] SMRT [63]
Presence of NRE at 3′ of ARE	RPA1 [66]
Inhibition of RNA Pol II recruitment	NRF2 [67]
Truncated NRF2, lacking transactivation domain	NRF2 [65]

transcriptional activity. For example, the nuclear export of BACH1 is triggered by antioxidant-induced phosphorylation of tyrosine 486 [64].

Interestingly, there are also regulatory mechanisms by which NRF2 can repress ARE-driven transcription. A caspase-cleaved, truncated NRF2, which lacks a transactivation domain, can suppress full-length NRF2 by the occupation of ARE [65]. Furthermore, over 400 genomic loci contain the ARE-NRE sequence, which can be repressed by ARE-bound NRF2 [66]. It is achieved by binding RPA-1 to NRE sequences at 3' of ARE. The suppressor activity of NRF2 may also reach beyond ARE motif. NRF2 inhibits the expression of proinflammatory cytokines by blocking RNA Pol II recruitment [67] (Table 2).

NRF2 transcriptional activity requires the integration of many cellular pathways, and many inhibitory mechanisms prevent NRF2-driven transcription. Recently, NRF2 functional interactome was created, revealing new plausible regulation of ARE-related transcription [68]. It could provide insights into the regulatory mechanisms governing the expression of the myriad NRF2-dependent genes. Finally, it is worth remembering that prolonged NRF2 presence in the nucleus is not

simultaneous with the increased transcript. Interestingly, NRF2 shuttles in and out of the nucleus during stress, which permits the response's adjustment. Notably, the prolonged NRF2 presence in the nucleus shuts down the transcription. Therefore, such translocational oscillations are valuable [69].

1.6. Fact 6: NRF2-responsive genes are modulated by other transcription factors too

NRF2 transcriptional activation is often examined with one or two surrogate markers such as expression of *HMOX1*, encoding Heme Oxygenase-1 (HO-1) or *NQO1*, encoding NADP(H) Quinone Oxidoreductase 1 (NQO1). However, these and other NRF2 target genes have a complex transcriptional regulation that involves additional transcription factors.

HMOX1 was initially considered a heat shock protein [70], already indicating a complex regulation. A deep analysis of 15 kb upstream of the murine coding sequence demonstrated the existence of responsive elements for AP1, members of the heat shock factor family and HIF1α, besides NRF2 [70,71]. Moreover, *HMOX1* expression is also regulated by SP1 and NFκB transcription factors [72,73]. Concerning NRF2, it has been found that bZip protein BACH1 represses the AREs of *HMOX1* in heterodimerization with small MAFs. BACH1-repression is dominant over NRF2-activation for *HMOX1* transcription, and inactivation of BACH1 is a prerequisite for *HMOX1* induction by allowing NRF2 molecules already existent in the nucleus to bind to AREs [74]. It has even been shown that, in particular conditions, the effect of BACH1 on *HMOX1* expression could be independent of NRF2 [75]. Several electrophiles traditionally considered to activate NRF2 might elicit part of their activity by inhibiting BACH1 [71,76].

NQO1 is very often used as a marker gene of NRF2 activity. However, it should not be used as the sole indicator. Single-cell transcriptome analysis of different murine organs reveals that the *Nfe2l2* expression differs significantly between tissues, but *Nfe2l2* seems to be constantly expressed among many organs. The highest level of *Nfe2l2* transcript can be seen in the urothelial cells of the bladder, also epidermal cells of the tongue, epithelial cells of the large intestine, epidermal cells of the mammary gland, myeloid cells of the heart and fat, mesenchymal cells of the fat, mammary gland, trachea, diaphragm, lung, microglia, and endothelial cells of the heart, fat, aorta (Fig. 8). The expression patterns

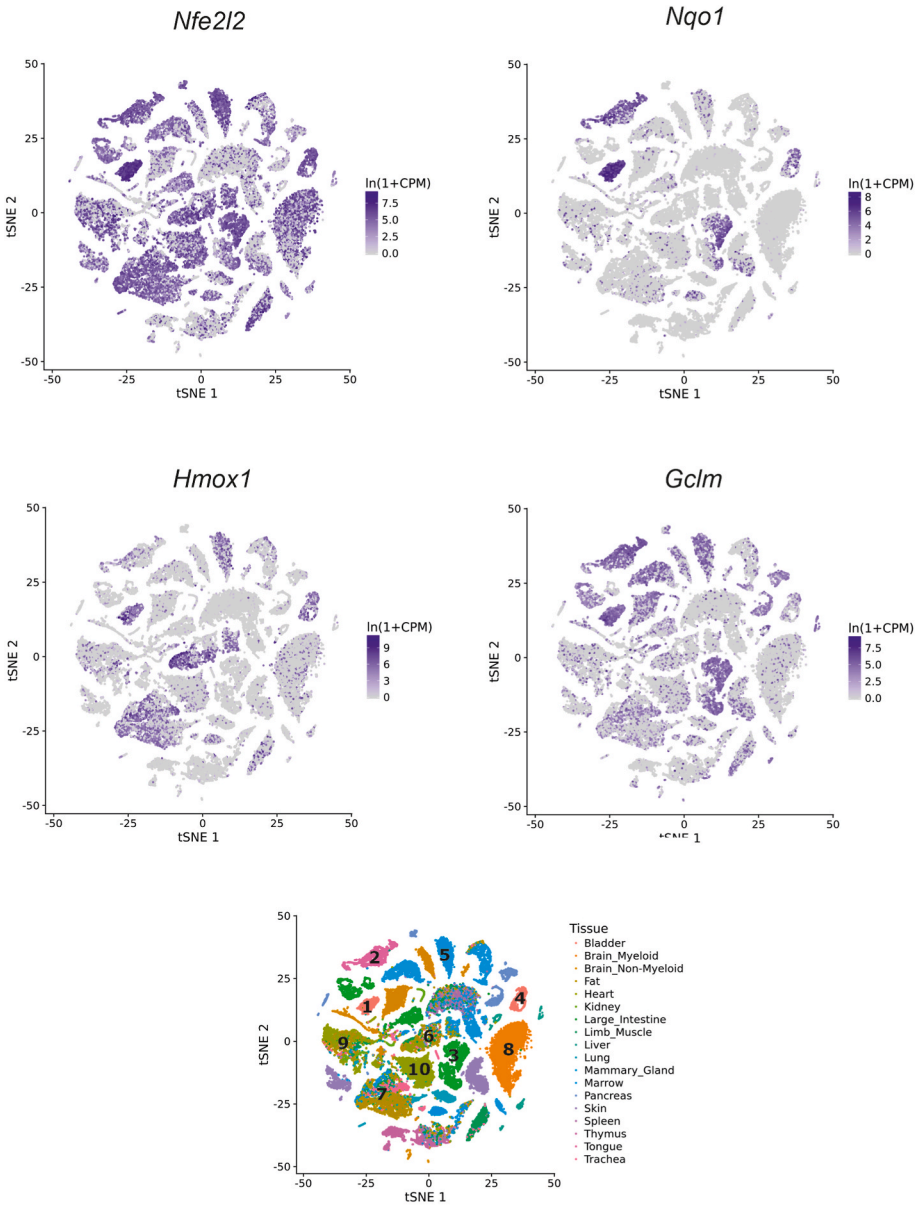


Fig. 8. Single-cell analysis of the expression of *Nfe2l2, Nqo1, Hmox1* and *Hmox1* and *Gclm* in different tissues. The expression level of genes was assessed by FACS-based full-length transcript analysis. The plots were generated based on data published by the Tabula Muris Consortium [132], which conducted a transcriptomics profiling of 100 000 single cells from 20 different organs. The bottom plot represents a t-SNE (t-Distributed Stochastic Neighbour Embedding) visualisation of cells coloured by organ. The upper and middle plots represent the *Nfe2l2, Nqo1, Hmox1* and *Gclm* expression patterns, with each dot corresponding to the cell of origin depicted in the organ plot. 1 – urothelial cells of the bladder, 2 – epidermal cells of the tongue, 3 – epithelial cells of the large intestine, 4 – bladder cells, 5 – epidermal cells of the mammary gland, 6 – myeloid cells of the heart and fat, 7 – mesenchymal cells of the fat, mammary gland, trachea, diaphragm, lung, 8 – microglia, 9 – endothelial cells of the heart, fat, aorta, 10 – fibroblasts of the heart.

of *Nqo1* and *Nfe2l2* plotted together show little correlation between those two genes (Fig. 8). Similar discrepancies can be observed for *Hmox1* and *Gclm* overlaid on *Nfe2l2* (Fig. 8). Moreover, the expression of these three NRF2 target genes is much more limited than that of *Nfe2l2*. NRF2 activity is regulated mainly at the protein level. Therefore discrepancy between NRF2 and target genes at the mRNA level is reasonable. NRF2 protein is detected at high levels in the liver (gene cards.org). It is not the case for NQO1, for which relatively high levels are observed in numerous organs, excluding blood and immune cells. HO-1 is the most abundant in the spleen and bone, whereas GCLM protein level is also high in the bone. Generally, a spatial discrepancy between these genes and proteins exists.

Noteworthy, *NQO1* can be transactivated by several transcription factors. Molecularly, in the promoter region of the *NQO1* gene, there are many *cis*-acting sites: ARE (antioxidant RE), XRE (xenobiotic RE), AP1 (immediate early response) and AP2. They serve as a consensus sequence for transcription factors such as NRF1, NRF2, AhR, AP1, AP2, and NF-κB induced in response to various stimuli [77–87]. Of note, the spatial organisation between the binding sites differs between species [79,83]. Out of these binding sites, ARE has the most pronounced impact on *NQO1* expression [80,84]. In this consensus sequence, two subelements can be distinguished: 12-O-tetradecanoylphorbol-13-acetate response element (TRE) and TRE-like, which mediate the basal transcription of *NQO1* [88,89]. The ARE-driven expression is mainly driven by NRF1, NRF2 and members of the AP1 family. Notably, a mutation in AP1 sites results in the loss of expression of *NQO1* in human cells [82]. Such a battery of possible regulators indicates redundancy in *NQO1* regulation patterns. It may explain the presence of *Nqo1* in NRF2 KO mice (Fig. 9A). Noteworthy, the level of *Nqo1* mRNA does not differ between WT and NRF2 KO tissue samples. However, in the case of the liver some tendency might be observed ($p = 0.061$) (Fig. 9A). In contrast to *Nqo1*, we found significant downregulation of *Hmox1* in the spleen, aorta and colon of NRF2 KO mice in basal conditions (Fig. 9B). Similar pattern was observed for *Gclm* transcripts (Fig. 9C). Noteworthy, the analysis of mRNA levels in organs requires snap-freezing of samples. Otherwise, induction of NRF2 target genes might be observed due to hyperoxic stress, which could generate experimental artefacts.

NQO1 and *HMOX1* represent two of the "default NRF2 program" genes at the intersection of NRF2 transcriptional targets induced by various stimuli [33]. Since they can be regulated by several transcription factors, not only NRF2, therefore, we suggest that additional NRF2 target genes should be examined, such as glutathione metabolism genes *GCLC* and *GCLM, GSR, AOX, AKR1B10,* etc. [90–92]. Since multiple transcription factors also regulate those genes in addition to NRF2, such as BACH1, HIF-1α and NF-κB in the case of *GCLM* [93–95], verification of data with chromatin immunoprecipitation assay or reporter assay would be an excellent choice.

1.7. Fact 7: electrophilic activators of NRF2 have off-targets

The chemical basis of electrophilic activation of NRF2 is that these molecules make adducts with specific cysteines in KEAP1. A very relevant electrophile sensor is C151, located at the IVR region of KEAP1, which upon adduct formation, prevents the correct interaction of KEAP1 with the ubiquitin E3 ligase complex (Cullin3/RBX1) [96–98]. However, sulfhydryl reactions also take place with cysteines of other proteins. For instance, PTEN is a phosphatase that contains redox-sensitive cysteine (C124) in its catalytic centre and is susceptible to adduct formation with several electrophilic molecules that activate NRF2 [99]. tBHQ, a food preservative that has been widely used as NRF2 activator, reacts with C124, inhibiting PTEN and therefore altering the balance between kinase (phosphatidylinositol 3 kinase) and phosphatase (PTEN) and propelling the AKT signaling pathway [100]. Dimethyl fumarate (DMF), the only FDA and EMA-approved drug activating NRF2, is used to treat remitting relapsing multiple sclerosis and psoriasis. However, some neuroprotective actions of this compound are NRF2-independent [101], and mass spectrometry of thiol modification identified 24 novel proteins modified by DMF in neurons and astrocytes [102].

The discovery that many dietary products contain hormetic NRF2-activating compounds started long-lasting research depicting the

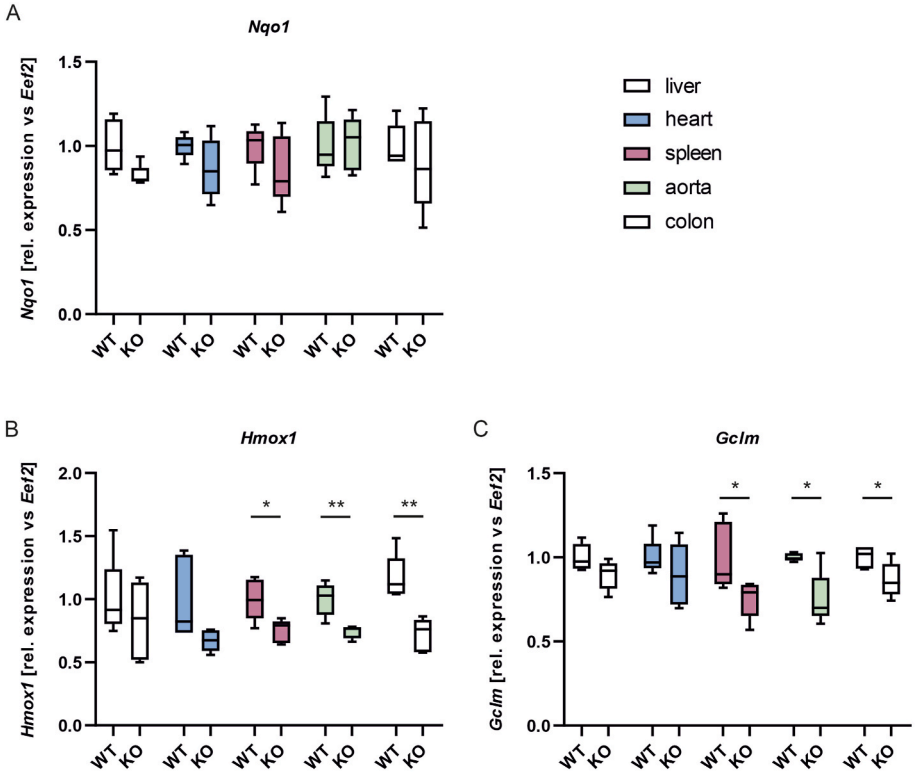


Fig. 9. *Nqo1, Hmox1 and Gclm* relative expression in organs isolated from NRF2 WT and KO mice. Murine organs: colon, aorta, spleen, heart, and liver were snap-frozen in liquid nitrogen. Total RNA was isolated using Chomczynski's method, followed by qPCR. *Eef2* served as a constitutive gene. Primer sequences: *Nqo1* F: 5′-GCTCGTAGCAGGATTTGCCT-3′, R: 5′-CAGGATGCCA CTTCTGAATCG-3'; *Hmox1* F: 5′-TTCTTCACCTTCC CCAACAT
TG-3′, R: 5′-CAGCTCCTGCAACTCCTCAAA-3'; *Gclm* F: 5′-GTTGCTATAGGCACCTCT
GA-3′, R: 5′-GTCAAATCTGGTGGCATCAC-3'; *Eef2* F: 5′-GACATCACCAAGGGTGTG
CA-3′, R: 5′-TCAGCACACTGGCATAGAGG-3'. 5 mice per group. *$p < 0.05$ vs WT in each tissue, **$p < 0.01$ vs WT in each tissue.

preventive role of NRF2 in many diseases or cellular contexts. One such deeply researched compound is sulforaphane (SFN). It has a highly electrophilic isothiocyanate (—NCS) group, which promotes the reaction with nucleophilic sulphur-, nitrogen-, and oxygen-containing groups (reviewed in Ref. [103]). Therefore, due to this high reactivity, it may modify many cellular proteins. Indeed, profiling SFN's cell-dependent targets revealed more than 500 modified proteins involved in the regulation of inflammation, kinase signalling or oxidative stress [104,105]. Some of these targets (*NFκB, MIF*) were identified independently [106,107]. In general, the higher the concentration of SFN is, the more modified proteins there are. The NRF2-targeting effect can only be considered in low concentrations (<5 μM). However, in this range, apart from KEAP1, macrophage migration inhibitory factor (MIF) may be modified [105]. Sulforaphane is a potent competitive inhibitor of CYP2E1 [108]. Notably, SFN binds major and minor grooves of DNA, ribonucleotides and nucleic acid phosphates [109]. The metabolites of SFN also have a cellular function as they may inhibit HDACs [110].

Based on this, SFN is not a specific NRF2-activator. Other well-known NRF2 inducers also have off-targets; for example, oleanane triterpenoid and dimethyl fumarate can bind to crucial signalling proteins mTOR and PKCθ, respectively [111,112]. Apart from mTOR, proteomic profiling of potential targets of oleanane triterpenoid CDDO-Imidazolide (CDDO-Im), a close variant of bardoxolone methyl, identified 576 putative binding proteins [112]. Strikingly, the phase III clinical trial for treating chronic kidney disease with bardoxolone methyl (CDDO-Me), a very potent electrophilic compound activating NRF2, was terminated because of an increase in heart failure events related to fluid retention, that mimicked the effect of endothelin receptor antagonist [113,114]. In human microvascular endothelial cells, a comparison among the effects of bardoxolone methyl vs. SFN and DMF indicated that, although the three molecules activate NRF2, only bardoxolone methyl exhibited detrimental effects on endothelin-1 release or mitochondrial potential, further supporting off target effects [115]. Therefore, when using such compounds, the researchers should be more careful in concluding on the NRF2-dependence and verify data interpretation with knockdown/dominant-negative/knockout experimental settings.

1.8. Fact 8: low NRF2 activity is not always associated with oxidative stress and damage

NRF2 is a transcriptional player that mediates cellular response to oxidative stress. Therefore, intuitively, one may expect that the inhibition of its transcriptional activity should result in oxidative damage. On the other hand, NRF2 KO mice, unless challenged, are viable and do not manifest apparent defects [90,116]. NRF2-deficient cells, generated by RNA interference or CRISPR/Cas9, are also viable [4,117–119], in contrast to cells isolated from NRF2 KO mice [4]. Many articles report that NRF2 deficiency causes increased ROS generation and oxidative stress. However, the effect of NRF2 signalling deficiency on the oxidative status might be cell type-dependent. Peering closer, NRF2 deficiency in endothelial cells is associated with a mild increase in oxidants level detected by fluorescent probes or, more specifically, by HPLC, but not excessive oxidative stress and damage assessed by oxidated glutathione level or lipid peroxidation. Conversely, the total glutathione level decreases, implying reduced antioxidative potential [6,120–122]. Mouse embryonic fibroblasts (MEFs) isolated from NRF2 KO animals do not show increased ROS formation, in contrast to glioneuronal cells [123]. Similar discrepancies in the oxidative status can be found in organs, with the liver having the highest but mild oxidative burden [120] and the aorta being protected from oxidative damage [6].

There are several explanations for these findings. The NRF2 target genes possess ARE, which can be recognized by many other transcription factors (as described above and in Ref. [124]). Secondly, NRF2 deficiency is associated with thiol modification and increased levels of NO-producing enzymes in the heart and aorta, the latter protected against oxidative stress [120]. Molecularly, the NO-related protective modification of thiols is S-nitrosation. The mild prooxidant environment upon NRF2 inhibition could facilitate the covalent attachment of NO^+ to cysteine and S-nitrosation [6,125]. Importantly, KEAP1 is a crucial regulator of S-nitrosation and may constitute a novel S-nitrosation forming complex. Furthermore, the KEAP1-related S-nitrosation of NOX4 protects NRF2-deficient endothelial cells from oxidative damage and apoptosis [6]. For further details regarding KEAP1-driven NO regulation, please refer to Ref. [19]. Overall, the interplay between NO- and oxidative stress-related cellular components may constitute a compensatory mechanism, protecting NRF2-lacking endothelial cells from oxidative stress. The protective effect can also depend on the NOX4 level, as in the case of NRF2 KO MEFs [123].

1.9. Fact 9: NRF2 and KEAP1 have a broad interactome

The quest for NRF2 transcriptional repression permitted the identification of KEAP1 [18], which is a hallmark interactor for NRF2. Indeed, the ETGE-related binding affinity between these two proteins is very high. This interaction is preferential with the significant concentration of these proteins in the cytoplasm [30]. However, biochemical and high-throughput analyses revealed a large KEAP1 interactome (the number differs: 260 in BioGrid, 179 in IntAct). These interactors possess ETGE-like motifs and were uncovered during the investigation of the modes of NRF2 activation. They mediate different cellular processes [19], permitting the KEAP1 involvement in the governance of cellular fate, especially in regulating actin cytoskeleton, proteostasis or mitochondrial function. The impact of KEAP1 on cellular homeostasis is primarily exposed in NRF2-deficient cells. There, KEAP1 exerts a detrimental role, leading to premature ageing, protein modifications and aggregation, and mitochondrial impairment [4–6,20]. In light of the above, NRF2 can be considered a KEAP1 repressor, sequestering it to prevent interaction with other proteins. Moreover, similarly to KEAP1, NRF2 also possesses a significant interactome, consisting mainly of nuclear proteins [68]. The proteins that interact with the Neh4 and Neh5 (transactivation domain) and Neh1 (DNA binding domain) domains may be particularly important for the transcriptional activity of NRF2. In addition to CBP and p300 histone acetyltransferases, Neh4 and Neh5 domains interact with other factors influencing chromatin remodelling, such as BRG1 [126] and SMRT [63], and mediator subunits, with MED16 directly bridging NRF2 to the mediator complex [127]. Other significant Neh4 and Neh5 binders include HRD1, mediating NRF2 degradation [128] and RAC3, a co-activator of NRF2 [129]. The expression profile of NRF2 target transcripts can also be modulated by numerous bZIP transcription factors interacting with Neh1 domain of NRF2 [68]. The broad interactome of NRF2 is worth noting when interpreting experimental data.

2. Concluding remarks

NRF2/KEAP1 system has been recognized as a master orchestrator of cellular stress response and a critical defence mechanism. This field does not raise much controversy, and research data is relatively consistent. However, some experimentally important nuances may affect data understanding and interpretation. Noteworthy, the awareness of unique out-of-the-box mechanisms related to NRF2/KEAP1 regulation and activity may broaden opportunities for modulation of the NRF2/KEAP1 system in translational research.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.08.044.

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