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Running head: Nrf1 is a nuclear envelope-bound transcription factor

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ABSTRACT

In rat liver RL-34 cells, endogenous Nrf1 (NF-E2-related factor 1) is localized in the endoplasmic reticulum (ER) where it exists as a glycosylated protein. Electron microscopy has demonstrated that ectopic Nrf1 in COS-1 cells is located in the ER and the nuclear envelope (NE). Subcellular fractionation, together with a membrane proteinase protection assay, revealed that Nrf1 is an integral membrane protein with both luminal and cytoplasmic domains. The N-terminal 65 residues of Nrf1 direct its integration into the ER and NE membranes and tether it to a Triton X-100-resistant membrane microdomain that is associated with lipid rafts. The activity of Nrf1 was increased by the electrophile *tert*-butyl hydroquinone (tBHQ) probably through an N-terminal domain-dependent process. We found that the NST (Asn/Ser/Thr-rich) domain, along with AD1 (acidic domain 1), contributes positively to the transactivation activity of full-length Nrf1. Further, the NST domain contains seven putative −Asn−X−Ser/Thr− glycosylation sites and when glycation was prevented by substituting all of the seven Asn to either Gln ($Nrf1^{1\text{-}7\times N/Q}$) or Asp ($Nrf1^{1\text{-}7\times N/D}$), the former multiple point mutant possessed less activity than the wild-type factor whereas the latter mutant exhibited substantially greater activity. Lastly, the ER stressors tunicamycin, thapsigargin and brefeldin A were found to inhibit basal Nrf1 activity by ~25%, and almost completely prevented induction of Nrf1-mediated transactivation by tBHQ. Collectively, these results suggest that the activity of Nrf1 critically depends on its topology within the ER, and that this is modulated by redox stressors, as well as its glycosylation status.

INTRODUCTION

Nuclear factor-erythoid 2 p45 subunit-related factor 1 (Nrf1) belongs to the family of cap 'n' collar (CNC) basic-region leucine zipper (bZIP) transcription factors [1, 2]; it should be noted that the CNC/bZIP Nrf1 protein is distinct from nuclear respiratory factor 1, a palindromic DNA sequence-binding protein [3], that unfortunately shares the same abbreviation in the scientific literature. The CNC/bZIP factor Nrf1 is widely expressed in mouse and human tissues [1, 2, 4, 5] and regulates cytoprotective genes through antioxidant response elements (AREs) in their promoter regions [6-8]. Genes transactivated by Nrf1 include those encoding the glutamate cysteine ligase catalytic and modifier subunits, which together catalyze the rate-limiting step in glutathione (GSH) biosynthesis, as well as NAD(P)H:quinone oxidoreductase 1 (NQO1), haem oxygenase 1, ferritin and metallothionein [9-12]. Transcriptional activation of ARE-driven genes by Nrf1 prevents oxidative stress *in vivo* [9].

In addition to Nrf1, the related CNC/bZIP factor Nrf2 also controls ARE-driven genes [5, 13, 14]. Whilst it might be anticipated that Nrf1 and Nrf2 fulfill similar roles, gene-targeting experiments have revealed that the two factors have distinct biological functions. Global knockout of Nrf1 is lethal to mouse embryos [15, 16], and foetal livers from *Nrf1*$^{-/-}$ mice exhibit increased apoptosis resulting from endogenous oxidative stress [17]. Liver-specific disruption of *Nrf1* in neonatal mice results in the development of non-alcoholic steatohepatitis at 4 weeks, and pre-neoplastic hepatic lesions by 4 months of age [18]. By contrast, *Nrf2*$^{-/-}$ mice develop normally and their livers show no overt evidence of steatohepatitis [19]. The major *in vivo* role for Nrf2 appears to be adaptation to redox stress as it mediates induction of the ARE-gene battery by thiol-active chemicals [5]. These results suggest that Nrf1 is indispensable for the

constitutive expression of certain critical ARE-driven genes during liver development, whereas Nrf2 makes less of a contribution to the basal expression of these genes.

It is plausible that the distinct *in vivo* roles of Nrf1 and Nrf2 are determined, at least in part, by differences in their subcellular localization [20, 21]. We have reported previously that Nrf1 is anchored to the endoplasmic reticulum (ER) through an N-terminal signal targeting sequence that encompasses NHB1 (N-terminal homology box 1, amino acids 11-30) within its NTD (N-terminal domain, amino acids 1-124) [22]. We have also demonstrated that Nrf1 is glycosylated in the ER through its NST (Asn/Ser/Thr-rich) domain that lies between amino acids 296 and 403. Although the significance of this post-translational modification is unclear, Nrf1 protein within the ER is glycosylated and has a molecular mass of ~120 kDa, whereas Nrf1 protein in the nucleus does not appear to be glycosylated and has a molecular mass of ~95 kDa [22]. Transcription factor Nrf2 is smaller than Nrf1, and contains neither the NTD nor a NST domain. Unlike Nrf1, Nrf2 is not targeted to the ER nor is it glycosylated [20, 22]. Thus Nrf1 and Nrf2 may be activated by redox signals, or other stimuli, in different subcellular compartments.

It is unclear how Nrf1 migrates from the ER to the nucleus. One possibility is that it is released from the ER in response to an appropriate stimulus, and thereafter translocates to the nucleus through the cytoplasm. We have however found no evidence that this CNC/bZIP factor is proteolytically cleaved within its NTD [22]. Another possibility is that Nrf1 is tethered within the ER but is sorted to the nuclear envelope (NE), by an unknown mechanism, whereupon it is able to gain access to chromatin. An obvious shortcoming of the second model is that it has not yet been shown whether Nrf1 can associate with the NE.

The original discovery that Nrf1 can activate ARE-driven gene expression was made in the context that both it and Nrf2 are capable of mediating induction of gene expression by *tert*-butyl hydroquinone (tBHQ) [6], a redox-cycling electrophilic quinone that can form conjugates with GSH [23]. It is not however known if the ability of Nrf1 to mediate gene induction by tBHQ is accompanied by alterations in its glycosylation status. More recently, Wang and Chan [21] reported that tunicamycin (TU), an inhibitor of Asn-linked glycosylation, decreases the molecular mass of Nrf1 from ~120 kDa to ~95 kDa, and that this is associated with an increased nuclear accumulation of the transcription factor. Whilst TU is known to produce ER stress [24], it is not known whether the decrease in molecular mass of Nrf1 affected by TU is due to ER stress or is simply due to failure to glycosylate the CNC/bZIP protein. Importantly, it is also not known whether TU treatment increases the transactivation activity of Nrf1. In order to evaluate whether ER stress *per se* activates Nrf1, it would be helpful to known whether other classic stressors such as thapsigargin (TG) and brefeldin A (BFA) also produce a decrease in the mass of Nrf1, and whether such an event is accompanied by an increase in ARE-driven gene expression.

To provide a better understanding of the significance of targeting Nrf1 to the ER, we have examined in the present study whether: i) the subcellular localization, the molecular mass, the abundance and the transactivation activity of Nrf1 is influenced by the ER stressors TU, TG and BFA and/or the electrophile tBHQ; ii) Nrf1 is completely integrated into the ER membrane; iii) the loss of Asn-glycosylation sites increases Nrf1 activity; iv) Nrf1 can localize to the NE and/or the nucleoplasm.

EXPERIMENTAL

Chemicals and other reagents

These were all of the highest quality available and were readily available commercially. The ER extraction kit and all chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK). Endoglycosidase (Endo) H, peptide:N-glycosidase (PNGase) F and proteinase K (PK) were obtained from New England Biolabs (Hitchin, UK). Rabbit polyclonal antibodies against calreticulin (CRT, residues 405-417) and green fluorescent protein (GFP) were bought

from Calbiochem (San Diego, USA) and Abcam PLC (Cambridge, UK), respetively. Antibodies against ATF6α and GRP78 were from Santa Cruz Biotechnology (Santa Cruz, USA). Mouse monoclonal antibodies against the V5 epitope and DsRed, a *Discosoma sp*. red fluorescent protein, were from Invitrogen Ltd. (Paisley, UK), whereas those against Sec61α and Lamin A/C were supplied by Upstate (Dundee, UK). Antiserum against residues 292-741 of Nrf1 was raised in female New Zealand white rabbits [22].

Expression Constructs

The cloning of mouse Nrf1 cDNA has been described previously [20]. Polymerase chain reaction (PCR) amplification and site-directed mutagenesis were performed to create cDNA encoding various Nrf1 mutants [25], and these were also cloned into the KpnI/XbaI site of pcDNA3.1/V5His B. All oligonucleotide primers in this study were synthesized by MWG Biotech (Ebersberg, Germany) and are listed in supplemental Table S1.

A fusion expression vector pDsRed2-GFP was made by inserting the cDNA for GFP into the KpnI/XmaI site of plasmid pDsRed2-C1 (Clontech Laboratories, Mountain View, CA, USA). Nucleotide sequences encoding either full-length Nrf1 protein or mutants lacking different portions of its C-terminus were inserted between the cDNAs for DsRed2 and GFP within the pDsRed2-GFP expression vector through either the SalI/KpnI or Hind III/KpnI multiple cloning sites. In these constructs, the first Kozak's consensus translation initiation sites in both Nrf1 and GFP were eliminated by site-directed mutagenesis to avoid translation of unfused Nrf1 and/or GFP. As a result, the constructs directed the expression of a single fusion fluorescent polypeptide with Nrf1 sandwiched between DsRed and GFP (called DsRed/Nrf1/GFP herein).

A series of Gal4/Nrf1 expression constructs were generated by ligating Nrf1-encoding cDNA sequences to the 3′-end of cDNA encoding the Gal4 DNA-binding domain (called Gal4D), within pcDNA3.1Gal4D-V5, through the BamHI/EcoRI site [20]. The fidelity of all cDNA products was confirmed by sequencing.

Cell culture, transfection and luciferase reporter assays

Monkey kidney COS-1 and rat liver RL-34 epithelial cells were grown for 24 h in DMEM (Dulbecco's modified Eagle's medium) [20]. After the cells reached 70% confluence, they were transfected with expression constructs for Nrf1 and its mutants, along with an ARE-driven luciferase reporter plasmid [26], using Lipofectamine 2000 (Invitrogen). In some experiments, COS-1 cells were cotransfected with an ATF6α expression construct and a *5×ATF6GL3/UPRE-Luc* reporter plasmid, both of which were a gift from Dr. L. H. Glimcher, Department of Immunology and Infectious Diseases, Harvard School of Public health [27]. The pRL-TK plasmid (Promega Co., Southampton, UK) encoding renilla, as well as pcDNA4/HisMax/*lacZ* (Invitrogen) encoding β-galactosidase (β-gal), was used to control for transfection efficiency. In some experiments, transfected cells were treated for 24 h with chemicals before reporter gene activity was measured as described previously [20].

Nrf1 siRNA transfection and TaqMan® quantitative real-time PCR

The siRNA oligonucleotides against Nrf1 were designed using an online program from MWG Biotech, according to the current guidelines for effective knockdown by this method. A pair of rat Nrf1-targeting siRNAs, 5'-AAAAUCAUCAACCUGCCUGUAGAAGAA-3' (sense) and 5'-UUC UUCUACAGGCAGGUUGAUAGUUUU-3' (antisense), and an additional pair of the scrambled siRNAs, 5'-GACGAGCGGCACGUGCACA-3' (sense) and 5'-UGUGCACGUGCCGCUCGUC-3' (antisense) were provided by MWG Biotech in a freeze-dried, pre-annealed, HPLC-purified form. Rat liver RL-34 cells were transfected with these siRNAs (20 and 50 nmol/l) by incubating them overnight in a reaction mixture containing 10 μl Lipofectamine 2000 in 2 ml of serum-free Opti-MEM1 medium (Invitrogen). Approximately 24 h after transfection, RL-34 cell lysates were prepared, filtered through a

QIAshredder™column (Qiagen, West Sussex, UK), and total RNA extracted using an RNeasy® mini kit (Qiagen). After contaminant genomic DNA was removed through on-column DNase digestion, 2 μg of total RNA was reverse-transcribed into cDNA using Omniscript (Qiagen). The levels of Nrf1 mRNA in cell lysates were determined by TaqMan® RT-PCR chemistry using 18S rRNA as an internal control; the Nrf1 primers and probe are listed in Table S1.

Immunocytochemistry, confocal and electron microscopy

These experiments were performed as described previously [20, 22, 28].

Subcellular and subnuclear fractionations

The intact ER-rich, microsome-containing membrane and cytosolic fractions were prepared as described previously [22, 29]. The whole nuclei fraction, along with its surrounding NE, was purified from COS-1 cell homogenates by the method of Harfors and Bonifacino [30]. Cell pellets were resuspended in 4 volumes of 1 × isotonic extraction buffer [10 mM Hepes, pH 7.8, containing 250 mM sucrose, 1 mM EGTA, 1 mM EDTA and 25 mM KCl supplemented with 1% (v/v) Complete protease inhibitor cocktail (CPIC)]. The resuspended cells were homogenized by repetitive passage through a 23-gauge needle using 20 strokes. The nuclear (N) fraction was purified from cell homogenates in 3 volumes of STM-N buffer [250 mM sucrose, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5% (v/v) NP-40, supplemented with 1 mM dithiothreito1 (DTT) and 1% CPIC]. The salt-extracted nuclear fraction was prepared according to a previously described method [31, 32]. In this case, purified nuclei were incubated with an extraction buffer [10 mM Hepes-KOH, pH 7.4, 0.42 M NaCl, 2.5% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.5 mM sodium EDTA, 0.5 mM EGTA, 1 mM DTT, 1 mM phenylmethylsufonyl fluoride (PMSF), and 1% protease inhibitors mixture]. After being gently rotated for 60 min at 4 °C, the mixture was centrifuged at 14,000 × *g* for 5 min at 4°C and the resulting supernatant that was saved is referred to as salt-extracted nuclei.

The NE fraction was obtained by the high-ionic-strength method of Kaufmann *et al* [33]. Briefly, purified nuclei were suspended at a concentration of 5 mg DNA/ml in the SMT buffer [250 mM sucrose, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, supplemented with 1 mM PMSF and 1% CPIC], before being gently homogenized by passing the mixture through a 25-gauge needle with 10 strokes. The nuclear homogenate (1 ml) was incubated with both DNase I and RNase at 250 μg/ml for 1 h at 4°C, and was then sedimented by centrifugation at 1,000 × *g* for 10 min at 4°C. The resulting pellet was resuspended in the same volume of MT buffer [0.2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, containing 1 mM PMSF and 1% CPIC], and was subsequently mixed with 4 volumes of high-NaCl buffer [2 M NaCl, 0.2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, supplemented with 1% (v/v) 2-mercaptoethanol, 1 mM PMSF and 1% CPIC]. After stirring for 30 min, the NE was pelletted by centrifugation at 1,600 × *g* and 4 °C for 30 min. The inner nuclear membrane (INM)-rich fraction from the NP-40-containing cell homogenates was prepared by the above purification procedure.

Membrane proteinase protection reactions

This was carried out to determine whether significant portions of Nrf1 are buried within the ER membrane and thereby protected against PK digestion [34]. Briefly, either intact ER-rich or microsome-containing membrane fractions were purified and resuspended in 100 μl of 1 × isotonic buffer [10 mM Hepes, pH 7.8, containing 250 mM sucrose, 1 mM EGTA, 1 mM EDTA and 25 mM KCl]. An aliquot (30 μg of protein) of the membrane-containing preparation was incubated for 30 min on ice with PK at a final concentration of either 50 or 100 μg protein/ml in either the presence or absence of 1% (v/v) TX. The reactions were stopped by incubation at 90°C for 10 min following the addition of 1 mM PMSF.

Deglycosylation reactions, co-immunoprecipitation and western blotting

These experiments were carried out as described previously [20, 22, 35, 36]. On some occasions, antibody-blotted nitrocellulose membranes were washed for 30 min with stripping buffer [7M guanidine hydrochloride, 50 mM glycine, 0.05 mM EDTA, 0.1 M KCl and 20 mM 2-mercaptoethanol at pH 10.8] before being re-probed with an additional primary antibody [37].

Statistical analysis

Nrf1-mediated luciferase transactivation activity from *P_{TK}nqo1-ARE-Luc* and *P_{SV40}GSTA2-6×ARE-Luc* reporter constructs was calculated after subtraction of the background activity obtained from an empty pGL3-promoter plasmid, and the data are shown as a fold change (mean ± S.D) of the activity obtained following transfection with an expression vector for the CNC/bZIP factor when compared with that obtained following transfection with an empty pcDNA3.1/V5 His B vector. The data presented in this study are typical of at least 3 different independent culture experiments undertaken on different days, each of which was performed in triplicate. The significance of differences in the luciferase activity was determined using the unpaired Student's *t* test and is shown as a *p* value.

RESULTS

Endogenous Nrf1 is localized in the ER and modified by Asn-glycosylation

We have reported previously that ectopic V5-tagged Nrf1 is targeted to the ER [20, 22]. To confirm that endogenous Nrf1 is also localized in this organelle, we performed immunocytochemistry using an antibody raised against residues 292-741 of the CNC/bZIP protein. Confocal microscopy showed that Nrf1 is located primarily in the extra-nuclear compartment of RL-34 cells and yielded a staining pattern similar to that obtained for the ER marker calreticulin (Figure 1A). To help support the immunocytochemical data, knockdown experiments were performed. Extra-nuclear staining for Nrf1 was markedly diminished in RL-34 cells following their transfection with siRNA targeted against the factor (Figure 1B). TaqMan® chemistry revealed that 50 nmol/l siRNA against Nrf1 reduced the level of its mRNA to approximately 40% of the amount obtained following mock transfection (Figure 1C). Western blotting of proteins resolved by LDS (lithium dodecyl sulfate)-NuPAGE with anti-Nrf1 serum revealed that the ER-enriched fraction contained two immnunoreactive polypeptides with estimated molecular masses of ~55 kDa and ~120 kDa (Figure 1D, *lane 2*). The relative amounts of both Nrf1 proteins were decreased by knockdown with a specific siRNA to 40%–50% of the levels obtained from transfection with a scrambled siRNA (Figure 1D, *c.f. lanes 3 with 2*). Following *in vitro* deglycosylation with Endo H, the size of the ~120-kDa Nrf1 electrophoretic band in the ER decreased to ~95 kDa, whereas the ~55-kDa Nrf1 protein was unaffected (Figure 1D, *c.f. lanes 4 with 5*). These results demonstrate that in RL-34 cells endogenous full-length Nrf1 is located in the ER where it exists as a glycoprotein.

Nrf1 is not activated by ER stressors

To test whether Nrf1 is activated by ER stressors, COS-1 cells were cotransfected with an Nrf1 expression construct and an ARE-Luc reporter plasmid; as a positive control, COS-1 cells were also co-transfected with an ATF6α expression construct and an UPRE (unfolded protein response element)-Luc reporter plasmid. After 18 h recovery from transfection, the cells were treated for 24 h with TU, TG or BFA. Examination of the *P_{TK}nqo1-ARE-Luc* reporter activity showed that none of the ER stressors increased the ability of Nrf1 to transactivate gene expression (Figure 2A, *upper panel*). Indeed, TU, TG and BFA diminished Nrf1 activity to between 65% and 75% of that observed when the cells were treated by vehicle alone, i.e. 0.1% dimethyl sulfoxide (DMSO). These compounds did however increase the expression of GRP78, a chaperone protein induced by ER stress (Figure 2A, *lower panel*). Whilst these ER stressors

did not increase ARE-Luc activity they did increase ATF6α-mediated UPRE-driven luciferase gene activity about 2-fold (supplemental Figure S2). Furthermore, activation of ARE-driven luciferase activity was significantly inhibited by the presence of ALLN (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal), a proteasome inhibitor.

Western blotting confirmed the report of Wang and Chan [21] that treatment with TU resulted in an increase in the amount of the ~95-kDa Nrf1 polypeptide and a corresponding relative decrease in the level of the ~120-kDa protein. However, this increase in the ~95-kDa Nrf1 isoform was not accompanied by an increase in ARE-driven reporter gene activity (Figure 2A), indicating that failure to glycosylate Nrf1 is not sufficient to affect transactivation of target genes. Immunoblotting also showed that the level of the ~120-kDa Nrf1 polypeptide, rather than the ~95-kDa protein, increased following treatment with TG or BFA (Figure 2A, *middle left panel*), but in neither case did this enhance ARE-driven luciferase activity.

Subcellular localization of Nrf1 is unaffected by ER stressors

Immunocytochemistry revealed that treatment with TU, TG or BFA caused a modest decrease in nuclear accumulation of Nrf1, when compared with the staining pattern of Nrf1 in vehicle (DMSO)-treated cells (Figure 2C). Following co-treatment with TU plus ALLN, or treatment with ALLN alone, we found that Nrf1 was located primarily in the extra-nuclear ER subcellular fraction in 95% and 99% of the cells, respectively (Figure 2C). These results suggest that TU, TG and BFA did not markedly influence retention of Nrf1 in the ER, and are consistent with the failure, noted above, of the stressors to stimulate Nrf1-mediated expression of ARE-driven genes. Treatment with ALLN produced an increase in the amount of the ~120-kDa Nrf1 protein, suggesting that its abundance may be controlled by the proteasome.

Nrf1 is activated by tBHQ and the activation is inhibited by ER stressors

Nrf1 has been reported to mediate gene induction by tBHQ [6]. We sought to confirm this finding and test whether the localization of Nrf1, its abundance or its size is influenced by the hydroquinone. Figure 2B shows that treatment of COS-1 cells with 50 μM tBHQ produced a significant increase in transactivation of P_{SV40}*GSTA2-6×ARE-Luc* by Nrf1 from ~14-fold to 28-fold. A dose-dependent increase in Nrf1-mediated transactivation of P_{TK}*nqo1-ARE-Luc* was observed following treatment of COS-1 and RL-34 cells with tBHQ (supplemental Figures S3A and B). Furthermore, Nrf1-mediated induction of ARE-driven gene expression by tBHQ was also observed in *Nrf2*$^{-/-}$ mouse embryonic fibroblasts (supplemental Figure S9B). Immunocytochemistry revealed that following treatment of COS-1 cells with 50 μM tBHQ, ectopic Nrf1 gave predominantly nuclear staining in approximately 65% of the cells examined, with only 35% of cells showing primarily extra-nuclear staining (Figure 2C).

Induction of Nrf1-mediated transactivation by tBHQ was almost completely abolished by the ER stressors TU, TG or BFA, as well as by ALLN (Figure 2B, *upper panel*). Western blotting showed no change in the protein electrophoresis patterns of Nrf1 expressed in tBHQ-treated cells, as compared to the vehicle (DMSO)-treated cells (Figure 2B, *lower panels*). A single ~95-kDa Nrf1 protein band was observed in total lysates of cells that were co-treated with TU and tBHQ. When compared with treatment with tBHQ alone, the abundance of the ~120-kDa Nrf1 protein increased in tBHQ and TG co-treated cells, but it was unaffected by co-treatment with BFA. The results indicate that these ER stressors probably inhibit Nrf1 activity by blocking its glycosylation and/or deglycosylation reactions and that this in turn prevents its transport to the nucleus.

Activation of Nrf1 by tBHQ requires the presence of its NTD

It is not known which region of Nrf1 is responsible for its activation by tBHQ. As Nrf1 is negatively regulated by its NTD [20], we tested whether loss of repression by this domain represents the mechanism by which tBHQ can increase

gene transactivation through the CNC/bZIP factor. Expression constructs were therefore prepared for a series of Nrf1 mutants in which internal contiguous sequences were sequentially deleted from its NTD. Rat liver RL-34 cells were transfected with these constructs along with an ARE-Luc reporter plasmid. After recovery from transfection, the cells were treated for 24 h with either DMSO or 50 μM tBHQ. Under basal conditions, most of the Nrf1 mutants produced levels of luciferase activity that were similar to that produced by the wild-type factor. Consistent with our previous investigations into the signal peptide sequences associated with NHB1 [22], Nrf1$^{\Delta 2\text{-}10}$ exhibited a low basal luciferase activity whereas Nrf1$^{\Delta 11\text{-}22}$ possessed substantially higher basal luciferase activity than wild-type Nrf1 (supplemental Figure S3C). Some of the other Nrf1 mutants, such as Nrf1$^{\Delta 23\text{-}30}$, Nrf1$^{\Delta 96\text{-}106}$ and Nrf1$^{\Delta 107\text{-}124}$, that lacked different portions of the NTD, produced similar basal luciferase activity as the wild-type protein. Others however, including Nrf1$^{\Delta 31\text{-}80}$, Nrf1$^{\Delta 66\text{-}80}$, Nrf1$^{\Delta 81\text{-}90}$ and Nrf1$^{\Delta 81\text{-}106}$ exhibited higher basal luciferase activity than the wild-type factor. This increase in basal activity was accompanied by a significant reduction in the ability of tBHQ to induce *luciferase*; in particular, the Nrf1$^{\Delta 31\text{-}80}$, Nrf1$^{\Delta 66\text{-}80}$ and Nrf1$^{\Delta 81\text{-}90}$ mutants failed to demonstrate an increase in transactivation activity upon treatment with the hydroquinone (supplemental Figure S3C). Western blotting showed that in the levels of these proteins did not change following treatment with tBHQ (supplemental Figure S3D). Collectively, these results suggest amino acids 31-90 contribute to the negative regulation of Nrf1 but this inhibition is partially alleviated by tBHQ. One interpretation of these data is that residues between NHB1 and NHB2 control the topology of Nrf1 in the ER, and that tBHQ influences the protein fold of this region. Presumably the Nrf1$^{\Delta 31\text{-}80}$, Nrf1$^{\Delta 66\text{-}80}$ and Nrf1$^{\Delta 81\text{-}90}$ mutants are inserted incorrectly into ER membranes and are therefore neither efficiently inhibited by the mutated NTD nor activated by tBHQ.

Nrf1 is both positively and negatively regulated through its NST domain

Nrf1 exists in the nucleus as a ~95-kDa non-glycosylated protein [22]. Surprisingly, we discorved that treatment of cells with TU increased the amount of ~95-kDa Nrf1 protein whilst inhibiting its transactivation activity by approximately 25% (Figure 2A). We therefore examined whether failure to glycosylate or deglycosylate the CNC/bZIP factor influences its activity. To this end, we studied mutants of Nrf1 lacking its entire NST domain or only the glycosylation consensus sites within this region. As shown in Figure 3, the Nrf1$^{\Delta 299\text{-}400}$ mutant exhibited less transactivation activity in COS-1 cells (*panel* A) and RL-34 cells (*panel* B) than wild-type Nrf1, suggesting that the NST domain makes a positive contribution to the activity of the CNC/bZIP factor. Furthermore, deletion of residues 299-400 also prevented the increased transactivation that was observed upon disruption of NHB1 (c.f. Nrf1$^{\Delta 11\text{-}30:\Delta 299\text{-}400}$ with Nrf1$^{\Delta 11\text{-}30}$), indicating the NST domain is required for transactivation activity of a mutant factor that translocates directly to the nucleus. Our data also indicate that the NST domain primarily controls basal gene expression but is not required for Nrf1-mediated induction of ARE-driven gene expression by tBHQ (Figure 3B). This domain contains three Asn-glycosylation consensus sites (Asn^{300}, Asn^{319} and Asn^{331}) between residues 299-333 and deletion of this region gave rise to a mutant with an activity similar to that of Nrf1$^{\Delta 299\text{-}400}$. By contrast, deletion of residues 364-400, which contains four potential Asn-glycosylation sites (Asn^{371}, Asn^{376}, Asn^{394} and Asn^{398}), produced an Nrf1 mutant protein with a modestly increased transactivation activity (Figure 3A). To allow a more precise understanding of the effect that glycosylation and/or deglycosylation has on Nrf1 function, each of these seven asparagine residues in the NST domain was substituted with aspartic acid or glutamine to yield two multiple point mutants, called Nrf1$^{1\text{-}7xN/D}$ and Nrf1$^{1\text{-}7xN/Q}$. The latter represents a non-glycosylated version of Nrf1, whilst the former is considered to represent a deglycosylated version of the CNC/bZIP protein. Western blots of lysates from COS-1 cells that expressed either Nrf1$^{1\text{-}7xN/D}$ or Nrf1$^{1\text{-}7xN/Q}$ and had been digested with PNGase F confirmed that the mutant proteins were not glycosylated (Figure 3C, *lower panel*). Most importantly, when compared with wild-type Nrf1, the Nrf1$^{1\text{-}7xN/D}$ mutant

exhibited significantly higher activity than its wild-type counterpart ($p < 0.001$), whereas Nrf1$^{1\text{-}7xN/Q}$ possessed modestly less activity ($p < 0.05$) (Figure 3C, *upper panel*). These results suggest that Nrf1 is positively regulated by its NST domain, but that Asn-glycosylation within this region represses its activity. Once glycosylated, Nrf1 can then be activated by deglycosylation of the glycan-linked asparagines, a reaction that results in each of them being converted into aspartic acid.

To evaluate independently whether the NST domain contributes to gene transactivation, a Gal4 reporter assay was performed. Expression constructs were made in which residues 1-607 of Nrf1 were fused to the C-terminus of the DNA-binding domain of Gal4. Following transfection into COS-1 cells, it was found that those constructs lacking the entire NST domain produced a significantly reduced *UAS-Luc* reporter gene activity than those with this domain (c.f. Gal4D/Nrf1$^{297\text{-}607}$ with Gal4D/Nrf1$^{403\text{-}607}$) (Figure 3D); no changes in their protein levels were observed (Supplemental Figure S4). These findings indicate that NST functions as a transactivation domain in the context of a Gal4/Nrf1 fusion protein; this fusion protein does not contain an ER-targeting signal and is presumably not Asn-glycosylated.

The Neh5L subdomain within AD1 contributes to the activity of Nrf1

In Nrf1, AD1 resides between amino acids 125 and 298. Characterization of a series of internal deletion mutants within AD1 revealed that amino acids 125 to 170 at the N-terminal end of this domain, residues 280-298 at the C-terminal end and, to a lesser extent, its adjoining amino acids 261-279, are essential for transactivation (Figure 4A, *left panel*). By contrast, deletion of portions of AD1 between residues 171-260 appeared to contribute little to the activity of Nrf1.

Whilst deletion of residues 125-170 in Nrf1 greatly diminished basal activity, the mutant factor still respond to tBHQ. Deletion of amino acids 280-298 completely abolished both the basal activity of the mutant and its ability to transactivate an ARE-driven reporter gene in the presence of tBHQ (Figure 4A, *right panel*). Within Nrf1, amino acids 280-298 comprise the sequence DLEQQWQDLMSIMEMQAME, and this resembles the Neh5 domain of Nrf2 that, along with Neh4, allows gene transactivation through binding the coactivator CBP (CREB binding protein) [38].

The finding that amino acids 171-260 of Nrf1 make little contribution to its activity (Figure 4A, *left panel*) is surprising because residues 171 to 183 in Nrf1 are closely similar to the DIDLID element that acts as the core of an activation domain in the *C. elegans* transcription factor Skn1 [39]; this element is also represented in Nrf2 (Figure 4B). Immediately adjacent to the DIDLID element, Nrf1 contains a DLG motif (residues 184-186) as well as a more distal ETGE motif (residues 234-237). Both the DLG and ETGE motifs are present in Nrf2 where they serve respectively as low- and high-affinity Keap1-binding sites that allow the CNC/bZIP protein to be ubiquitylated by Cul3/Rbx1 [40]. Redox stressors, such as tBHQ, prevent ubiquitylation of Nrf2 by causing structural changes in the $BC_3B^{Keap1/Keap1}$ complex. In order to test whether the DIDLID element, the DLG motif or the ETGE motif are necessary for Nrf1-mediated induction of ARE-driven gene expression by tBHQ, we prepared expression constructs for Nrf1$^{\Delta 171\text{-}180}$, Nrf1$^{\Delta 184\text{-}186}$ and Nrf1$^{\Delta 234\text{-}237}$; in the case of the DIDLID element, only its N-terminal portion was deleted because the region of the DLG motif required to interact with Keap1 probably overlaps with the C-terminal part of the DIDLID element. Examination of these mutants revealed that neither the basal activity of Nrf1 nor its ability to mediate induction of ARE-driven gene expression by tBHQ were altered significantly by deletion of the DIDLID element (Figure 4B, *left panel*). These results suggest that the DIDLID element is not necessary for the transactivation activity of Nrf1. Deletion of neither the DLG motif nor the ETGE motif from Nrf1 increased either basal or tBHQ stimulated ARE-driven gene expression (Figure 4B). As shown in supplementary Figure S5, no change in the levels of Nrf1 protein was observed upon deletion of DLG or ETGE by western blotting. These results are consistent with previous data that the DLG and ETGE motifs within AD1 do not direct Nrf1 to the Keap1-dependent degradation

pathway [20].

Nrf1 is an integral membrane protein with transmembrane and extra-membrane regions

To evaluate the extent to which Nrf1 is integrated into the ER membrane, proteinase K (PK) protection reactions were performed. Figure 5A shows that following PK digestion for 30 min, the abundance of endogenous ~120-kDa Nrf1 protein in the ER of RL-34 cells decreased significantly, and that this was associated with a marked increase in the abundance of a ~55 kDa Nrf1 protein, along with a digested peptide ladder between ~36 kDa and ~55 kDa (Figure 5A). These ~36-kDa and ~55-kDa Nrf1 products were almost completely digested by PK following addition of 1% Triton X-100 (TX), a detergent that can solubilize general lipid-disordered membrane. Moreover, when a similar PK protection assay was performed using ER membranes from COS-1 cells expressing ectopic Nrf1, western blotting with a V5 antibody failed to identify an epitope-tagged product between ~55 kDa and ~120 kDa (supplemental Figure S6). By contrast, neither the abundance nor the electrophoretic mobility of calreticulin, an ER luminal protein, was affected by PK digestion. These results indicate that although Nrf1 is translocated into the ER lumen and then inserted into the membrane, a significant portion of this CNC/bZIP protein remains exposed to the cytoplasm.

Our previous work has suggested that Nrf1 is secured in the ER through its NHB1 which acts as a transmembrane anchor [22]. To test the hypothesis that the N-terminus of Nrf1 is exposed to the cytoplasm, we prepared ER membranes from COS-1 cells that expressed a DsRed/Nrf1/GFP sandwich fusion protein. Immunoblotting revealed a polypeptide of ~150 kDa that cross-reacted with an antibody against DsRed in ER membranes of COS-1 cells that expressed the sandwich fusion protein (Figure 5B). However, following incubation with PK, probing with an antibody against DsRed failed to identify a cross-reacting band, indicating that the N-terminus of Nrf1 is exposed to the cytoplasmic milieu and thus is not protected from proteolysis by the ER membrane (Figure 5B, *lower panel*). To test whether the C-terminus of Nrf1 is also exposed to the cytoplasm we probed the PK digests with an antibody against GFP, and in this case a 32-kDa polypeptide was identified; by contrast, free GFP migrated with a molecular mass of 28 kDa during LDS-NuPAGE. Significantly, the 32-kDa form was still resistant to digestion by PK even in the presence of 1% TX (Figure 5B, *upper panel*). These data suggest that a C-terminal transmembrane peptide of Nrf1 which is fused to GFP can integrate into the TX detergent-resistant membrane microdomain (DRM) with an N_{cyt}/C_{lum} orientation (i.e., its N- and C-terminal flanking regions reside in the cytoplasmic and luminal sides, respectively).

Further western blotting experiments using either DsRed or GFP antibodies revealed that a polypeptide of about 62 kDa was recovered in the ER fractions of COS-1 cells which had been transfected with an expression construct for a fusion protein in which the N-terminal 65 residues of Nrf1 (N65) were sandwiched between DsRed and GFP (Figure 5C). This fusion protein was partially digested by PK, but the degradation was not increased by TX. A weak PK-digested band of 32 kDa cross-reacted with an antibody against GFP, but not DsRed, and its abundance was also unaffected by TX. These results indicate that a fraction of the DsRed/N65/GFP fusion protein is correctly integrated into the DRM through an appropriately folded transmembrane region (i.e., NHB1) with an N_{cyt}/C_{lum} orientation. However, the remaining fraction of this fusion protein may be incorrectly folded such that NHB1 is unable to span the membrane and the N-terminus is buried within the ER, thereby allowing DsRed to escape proteolysis by PK.

The TM1 region of Nrf1 determines its membrane integration

Bioinformatic searches for topogenic signals within Nrf1 protein revealed that residues 7-26, 374-393 and 707-725 could form three membrane-spanning α-helices. As shown in Figure 6A, we have designated these TM1, TMi and TMc (also see supplemental Figure S8). The TM1 sequence has been reported previously to be necessary to target and anchor Nrf1 to the ER membrane [22]. The presence of the putative TMi sequence between residues 374-393 suggests that the NST domain, along with AD1 and the C-terminal portion of NTD, is normally positioned within the lumen of

the ER. This is consistent with the fact that glycosylation of Nrf1 occurs through its NST domain. The position of a membrane-spanning peptide in the C-terminal part of the NST domain suggests that AD2, and the SR (serine-rich), Neh6L (Neh6-like), CNC and bZIP domains are partitioned in the cytoplasmic side of the ER. The functional significance of TMc with an N_{cyt}/C_{lum} orientation is supported by the membrane PK protection reactions described above (Figure 5B).

Confocal microscopy revealed that approximately 72% of COS-1 cells transfected with an expression construct for the Nrf1$^{\Delta 11\text{-}30}$ mutant, which lacks most of the TM1 sequence, showed primarily nuclear staining; however, about 28% of cells expressing Nrf1$^{\Delta 11\text{-}30}$ gave a predominantly extra-nuclear staining pattern (Figure 6B). This finding suggests that Nrf1 can be targeted to extra-nuclear compartments through other motifs besides TM1. This assumption is supported by the additional observation that ~25% of cells expressing Nrf1$^{\Delta 11\text{-}30:\Delta 364\text{-}400}$, in which both TM1 and TMi were disrupted, gave a predominantly extra-nuclear stain; it should be noted that the immunoreactive stain produced by this mutant protein was distinct from that of ER/DsRed, suggesting it was not targeted specifically to the ER.

Western blotting of LDS-NuPAGE gels failed to show the presence of a ~120-kDa Nrf1 band in the ER fraction from COS-1 cells transfected with an expression construct for Nrf1$^{\Delta 11\text{-}30}$ or Nrf1$^{\Delta 11\text{-}30:\Delta 364\text{-}400}$, but rather a weak band of between 90 kDa and 95 kDa was observed (Figure 6C). Two closely migrating bands of estimated 90 kDa and 110 kDa were detected predominantly in the ER of cells transfected with the Nrf1$^{\Delta 686\text{-}741}$ expression construct. By contrast, Nrf1$^{\Delta 364\text{-}400}$ gave rise to a major band of 95 kDa that was present primarily in the membrane fraction.

Nrf1 is localized in the NE and can heterodimerize with small Maf protein

It is not known how Nrf1, once anchored in the ER, gains access to its target genes. Immuno-electron microscopy of ectopic V5-tagged wild-type Nrf1 revealed that it was associated with both the ER and the NE (supplemental Figures S7A and B). In addition to the ER lumen, the ER membrane and the inner nuclear membrane (INM) stained positively for Nrf1. Confirmation of this finding was sought by immunoblotting subcellular and subnuclear fractions. This showed that the glycosylated ~120-kDa Nrf1 protein was primarily present in the intact ER fraction, whereas non-glycosylated and/or deglycosylated ~95-kDa Nrf1 protein was found predominantly in the total membrane, cytosolic and nuclear fractions (Figures 7A and B). Both the ~95-kDa and ~120-kDa polypeptides co-existed in either the intact NE or whole nuclei (Figure 7C). It is noteworthy that ~95-kDa Nrf1 protein was recovered principally in the INM-containing fraction. Neither the ~95-kDa nor ~120-kDa forms of Nrf1 were found in the salt-extracted soluble nuclear fraction, but instead a short polypeptide of approximately ~55 kDa was detected in this fraction (Figure 7C). The short Nrf1 isoform was also recovered in whole nuclei, but not in the NE, INM, ER, or membrane fractions. Together, these results indicate that the ~120-kDa Nrf1 glycoprotein exists in the lumen of the ER and the NE, and once inserted into the membranes, it may be deglycosylated to form the ~95-kDa protein during sorting from the ER to the INM. By contrast, the soluble ~55-kDa Nrf1 was detected primarily in the nucleoplasm.

Co-immunoprecipitation followed by western blotting revealed that both the ~120-kDa glycoprotein produced by the expression construct for Nrf1$^{\Delta 2\text{-}10}$ and the non-glycated ~95-kDa protein produced from the expression construct for Nrf1$^{\Delta 11\text{-}22}$ were co-precipitated by antibodies against the small MafK protein (Figure 7D, *middle panel*). Nrf1$^{\Delta 1\text{-}296}$ gave rise to a ~55-kDa polypeptide (Figure 7E) and exhibited less activity than full-length Nrf1 (supplemental Figure S9), but this isoform was unstable in the immunoprecipitation buffer and was processed into two polypeptides of ~43 kDa and 46 kDa (Figure 7D, *lane 4*). The ~46-kDa polypeptide, like the ~95-kDa and the ~120-kDa Nrf1 isoforms, was also co-precipitated by MafK antibodies. Conversely, MafK protein was pulled-down by antibodies against residues 292-741 of Nrf1 that were incubated with lysates expressing the CNC/bZIP protein isoforms (e.g., Nrf1 of 46, 55, 95 or 120 kDa). These results indicate that all Nrf1 isoforms can heterodimerize with small Maf proteins

through their bZIP domains.

The N-terminal 65 residues of Nrf1 are bound to the NE membrane

To test which region of Nrf1 directs its integration into the NE membrane, residues 66-741 of this CNC/bZIP protein were progressively truncated from its C-terminus in the context of expression constructs in which portions of Nrf1 were sandwiched between DsRed and GFP (Figure 8A). Western blotting of the nuclear membrane revealed that all Nrf1-containing fusion proteins were recovered in the NE fraction from COS-1 cells. Confocal microscopy showed that a fusion protein comprising just DsRed-GFP yielded a uniform green fluorescent stain that matched that of the red fluorescent stain throughout the cells (Figure 8B). Following transfection of COS-1 cells with five expression constructs for the sandwiched Nrf1-fluorescent fusion proteins and its truncated mutants, a predominant green fluorescent stain was observed in the extra-nuclear compartments (e.g., the ER), with an obvious enrichment surrounding the nucleus (e.g., NE) (Figure 8B). It should however be noted that these transfected cells gave a generally weak red fluorescent stain only in the extra-nuclear compartment, with no obvious nuclear stain. These results indicate that the N-terminal 65 amino acid region of Nrf1 contains a determinant that allows it to be integrated into the NE membrane. The GFP portion of the fusion protein appears to fold correctly in the ER lumen. By contrast, the DsRed protein attached to the N-terminus of Nrf1 appears to be partitioned principally in the extra-luminal side of the membrane. Under these conditions only a minor portion of DsRed may be properly folded, though it gives a satisfactory western blot signal.

DISCUSSION

Nrf1 is a membrane-spanning glycoprotein with cytoplasmic and luminal regions

Subcellular fractionation, together with membrane PK protection reactions, has revealed that Nrf1 possesses two sequences at either end of the protein that can interact with membranes. These two hydrophobic peptides are predicted to form α-helical secondary structures. One of them is formed by residues 7-26 (called TM1) and the other is formed by residues 707-725 (called TMc). The former of these, TM1, is an essential part of the signal anchor sequence of Nrf1 that tethers it to the ER membrane [22]. Herein, we have demonstrated that TM1 determines the integration of Nrf1 into the ER and it appears to be orientated in an N_{cyt}/C_{lum} fashion across the membrane. Our analyses have shown that TM1, in the context of a DsRed/N65/GFP sandwich fusion protein, does not span the membrane correctly (Figure 5C), although it is capable of doing so in the context of DsRed/NTD/GFP (data not shown) and DsRed/Nrf1/GFP fusion proteins (Figure 5B). These findings suggest that the correct insertion of TM1 into the ER may require an auxiliary secondary element located within the NTD. The exact location of such an element is unclear, but it is likely to be situated adjacent to the NHB1 sequence. The existence of an auxiliary secondary structure is consistent with predictions based on the double-spanning model of co-translational ER integration [41]. We propose that the TM1 region and its adjoining sequence function in concert to regulate Nrf1 negatively, based on our finding that three mutants lacking different portions of the NTD, $Nrf1^{\Delta 11\text{-}22}$, $Nrf1^{\Delta 31\text{-}80}$ and $Nrf1^{\Delta 66\text{-}80}$, exhibit higher transactivation activity than wild-type Nrf1. Moreover, processive deletion of increasing N-terminal regions of the NTD, from amino acids 2 to 90, results in a progressive increase in Nrf1 activity [22].

The TMc α-helix identified in this study (residues 707-725) has not been reported previously. We propose it is inserted into the membrane with an N_{cyt}/C_{lum} orientation. Bioinformatic analyses predict that TMc flanks a large cytoplasmic portion of Nrf1 (including AD2, SR, Neh6L, CNC and bZIP) that will be partitioned into the nucleoplasm after the factor migrates to the NE. It will be interesting to discover whether it is necessary for TMc to be released from the nuclear envelope in order for the bZIP domain to gain access to DNA.

In view of the N_{cyt}/C_{lum} orientation of TM1 and the similar predicted orientation of TMc, it is apparent that Nrf1 contains at least one other transmembrane region. This intermediate transmembrane (TMi) region must be located between the TMc-connecting cytoplasmic portion of Nrf1 and its NST domain; this supposition is based on the fact that Nrf1 is glycosylated through its NST domain, a reaction which is known to occur at the luminal side of the ER [42]. The best candidate for this TMi region is the SLNSTFGSTNLAGLFFPSQL sequence between residues 374-393). It is intriguing that this peptide includes a potential glycosylation site at Asn^{376} and is flanked on both sides by possible glycosylation sites (Asn^{371}, Asn^{394} and Asn^{398}). It is possible that the extent of glycosylation controls the ability of the 374-393 peptide to span the ER membrane and that this somehow controls the activity of Nrf1.

Mechanisms responsible for sorting Nrf1 to the NE

Immuno-electron microscopy and subcellular fractionation have shown that Nrf1 can localize to the NE. Our finding that full-length Nrf1 could not be solubilized by salt extraction of the subnuclear fraction indicates it is an integral INM protein. At present it is not known how Nrf1 is sorted from the ER and directed to the INM. Several models can be proposed to explain this process. For example, Nrf1 may travel during interphase through the ER membrane to the NE, and diffuse passively within the plane of the membrane from the outer nuclear membrane to the INM [43]. Alternatively, Nrf1 could be sorted to the NE through post-mitotic membrane-related events [44]. From our PK protection experiments, we deduce that Nrf1 can associate with a membrane microdomain that is resistant to Triton X-100 [45]. The detergent-resistant membrane (DRM) is likely to represent a coalesced lipid-ordered microdomain (e.g., lipid raft and caveolae) where phospholipids are much more tightly packed with cholesterol and sphingolipids than their surrounding non-raft regions of the bilayer [46]. We propose that Nrf1 may be located within DRM structures and ferried in a lipid raft along the continuous membrane from the ER to the NE through an as-yet-unidentified mechanism. Another possibility is that Nrf1 may be integrated into raft-like caveolae for post-mitotic NE sorting [47]. In addition to its transmembrane regions, Nrf1 contains several potential sorting determinants, including luminal Asn-glycan and cytoplasmic di-Leu and Tyr-based motifs, that have been shown to sort other proteins [48]. Following trafficking of Nrf1 to the NE, the factor may flip-flop in the sphingolipid-rich nuclear membrane, as the constituent lipids and other membrane proteins in the DRM move in and out of the microdomain with different partitioning kinetics [46].

Once in the nucleus, it is unclear how Nrf1 gains access to the promoters of target genes and the transcriptional machinery. It is possible that Nrf1 might undergo retro-translocation from the INM to the nucleoplasm, but this appears unlikely as we detected relatively little ectopic Nrf1 in salt buffer nuclear extracts, and we have also obtained no evidence that the factor can be cleaved from its N-terminal anchor sequence. It therefore seems most probable that Nrf1 undergoes some sort of topological change within the NE that allows its bZIP domain to dimerize with small Maf proteins and bind to ARE enhancers in gene promoters.

The transactivation activity of full-length Nrf1 is primarily due to its AD1 and NST domains

Both AD1 and AD2 have been reported to contribute to the transactivation activity of Nrf1 [49, 50]. In this study we have found that AD1 accounts for the major portion of transactivation activity of full-length Nrf1, whereas AD2 contributes little to its activity. Within AD1 we found that residues 125-170 and residues 261-298 are principally responsible for the transactivation activity of Nrf1. It is noteworthy that residues 261-298 share substantial sequence identity with the Neh5 domain of Nrf2 that is responsible for binding the coactivator CBP [38]. It is therefore probable that this Neh5-like region contributes to the activity of Nrf1 by interacting with CBP.

In addition to AD1, the NST domain of Nrf1 also contributes to transactivation. Amongst the domains of Nrf1, NST is uniquely glycosylated. The significance of glycosylation and/or deglycosylation reactions in the activation of

Nrf1 is not understood. However, given the fact that the NST domain lies between AD1 and AD2, it could play a pivotal role in controlling the repartitioning of these two acidic domains into the nucleoplasm. As glycan can influence the topogenesis of membrane protein [51] and can also function as a signal for retro-translocation [52], an interesting hypothesis is that membrane topogenesis of Nrf1 and its transactivation activity are regulated by an NST glycosylation and/or a deglycosylation event. It is possible that Nrf1 is a dual topology protein that can adopt several conformational states within the membrane, as has been described for prion protein [53].

A model to describe the post-synthetic processing of Nrf1

Nrf1 is synthesized as a membrane protein that is targeted to the ER [22]. Once anchored to the ER membrane through its TM1 region, the remaining portion of Nrf1 is translocated into the ER lumen, where it is Asn-glycosylated through its NST domain. The ~120-kDa glycoprotein probably represents a low activity precursor; this is based on our observation that the Nrf1$^{\Delta 2\text{-}10}$ mutant is abundant but has little activity (supplemental Figure S3C). We hypothesize that the ~120-kDa precursor protein is inserted into the ER membrane through the TM1, TMi and TMc regions and that its CNC/bZIP domain is repartitioned into the cytoplasm whilst the NST-adjoining regions may be retained in the ER lumen (supplemental Figure S8, *lower panel*). The ~120-kDa precursor may be sorted from the ER into the INM, but it remains to be identified whether Nrf1 contains an INM-sorting motif that is recognized by importin-α-16 [54]. An additional fraction of the precursor protein may also be inserted from the perinuclear lumen into the INM. During the process when the luminal portion of the ~120-kDa precursor glycoprotein is retro-translocated into the nucleoplasm, it is deglycosylated to become an active ~95-kDa isoform. We found that the deglycated ~95-kDa protein was detected primarily in the INM. Once the transactivation domains of Nrf1 (e.g., AD1 and NST) are re-localized to the nucloplasm, they can gain access to the transcriptional machinery to allow full transcriptional activation of ARE-driven genes.

A ~55-kDa polypeptide was observed during LDS-NuPAGE of lysate from COS-1 cells expressing Nrf1$^{\Delta 1\text{-}296}$ that lacked both the NTD and AD1; this isoform migrated with an apparent molecular mass of ~65-kDa during the Laemmli SDS-PAGE. It seems likely that this smaller Nrf1 isoform arises through translation from an internal methionine codon situated between residues 289 and 297 as suggested previously [2, 49]. In this case, the ~55-kDa isoform lacks the N-terminal signal anchor sequence and thus is unlikely to be targeted to the ER, but it could contain the necessary nuclear localization signal sequence within the bZIP domain to allow its nuclear import. This Nrf1 isoform was originally cloned as a transcriptional activator, designated locus control region-factor 1 (LCR-F1) [50]. It contains both the NST domain and AD2 and data we have presented show that they function as transactivation domains (Figure 3D and also supplemental Figure 9), though as it lacks AD1 it would be less active than the ~95-kDa protein.

Interestingly, in this study we also found an endogenous ~55-kDa Nrf1 isoform in RL-34 cells that was associated with the ER. Its abundance increased following digestion with PK. This indicates that the ~55-kDa Nrf1 isoform is a luminal ER protein that is probably produced through a proteolytic cleavage occurring around the NST domain. It might also be further proteolytically processed to become a shorter form of between ~30 kDa and 46 kDa. These cleavage products might be released from the membrane to the lumen and then retro-translocated into the nucleoplasm. However, only those polypeptides lacking all three transactivation domains AD1, NST and AD2 could function as dominant-negative forms (supplemental Figure S9). This hypothesis is consistent with the data reported previously [55].

Cross-talk between Nrf1 and Nrf2

There are two levels at which Nrf1 may influence transactivation of ARE-driven gene expression. Firstly, as the *Nrf2*

gene promoter contains two ARE sequences [56], it is possible that Nrf1 transcriptionally regulates *Nrf2*. Further experiments are required to evaluate this possibility. Secondly, as both Nrf1 and Nrf2 bind to ARE enhances, it is possibly they antagonize each other through mutual competition for binding sites. Through forced expression of Nrf1 and/or Nrf2 in *Nrf2*$^{-/-}$ mouse embryonic fibroblasts, we have found no evidence that either factor interferes with the ability of the other to transactivate ARE-driven gene under basal conditions or following treatment with tBHQ (supplemental Figure S9).

Nrf1 is activated by tBHQ rather than ER stressors

We have found that Nrf1 is an INM-bound transcription factor and it is not involved in the ER unfolded protein response. However, the activity of Nrf1 was increase by redox stress affected by tBHQ. Such stress is likely to arise through oxidization of tBHQ, possibly catalysed by cytochrome P450 enzymes in the ER, to the thiol-reactive metabolite 2-*tert*-butyl-1,4-benzoquinone [57]. As Nrf1 is located in the ER, it is possible that one of its roles is to maintain redox homeostasis in this organelle. This prediction assumes that the genes of some ER-resident proteins contain AREs. One such candidate is NADPH cytochrome b5 oxidoreductase, which has been proposed to protect against the accumulation of reactive oxygen species. The expression of this reductase can be induced by tBHQ, and its gene promoter contains an ARE enhancer [58]. It seems likely that it will be regulated in part by Nrf1. One possible scenario is that metabolism of tBHQ in the ER produces redox stress, which activates Nrf1 leading to induction of NADPH cytochrome b5 oxidoreductase which then provides the ER with greater protection against reactive oxygen species.

We have provided evidence that tBHQ may activate Nrf1 by antagonizing the inhibitory effects of residues 31-90 within its NTD (supplemental Figure S3C). It is not known how this is achieved. However, residues 31-90 of Nrf1 contain two potential tyrosine phosphorylation motifs, ^{62}LDGYGIHPK^{70} and ^{74}LDNYFTARR^{82}, that exist within potential cholesterol recognition amino acid consensus sequences (-L/V-X_{1-5}-Y-X_{1-5}-K/R-) [59]. Our data show that Nrf1 is integrated into Triton X-100 detergent-resistant regions within membranes, and it is also located in the INM. These observations suggest that Nrf1 might be incorporated into raft-like detergent-resistant membranes as a consequence of it being able to bind cholesterol and/or sphingolipids before being ferried to the INM. It is possible that tBHQ activates Nrf1 because it stimulates tyrosine phosphorylation within the cholesterol recognition motifs thereby altering its ability to associate with cholesterol and sphingolipids, and thus increases the trafficking of Nrf1 from the ER to the NE.

We found that although treatment of cells with TU, TG and BFA increased the abundance of Nrf1 protein, these ER stressors partially inhibited its basal transactivation activity by ~25%, and almost completely prevented Nrf1-mediated induction of ARE-driven gene expression by tBHQ. This finding suggests that under ER stress conditions, Nrf1 may be incorrectly folded within the membrane, which probably affects its membrane trafficking and sorting in the INM. It is known that Asn-glycosylation is necessary for correct folding and proper topogenesis of membrane proteins and that upon deglycosylation the Asn residues to which the sugars were attached are each converted to Asp [51, 60]. Herein, we mutated these seven putative Asn residues to Gln or Asp. The Nrf1$^{1\text{-}7\times N/Q}$ mutant represents a non-glycosylated protein and it was found to possess less activity than wild-type protein, whereas Nrf1$^{1\text{-}7\times N/D}$ represents a deglycosylated protein and it was shown to exhibit substantially greater activity than the wild-type factor. Therefore, one explanation as to why TU, TG and BFA prevented tBHQ from activating Nrf1 is that they stopped the CNC/bZIP factor from integrating correctly into the ER membrane, either directly or because they interfered with glycosylation and/or deglycosylation reactions.

Concluding comments

Endogenous Nrf1, like its ectopic protein, is targeted to the ER where it is Asn-glycosylated. We have also found that Nrf1 is localized in the NE membrane. As Nrf1 in the ER is essentially entirely glycosylated but it largely deglycosylated in the INM, our data imply that deglycosylation of the transcription factor could be a pivotal regulatory event. Further studies are required to provide information about how glycosylation and deglycosylation of Nrf1 is controlled. Given the location of Nrf1 in the ER, and if the fact that the ER is uniquely susceptible to the formation of reactive oxygen species through certain types of uncoupling reactions, it appears likely that the CNC/bZIP protein mediates protection against oxidative stress that arises in the organelle. Whether glycosylation and/or deglycosylation of Nrf1 is required by oxidative stress is not known.

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FOOTNOTES

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The abbreviations used: AD1, acidic domain 1; ARE, antioxidant response element; BFA, brefeldin A; bZIP, basic region-leucine zipper; CNC, cap 'n' collar; CPIC, complete protease inhibitor cocktail; DRM, detergent-resistant membrane microdomain; DsRed, *Discosoma sp*. red fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; INM, inner nuclear membrane; LDS, lithium dodecyl sulfate; N65, N-terminal 65 residues; NE, nuclear envelope; Neh5L, Neh5-like subdomain; NHB1, N-terminal homology box 1; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf, nuclear facter-erythroid 2 p45 subunit-related factor; NST, Asn/Ser/Thr-rich; NTD, N-terminal domain; PK, proteinase K; tBHQ, *tert*-butyl hydroquinone; TG, thapsigargin; TM, transmembrane region; TU, tunicamycin; TX, Triton X-100.

FIGURE LEGENDS

Figure 1 Endogenous Nrf1 is localized and Asn-glycosylated in the ER.
(A) RL-34 cells (3×10^5) were grown for 24 h on a cover-slip in 6-well plates. After the cells were fixed and permeabilized, they were examined by immunocytochemistry using primary antibodies against Nrf1 and calreticulin (CRT). The confocal images were visualized with a green-fluorescent Alexa-Fluor 488 (FITC)-labelled secondary antibody against rabbit IgG (also see supplemental Figure S1 in colour). **(B)** RL-34 cells were transfected using 1 ml of serum-free Opi-TEM1 medium containing 50 nmol/l of either Nrf1-targeted or scrambled (Scram) siRNA with 10 μl of Lipofectamine 2000. Approximately 48 h after transfection, the cells were fixed, permeabilized and then subjected to immunocytochemistry with either primary anti-Nrf1 serum or pre-immune serum. The green images were obtained as described above and merged with blue DAPI images. **(C)** Approximately 24 h after transfection with either Scrm (50 nmol/l) or Nrf1-targeted siRNA (20 or 50 nmol/l), total RNA was extracted from the RL-34 cells and TaqMan chemistry performed as described. The level of Nrf1 mRNA was normalized to the level of 18S rRNA and is shown as a fold change (mean ± S.D) from three independent experiments. An arbitrary value of 1 was given to the normalized level of Nrf1 mRNA measured in cells that had been mock transfected with lipofectamine 2000 alone (Lipo). Statistical significance was determined by the unpaired *t*-test and is shown as a p-value. **(D)** Forty-eight h following transfection of RL-34 cells with siRNA (*left panel*), intact ER fractions were prepared, proteins resolved using a 4-12% LDL-NuPAGE Tris-Bis buffer system and immunoblotted with Nrf1 antibody. Lane 1 represents lysates from COS-1 cell expressing ectopic Nrf1. Lanes 2 and 3 represent ER fractions from RL-34 cells transfected with either Scrm or Nrf1 siRNAs. Lanes 4 and 5 show immunoblots of ER fractions from RL-34 cells that either had been incubated with 500 units of Endo H (lane 5) or had been incubated in the absence of Endo H (lane 4). The same nitrocellulose membranes blotted with the Nrf1 antibody were stripped and re-probed with a CRT antibody to verify that equal amounts of protein were loaded. The arrows indicate Nrf1 isoforms of ~120, 95 and 55 kDa.

Figure 2 ER stressors neither increase the activity of Nrf1 nor influence its subcellular localization
(A) COS-1 cells were transfected with 1.2 μg of Nrf1/pcDNA3.1/V5 His B, along with 0.6 μg of P_{TK}*nqo1-ARE-Luc* and 0.2 μg of the β-gal reporter plasmids. The cells were treated for 24 h with DMSO (0.1%), TU (1 μg/ml), TG (1 μM), BFA (1 μmol/l), ALLN (5 μg/ml) either individually or in combination. Subsequently, the cell lysates were subject to luciferase reporter assay (*upper panel*) and western blotting with antibodies against the V5 epitope or GRP78 (*lower panels*). The arrow indicates an Nrf1 polypeptide of ~95 kDa. **(B)** COS-1 cells were transfected with 1.2 μg of Nrf1/pcDNA3.1/V5 His B, along with 0.6 μg of P_{SV40}*GSTA2-6×ARE-Luc* and 0.2 μg of the β-gal reporter plasmids. Eighteen h after transfection, the cells were co-treated with the same concentrations of the above agents together with tBHQ (50 μmol/l). Luciferase reporter gene activity is shown as a fold change (mean ± S.D) from three independent experiments each in triplicate. The significance of differences was determined by the unpaired *t*-test and is shown as a p-value. Total cell lysates were resolved using LDS-NuPAGE containing either 4-12% polyacrylamide in the Tris-Bis buffer (middle panel) or 7% polyacrylamide in the Tris-Acetate buffer (lower panel). **(C)** COS-1 cells were transfected with expression constructs for V5-tagged Nrf1 (1.3 μg DNA of each) and allowed to recover for 24 h before they were treated for an additional 24 h with the same chemicals as those used in panel A. Subcellular location of proteins was examined by immuocytochemistry followed by confocal imaging. FITC-labelled second antibody was used to locate V5-tagged proteins. Nuclear DNA was stained by DAPI. The ER/DsRed gave a red image in the ER. The merge signal represents the results obtained when the three images were superimposed. The corresponding quantitative data shown here were calculated by

determining the percentage of cells in which the extra-nuclear stain, i.e. cytoplasmic plus ER (called simply C) was greater than or equal to the nuclear stain (called N), as opposed to the percentage of cells in which the extra-nuclear stain was less than the nuclear stain. Bar = 20 μm.

Figure 3 The NST domain positively regulates Nrf1

(**A**) Expression constructs for wild-type Nrf1 or mutants lacking portions of the NST domain (1.2 μg DNA of each) were each transfected into COS-1 cells together with 0.6 μg of P_{TK}*nqo1*-ARE-Luc and 0.2 μg of either the pRL-TK or β-gal reporter plasmids, as indicated. Approximately 36 h after transfection, the cells were harvested and luciferase reporter assays performed. Statistical significance of the luciferase data was determined as described in the legend of Figure 2 (**B**) RL-34 cells were cotransfected with each of the indicated expression constructs along with the P_{SV40}*nqo1*-ARE-Luc and pRL-TK or β-gal reporter plasmids, before being treated with 0.1% DMSO or 50 μM tBHQ. Luciferase reporter assay was carried out 24 h later. Cell lysates were immunoblotted with antibodies against the V5 epitope. (**C**) Mutations were introduced into the expression construct for Nrf1 that resulted in individual Asn residues within the NST domain being replaced with either Asp or Gln as indicated. These were each co-transfected into COS-1 cells, along with P_{TK}*nqo1*-ARE-Luc and pRL-TK or β-gal reporter plasmids, and luciferase activity was measured. Total cell lysates were (+) or were not (-) incubated for 1 h with 500 units of PNGase F, resolved using 4-12% LDS/NuPAGE Tris-Bis and immunoblotted with antibodies against V5 (*bottom panel*). (**D**) COS-1 cells were cotransfected with each of expression constructs for the various Gal4D/Nrf1 fusion proteins (shown on the left-hand side) together with P_{TK}*UAS*-Luc and pRL-TK plasmids. Luciferase was measured 24 h later. These Gal4D/Nrf1 proteins were visualized by western blotting (supplemental Figure S4).

Figure 4 Nrf1 is positively regulated by subdomains within AD1

(**A**) Left-hand side, COS-1 cells were transfected with 1.2 μg of an expression construct for either wild-type Nrf1 or mutants lacking different portions across AD1, together with 0.6 μg of P_{TK}*nqo1*-ARE-Luc plus 0.2 μg of either the pRL-TK or β-gal reporter plasmids. Approximately 36 h after transfection, the cells were harvested and luciferase reporter assays carried out. Luciferase activity was analyzed as described in the Experimental section. Right-hand side, RL-34 cells were transfected with 1.2 μg of either an expression construct for wild-type Nrf1 or mutants lacking the N-terminal part or the C-terminal part of AD1 together with 0.6 μg of P_{SV40}*nqo1*-ARE-Luc along with 0.2 μg of the pRL-TK or β-gal reporter plasmids; note the sequence homology between the C-terminal part of AD1 and the Neh5 domain of Nrf2 is shown at the top. Eighteen h after transfection, the RL-34 cells were treated with 0.1% DMSO or 50 μM tBHQ for 24 h, and thereafter, were subject to luciferase reporter assays and western blotting with antibodies against the V5 epitope**.** The amount of protein added to each electrophoresis sample well was adjusted to ensure equal loading of β-gal activity. (**B**) RL-34 cells were cotransfected with expression constructs for either wild-type Nrf1 or mutant protein lacking the DIDLID element, the DLG motif or the ETGE motif, as shown in the sequence alignment, along with P_{SV40}*nqo1*-ARE-Luc and pRL-TK or β-gal reporter plasmids, before being treated with 0.1% DMSO or 50 μM tBHQ. ARE-driven luciferase activity was calculated and analyzed as described in the Experimental section.

Figure 5 Nrf1 is targeted into Triton X-100 detergent-resistant membrane microdomains through its N-terminal region

(**A**) The intact ER-rich fraction was purified from RL-34 cells, and after washing the fractions were resuspended in an isotonic extraction buffer. These membrane samples were incubated for 30 min either with proteinase K (PK) (+) at a final concentration of 100 μg/ml or without PK (–) in the presence (+) or absence (–) of 1% (v/v) Triton X-100 (TX). After the reaction was stopped, endogenous Nrf1 was analyzed by 4-12% NuPAGE Bis-Tris buffer system followed by western blotting with antibodies against residues 292-741 of mouse Nrf1. The same sample blotted nitrocellulose membrane was reprobed with an additional antibody against CRT (calreticulin), to confirm integrity of the membrane. Endogenous Nrf1 polypeptides of ~120, 55 and 36 kDa are indicated by arrows. (**B** and **C**) The ER fraction was purified from COS-1 cells that were transfected with an expression construct for fusion proteins in which full-length Nrf1 (**B**) or its N-terminal 65 residues (N65) (**C**) were sandwiched between DsRed and GFP. These fractions were incubated with either 50 μg/ml (+) or 100 μg/ml (++) of PK in the presence (+) or absence (–) of 1% TX. The reaction products were visualized by western blotting with antibodies against GFP or DsRed.

Figure 6 The first transmembrane (TM1) region determines membrane topology of Nrf1

(**A**) Diagrammatic representation of the predicted three transmembrane regions, called TM1 (residues 7-26), TMi (residues 374-393) and TMc (residues 707-725). For further details of predicted α-helices, please see supplemental Figure S8. (**B**) COS-1 cells were cotransfected with each of expression constructs expressing wild-type Nrf1 and deletion mutants, together with an ER/DsRed construct. Subcellular localization of these proteins was determined by immunocytochemistry, and quantitative data of confocal images were calculated as described in the legend to Figure 2C. Bar = 20 μm. (**C**) COS-1 cells that were transfected with the indicated constructs were subjected to subcellular fractionation. The V5-tagged fusion proteins in the intact ER and microsome-rich membrane (M) fractions were resolved by NuPAGE containing 4-12% polyacrylamide gradient and a Bis-Tris buffer system and visualized by western blotting.

Figure 7 Nrf1 is localized in the ER and the NE membranes and can heterodimerize with small Maf protein

(**A** to **C**) COS-1 cells expressing wild-type Nrf1 were subjected to subcellular and subnuclear fractionations. Nrf1-V5 protein in the different fractions was resolved using 4-12% NuPAGE Bis-Tris and visualized by western blotting. Abbreviations: Cyto, cytosol fraction from the 100,000 × *g* supernatant; ER, intact ER-enriched fraction obtained from sucrose gradient-density purification; INM, inner nuclear membrane-rich fraction isolated from the purified nuclear fraction; M, membrane factions containing microsomes pelleted by centrifugion at 100,000 × *g*; N, nuclei purified from NP-40 homogenates; NE, nuclear envelope isolated from the purified whole nuclei fraction; SN, salt-extracted nuclear fraction; T, total cell lysates; WN, whole nuclei with the intact surrounding NE using a detergent-free purification procedure. Blotting with antibodies against Sec61α, CRT and Lamin A/C was undertaken to confirm the efficacy of subcellular fractionation. (**D**) COS-1 cells were co-transfected with an expression construct for MafK, along with an expression construct for wild-type Nrf1 (lane 1), Nrf1$^{\Delta 2\text{-}10}$ (lane 2), Nrf1$^{\Delta 11\text{-}22}$ (lane 3) or Nrf1$^{\Delta 1\text{-}296}$ (lane 4). Total cell lysates were subjected to co-immunoprecipitation (IP) with antibodies against Nrf1 or

small Maf, followed by western blotting (WB) with antibodies against the V5 epitope. The Input samples indicate total lysate incubated with IP buffer alone. (**E**) Total lysates of COS-1 cells expressing Nrf1 or Nrf1$^{\Delta 1\text{-}296}$ were harvested in the LDS sample buffer, and then subjected to western blotting with the V5 antibody. Ectopic Nrf1 polypeptides of ~120, 95, 55 and 46 kDa are indicated by the solid-headed arrows (**A** to **E**).

Figure 8 Nrf1 can associate with the NE through its N-terminal 65 residues

(**A**) NE fractions were purified from COS-1 cells expressed various fusion proteins that comprised different portions of Nrf1 sandwiched between DsRed and GFP. The presence of the ectopic fusion proteins was examined by western blotting with a GFP antibody**.** The sample T represents total cell lysates. (**B**) Expression constructs for Nrf1-containing sandwich fusion proteins (1.3 μg DNA of each) were transfected into COS-1 cells. Subsequent immuocytochemistry and confocal imaging were performed as described in the legend for Figure 2C.

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FIGURE 1

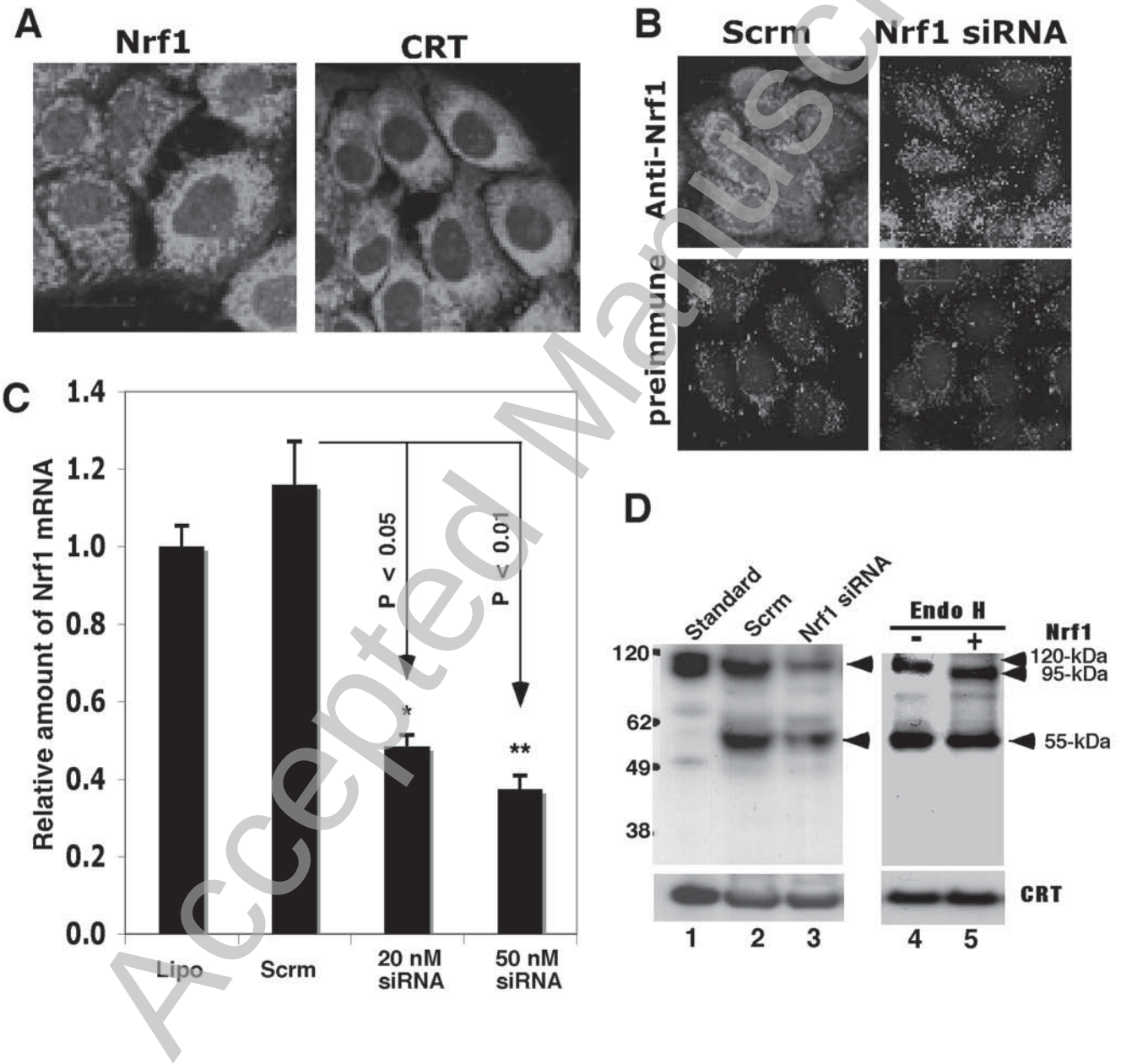

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FIGURE 2

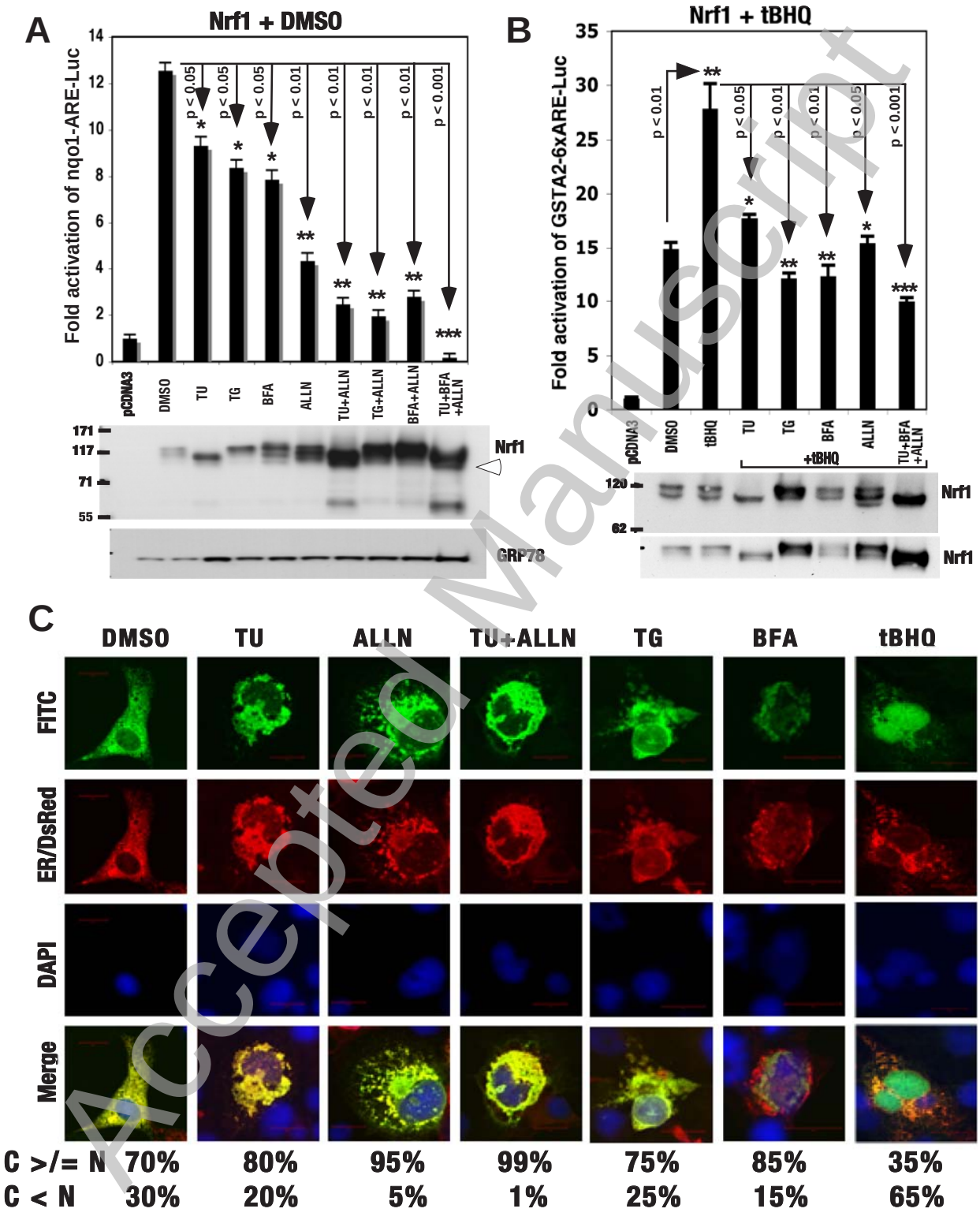

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FIGURE 3

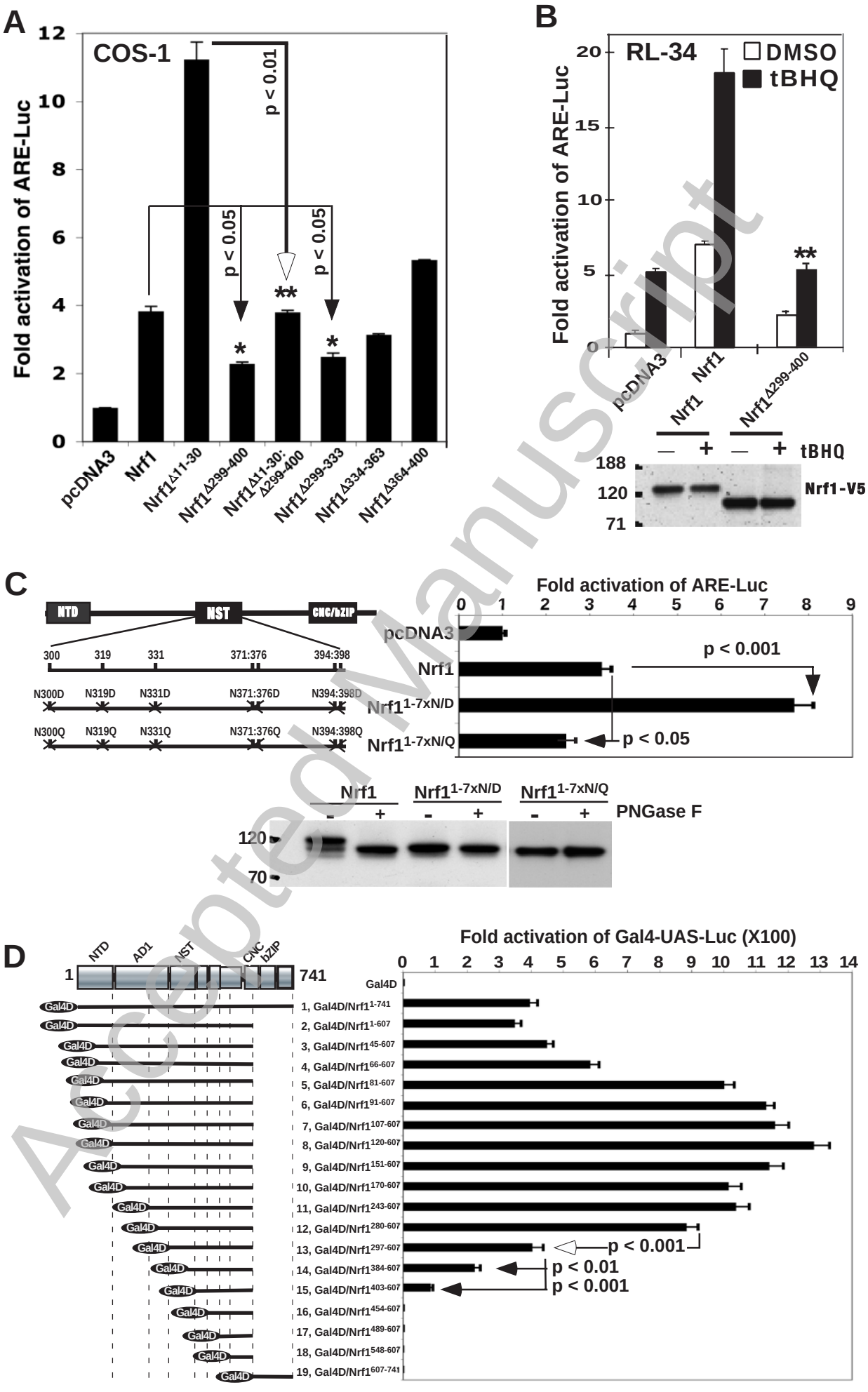

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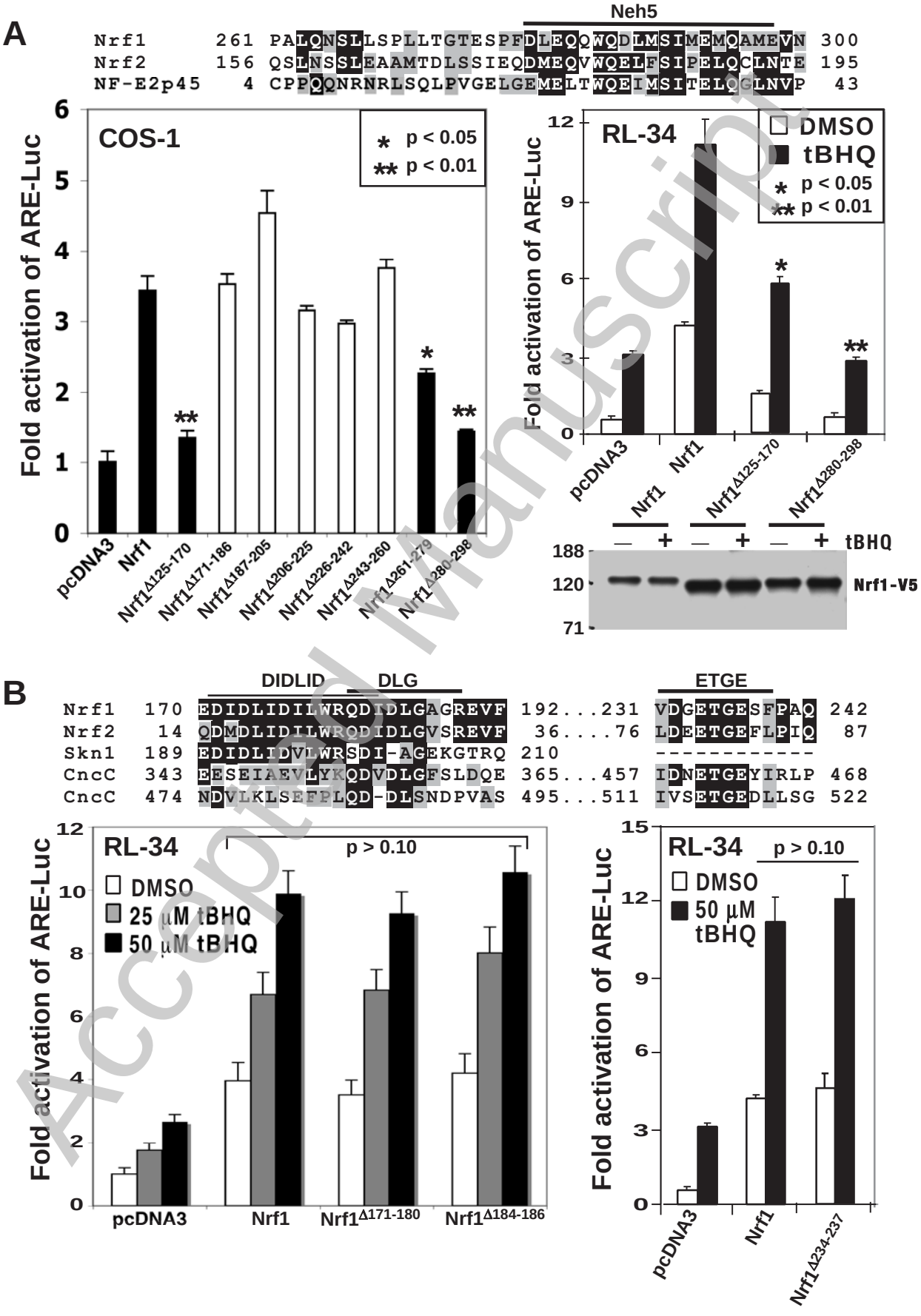
FIGURE 4



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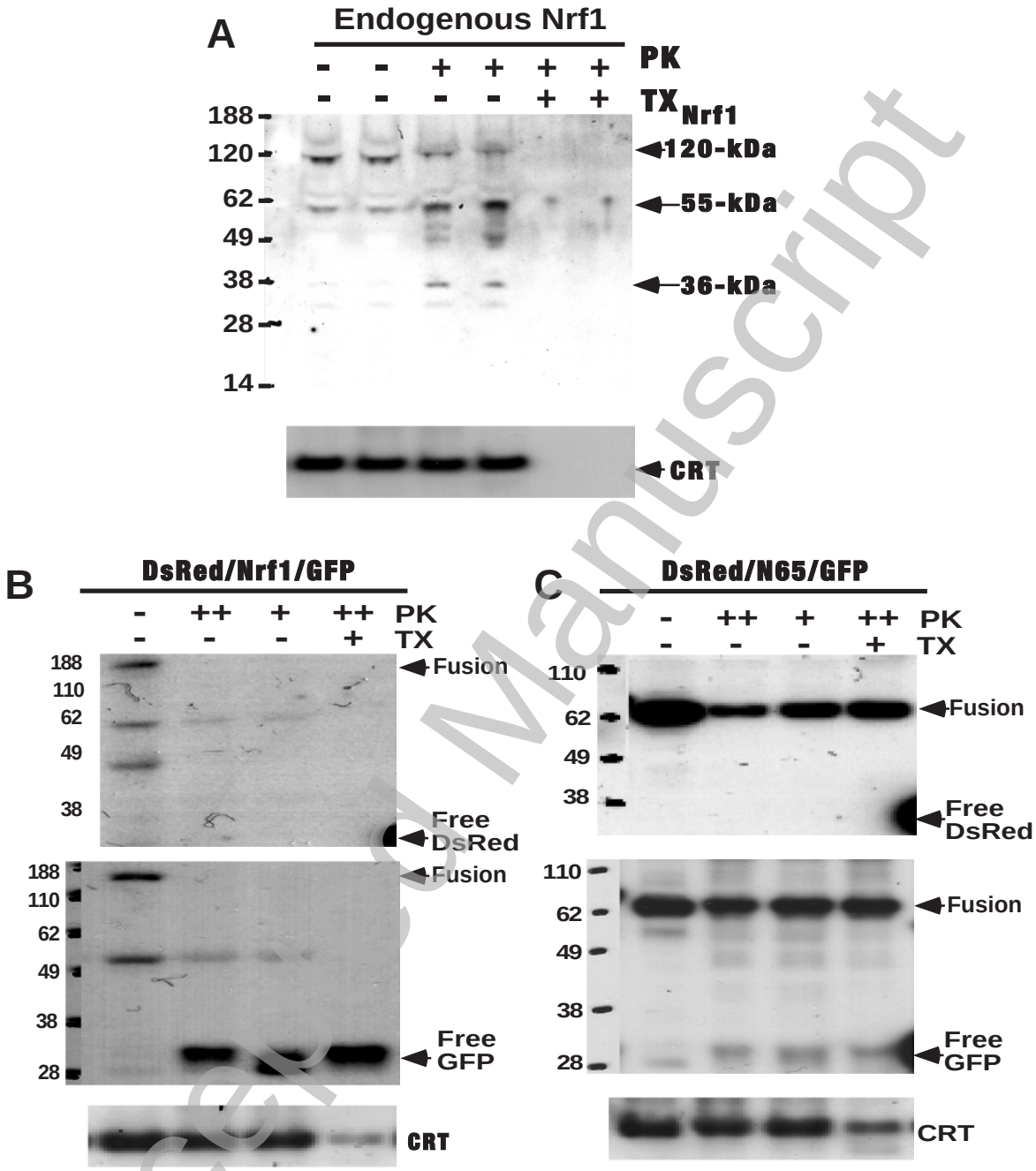

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FIGURE 6

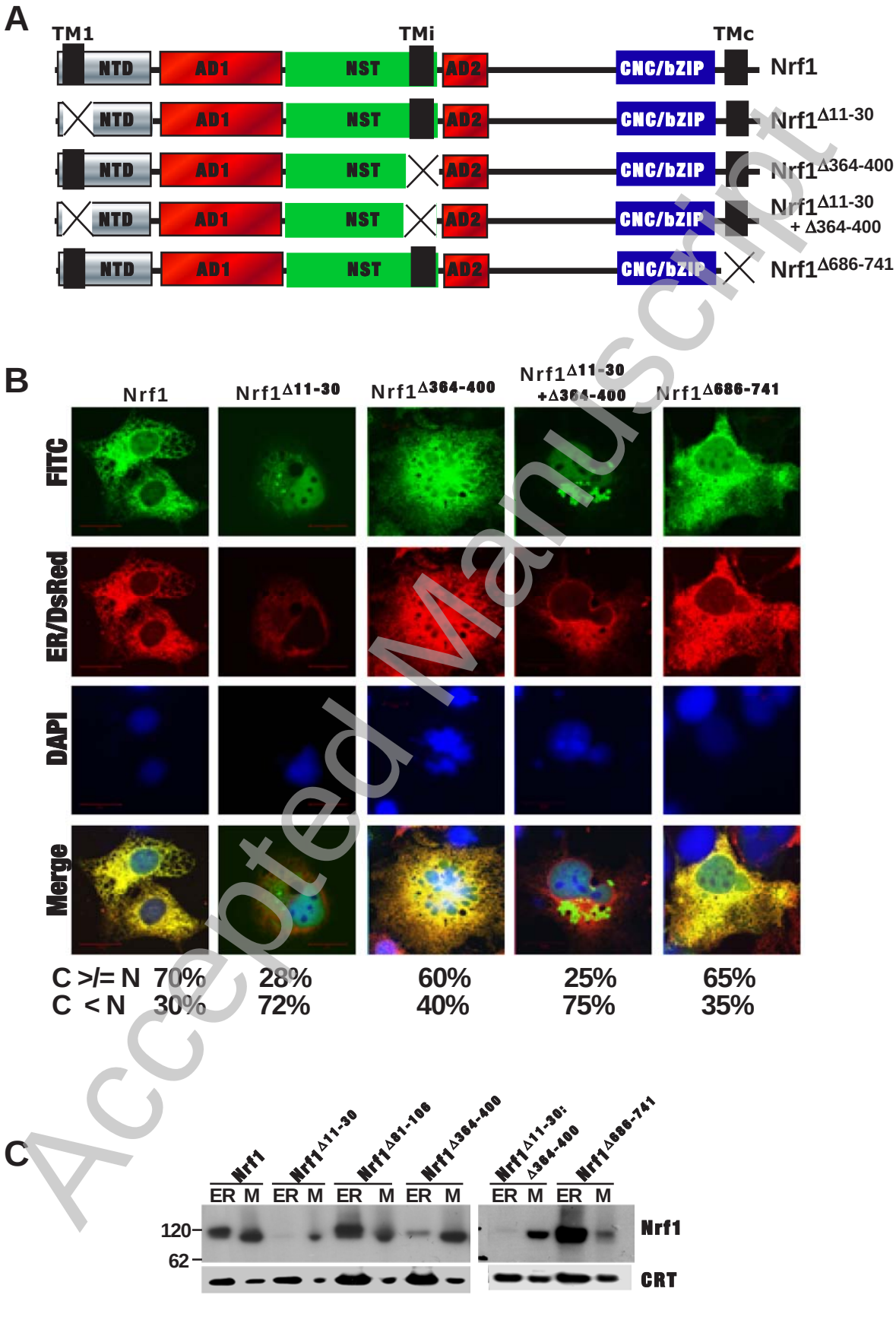

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FIGURE 7

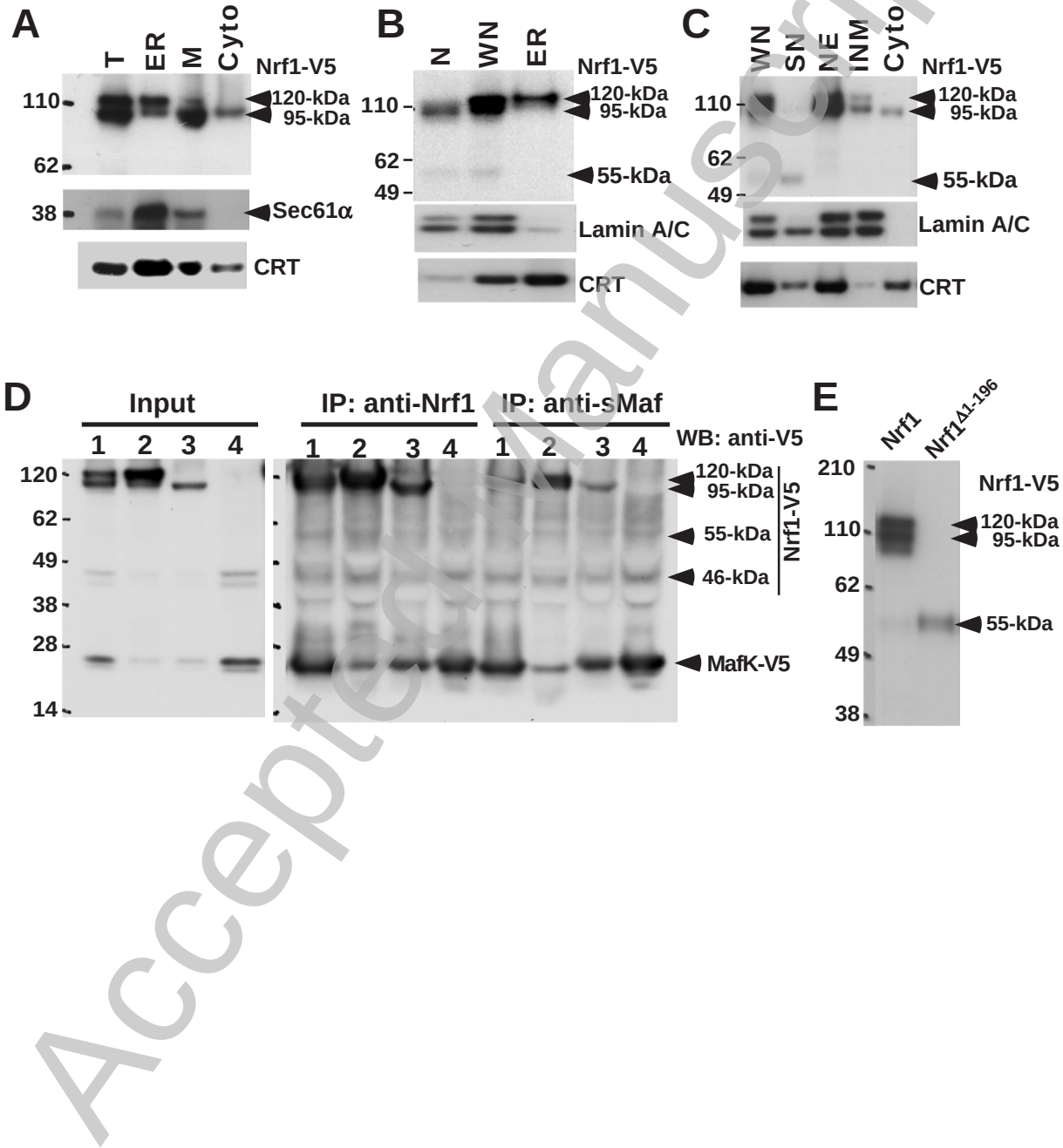

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FIGURE 8