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RESEARCH ARTICLE

NF-E2-related factor 2, a key inducer of antioxidant defenses, negatively regulates the *CFTR* transcription

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Abstract A few studies have clearly indicated that oxidative stress suppresses the cystic fibrosis transmembrane conductance receptor (CFTR) function and expression. However, the mechanisms by which this occurs are still poorly understood. To clarify this effect, we investigated the role of NF-E2-related factor 2 (Nrf2) transcription factor, a key cellular sensor of oxidative stress. A conserved antioxidant response element (ARE) in the *CFTR* minimal promoter, which binds Nrf2, has been identified. Surprisingly, Nrf2 exerts an unexpected repressive role on the *CFTR* gene promoter activity. To decipher the molecular mechanisms involved, we evaluated the role of YY1 in the Nrf2-mediated transcriptional activity and showed cooperation between these two factors. We demonstrated that Nrf2 promotes YY1 nuclear localization and increases its binding to the *CFTR* promoter. To our knowledge, this study is the first to report a repressor role of Nrf2 through the cooperation with YY1 and contributes to clarify the cascade events leading to the oxidative stress-suppressed *CFTR* expression.

Keywords Oxidative stress · Cystic fibrosis · Transcription · Nrf2 · YY1 · CFTR

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Introduction

Cystic fibrosis (CF) is caused by the loss of functional cystic fibrosis transmembrane conductance regulator (CFTR or ABCC7), an epithelial Cl^- channel. CFTR exhibits tightly regulated expression. Although some important advances in identifying DNA elements controlling tissue-specific *CFTR* transcription in DHS sites have been performed [1–4], the promoter region remains poorly explored [5, 6]. Recently, few studies have enlightened the role of oxidative stress in the regulation of CFTR function and expression. Cigarette smoke and pyocyanin, a compound secreted by *Pseudomonas aeruginosa*, were shown to inhibit chloride secretion in human bronchial epithelium, suggesting that oxidants may modulate CFTR function [7–9]. Using oxidants, Cantin et al. [10] demonstrated that oxidative stress suppresses *CFTR* mRNA expression. Recently, ozone stress has also been shown to decrease *CFTR* expression in human airway epithelium [11]. However, the molecular events governing the inhibition of *CFTR* expression by various exogenous oxidative stress inducers remain unclear.

Oxidative stress-responsive transcription is regulated in part through the *cis*-acting promoter sequence called antioxidant response element (ARE) [12]. Several molecules, such as nuclear factor-E2-related factor (Nrf2), c-Jun, ATF4, Bach1, small-Maf proteins, were shown to bind to the ARE sequence [13, 14]. Of these, Nrf2 is extensively

proven to be a strong activator of ARE-mediated gene expression [13]. Nrf2 plays a critical role in stress-induced transcriptional up-regulation of cytoprotective genes. In particular, Nrf2 coordinates induction of anti-oxidant and phase 2 metabolizing enzymes encoding genes [15]. Under homeostatic unstimulated conditions, Nrf2 remains sequestered in the cytosol by interacting with Keap1. However, the exposure to oxidative stress leads to dissociation of Nrf2 from Keap1 and allows its migration into the nucleus [16].

The role of Nrf2 in chronic inflammatory pulmonary diseases has received increasing interest. Cho et al. [17] have shown that the lungs from Nrf2−/− mice are more susceptible to inflammation, fibrosis, and injury. A recent study has demonstrated that patients with pulmonary emphysema have an altered Nrf2/Keap1 equilibrium with decreased Nrf2 and increased Keap1 levels [18].

Interestingly, Nrf2 has been shown to induce the expression of genes of the same family than CFTR, the ABC transporters [19, 20]. However, the putative implication of the Nrf2-ARE signal pathway has never directly been addressed in the *CFTR* transcription. By using prediction tools, we identified a conserved functional ARE-like element located downstream the major transcriptional start site in the minimal *CFTR* promoter. We found that the transcription factor Nrf2 binds to the ARE-like sequence and surprisingly inhibits the *CFTR* expression. In the aim to elucidate the molecular mechanisms surrounding this effect, a more detailed in silico analysis of *cis*-regulatory elements in the *CFTR* minimal promoter identified an YY1-binding site embedded within the ARE sequence. We demonstrated a co-operation between Nrf2 and YY1 and suggested YY1 as a new partner of Nrf2.

Materials and methods

Reagents and treatment

Curcumin and *tert*-Butylhydroquinone (*t*-BHQ) were purchased from Sigma-Aldrich (Lyon, Fr). Unless indicated, Beas2B cells were challenged either with vehicle (DMSO), 6.25 μM of curcumin or 10 μM of *t*-BHQ for 24 h. Anti-HA antibody (clone 12C5) was obtained from Roche Applied Science (Meylan, France). DTBP (dimethyl 3,3′-dithiobispropionimidate-2HCl) was purchased from Perbio Science (Brebières, Fr). Antibodies against Nrf2 (H300X, C20X) or YY1 (H414X), were obtained from Santa-Cruz Biotechnology (Tebu-bio, Le Perray en Yvelines, France). Human HP validated siRNAs for Nrf2 (SI03246614 and SI03246950) and unspecific siRNA (1022076) were purchased from Qiagen SA (Courtaboeuf, France).

Computational analyses

A position weight matrix statistical model of the ARE site was previously published by Wang et al. [21]. Search for putative ARE motifs within the human minimal *CFTR* promoter was performed using two computer programs ConSite (http://www.phylofoot.org/) and TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess). To avoid a bias introduced by palindromic or internally repetitive *cis*-regulatory elements, overlapping matches including on opposite DNA strands were defined as a single match. To assess the importance of the putative ARE motif identified in the minimal *CFTR* promoter, we performed multiple sequence alignments of the sequences upstream the ATG of various mammals using ClustalW global alignment tool (http://clustalw.genome.ad.jp/).

Plasmids DNA

pGL3-basic containing the *CFTR* minimal promoter noted CFTRmp has been previously described [22]. pEF-Nrf2, pEF-Nrf2-DN corresponding to the dominant negative form, and p3xARE-luc were generously given by J. Alam (Department of Molecular Genetics, Ochsner Medical Center, New Orleans, Louisiana, USA). *NAD(P)H dehydrogenase, quinone 1* (*NQO1*) *NQO1*-luc reporter plasmid was a gift from R. Faraonio (Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Napoli, It.). pCMV2*β*-Flag-Keap1 was kindly provided by M. Freeman (Department of radiation Oncology, Vanderbilt University School of Medicine, Nashville, USA). pcDNA3-YY1 vectors was previously described [5].

Cell culture and transient transfections

Beas2B, human bronchial epithelial cells expressing endogenous *CFTR* obtained from ATCC were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Eurobio, Courtaboeuf, Fr.), 2 mM L-glutamine and 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Invitrogen, Cergy Pontoise, France) and kept at 37°C under 5% CO_2.

Cells were transiently transfected using Fugene6 reagent (Roche diagnostics, Meylan, France) according to the manufacturer's recommendations. At least two different maxipreps were used for each vector. Luciferase assays were performed as previously reported [5]. All luciferase activities represented the result of at least four independent experiments performed in triplicates. When indicated, Beas2B cells were challenged either with vehicle (DMSO), 6.25 μM of curcumin for 24 h or 50 μM of *t*-BHQ for 4h.

Chromatin immunoprecipitation assays

ChIP assays were performed as previously described [5] with minor modifications. To stabilize DNA–protein complexes, Beas2B cells were treated with 5 mM of DTBP at room temperature for 30 min, prior to harvest [23]. The cross-linking reaction was terminated by washing the cells with buffer containing 150 mM NaCl and 100 mM Tris–HCl. Purified crosslinked chromatin was immunoprecipitated using either anti-Nrf2 (C20X), anti-HA or control IgG antibody. Input or immunoprecipitated DNA from various ChIPs were analyzed by PCR using GC-rich Accuprime PCR kit (Invitrogen, Cergy Pontoise, France). The sequences of *CFTR* minimal promoter primers were previously described [22]. *NQO1* and *Caspase 8* promoters were used as positive and negative PCR control, respectively.

For quantitative ChIP assays, 3 µl of immunoprecipitated- and input-DNA were used with 5 µl of LightCycler® 480 DNA SYBR Green I Master (Roche-Applied Science, Meylan, France) and real-time PCR was performed on a LightCycler 480 (Roche-Applied Science, Meylan, France). *NQO1* and *Glucocorticoid Receptor* (GR) promoters were used as Nrf2 and YY1 positive control, respectively. All quantitative PCR were carried out in triplicates. Experiments were repeated at least three times, averaged, and expressed relative to the input signal and to negative control.

Co-immunoprecipitation and western blot

To preserve intracellular protein complexes, cells were treated with 5 mM DTBP. Total protein extracts from Beas2B cells were prepared using RIPA buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and 5 mM EDTA) plus protease inhibitors cocktail. Immunoprecipitations were performed using 3 µg of indicated polyclonal antibodies. Extensively washed immunoprecipitates were then separated in SDS-PAGE gel and analyzed by western blotting as previously described [5]. Densitometric analyses were performed by using QuantityOne software (Biorad, Marnes-la-Coquette, France).

RNA extracts and RT-PCR

Total RNA from epithelial cells was isolated using RNeasy plus kit (Qiagen, Courtaboeuf, France). Reverse transcription was performed with 1 µg (for *NQO1* gene) or 3 µg (for *CFTR* gene) of total RNA, 300 ng of random hexamers (Invitrogen, Cergy Pontoise, France), 10 mM dNTPs, 1 µl of RNasin RNase inhibitor (Promega, Charbonnieres, France), 2 µl of DTT at 0.1 M, 4 µl of first strand buffer (Invitrogen), 200 units of M-MLV RT (Invitrogen), and nuclease-free water (Promega) in a final reaction volume of 20 µl. Two negative controls, one without RNA-template and the other with reverse transcriptase omitted, were included to rule out contamination and amplification of genomic DNA, respectively. The RT reaction was firstly incubated for 10 min at 25°C followed by first strand synthesis (40 min at 42°C) and heat-inactivated (at 72°C for 3 min). One-microliter aliquots of the cDNA synthesis reaction mixture were used for PCR analyses. The mRNA expression levels were analyzed by multiplex PCR with human-specific primers for *HPRT* gene using multiplex kit (Qiagen) according to the manufacturer's instructions. Co-amplified *HPRT* mRNA allowed a relative estimate of *CFTR* mRNA levels. The RT-PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide. Relative quantification of mRNA was performed by densitometric analyses of ethidium bromide-stained fragments using QuantityOne software (Biorad).

Statistical analyses

Transfection data were expressed as mean ± SE of at least three independent triplicates. Paired comparisons were made using Student's *t* test. Data were considered statistically significant at $p < 0.05$ (*). Statistical analyses were done with GraphPAD Prism software.

Results

Curcumin and *t*-BHQ diminish the CFTR expression

To evaluate the role of Nrf2-mediated oxidative stress signaling pathway as putative regulator of the *CFTR* expression, curcumin, a component promoting the Nrf2 nuclear localization and *t*-BHQ, a widely used oxidative stress inducer were employed [24–27]. In the first set of experiments, we verified the effects of these drugs on the Nrf2 localization in a bronchial epithelial cell line used. Immunoblot analyses of the nuclear and cytosolic fractions from treated-Beas2B cells showed a decline in the cytoplasm contrasting with an increase in nuclear Nrf2 levels (Fig. 1a). In the second set of experiments, we evaluated the effects of these drugs on both *CFTR* transcriptional activity and mRNA levels. As controls, we performed the experiments with the well-known Nrf2-regulated gene *NAD(P)H dehydrogenase, quinone 1* (*NQO1*) and a control plasmid containing three tandemly repeated ARE consensus sequences (3xARE). Exposure of Beas2B cells to curcumin and *t*-BHQ resulted in a significant decrease of the *CFTR* transcriptional activity (Fig. 1b). As previously described [28, 29], the 3xARE- and *NQO1*-driven

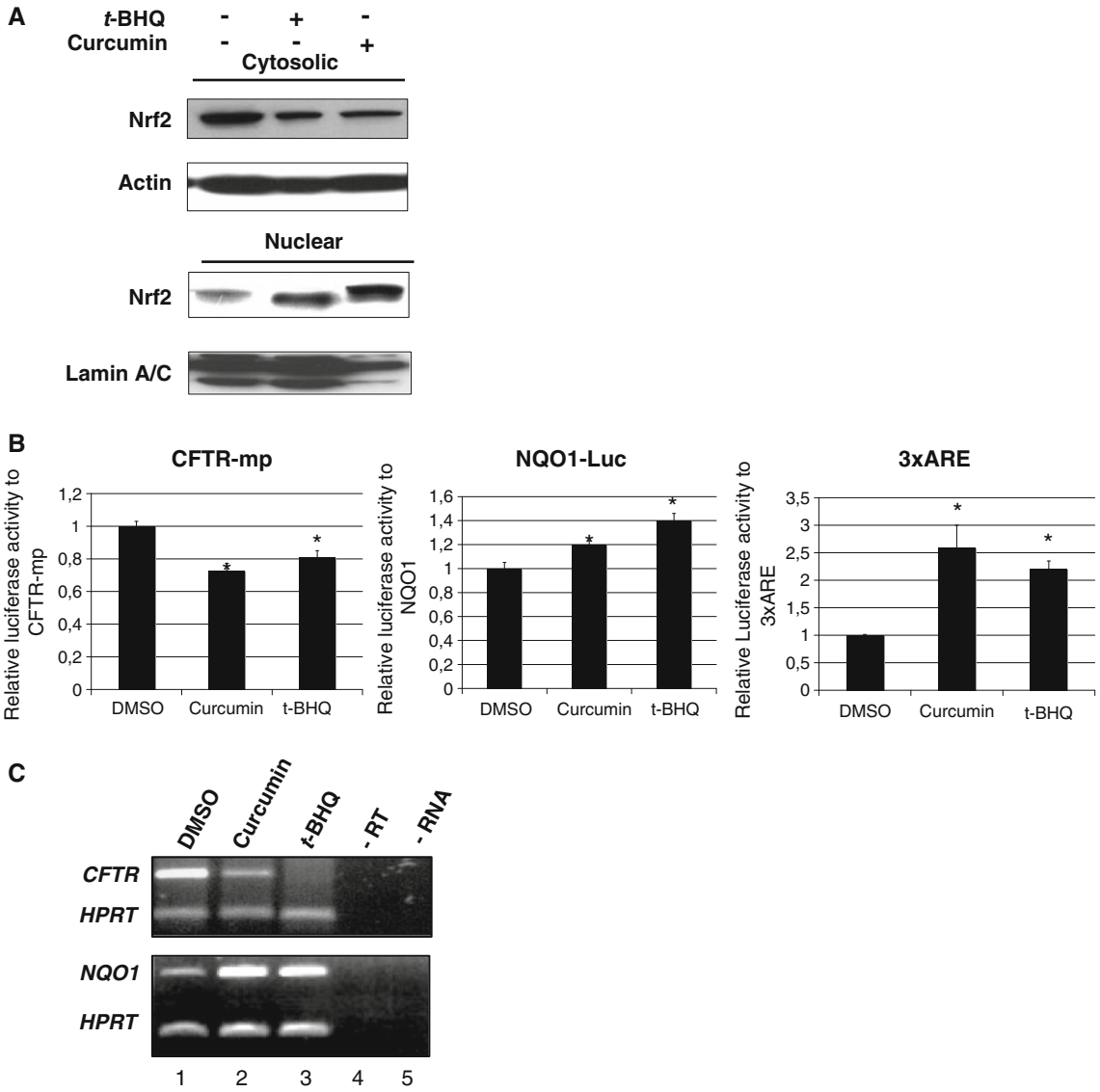

Fig. 1 Curcumin and *t*-BHQ diminishes *CFTR* expression in Beas2B cells. **a**, **b**, and **c** Beas2B cells were challenged either with vehicle (DMSO), curcumin or *t*-BHQ, pro-oxidants known to induce oxidative stress. **a** After challenge, nuclear and cytoplasmic proteins were extracts and analyzed by western blot. Proteins from cells treated with vehicle were used as a control. Actin and Lamin A/C were used as controls for normalization of protein quantity in cytoplasmic and nuclear extracts, respectively. **b** Cells were co-transfected with reporter plasmid expressing luciferase driven either by *CFTR* minimal promoter (*CFTR-mp*), *NQO1* or three repeated *ARE* sequences and pRL-SV40 expressing *renilla* was used for normalization of transfection efficiency. Cells were treated by curcumin for an additional 24-h period or by *t*-BHQ for 4 h. Cells were lysed and analyzed for both *firefly* and *renilla* luciferase activities. Data are means ± SE. $*p < 0.05$ versus vehicle treatment. **c** Total mRNA was extracted from cells treated by pro-oxidants. Transcripts levels for *CFTR*, *NQO1*, and *HPRT* were assessed by multiplex RT-PCR as described under "Materials and methods"

luciferase activities were greatly enhanced in response to curcumin and *t*-BHQ. To further confirm the effects of these drugs on the *CFTR* transcription, mRNA were then extracted and analyzed by RT-PCR. Curcumin and *t*-BHQ reduced the amount of *CFTR* mRNA and increased the *NQO1* mRNA [30] (Fig. 1c, lane 2 and 3, respectively).

These findings show that the increase of nuclear Nrf2 by curcumin and *t*-BHQ is associated with a decrease of *CFTR* transcriptional activity and mRNA. Together, this data confirm that oxidative stress alters *CFTR* expression and suggest that Nrf2 could play a role in this regulation.

The conserved ARE-like motif in the CFTR minimal promoter binds Nrf2

To identify putative ARE elements within the *CFTR* minimal promoter, computer sequence analyses were performed. Using Consite and TESS software, we identified an

ARE-like element at position +15 to +24 of the *CFTR* promoter (the +1 was taken as the major transcription start site described by Chou et al. [31]). As shown in Fig. 2a (*upper*), this *CFTR*-ARE-like element 5′-ATGACATCA CA-3′ diverged slightly at the nucleotide 9 from the published ARE consensus sequence 5′-RTKAYnnnGCR-3′ (where R = A or G, K = G or T, Y = C or T) (*lower*) [32]. To assess the putative relevance of this *CFTR* ARE-like

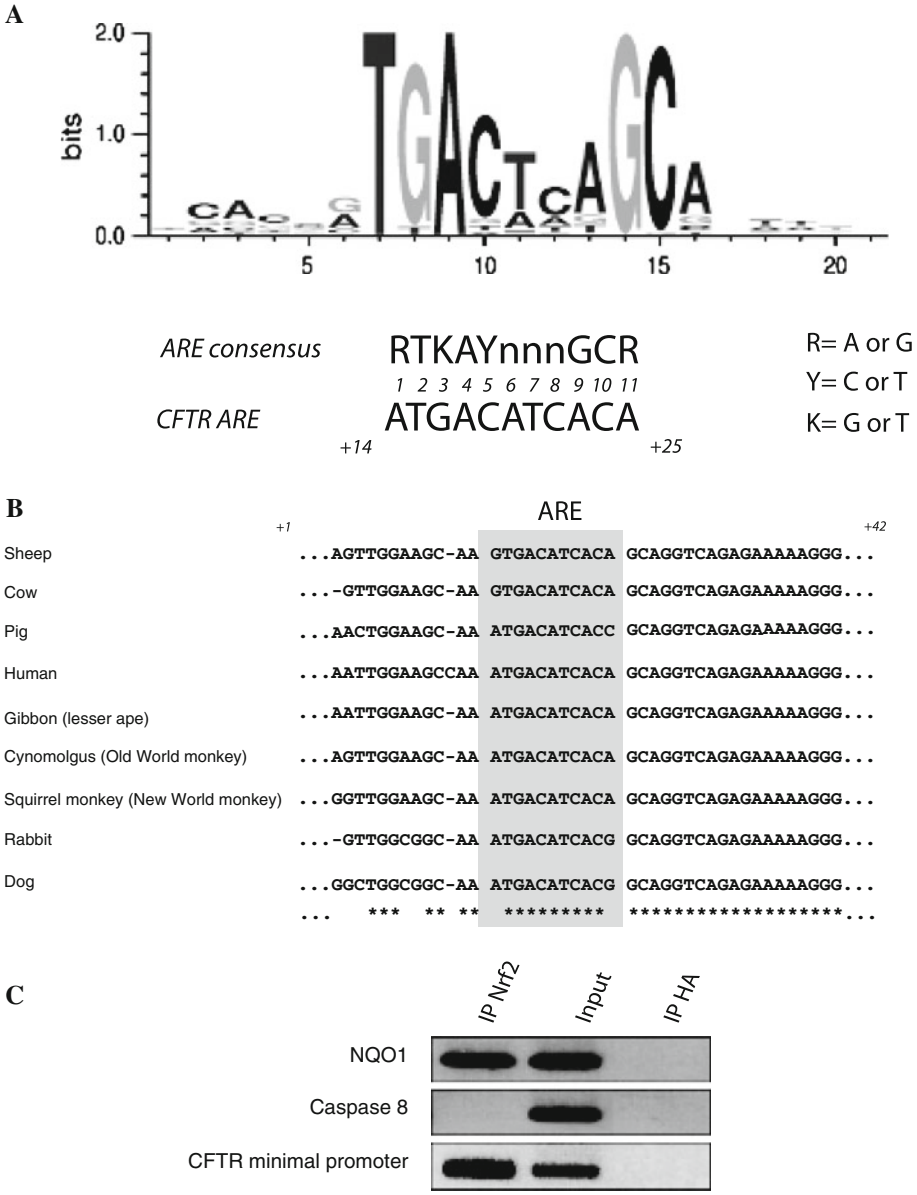

Fig. 2 The highly conserved *CFTR*-ARE sequence binds Nrf2. **a** *Upper* Visualization of the *CFTR*-ARE-like binding site: Logo of the ARE consensus sequence previously published by Wang et al. [21] displaying the critical nucleotides for the Nrf2 binding. The height of each stack of *letters* is the information content (sequence conservation) in bits per base. *Lower* The "core" ARE consensus was exhibited for comparison with the *CFTR*-ARE-like motif identified in the 5′UTR region between +14 and +25 nucleotide position. The position is relative to the transcriptional initiation site described by Chou et al. [31]. '*n*' is used to denote any nucleotide. *R*, *Y* and *K* are the abbreviations used follow standard IUPAC nomenclature indicated on the *right*. **b** An alignment view of the *CFTR*-ARE-like element of Cynomolgus, Gibbon, Squirrel Monkey, Rabbit, Pig, Sheep, Cow, and Dog promoter regions. *Dashes* indicate gaps that were introduced to maximize alignment. *Stars* indicate nucleotide completely conserved between species. The *CFTR*-ARE-like motif is *shaded*. **c** The in vivo specificity for Nrf2 binding to the ARE-like element of *CFTR* gene in Beas2B cells. ChIP was carried out as described in "Materials and methods". The cross-linked chromatin was immunoprecipitated with anti-HA (used as negative immunoprecipitation control) or anti-Nrf2 antibody. DNAs were analyzed by PCR with oligo-primers for the site of Nrf2 in the upstream of *NQO1* gene (positive control), the promoter region of *Caspase 8* gene (negative control) and ARE binding site in *CFTR*

motif, we performed a comparison of sequence homologies between several orthologous *CFTR* minimal sequences using the ClustalW program. As illustrated in Fig. 2b, we found that the ARE-like motif is remarkably conserved in all tested species. The replacement of the A by a G in the first nucleotide in sheep and cow and in the last nucleotide in rabbit and dog conserved the ARE motif as the two nucleotides could occur at these positions [32].

The occupancy of *CFTR* promoter by Nrf2 was investigated in vivo by using chromatin immunoprecipitation assays. As shown in Fig. 2c, we detected Nrf2 binding on the minimal *CFTR* promoter region and the *NQO1* sequence used as a positive control. No enrichment was detected with the anti-HA antibody and the caspase 8 promoter used as immunoprecipitation and PCR negative controls, respectively.

Inhibition of the basal *CFTR* transcriptional activity by Nrf2 in bronchial epithelial cells

To examine the function of Nrf2 protein in the *CFTR* transcriptional regulation, we performed transient co-transfection assays with a reporter construct containing the *CFTR* minimal promoter and Nrf2 expression vectors. While, to our knowledge, Nrf2 has always been reported to be associated with induction of gene expression, it surprisingly inhibited the basal *CFTR* transcriptional activity in Beas2B cells (Fig. 3a). This last effect was abolished by using the Nrf2-dominant negative form deleted of the N-terminal activation region, previously described [33]. To strengthen the result that Nrf2 is capable of causing a down-regulation of *CFTR* reporter gene, we performed experiments with other luciferase reporter plasmids, 3xARE and *NQO1* used as ARE-mediated transcriptional up-regulated controls (Fig. 3a). As expected, enforced expression of Nrf2 activated the luciferase expression driven by either 3xARE sequence or *NQO1* promoter (Fig. 3a). Co-expression of these luciferase reporter plasmids with the Nrf2-dominant negative expression vector significantly attenuated Nrf2-mediated activity. To verify whether this effect could be dependent on the cell-type and on the presence of CFTR functional protein, we evaluated also the role of Nrf2 on the *CFTR* transcription in Caco-2, colic epithelial cells expressing endogenous *CFTR* and in the IB-3 cells, a CF-derived cells line expressing no functional CFTR protein. Similar repression of the *CFTR* transcription was observed in Caco-2 cells and in IB-3 cells, indicating that the inhibitory effect of Nrf2 on the *CFTR* expression was not dependent on the considered cell-type (data not shown).

The direct demonstration of Nrf2-negative role in the regulation of *CFTR* expression was brought by using siRNA assays. The efficiency of mixed siRNAs against Nrf2 was first verified by western blot. Transient transfections of Nrf2-siRNAs (25 nM for 48 h) reduced Nrf2 protein levels by approximately 40% compared to a non-specific siRNA control (Fig. 3b, *left panel*). Then, negative unspecific and Nrf2-specific siRNAs were co-transfected into Beas2B cells with reporter luciferase plasmids containing either *CFTR* promoter, *NQO1* or 3xARE promoter. As shown in Fig. 3b (*right panel*), a decrease of the endogenous Nrf2 level exhibited enhanced *CFTR* transcriptional activity, whereas it inhibited the ARE-dependent transcription from 3xARE and *NQO1* luciferase constructs.

We next evaluated the effect of Nrf2 on the *CFTR* expression at the mRNA level by using RT-PCR. In this aim, we performed a multiplex RT-PCR with *CFTR*- and *HPRT*- specific primers. As shown in Fig. 3c, enforced Nrf2 expression decreased mRNA transcripts for *CFTR*, whereas they substantially increased mRNA transcripts for *NQO1*.

Together, these results demonstrated for the first time that Nrf2 functions as an inhibitor for the *CFTR*-ARE-mediated transcriptional activity in bronchial epithelial cells.

Nrf2 cooperates with YY1 for the repression of the CFTR transcriptional activity

To gain further insight into the Nrf2 inhibitory role on the *CFTR* transcription, a comprehensive in silico analysis of the *cis*-regulatory elements in the *CFTR* minimal promoter was made. Because YY1 was previously described as a strong repressor of the CFTR transcription [5], we are more interested in an YY1-binding site embedded within the ARE sequence (Fig. 4a). In order to evaluate the role of YY1 on the Nrf2-decreased *CFTR* transcription, overexpression and siRNA studies were performed. As shown in Fig. 4b, the co-transfection of YY1 with Nrf2 strengthened the Nrf2-driven repression of *CFTR*, suggesting a cooperation between these repressors. The role of YY1 in Nrf2-mediated inhibition was further investigated by knockdown experiments with increasing amounts of mixed Nrf2 siRNAs and a fixed concentration of YY1 protein (Fig. 4c) or vice versa (Fig. 4d). The Nrf2 silencing completely abolished the YY1-mediated *CFTR* repression (Fig. 4c) and increasing amounts of YY1siRNA relieved the Nrf2-mediated inhibition of the *CFTR* transcription (Fig. 4d). Together, these data evidence a cooperation between Nrf2 and YY1.

Nrf2 potentiates the YY1 binding

Nrf2 proteins have been shown to function as obligate heterodimers by pairing with numerous partners [34].

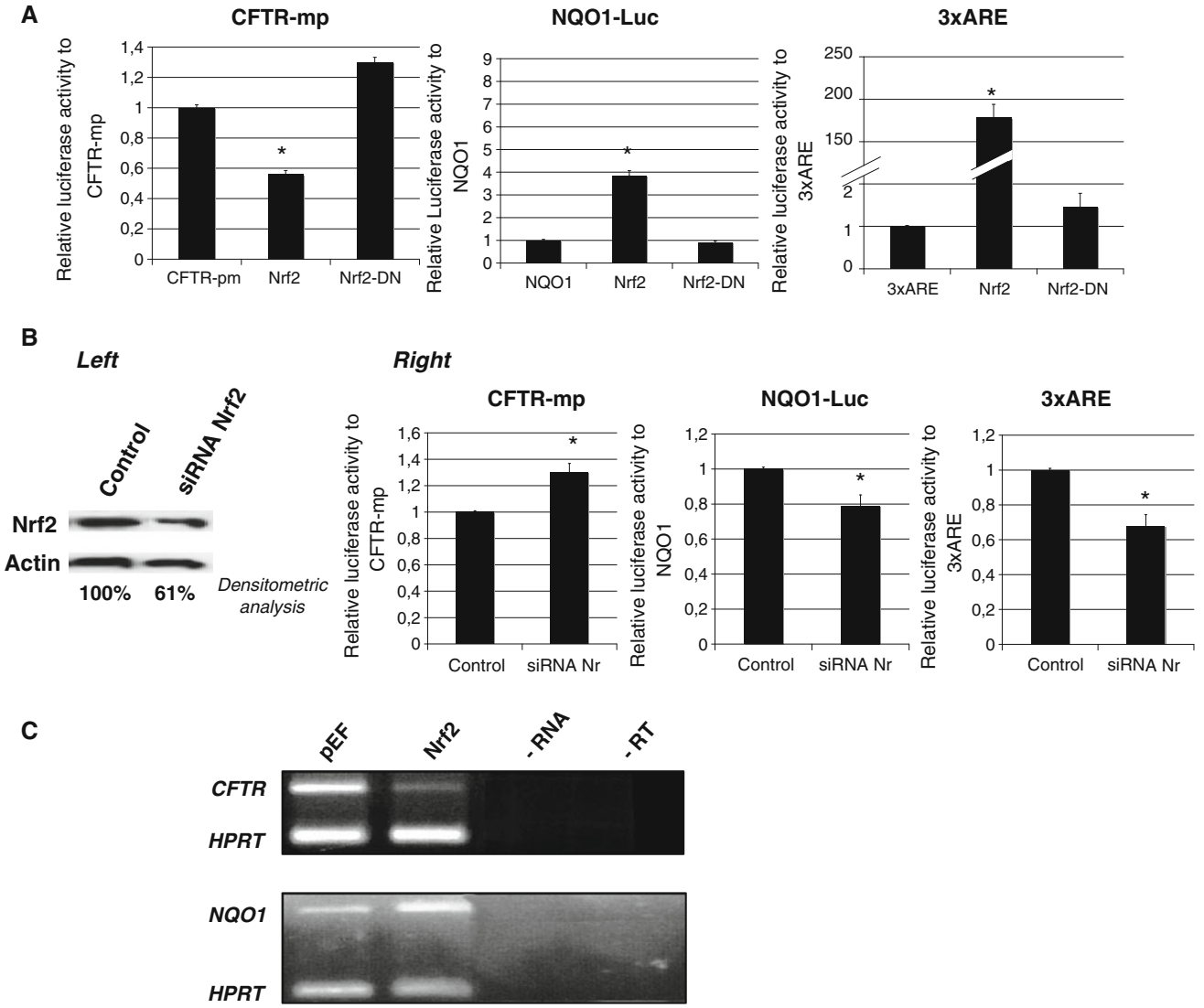

Fig. 3 Role of Nrf2 on the *CFTR* gene transcription. **a** Bronchial epithelial cells lines Beas2B expressing endogenous *CFTR* were transfected with luciferase reporter plasmids under the control of the *CFTR* minimal promoter, the *NQO1* promoter or the tandemly repeated ARE sequence. Nrf2 or Nrf2 dominant negative form (Nrf2-DN) were overexpressed. pRL-SV40 encoding *renilla* luciferase was used as an internal control. The cells were harvested 48 h after transfection and assayed for luciferase activity. The values represent mean ± SE of at least four independent triplicates. **b** Down-regulation of Nrf2 increases the *CFTR* transcriptional activity. Cells were transfected with either an unspecific siRNA negative control or mixed siRNAs directed against Nrf2 mRNA. Total protein extracts were done and analyzed by western blot (*Left panel*). Representative immunoblot of Nrf2 proteins from cells transfected with Nrf2-siRNAs. Nrf2 gene silencing activity of siRNAs was estimated from ratios of Nrf2 protein level/Actin protein level. Transfection with an unspecific siRNA was used as a control and the Nrf2/Actin ratio was set to 100%. (*Right panel)* The luciferase activities were analyzed 48 h post-transfection. Data are means ± SE of at least four independent experiments. *$p < 0.05$ versus unspecific control siRNA. **c** Beas2B cells were transiently transfected with Nrf2 expression vector or its corresponding empty vector (pEF). After 48 h, total mRNA was extracted and expression of the *CFTR*, *NQO1*, and *HPRT* mRNA was analyzed by multiplex RT-PCR. Negative controls are noted as follows: No RT for the absence of reverse transcriptase and No RNA for an H_2O no-template reaction

Therefore, we hypothesized that Nrf2 could act in cooperation with YY1 by facilitating its binding to the DNA. To investigate this possibility, we performed a series of quantitative chromatin immunoprecipitation assays from cells transfected with Nrf2 expression vector or its corresponding empty vector used as a control. The *NQO1* and the *Glucocorticoid Receptor* (*GR*) promoter regions were used as Nrf2 and YY1 positive controls, respectively [35, 36]. The analysis of the results showed that overexpression of Nrf2 significantly increased the YY1 binding on *CFTR* minimal promoter sequence but also on *NQO1* and *GR* promoters (Fig. 5).

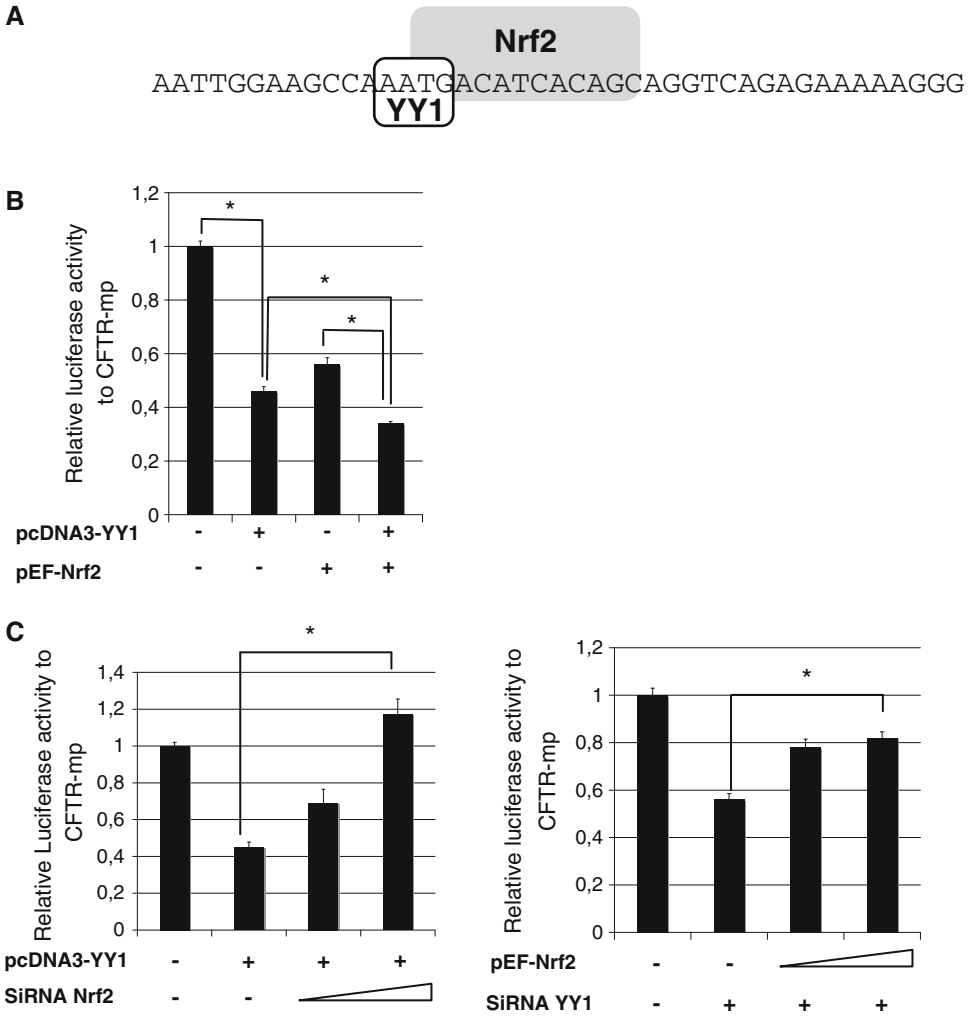

Fig. 4 YY1 cooperates with Nrf2. **a** Schematic representation of the *CFTR* composite YY1/ARE-like *cis*-acting element. Binding sites for Nrf2 and YY1 were predicted by Consite and TESS programs. **b** Reporter plasmid containing the luciferase gene under the control of *CFTR* promoter were co-transfected with Nrf2 and YY1 expression plasmids alone or in combination. Luciferase activities were evaluated on lysates 48 h post-transfection. Each *error bar* represents standard deviation calculated from at least three independent triplicates. **c**, **d** Beas2B cells were co-transfected with unspecific siRNA or increasing concentration of Nrf2 siRNA in presence of fixed quantity of YY1 expression plasmids (**c**) and vice versa (**d**). Transfected cells were harvested and analyzed for luciferase activity

Nrf2 promotes the YY1 nuclear localization

As both Nrf2 and YY1 are regulated through a shuttling between the cytoplasm and the nucleus [37–39], we sought to determine whether Nrf2 could favor the YY1 nuclear localization. First, we performed co-immunoprecipitation experiments to evaluate a putative interaction between Nrf2 and YY1. Immunoprecipitation with an anti-Nrf2 antibody followed by immunoblotting with an anti-YY1 antibody and vice versa showed that Nrf2 interacts with YY1 in Beas2B cells (Fig. 6a). Then, western-blot experiments with extracts transfected with either Nrf2 or YY1 were done. Because of differences in total quantities of proteins as shown by immunoblots against lamin A/C and actin proteins, we performed a densitometric analysis. Immunoblot analyses showed that enforced expression of Nrf2 diminished the cytosolic fraction of YY1 while it increased the YY1 protein level in the nucleus (Fig. 6b). These results suggest that Nrf2 favors the YY1 nuclear localization.

Keap1 relieves the YY1-mediated repression by decreasing its nuclear localization

As Keap1 is known to retain Nrf2 in the cytoplasm, to ascertain whether the Nrf2-mediated *CFTR* repression was based on promotion of YY1 nuclear localization, we evaluated the role of Keap1 in the YY1-mediated *CFTR* transcriptional inhibition. First, we verified the effect of Keap1 on the Nrf2 localization and transcriptional activity. So, we performed western-blot analyses with Keap1-overexpressed cytosolic and nuclear extracts. Compared to the empty vector, we observed an increase of Nrf2 amounts in cytosolic extracts from Beas2B cells transfected with Keap1 expression vector. By contrast, transfection of Keap1 expression vector decreased the Nrf2 quantity in nuclear extracts (Fig. 7a). These results suggested that Keap1 sequestered Nrf2 in the cytoplasm in Beas2B cells as it has been previously described in other cell lines [16]. To provide further insights into the role of Keap1 on the Nrf2-mediated transcription, we performed multiple

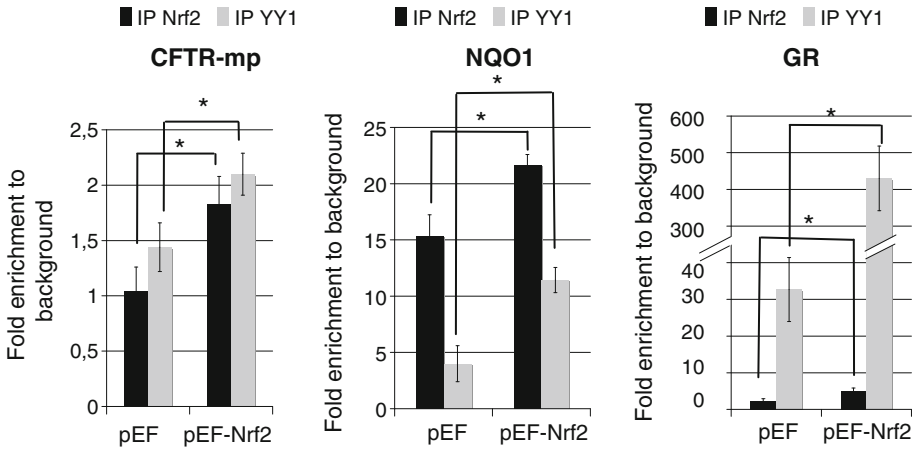

Fig. 5 Nrf2 facilitates the YY1 binding to the CFTR minimal promoter. Beas2B cells were transfected with Nrf2 expression vector or its corresponding empty vector for 48 h. Then, sonicated-chromatin was immunoprecipitated with either anti-Nrf2, anti YY1, or no-specific antibody. DNA from immunoprecipitates and input DNA (which represents 1% of total chromatin) were analyzed by quantitative PCR using primers amplifying the minimal *CFTR* promoter or the *NQO1* promoter (as Nrf2 positive control) or the *GR* promoter (as a YY1 positive control). Data are expressed as fold enrichment of DNA associated with indicated immunoprecipitated antibody relative to a 1/100 dilution of input chromatin. Specific binding was determined by subtracting binding with rabbit IgG. Each *error bar* represents standard deviation calculated from at least three independent triplicates

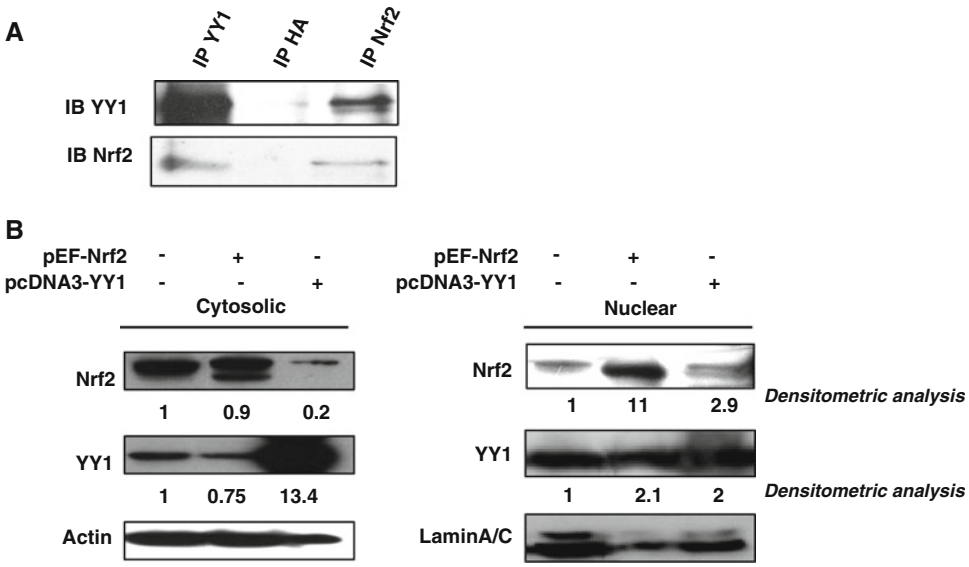

Fig. 6 Nrf2 promotes YY1 nuclear localization. **a** Whole-cell extracts were immunoprecipitated with anti-YY1, anti-Nrf2, or anti-IgG (negative control) antibody and visualized by western-blot analysis with indicated antibodies. **b** Cells were transiently transfected with plasmids expressing Nrf2 and YY1. Nuclear and cytosolic proteins are extracted and analyzed by western blot with indicated antibodies. Anti-Actin and anti-Lamin A/C were used as markers for cytosolic and nuclear extracts, respectively

co-transfections with their respective expression vectors. Supplying exogenous Keap1 significantly inhibits the Nrf2 transcriptional activity in all luciferase constructs used (Fig. 7b).

These data demonstrated that Keap1 decreased the amount of Nrf2 in the nucleus and affected the Nrf2-driven repression of the *CFTR* gene transcription.

Then, we transfected Beas2B cells with the Keap1 expression vector and analyzed the YY1 protein expression in cytosolic and nuclear extracts by western blot. Overexpression of Keap1 led to an increase in cytoplasmic YY1 levels contrasting with a decline in the nucleus (Fig. 7c). Next, we evaluated the role of Keap 1 in the YY1 repressive effect by using co-transfection experiments with the two expression vectors. As shown in Fig. 7d, overexpression of Keap1 severely affected the *CFTR* transcriptional inhibition due to YY1. Collectively, these results show that Nrf2 promotes YY1 nuclear localization.

Discussion

CF is characterized by an exaggerated inflammatory response and repetitive infections resulting in the destruction of the lung. Chronic inflammatory and infections lead to a disequilibrium of the oxidants/antioxidants balance. Increasing evidence has demonstrated that oxidative stress suppresses *CFTR* expression [7–9]. However, the molecular

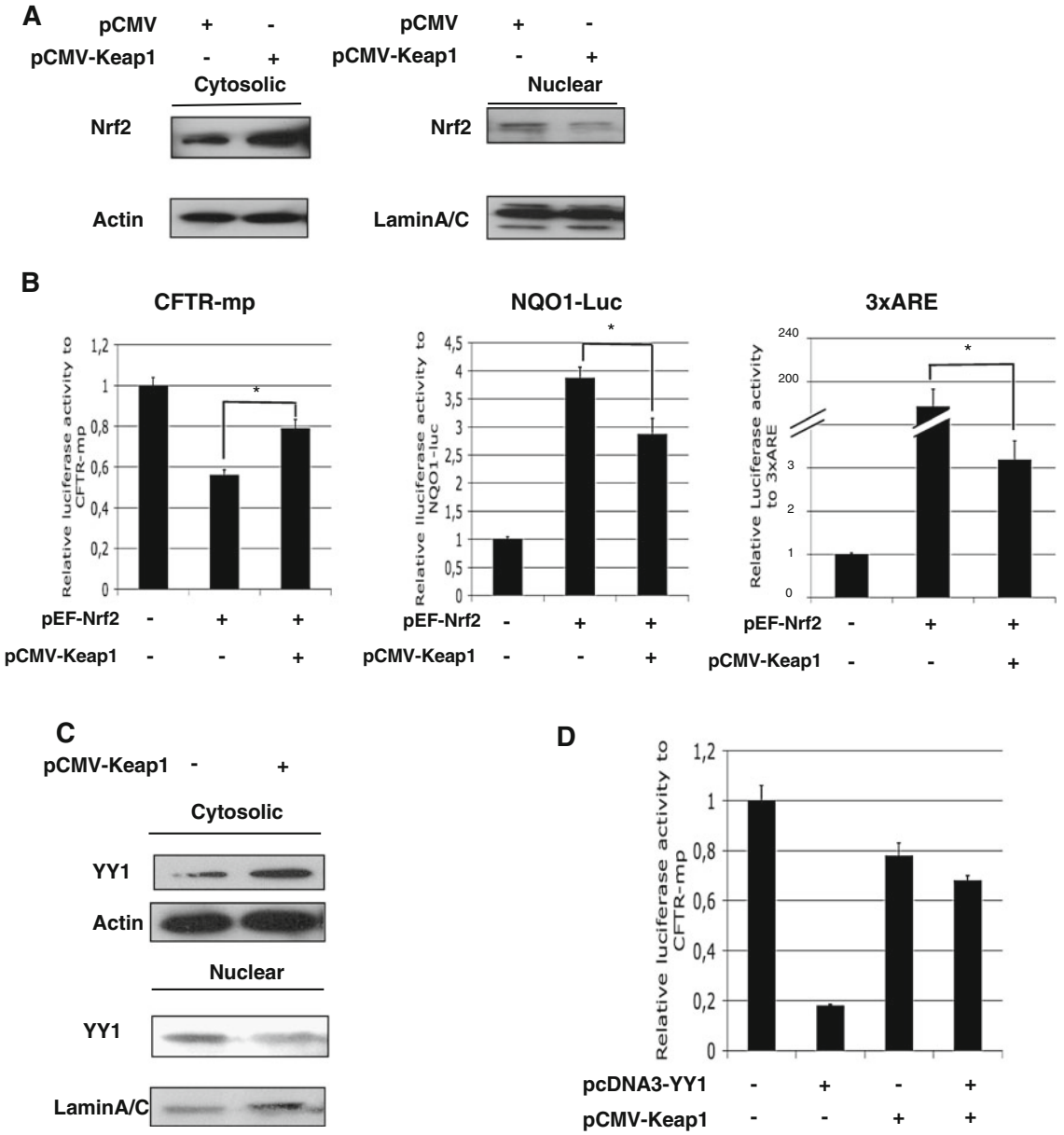

Fig. 7 Keap1 relieves the YY1-mediated repression by decreasing its nuclear localization. **a**, **c** Beas2B cells were transfected with either pCMV or pCMV-Keap1 expression vector. The nuclear and cytosolic extracts were prepared and subjected to western blot analysis with indicated antibodies. **b** Cells were co-transfected with indicated expression vectors and reporter constructs containing the *CFTR* minimal promoter, the *NQO1* or the 3xARE consensus sequences. **d** Beas2B cells were transiently co-transfected with YY1 and Keap1 expression plasmids alone or in combination. **b**, **d** Luciferase assays were performed on lysates as described in Materials and Methods. Data are the results of four independent experiments and are expressed as means ± SE. * $p < 0.05$ versus empty vectors

mechanisms leading to this effect are not well understood. To expand our knowledge about these topics, we postulated that Nrf2, a well-known oxidative stress-induced transcription factor, may affect the *CFTR* expression.

Using two Nrf2-activating drugs, curcumin and *t*-BHQ, we confirmed by RT-PCR that oxidative stress suppresses the *CFTR* expression in Beas2B cells and hypothesized that Nrf2 could be a key regulator of this effect (Fig. 1). Curcumin decreased the *CFTR* transcriptional activity whereas *t*-BHQ had no significant effect on the basal transcriptional activity. The effects of curcumin and *t*-BHQ on the *CFTR* gene transcription suggest that there is more than a single mechanism for activation of the ARE/Nrf2 transcription system. Indeed, a recent study proposes a model in which Keap1-Nrf2 responds to diverse chemical Nrf2-activating compounds by disparate mechanisms constituting several molecular networks [40].

We identified a conserved ARE-like element in the *CFTR* minimal promoter (Fig. 2) slightly diverging at the nucleotide 9 from the published ARE consensus sequence

[32]. However, Nioi et al. [35] have recently demonstrated that the G at this position was not essential for the Nrf2-enhanced activity. Chromatin immunoprecipitation assays showed that Nrf2 binds to the *CFTR* minimal promoter in vivo. Surprisingly, overexpression and siRNAs approaches demonstrated for the first time an inhibitory role of Nrf2 (Fig. 3). Collectively, these findings prove the biological importance of Nrf2 on the *CFTR* expression through its binding to the minimal *CFTR* promoter. Nrf2 is known to essentially activate genes involved in the anti-oxidant response. These experiments illustrate new features concerning the role of Nrf2 in a non-cytoprotective gene and extended the interest of studying its role in the regulation of other genes. The repressive role of Nrf2 is consistent with other investigations, which showed that oxidative stress induced by various drugs or ozone suppressed the *CFTR* expression [10, 11, 41].

This discovery raised our interest in further exploring the underlying mechanisms resulting in the Nrf2-mediated *CFTR* repression. A more accurate in silico analysis of the *CFTR* ARE-like sequence identified an YY1 binding site overlapping to the ARE element. As we have previously shown that YY1 is a strong repressor of *CFTR* expression [5], we checked the combined role of Nrf2 and YY1. We demonstrated that Nrf2 cooperates with YY1 to repress *CFTR* transcription by facilitating its localization into the nucleus and thus its binding (Fig. 8). However, the precise mechanism of this co-operation remains unclear. While further studies are required to characterize the Nrf2-mediated *CFTR* repression, this data suggest that Nrf2 acts by promoting the binding of YY1 repressor protein. Another possibility is the displacement of additional unidentified positive factors by the Nrf2/YY1 binding, which contributes to the negative effect. Indeed, numerous transcription factors have been shown to compete with Nrf2 for ARE-binding. For instance, Nrf1 activates the *NQO1*-ARE [42] and could compete with Nrf2 for interaction with small Maf proteins and binding to the ARE element [43]. Other good candidates could be the C/EBPs family transcription factors. Interestingly, a site for the strong activator C/EBPδ embedded in the ARE motif was previously described [44]. Moreover, C/EBPα has been shown to antagonize the Nrf2 effect by competing for DNA binding [45].

This study gives novel insights into the mechanisms underpinning the *CFTR*-altered expression by oxidative stress. Although not discussed explicitly in this paper, it is expected that information, resulting from this work, will provide guidance to more detailed studies on the mechanisms leading to the oxidative stress-caused bronchial obstruction in several chronic inflammatory pulmonary diseases [18, 46, 47]. In the context of the above data, *CFTR* could be a physiologically important regulator of the response to oxidative stress. As CFTR has been shown to play an essential role for the glutathione transmembrane export [48], a major anti-oxidant in lung, the down-regulation of *CFTR* by Nrf2 could be a part of a cyto-protective response to oxidative stress and contribute to development of CF-like symptoms in patients with chronic lung inflammatory disorders [49]. Moreover, a recent work suggest that Nrf2 plays a role in the pathogenesis of chronic inflammation in cystic fibrosis by modulating H_2O_2 production [50]. We believe that our findings provide important clues for the design of future therapies for the lung inflammatory diseases and for treatment involving the molecular targeting of Nrf2. Given that there are both beneficial and adverse effects of Nrf2 activity, caution will therefore be needed when using antioxidants for prevention or therapy.

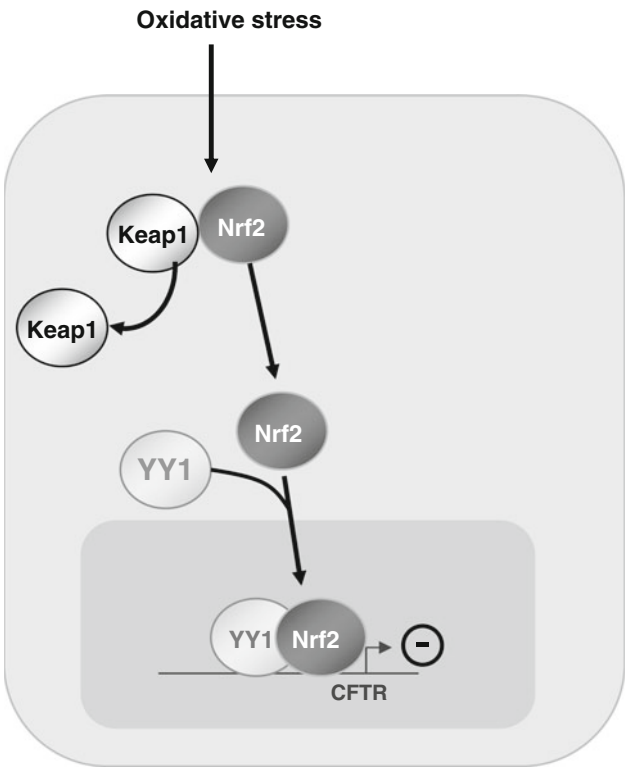

Fig. 8 Model of Nrf2-mediated CFTR transcriptional regulation. Under stress conditions, Keap1 releases Nrf2, which migrates in the nucleus. Nrf2 binds to YY1 and promotes the YY1 nuclear localization. Together, they bind to the *CFTR* minimal promoter to repress the *CFTR* transcriptional activity

In conclusion, we demonstrate, for the first time, an inhibitory role of Nrf2 through the cooperation with YY1 in the transcriptional regulation of a non-cytoprotective gene. While diverse *cis*-acting elements are identified mainly outside the *CFTR* promoter, the exact mechanism involved in the tightly regulated *CFTR* gene expression remains obscure. Our findings point to a novel pertinent mechanism for *CFTR* transcriptional control and open new opportunities in understanding how oxidative stress contributes to disease progression.

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