



Carbon dioxide-dependent regulation of NF- κ B family members RelB and p100 gives molecular insight into CO₂-dependent immune regulation

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CO₂ is a physiological gas normally produced in the body during aerobic respiration. Hypercapnia (elevated blood pCO₂ > ≈50 mm Hg) is a feature of several lung pathologies, e.g. chronic obstructive pulmonary disease. Hypercapnia is associated with increased susceptibility to bacterial infections and suppression of inflammatory signaling. The NF- κ B pathway has been implicated in these effects; however, the molecular mechanisms underpinning cellular sensitivity of the NF- κ B pathway to CO₂ are not fully elucidated. Here, we identify several novel CO₂-dependent changes in the NF- κ B pathway. NF- κ B family members p100 and RelB translocate to the nucleus in response to CO₂. A cohort of RelB protein-protein interactions (e.g. with Raf-1 and I κ B α) are altered by CO₂ exposure, although others are maintained (e.g. with p100). RelB is processed by CO₂ in a manner dependent on a key C-terminal domain located in its transactivation domain. Loss of the RelB transactivation domain alters NF- κ B-dependent transcriptional activity, and loss of p100 alters sensitivity of RelB to CO₂. Thus, we provide molecular insight into the CO₂ sensitivity of the NF- κ B pathway and implicate altered RelB/p100-dependent signaling in the CO₂-dependent regulation of inflammatory signaling.

Oxygen (O₂) and carbon dioxide (CO₂) are the substrate and product of aerobic respiration, respectively. Atmospheric CO₂ levels have recently exceeded 400 ppm (0.04%) with the highest ever daily average CO₂ recorded at Mauna Loa Observatory in April, 2016 (409.44 ppm). Although the levels of this greenhouse gas are rising, the levels in the atmosphere are still much lower than that experienced within respiring organisms. CO₂ is produced as a consequence of aerobic respiration during the pre-Krebs and Krebs cycle reactions. Thus, in normocapnia the normal pCO₂ in the human circulation is ~40 mm Hg. Because the main mechanism through which CO₂ is removed

from the body is via exhalation, the circulating pCO₂ is closely related to lung function and ventilation. Hyperventilation can result in lower than normal levels of CO₂ (hypocapnia), and hypoventilation and/or chronic lung diseases such as chronic obstructive pulmonary disease and cystic fibrosis result in elevated levels of CO₂ (hypercapnia) (1). In patients, the degree of hypercapnia can be profound with arterial pCO₂ values in excess of 100 mm Hg recorded in exacerbated COPD² (2). Hypocapnia is associated with a worse outcome in COPD (3) and worsens cerebral ischemia (4). Interestingly, hypercapnia is also associated with a worse outcome in COPD (3) with additional deleterious consequences reported in terms of muscle dysfunction (5) and immunosuppression (6). This evidence indicates that carbon dioxide is not merely a waste product of metabolism and that like oxygen it can elicit a specific repertoire of transcriptional events in a dose-dependent fashion (1, 7). In particular, genes associated with inflammation, immunity, and metabolism appear to be CO₂-sensitive and that sensitivity of these cohorts of genes to CO₂ is evolutionarily conserved (8–10). The mechanisms, however, are not well characterized. We and others have previously demonstrated sensitivity of the NF- κ B pathway to CO₂ (11–16), and elevated CO₂ is associated with suppression of pro-inflammatory cytokines in a number of different settings (11, 12, 17). The current state of the art is that hypercapnia may be damaging in the context of infection (6, 18) due to immunosuppression. Conversely, hypercapnia may be of benefit in the context of destructive inflammation (19, 20) due to suppression of inflammatory signaling (1, 21). Given the importance of the NF- κ B pathway in the regulation of immune and inflammatory signaling, we hypothesize that CO₂-dependent alterations in NF- κ B are important in determining inflammatory signaling over a range of pCO₂ values from hypocapnia to normocapnia to hypercapnia. Our previous work has implicated members of the non-canonical NF- κ B pathway (IKK, inhibitor of κ B kinase (IKK α) and RelB) as being particularly sensitive to changes in CO₂ in the basal (unstimulated) state (11, 12). This study gives molec-

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This article contains [supplemental Figs. S1–S5 and Mass spectrometry raw data file](#).

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² The abbreviations used are: COPD, chronic obstructive pulmonary disease; MEF, in mouse embryonic fibroblast; IP, immunoprecipitation; F, forward; R, reverse; h, human; LFQ, label-free quantification; IKK, inhibitor of κ B kinase; EMBL-EBI, European Molecular Biology Laboratory-European Bioinformatics Institute.

CO₂-dependent regulation of RelB

ular insight into CO₂-dependent modulation of the NF- κ B transcription factor RelB.

NF- κ B is a family of five transcription factors: p50, p65, p52, RelB, and c-Rel. Of these, p65 is the transcriptionally active component of the canonical p65-p50 heterodimer that is activated downstream of the heterotrimeric IKK α,β,γ complex (22). On the other hand, RelB is the transcriptionally active component of the non-canonical RelB-p52 heterodimer that is activated downstream of an IKK α homodimer complex (23). Canonical signaling is associated with the regulation of classical pro-inflammatory gene activation, *e.g.* TNF α , IL-6, and IL-1 (mainly via p65/p50 heterodimers), although non-canonical NF- κ B signaling is associated with regulation of genes involved in lymphogenesis development (24, 25) (mainly via RelB/p52 heterodimers). Both canonical and non-canonical NF- κ B family members demonstrate sensitivity to CO₂ in the stimulated state (11); however, non-canonical family members IKK α and RelB appear much more sensitive to CO₂ in the basal (non-ligand stimulated) state (12).

RelB has been dubbed “an outlier in leukocyte biology” (23) and is relatively less well characterized than p65. RelB uniquely possesses an N-terminal leucine zipper domain that affects the ability of RelB to activate transcription of target genes (26); furthermore, it is postulated that RelB can activate a more diverse array of NF- κ B consensus binding sequences than other family members (27). The RelB C-terminal contains a transactivation domain, which is conserved among RelA, RelB, and c-Rel (25).

Pathways regulated by RelB include those involved in the following: (i) immune development and signaling (24, 28); (ii) response to xenobiotics (29); (iii) chromatin remodeling, *e.g.* RelB can associate with the ATP-dependent SWI/SNF nucleosome-remodeling complex (30); (iv) circadian rhythms (31); and (v) cellular metabolism (23). Thus, RelB can modulate an array of cellular responses. The molecular mechanisms underpinning these effects are not yet fully appreciated. Taken together, it is clear that RelB differs significantly from other NF- κ B family members in terms of structure, regulation, and target gene expression. Indeed, the relative contribution of both RelA and RelB to lymphotoxin-induced gene expression reveals both overlapping and subunit-specific target genes (32).

RelB is a labile protein subject to significant post-translational modification. Elegant biochemical studies have illustrated that RelB is a target for phosphorylation (33, 34), ubiquitination (35), sumoylation (36), signal-induced degradation, and cleavage (33, 37). These modifications regulate RelB function as well as protein-protein interactions. The protein-protein interaction between RelB and p100 is particularly important for their respective functions (34). p100 acts as an inhibitor of RelB as well as facilitating a “co-operative stabilizing state” between the two proteins (23). p100 in addition to stabilizing and inhibiting RelB has been shown to inhibit canonical NF- κ B-dependent transcription via sequestration of RelA-p50 dimers (38). Recent work has highlighted the association of RelB (along with other NF- κ B subunits) with p100 as part of a high-molecular-weight repressive “kappaBsome” (39, 40).

Finally, a recent study identified RelB mRNA expression as being associated with acid-base and cardiovascular features in

patients with exacerbated COPD (41), suggesting a functional regulation of this NF- κ B family member in a serious disease where hypercapnia is prevalent. The detailed mechanisms underpinning the CO₂-dependent modulation of p100 and RelB along with the downstream consequences for NF- κ B-dependent signaling beyond this have not yet been elucidated and are the focus of this study.

Results

Elevated CO₂ causes a cellular re-organization of the NF- κ B family members RelB and p100

We have previously reported that exposure of cells to elevated CO₂ induces RelB nuclear localization and cleavage in a number of cell types. The mechanisms underpinning this CO₂-dependent modulation of immune signaling are not fully understood and are a focus of this study. Here, we demonstrate in mouse embryonic fibroblasts (MEF) CO₂-dependent nuclear localization and cleavage of RelB that are evident over a range of CO₂ conditions (0.03, 5, and 10% CO₂) (Fig. 1, A and D). We observed a very similar pattern in lung epithelial A549 cells (supplemental Fig. S1, A–C). This CO₂ dose-dependent regulation of NF- κ B from hypocapnia, to normocapnia, to hypercapnia highlights the importance of considering the impact of microenvironmental CO₂ concentrations in a range of conditions.

Given that RelB functionally interacts with p100 within the non-canonical NF- κ B pathway, we next investigated whether p100 also demonstrated sensitivity to CO₂. Interestingly, we observed sensitivity of the p100 subunit to CO₂. To our knowledge, this is the first time that p100 sensitivity to CO₂ has been reported. p100 markedly translocates to the nucleus following exposure to 5% CO₂ and 10% CO₂ (Fig. 1, A and E). We observed a very similar pattern in lung epithelial A549 cells (supplemental Fig. S1, A, D, and E) with a more marked difference observed between 5 and 10% CO₂ in these cells. Thus, in the basal (unstimulated) state members of the non-canonical NF- κ B family undergo cellular modification and re-organization by CO₂ exposure that does not appear to be a consequence of decreased pH_e, or pH_i, which remained consistent under our experimental conditions (supplemental Fig. S2).

RelB is cleaved at its C terminus in response to elevated CO₂

We next developed an overexpression system to test RelB sensitivity in HEK cells. The purpose of this was to allow us to perform mass spectrometric interactome studies in cells exposed to elevated CO₂, looking at RelB and its associated proteins (*e.g.* p100/p52). HEK cells that had been transfected with a full-length N-terminal FLAG-tagged RelB construct (Fig. 2A), re-capitulated the previously observed RelB response to elevated CO₂ (Fig. 1, A and D, and supplemental Fig. S1, A–C) (12). Interestingly, the banding pattern for RelB and FLAG from the HEK cells was almost identical (Fig. 2, B and C) suggesting that RelB is cleaved at its C terminus. Thus, we observed CO₂-dependent regulation of endogenous NF- κ B family members p100 and RelB and also re-capitulated CO₂-dependent cleavage of recombinant RelB. Our next experiments were performed to gain insight into the mechanisms governing RelB sensitivity to CO₂ as well as the consequences for RelB protein-protein interactions.

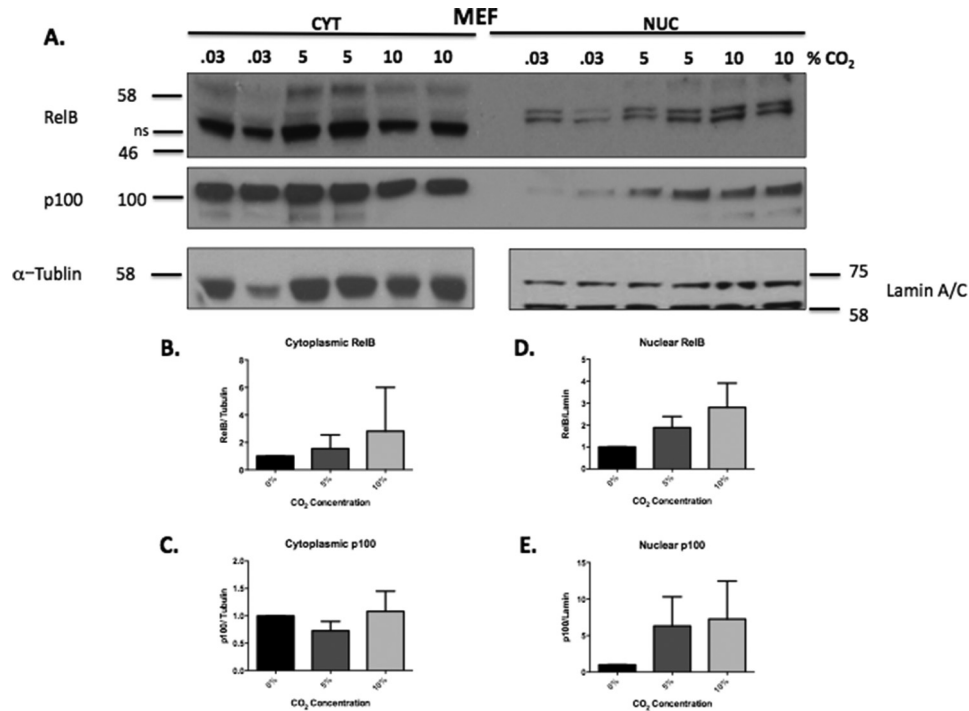


Figure 1. Elevated CO₂ causes a cellular re-organization of the NF-κB family members RelB and p100. *A*, MEF were exposed to 0.03, 5, or 10% CO₂ in pH-buffered media for 75 min prior to preparation of cytosolic (*Cyt*) and nuclear (*Nuc*) protein fractions. Lysates were immunoblotted using specific antibodies against RelB, p100/p52, lamin, and α-tubulin. *ns* denotes a nonspecific cytosolic band. *B*, densitometric quantification of cytoplasmic RelB relative to α-tubulin. *C*, densitometric quantification of cytoplasmic p100 relative to α-tubulin. *D*, densitometric quantification of nuclear RelB relative to lamin. *E*, densitometric quantification of nuclear p100 relative to lamin. Data representative of *n* = 3 experiments.

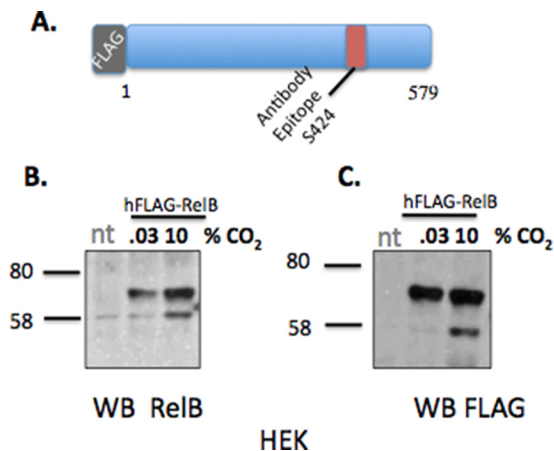


Figure 2. RelB is cleaved at its C terminus in response to elevated CO₂. *A*, schematic indicating the structure of the hFLAG-RelB construct and the region on this protein to which our RelB antibodies are directed (Ser-424). *B*, HEK cells transiently transfected with hFLAG-RelB were exposed to 0.03 or 10% CO₂ in pH-buffered media for 75 min prior to preparation of nuclear protein fractions. Lysates were immunoblotted using specific antibodies against RelB and FLAG (*C*). Data are representative of *n* > 3 experiments. *nt*, non-transfected.

RelB interactome is altered by CO₂ exposure

RelB expression and cellular localization are altered in response to CO₂ (Figs. 1, *A* and *D*, and 2, *B* and *C*, and [supplemental Fig. S1, A–C](#)) (12). Thus, we hypothesized that these changes were being driven at least in part by altered RelB-protein-protein interactions in the different CO₂ environments. We tested this hypothesis in an unbiased and quantitative manner. We first overexpressed FLAG-RelB in HEK cells, exposed the cells to ambient or elevated CO₂ under pH-buffered condi-

tions, and performed an immunoprecipitation for FLAG. Precipitated proteins were then analyzed by mass spectrometry (Fig. 3*A*). Proteins of interest were then determined by meeting specific stringency thresholds for (i) enrichment over control (non FLAG-RelB-expressing cells) and (ii) evidence for CO₂ sensitivity in the RelB-specific protein interactions (2-fold difference up or down) (Fig. 3*B* and [supplemental mass spectrometry files](#)). Several known RelB-interacting proteins were identified by the screen, which supports the sensitivity of our experimental approach (Fig. 3*C*). For example, IκBα, importin α-7, and the ubiquitin C-terminal hydrolase-7 are the most enriched, the 50th most enriched, and the 90th most enriched interaction, respectively (www.ebi.ac.uk/intact). EMBL-EBI IntAct Molecular Interaction Database was searched using the search-term “RelB.” Regarding CO₂ sensitivity, 25 proteins met or exceeded the thresholds for being a FLAG-RelB-associated protein that were differentially associated with RelB in a CO₂-dependent manner. These proteins are listed in [supplemental Fig. S3](#), with a selection of proteins illustrated in Fig. 4. The seven proteins demonstrated increased association with FLAG-RelB, and 18 proteins demonstrated decreased association with FLAG-RelB at 10% CO₂ compared with ambient CO₂. Interestingly, when Gene Ontology analysis was performed on the 25 proteins that were found to have differential interactions with RelB in a CO₂-dependent manner, there was a strong enrichment of proteins involved in both protein transport and nucleic acid binding ([supplemental Fig. S4](#)). These unbiased data support the concept that RelB translocates to the nucleus in a CO₂-dependent manner, conceivably facilitated by importin proteins. Selected proteins were chosen for validation of the

CO₂-dependent regulation of RelB

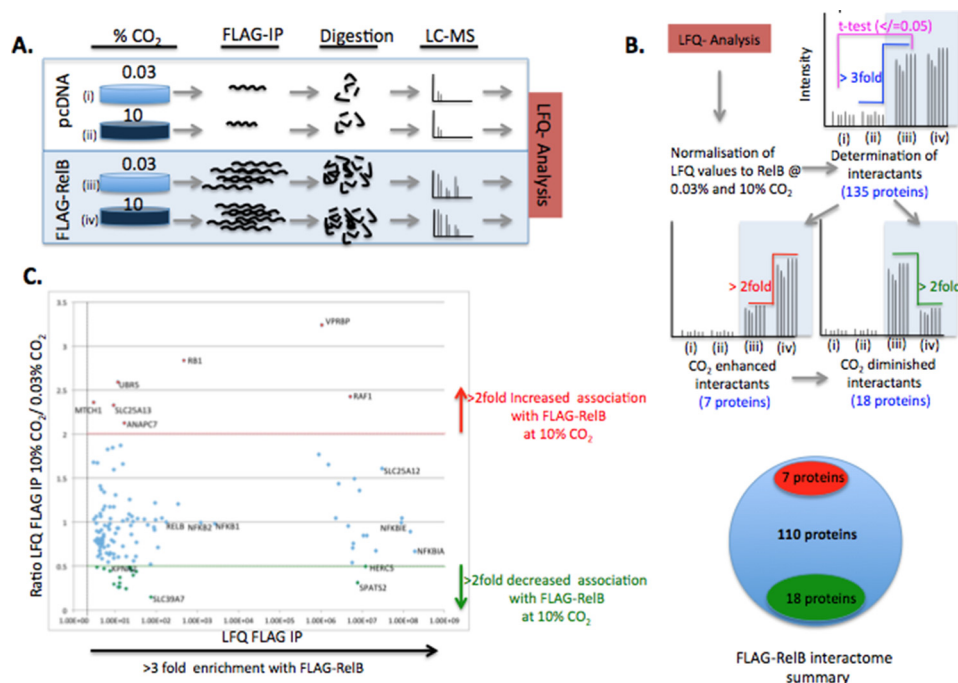


Figure 3. Mass spectrometric analysis of RelB protein-protein interactions. *A*, schematic illustrating the experimental workflow leading to the identification of proteins associated with FLAG-RelB. *B*, schematic illustrating the filtering strategy of the LFQ data to identify *bona fide* RelB interactions and RelB interactions that are altered by exposure to 10% CO₂. *C*, scatter plot of the 135 RelB protein interactions plotted for LFQ FLAG IP intensity (degree of enrichment) versus ratio LFQ FLAG IP 10% CO₂/0.03% CO₂ (CO₂ sensitivity). CO₂ enhanced interactions are shown in red; CO₂ diminished interactions are shown in green, and other RelB interactions are shown in blue.

MS/MS screen by FLAG-RelB overexpression coupled to conventional Western blotting. RelB interactions with p100 were not affected by CO₂ exposure (supplemental Fig. S5A). IκBα had reduced association with RelB at 10% CO₂ in several experiments (supplemental Fig. S5), whereas Raf-1 had increased association at 10% CO₂ (supplemental Fig. S5C). Thus, the data from these IP Western blotting experiments support the IP MS/MS data. Of note, SMARCD2 was markedly enriched with FLAG-IP, but it was not consistently different in 10% CO₂ by IP Western blotting (supplemental Fig. S5D). Thus, the IP Western data largely validate the mass spectrometry data, with the MS/MS approach likely more sensitive than IP Western blotting approaches. However, given the SMARCD2 data, we suggest caution in interpreting the data from the less enriched proteins due to a risk of false positives. CO₂-dependent changes were more reproducibly validated by Western blotting for highly enriched interactors (e.g. IκBα, p100, and Raf-1).

Amino acids 484–503 are involved in CO₂-dependent processing of RelB

Having identified the CO₂-dependent nuclear translocation and processing of RelB (Fig. 1), we next sought to identify the region of RelB that was being cleaved. First, data from Fig. 2, *B* and *C*, suggested that RelB was being cleaved in its C-terminal region downstream of Ser-424 (the RelB antibody epitope). Second, the lower molecular weight form of RelB observed in 10% CO₂ revealed a relatively small (≈10–15 kDa) increase in electrophoretic mobility. Thus, we performed sequential mutagenesis of the C-terminal region of RelB spanning from before the RelB antibody epitope (Ser-404) (as a control) to beyond where we thought the cleavage site to likely be (Val-524). Thus, to test

our hypothesis and gain molecular insight into the effects of CO₂ on RelB, we generated six mutants that individually deleted 20 amino acid segments of human RelB spanning from amino acids 404–524 (Fig. 5A). These mutants were screened for CO₂ sensitivity alongside a full-length RelB control. As expected, Δ404–423 (which is N-terminal to the Ser-424 RelB antibody epitope and therefore serves as an internal control) demonstrated CO₂ sensitivity analogous to that of wild-type RelB. Furthermore, this CO₂ sensitivity of Δ404–423 was blocked by pre-treatment with the proteasome inhibitor (as observed for wild-type RelB). Similarly, deletions in the regions 424–443, 444–463, and 464–483 demonstrated the same CO₂ sensitivity and MG-132 sensitivity as the wild-type FLAG-RelB construct. Interestingly, the Δ484–503 mutant did not demonstrate CO₂-dependent cleavage, whereas CO₂ and MG-132 sensitivity was restored in the downstream Δ504–524 mutant (Fig. 5B). Interestingly, these 20 amino acids (LLDDGFAYDPTAPTLFTMLD) reside within a highly conserved region of the protein. Taken together, these data suggest that amino acids 484–503 are within the cleavage site of RelB and/or are involved in transducing the CO₂-dependent cleavage of RelB.

RelB Δ484–579 has altered NF-κB-dependent transcriptional activity

Having identified that absence of amino acids 484–503 rendered RelB insensitive to CO₂-dependent cleavage, we hypothesized that under conditions of elevated CO₂ there is an enriched population of C-terminally truncated RelB in the nucleus. We further hypothesized that the truncated form of RelB has altered transcriptional activity and contributes to

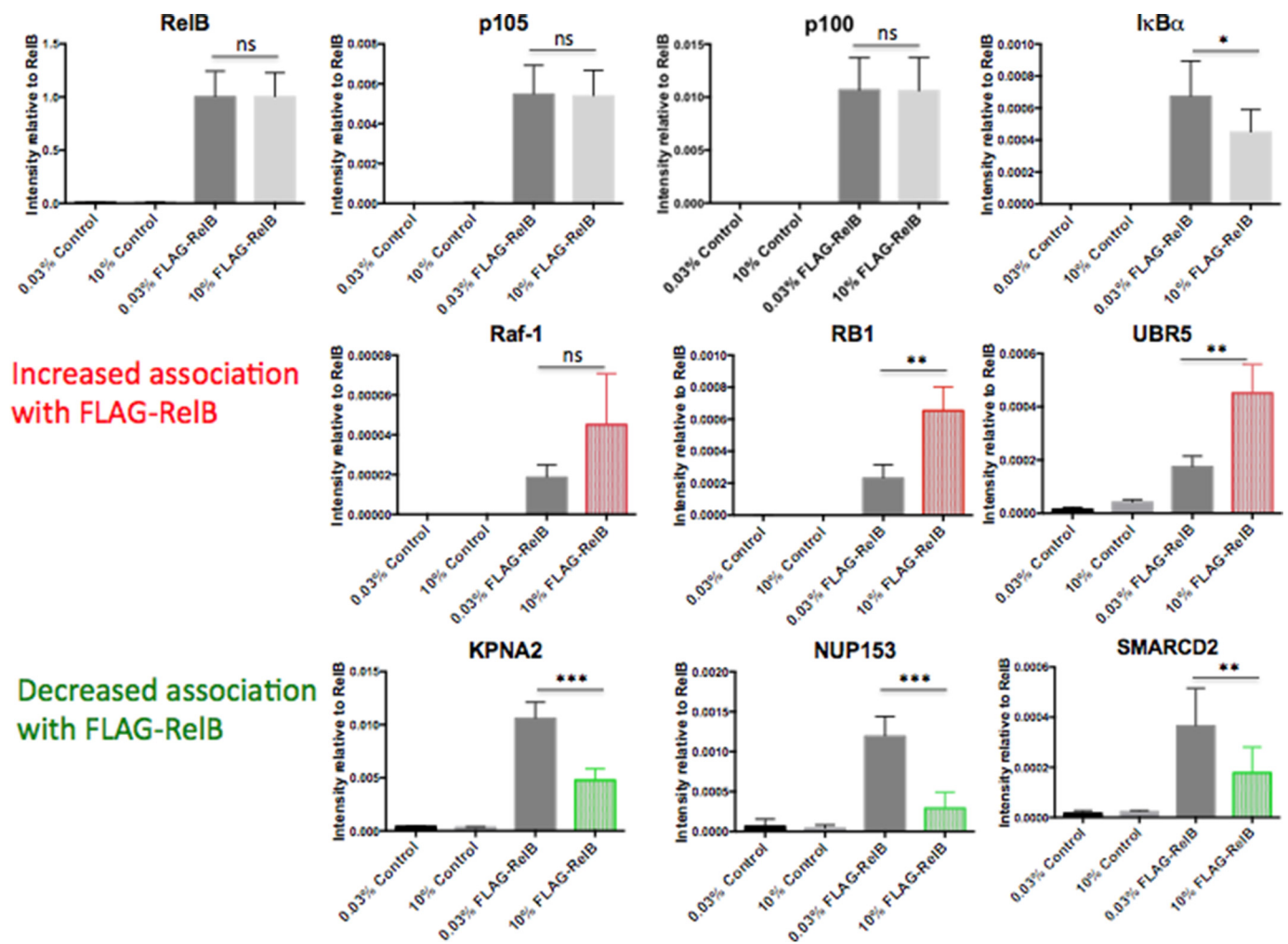


Figure 4. RelB interactome is altered by CO₂ exposure. HEK cells transiently transfected with pcDNA control plasmid or hFLAG-RelB were exposed to 0.03 or 10% CO₂ in pH-buffered media for 75 min prior to preparation of whole-cell lysates. Lysates were immunoprecipitated using FLAG-agarose, and precipitated proteins were analyzed by mass spectrometry. Data shown are protein LFQ-intensity values of selected proteins identified in the negative control and the FLAG-RelB IP at 0.03 and 10% CO₂. Selected proteins demonstrating increased association with FLAG-RelB at 10% CO₂ are highlighted in red, selected proteins demonstrating decreased association with FLAG-RelB at 10% CO₂ are highlighted in green. Data are representative of mean peptide intensity values \pm S.D. relative to RelB for three biological replicates and two technical replicates per treatment. Statistical analysis comparing FLAG-RelB at 0.03 and 10% CO₂ was performed using a Student's *t* test with a *p* value \leq 0.05 deemed significant. *ns*, not significant. *, *p* \leq 0.05; **, *p* \leq 0.01; ***, *p* \leq 0.001.

CO₂-dependent alterations in gene expression. To test the hypothesis that a truncated form of RelB has altered signaling capabilities, we generated a C-terminally truncated form of FLAG-RelB (RelB Δ 484–579 also known as RelBshort (Fig. 6A)) and compared it with wild-type FLAG-RelB in an NF- κ B-luciferase assay. TNF α -significantly increased NF- κ B-luciferase activity at 0.1 and 1 ng/ml.

Overexpression of RelBshort led to reduced TNF α -stimulated NF- κ B-luciferase activity compared with wild-type RelB indicating altered transcriptional activity (Fig. 6B). Thus, overexpression of a truncated form of RelB (that mimics the form of RelB that is enriched in the nucleus at 10% CO₂) alters cytokine-stimulated NF- κ B-dependent transcriptional activity.

Loss of p100 impairs the CO₂-dependent nuclear localization of RelB

To investigate the mechanisms underpinning CO₂-dependent nuclear localization of RelB further, we focused on the

interaction between p100 and RelB. p100 and RelB regulate each other's stability (34). Furthermore, p100 is known to have an inhibitory role on NF- κ B signaling (38) and play a key role in the inhibitory kappaBosome (39, 40). Our earlier data demonstrated that despite a marked nuclear accumulation of RelB in response to CO₂, the interaction with p100 remained relatively constant (Figs. 3C and 4 and supplemental Fig. S5A). This suggested that p100 might also become nuclearly localized in response to elevated CO₂ and contribute to the CO₂-dependent effect on NF- κ B signaling. Indeed, we observed clear p100 nuclear localization in response to elevated CO₂ (Fig. 1, A and E, and Fig. S1, A and D). These data suggest the possibility of RelB and p100 translocating to the nucleus together as part of a complex. Given the role of p100 in RelB stability (34), we hypothesized that p100 might be required to confer CO₂ sensitivity on RelB. To test this hypothesis, we compared the pattern of CO₂-dependent RelB nuclear localization in wild-type MEF and in MEF deficient in "canonical" p105 (*NFKB1*) as well as "non-

CO₂-dependent regulation of RelB

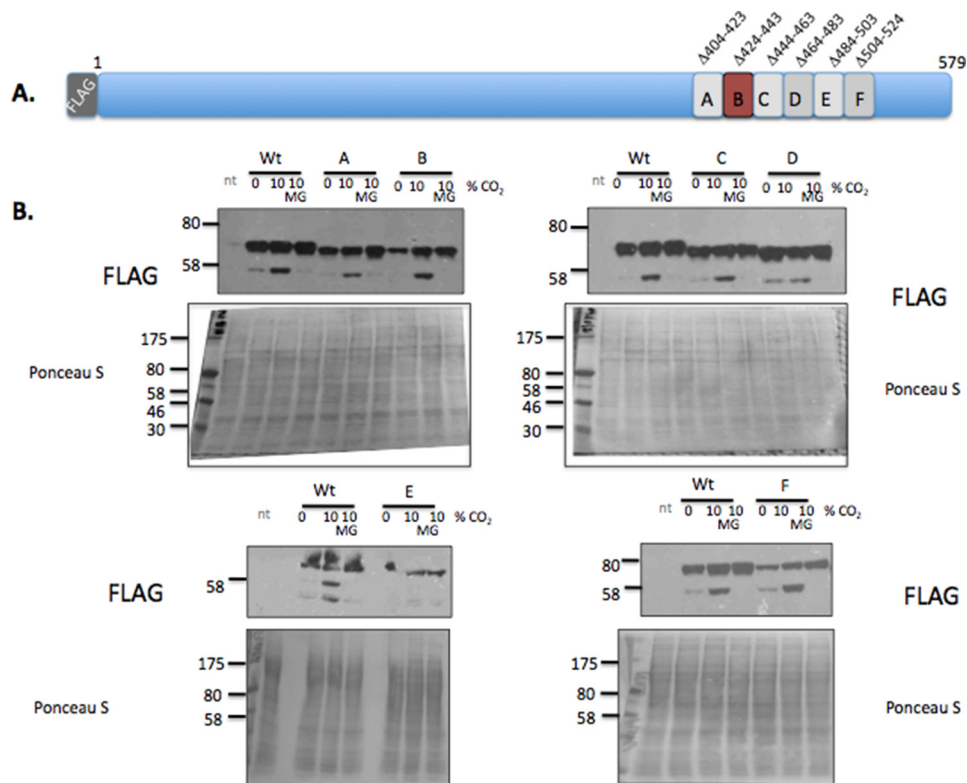


Figure 5. Amino acids 484–503 are involved in CO₂-dependent processing of RelB. A, HEK cells transiently transfected with full-length FLAG-RelB or one of six 20-amino acid deletions of RelB (B) were exposed to 0.03 or 10% CO₂ in pH-buffered media for 75 min ± Mg-132 (10 μM) prior to preparation of nuclear lysates and immunoblotting using a FLAG antibody and Ponceau S staining of the nitrocellulose membrane. Data are representative of > 3 experiments.

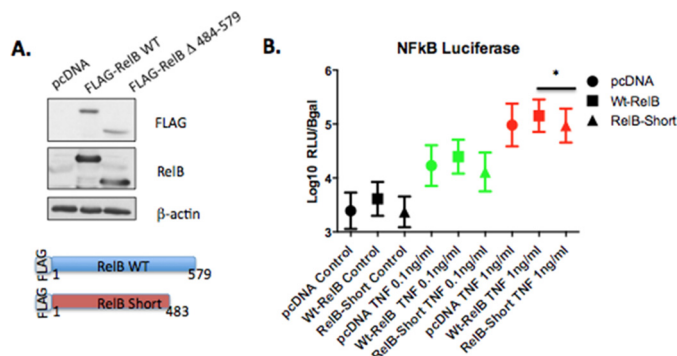


Figure 6. RelB Δ484–579 has altered NF-κB-dependent transcriptional activity. A, HEK cells were transiently co-transfected with pcDNA control, full-length FLAG-RelB (Wt), or RelBshort (FLAG-RelB Δ484–579) in addition to a κB-luciferase promoter reporter construct and a β-galactosidase reporter. B, cells were treated with TNFα (0, 0.1, and 1 ng/ml) for 24 h at 5% CO₂. Relative light units (RLU) were normalized to β-galactosidase absorbance for each sample. These non-parametric data were normalized by transforming the data by log₁₀. Data presented are mean ± S.E. for n = 4 experiments. Statistical analysis was performed using repeated measures one-way ANOVA, with Tukey's multiple comparisons test. A p value ≤ 0.05 was deemed significant (*, p ≤ 0.05).

canonical" p100 (NFKB2). Interestingly, the p100^{-/-} MEF demonstrated an aberrant pattern of nuclear RelB, compared with both the wild-type and p105^{-/-} MEF. In p100^{-/-} MEF, the full-length form of RelB was not enriched in the nucleus in response to elevated CO₂; however, the lower molecular weight form of RelB was observed (Fig. 7, B, D, and F). Taken together, this suggests that p100 NF-κB is sensitive to CO₂ and that it is required for the normal distribution of RelB in the nucleus under conditions of elevated CO₂.

Discussion

Alterations in CO₂ levels are increasingly being associated with human pathologies, e.g. COPD, where either hypo- or hypercapnia increases the hazard ratio for death (3). This observation is supported by *in vivo* experimentation illustrating the deleterious consequences of elevated CO₂ in the context of infection (6) and in a clinical trial where reducing hypercapnia in COPD patients was beneficial (43). Against this background, it is perhaps counterintuitive that therapeutic hypercapnia is being investigated in the context of single lung ventilation (44). There is, however, emerging evidence for elevated CO₂ being associated in a better outcome in models of inflammation (19), ventilator-induced lung injury (45, 46), skin graft survival (47), and stretch-induced epithelial injury (48). The mechanisms reconciling these seemingly opposing outcomes in hypercapnia are not fully elucidated and are a basis for this study.

Studies in both model organisms as well as human cells and tissues have implicated a role for altered NF-κB-dependent signaling in response to different CO₂ concentrations (9, 11, 12, 14, 15, 46, 49, 50). Members of the non-canonical or "alternative" NF-κB family (IKKα and RelB) have been reported to be CO₂-sensitive (11, 12), as have genes downstream of the *Drosophila* orthologue *Relish* in flies (9). Thus, we focused our attention on the transcriptionally active component of the non-canonical NF-κB pathway to gain insight into CO₂ sensing and how CO₂ affects NF-κB signaling.

Our data suggest a significant re-arrangement of RelB and p100 within the cell under conditions of elevated CO₂. Mass spectrometry analysis of immunoprecipitated RelB reveals 135

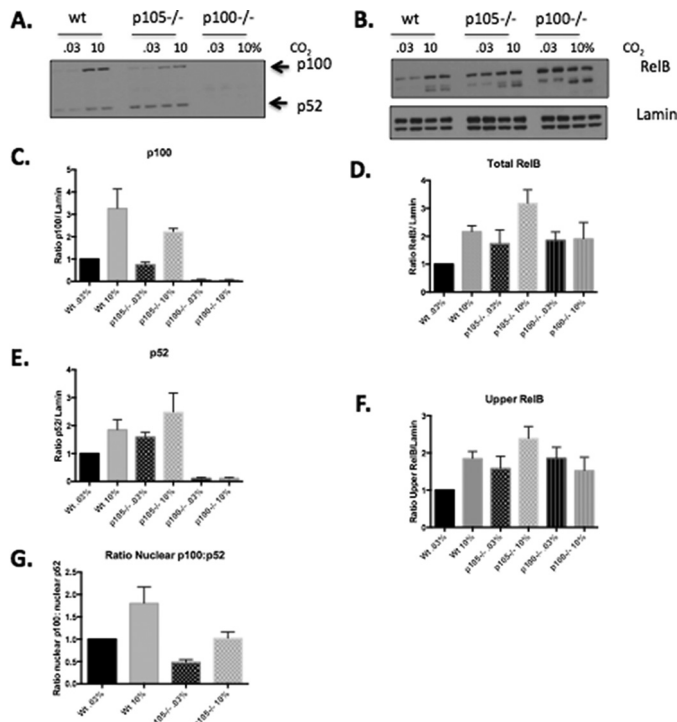


Figure 7. Loss of p100 NF- κ B impairs the CO₂-dependent nuclear localization of RelB. MEF (WT *NFkB1*^{-/-} (*p105*^{-/-}) and *NFkB2*^{-/-} (*p100*^{-/-})) were exposed to 0.03 or 10% CO₂ in pH-buffered media for 75 min prior to preparation of nuclear lysates. Lysates were immunoblotted using (A) p100/p52, (B) RelB and lamin-specific antibodies. Densitometric analysis was performed to determine the ratio of (C) p100, (D), total RelB, (E) p52, and (F) upper band of RelB relative to lamin. Data shown are the mean protein expression \pm S.E. relative to control (WT MEF exposed to 0.03% CO₂). G, densitometric analysis of the ratio of nuclear p100 relative to nuclear p52 in response to elevated CO₂. Densitometric data are presented as mean \pm S.E. for $n = 4$ experiments.

proteins that are 3-fold-enriched compared with control. Of these, several *bona fide* interactors (p100, I κ B α , and Raf-1) were found to be significantly enriched by mass spectrometry and separately validated by conventional immunoprecipitation coupled to Western blots. Approximately 19% of these proteins were differentially associated with RelB in a CO₂-dependent manner. Thus, most RelB interactions were not significantly changed by CO₂. This is an interesting observation given that we can clearly observe marked nuclear translocation of RelB in response to elevated CO₂. This suggests that a subpopulation of RelB is actually moving into the nucleus in response to CO₂ and/or that when RelB moves into the nucleus, it does so as a complex with a number of other proteins, including p100, for example. This proposed scenario might also explain why the ratio of RelB/p100 is unchanged by CO₂. What is clear from the mass spectrometry experiment is that there is a difference in the degree of RelB interaction with proteins associated with the nucleus regarding nuclear shuttling (importins), nuclear pore, and DNA binding, for example (SMARCD2 and RB1), upon exposure to CO₂. RelB has previously been shown to have a physical association with members of the KPNA family (51). Our observed decrease in association between RelB and α -importin proteins suggests the possibility that RelB is associated with the importin α complex in advance of a stimulus, and following CO₂ exposure this interaction is reduced, as a subpopulation of RelB translocates to the nucleus. Taken together,

these data point to a selective re-arrangement of RelB with its interacting partners in response to CO₂, which facilitates localization to the nucleus and may interfere with existing RelB-DNA-binding complexes.

RelB is subject to multiple post-translational modifications as well as cleavage by a variety of enzymes (33, 52, 53). Our data indicate that RelB is cleaved at its C terminus (Fig. 2, B and C). Scanning mutagenesis was employed to determine the precise site involved, to gain insight into how CO₂ modulates NF- κ B-dependent signaling. Here, we report that a Δ 484–503 deletion mutant of RelB demonstrates aberrant CO₂-dependent processing (Fig. 5B). The 484–503 region is C-terminal to the RelB nuclear localization motif, which explains why both full-length and truncated forms of RelB can accumulate in the nucleus in a CO₂-dependent manner. Furthermore, this site is distinct from other sites that have previously been reported to control RelB processing, for example Asp-205 (54), Thr-84/Ser-552 (53), and Arg-85 (52). Thus, we propose that this 20-amino acid CO₂-responsive domain of RelB is involved in CO₂-dependent cleavage of RelB. The crystal structure for this region of the RelB protein has not yet been solved, and *in silico* structural predictions of this region are of low confidence. However, region 484–503 does lie within the C-terminal transactivation domain of RelB (55). This suggests that RelB proteins deficient in the region C-terminal to the cleavage site may have impaired transcriptional activity. Previous studies have demonstrated the requirement of both N- and C-terminal regions of RelB in the presence of p50-NF- κ B for full transactivation (26).

Thus, our data suggest that under conditions of elevated CO₂ RelB is cleaved and that both full-length and truncated forms of RelB can then translocate to the nucleus. It is unlikely that both of these forms of RelB have identical transcriptional activity, and consequently, we generated a RelBshort construct to test this. This truncated form of RelB (which mimics a form of RelB generated in hypercapnia) has impaired NF- κ B-dependent transcriptional activity compared with full-length RelB (Fig. 6B).

Finally, given the known reciprocal role of p100 in stabilizing RelB, we demonstrated for the first time a profound nuclear localization of p100 in response to elevated CO₂ (Figs. 1 and 7, A and C, and [supplemental Fig. S1, A and D](#)). This is consistent with our mass spectrometry data indicating that the ratio of RelB/p100 is unchanged at 10% CO₂ and that the two proteins may translocate to the nucleus as a complex. Thus, using MEF deficient in p100, we investigated the requirement of p100 on the RelB response to elevated CO₂. Interestingly, loss of p100 significantly altered the CO₂-dependent nuclear localization profile of RelB in the nucleus (Fig. 7, B and D). Notably, a lower molecular weight form of RelB was still evident in the nucleus in response to CO₂ in p100^{-/-} MEF. However, the full-length form of RelB that normally accumulates under those conditions did not (Fig. 7, B and F). Together, this suggests that p100 is important in the regulation of the RelB response to elevated CO₂ but is dispensable for the cleavage. Thus, nuclear p100 localization appears to be a key event in coordinating the NF- κ B-dependent response to elevated CO₂. Interestingly, although we observed both p100 and p52 translocation to the

CO₂-dependent regulation of RelB

nucleus in response to CO₂, they did not accumulate to the same extent, with p100 relatively more enriched in the nucleus at 10% CO₂ compared with p52 (Fig. 7G and supplemental Fig. S1F). This observation first suggests that elevated CO₂ is not driving the non-canonical processing of p100 to p52 and second that a repressive p100- and RelB- containing complex is enriched in response to CO₂.

In summary, hypercapnia is a feature of a number of pathologies and is known to modulate innate and immune signaling. A role for the NF- κ B pathway downstream of hypercapnia has been proposed; however, the mechanisms are not fully elucidated. Targeting CO₂-dependent signaling may represent a new anti-inflammatory strategy in the treatment of human disease; however, the molecular mechanisms downstream of CO₂ need to be more fully described. Recently, RelB has been proposed as a potential novel marker of health outcomes in exacerbated COPD (57), a condition linked to hypercapnia. Here, we show that RelB is a CO₂-sensitive transcription factor that undergoes a complex cellular rearrangement under conditions of elevated CO₂. RelB demonstrates decreased association with importin proteins, CO₂-dependent cleavage at its C-terminal that requires amino acids 484–503, and translocates to the nucleus both as a full-length protein and as a cleaved short form. RelBshort (a truncated form of RelB) demonstrates decreased NF- κ B-dependent transcriptional activity compared with wild type, which may be due to impairment of its transactivation domain and consequent ability to bind nuclear proteins. The RelB interactome is altered in response to CO₂, but several RelB-protein interactions are maintained at 10% CO₂, e.g. interactions with p100. p100, like RelB, also translocates to the nucleus under conditions of elevated CO₂, and loss of p100 impairs RelB nuclear localization when CO₂ levels are elevated. Interestingly, in a recent cohort study of patients requiring acute mechanical ventilation, PaCO₂ was an independent predictor of survival to hospital discharge over a linear range of PaCO₂ pressures from hypocapnia (<35 mm Hg) to hypercapnia (66–75 mm Hg) (42). Thus, a better understanding of the molecular mechanisms underpinning CO₂-dependent NF- κ B regulation will enhance our understanding of human pathologies where hypercapnia is a feature and help to develop CO₂-dependent therapeutic strategies.

Materials and methods

Cell culture and exposure to different CO₂ environments

Human embryonic kidney, mouse embryonic fibroblast, and A549 cells were cultured at ambient O₂ and 5% CO₂ and maintained in a humidified tissue culture incubator prior to exposure to the conditions indicated in the individual experiments.

Temperature was maintained at 37 °C in a humidified environment. CO₂ incubation was achieved by exposure of cells to preconditioned medium in an environmental chamber (COY Laboratories) set at 5 or 10% CO₂ with a balance of air. Ambient CO₂ experiments were carried out in a 37 °C humidified incubator with room air.

For experiments involving exposure to 0.03, 5, and 10% CO₂, pH buffering was achieved by supplementing high glucose DMEM powder (D1152 Sigma) with different amounts of

NaHCO₃ as described previously (12). Media were then reconstituted, filter-sterilized, and supplemented with FCS (10%) and penicillin/streptomycin. NaCl was supplemented to correct for osmolality differences. Taken together, this approach can maintain pH_e over a range of CO₂ concentrations (0.03–10%).

Western blot analysis

Nuclear, cytosolic, whole-cell, or immunoprecipitated lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described previously (11). Primary antibodies against RelB (4954) (Figs. 1, 6, and 7), RelB (4922) (supplemental Fig. S1), p100 (4882), and lamin (4777) (Cell Signaling Technology), α -tubulin (sc-8035) (Santa Cruz Biotechnology), and FLAG (F7425) and β -actin (A5316) (Sigma) were used, as well as species-specific HRP-conjugated secondary antibodies.

Molecular cloning/mutagenesis

hFLAG-RelB in a pCR3 (Invitrogen) backbone underwent site-directed mutagenesis of its C-terminal region using QuikChange XL mutagenesis kit according to the manufacturer's instructions. The QuikChange primer design tool was used to generate a series of deletion mutants using the following specific primers: mutant Δ 404–423 F, 5'-ctcgcgaccatgacgctctgacccccca-3', and Δ 404–423 R, 5'-tgggggtcagagctgtcatggtcgcgag-3'; B mutant Δ 424–443 F, 5'-cttggggagctgaccacttctctgcccaac-3', and Δ 424–443 R, 5'-gttgggcaggaagtgttgcagctcccccaag-3'; C mutant Δ 444–463 F, 5'-ccggccatctgacacctgacttctctct-3', and Δ 444–463 R, 5'-agagaagaagtcagggtccaggatggcccg-3'; mutant Δ 464–483 F, 5'-ccctgctccagacctctggacgatgg-3', and Δ 464–483 R, 5'-ccatcgtccaggaggtctggcagcaggg-3'; mutant Δ 484–503 F, 5'-gcgggcctgacctgctgcccc-3', and Δ 484–503 R, 5'-gggggcagcaggtcaggccccg-3'; F mutant Δ 504–523 F, 5'-ttcaccatgctggacgtgttggggagacc-3', and Δ 504–523 R, 5'-ggtctcccaaccagctccagcatggtgaa-3'; and RelBshort Δ 484–579 F, 5'-ccctggcgggctgactagaagctgaattctg-3', and Δ 484–579 R, 5'-cagaattcaagctttagtcaggccccccaggg-3'. Mutations were confirmed by sequencing using forward T7 and/or pCR3.1-BGHrev primers.

Transfection

Cells were transfected using plasmid DNA, OptiMEM1 serum-free media (Gibco), and Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions in antibiotic-free media. Plasmids for RelB and RelB mutants are described above.

Immunoprecipitation

HEK cells overexpressing recombinant FLAG-tagged proteins were lysed in whole-cell lysis buffer (1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl₂) and incubated with anti-FLAG M2 affinity gel (7.5–15 μ l/Eppendorf) end-over-end with rotation at 4 °C for 1–2 h. Samples were centrifuged at 500 rpm for 1 min to pellet the beads, which were then washed two times in lysis buffer and two times in wash buffer (lysis buffer without Triton X-100) with centrifugation in between each wash step. Beads were then incubated with

NuPAGE sample buffer (≈ 30 – $60 \mu\text{l}$) and boiled for 5 min. The supernatant was collected, supplemented with 100 mM DTT, and boiled for a further 5 min. Samples were then frozen at -20°C or immediately run on a Western blot.

Mass spectrometry

Following immunoprecipitation, samples were treated as followed for MS analysis. After washing twice with 300 μl of ice-cold PBS, beads with bound proteins were eluted in two steps. First, 60 μl of eluting buffer I (50 mM Tris-HCl (pH 7.5), 2 M urea, and 50 $\mu\text{g}/\text{ml}$ trypsin (modified sequencing grade trypsin); Promega) were incubated while shaking at 27°C for 30 min, and second, 25 μl of elution buffer II (50 mM Tris-HCl (pH 7.5), 2 M urea, and 1 mM DTT) was added twice. Both supernatants were combined and incubated overnight at room temperature. Samples were alkylated (20 μl of iodoacetamide, 5 mg/ml, 30 min in the dark). Then, the reaction was stopped with 1 μl of 100% trifluoroacetic acid (TFA), and 100 μl of the sample was immediately loaded into equilibrated hand-made C18 StageTips containing Octadecyl C18 disks (Supelco). Samples were desalted by using two times 50 μl of 0.1% TFA and eluted with two times 25 μl of 50% acetonitrile and 0.1% TFA solution. Final eluates were combined and concentrated until the volume was reduced to 5 μl , using a CentriVap concentrator (Labconco). Samples were diluted to obtain a final volume of 12 μl by adding 0.1% TFA. The samples were run on a Q-Exact mass spectrometer (Thermo Fisher Scientific) connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system (Thermo Fisher Scientific). Each sample was loaded onto Biobasic Picotip Emitter (120 mm length, 75 μm internal diameter) packed with ReproCil Pur C18 (1.9 μm) reverse phase media column and was separated by an increasing acetonitrile gradient, using a 53-min reverse phase gradient at a flow rate of 250 nl/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 220°C and a capillary voltage of 1,900 V applied to the capillary. All data were acquired with the mass spectrometer operating in automatic data-dependent switching mode. A high-resolution-MS scan (350–2,000 Da) was performed using the Orbitrap to select the 12 most intense ions before MS/MS analysis using the ion trap. Raw files were analyzed, and relative protein concentration and identifications were determined by label-free quantification using the MaxQuant software suite (9). MS/MS spectra were searched against the human UniProt database. Triplicate biological samples for each treatment were performed in each case. Each individual biological sample was then run in duplicate on the mass spectrometer. Each of the triplicate biological samples was considered as an individual n -number for the purposes of this experiment and to determine statistical significance. We have used a similar approach previously (56).

Filtering of mass spectrometry/mass spectrometry data

Protein mass spectrometry label-free quantification (LFQ) intensity values were normalized in each replicate for the respective experimental treatments. To discriminate specific FLAG-RelB-associated interactions from nonspecific FLAG-agarose interactions, we defined several inclusion criteria.

Enriched interactions—Proteins that were enriched >3 -fold in the 0.03% CO₂ and 10% CO₂ FLAG-RelB sample were compared with their respective pcDNA control sample at 0.03 and 10% CO₂ with a p value of ≤ 0.05 in each case. 135 proteins were enriched with FLAG-RelB using this analysis.

CO₂-sensitive interactions—Proteins that were enriched as under “Enriched interactions” above were additionally filtered for CO₂ sensitivity (ratio FLAG-RelB 10% CO₂/FLAG-RelB 0.03% CO₂, >2 or <0.05) (2-fold difference up or down). 25 proteins had a CO₂-sensitive interaction with FLAG-RelB using this analysis.

Pathway analysis of mass spectrometry/mass spectrometry data

25 CO₂-sensitive protein interactions (see under “CO₂-sensitive interactions” above for filtering of mass spectrometry data and supplemental Fig. S1) were analyzed for protein class using Panther bioinformatic software (www.pantherdb.org)³ (58).

Densitometry

Densitometric analysis was carried out using ImageJ software to determine band size/intensity of target proteins on Western blottings and normalized to respective controls, e.g. α -tubulin, lamin A/C, or FLAG.

Luciferase assay

HEK cells were seeded on 24-well plates (50,000 cells/well). 24 h later, cells were co-transfected with NF κ B-Luc PEST (Promega) and β -galactosidase control plasmid along with pcDNA or WT hFLAG-RelB or FLAG-RelBshort mutant RelB. 24 h later cells were treated \pm TNF α (0.1–1 ng/ml) (Sigma) for 24 h prior to lysis. Lysate was incubated with luciferase substrate (Promega), and chemiluminescence was detected on a BIO-TEK Synergy-HT plate reader.

pH_i assay

This experiment was performed as described previously (11). Briefly, cells were washed in OptiMEM1 (Gibco) serum-free media and loaded with 5 μM 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Molecular Probes Code B1170) in OptiMEM1 for 30 min at 37°C , 21% O₂, 5% CO₂. Dye was removed, and cells were incubated in full DMEM for 30 min at 37°C , 21% O₂, 5% CO₂. Cells were then exposed to pre-equilibrated buffered media at 0.03% CO₂, 5 or 10% CO₂ for 75 min. Following exposure, cells were removed and immediately assayed in a fluorescent plate reader at room temperature at 21% O₂, ambient CO₂. The fluorophore was excited at 485 nm (λ_1) and 444 nm (λ_2), and emission was recorded at 538 nm in each case. The ratio λ_1/λ_2 is directly proportional to intracellular pH, which was confirmed using a standard curve of nigericin (Sigma)-permeabilized cells exposed to a high potassium buffer (KCl (140 mM), MgCl₂ (1 mM), CaCl₂ (2 mM), D-glucose (5 mM)) adjusted to a range of pH values (pH 5–8) using MES (20 mM)-acidifying solution or Tris base (20 mM) alkaline solution.

³ Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.

Author contributions—C. E. K., C. C. S., J. R., A. C. S., A. von K., and E. P. C. designed, performed, and analyzed experiments. E. P. C. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the manuscript.

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