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RasGRF couples Nox4-dependent endoplasmic reticulum signaling to Ras

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Abstract

Objectives—In response to ER stress, endothelial cells initiate corrective pathways such as the unfolded protein response (UPR). Recent studies suggest that reactive oxidant species (ROS) produced on the ER may participate in homeostatic signaling through Ras in response to ER stress. We sought to identify mechanisms responsible for this focal signaling pathway.

Approach and Results—In endothelial cells we found that ER stress induced by tunicamycin activates the NADPH oxidase Nox4 focally on the ER surface but not on the plasma membrane. Ras activation is also restricted to the ER, occurs downstream of Nox4, and is required for activation of the UPR. In contrast, treatment with the growth factor VEGF results in Ras activation and ROS production confined instead to the plasma membrane and not the ER, demonstrating local coupling of ROS-Ras signals. We further identify the calcium-responsive, ER resident guanyl exchange factors RasGRF1 and RasGRF2 as novel upstream mediators linking Nox4 with Ras activation in response to ER stress. Oxidation of the calcium pump SERCA and increases in cytosolic calcium caused by ER stress are blocked by Nox4 knockdown, and reduction in cytosolic free calcium prevents both Ras activation and the UPR.

Conclusions—ER stress triggers a localized signaling module on the ER surface involving Nox4-dependent calcium mobilization, which directs local Ras activation through ER-associated, calcium-responsive RasGRF.

Keywords

Ras; ROS; Nox; Nox4; ER stress; tunicamycin; autophagy; RasGRF1; RasGRF2

INTRODUCTION

The integrity of client protein folding within the ER is exquisitely sensitive to a variety of changes in the environment, including nutrient availability, calcium levels, mechanical stimuli, and redox state. Consequently, a number of cellular stress programs that respond to

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DISCLOSURES

None.

such perturbations are integrated with ER stress sensing and signaling pathways. Proteins that control cell cycle entry, apoptosis, calcium flux, metabolism, and autophagy decorate the ER endomembrane. These proteins allow the ER to respond to proteotoxic and oxidative stress, loss of anchorage, insufficient or excessive nutrient availability, increases in synthetic demands, and other intrinsic and extrinsic stressors [1–4]. The ER surface therefore functions as an important integrative signaling domain to organize stress response signaling.

The Nox family of NADPH oxidases and the Ras GTPases both have broad signaling functions including the response to various cellular stresses. Accurate subcellular routing of both protein families to discrete signaling platforms such as the ER is critical to transducing appropriate responses. Restriction of the single Ras gene product of the yeast *Schizosaccharomyces pombe* to the plasma membrane, for instance, supports the mating and starvation responses but not cell shape changes, while ER-restricted Ras exclusively controls cell morphology [5]. In mammalian cells, ER-resident Ras isoforms are activated by growth factors and differentially activate downstream pathways compared to Ras proteins residing on other membrane domains [6]. Nox proteins are likewise targeted to different subcellular structures in order to direct diverse signaling outputs [7]. For example, Nox4 localized to focal adhesion sites controls focal adhesion turnover and traction force generation [8]. In contrast, nuclear envelope-localized Nox4 promotes export of HDAC4 to activate NFAT, and mitochondrial Nox4 oxidizes aconitase-2 and citrate synthase, signaling mitochondria-dependent death [9, 10].

A significant fraction of Nox4 resides on the ER endomembrane, where it participates in ER signaling. ER-localized Nox4 terminates EGF signaling through oxidative inactivation of the ER resident phosphatase PTP-1B, and glucose deprivation activates autophagy through ROS produced by ER-associated Nox4 [11, 12]. Previously, we demonstrated that a Nox4-Ras pathway acts locally to activate the unfolded protein response (UPR) and initiate autophagy as a protective response against specific ER stressors [13]. However, the molecular basis for the coupling of Nox4 and Ras in response to ER stress is not known, nor is the significance of their shared residence on the ER surface. Here, we find that ER stress initiates a Nox4-dependent calcium signal which in turn restricts Ras activation to the ER and not plasma membrane through the calcium-responsive, ER-localized guanyl exchange factor RasGRF. This novel signaling pathway therefore integrates ROS, calcium, and Ras signaling in response to ER stress through regional interaction of ER-based proteins.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

Nox4 mediates ER-localized ROS production

We first examined sites of ROS production during ER stress following treatment of HUVEC with the well-known ER stressor tunicamycin. Both Nox4 and Ras are anchored to the plasma membrane as well as endomembranes such as the ER, thus we focused our attention on focal production of ROS at these two subcellular membrane domains. To spatially

distinguish sites of oxidant production we employed HyPer, a H_2O_2-specific ratiometric sensor composed of cpYFP inserted into the regulatory domain of OxyR [14]. We have previously shown that HyPer-ER, which contains an ER leader sequence and C-terminal retention peptide, accurately targets the endothelial ER and reports ER H_2O_2 levels [13]. Consistent with prior observations [13], we found increases in H_2O_2 levels within the ER by 16 h of treatment with tunicamycin, and further, that RNAi-dependent knockdown of Nox4 abrogated tunicamycin-dependent increases in ER H_2O_2 production (Figure 1A–B, Figure I A). To interrogate the plasma membrane for focal ROS production, we fused HyPer to the C-terminal membrane targeting domain of Rit (HyPer-Rit). This domain localizes exclusively to the plasma and not ER membrane, and unlike CAAX-containing proteins it does not require posttranslational modifications and does not direct trafficking through ER endomembranes [5]; it is therefore insensitive to ER integrity. ER stress induced by tunicamycin had no effect on HyPer-Rit ratiometric signals, indicating a lack of increase in H_2O_2 production at the plasma membrane (Figure 1A–B). To verify our ability to spatially discriminate sites of ROS production, we stimulated cells with VEGF, which has also been linked with Nox4 signaling [15]. Like nearly all plasma membrane receptor-triggered events, VEGF membrane signaling proceeds over minutes rather than hours; thus, VEGF-induced ROS production was found within 5 min, on a different time course than tunicamycin. Notwithstanding, addition of VEGF resulted in the opposite spatial activation pattern in that H_2O_2 levels increased at the plasma membrane but not at the ER (Figure 1C–D). Thus, tunicamycin-induced ER stress initiates ROS production specifically at the ER and not plasma membrane through Nox4.

Tunicamycin initiates Nox4-dependent Ras activation on the ER

Using a Ras-GTP pulldown assay, we found that activation of Ras accompanied tunicamycin-induced ER stress (Figure 2A). Ras activation was blocked by expression of ER-targeted catalase or by Nox4 knockdown, both consistent with activation of Ras by H_2O_2 on the ER endomembrane (Figure 2A, Figure I B). To provide further support for ER-restricted Ras activation, we expressed a triconcatenated Ras binding domain of Raf fused to GFP (RBD3(R59A)-GFP), which reports sites of activation of endogenous Ras [16]. Coexpression of this probe with ssRFP-KDEL, which marks the ER, revealed that tunicamycin treatment caused translocation of RBD3(R59A)-GFP to the ER (Figure 2B). Quantification confirmed colocalization of this Ras-GTP-avid probe with the ER which was diminished by Nox4 knockdown. Further, knockdown of K-Ras abrogated translocation of RBD3(R59A)-GFP to the ER, supporting its ability to report sites of Ras activation (Figures II A–D). In contrast, using a viable membrane stain to mark plasma membranes, we found that VEGF stimulation but not tunicamycin induced RBD3(R59A)-GFP translocation to the plasma membrane, indicating Ras activation on plasma membranes by VEGF but not ER stress (Figure 2C). Thus spatial compartmentalization of Ras activation parallels that of ROS generation in an agonist-specific fashion.

Of note, levels of Nox4 protein did not appreciably change in response to tunicamycin treatment for up to 24 h, suggesting activation of the oxidase (Figure III A). Currently, mechanisms of Nox4 activation are incompletely understood. The only clear modifier of Nox4 activity is polymerase-δ-interacting protein 2 (Poldip2), which associates strongly

with focal adhesions and stress fibers [8, 17] but is not known to be present in the ER. Accordingly, knockdown of Poldip2 had no effect on Ras activation (Figures III B–C), suggesting Nox4 activation by an alternate pathway.

RasGRF controls ER stress-induced Ras activation upstream of the UPR

Activation of the UPR has been linked to pathways downstream of Ras [13, 18, 19]. Indeed, knockdown of K-Ras blocked induction of BiP and CHOP, as well as phosphorylation of eIF2α, suggesting that the Nox4/Ras signaling pathway controls the UPR in response to tunicamycin-induced ER stress (Figure 3A–C). As an independent marker of sustained ER stress, we noted the conversion of LC3-I to LC3-II, a marker of autophagy which is known to be triggered following prolonged ER stress states [13, 20, 21] (Figure 3D). LC3-II formation was also reversed by K-Ras knockdown, indicating that ER Ras coordinates multiple responses to ER stress. To further support the link between ER ROS, Ras, and the UPR, we found that ER-targeted catalase also effectively blocked BiP and CHOP induction, eIF2α phosphorylation, and LC3-I to LC3-II conversion (Figure IV A–C).

We hypothesized that an ER-resident Ras guanyl exchange factor (GEF) was specifically activated during ER stress, leading to Ras activation. Of the known Ras GEFs, RasGRF1 and RasGRF2 have been shown to associate with both plasma membrane and ER endomembranes, although their function at ER sites is unknown [22]. In HUVECs, iodixanol density gradients demonstrated that RasGRF cosedimented quantitatively with ER fractions, marked by BiP, with a lesser amount cosedimenting with plasma membrane fractions, marked by caveolin, Rit, and Na-K ATPase (Figure 4A). Further, knockdown of either RasGRF1 or RasGRF2 alone markedly reduced Ras activation by tunicamycin (Figure 4B). The RasGRF isoforms are known to require homo or heterodimerization for full activation [23], suggesting a possible explanation for the pronounced effect of single isoform knockdowns. In contrast, knockdown of SOS1, known to associate with the plasma membrane, had no effect on ER stress-induced Ras (Figure 4C). Further, a time course study indicated that Ras becomes activated by 8 h of tunicamycin treatment but not at early time points, precluding the involvement of SOS1 at earlier time points (Figure V A). Accordingly, SOS1 knockdown did not significantly affect Ras activation at any time point (Figure V B). Consistent with the activation of plasma membrane Ras by VEGF, SOS1 knockdown completely blocked Ras activation by VEGF (Figure 4C).

As further confirmation that ER-associated Ras GRFs act upstream of the UPR, we found that knockdown of RasGRF1 or RasGRF2 also prevented tunicamycin-induced eIF2α phosphorylation, BiP and CHOP induction, and LC3-I to LC3-II conversion (Figure 5A). In addition, overexpression of Nox4 and its requisite partner $p22^{phox}$ caused Ras activation (Figures 5B and VI A), eIF2α phosphorylation, BiP and CHOP induction, and LC3-I to LC3-II conversion (Figure 5C). Nox4-induced Ras activation, as well as induction of the UPR and autophagy, were blocked by knockdown of RasGRF1 or RasGRF2 (Figure 5B–C), confirming the transduction of Nox4-dependent ER stress signals by RasGRF. In contrast, knockdown of RasGRF1 or RasGRF2 had no effect on VEGF-induced Ras activation (Figure VI B). Together, these data indicate that tunicamycin-induced ER stress triggers

focal activation of Ras through Nox4 and ER-associated RasGRF, leading to downstream initiation of the UPR.

Nox4 triggers Ca^{2+}-induced ER signaling

RasGRF is known to respond to calcium through the binding of calcium-calmodulin to the RasGRF IQ domain, consistent with its calcium-sensitive activation in neuronal cells [24]. We therefore hypothesized that calcium may link Nox4 with Ras activation through RasGRF. Tunicamycin increased cytosolic free calcium in a perinuclear space consistent with an ER distribution (Figure VII A). Increases in cytosolic calcium following treatment with tunicamycin were markedly reduced by Nox4 knockdown as well as ER-targeted catalase (Figure 6A, Figure VII B), confirming that Nox4-dependent H_2O_2 acts upstream of calcium signals and possibly RasGRF activation. Consistent with this scenario, reduction of cytosolic calcium levels using BAPTA-AM blocked tunicamycin-induced activation of Ras (Figure 6B) as well as eIF2α phosphorylation, BiP and CHOP induction, and LC3-I to LC3-II conversion (Figure 6C). Oxidants are known to induce increases in cytosolic free calcium, in large part through cysteine oxidation of ER-associated calcium transporters. Of known oxidant-sensitive calcium transport modulators, very low levels of the ryanodine and IP_3 receptors were found. However, significant amounts of sarcoendoplasmic reticulum calcium ATPase (SERCA) were detected. Using 5-IAF to label reactive (reduced) cysteine residues, tunicamycin was found to markedly increase cysteine oxidation of SERCA (corresponding to reduced 5-IAF labeling) (Figure 6D). Knockdown of Nox4 restored levels of reduced cysteine, indicating that SERCA is a direct oxidation target of Nox4. Taken together, our data demonstrate that ER stress triggers a Nox4-Ca^{2+}-RasGRF-Ras signaling module on the ER surface which is required to activate an ER stress response through the UPR.

DISCUSSION

In this study, we found a novel basis for the functional coupling of Nox4 and Ras using tunicamycin to study the specific example of ER stress in endothelial cells. We found first that despite the co-occupancy of Nox4 and Ras on different subcellular membrane domains, ROS production and Ras GTP loading colocalize specifically on the ER surface in response to tunicamycin. In support of the general importance for local ROS-Ras coupling, we found that VEGF, known to initiate signaling on the plasma membrane, caused ROS production and Ras activation restricted instead to the plasma and not ER membrane, demonstrating agonist-specific spatial coactivation. Following ER stress, the mechanism of Nox4 activation remains unclear. While expression of this oxidase is clearly inducible, Nox4-dependent increases in ROS production have been shown to occur within minutes in response to angiotensin II, insulin, VEGF, arachidonic acid, and mechanical stress [25–28]. Thus activation of a latent pool of ER-associated Nox4 is likely to occur, although the precise mechanism for activation remains unknown.

Secondly, our data indicate that the Ras-selective GEFs RasGRF1 and RasGRF2 activate ER signaling upstream of Ras. These GEFs are predominantly associated with the ER and nuclear envelope, with some plasma membrane association, and are excluded from the Golgi [22]. RasGRF1/2 are best known as regulators of neuronal differentiation and synaptic

plasticity during memory consolidation, and are activated in association with calcium transients following synaptic receptor activation [24, 29–31]. More recently, participation of RasGRF1/2 in T-cell activation has also been described [32, 33]. However, despite their strong ER localization, these RasGRFs have not previously been demonstrated to participate in ER signaling. Our data suggest that their location on the ER endomembrane and their sensitivity to calcium signals confer unique properties to rapidly and specifically respond to ER stress, a function not previously assigned to the RasGRFs.

The activation of specific GEFs targeted to different subcellular domains is an important cellular strategy to compartmentalize Ras activation, which in turn discriminates downstream signaling events. Differential targeting of the Ras GEFs Ste6 and Efc25, for instance, distinguishes Ras involvement in yeast mating versus morphogenic signaling [34], and Golgi-restricted RasGRP1 directs Ras signaling to the Golgi apparatus of T-cells as opposed to the plasma membrane [35]. Our data suggest that the initiation of ER stress signaling requires a similar arrangement whereby ER-localized RasGRF confines Ras activation to the ER surface and assures an appropriate ER-based response.

Finally, we found that the role of Nox4 in this cascade appears to be the triggering of calcium signals that are required for RasGRF activation. The initiation of calcium signals and ROS production are now known to be tightly linked, as all major calcium-handling molecular complexes are redox-sensitive [36]. Our studies indicate that Nox4 targets SERCA in response to ER stress. Cysteine oxidation of SERCA, which constitutively pumps calcium from the cytosol into the ER, inhibits its activity and raises cytosolic calcium. Besides SERCA, it is possible that other proteins which regulate transport of calcium across the ER membrane are targeted by Nox4-derived H_2O_2.

From an evolutionary standpoint, genes encoding Nox and Ras arose together in simple eukaryotes to control overlapping signaling pathways, particularly those which respond to environmental stresses. Following nutrient deprivation, for instance, both filamentous fungi and slime molds form spore-bearing fruiting bodies, a process requiring both Nox and Ras genes [37–41]. Likewise, penetration of rice plants by the filamentous fungus *Magnaporthe grisea* requires coactivation of both Ras and Nox proteins within a specialized appendage, the appressorium [42, 43]. Recently, an ancient Nox protein, Yno1p, was found in yeast cells to be quantitatively associated with the ER and to relay mitochondrial stress signals in collaboration with Ras [44]. Therefore, the cooperation we observe between Ras and Nox4 in ER stress signaling may exemplify a conserved interaction between these two spatiotemporally co-regulated protein families in stress response pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

Nox	NADPH oxidase
GRF	Guanine nucleotide releasing factors
HUVEC	Human umbilical vein endothelial cells
ROS	Reactive oxidant species
SERCA	Sarcoendoplasmic reticulum calcium ATPase
UPR	Unfolded protein response

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HIGHLIGHTS

- ER stress caused by tunicamycin initiates a calcium signal downstream from Nox4.
- RasGRF1/2 is an ER resident, calcium-dependent Ras GEF which is activated by ER stress.
- RasGRF1/2 activates Ras on the ER surface, causing local coupling of ROS and Ras on the ER surface.

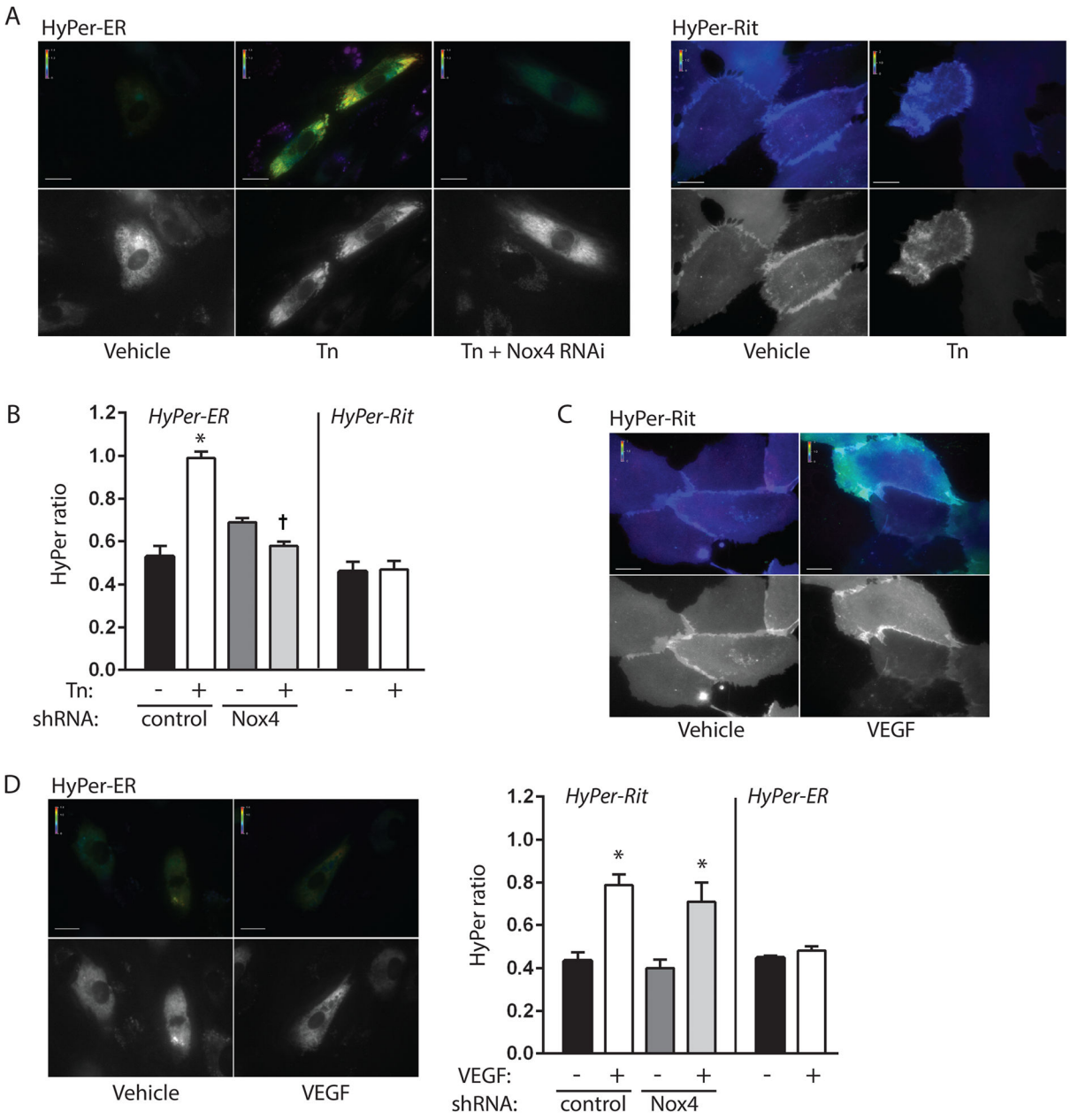

Figure 1. Focal production of ROS by tunicamycin and VEGF

A. HUVEC expressing control or Nox4 shRNA were transduced with HyPer-ER and exposed to vehicle (DMSO) or tunicamycin (Tn, 10 μg/ml) for 16 h. Pseudocolored ratiometric images for excitation wavelengths 492/405 nm are shown in top panels. Grayscale images at bottom show 405 nm excitation images to document HyPer-ER expression. B. Quantification of HyPer intensity ratios for indicated conditions. *P<0.001 compared to vehicle with control shRNA, † P<0.001 compared to Tn with control shRNA, mean ± SEM of 18–39 determinations. C, D. Ratiometric images of HUVEC expressing HyPer-Rit (C) or HyPer-ER (D) and stimulated with saline or VEGF (50 U/ml) for 3–10 min. Bar graphs in D show quantification of HyPer intensity ratios for indicated conditions.

*P<0.01 compared to vehicle with corresponding control or Nox4 shRNA, mean ± SEM of 11–20 determinations. All scale bars are 20 μm.

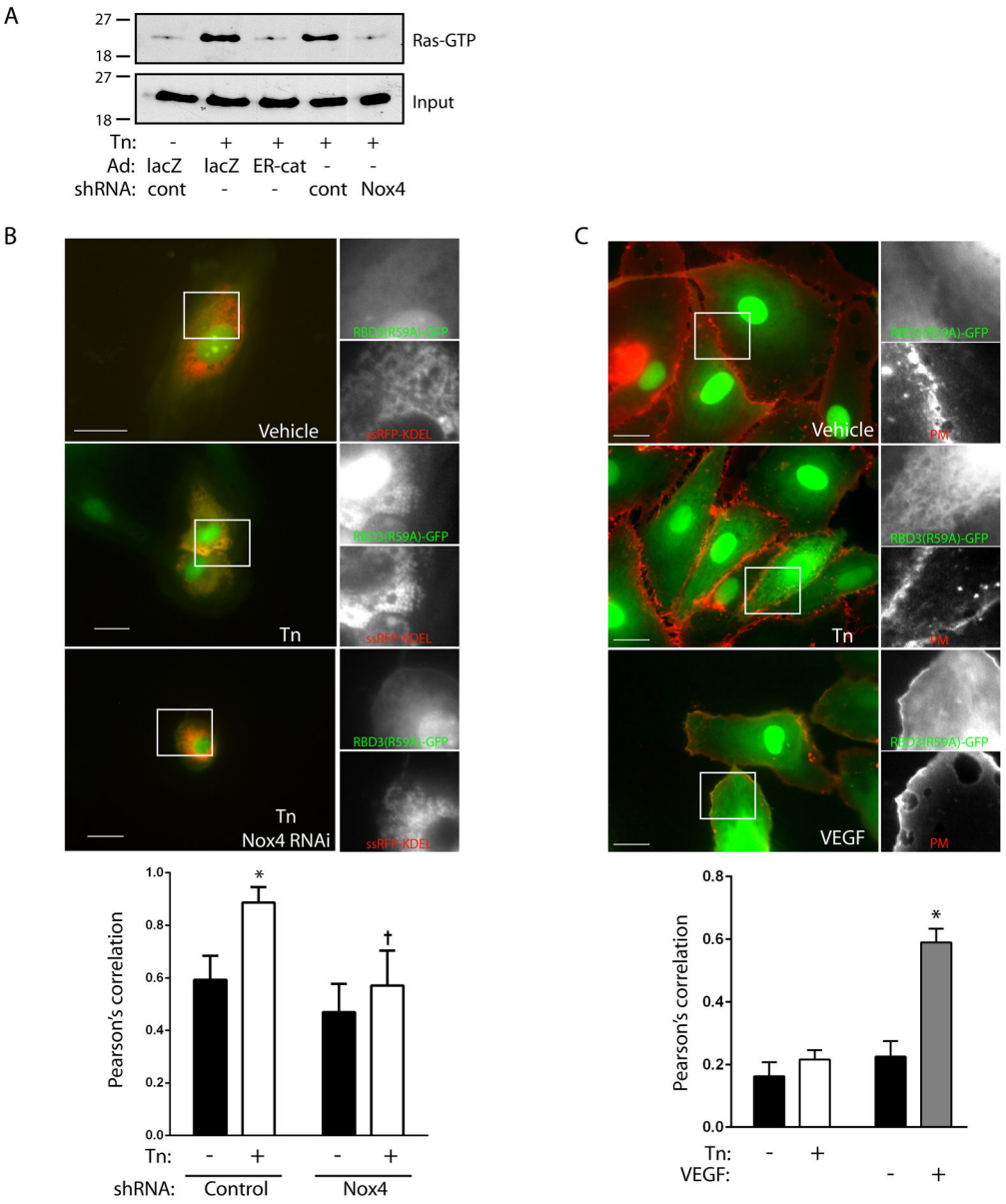

Figure 2. Ras activation by tunicamycin is restricted to the ER

A. HUVEC expressing control or Nox4-targeted shRNA were transduced with lacZ or ER-targeted catalase (ER-cat) and treated with tunicamycin (10 μg/ml) for 16 h. Ras activity was assessed by pulldown, with input shown in lower panel. Representative of two separate experiments. B. Representative photomicrographs of cells cotransfected with RBD3(R59A)-GFP and the ER marker ssRFP-KDEL, under the conditions as labeled. Inset shows magnification of green and red channels separately. Quantification of colocalization for the red and green channels for whole-cell regions of interest are shown in bar graph below. *P<0.001 compared to vehicle with control shRNA, † P<0.001 compared to Tn with control shRNA, mean ± SEM of 9 fields. C. Cells were transduced with RBD3(R59A)-GFP (green channel), stimulated with Tn (10 μg/ml, 16 h) or VEGF (50 U/ml, 5 min), and plasma membranes stained (PM, red channel). Bar graph shows quantification of red/green

colocalization for membrane regions of interest. *P<0.001 compared to vehicle. Mean ± SEM of 15–20 determinations. All scale bars are 20 μm.

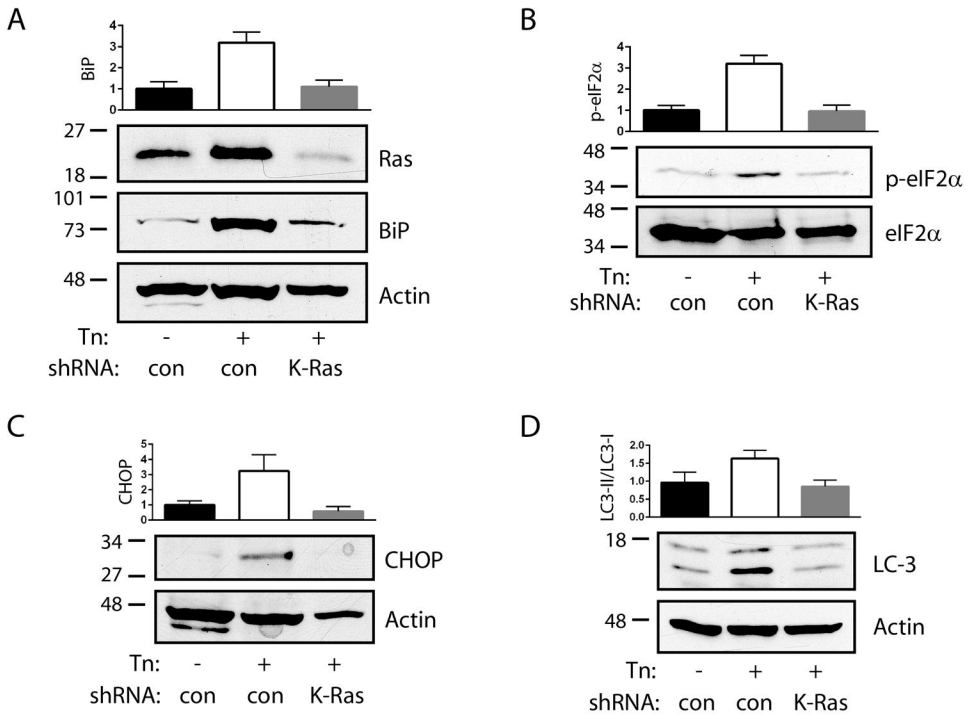

Figure 3. K-Ras mediates the UPR

A. Cells expressing control or K-Ras shRNA were stimulated with tunicamycin (Tn, 10 μg/ml for 16 h). Ras and BiP protein expression are shown. K-Ras knockdown was 85% efficient. Bar graph shows BiP levels, mean ± SEM of 3 determinations. B–D. Cells expressing control or K-Ras shRNAs were stimulated with Tn and eIF2α phosphorylation (B), CHOP (C), and LC3-I (upper band, D) to LC3-II (lower band, D) conversion were determined. Bar graphs show mean ± SEM of 3 determinations.

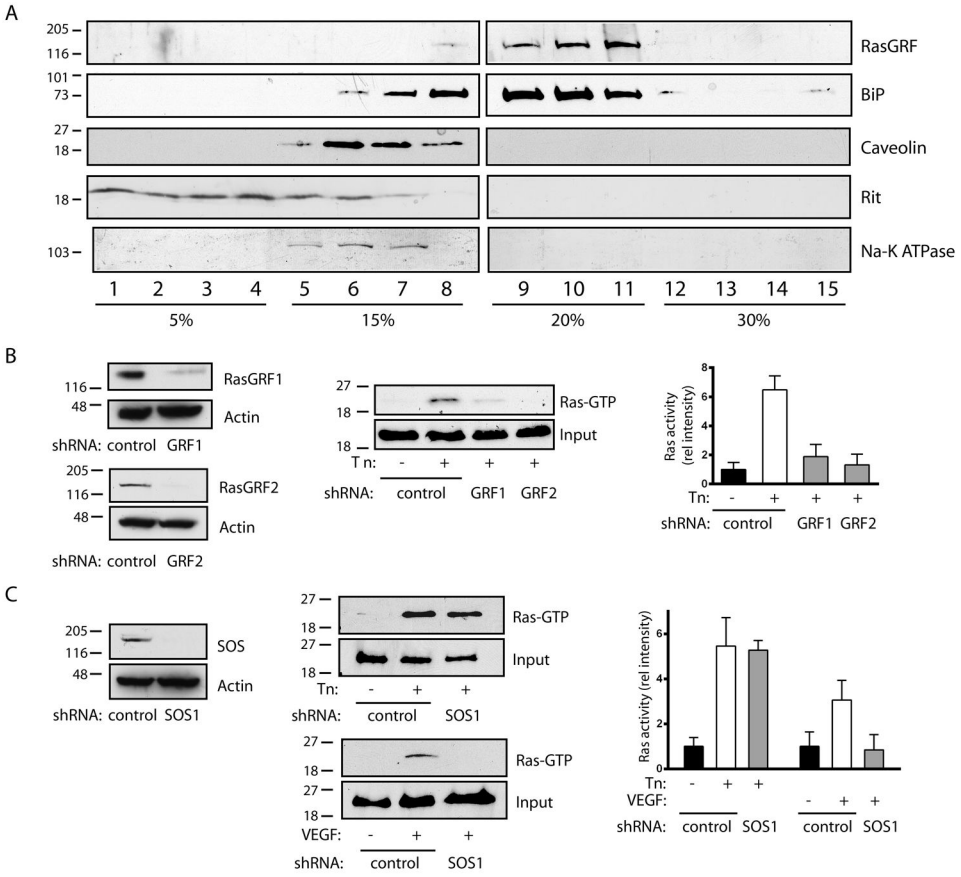

Figure 4. RasGRF is required for tunicamycin-induced Ras activation

A. HUVEC were subjected to discontinuous iodixanol gradient fractionation. RasGRF cosedimented with ER fractions (9–11, marked by BiP), and not plasma membrane fractions (5–7, marked by caveolin, Rit, and Na-K ATPase). B. Effect of shRNAs against RasGRF1 and RasGRF2 on protein expression is shown in left panels. Cells expressing control or RasGRF shRNAs were stimulated with Tn (10 μg/ml for 16 h) and assessed for Ras activity. Representative pulldowns in center panel, graph at right shows Ras activity, mean ± SEM of 3 determinations. Knockdowns were 77% (RasGRF1) and 93% (RasGRF2) efficient. C. Effect of shRNA against SOS1 on protein expression shown in left panel. Cells expressing control or SOS1 shRNA were stimulated with Tn (10 μg/ml for 16 h) or VEGF (50 U/ml, 5 min) and assessed for Ras activity. Representative pulldowns in center panel, graph at right shows Ras activity mean ± SEM of 3 determinations. SOS1 knockdown was 98% efficient.

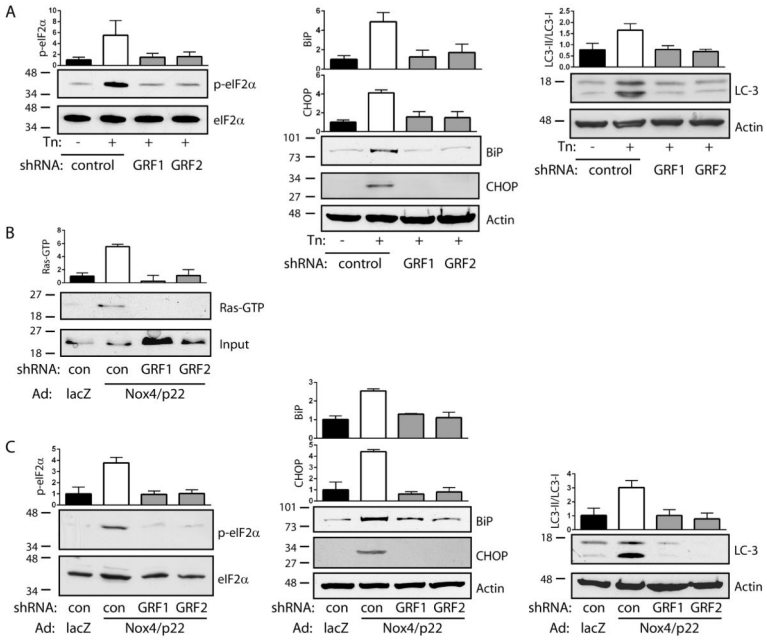

Figure 5. RasGRF mediates Nox4-dependent ER stress signaling

A. Cells expressing control, RasGRF1, or RasGRF2 shRNAs were stimulated with Tn (10 μg/ml for 16 h) and eIF2α phosphorylation (left), BiP and CHOP expression (center), and LC3-I to LC3-II conversion (right) were determined. Bar graphs are mean ± SEM of 3 determinations. B. Cells expressing control, RasGRF1, or RasGRF2 shRNAs were transduced with lacZ or Nox4/p22^{phox} and Ras activity was assessed by pulldown. Bar graph is mean ± SEM of 3 determinations. C. Cells were treated as in (B) and eIF2α phosphorylation (left), BiP and CHOP expression (center), and LC3-I to LC3II conversion (right) were determined. Bar graphs are mean ± SEM of 3–4 determinations.

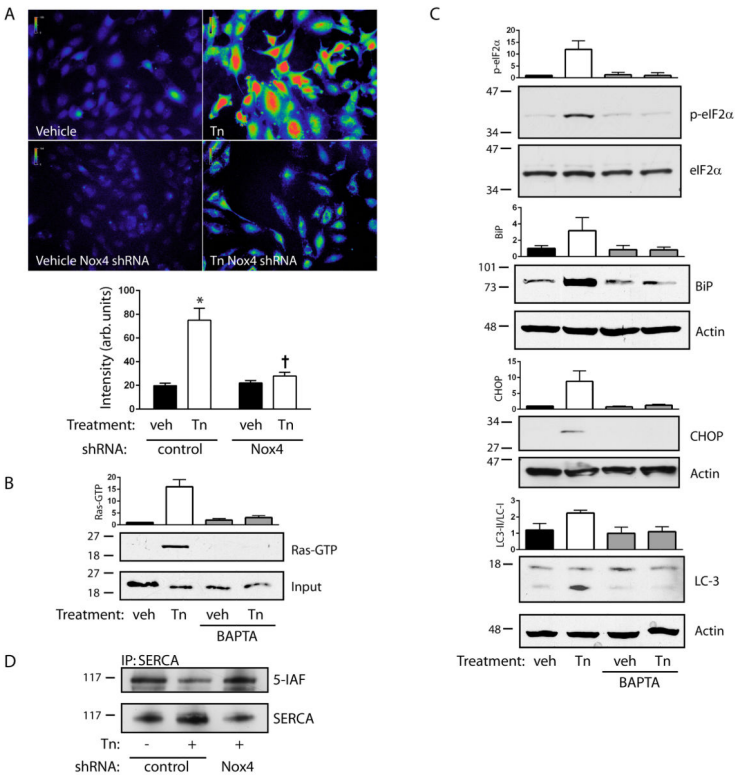

Figure 6. Calcium mediates Ras-dependent ER signaling

A. Fluo-3-AM fluorescence intensities were measured following treatment of control or Nox4 shRNA-expressing cells with Tn (10 μg/ml, 16 h). Top panels show representative pseudocolored Fluo-3-AM images, bar graph below shows mean ± SEM of 50 determinations; *P<0.001 compared to vehicle with control shRNA, † P<0.001 compared to Tn with control shRNA. B. Cells were preloaded with BAPTA-AM and treated with Tn. Ras activity was assessed by pulldown. Bar graph is mean ± SEM of 3 determinations. C. Cells were treated as in (B) and eIF2α phosphorylation, BiP and CHOP expression, and LC3-I to LC3-II conversion were determined. Bar graphs are mean ± SEM of 3 determinations. D. Cells expressing control or Nox4 shRNA were treated with Tn and reactive cysteines were alkylated with 5-iodoacetamidofluorescein (5-IAF). Lysates were immunoprecipitated for SERCA and immunoblotted for either SERCA or fluorescein.