Supplementary Figure 1 Effect of BFA and other compounds on ARF4 and ARF5 knockdown cells. (a) ARF4 knockdown validation of PANC1 and A549 cells infected with ARF4- or control-hairpins. Blots are representative of two or more independent experiments. (b) U251 cells lentivirally-transduced with control or ARF4 shRNAs were seeded in 96-well plates and treated for 3 days with or without indicated drugs before assessing cell viability using the CTG assay. ARF4 KD cells are significantly protected against BFA and GCA but not against TM, TG, or A23187 compared with control cells. 12 wells were measured of each genotype, p<10⁻¹³ for both shARF4 hairpins compared to control hairpins (student's t-test). Drug concentrations used and abbreviations are: 30 ng/mL Brefeldin A (BFA), 3 μM Golgicide A (GCA), 500 ng/mL Tunicamycin (TM), 5 nM Thapsigargin (TG) and 1 μg/mL A23187. Two independent experiments were performed. (c) ARF4 or ARF5 depletion in HeLa cells causes resistance and hypersensitivity to BFA, respectively. Cells were left untreated or treated for 3 days with 7.5 ng/mL BFA, and the survival ratio was calculated by counting the number of surviving cells in the presence and absence of BFA. It is likely that the ARF4 antibody used for Western blotting also weakly recognizes ARF5, which is 90% identical to ARF4 (full length ARF4 was used as immunogen for antibody production) as there seems to be a slight ARF4 signal reduction upon ARF5 depletion in this particular cell line. Four wells for the control and shARF4 hairpins were measured and two wells for the individual shARF5 hairpins, p<0.0001 for both ARF4 shRNAs (student's 2-tailed t-test). (d) KD of ARF5 in PANC1 cells makes cells more BFA-sensitive similar as in (c). Treatment duration was 4 days and 30 ng/mL BFA was used. Viability score was calculated as in (c), n=4 wells for controls and n=2 wells for each shARF5 hairpin.
Supplementary Figure 2 Protection against BFA treatment upon loss of ARF4. (a, b) Golgi morphology is preserved in ARF4-depleted HeLa cells but not control cells upon treatment with low BFA concentrations. Shown are representative (two independent experiments) IF images of cells stained for the cis-Golgi marker GM130 and DNA (Hoechst). BFA treatment disrupts the Golgi complex in control but not in ARF4 KD cells. (a) Cells left untreated, and (b) cells treated for one hour with 40 ng/mL BFA. (c) Pulse/chase labeling experiment following MHC class I receptor trafficking (revealed by immunoprecipitation with the W6/32 antibody) with or without BFA treatment demonstrates that a functional secretory pathway persists in ARF4 KD HeLa cells despite the presence of 50 ng/mL BFA. (d) Short term treatment of A549 cells transduced with indicated hairpins with a high (1 μg/mL) BFA dose disperses the Golgi both in control and ARF4 KD cells. Experiment was done twice.
Supplementary Figure 3 Co-knockdowns of ARF4 and ARF5 or ARF3 and their effects on survival following BFA treatment. (a) ARF4 ARF5 co-depletion makes A549 cells less resistant to BFA in comparison to DKD controls. Cells were treated for 3 days with 20 ng/mL BFA or left untreated, and viable cells were counted in both conditions to calculate the survival ratios. Two wells for each DKD combination and condition were tested. (b) A549 cells simultaneously depleted for ARF3 and ARF4 display the ARF4 single KD phenotype indicating a negligible role of ARF3 for BFA resistance upon loss of ARF4. Two wells of each DKD combination and condition were measured. Viability was calculated as in (a). (a, b) Western blotting confirms KD of the respective ARFs in the DKD cells.
Supplementary Figure 4 Effect of Golgi stress treatments and CREB3 expression on ARFs and ARF-GEFs. (a) Shown are quantitative real-time PCR results of BFA treated and vehicle treated cells (treatment duration was 29 hours, and cells were either vehicle-treated or with 20 ng/mL BFA). P-values (student’s 2-tailed t-test) are as follows: for GBF1, p<0.01 and p<0.004 (A549 and HeLa, respectively); BIG1, p<0.05 and p<0.009 (A549 and HeLa, respectively); BIG2, p<0.001 and p<0.004 (A549 and HeLa, respectively). RNA of three wells per genotype and condition was extracted, and two Q real-time PCR reactions were run simultaneously (technical replicates) to derive the average value for the individual genes. (b) HeLa and A549 cells were co-treated with BFA and two different doses of the protein synthesis inhibitor Cycloheximide (CHX) for 29 hours. The experiment was performed twice. (c) Stable Flag-CREB3 and control (Flag-γTubulin) protein-expressing HeLa cells were left untreated or treated for 24 hours with 10 ng/mL BFA before cell lysis, and the lysates tested for ARF isoforms and ER stress marker expression by immunoblotting. Blots are representative of two independent experiments. (d) A549 cells infected with control or CREB3 hairpins were treated for 24 hours with several Golgi stressors and the lysates probed with the indicated antibodies after SDS-PAGE. Experiment was done twice. Note the induction of ARF1 following CREB3 downregulation.
**Supplementary Figure 5** Involvement and regulation of CREB3 and S1P in Golgi stress-induced ARF4 induction. (a) Lentivirally-transduced HeLa cells stably expressing Flag-CREB3 were left untreated or treated for 23 hours with 20 ng/mL BFA, 5 μM GCA, 75 μM Exo1 or 10 μM Monensin before fixation and immunofluorescent staining to assess CREB3 localization and Golgi morphology. Shown are images of two representative experiments. (b) Simultaneous treatment of A549 cells with 10 ng/mL BFA for 28 hours and either of two different serine protease inhibitors (AEBSF or PF-429242) mitigates ARF4 upregulation in a dose-dependent manner. Western blots with independent samples were repeated twice. (c) Pharmacological inhibition of S1P in the presence of several Golgi stress agents decreases ARF4 expression compared with BFA only-treated HeLa cells. Treatment duration was 29 hours and PF-429242 was used at 10 μM. The experiment was done twice.
Supplementary Figure 6 Resistance of ARF4 KD cells to C. trachomatis and S. flexneri is mimicked by CREB3 loss of function and depends on ARF5 and GBF1. (a) HeLa cells transduced with lentiviral control or ARF4 hairpins were infected with C. trachomatis, and cells were lysed 30 or 36 hours after infection. Blots (representative of two independent experiments) were probed with indicated antibodies. Ctr. HSP60 refers to C. trachomatis HSP60. (b) HeLa ARF4 KD and control cells were infected with Chlamydia, and 30 hours post-infection cells were fixed and stained for Chlamydia HSP60 (red), GM130 (green) and DNA (Hoechst; blue). Arrows point to Chlamydia-infected cells and the effects on the Golgi apparatus. Representative images of two independent experiments are presented. (c) HeLa DKD cells were infected with C. trachomatis and bacterial progeny tested for their ability to form inclusions in wild type HeLa cells (48 hours post-infection). Statistically significant differences (p<0.05) using one-way ANOVA were observed between ARF4 DKD control cells and ARF4 GBF1 DKD cells; four wells were measured per genotype. The graphs show the mean IFUs/well ± S.D. (d) Immunoblots of cell lysates from HeLa DKD cells used in (c). Two independent experiments were performed yielding similar results. (e) Loss of CREB3 impairs S. flexneri growth (gentamicin protection assay was done 20 hours post-infection) and inhibits C. trachomatis reproduction (analysis done 40 hours post-infection). Both experiments have been repeated twice with 3-4 plates of each genotype, p≤0.05 using Student’s t-test or one-way ANOVA test. Displayed are the mean values ± S.D. (f) Western blot analysis of HeLa CREB3 KD cells used for S. flexneri and C. trachomatis infection as shown in (e). Cell lysates were derived from both untreated cells and cells treated with 10 ng/mL BFA for 27 hours.
Supplementary Figure 7 Knockdown of ARF1 or GBF1 causes CREB3 translocation into the nucleus. (a) A549 cells infected with shLUC, shARF1 or shGBF1 lentiviral hairpins were transfected with Flag-CREB3 cDNA to assess CREB3 subcellular localization. BFA treatment of control cells overexpressing CREB3 led to nuclear enrichment of CREB3 thereby validating the transient transfection assay. ShLUC control cells were left untreated or treated with 20 ng/mL BFA for 24 hours, and the remaining images reflect untreated conditions. The Golgi apparatus was visualized with GM130 and nuclei were stained with Hoechst. (b) Assessment of Golgi morphology and KD validation of GBF1 KD cells using anti-GBF1 antibody. (a, b) IF images are representative of two independent experiments.
Supplementary Figure 8 continued
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