

Hypoxia reduces endoplasmic reticulum (ER)-to-Golgi protein trafficking and increases cell death by inhibiting the adaptive unfolded protein response in mouse beta cells

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Abstract

Aims/hypothesis Hypoxia may contribute to beta cell failure in type 2 diabetes and islet transplantation. The adaptive unfolded protein response (UPR) is required for endoplasmic reticulum (ER) homeostasis. Here we investigated whether or not hypoxia regulates the UPR in beta cells and the role the adaptive UPR plays during hypoxic stress.

Methods Mouse islets and MIN6 cells were exposed to various O₂ tensions. *Ddit3*, *Hif1α* and *Hspa5* were inhibited using siRNA. *Hspa5* was overexpressed. *db/db* mice were used.

Results Hypoxia-response genes were upregulated *in vivo* in the islets of diabetic, but not prediabetic, *db/db* mice. In isolated mouse islets and MIN6 cells, O₂ deprivation (1-5% vs 20%; 4-24h) markedly reduced the expression of adaptive UPR genes, including *Hspa5*, *Hsp90b1*, *Fkbp11* and spliced *Xbp1*. Coatamer protein complex genes (*Copa*, *Cope*, *Copg*, *Copz1* and *Copz2*) and ER-to-Golgi protein trafficking were also reduced, whereas apoptotic genes (*Ddit3*, *Atf3* and *Trb3*), JNK phosphorylation and cell death were increased. Inhibition of JNK, but not *Hif1α*, restored adaptive UPR gene expression and ER-to-Golgi protein trafficking while protecting against apoptotic genes and cell death following hypoxia. *Ddit3* inhibition delayed the loss of the adaptive UPR and partially protected against hypoxia-induced cell death. The latter response was prevented by *Hspa5* inhibition. Finally, *Hspa5* overexpression significantly protected against hypoxia-induced cell death.

Conclusions/interpretation Hypoxia inhibits the adaptive UPR in beta cells via JNK and DDIT3 activation, but independently of *Hif1α*. Downregulation of the adaptive UPR contributes to reduced ER-to-Golgi protein trafficking and increased beta cell death during hypoxic stress.

Keywords

Apoptosis

Beta cell

Hypoxia

Islet of Langerhans

Protein trafficking

Type 2 diabetes

Unfolded protein response

Abbreviations

ATF Activating transcription factor

Cop Coatamer protein complex

DDIT3 DNA-damage inducible transcript 3

Dnajc3 DnaJ (Hsp40) homolog, subfamily C, member 3

EIF2A Eukaryotic translation initiation factor 2 α

ER Endoplasmic reticulum

Fkbp11 FK506 binding protein 11

JNK c-Jun N-terminal kinase

Hif1 α Hypoxia-inducible transcription factor 1 α

HSPA5 Heat shock 70kDa protein 5

HSP90B1 Heat shock 90kDa protein beta

ISR Integrated stress response

PERK PKR-like ER kinase

UPR Unfolded protein response

VSVG Vesicular stomatitis viral glycoprotein

XBP1 X-box binding protein 1

Introduction

Chronic exposure to elevated glucose levels exerts toxic effects in beta cells, a concept known as glucotoxicity [1]. Glucotoxicity contributes to the deterioration of functional beta cell mass in type 2 diabetes. However, the precise mechanisms involved are not understood.

Hypoxia occurs when demand for O₂ exceeds supply leading to a drop in tissue pO₂. Beta cells are sensitive to hypoxic stress because their function depends heavily on the acceleration of mitochondrial metabolism upon glucose stimulation to generate ATP [2]. Glucose stimulation of beta cell oxygen consumption generates intracellular hypoxia leading to activation of hypoxia-inducible factors (HIFs) and upregulation of HIF-target genes in rodent and human islets (reviewed in [1]). The presence of hypoxia *in vivo* in the islets of animal models of type 2 diabetes [3-8] supports a role for hypoxic stress in beta cell apoptosis. Moreover, severe hypoxia in islet grafts contributes to beta cell apoptosis in the early post-transplantation period [9;10]. However, the mechanisms underlying the deleterious effects of hypoxia in beta cells are unclear.

The endoplasmic reticulum (ER) is highly active in beta cells due to heavy engagement in the synthesis, processing and secretion of insulin. This function relies on a finely tuned adaptive unfolded protein response (UPR) [11]. The accumulation of unfolded proteins (ER stress), occurring as a result of an imbalance between ER folding capacity and demand, triggers the UPR via three main sensors, namely PKR-like ER kinase (PERK), inositol requiring kinase 1 (IRE1) and activating transcription factor (ATF) 6. Activation of PERK and the ensuing phosphorylation of eukaryotic translation initiation factor 2 (EIF2A) leads to rapid and transient reduction in global protein synthesis to attenuate ER load. IRE1 triggers the unconventional splicing of *Xbp1* mRNA generating X-box binding protein 1 (XBP1s) transcription factor. XBP1s, together with ATF6, stimulates the expression of genes that enhance ER capacity as well as the degradation of unfolded proteins. However, irresolvable

ER stress triggers apoptosis via several effectors including JNK, *Ddit3* (also known as *Chop/Gadd153*), *Atf3* and *Trb3* [11-13]. Interestingly, recent studies suggest that a progressive decline in the adaptive UPR characterises beta cell failure in type 2 diabetes [14-16] as well as in islet transplantation [17]. Therefore, knowledge of the regulatory mechanisms controlling the adaptive UPR is crucial.

In the present study, we demonstrate for the first time that hypoxia downregulates the adaptive UPR specifically in beta cells. We show that inhibition of the adaptive UPR is associated with reduced ER-to-Golgi protein trafficking and beta cell death during hypoxic stress. Mechanistically, we show that hypoxia mediates these responses via JNK and DDIT3 activation, but independently of *Hif1α*. Our novel findings suggest that hypoxia may provide a cellular mechanism for failure of the adaptive UPR during the progressive loss of functional beta cell mass in type 2 diabetes.

Methods

Reagents

Control Non-Targeting and ON-TARGETplus SMARTpool siRNAs and transfection reagent DharmaFECT3 were from Thermo Fisher Scientific (Lafayette, CO, USA). JNK inhibitor II SP600125 and NanoJuice transfection reagents were from Merck (Kilsyth, VIC, Australia).

Mice

C57BL/KsJ *db/db* mice and their age-matched lean control mice (C57BL/KsJ) were obtained from the Garvan Institute breeding colony (Australian BioResources, Moss Vale, NSW, Australia). For islet culture [14], 8-10 week-old C57BL/6J mice were taken from the Garvan Institute breeding colony, which was derived from The Jackson Laboratory colony that harbors an *Nnt* mutation (Bar Harbor, ME, USA). Islets were isolated as previously described [14]. Some experiments were performed on a subset of mice used in our previous study [14].

All experiments were approved by the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee.

Cell culture and treatment

MIN6 beta cells (P26-39) and MEF cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 25 mmol/l glucose, 10 mmol/l HEPES, 10% FCS, 50 units/ml penicillin, and 50 µg/ml streptomycin in the presence of normoxia (20% O₂) or hypoxia (1% O₂) using hypoxic chambers (Billups-Rothenberg, Del Mar, CA, USA). Cells were transfected with control, *Ddit3*, *Hif1a* or *Hspa5* siRNA. EGFP or HSPA5 expression vectors [18] were transfected into cells using NanoJuice transfection reagent. JNK inhibitor II (JNKi; 10 µmol/l) was used to inhibit JNK activation. Cell death was determined with the use of a Cell Death Detection ELISA (Roche Diagnostics, Castle Hill, NSW, Australia) [19].

Real-time RT-PCR

Real-time RT-PCR was performed as previously described [14]. Primer sequences are listed in Supplementary Table 1. The value obtained for each specific gene product was normalised to the control gene cyclophilin A and expressed as a fold change of the value in control condition. *Xbp1* mRNA splicing measurement was performed as previously described [19].

Protein analysis

Western blotting and immunostaining were performed as previously described [7;20]. Western blot bands were quantified by densitometry and normalised to total protein or loading control (actin, tubulin or 14-3-3). Antibodies are listed in Supplementary Table 2. For immunofluorescence experiments, images were acquired using Leica SP8 confocal microscope.

Trafficking assay

ER-to-Golgi protein trafficking was assessed using the temperature-sensitive mutant vesicular stomatitis viral glycoprotein (VSVG) assay [21;22]. MIN6 cells seeded on coverslips were

transfected with VSVG protein tagged with green fluorescent protein (GFP). Cells were cultured in the presence of 20% or 1% O₂ for 24h at 40°C to retain the VSVG in the ER. After addition of cycloheximide (20 µmol/l) to prevent novel VSVG synthesis, trafficking was commenced by transfer to the permissive temperature of 32°C. VSVG localisation was scored using confocal images according to the scale: 1, exclusively ER; 2, mostly ER with some Golgi; 3, equivalent ER and Golgi; 4, mostly Golgi with some ER; 5 exclusively Golgi [22].

Statistical analysis

Results are means±SEM for the indicated number of experiments. Statistical significance was assessed by unpaired two-tailed student t-test, one-way ANOVA and a post-test of Newman-Keuls or two-way ANOVA and a post-test of Bonferroni.

Results

Hypoxia-response genes are upregulated in the islets of diabetic, but not in prediabetic, db/db mice In *db/db* mice, blood glucose levels were increased at 16 weeks (average blood glucose levels: control 8.1 ± 0.5 , $n=9$, *db/db* 26.0 ± 1.8 mmol/l, $n=7$; $p<0.001$) but not at 6 weeks of age (control 8.5 ± 0.2 , $n=16$, *db/db* 9.1 ± 0.3 mmol/l, $n=8$; NS) compared to age-matched lean C57BL/KsJ control mice [14]. The mRNA levels of hypoxia-response genes *Adm*, *Tpi1*, *Gapdh* and *Eno1* were markedly upregulated *in vivo* in the islets of diabetic *db/db* mice in comparison to age-matched control mice (Fig. 1a-d). In contrast, there were no significant changes in hypoxia-response genes in the islets of prediabetic *db/db* mice (Fig. 1a-d). We have previously demonstrated the time-dependent downregulation of UPR genes important for adaptation to ER stress in the islets of diabetic *db/db* mice [14]. Taken together, our studies demonstrate a novel *in vivo* association between islet hypoxia and the failure of the adaptive UPR in diabetic mice.

Hypoxia inhibits adaptive UPR gene expression in beta cells MIN6 cells were treated with normoxia (20% O₂) or hypoxia (1% O₂) for 24h. Hypoxia markedly reduced *Xbp1* mRNA splicing as well as the mRNA levels of adaptive UPR genes, including chaperones *Hspa5* (*Bip*) and *Hsp90b1* (*Grp94*) and the peptidyl prolyl isomerase *Fkbp11* (Table 1). In contrast, hypoxia strongly upregulated the mRNA levels of apoptotic genes *Ddit3*, *Atf3* and *Trb3* (Table 1). As expected, hypoxia strongly upregulated the mRNA levels of HIF-target genes *Adm*, *Aldoa*, *Hmox1* and *Tpi1* (Table 1). In agreement with these findings, hypoxia reduced the protein levels of XBP1s by more than 90%, whereas p-IRE1A was unchanged (Fig. 2a,b). In addition, the protein levels of full-length and cleaved ATF6 were reduced by about 20 and 50%, respectively (Fig. 2a,b). Furthermore, the protein levels of heat shock 70kDa protein 5 (HSPA5) and heat shock 90kDa protein beta (HSP90B1) were reduced by more than 50% and 70%, respectively (Fig. 2a,b). Moreover, hypoxia significantly reduced PERK phosphorylation, whereas EIF2A phosphorylation was increased (Fig. 2a,c) suggesting possible activation of the integrated stress response (ISR) and an upstream EIF2A kinase other than PERK [23]. We therefore checked phosphorylation of double stranded RNA-dependent protein kinase (PKR) and found it was increased by hypoxia (Fig. 2a,c). Finally, hypoxia markedly increased proapoptotic effectors including DNA-damage inducible transcript 3 (DDIT3) and cleaved caspase 3 protein levels and JNK1/2 phosphorylation in association with increased cell death (Fig. 2a,c,d).

The effects of hypoxia on the adaptive UPR were confirmed in isolated primary mouse islets. Hypoxia (1% O₂ for 12h) significantly downregulated *Fkbp11*, the chaperone *Dnajc3* (*p58^{IPK}*) and the co-chaperone *Dnajb9*, and tended to reduce *Hspa5* and the protein disulfide isomerase *Pdia4* (*Erp72*) (Table 2). In contrast, hypoxia strongly upregulated the mRNA levels of apoptotic genes *Ddit3*, *Atf3* and *Trb3* as well as HIF-target genes in mouse islets (Table 2). Analysis of dispersed mouse islets exposed to hypoxia for 24h revealed a marked

downregulation of KDEL-containing ER proteins in beta cells (Fig. 3). The anti-KDEL antibody detects HSPA5, HSP90B1 and PDIA6 (ESM. 1).

To assess whether the effects of hypoxia on the UPR in beta cells were cell-specific, we performed studies using MEF cells (exposed to 20% or 1% O₂ for 24h). We found that, in opposition to findings in beta cells, hypoxia significantly increased the mRNA levels of adaptive UPR genes *Hspa5*, *Hsp90b1*, *Fkbp11* and *Pdia4* in MEF cells (ESM. 2b). Apoptotic and HIF-target genes were also increased (ESM. 2a,c). These findings suggest that hypoxia activates canonical ER stress response pathways in MEF cells and that its inhibitory effects on the adaptive UPR are specific in beta cells.

Hypoxia time-dependently inhibits the adaptive UPR without transient activation in beta cells

Hypoxia time-dependently decreased the mRNA levels of adaptive UPR genes without detection of transient activation (Fig. 4a-d). On the other hand, hypoxia time-dependently increased the mRNA levels of *Ddit3* (Fig. 4e). In association with the changes in the adaptive UPR, XBP1s protein levels were strongly and rapidly reduced (Fig. 4f and ESM. 3). ATF6c protein levels were also downregulated with time (Fig. 4f and ESM. 3), whereas EIF2A phosphorylation and cleaved caspase 3 and DDIT3 protein levels were progressively increased (Fig. 4f and ESM. 3).

Hypoxia reduces coatamer complex gene expression and ER-to-Golgi protein trafficking

UPR coordination of the secretory pathway and ER-Golgi vesicular transport is fundamental in professional secretory cells [24;25]. In parallel with the attenuation of the adaptive UPR, hypoxia (1% O₂) reduced the mRNA levels of coatamer complex genes *Copa*, *Cope*, *Copg*, *Copz1* and *Copz2* in MIN6 cells and mouse islets (Fig. 5a,b). We also tested whether hypoxia alters ER-to-Golgi protein trafficking using a VSVG reporter assay [26]. The temperature-sensitive VSVG mutant protein is retained in the ER at 40°C (ESM. 4) and translocates to the Golgi after the switch to the permissive temperature of 32°C [21]. Interestingly, in

comparison to the normoxic condition, cells treated with hypoxia (1% O₂) retained substantial VSVG in the ER (Fig. 5c,d), thereby demonstrating that hypoxia delays ER-to-Golgi protein trafficking. ER morphology did not differ in cells treated with normoxia and hypoxia (ESM. 5). The positive control ER stress inducer, tunicamycin, strongly inhibited ER-to-Golgi trafficking of VSVG (Fig. 5c,d). These results demonstrate for the first time that hypoxia impairs ER-to-Golgi protein trafficking in beta cells in association with the downregulation of adaptive UPR and Cop genes. Similar results were obtained using 5% O₂ (ESM. 6 and 7), whereas with 10% O₂, hypoxia response gene and *Ddit3* mRNA levels were only slightly increased and the majority of the adaptive UPR and Cop genes were unchanged, with the exception of slightly reduced *Copz1* mRNA levels (ESM. 8 and 9).

Hypoxia downregulates the adaptive UPR independently of HIF1A activation Hypoxia triggers a transcriptional program including gene repression via the stabilisation of HIF1/2A [27;28]. We therefore examined the role of HIF1A in hypoxia-mediated downregulation of adaptive UPR. Inhibition of *Hif1α* using siRNA prevented the upregulation of HIF-target genes after hypoxia (ESM. 10a-d), but did not affect hypoxia-mediated downregulation of adaptive UPR genes *Hspa5*, *Hsp90b1*, *Fkbp11* and *Pdia4* (ESM. 10e-h). On the other hand, *Hif1α* inhibition potentiated the hypoxia-mediated increases in *Ddit3*, *Atf3* and *Trb3* mRNA levels and cell death (ESM. 10i-l). These results show that *Hif1α* activation is not required for the decline of adaptive UPR gene expression during hypoxia.

JNK activity is partially responsible for downregulation of the adaptive UPR, reduced ER-to-Golgi trafficking and increased beta cell death following hypoxia JNK has been implicated in beta cell apoptosis under various stress conditions [19;29-34], but has not previously been investigated in the context of islet hypoxia. Here we tested whether JNK activity influences beta cell responses to hypoxia. Strikingly, treatment of MIN6 cells with JNKi completely protected against the hypoxia-mediated reductions in *Hspa5* and *Hsp90b1* mRNA levels, and

partially restored *Fkbp11* and *Dnajc3* mRNA levels (Fig. 6a-d). In agreement, inhibition of JNK activity prevented the hypoxia-mediated reduction of KDEL immunostaining (Fig. 6e). These changes were accompanied by the partial normalisation of XBP1s, PKR and EIF2A phosphorylation, DDIT3 and cleaved caspase 3 protein levels (Fig. 6f and ESM. 11) as well as reduced cell death (Fig. 6g). Additionally, JNK inhibition either partially or completely protected against hypoxia-mediated reductions in Cop mRNA levels (Fig. 6h-k) and ER-to-Golgi protein trafficking (Fig. 6l,m). These novel findings indicate that JNK activation is necessary for alterations in the UPR during hypoxic stress.

Ddit3 induction is required for hypoxia-mediated downregulation of the adaptive UPR Since DDIT3 can act as a negative transcriptional regulator [35;36] and its expression was lowered by JNK inhibition (Fig. 6f), we examined the influence of *Ddit3* in the hypoxia-mediated changes in the adaptive UPR. Interestingly, siRNA-mediated inhibition of *Ddit3* expression (Fig. 7a and ESM. 12) upregulated the mRNA levels of adaptive UPR genes *Hspa5*, *Hsp90b1* and *Fkbp11* under normoxia and delayed their downregulation following hypoxia (Fig. 7b-d). This was associated with a partial reduction in hypoxia-induced cell death (Fig. 7e). We then tested whether the protective effect of *Ddit3* inhibition on hypoxia-induced cell death was due to changes in the adaptive UPR using knockdown of both *Ddit3* and *Hspa5*. We found that the protective effect of *Ddit3* inhibition was abolished with the concomitant knockdown of *Hspa5*. These findings demonstrate that downregulation of the adaptive UPR is required for *Ddit3*-dependent cell death during hypoxia (Fig. 7e).

Overexpression of Hspa5 significantly protects against hypoxia-induced beta cell death To further confirm the role of adaptive UPR downregulation in hypoxia-induced beta cell death, we examined the effects of restoring expression of the ER chaperone HSPA5 during hypoxia. HSPA5 overexpression (Fig. 8a,b) did not affect the expression of adaptive UPR components *Hsp90b1* or *Fkbp11* (Fig. 8c,d), but downregulated DDIT3 after hypoxia (Fig. 8a,e). Hypoxia-

induced apoptosis was significantly reduced in HSPA5-overproducing versus control (EGFP-producing) MIN6 cells (Fig. 8f). These data are the first indication that the decline of the adaptive UPR is at least partially required for hypoxia-induced cell death in beta cells.

Discussion

We demonstrate here that hypoxia strongly downregulates the protective adaptive UPR in beta cells. We further show that downregulation of the adaptive UPR is associated with impaired ER-to-Golgi protein trafficking and increased beta cell death during hypoxic stress. These findings indicate for the first time that hypoxia may contribute to the decline of the adaptive UPR in type 2 diabetes islets [14-16] and in islet graft failure [17]. Furthermore, our results provide JNK and DDIT3 activation as molecular mechanisms for downregulation of the adaptive UPR during hypoxic stress.

Aberrant UPR in beta cells during hypoxia Hypoxia may contribute to beta cell dedifferentiation and loss in type 2 diabetes [7;37] and after islet transplantation [9]. In the present study, we demonstrate a novel *in vivo* association between hypoxia and downregulation of the adaptive UPR in islets of diabetic mice. The upregulation of hypoxia response genes solely in the islets of diabetic mice (Fig. 1) is paralleled by reduced expression of a group of adaptive UPR genes (*Hspa5*, *Hsp90b1*, *Pdia4* and *Dnajc3*; averaged mRNA levels were reduced by $39.2 \pm 9.0\%$ in the islets of diabetic compared with prediabetic *db/db* mice, $n=8-12$, $p<0.01$ [14]). Our findings suggest that the deleterious effects of islet hypoxia include downregulation of the adaptive UPR in type 2 diabetes. In non-beta cells (ESM. 2 and [38-40]), hypoxia activates canonical UPR signaling, including increased PERK/EIF2A phosphorylation, *Xbp1* mRNA splicing and upregulation of ER chaperones. The atypical response to hypoxia in beta cells is evidenced by the absence of even transient activation of the adaptive UPR. Perhaps this is the result of an already high basal activity of the adaptive

UPR in specialised secretory beta cells in comparison to non secretory cells. Interestingly, despite the reduction of PERK phosphorylation, PKR and EIF2A phosphorylation and DDIT3 expression were increased (Figs. 2 and 4), suggesting that hypoxia activates the ISR [23;41;42] (Fig. 8g).

Hypoxia impairs ER-to-Golgi protein trafficking in beta cells The ER is the primary compartment of the secretory pathway. After proper folding and posttranslational modifications, cargo proteins undergo subsequent trafficking between the ER-Golgi compartments to receive additional modifications before vesicular transport to the plasma membrane and secretion. Our results clearly demonstrate that hypoxia slowed ER-to-Golgi protein trafficking. Whether defective trafficking is up or downstream of ER stress is not clear. The downregulation of chaperones, co-chaperones and foldases may exacerbate the accumulation of misfolded proteins in the ER leading to reduced ER-to-Golgi trafficking (Fig. 8g). Indeed, tunicamycin, which directly induces ER stress by inhibiting protein glycosylation, was sufficient to cause strong inhibition of ER-to-Golgi trafficking (Fig. 5). Alternatively, under hypoxic conditions, low cellular energetic status or reduced expression of ER-Golgi vesicle transport genes may initiate the trafficking defect, which in turn exacerbates ER stress because of the build-up of protein in the ER lumen (Fig. 8g). Interestingly, hypoxia reduced the mRNA levels of Cop genes (Fig. 5), several of which were also reduced in the islets of diabetic *db/db* mice (ESM. 13). These genes are regulated by XBP1s [24;25] and are correlated with *Xbp1* mRNA splicing following glucose stimulation in beta cells [43]. A recent study showed that impaired Golgi-to-ER retrograde transport can induce beta cell ER stress [44]. Moreover, blockage of ER-to-Golgi protein trafficking causes accumulation of proteins in the ER and thus ER stress in beta cells chronically exposed to saturated fatty acids [21;22;45].

Downregulation of the adaptive UPR links JNK-DDIT3 activation with beta cell apoptosis during hypoxic stress We have identified JNK as a crucial regulator of the UPR and beta cell survival during hypoxia. JNK activation reduces the adaptive UPR, Cop gene expression and ER-to-Golgi trafficking while promoting beta cell apoptosis following hypoxia (Fig. 6). We further show that JNK activation contributes to the downregulation of Cop gene expression in the islets of diabetic *db/db* mice (ESM. 13). Although JNKi is regarded as a specific inhibitor of JNK activation, we cannot rule out the potential of an off target effect. JNK is a well-established stress-activated proapoptotic effector in beta cells [29-34]. It also plays a role in the regulation of the UPR following pro-inflammatory cytokine stimulation [19;46]. Hypoxia-induced JNK activation appears to occur independently of IRE1 (Fig. 2). Interestingly, hypoxia increased the expression of inflammatory genes (ESM 14), raising the possibility that hypoxia activates JNK via inflammatory signalling. Moreover, ISR components (PKR and EIF2A phosphorylation and DDIT3 expression) were reduced by JNK inhibition during hypoxia (Fig. 6). DDIT3 is an established modulator of beta cell apoptosis [47], including that induced by hypoxia (Fig. 7 and [8;37]), an effect that we show was dependent on the decline of adaptive UPR (Fig. 7). HSPA5 overexpression only partially protects against the induction of *Ddit3* and apoptosis during hypoxia (Fig. 8). However, other ER chaperones and protein folding enzymes are downregulated during hypoxia, which may reduce the impact of these factors when addressed individually.

Thus, our results identify a crucial role of DDIT3 downstream of JNK in hypoxia-mediated apoptosis and downregulation of the adaptive UPR. Other effectors must be involved, potentially generated via inflammatory (ESM. 14) and/or metabolic pathways. The UPR during hypoxia resembles the beta cell response to low non-stimulatory glucose concentrations where ATP and ER [Ca^{2+}] levels and protein synthesis are reduced [43;48-50]. However, the inhibitory effect of hypoxia on the adaptive UPR is observed even in the

presence of low glucose concentrations (not shown) suggesting additional mechanisms. Our studies suggest that HIF1A, an important regulator of cell survival and gene expression during hypoxia [27;28;51], is not required for downregulation of the UPR. Finally, by demonstrating that overproduction of the ER chaperone HSPA5 partially protects against hypoxia-induced apoptosis, we provide the first indication that downregulation of the adaptive UPR contributes to hypoxic stress in beta cells, and by extension to beta cell failure in type 2 diabetes.

In conclusion, we have demonstrated for the first time that hypoxia inhibits the adaptive UPR and ER-to-Golgi protein trafficking in beta cells. The decline of the adaptive UPR is associated with increased beta cell death during hypoxia. Hypoxia might therefore contribute to the failure of the adaptive UPR and the progressive loss of functional beta cell mass in type 2 diabetes. The identification of the hypoxia-JNK-DDIT3 axis in adaptive UPR failure may help for the development of therapeutic strategies to protect beta cells in type 2 diabetes and after islet transplantation.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement MB and DRL conceived and designed experiments, acquired and analysed data and wrote the manuscript. ELM, JYC, JL, PKW, JCJ and JEG designed experiments, acquired and analysed data and critically reviewed the manuscript. All authors approved the final version of the manuscript. DRL is the guarantor of this work.

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Table 1. Hypoxia reduced the mRNA levels of adaptive UPR genes in MIN6 cells. MIN6 cells were cultured in the presence of normoxia (20% O₂) or hypoxia (1% O₂) for 24h. mRNA levels are expressed as fold change of the level in control (normoxia). *Xbp1* mRNA splicing is expressed as the ratio of spliced over total mRNA. Unpaired two-tailed student t-test; **p*<0.05, ***p*<0.01, *p*<0.001 vs normoxia. Data are mean ± SEM from 4 independent experiments.

	Normoxia	Hypoxia
HIF-target genes		
<i>Adm</i>	1.0 ± 0.30	119.5 ± 34.2*
<i>Aldoa</i>	1.0 ± 0.07	6.92 ± 0.59***
<i>Tpi1</i>	1.0 ± 0.10	6.39 ± 0.09***
<i>Hmox1</i>	1.0 ± 0.09	5.07 ± 1.39*
Adaptive UPR genes		
<i>Xbp1s/t</i>	0.47 ± 0.01	0.31 ± 0.02***
<i>Hspa5 (Bip)</i>	1.0 ± 0.07	0.67 ± 0.06*
<i>Hsp90b1 (Grp94)</i>	1.0 ± 0.06	0.53 ± 0.02***
<i>Fkbp11</i>	1.0 ± 0.06	0.17 ± 0.01***
Apoptotic genes		
<i>Ddit3 (Chop)</i>	1.0 ± 0.21	17.85 ± 4.42**
<i>Atf3</i>	1.0 ± 0.17	10.36 ± 3.16*
<i>Trb3</i>	1.0 ± 0.06	8.72 ± 1.66**

Table 2. Hypoxia reduced the mRNA levels of adaptive UPR genes in mouse islets. Isolated mouse islets were cultured in the presence of normoxia (20% O₂) or hypoxia (1% O₂) for 12h. mRNA levels are expressed as fold change of the level in control (normoxia). Unpaired two-tailed student t-test; **p*<0.05, ***p*<0.01, *p*<0.001 vs normoxia. Data are mean ± SEM from 3 independent experiments.

	Normoxia	Hypoxia
HIF-target genes		
<i>Adm</i>	1.0 ± 0.63	44.5 ± 3.04***
<i>Aldoa</i>	1.0 ± 0.31	4.03 ± 0.47**
<i>Tpi1</i>	1.0 ± 0.16	8.24 ± 0.89**
<i>Hmox1</i>	1.0 ± 0.36	26.0 ± 2.93**
Adaptive UPR genes		
<i>Hspa5 (Bip)</i>	1.0 ± 0.16	0.76 ± 0.08
<i>Fkbp11</i>	1.0 ± 0.03	0.55 ± 0.02***
<i>Pdia4 (Erp72)</i>	1.0 ± 0.23	0.64 ± 0.08
<i>Dnajc3 (p58^{IPK})</i>	1.0 ± 0.08	0.64 ± 0.01*
<i>Dnajb9</i>	1.0 ± 0.12	0.56 ± 0.03*
Apoptotic genes		
<i>Ddit3 (Chop)</i>	1.0 ± 0.08	8.80 ± 1.93*
<i>Atf3</i>	1.0 ± 0.12	4.93 ± 0.91*
<i>Trb3</i>	1.0 ± 0.07	2.32 ± 0.30*

Figures

Figure1

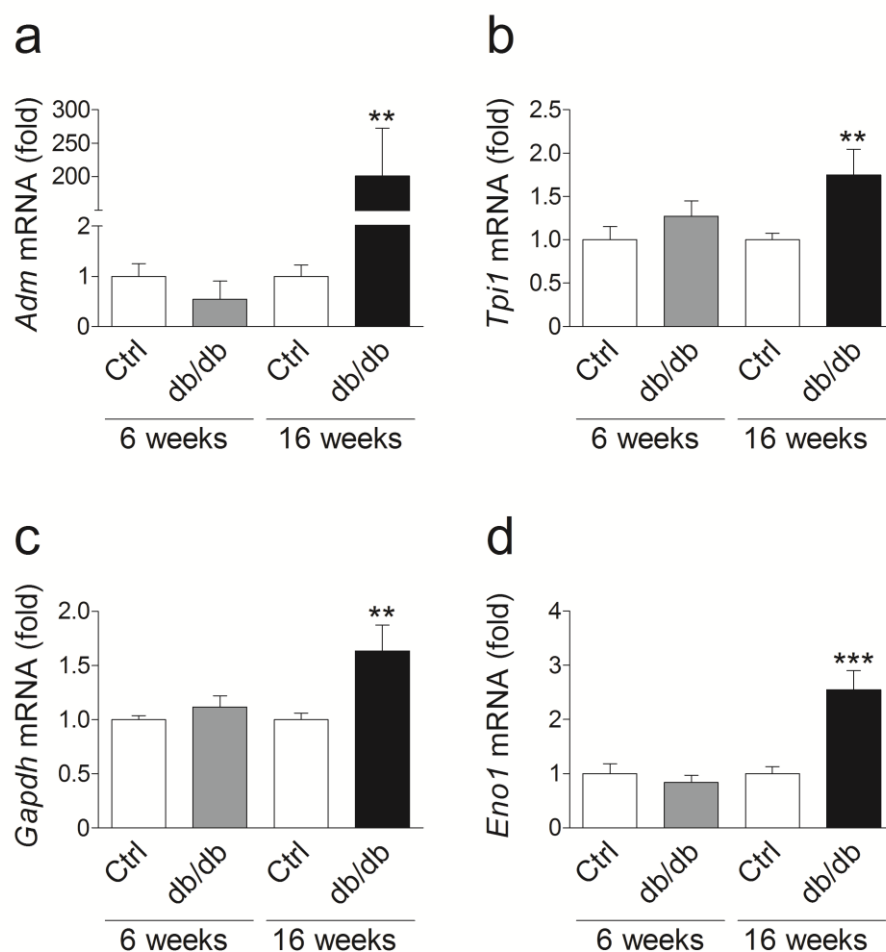


Fig. 1 HIF-target gene mRNA levels are upregulated in the islets of diabetic, but not prediabetic, *db/db* mice. Changes in the mRNA levels of *Adm*, *Tpi1*, *Gapdh* and *Eno1* in the islets of control C57BL/KsJ (Ctrl, white bars, 6 and 16 weeks of age), prediabetic *db/db* (grey bars, 6 weeks of age) and diabetic *db/db* (black bars, 16 weeks of age) mice. $n=8-14$ animals per group. ** $p<0.01$, *** $p<0.001$ vs control at the same age.

Figure 2

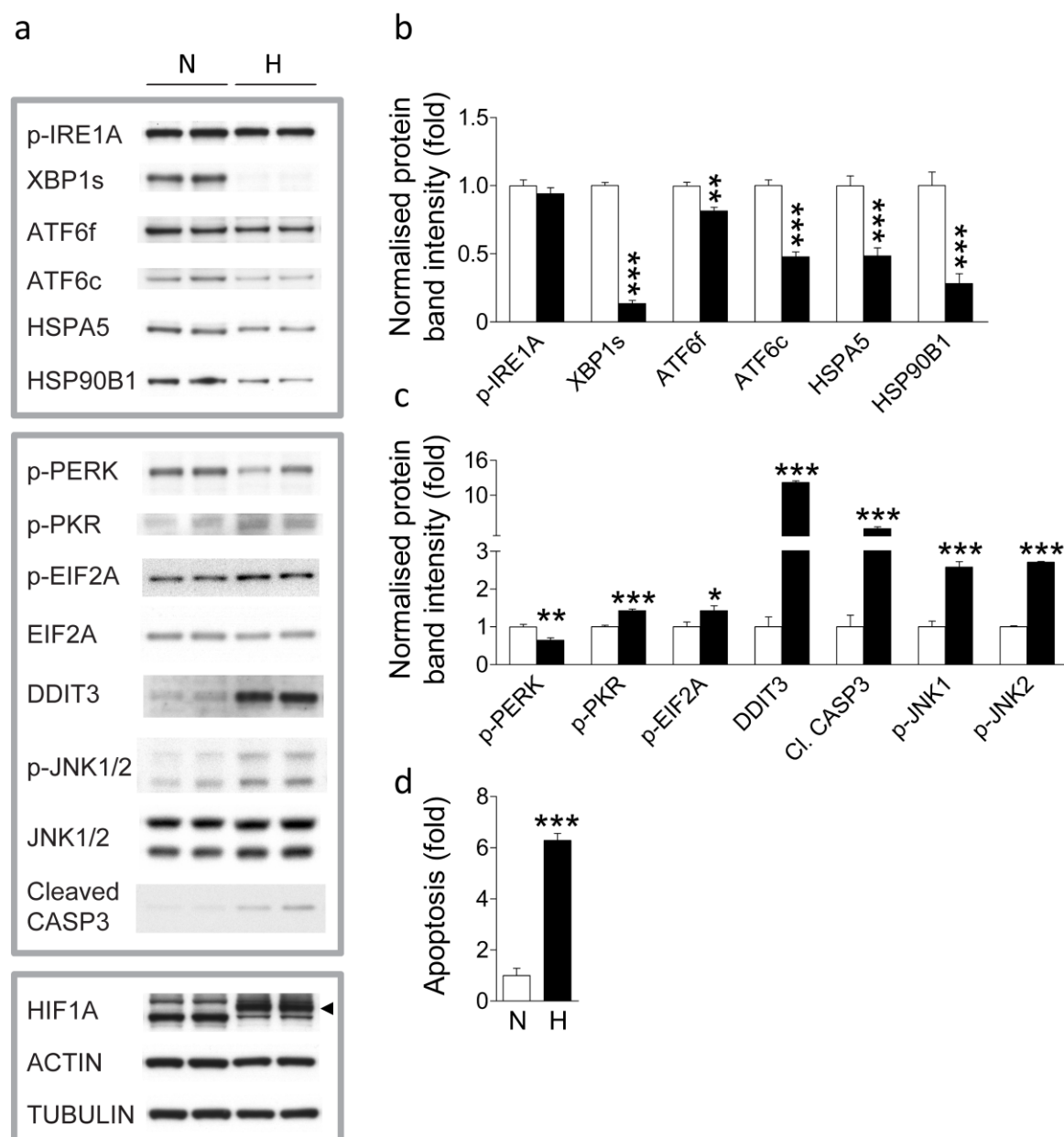


Fig. 2 Hypoxia decreases adaptive UPR protein expression and upregulates proapoptotic protein expression and apoptosis. MIN6 cells were cultured for 24h in the presence of normoxia (N, 20% O₂, white bars) or hypoxia (H, 1% O₂, black bars). Changes in (a-c) protein levels and (d) apoptosis. $n=3-5$ experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs normoxia.

Figure 3

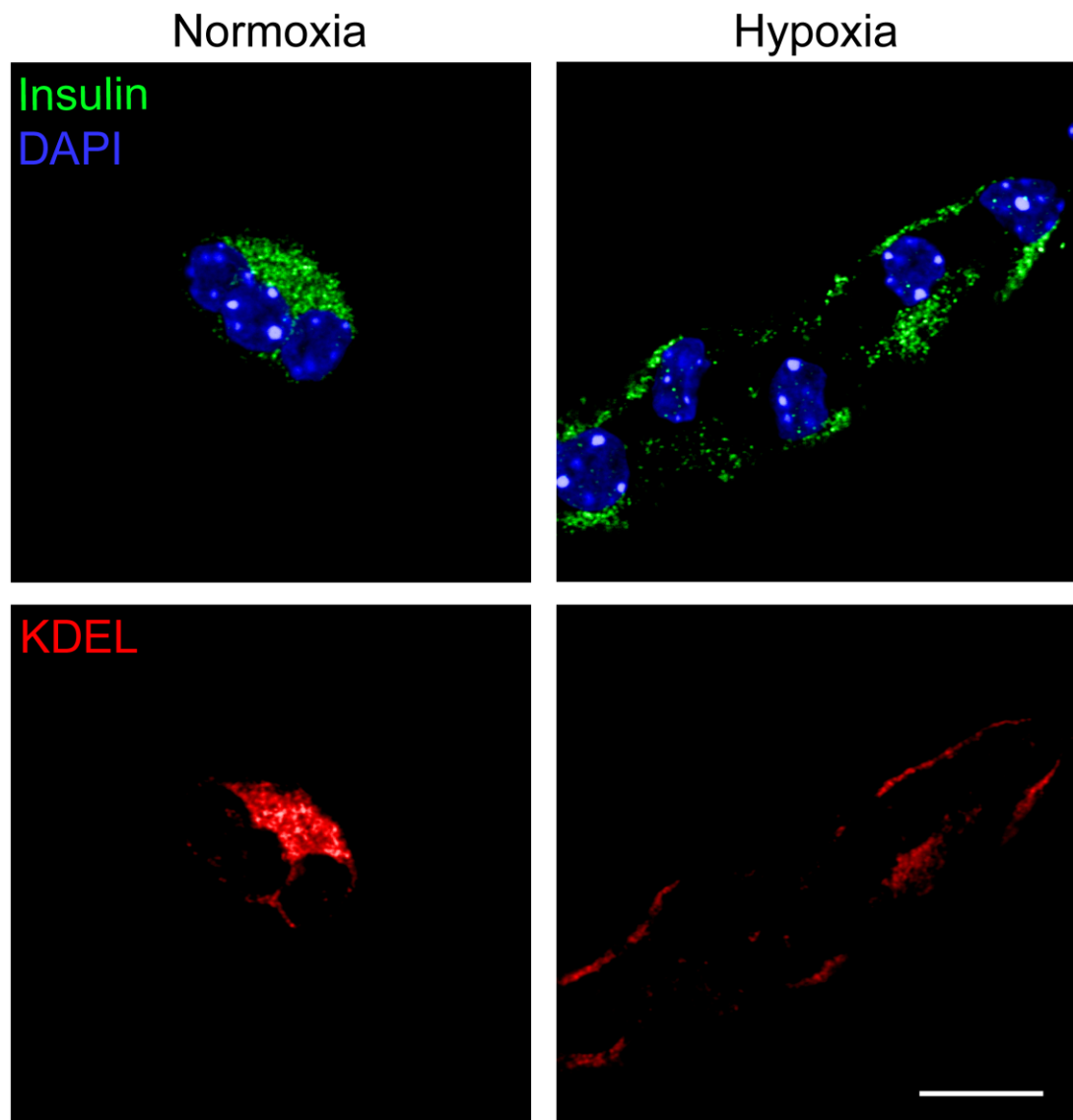


Fig. 3 Hypoxia decreases KDEL-containing ER proteins in primary beta cells. Immunostaining of KDEL in dispersed mouse islets cultured for 24h in the presence of normoxia (20% O₂) or hypoxia (1% O₂). Images are representative of three experiments. Scale bar, 10 μ m.

Figure 4

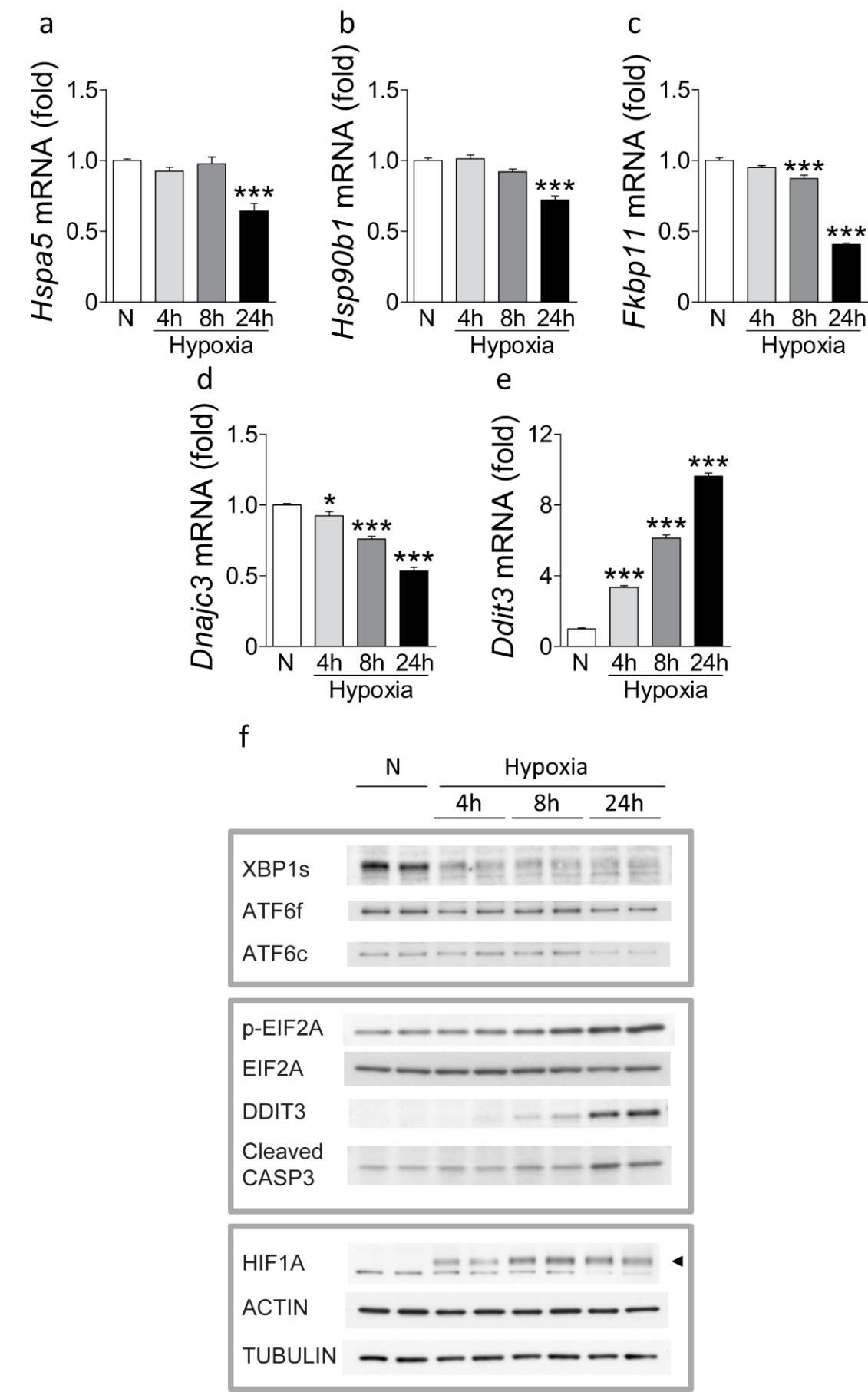


Fig. 4 Hypoxia time-dependently decreases adaptive UPR gene expression without transient activation, while increasing apoptotic gene expression. MIN6 cells were cultured for 4-24h in the presence of normoxia (N, 20% O₂) or hypoxia (1% O₂). Changes in (a-e) mRNA and (f) protein levels. *n*=4 experiments. **p*<0.05, ****p*<0.001 vs normoxia.

Figure 5

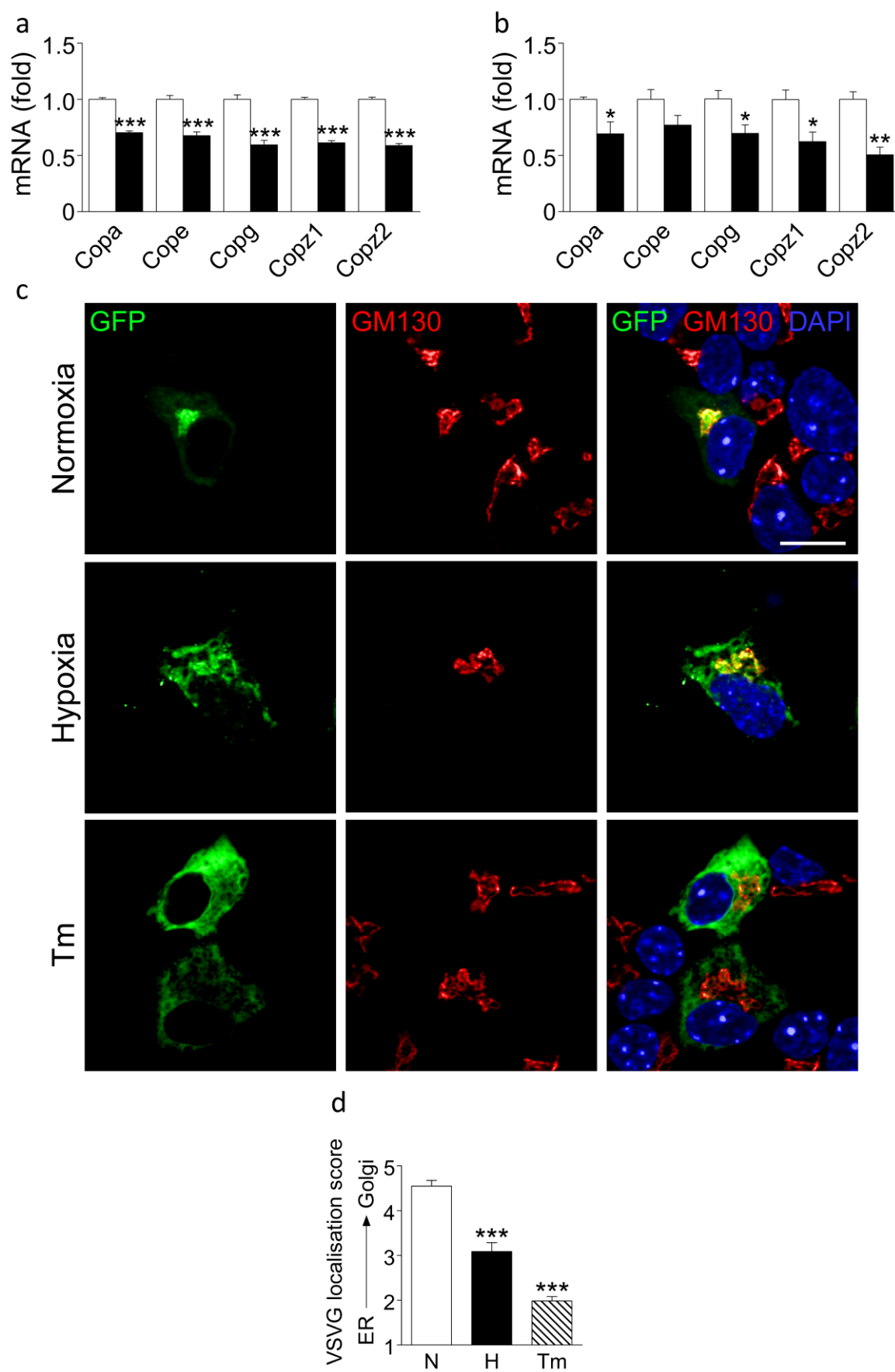


Fig. 5 Hypoxia reduces Cop gene expression and delays ER-to-Golgi protein trafficking. (a,c,d) MIN6 cells and (b) mouse islets were cultured in the presence of normoxia (N, 20% O₂, white bars) or hypoxia (H, 1% O₂, black bars). (a,b) Changes in Cop gene mRNA levels. (c) Representative images of VSVG-GFP and Golgi marker GM130 at 32°C. Tm: tunicamycin treatment (5µg/ml; 20% O₂). Scale bar, 10 µm. (d) VSVG localisation score. *n*=3-4 experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 vs normoxia.

Figure 6

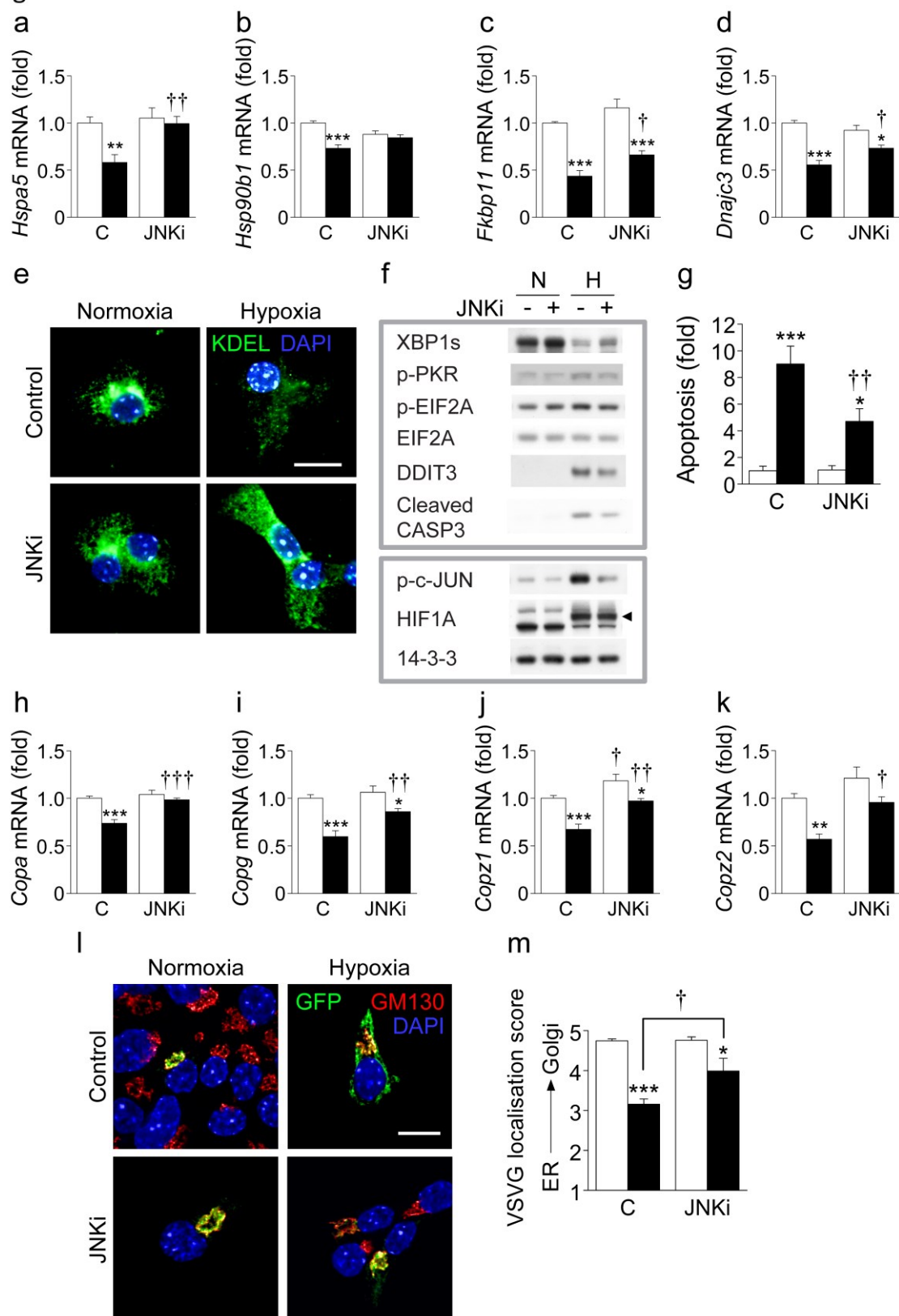


Fig. 6 JNK inhibition protects against downregulation of adaptive UPR and Cop gene expression, defective ER-to-Golgi protein trafficking and apoptosis due to hypoxia. MIN6

cells were cultured under normoxia (20% O₂, white bars) or hypoxia (1% O₂, black bars) in the presence of DMSO (C, control) or JNK inhibitor (JNKi; 10 µmol/l). Changes in (a-d) adaptive UPR gene expression, (e) KDEL immunostaining, (f) protein levels, (g) apoptosis, (h-k) Cop gene expression, (l) VSVG-GFP subcellular localisation at 32°C and (m) VSVG localisation score. Scale bar, 10 µm. *n*=3-5 experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 vs normoxia. †*p*<0.05, ††*p*<0.01, †††*p*<0.001 vs control.

Figure 7

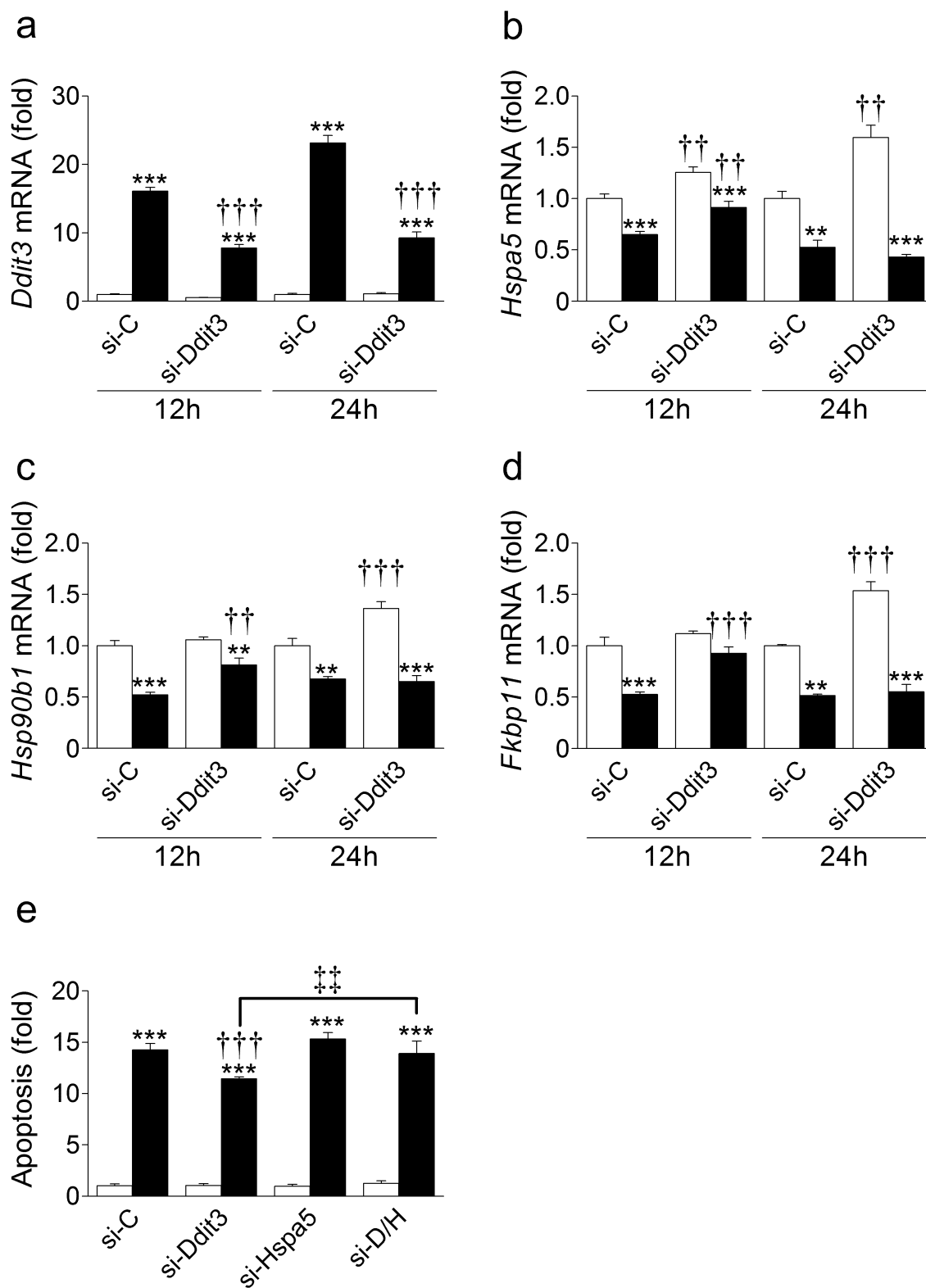


Fig. 7 *Ddit3* contributes to hypoxia-induced apoptosis by downregulating the adaptive UPR. MIN6 cells transfected with either control siRNA (si-C) or siRNA against *Ddit3*, *Hspa5* or both (si-D/H) were cultured for 12-24h in the presence of normoxia (20% O₂; white bars) or hypoxia (1% O₂; black bars). Changes in (a-d) mRNA levels and (e) apoptosis. *n*=3-7 experiments. ***p*<0.01, ****p*<0.001 vs normoxia. ††*p*<0.01, †††*p*<0.001 vs control siRNA. ‡‡*p*<0.01 vs *Ddit3* siRNA.

Figure 8

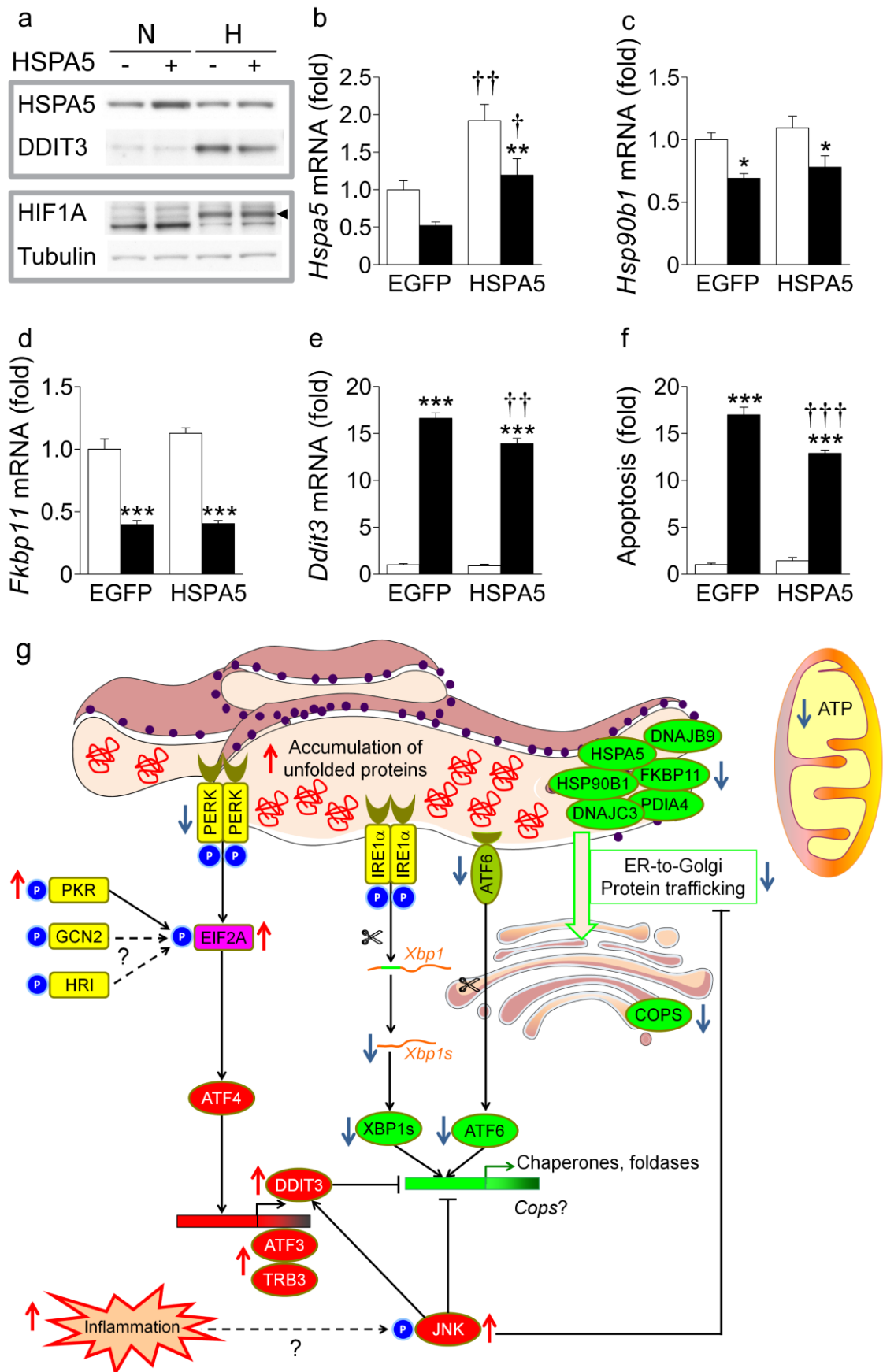
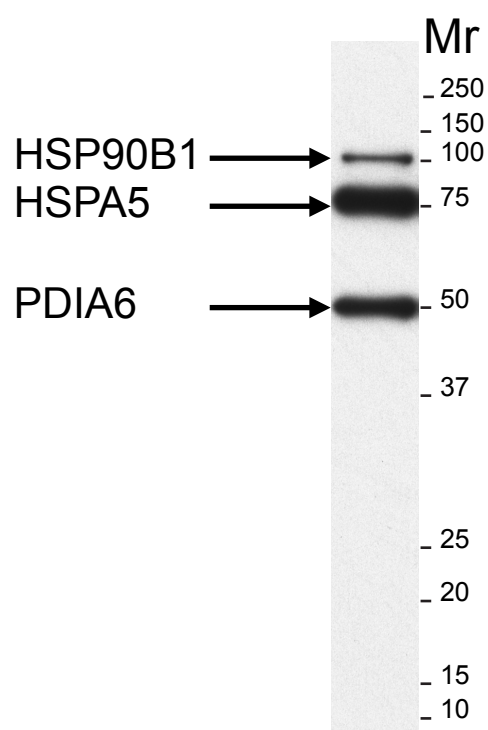


Fig. 8 Overexpression of *Hspa5* partially protects against hypoxia-induced apoptosis. MIN6 cells transfected with either EGFP or HSPA5 encoding vector were cultured for 24h in the presence of normoxia (20% O₂; white bars) or hypoxia (1% O₂; black bars). Changes in (a) protein, (b-e) mRNA levels and (f) apoptosis. *n*=3-4 experiments. ***p*<0.01, ****p*<0.001 vs normoxia. ††*p*<0.01, †††*p*<0.001 vs control siRNA. (g) The proposed model: Hypoxia inhibits adaptive UPR gene expression and ER-to-Golgi protein trafficking in beta cells via the activation of JNK and DDIT3. Hypoxia-mediated reductions in ER folding capacity, ATP production and ER-to-Golgi protein trafficking lead to the accumulation of unfolded proteins, irresolvable ER stress and ultimately beta cell death. Arrow's direction indicates the effect of hypoxia.

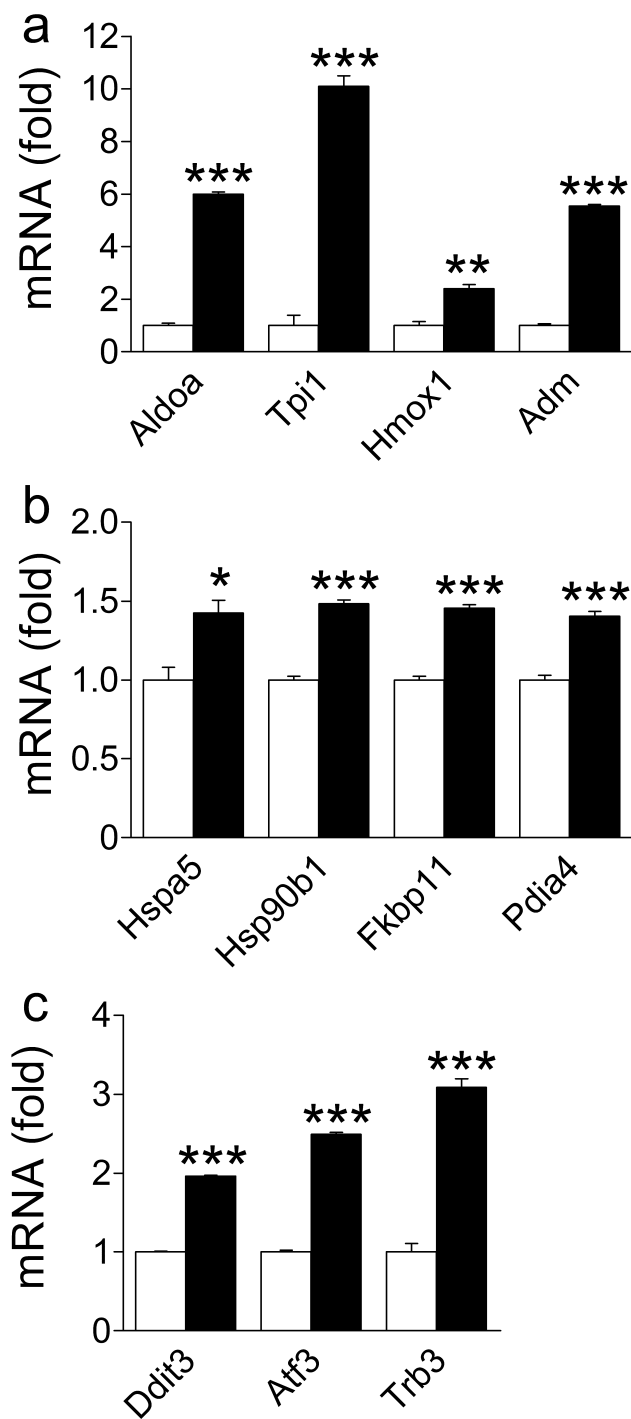
ESM Fig. 1



ESM Fig. 1

Western blot analysis of KDEL containing proteins in mouse islets shows the presence of three bands at ~94, ~78 and ~55 kDa corresponding to HSP90B1, HSPA5 and PDIA6. Blot is representative of three experiments.

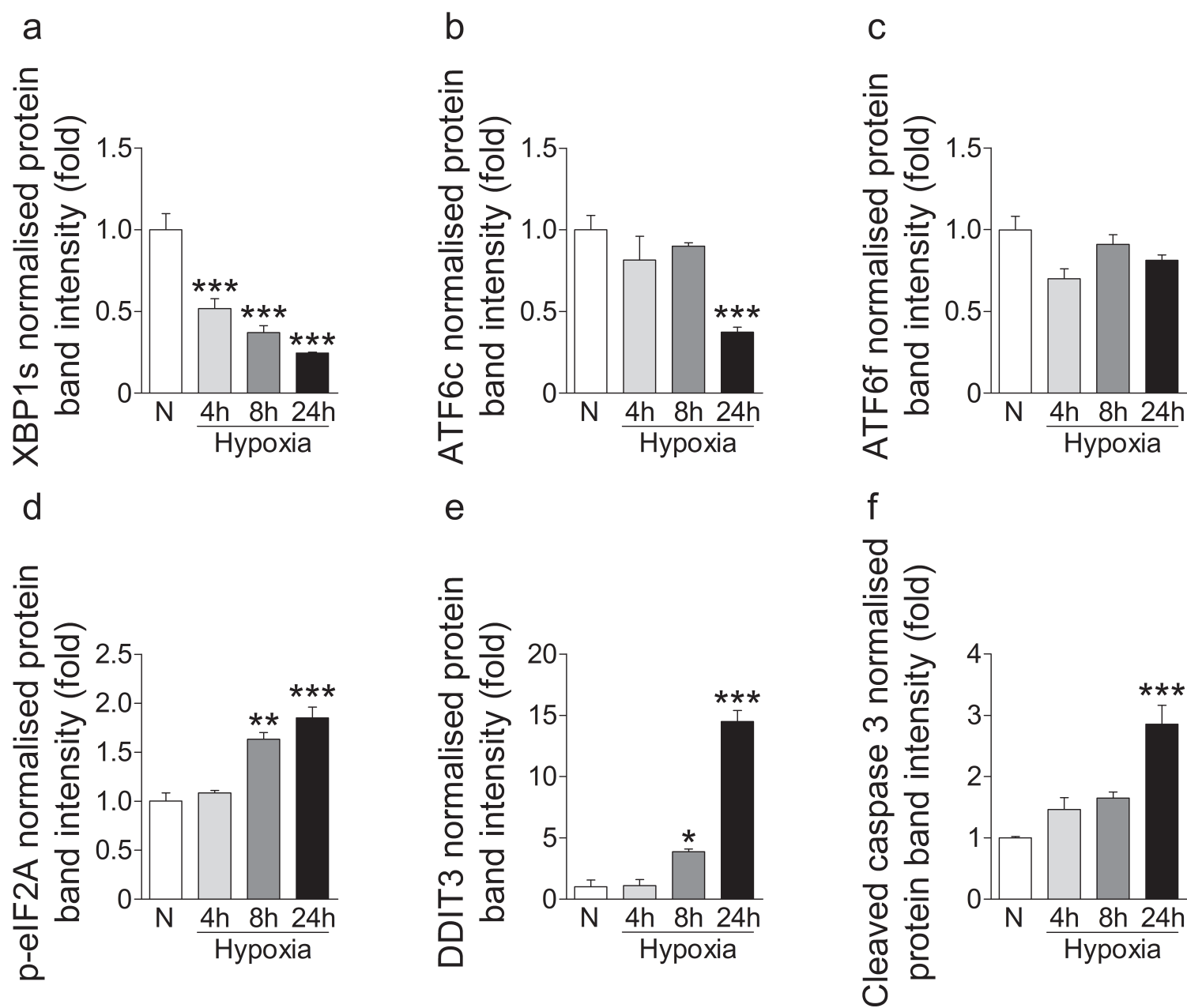
ESM Fig. 2



ESM Fig. 2 Hypoxia activates canonical ER stress response pathways in MEF cells

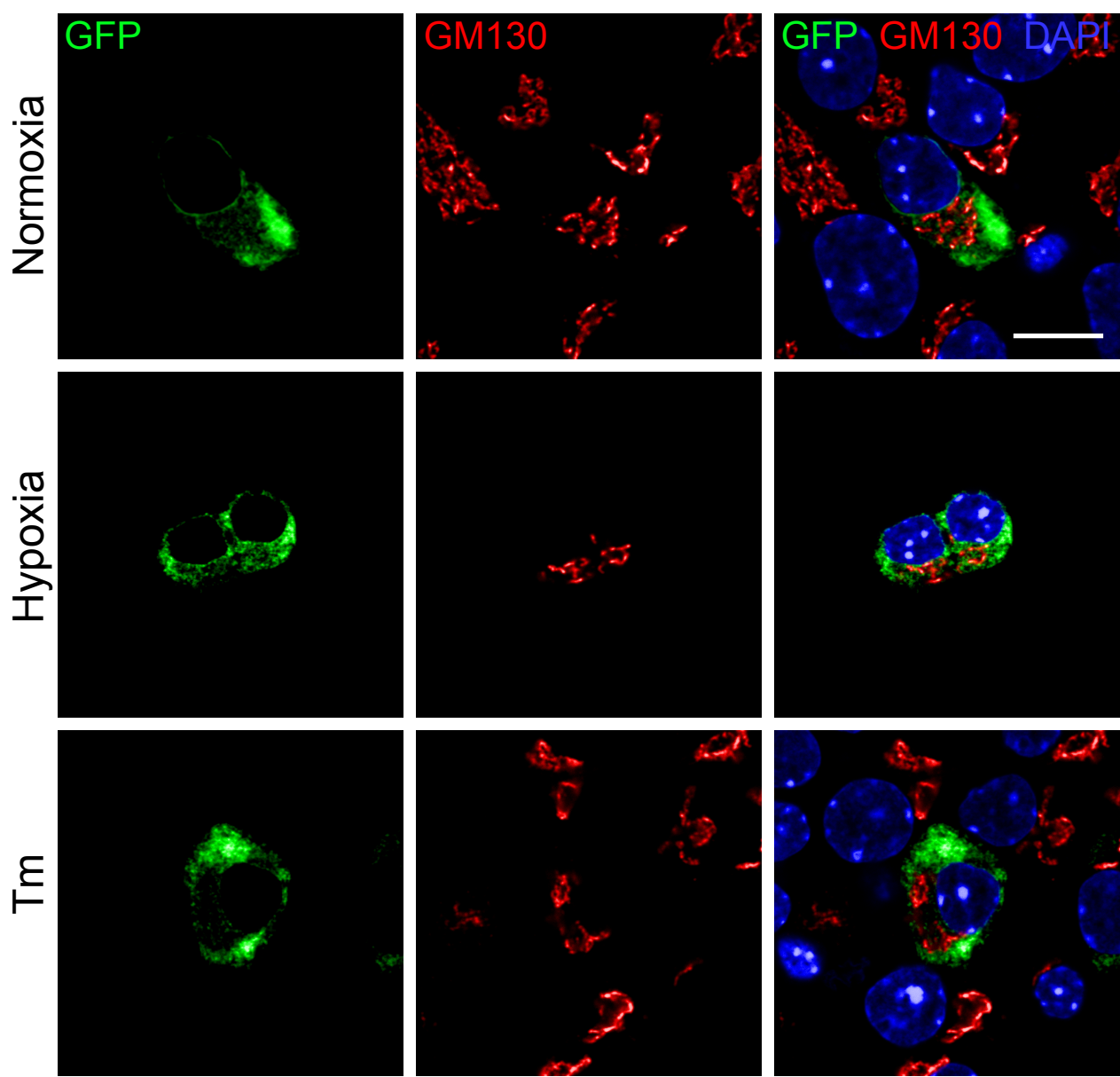
MEF cells were cultured in the presence of normoxia (20% O₂; white bars) or hypoxia (1% O₂; black bars) for 24h. Changes in the mRNA levels of (a) HIF-target genes (b) adaptive UPR genes and (c) apoptotic UPR genes, normalised to cyclophilin A mRNA levels and expressed relative to the normoxia condition. Data are means±SEM for three experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for the effect of hypoxia (unpaired two-tailed Student t-test).

ESM Fig. 3



ESM Fig. 3 Hypoxia time-dependently inhibits the adaptive UPR

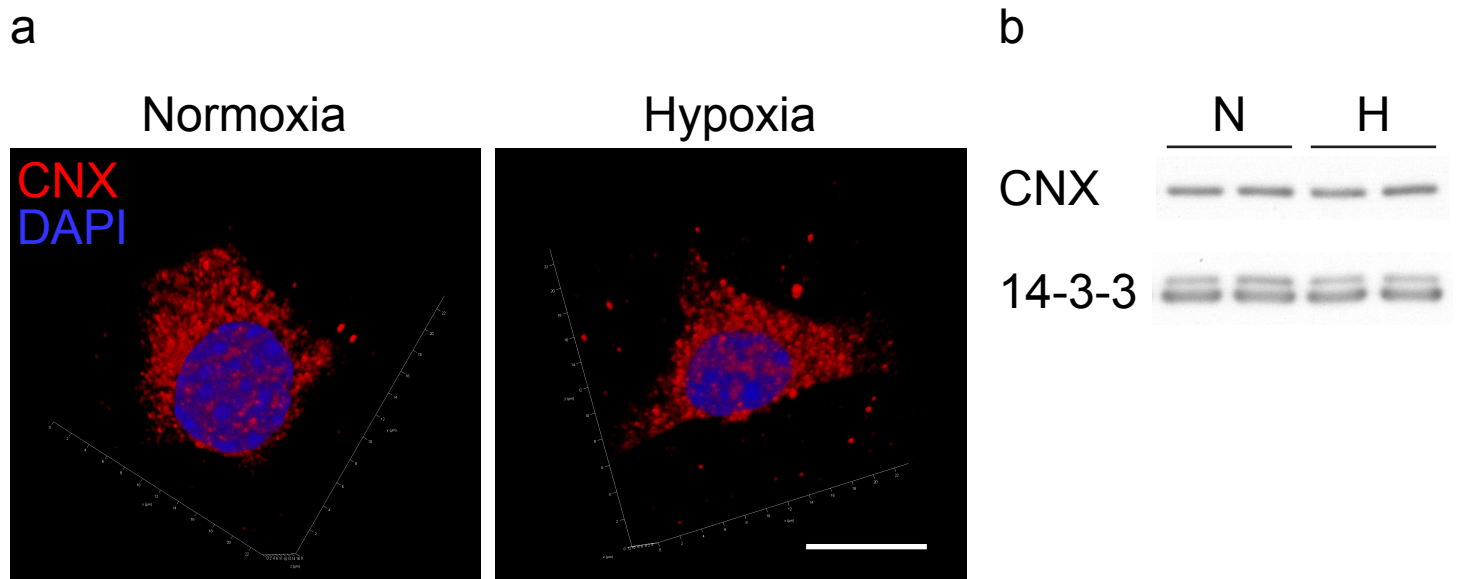
MIN6 cells were cultured for 4-24h in the presence of normoxia (N; 20% O₂; white bars) or hypoxia (1% O₂) for 24h. (a-f) Changes in the protein levels of XBP1s, ATF6c, ATF6f, p-eIF2A, DDIT3 and cleaved caspase 3 were analysed by western blot, quantified by densitometry and normalised to total protein or loading controls. Data are means±SEM for four experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 for the effect of hypoxia (one-way ANOVA + Newman-Keuls posttest).



ESM Fig. 4 The temperature-sensitive VSVG mutant protein is retained in the ER at 40°C

MIN6 cells were cultured in the presence of normoxia (20% O₂) or hypoxia (1% O₂) for 24h at 40°C. Representative images of VSVG-GFP and Golgi marker GM130. Tm: tunicamycin treatment (5μg/ml; 20% O₂). Images are representative of three experiments. Scale bar, 10 μm.

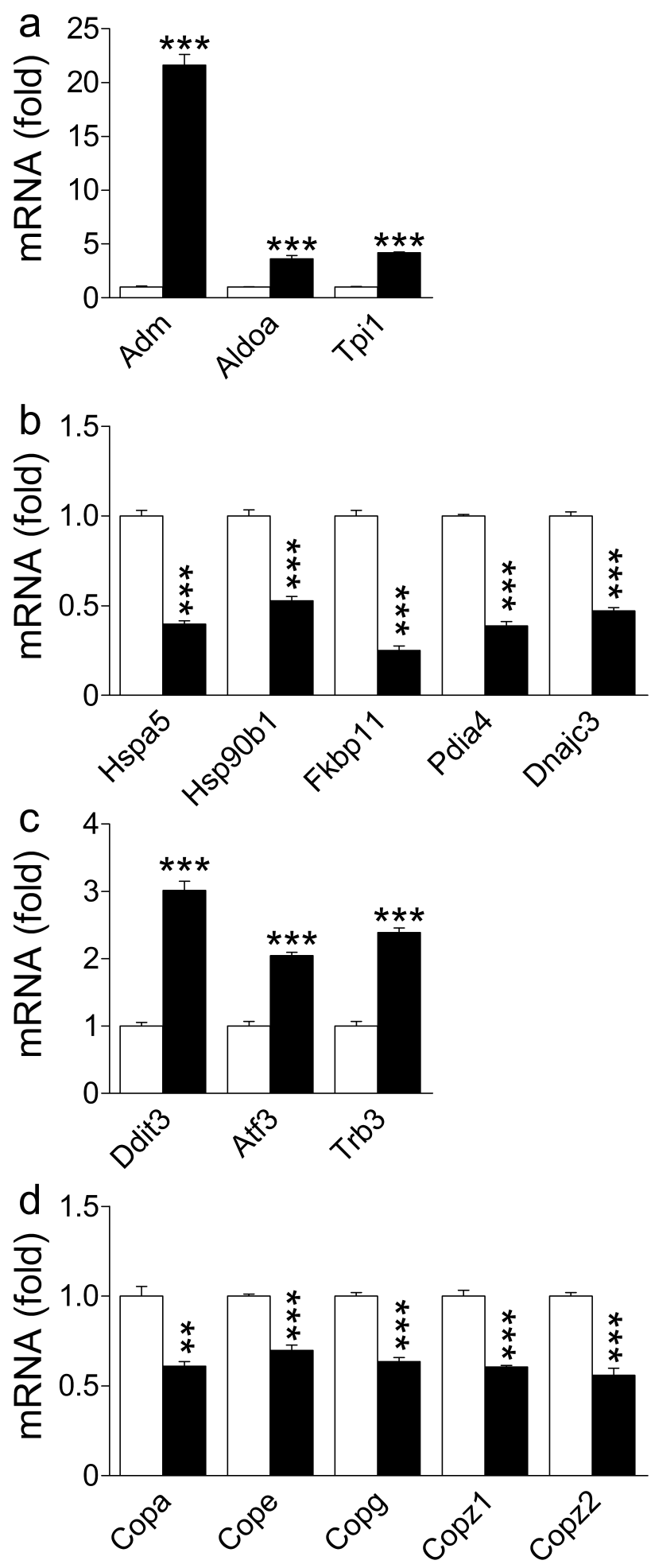
ESM Fig. 5



ESM Fig. 5 ER morphology did not differ in cells treated with normoxia and hypoxia

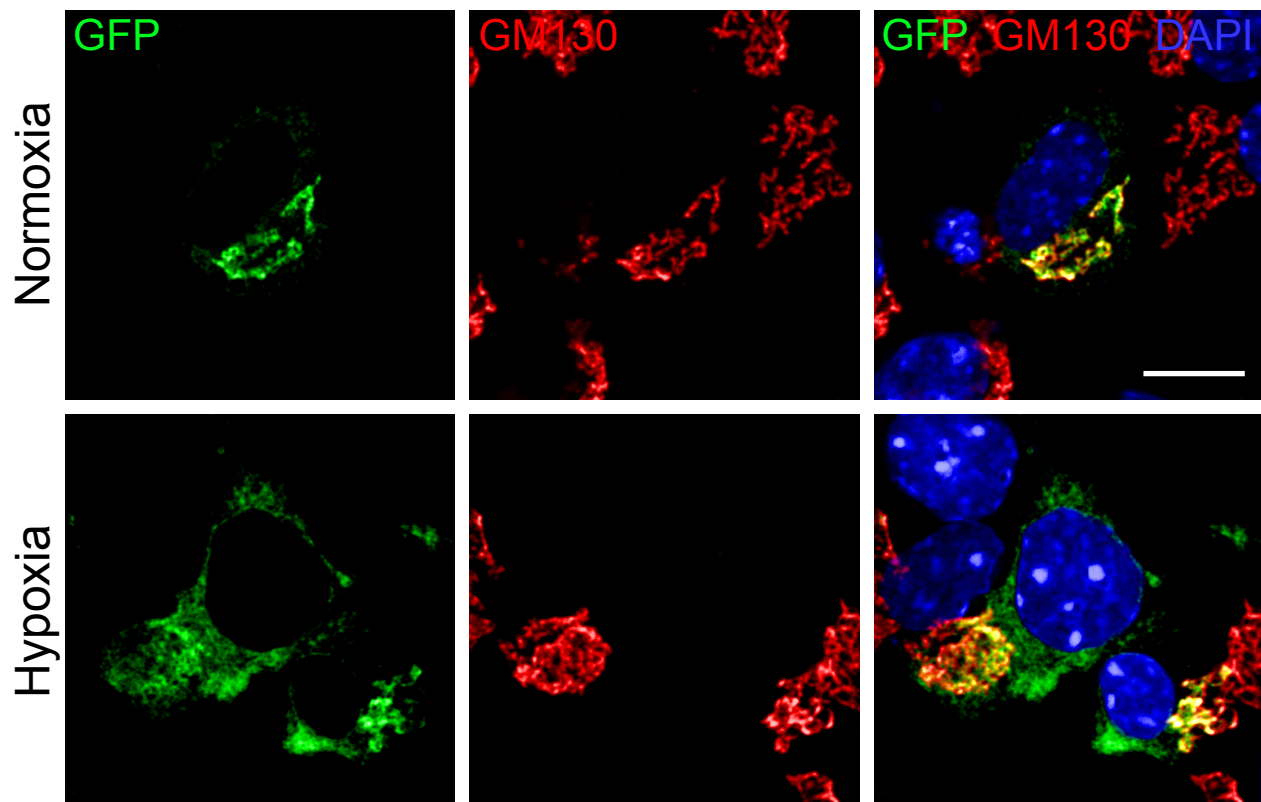
MIN6 cells were cultured in the presence of normoxia (N; 20% O₂) or hypoxia (H; 1% O₂) for 24h. (a) Representative 3D projections of z-stack images of calnexin (CNX) immunostaining and (b) changes in CNX protein levels. $n=3-4$ experiments. Scale bar, 10 μm .

ESM Fig. 6



ESM Fig. 6 Hypoxia induced by 5% O₂ reduced the mRNA levels of adaptive UPR and Cop genes in MIN6 cells

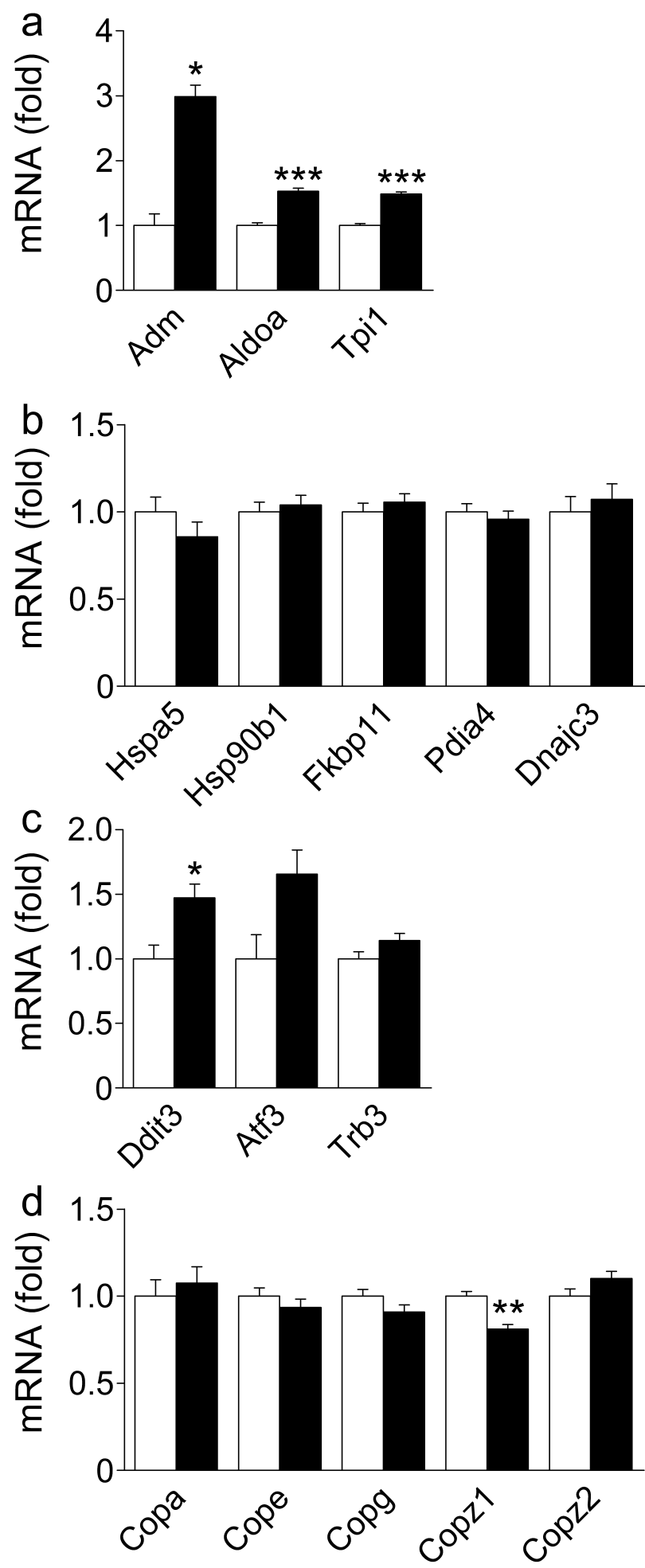
MIN6 cells were cultured in the presence of normoxia (20% O₂; white bars) or hypoxia (5% O₂; black bars) for 24h. Changes in the mRNA levels of (a) HIFtarget genes, (b) adaptive UPR genes, (c) apoptotic genes and (d) Cop genes, normalised to cyclophilin A mRNA levels and expressed relative to the normoxia condition. Data are means±SEM for three experiments. **p<0.01, ***p<0.001 for the effect of hypoxia (unpaired two-tailed Student t-test).



ESM Fig. 7 Hypoxia induced by 5% O₂ impairs ER-to-Golgi protein trafficking in MIN6 cells

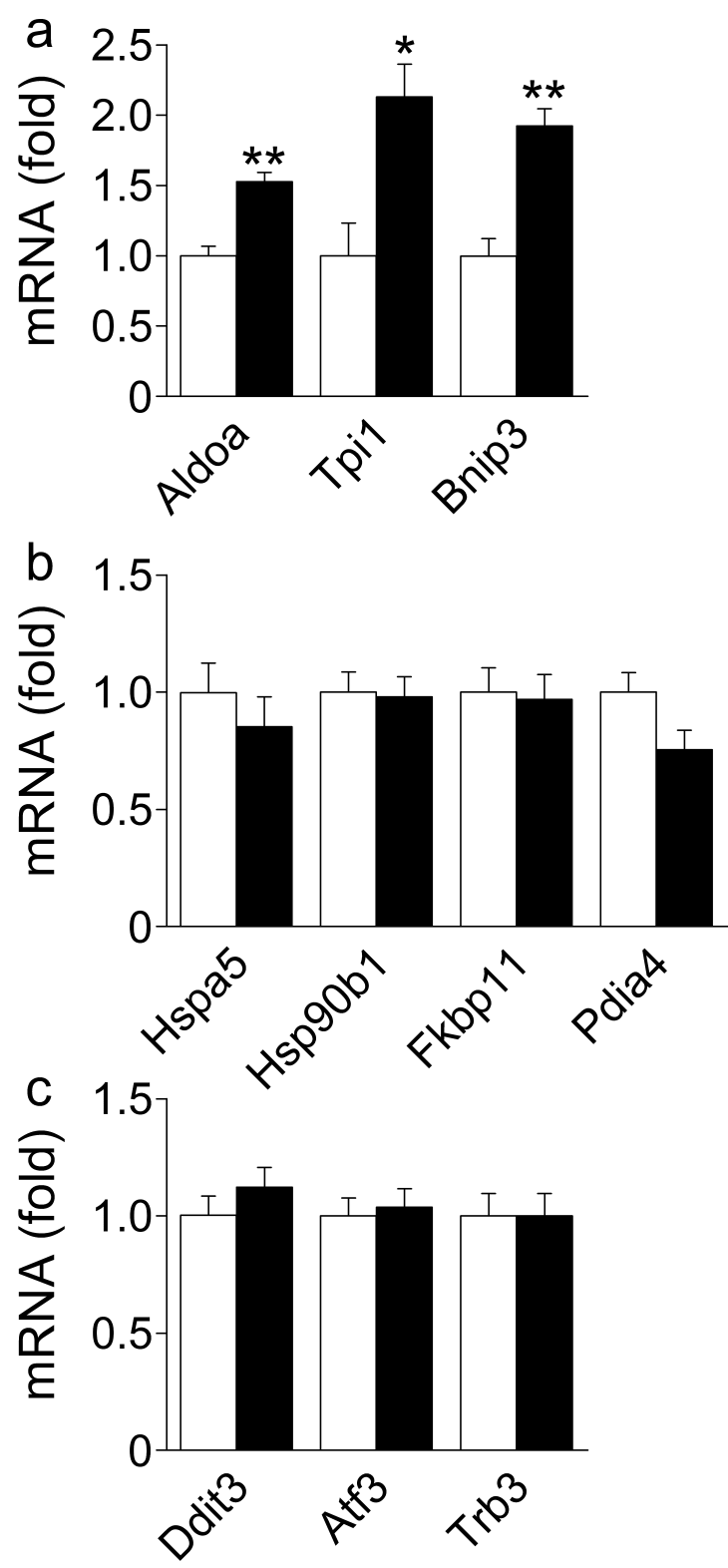
MIN6 cells were cultured in the presence of normoxia (20% O₂) or hypoxia (5% O₂) for 24h. Representative images of VSVG-GFP and Golgi marker GM130 at 32°C. Images are representative of three experiments. Scale bar, 10 μm.

ESM Fig. 8



ESM Fig. 8 Effects of mild hypoxia on UPR gene expression in MIN6 cells

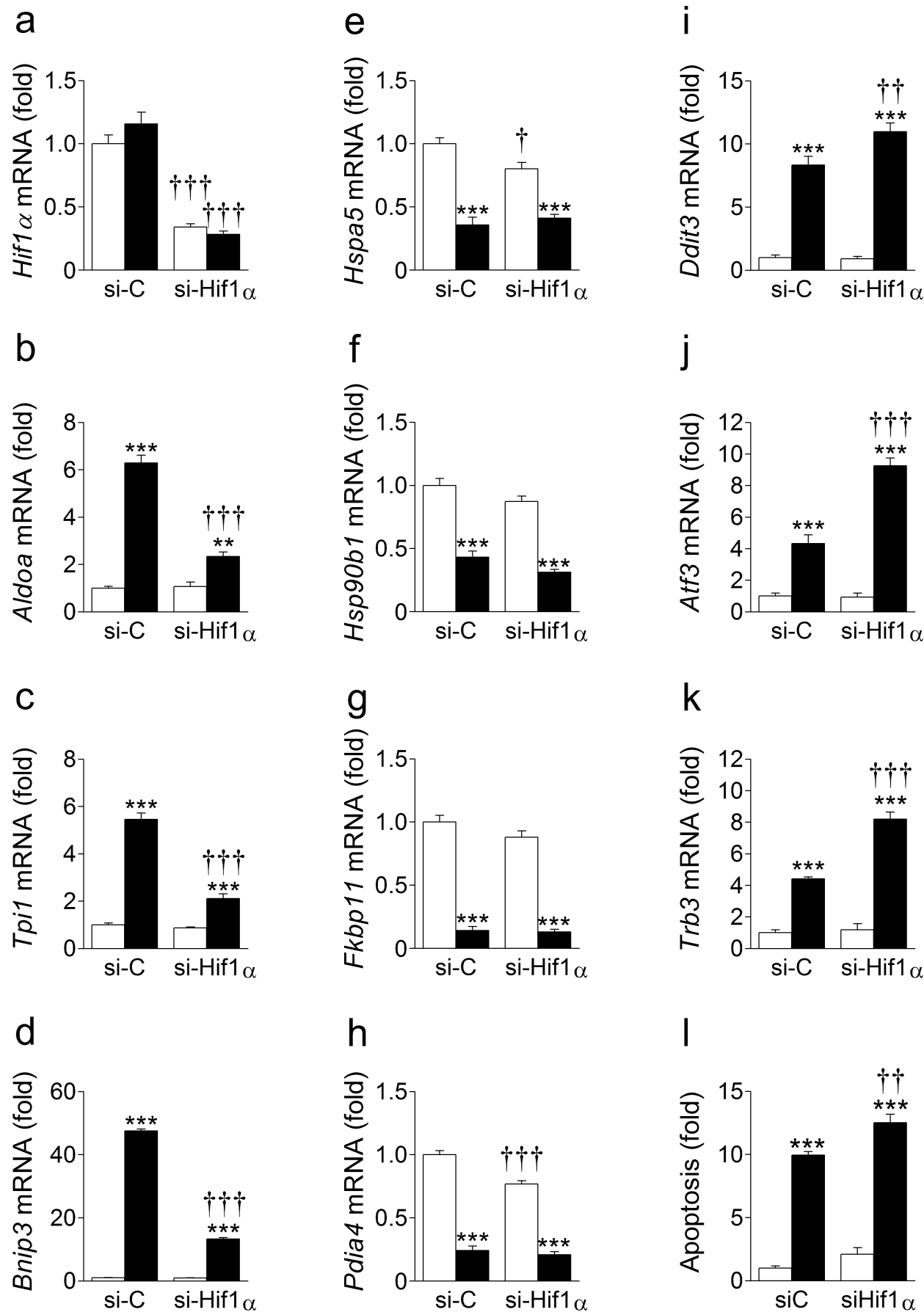
MIN6 cells were cultured in the presence of normoxia (20% O₂; white bars) or hypoxia (10% O₂; black bars) for 24h. Changes in the mRNA levels of (a) HIF-target genes, (b) adaptive UPR genes, (c) apoptotic genes and (d) Cop genes, normalised to cyclophilin A mRNA levels and expressed relative to the normoxia condition. Data are means±SEM for three experiments. * p <0.05, ** p <0.01, *** p <0.001 for the effect of hypoxia (unpaired two-tailed Student t-test).



ESM Fig. 9 Effects of mild hypoxia on UPR gene expression in mouse islets

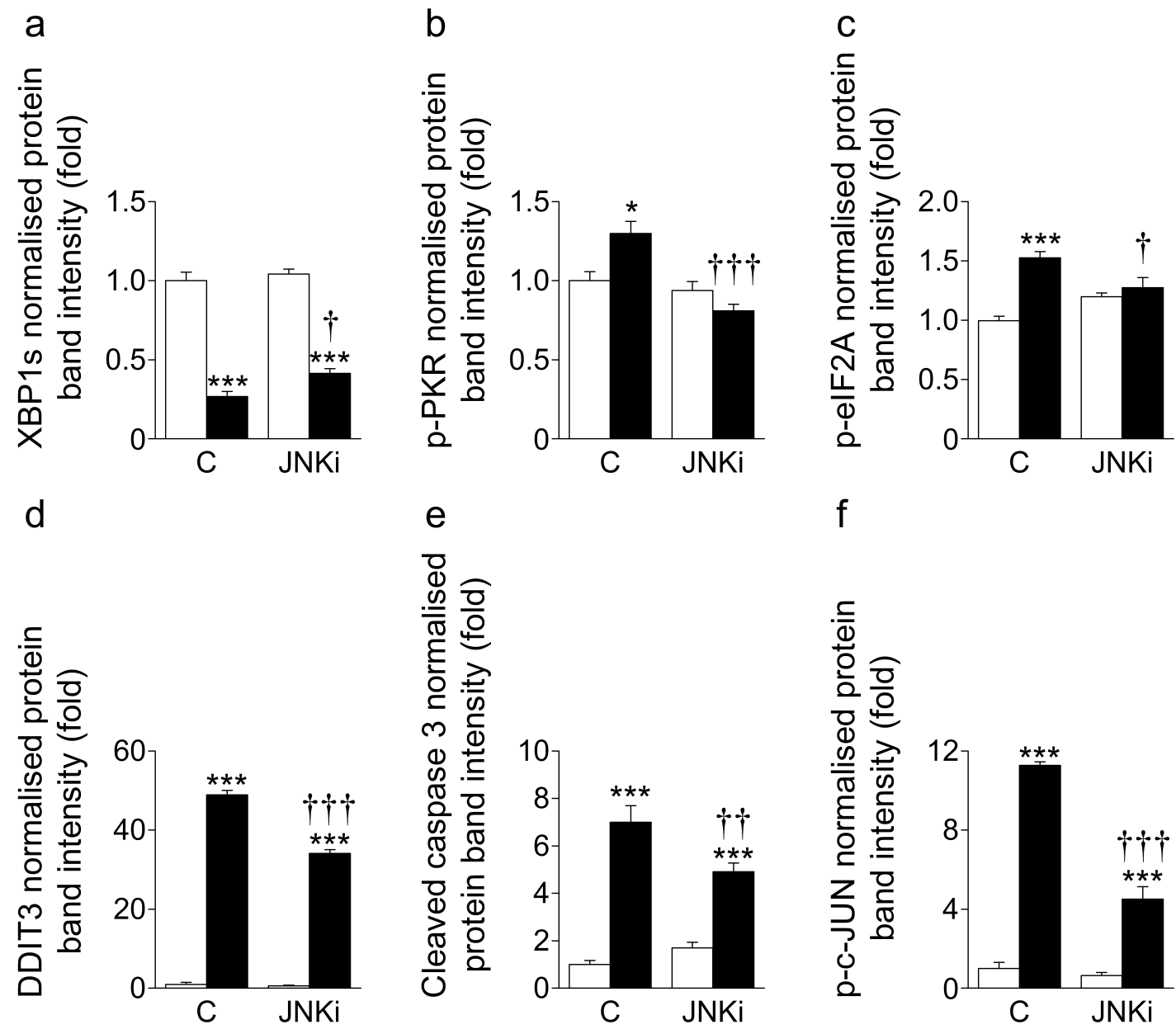
Mouse islets were cultured in the presence of normoxia (20% O₂; white bars) or hypoxia (10% O₂; black bars) for 24h. Changes in the mRNA levels of (a) HIF-target genes, (b) adaptive UPR genes and (c) apoptotic genes, normalised to cyclophilin A mRNA levels and expressed relative to the normoxia condition. Data are means±SEM for four experiments. **p*<0.05, ***p*<0.01 for the effect of hypoxia (unpaired two-tailed Student t-test).

ESM Fig. 10



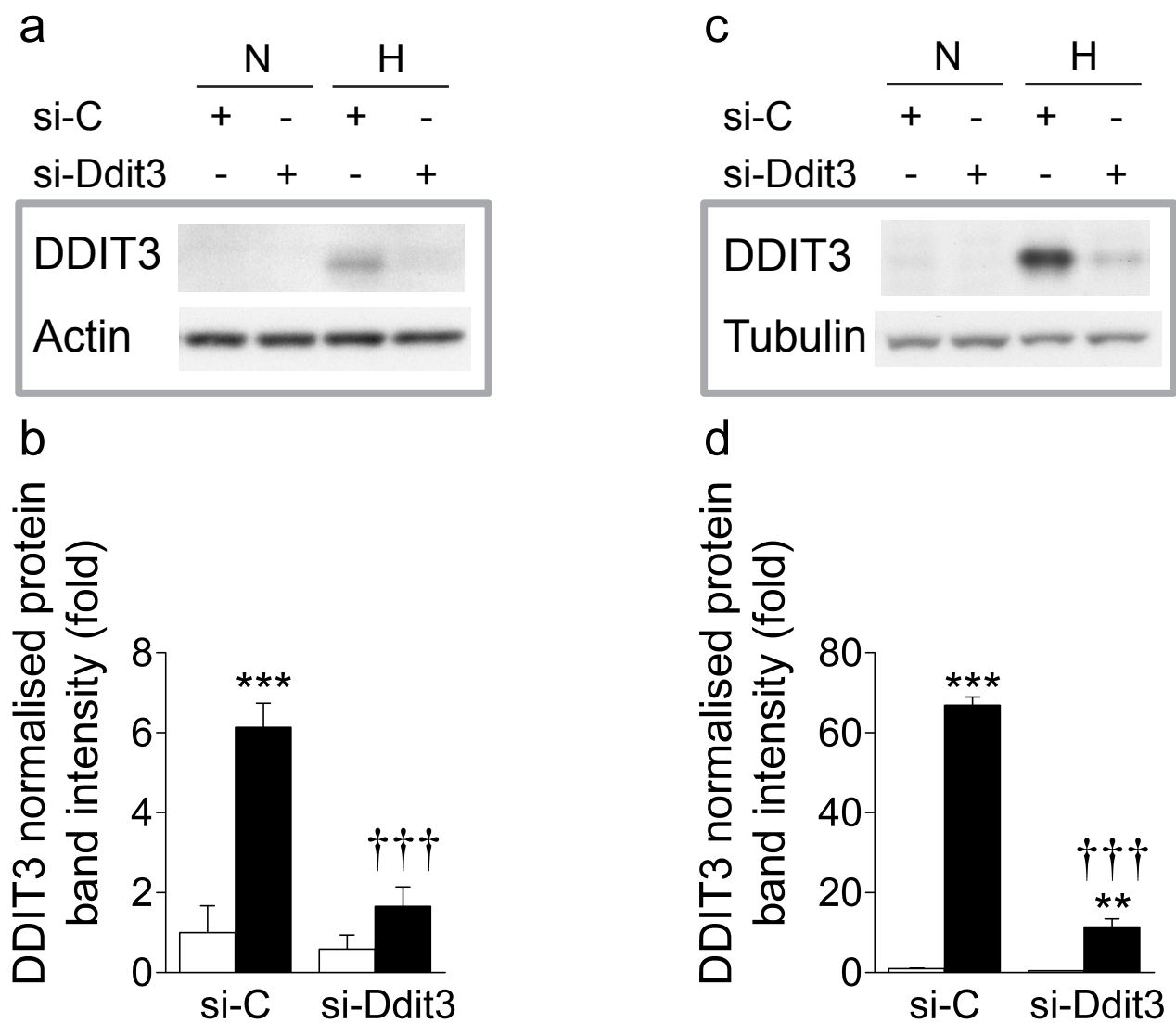
ESM Fig. 10 Hypoxia downregulates the adaptive UPR independently of HIF1A activation

MIN6 cells transfected with either control siRNA (si-C) or siRNA against *Hif1 α* (si-Hif1 α) were cultured in the presence of normoxia (20% O₂; white bars) or hypoxia (1% O₂; black bars) for 24h. Changes in the mRNA levels of (a-d) HIF-target genes, (e-h) adaptive UPR genes and (i-k) apoptotic genes normalised to cyclophilin A mRNA levels and expressed relative to the normoxia condition. (l) Changes in apoptosis levels normalised to DNA content. Data are means $\pm$ SEM for 3-4 experiments. ** p <0.01, *** p <0.001 vs normoxia. † p <0.05, †† p <0.01, ††† p <0.001 vs control siRNA (two-way ANOVA+test of Bonferroni).



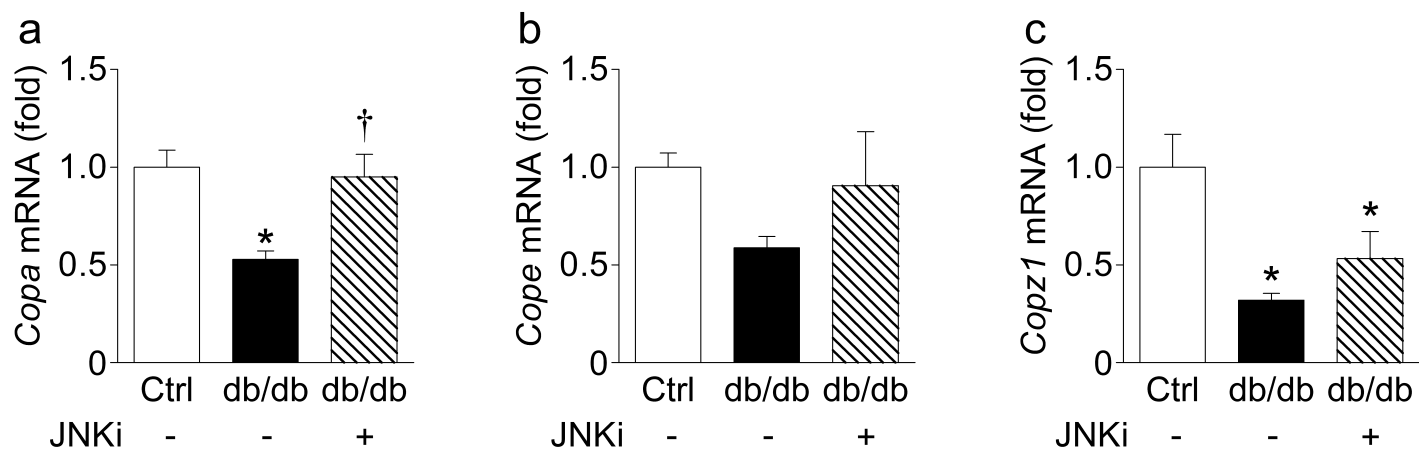
ESM Fig. 11 JNK inhibition protects against hypoxia-induced downregulation of the adaptive UPR and upregulation of the ISR

MIN6 cells were cultured in the presence of normoxia (20% O₂) or hypoxia (1% O₂) for 24h in the presence of DMSO (C, control) or JNK inhibitor (JNKi; 10 μmol/l). (a-f) Changes in the protein levels of XBP1s, p-PKR, p-eIF2A, DDIT3, cleaved caspase 3 and p-c-JUN were analysed by western blot, quantified by densitometry and normalised to the total protein or loading controls. Data are means±SEM for three to five experiments. * $p < 0.05$, *** $p < 0.001$ vs normoxia. † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs control (two-way ANOVA + Bonferroni posttest).



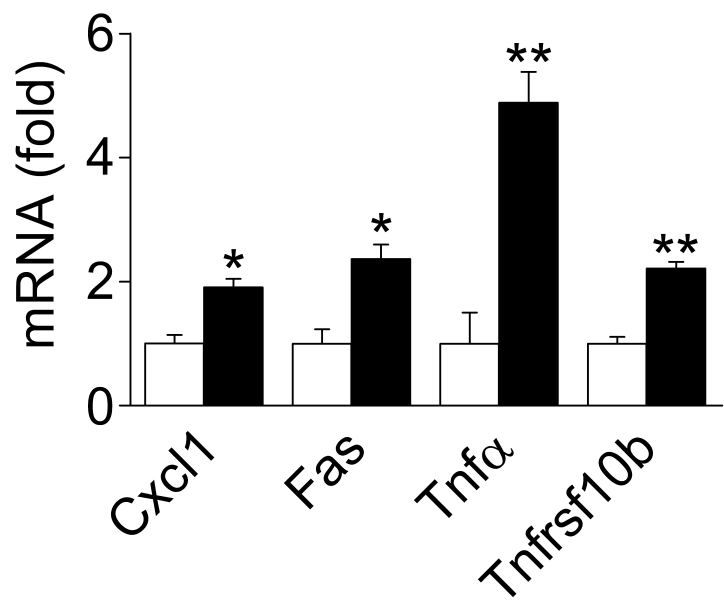
ESM Fig. 12 si-Ddit3 reduced DDIT3 protein levels.

MIN6 cells transfected with either control siRNA (si-C) or siRNA against *Ddit3* (si-Ddit3) were cultured in the presence of normoxia (N; 20% O₂; white bars) or hypoxia (H; 1% O₂; black bars) for 12-24h. Changes in protein levels of DDIT3 after (a, b) 12h or (c, d) 24h culture under hypoxia were analysed by western blot, quantified by densitometry and normalised to loading controls. Data are means±SEM for three experiments. ***p*<0.01, ****p*<0.001 vs normoxia. †††*p*<0.001 vs control siRNA. (two-way ANOVA + Bonferroni posttest).



ESM Fig. 13 JNK activation downregulates Cop gene expression in islets of diabetic *db/db* mice

Islets from C57BL/KsJ control (white bars) and diabetic *db/db* mice at 16 weeks of age were cultured in the absence (black bars) or presence of JNK inhibitor (JNKi; 20 μ mol/l; striped bars) for 24h. Changes in the mRNA levels of Cop genes were normalised to cyclophilin A mRNA levels and expressed relative to those in control mice. Data are means $\pm$ SEM for three experiments. * p <0.05 vs control. † p <0.05 vs *db/db* (one-way ANOVA + Newman-Keuls posttest).



ESM Fig. 14 Hypoxia increased the expression of inflammatory genes

Mouse islets were cultured in the presence of normoxia (20% O₂; white bars) or hypoxia (1% O₂; black bars) for 12h. Changes in the mRNA levels of inflammation genes were normalised to cyclophilin A mRNA levels and expressed relative to the normoxia condition. Data are means±SEM for three experiments. **p*<0.05, ***p*<0.01 for the effect of hypoxia (unpaired two-tailed Student t-test).

ESM Table 1. Sequences of oligonucleotide primers (5'-3')

Gene symbol	5' Oligonucleotide	3' Oligonucleotide
<i>Adm</i>	GCAATGCTTGTTGTCCAGCC	ACACACACACACACACACGGAAC
<i>Aldoa</i>	TCCATTGGCACCGAGAACAC	TTGATAACTTGGGGGAAGGGAC
<i>Atf3</i>	GCTGCCAAGTGTCTGAAACAAG	CAGTTTTCCAATGGCTTCAGG
<i>Bnip3</i>	CACTTCAGCAATGGCAATGGG	TATCTTGTGGTGTCTGGGAGCGAG
<i>Copa</i>	CAGATTCCATCACACTCTTTGACG	AACACCCCACTTTCATCCCAG
<i>Cope</i>	AAGTATGGCGTGGTCCTGGATG	ATGCTGTCCCTCTGGTTCTCACTG
<i>Copg</i>	AGGCTATGAGGATGAGTATGTGCTG	CGTGTGGGTGTTCTTGTTTTCTG
<i>Copz1</i>	AAGACCCATCGGACGGATAGTG	CATTTTTCTCAGCATCTGGCTC
<i>Copz2</i>	AGATAATGACGGGCGAAGGC	AAACAGGCAGGCAAGCACAGAC
<i>Cyclophilin (Cypa, Ppia)</i>	TGTGCCAGGGTGGTGACTTTAC	TGGGAACCGTTTGTGTTTGG
<i>Cxcl1 (Gro1)</i>	CAAACCGAAGTCATAGCCCACTC	TTGTCAGAAGCCAGCGTTCAC
<i>Ddit3 (Chop, Gadd153)</i>	TTCCTACTCTTGACCCTGCGTC	CACTGACCACTCTGTTTCCGTTTC
<i>Dnajc3 (p58^{IPK})</i>	AAGCCCGTGGAAGCCATTAG	GGTCATTTTCATTGTGCTCCTGAG
<i>Dnajb9</i>	GCAATGGGAGTCCTTTTGAACAG	TCCTGGAAGTGATGCCTTTGTC
<i>Eno1</i>	GATGGACGGCACAGAGAATAAATC	AGGCAGGATGACTTCAGGGTTG
<i>Fas</i>	AACCAGACTTCTACTGCGATTCTCC	CCTTTTCCAGCACTTTCTTTTCCG
<i>Fkbp11</i>	ACACGCTCCACATACTACACGG	ATGACTGCTCTTCGCTTCTCTCCC
<i>Gapdh</i>	CAAAATGGTGAAGGTCGGTGTG	TGATGTTAGTGGGGTCTCGCTC
<i>Hif1α</i>	CATCTCTCTGGATTTTGGCAGC	GAAGTGGCTTTGGAGTTTCCG
<i>Hmox1 (HO-1, Hsp32)</i>	CCACACAGCACTATGTAAAGCGTC	GTTCGGGAAGGTAAAAAAGCC
<i>Hspa5 (Bip, Grp78)</i>	AGGACAAGAAGGAGGATGTGGG	ACCGAAGGGTCATTCCAAGTG
<i>Hsp90b1 (Grp94)</i>	AAACGGCAACACTTCGGTCAG	GCATCCATCTCTTCTCCCTCATC
<i>Pdia4 (Erp72)</i>	AGTCAAGGTGGTGGTGGGAAAG	TGGGAGCAAAATAGATGGTAGGG
<i>Tnfα</i>	CCCCTTTACTCTGACCCCTTTATTG	AACCTGACCACTCTCCCTTTGC
<i>Tnfrsf10b (Dr5)</i>	TTGCGTTGCTGCTTGCTGTG	TGGTTGGAATGGCTGGTGTAGTC
<i>Trb3</i>	TCTTCAGCAACTGTGAGAGGACG	TCCAGACATCAGCCGCTTTG
<i>Tpi1</i>	CCTTCCATTGGTTTGGGCTG	AATACAGGGGCTTTGGCACC

Aliases of gene symbols given in parentheses

ESM Table 2. List of primary antibodies used in western blots and immunofluorescence

Antibody	Reference	Company	Fold dilution
α -TUBULIN	T90267	Sigma-Aldrich, St Louis, MO, USA	1000
β -ACTIN	A5441	Sigma-Aldrich, St Louis, MO, USA	1000
HIF1A	NB100-449	Novus Biologicals, CO, USA	500
Cleaved CASP3	9664	Cell Signaling Technology, Danvers, MA, USA	500
JNK	9252	Cell Signaling Technology, Danvers, MA, USA	1000
p-c-JUN	9164	Cell Signaling Technology, Danvers, MA, USA	500
p-EIF2A	9721	Cell Signaling Technology, Danvers, MA, USA	500
p-JNK	9251	Cell Signaling Technology, Danvers, MA, USA	500
p-PERK	3179	Cell Signaling Technology, Danvers, MA, USA	500
14-3-3	sc-629	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1000
DDIT3	sc-575	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1000
EIF2A	sc-11386	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1000
XBP1	sc-7160	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1000
p-IRE1A	PA1-16927	Thermo Scientific, FL, USA	1000
p-PKR	ab32036	Abcam, Cambridge, UK	500
GM130	610823	BD Transduction Labs, Franklin Lakes, NJ, USA	500
ATF6	ALX-804- 381-C100	Enzo Life Sciences, Farmingdale, NY, USA	250
Calnexin	SPA-865	Enzo Life Sciences, Farmingdale, NY, USA	500
KDEL	SPA-827	Enzo Life Sciences, Farmingdale, NY, USA	1000