

HIF1 α stability in hypoxia is not oxidant-initiated

Running title: Oxidants are dispensable for hypoxic HIF1 α stability

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Impact

The current findings address the redox regulation of hypoxia inducible factor 1 α (HIF1 α) stability in hypoxia by showing that cytosolic, mitochondrial or lipid ROS are not necessary for HIF1 α stabilization.

Abstract

Hypoxic adaptation mediated by HIF transcription factors requires mitochondria, which have been implicated in regulating HIF1 α stability in hypoxia by distinct models that involve consuming oxygen or alternatively converting oxygen into the second messenger peroxide. Here, we use a ratiometric, peroxide reporter, HyPer to evaluate the role of peroxide in regulating HIF1 α stability. We show that antioxidant enzymes are neither homeostatically induced nor are peroxide levels increased in hypoxia. Additionally, forced expression of diverse antioxidant enzymes, all of which diminish peroxide, had disparate effects on HIF1 α protein stability. Moreover, reduction of lipid peroxides by glutathione peroxidase-4 or superoxide by mitochondrial SOD, failed to influence HIF1 α protein stability. These data show that mitochondrial, cytosolic or lipid ROS were not necessary for HIF1 α stability, and favor a model where mitochondria contribute to hypoxic adaptation as oxygen consumers.

Keywords

Hypoxia, Mitochondria, Peroxide, Reactive oxygen species, HIF1 α stability, S-glutathionylation, VHL, HIF PHDs, Oxygen, Ischemia, Cancer, Stroke

Introduction

Over the past decade, our ability to monitor and manipulate reactive oxygen species (ROS) has grown enormously. These technological advances provide a novel view on how ROS interact with cells to modulate function. Specifically, ROS such as peroxide can act as cellular messengers. Messenger functions for ROS reflect their tight spatial control within cells. The tight spatial control of ROS has enabled their critical roles in growth factor signaling, inflammation, and regeneration (Jain et al. 2013, Lei and Kazlauskas 2014, Hameed et al. 2015). Specific signaling roles for ROS are facilitated by the existence of motifs in proteins such as phosphatases that render these proteins specifically susceptible to redox modulation (Bae et al. 1997, Lee et al. 1998, Salmeen et al. 2003).

An important area of biology where ROS signaling has been highly investigated but where no consensus has emerged is hypoxic adaptation. Seminal work from Semenza, Kaelin and Ratcliffe demonstrated that when oxygen tension falls below a critical threshold, that a family of enzymes dependent on oxygen, iron and 2-oxoglutarate known as the HIF Prolyl Hydroxylases (HIF PHDs) decrease their activity, leading to diminished hydroxylation of the alpha subunit of HIF transcription factors (Semenza and Wang 1992, Wang and Semenza 1993, Wang et al. 1995, Maxwell et al. 1999, Epstein et al. 2001, Ivan et al. 2001, Ivan et al. 2002). Diminished HIF1 α hydroxylation decreases recruitment of a key E3 Ubiquitin ligase, the von Hippel Lindau (VHL) protein, which allows HIF1 α to avoid proteasomal degradation. Stabilized HIF1 α dimerizes with its constitutively active partner to bind to hypoxia response elements in a coordinate gene

cassette that leads to hypoxic adaptation at a cellular, local and systemic level (Wang et al. 1995, Wood et al. 1996, Jiang et al. 1996).

Since the mitochondrial electron transport chain (ETC) generates reactive oxygen species via electrons that leak from ETC bind to oxygen to produce superoxide anions, the mitochondrial ROS production is tightly linked with availability of oxygen. Previous studies have shown that the dose response of HIF1 α stability to progressively lower oxygen concentrations required other factors besides oxygen to regulate HIF PHD activity (Chandel et al. 2000, Brunelle et al. 2005, Mansfield et al. 2005, Bell et al. 2007). Using then state of the art tools to monitor ROS, it was shown that during hypoxia, ROS were increasing and that pharmacological tools that nullified this increase in ROS diminished HIF1 α stability (Chandel et al. 2000). A compelling model emerged that hypoxia increases the flux of electrons via Rieske Iron Sulfur cluster proteins in complex III and this leads to an increase in mitochondrial ROS generation via the ubiquinone binding site near the outer aspect of the inner mitochondrial membrane (the Qo site) (Bell et al. 2007). Peroxide generated at this site could then diffuse through the outer mitochondrial membrane to inhibit HIF PHDs via either direct redox modulation of HIF PHDs (Bell et al. 2007) or activation of established redox sensitive p38 MAP kinase signaling (Emerling et al. 2005) to stabilize HIF1 α .

Since its inception, the role of mitochondria established by Chandel and Schumacker in HIF signaling has been validated (Agani et al. 2000, Mansfield et al. 2005, Taylor 2008). However, an alternate ROS-independent view of how mitochondria regulate HIF1 α stability in hypoxia has also been advanced. Pharmacological inhibition of ETC complexes or genetic knockdown of Rieske Fe/S proteins or cytochrome c not only inhibits mitochondrial ROS production (Brunelle et al. 2005, Chandel et al. 2000, Mansfield et al. 2005, Bell et al. 2007), it

also inhibits oxygen consumption. In this distinct scheme, inhibition of ETC function would alter the kinetics of availability of cytosolic oxygen levels. Indeed, several groups have provided data supporting the importance of respiratory chain driven mitochondrial oxygen consumption in dictating cellular oxygen gradients. These gradients have been hypothesized to decrease oxygen concentrations to levels required to inhibit enzymatic activity of oxygen dependent HIF PHDs-the key upstream regulator of HIF1 α stability (Doege et al. 2005, Hagen et al. 2003, Chua et al. 2010). There are a number of pathological conditions such as cerebral ischemia, acute kidney disease, ischemic heart disease, neonatal hypoxia-ischemic brain injury, cancer, and pulmonary arterial hypertension where both HIF1 α and ROS have been reported to be important players (Chen et al. 2011, Chen et al. 2012, Weidemann et al. 2008, Tomsa et al. 2019, Semenza 2014, Kibel et al. 2020, Sheldon et al. 2014, Qin et al. 2019, Semenza 2012, Sosa et al. 2013, Bryant et al. 2016, Demarco et al. 2010). However, the molecular interplay between ROS and HIF1 α are still not very clear. For instance, in ischemic stroke condition, ischemia-reperfusion injury leads to an increase in both ROS and HIF1 α . While HIF PHD inhibitors, which stabilizing HIF1 α and activate its downstream pathway, have shown significant benefit in providing the neuroprotection, decreasing ROS levels with antioxidants (which would decrease HIF1 α signaling if we consider ROS as an upstream regulator of HIF1 α stability) has also shown benefits from cerebral ischemia (Green and Shuaib 2006). This prevents from us from choosing ROS as a good clinical target for protection from ischemia-reperfusion injury. Thus, understanding the role of ROS in HIF1 α mediated adaptation has numerous clinical implications (Weidemann et al. 2008, Conde et al. 2012, Semenza 2014, Sheldon et al. 2014, Semenza 2012, Bryant et al. 2016).

In this manuscript, we leverage a host of complementary approaches that support the conclusion that peroxide is dispensable in regulated HIF1 α stability in hypoxia. Unexpectedly, our results suggest that HIF1 α stability in hypoxia is not oxidant-initiated.

Results

Mitochondria is involved in hypoxic HIF1 α stabilization in SH-SY5Y human neuroblastoma cells

In the current study, we leveraged the benefit of HIF-luciferase reporter as a surrogate marker of changes in endogenous HIF1 α protein stabilization in hypoxia. We first established the sensitivity and dynamic range of HIF-luciferase reporter. The HIF-luciferase reporter contains the oxygen dependent domain (ODD) of HIF1 α fused to luciferase (ODD-luciferase). We have previously demonstrated that this reporter behaves like endogenous HIF but does not influence endogenous HIF activity (Smirnova et al. 2010, Karuppagounder et al. 2013). We confirmed the same using adaptaquin (AQ), which we have previously established as a specific HIF PHD inhibitor (Karuppagounder et al. 2016). We found that endogenous HIF1 α protein levels could be monitored dynamically across a wide range of AQ concentrations (Figure 1A and 1B). As an independent measure, we also assayed the activity of HIF1 α -luciferase reporter by measuring luciferase activity. These assays showed that the HIF1 α -luciferase reporter measured by photometric luciferase activity possesses a high dynamic range and low coefficient of variation (Figure 1C). Moreover, we also assessed change in endogenous HIF transcriptional activity by measuring changes in the mRNA levels of HIF target genes, *Bnip3* and *Enolase 2*. We found dose dependent dynamic changes in the mRNA levels of HIF target genes, *Bnip3* and *Enolase 2* in response to increasing concentrations of AQ (Figures 1D and 1E). Indeed, changes in

HIF1 α -luciferase activity measurements with increasing doses of AQ showed a strong correlation with quantitative changes in endogenous HIF1 α protein levels measured by quantitative immunoblotting (Figure 1F) as well as quantitative changes in endogenous mRNA levels of HIF target genes, *Bnip3* and *Enolase 2* (Figures 1G and 1H). These findings confirmed ODD-luciferase activity as a sensitive and specific HIF1 α reporter.

Mitochondria has been previously shown to be involved in hypoxic HIF1 α stabilization in different cell lines (Chandel et al. 2000, Chua et al. 2010, Yang et al. 2012). However, we wanted to confirm if the same is true in SH-SY5Y human neuroblastoma cells. To this end, we treated SH-SY5Y cells with different mitochondrial electron transport chain (ETC) inhibitors such as rotenone (ETC complex I inhibitor), myxothizol (ETC complex III inhibitor), antimycin A (ETC complex III inhibitor), and sodium azide (ETC complex IV inhibitor) and exposed cells to normoxia or hypoxia in parallel for 4 hours and thereafter assessed ODD-luciferase activity in cell lysates. We found a dose dependent decrease in ODD-luciferase activity in response to increasing concentrations of all of the ETC complex inhibitors. These findings confirmed mitochondrial involvement in hypoxic HIF1 α stabilization SH-SY5Y cells.

Antioxidant enzymes are not homeostatically induced in hypoxia

Increases in ROS that are sufficient for signaling or toxicity trigger homeostatic transcriptional increases in antioxidant enzymes (Christman et al. 1989). To assess whether hypoxia results in similar homeostatic increases in antioxidant protein expression, we exposed human neuroblastoma (SH-SY5Y) cells to hypoxia for 8 hours and measured protein expression levels of peroxisomal, cytosolic and mitochondrial antioxidant enzymes including catalase, glutathione peroxidase-1 (GPX1), glutathione peroxidase-4 (GPX4), MnSOD, and

Peroxiredoxin-3 (PRDX3) (Figure 2A). The 8 hour time point was chosen to monitor homeostatic changes in antioxidant enzymes because this would be 6 hours following observable HIF1 α stability in hypoxia in SH-SY5Y cells, which provides adequate time for homeostatic increases to initiate transcriptional or post-transcriptional adaptations. At the 8 hour time point, the protein level of the peroxisomal antioxidant catalase, and antioxidants present in both cytosol and mitochondria, such as GPX1 and GPX4, showed no change in hypoxia with normalization to Actin (Figures 2B and 2C). However, since hypoxia induces HIF1 α -dependent mitophagy (Aminova et al. 2008, Zhang et al. 2008), mitochondrial mass is decreased with increasing duration of hypoxia, including decreases in mitochondrial DNA and proteins. Accordingly, we normalized distinct mitochondrially-targeted antioxidant enzymes to the level of citrate synthase, a mitochondrial protein. When normalized to citrate synthase, the expression levels of mitochondrial antioxidants, such as MnSOD and PRDX3, also did not change in hypoxia (Figures 2D and 2E). To establish whether these findings apply to non-transformed cells, we studied expression levels of antioxidant enzymes in hypoxia in primary neurons. Similar to neuroblastoma cells, antioxidant enzyme levels did not change in hypoxia in post-mitotic neurons (Figures 2F-2I). Although 8h hypoxic exposure did not show changes in various antioxidants, it is likely that levels of antioxidants might have changed before 8h time point. In order to explore this possibility, we assessed changes in above mentioned antioxidants for different time points such as 2h, 4h, and 8h. However, we again did not see changes in the protein levels of these antioxidants at any time point (Figure S1). Another alternative possibilities for not seeing changes in these antioxidants could be either not looking at right antioxidant(s) or changes in ROS levels being too small to induce detectable homeostatic changes in these antioxidants. Together, these indirect measures suggest that possible increase in ROS levels

during hypoxia are less likely. To further confirm if ROS levels increase in hypoxia or not, we directly measured real time changes in ROS levels in hypoxia by using a sensitive ROS marker, HyPer.

Peroxide levels do not increase during hypoxia

HyPer is a fusion protein composed of the peroxide-sensitive domain of the prokaryotic transcription factor, OxyR, and yellow fluorescent protein that is a reporter for cellular peroxide (Belousov et al. 2006). This reporter is not only sensitive and specific, but its activity is also ratiometric, which factors out differences in fluorescence due to cell geometry, path length, and reporter concentration. We have previously shown that enhanced pH buffering of the extracellular medium alleviates the putative effects of acidic pH during hypoxia on the reporter, and that fluorescent ratios can be calibrated in cells to known peroxide concentrations (Neal et al. 2016).

To monitor changes in the level of hydrogen peroxide (H_2O_2), we used real-time imaging of rat pancreatic islet cells in hypoxia with strong pH buffering. Pancreatic islet cells, rather than neurons or neuron-like cells, were used for initial analysis because mitochondrial peroxide in pancreatic islet cells increases in response in extracellular glucose levels, as measured using HyPer imaging. Elevated glucose, therefore, can be used as a positive control for mitochondrial peroxide increases. Additionally, elevated glucose increases insulin release in beta islet cells, which enables insulin release assays to establish cell viability if there is an absence of peroxide changes during hypoxia. Similar real-time measures of cell function are not readily available in primary neurons or SH-SY5Y neuroblastoma cells. Prior studies have also established that glucose-induced peroxide formation is derived from the mitochondria. Specifically, increased expression of mitochondrial catalase decreased glucose-induced HyPer signals (catalase

scavenges peroxide), as did decreased expression of mitochondrial SOD (MnSOD; the enzyme that converts superoxide to peroxide) (Neal et al. 2016). Finally, signaling levels of peroxide measured by HyPer are 1/20th those of peroxide levels required for toxicity (Neal et al. 2016), which demonstrates the sensitivity of peroxide measurements using HyPer in this cell type. Before assaying peroxide in hypoxia, we verified that two hours of hypoxia induced the established HIF1 α target genes *Kdm6b* and *Bnip3* in islet cells in our flow culture system (Figures 2J and 2K) (Choudhry and Harris 2018). This time point was selected to evaluate the role of peroxide in mediating the earliest changes in HIF1 α stability in hypoxia because it is well before mitochondrial autophagy is induced (Figure 2).

Accordingly, based on the sensitivity and specificity of the reporter assay, we were confident that mitochondrial peroxide was measureable in islet cells if it were increased in hypoxia. H₂O₂, NAD(P)H, and insulin secretion rates were measured simultaneously as a function of glucose concentration for two hours under hypoxic conditions (1% O₂) (Figure 2L). Increasing glucose concentration from 3 mM to 20 mM elicited expected increases in peroxide, NADPH levels, and insulin secretion rates under normoxia. When islet cells under the same glucose concentrations (20 mM) were exposed to 1% oxygen, H₂O₂ decreased by more than 80%. This reduction occurred in the midst of increased NAD(P)H levels. NADP(H) likely increased due to diminished utilization by the mitochondrial electron transport chain. Glucose-stimulated insulin secretion decreased by about 50% in response to hypoxia, but remained well above unstimulated rates, indicating that islets remained functional throughout the study. Indeed, restoring steady state O₂ levels (20%) resulted in expected increases in peroxide and insulin secretion with concomitant decreases in NAD(P)H, which indicates hypoxia delivered under the conditions of our experiments is not toxic to islet cells (Figure 2L).

To establish the generalizability of these findings to other cell types, we measured HyPer reporter fluorescence ratios in hypoxic condition in SH-SY5Y and Hep3B human hepatocellular carcinoma cells. Ratiometric imaging of both cell types showed no change in peroxide levels following two hours of hypoxia (Figures S2C and S2F), which was sufficient time to activate HIF1 α -dependent gene expression. This absence of changes in peroxide levels during hypoxia could not be attributed to a lack of HyPer reporter responsiveness to peroxide in these cell types since exogenous addition of peroxide (Figure S2G) following the hypoxic exposure led to expected, significant increases in reporter activity. We confirmed the hypoxic condition by measuring changes in mRNA levels of HIF target genes, *Bnip3* and *Enolase 2* in SH-SY5Y cells (Figures S2A and S2B) and Hep3B cells (Figures S2D and S3E). Together with our antioxidant protein expression results, these data suggest that mitochondrial peroxide either decreases or is unchanged by hypoxia in primary (pancreatic beta islets) and transformed cell types (SH-SY5Y, Hep3B), respectively.

HIF1 α stabilization is not oxidant-initiated in hypoxia

Prior studies showed that decreasing ROS by forced expression of individual antioxidant enzymes can decrease HIF1 α protein levels (Brunelle et al. 2005, Chandel et al. 2000). To confirm these findings with our HIF1 α -luciferase reporter, we forced expression of either catalase (a peroxide scavenger), GPX1 (a peroxide scavenger), or MnSOD (a superoxide scavenger and peroxide generator) (Figure 3A). SH-SY5Y cells expressing ODD-Luc were transduced with individual antioxidant enzymes encoded in distinct adenoviral constructs or an adenovirus encoding GFP only as a protein control. Seventy-two hours following infection, GFP

expression was observed in nearly 90% of SH-SY5Y cells (Figures S3A and S3B). Accordingly, cells were tested for specific enzyme activities of MnSOD, Catalase, or GPX1. These studies showed a seven-, eleven-, and three-fold increase in specific activity over GFP controls for MnSOD-, Catalase-, and GPX1-expressing cells, respectively (Figures S4F – S4H). Moreover, using a cell death assay with DNA binding Sytox blue dye (dead cell stain), we also confirmed the activities of these antioxidants under normoxia and hypoxia by indirectly assessing the protection conferred by these antioxidants from increasing concentrations of exogenous H₂O₂ treatment. As expected, catalase and GPX1 overexpression significantly enhanced protection under both normoxia and hypoxia while MnSOD did not improve protection as compared to GFP control (Figures S4D -S4E). As a final test of whether overexpression of these adenoviral constructs of antioxidants decrease oxidant production in SH-SY5Y cells or not, we measured 5, 6 carboxydichlorofluorescein fluorescence (a non-selective redox sensitive reporter) using flow cytometry. These experiments confirmed the ability of GPX1, PRDX3, and catalase to decrease steady state DCF oxidation, presumably resulting from oxidants generated physiologically (Figures S5A and S5B). However, there was no significant change in peroxide level in response to MnSOD expression despite a significant increase in the MnSOD enzyme activity and protein level. This was unexpected and could reflect compensatory activation of other antioxidants such as GPX1, GPX4 or Prdx3 in response to increased MnSOD activity. We verified that DCF loading and corresponding antioxidant effects were not different from normoxic or hypoxic cells, arguing against the possibility that our redox reporter or the antioxidant enzymes are behaving differently in normoxia and hypoxia (Figures S5D and S5E). Importantly, Chandel and other groups had added DCF before exposing cells to hypoxia. But, there was a concern that if changes in DCF signal are because of differential accumulation of DCF in hypoxic cells

as compared to normoxic cells or because of an increased oxidant signaling. To minimize the possibility of increased DCF accumulation during hypoxic exposure, we added DCF after exposing cells to normoxia/hypoxia. With this protocol, we found decrease in DCF signal in control GFP expressing cells with hypoxic exposure compared to control GFP expressing cells with normoxic exposure. Additionally, we did not find an increase in DCF signaling with H₂O₂ treatment in cells, which were exposed to hypoxia. This could be because of either decrease in ROS level during hypoxia, which likely increased with H₂O₂ to the level of GFP control under normoxia but didn't go further up or because of increased scavenging capacity of SH-SY5Y cells under hypoxia.

Having verified increases in individual antioxidant activities (MnSOD, GPX1, and Catalase) of overexpression antioxidant constructs, we examined the effect of these manipulations on HIF1 α stability. The mitochondrial peroxide model of HIF regulation predicts that MnSOD should increase HIF1 α stability in hypoxia, whereas GPX1 and catalase should diminish HIF1 α stability. In contrast to these predictions, we found that MnSOD had no effect on HIF1 α luciferase reporter activity, while Catalase increased reporter activity, and GPX1 decreased it (Figure 3B). To verify that our HIF1 α luciferase reporter accurately reflected endogenous HIF1 α levels, we performed quantitative fluorescence immunoblotting. These assays showed changes in endogenous HIF1 α protein are similar to those identified with the HIF1 α reporter (Figure 3C and 3D). From these findings, we conclude that HIF1 α levels are not correlated with mitochondrial peroxide production. Since catalase is a peroxisomal enzyme and GPX1 localizes to the cytosol and mitochondria, our results could not formally exclude the possibility that GPX1 localization to the mitochondria allowed it to decrease HIF1 α levels, while Catalase's inability to penetrate this compartment did not allow it to decrease HIF1 α levels. To address this possibility, we forced expression of PRDX3, a member of the peroxiredoxin family

of antioxidant enzymes that functions as a thioredoxin-dependent peroxide reductase in the mitochondria. Contrary to GPX1, PRDX3 increased levels of HIF1 α in hypoxia as measured by either HIF1 α luciferase reporter activity (Figure 3E) or quantitative fluorescence immunoblotting (Figures 3F and 3G). We also confirmed these findings in primary neurons (Figures S6A –S6D), where adenoviral constructs also effectively increased expression of the antioxidant enzymes (Figures S3C and S3D).

Since HIF1 α is a transcription factor, we asked if antioxidant led changes in HIF1 α protein levels correlate with a change in HIF-dependent transcription in neuroblastoma SH-SY5Y cells by examining *Enolase2* and *Bnip3* mRNA expression levels, which are two established HIF1 α target genes (Aminova et al. 2005, Poitz et al. 2014) . As expected, changes in *Enolase2* and *Bnip3* mRNA levels correlated with HIF1 α luciferase reporter levels (Figures 3H-3M).

Our findings did not exclude the possibility that antioxidants alter HIF1 α protein levels by differentially regulating either *Hif* mRNA synthesis or *Hif* mRNA stability. Accordingly, we monitored *Hif1 α* mRNA levels in the cells overexpressing Catalase, GPX1, and PRDX3. Quantitative PCR revealed that *Hif* mRNA levels were not changed in a manner that would contradict the observed ROS-independent changes in HIF1 α stability (Figures 4A and 4B). To confirm that changes observed in HIF1 α protein stability are related to changes in its half-life, we examined the stability of HIF1 α protein in the presence of cycloheximide, which suppresses de novo protein synthesis in cells pre-treated either with or without MG132. As expected, we found that Catalase and PRDX3, which increase HIF1 α protein levels, also increased HIF1 α half-life (Figures 4C, 4D and 4E). By contrast, GPX1, which diminished HIF1 α protein levels, decreased HIF1 α half-life (Figure 4D). Moreover, MG132 treatment significantly enhanced Hif-

1 α half-life in all cases showing that the antioxidant led changes in hypoxic Hif-1 α stabilization were the results of alterations in proteasomal degradation.

To establish whether our findings in primary neurons and neuroblastoma cells can be extended to non-neural cell types, we examined the ability of antioxidant enzymes capable of modulating peroxide levels to modulate HIF1 α stability in hypoxic Hep3B hepatocarcinoma cells and hypoxic HeLa cervical cancer cells. Forced expression of Catalase, GPX1, or PRDX3 using adenoviral vectors significantly increased in protein levels of each of the antioxidant enzymes in Hep3B or HeLa cells (Figures S7 and S8). Similar to primary neurons or neuroblastoma cells, we did not observe a uniform reduction in HIF1 α stability in hypoxia by enzymes whose common activity is to decrease peroxide in these non-neural cell types (Figures S7 and S8). Altogether, our findings suggest that peroxide levels are uncoupled from HIF1 α stability in neural and non-neural cells.

Neither Reactive Oxygen Species nor Reactive Lipid Species regulate HIF-1 α stability in hypoxia

Recent compelling evidence showed that reactive lipid species (RLS) are sufficient to drive HIF-dependent transcription via their effects on FIH inhibition without affecting HIF1 α stability (Masson et al. 2012). Accordingly, we forced expression of GPX4, a selenoprotein that neutralizes RLS (Figure 6A). To manipulate steady-state levels, we used GPX4 protein fused to an optimized destabilization domain (dd) from the prokaryotic dihydrofolate reductase gene. The dd domain destabilizes GPX4 protein unless trimethoprim (TMP, 10 μ M) is present (Figure 6B). TMP enhanced GPX4 levels in neuroblastoma cells expressing ddGPX4, but increasing GPX4 levels had no effect on hypoxia-induced HIF1 α stability (Figures 6C - 6E) or HIF1 α -dependent transcription (Figures 6F and 6G). We verified that GPX4 diminished RLS (Figure S5C) and

neutralized ferroptosis induced by glutamate, a form of cell death mediated by reactive lipid species that is abrogated by GPX4 (Figures 6H and 6I) (Tan et al. 1998). Altogether, these findings argue against a central role for hydrogen peroxide or lipid peroxides in mediating HIF1 α stabilization in hypoxia.

Discussion

Seminal studies have supported the notion that mitochondria are essential regulators of hypoxic adaptation possibly acting via their ability to generate peroxide (Agani et al. 2000, Chandel et al. 1998). In this paper, we show that HIF1 α stability mediated by HIF PHDs during hypoxia does not require peroxide. These data include our inability to detect an increase in peroxide during hypoxia (Figure 2L); the lack of homeostatic changes in antioxidant protein expression during hypoxia (Figures 2B-21I and Figure S1); and the failure of forced expression of antioxidant enzymes (Catalase, GPX1, and PRDX3) with the common ability to diminish cellular peroxide to influence HIF1 α stability and transcription in the same direction (Figure 3). Our findings agree with prior studies that showed: 1) that HIF PHDs are not inhibited by exogenously added peroxide (Chua et al. 2010), and 2) that forced expression of an alternative oxidase which directly transfers electrons from coenzyme Q to oxygen to form water maintains HIF1 α stability in hypoxia, despite reducing superoxide generation at Complex III (Chua et al. 2010). Our results cannot be attributed to differences in mitochondrial ROS generation by transformed versus primary cells, or to differences in neuron-like versus non-neural cells, as SH-SY5Y neuroblastoma cells and primary cortical neurons showed similar effects, as did Hep3B hepatocellular carcinoma cells and HeLa cervical cancer cells. While we cannot exclude the

possibility that culture conditions such as serum lots could reconcile our results with prior studies, in aggregate, the findings favor a role for mitochondria in modulating HIF1 α stability via their effects as oxygen consumers not as peroxide second messenger generators.

Peroxide scavengers have distinct effects on hypoxic HIF1 α stability

Prior studies have shown that in some cell types, ROS generation in hypoxia could be related to increased oxidant production or decreased defenses (Naranjo-Suarez et al. 2012). In order to address that the imbalance of oxidants and antioxidants plays the regulatory role in mediating hypoxia signaling, we forced expression of distinct antioxidant enzymes known to either decrease (GPX1, Catalase, and PRDX3) or enhance peroxide levels (MnSOD). Despite evidence for increased activity of the antioxidant enzymes studied using multiple experimental approaches, we found that HIF1 α stability and transcription did not correlate with effects on peroxide levels in neuroblastoma cells and primary neurons (Figures 3 and S6). Similar uncoupling was observed in non-neural cell types as well (Figures S7 and S8). The results uncouple peroxide generation in the mitochondria from HIF1 α stability via the HIF PHDs. It is likely that the GPX1, catalase, and PRDX3 influenced HIF1 α stability either via indirect but distinct effects on oxygen consumption, or alternatively via differential but direct effects on proteins that influence HIF1 α regulation.

HIF1 α stability under hypoxia is not regulated by reactive lipid species (RLS)

Recent studies have highlighted the potential role that RLS could play in regulating HIF transcription rather than HIF1 α stability. Accordingly, we forced expression of GPX4, a selenoprotein with known ability to neutralize reactive lipid species. Despite being active in combating RLS mediated ferroptotic death in transformed cells (Figure 6I), GPX4 had no effect on HIF1 α stability in hypoxia (Figures 6C – 6E) or HIF-dependent transcription (Figures 6F and

6G). These data suggest that while some RLS (e.g. tert-butyl hydroperoxide) are sufficient to activate HIF transcription, they are not necessary to stabilize HIF1 α or drive HIF1 α -dependent transcription in hypoxia.

Methods

Cell lines and In Vitro Tissue Culture Studies

Immature primary cortical neurons were isolated from CD1 mice embryos (embryonic day 15 [E15]) as previously described (Ratan et al. 1994) by following the protocol approved by IACUC at Weill Cornell Medicine. SH-SY5Y human neuroblastoma cells (purchased from ATCC) were cultured in DMEM/F-12 plus GlutaMAX medium added with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). HeLa cells and Hep3B cells (purchased from ATCC) were cultured in EMEM medium added with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

Islets were harvested from Sprague–Dawley male rats (~250g, Envigo, Huntingdon, Cambridgeshire, United Kingdom) anesthetized by an intraperitoneal injection of sodium pentobarbital (35 mg/230g rat). All procedures were approved by the University of Washington Institutional Animal Care and Use Committee. Islets were prepared and purified as previously described (Sweet et al. 2004) and then cultured at 37°C in RPMI Media 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) for specified times with the adenovirus coding the H₂O₂-sensitive dye (HyPer).

Adenoviral transduction and normoxia / hypoxia exposure

Adenoviral constructs of MnSOD, catalase, GPX1 and respective GFP control were purchased from University of Iowa, Viral Vector Core Facility. Adenoviral construct of ddGPX4 (GPX4 with destabilization domain) and the respective GFP control were obtained from ViraQuest, Inc. (North liberty, IA) and adenoviral constructs of PRDX3, GRX1 and respective GFP control were obtained from Vector Biolabs. For GPX4 construct, we took leverage of a novel technique recently developed by Thomas Wandless group (Iwamoto et al. 2010) to deliberately regulate the level of expression of a protein of interest. Adenoviral construct of ddGPX4 had an *E. coli* dihydrofolate reductase (ecDHFR) mutant (it was called as degradation domain) fused to its CMV promoter which displays Trimethoprim (TMP) dependent stability. Because of the fusion of degradation domain to the GPX4 promoter, GPX4 also displayed TMP dependent stability. Without TMP, GPX4 was rapidly degraded completely through proteasome but with increase in dose of TMP, GPX4 stability increases. Treatment of ddGPX4 expressing cells with 10 μ M TMP for 12h stabilized ddGPX4 very well. SH-SY5Y cells, HeLa cells or Hep3B cells were transduced with different adenoviral constructs at 500 MOI (Multiplicity of infection) incubated for 72h and primary immature cortical neurons (PCNs) were transduced with different adenoviral constructs at 100 MOI (Multiplicity of infection) for 48h. The maximal expression of these constructs was determined by expressing adenoviral construct of GFP at 500 MOI for 72h in SH-SY5Y cells and 100 MOI for 48h in PCNs, respectively, on slides and staining them with GFP antibody (Abcam, ab6556). 10 μ M TMP was added to ddGPX4 expressing cells and their respective control GFP expressing cells after 60h of adenoviral incubation for 12h in order to get stabilized GPX4 expression. Parallel sets without transduction of adenoviral constructs but probed with same GFP antibody were used as respective negative controls in each in-vitro models (Figure S3). Thereafter, one set was kept in normoxia (21% oxygen) and a parallel set in hypoxia (1%

oxygen) for 4h. For the study of changes in endogenous antioxidants, SH-SY5Y cells and primary cortical neurons were exposed to normoxia/hypoxia for a relatively longer time period, 8h so that we could visualize more clear difference under normoxic and hypoxic conditions.

In order to study real time changes in hydrogen peroxide in hypoxia condition, the adenovirus containing the cytosolic H₂O₂ sensor, pHyPer-cyto vector (FP942, Evrogen, Moscow, Russia) (Belousov et al. 2006) was generated by Vector Biolabs (Malvern, PA) as previously described (Karamanlidis et al. 2013). The H₂O₂ sensor was transduced in intact islets during incubation in RPMI media supplemented with 10% heat-inactivated fetal bovine serum and the adenoviruses at 100 MOI for 3 days at 37°C as previously optimized (Neal et al. 2016). SH-SY5Y cells and Hep3B cells were also transduced with the H₂O₂ sensor at 100 MOI in a similar manner.

Real-time epifluorescent imaging of intracellular H₂O₂

Real-time imaging experiments were carried out while islets were perfused using a commercially available temperature-controlled Bioptechs FCS2, a closed system, parallel plate flow chamber (Butler, PA) as previously described (Neal et al. 2016). After the islets were loaded into the perfusion chambers, the chamber was sealed and mounted onto the stage of a Nikon Eclipse TE-200 inverted microscope. KRB was pumped through the perfusion chamber at flow rates of 120µl/min using a Masterflex L/S peristaltic pump (Cole-Parmer, Vernon Hills, IL). Use of an artificial gas exchanger positioned on the inflow side of the perfusion chamber enabled rapid changes in the concentrations of dissolved oxygen by switching the source tank of gas between tanks containing 21% and 1% oxygen (balance 5% carbon dioxide and nitrogen)(Sweet et al. 2002). The HyPer signal was generated by dual fluorescence excitation via a xenon arc lamp (Lambda LS-1620, Sutter Instrument Company, Novato, CA) through either a 405/30nm or a

480/40nm bandpass filter and detected at 510nm through a longpass dichroic mirror with a cutoff below 500nm. The images were taken using a digital camera (Photometrics Cool Snap HQ2 CCD camera, Tucson, AZ) through a 40X Super Fluor Nikon objective (DIC H/N2). Data were expressed ratiometrically, where the excitation intensities at 480nm were divided by those obtained during excitation at 405nm. Similar procedure was used for real time monitoring of H₂O₂ in SH-SY5Y cells and Hep3B cells. In order to further confirm the specificity of HyPer signals, we treated Hep3B cells with the bacterial Streptolysin-O (which creates pores in the cell membranes) to selectively permeabilize the plasma membrane followed by their exposure to increasing concentrations of exogenous H₂O₂. Real time measurement of changes in the HyPer signals in response to exogenous addition of H₂O₂ confirmed the specificity of HyPer signal with regard to H₂O₂.

Real-time epifluorescent imaging of intracellular NAD(P)H

NAD(P)H autofluorescence was measured similarly to H₂O₂, except there was no need for loading with dye, and the excitation and emission wavelengths were 360 and 460nm, respectively [as previously described (Gilbert et al. 2008)]. To calibrate the relative fluorescence units (RFU), at the end of experiments, the steady state RFU in the presence of potassium cyanide (KCN) and, subsequently, FCCP was measured. The normalized fluorescence of NAD(P)H was then calculated as follows,

$$\% \text{ Reduced NAD(P)H} = 100 * (\text{RFU}_{\text{test}} - \text{RFU}_{\text{FCCP}}) / (\text{RFU}_{\text{KCN}} - \text{RFU}_{\text{FCCP}}) \quad (\text{Eq. 1})$$

where RFU_{FCCP} and RFU_{KCN} equals the average of the final 10 time points during which each agent was present.

Real-time measurement of insulin secretion rate

Outflow fractions from the flow system containing islets were collected in a fraction collection for

subsequent measurement of insulin [described previously (Sweet et al. 2004, Sweet et al. 2002). Insulin secretion rate was calculated as the flow rate (80ul/min) times the insulin concentration in the outflow Fractions, over the number of islets in the chamber [typically 50 (Sweet and Gilbert 2006)]. Insulin was measured by radioimmunoassay (RI-13K, EMD Millipore, Darmstadt, Germany) as per the manufacturer instructions.

ODD-luciferase activity assaay

The ODD-luciferase construct with pcDNA3.1 plasmid vector was constructed as previously described (Safran et al. 2006). The proline p402 and p564 present within the oxygen degradation domain (ODD) of HIF1 α , when hydroxylated by HIF-PHDs, allow its binding to the VHL protein that target it for proteasomal degradation. In this way, the stabilization of ODD can be used as a marker of HIF1 α stability (Safran et al. 2006, Smirnova et al. 2010). Because of the luciferase tagged with ODD, the increase in ODD stability leads to a proportional increase in the luciferase activity and this provides a very good way of measuring the HIF1 α stability in a quantitative manner with a wide dynamic range. To this end, we used SH-SY5Y cells stably expressing ODD-luciferase. These cells were made by co-transfecting ODD-luciferase plasmid along with a puromycin resistance plasmid in SH-SY5Y cells and stably transfected cells were positively selected in presence of 4 μ g/ml of puromycin. Luciferase activity was measured by luciferase assay kit (promega) using an LMaxII™ microplate luminometer (molecular Devices). ODD-luciferase activity was normalized to the protein content of each well measured by Bio-Rad DC™ protein assay kit.

Gene expression study

Total RNA was prepared from SH-SY5Y cells using the Nucleospin RNA kit (MACHEREY-NAGEL) and following their protocol. Real time PCRs were performed as a duplex reaction using

FAM labeled *Enolase2* (Human - Hs00157360_m1), *Bnip3* (Human - Hs00969291_m1), and *Hif1 α* (Human - Hs00153153_m1) gene expression assays (Thermo Fisher Scientific) and VIC labeled *human β actin* endogenous control probe (Human - 4326315E) or *RNA28S5* (Human - Hs03654441_s1) (Thermo Fisher Scientific) so that amplified mRNA can be normalized to *β actin* or *RNA28S5*. These experiments were performed using a 7500 Rea-time PCR system (Applied Biosystems) using standard PCR protocol and amplification conditions.

In order to measure gene expression in pancreatic islets exposed to normoxia or hypoxia, islets were first placed in incubators containing either 21% or 1% oxygen for 2h. At the end of this time, islets were lysed and total RNA was purified using RNeasy Mini Kit (Qiagen, Hilden Germany). *Bnip3*, *Kdm6b* and *rat Actin B* mRNA were measured by quantitative PCR using FAM labeled *Bnip3* (Rat - Rn00821446_g1), *Kdm6b* (Rat - Rn01471506_m1) gene expression assays and VIC labeled *rat β actin* endogenous control probe (Rat - 4352340E), all purchased from Thermo Fisher Scientific. These experiments were performed on Mx3005P® Multiplex QPCR System (Stratagene, La Jolla, CA) with samples loaded in triplicate using ~ 100ng of total RNA.

Enzyme activity assay

For GPX1, catalase, and MnSOD activity assay, cells in each sample expressing respective adenoviral constructs were collected, lysed and used for respective enzyme activity assay following the protocols of GPX1, catalase, and SOD assay kit from Biovision. Total protein was measured using the Bio-Rad DC protein assay kit. The enzyme activity was normalized to the protein concentration for each sample.

Cell viability assay

In order to test the functional activity of GPX4, immature primary cortical neurons (E15) were isolated from mice embryos and plated at 10^6 cells/ml in 96 well plate. The next day, cells were

transduced with GPX4 adenoviral constructs at 100 MOI. After 24h incubation, cells were treated with glutamate analog, homocysteic acid (HCA) (5mM) which inhibits Xc⁻ transporter, thereby inhibiting cysteine uptake which leads to glutathione depletion and increase in intracellular oxidative stress. Cells were also treated with 10µM TMP at the same time to stabilize GPX4 protein. The next day, cell viability was assessed by the MTT assay (Promega) to understand whether ddGPX4 is functionally active to show its protective effect by decreasing oxidants under oxidative stress.

Cell death assay

SH-SY5Y ODD-luc cells overexpressing different adenoviral constructs of antioxidants were treated with increasing concentrations of H₂O₂ (1mM – 10mM) and then were exposed to normoxia or hypoxia in parallel for 4 hours and then plates were taken out and media was removed gently and 1xPBS containing 10µM Sytox blue dye (Nucleic acid stain that easily penetrates cells with compromised membranes and binds to DNA) was added in each well and incubated at room temperature for 15 min and then fluorescence was measured using fluorescence plate reader with excitation/emission maxima at 440/480 nm.

ROS measurement through DCF flow cytometry

To measure changes in ROS levels, molecular probe DCFDA (2', 7' – dichlorofluorescein diacetate) was used. After exposing parallel sets of SH-SY5Y ODD-Luc cells to either normoxia or hypoxia for 4 h, media was removed, cells were washed with 1xPBS, and then 1xPBS with 20µM DCFDA was added in each well. Additional parallel sets of cells from both conditions were also treated with 5mM H₂O₂ at the same time and were used as positive controls. Plates from both normoxia and hypoxia were then incubated at room temperature for 30 min in normoxic condition. DCFDA diffuses through the cell membrane and is deacetylated by intracellular

esterases to a non-fluorescent form which is later oxidized by ROS into 2', 7' - dichlorofluorescein (DCF) which is a highly fluorescent compound. After incubation, fluorescence was measured through flow cytometry at the wavelength of excitation, 485nm and emission, 535nm, respectively. Production of ROS was measured as mean fluorescence index multiplied by respective cell counts and was expressed as fold change with respect to control.

Immunoblotting

Protein extracts were prepared using 1% triton buffer containing protease inhibitor cocktail, MG132, PMSF, and DTT and were separated by SDS-PAGE, transferred onto nitrocellulose membrane and probed with antibodies against GFP (Cell Signaling Technology; 2555), MnSOD (Sigma-Aldrich; HPA001814), catalase (Sigma-Aldrich; C0979), GPX1 (Cell Signaling Technology; 3286S and Novus Biologicals; NBP1-33620), GPX4 (LSBio; LS B1596), PRDX3 (Novus Biologicals; NBP2-19777), Luciferase (Santa Cruz Biotechnology; sc-74548), HIF1 α (Novus Biologicals; NB100-479 and NB100-105), Citrate synthase (Cell Signaling Technology; 14309S) antibodies were used for immunoblotting. Nuclear-cytoplasmic fractionation was done using NE-PER Nuclear and Cytoplasmic Extraction kit (ThermoFisher Scientific, Catalog number: 78835) and nuclear fraction was used for the immunoblotting of HIF1 α using the monoclonal antibody (NB100-105) while the whole cell extract was used for the immunoblotting of HIF1 α using polyclonal antibody (NB100-479) and all other immunoblots.

Quantification and Statistical Analysis

All experiments were performed as at least three independent sets and data were displayed as means \pm standard deviation (SD). Statistical significance were assessed in GraphPad Prism using either Student's t tests to compare values between two specific groups or one-way ANOVA followed by Dunnett's post-hoc test/Tukey's Post-hoc test to compare the values of more than

two groups or two-way ANOVA followed by Bonferroni's post-hoc test to compare the values of two groups under two different conditions at a given time. Statistical details for each figures can be found in their respective figure legends. The p value of 0.05 or less was considered statistically significant in all of statistical analyses.

Online Supplemental material

Fig. S1 shows that hypoxic exposure of SH-SY5Y cells for different time periods such as 2h, 4h, and 8h do not induce homeostatic increase in antioxidant enzymes. Fig.S2 provides additional evidence in SH-SY5Y cells or Hep3B cells that hypoxia does not increase peroxide levels. Fig. S3 shows a validation of the degree of expression of transduced transgenes encoded within adenoviral vectors in SH-SY5Y cell and primary cortical neurons (PCNs) using adenoviral particles encoding GFP. Fig. S4 shows the validation of the enzyme activities of various antioxidants in normoxia and hypoxia. Fig. S5 shows the validation of the functional activity of antioxidants to decrease reactive oxygen species. Fig. S6 provides the additional evidence in primary neurons that the stabilization of HIF1 α is not oxidant-initiated in hypoxia. Fig. S7 provides additional evidence in HELA cells that the stabilization of HIF1 α is not oxidant-initiated in hypoxia. Fig. S8 provides another additional evidence in Hep3B cells that the stabilization of HIF1 α is not oxidant-initiated in hypoxia.

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Author Contributions

AK and RRR conceived and designed the research and AK performed most of the experiments. MV performed some initial experiments and SSK helped in optimizing HIF1 α blot. IG helped with enzyme activity assay, AAS helped with ROS measurement and JTP helped with glutathione redox measurement. AR, WW and IRS performed experiments in islets and helped with writing the same part in the manuscript. AK analyzed, compiled the data and prepared figures. JWC helped with the preparation of figures. AK and RRR wrote the manuscript. All co-authors discussed the results and contributed in the final editing of the manuscript.

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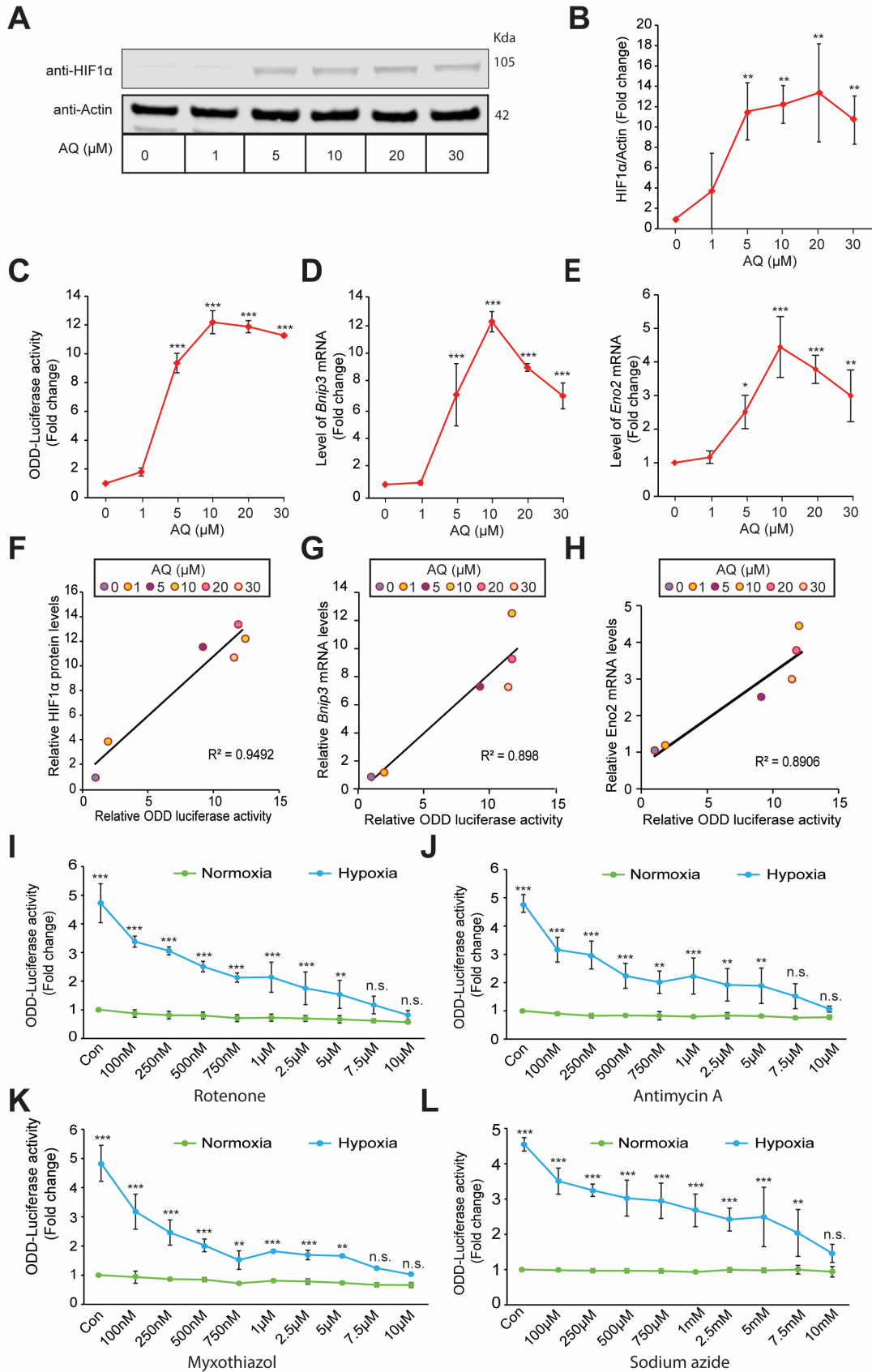


Figure 1. Mitochondrial electron transport chain is involved in hypoxic HIF1 α stabilization in human neuroblastoma cells. Experiments A - H establish specificity and sensitivity of HIF-reporter activity (i.e. ODD-Luciferase activity) while experiments I-L establish involvement of mitochondrial ETC in the hypoxic HIF1 α stabilization. SH-SY5Y ODD-Luc. cells that stably overexpressed ODD (HIF Oxygen Dependent Domain-luciferase fusion) were treated with increasing concentrations of adaptaquin (AQ, 1 μ M – 30 μ M) for 3 h and were, thereafter, processed for assessment of either changes in HIF protein level via immunoblotting (**A, B**) or changes in HIF reporter luciferase activity (i.e. ODD-luciferase activity) (**C**) or changes in expression of HIF1 α target genes, *Enolase 2* and *Bnip3* (**D, E**). (**F**) Correlation between relative changes in HIF reporter luciferase activity (i.e. ODD-luciferase activity) and relative changes in HIF1 α protein. (**G, H**) Correlation between relative changes in HIF reporter luciferase activity (i.e. ODD-luciferase activity) and relative changes in HIF1 α target genes, *Enolase 2* and *Bnip3*. (**I-L**) SH-SY5Y ODD-Luc. cells were treated with increasing concentrations of rotenone (Complex I inhibitor), antimycin A (Complex III inhibitor), myxothiazol (Complex III inhibitor), and sodium azide (Complex IV inhibitor) and then were exposed to either normoxia or hypoxia for 4h. Thereafter, HIF reporter luciferase activity was measured in cell lysates. All experiments were performed in three independent sets. The statistical analyses were performed using One way ANOVA with Dunnett post-test (B-E) and Student's t test (J-L). (B-E) (n.s.), (*), (**), and (***) indicate non-significant difference and the statistical differences of p<0.05, p<0.01, and p<0.001 with respect to normoxia control. Two way ANOVA with Bonferroni's post-test was used for all statistical analyses (I-L). (n.s.), (**), and (***) indicate non-significant difference or statistical differences of p<0.01 and p<0.001 with respect to respective control or treatment concentration under normoxia.

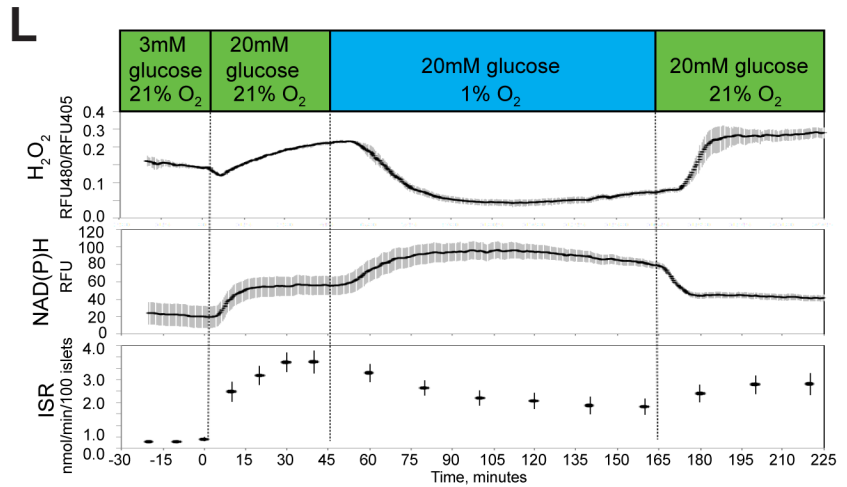
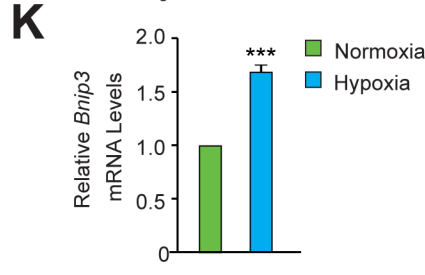
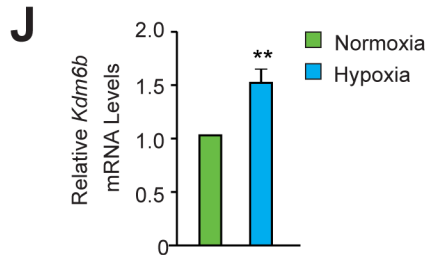
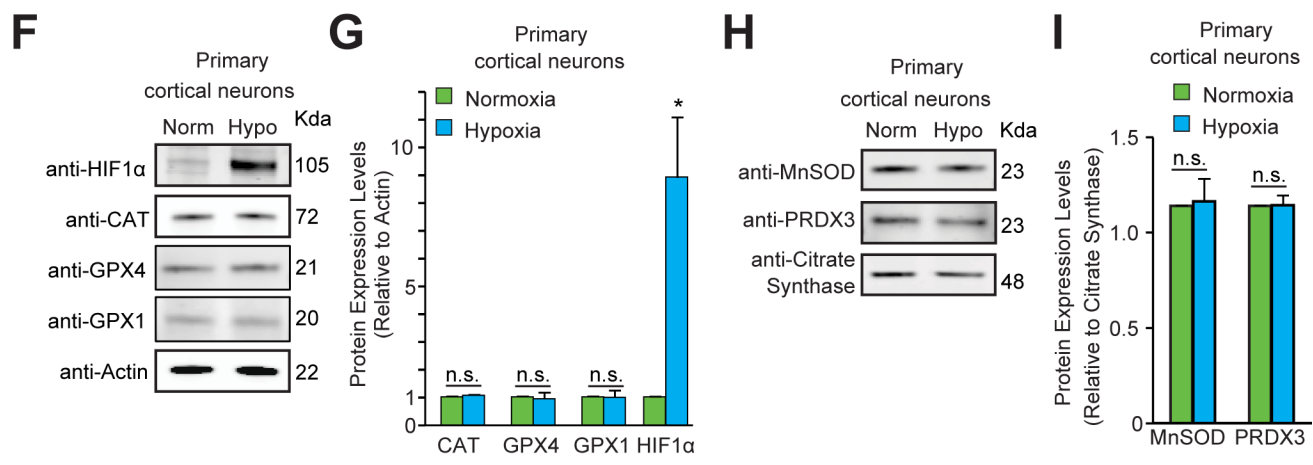
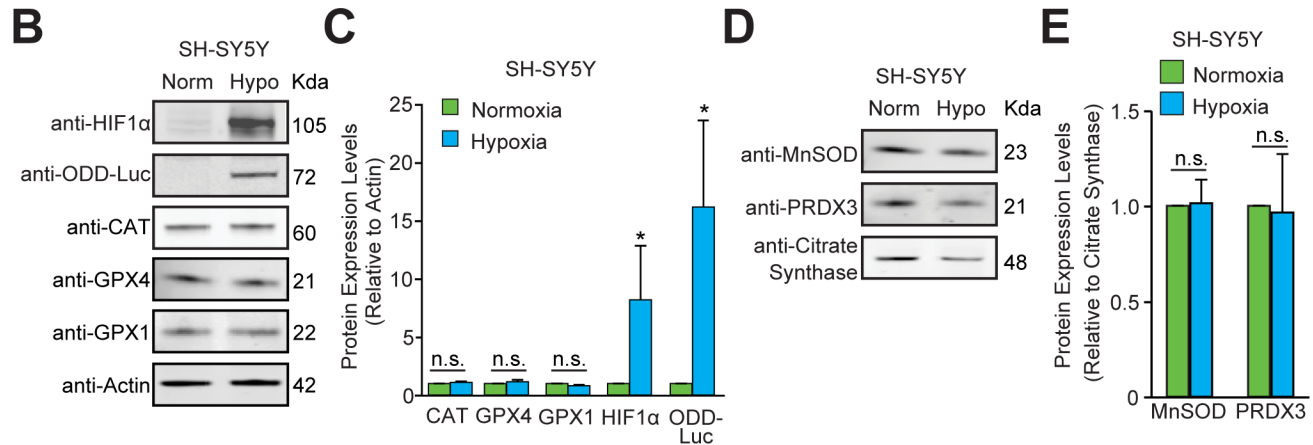
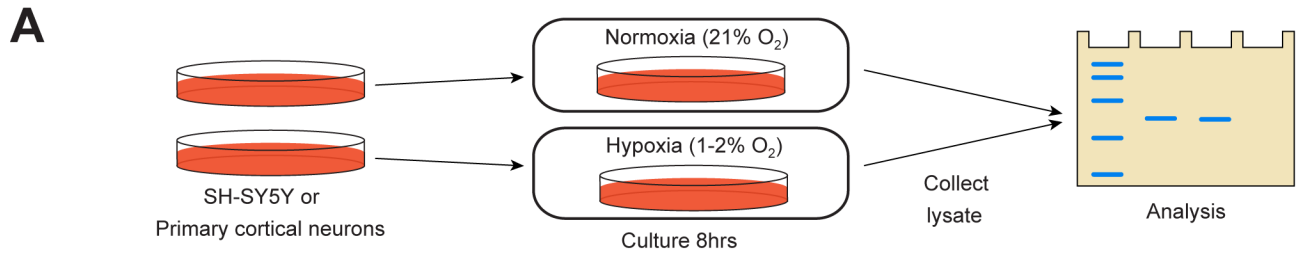


Figure 2. Hypoxia does not induce homeostatic increases in antioxidant enzymes or increase peroxide levels (A) Experimental approach employed to examine changes in the protein levels of endogenous antioxidant enzymes in hypoxia. Immunoblots or densitometric analyses of catalase (peroxisome), GPX1 and GPX4 (cytosol and mitochondria) or MnSOD and PRDX3 (mitochondria) in SH-SY5Y ODD Luc. cells **(B-E)** that stably overexpressed ODD (HIF Oxygen Dependent Domain-luciferase fusion) or PCNs **(F-I)** exposed to normoxia or hypoxia for 8h. The protein levels of catalase, GPX4, and GPX1 were normalized to actin while those of MnSOD and PRDX3 were normalized to the mitochondrial protein, citrate synthase. A monoclonal antibody to luciferase (indicated as anti-ODD-Luc. in the figure) was used to detect changes in ODD-luciferase protein levels in SH-SY5Y ODD-Luc. cells. **(J-K)** Pancreatic islets were exposed to hypoxia for 2h and then were lysed and processed for mRNA expression analysis of HIF1 α target genes, *Kdm6b* and *Bnip3*. The densitometric data and gene expression data were pooled from three independent experiments in the form of mean \pm SD. The statistical analyses of densitometric data and gene expression data were done using Student's t test (B-K). (n.s.) indicates non-significant difference, (**) indicates $p < 0.01$ and (***) indicates $p < 0.001$ with respect to respective normoxia controls. **(L)** Hypoxia leads to large decrease in H₂O₂ levels (top), increased NAD(P)H (middle), and decreased insulin secretion (bottom). Glucose stimulation by 20 mM glucose was added as reference, and oxygen levels were changed using an artificial gas equilibration device placed inline in the flow system. All three experiments were carried out separately, but using the same flow culture system.

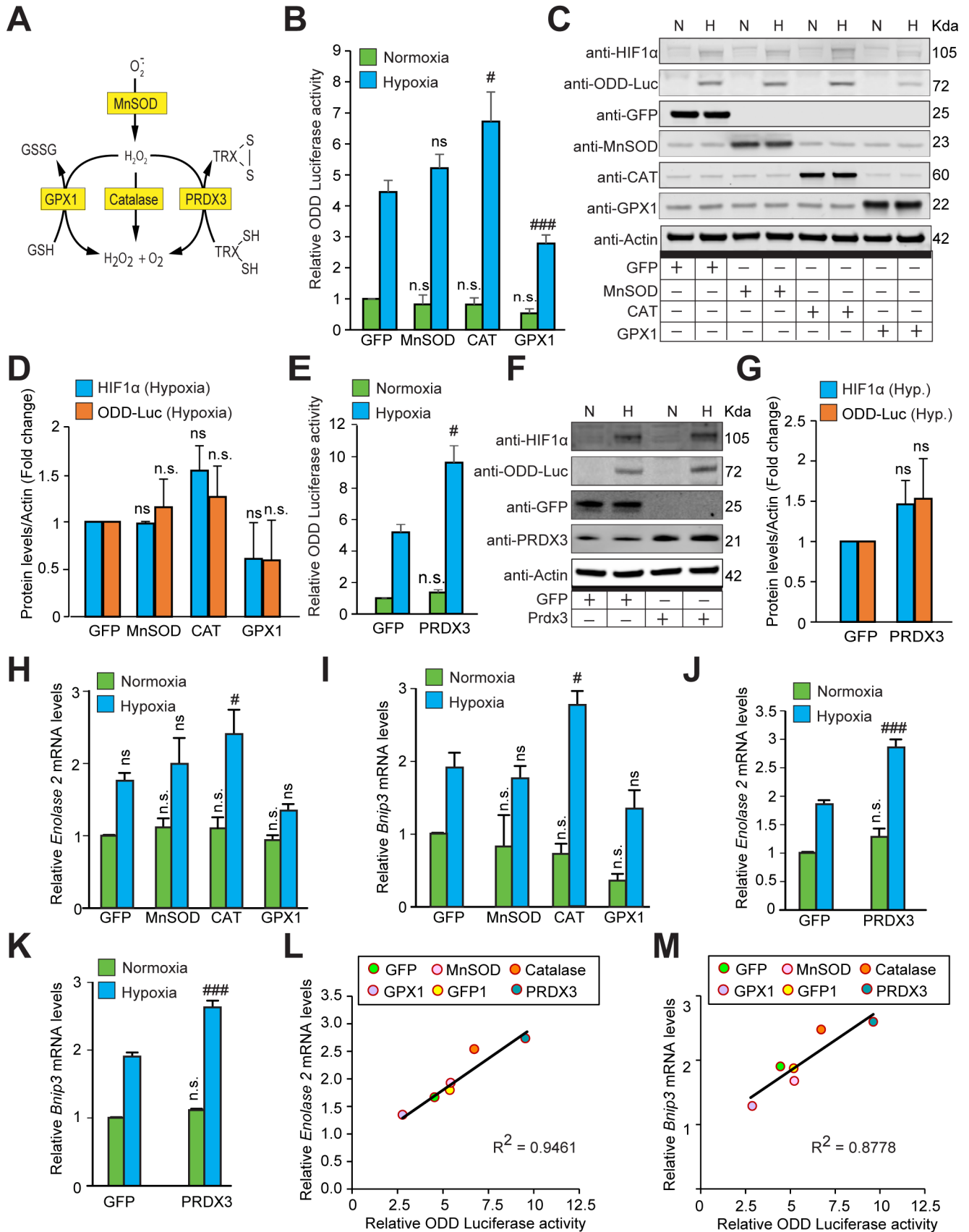


Figure 3. The stabilization of HIF1 α and its transcriptional activity are not oxidant-initiated in hypoxia. (A) A schematic diagram showing known mechanisms of H₂O₂ detoxification by peroxisomal, cytosolic and mitochondrial antioxidants. (B-K) SH-SY5Y cells stably expressing ODD-luciferase were transduced with adenoviruses encoding distinct antioxidant enzymes for 72h and then exposed to normoxia or hypoxia in parallel and were either processed for luciferase activity assay (A measure of quantitative changes in ODD) (B and E) or immunoblot analysis (A measure of quantitative changes in protein levels of ODD and HIF1 α) (C, D, F, and G) or gene expression analyses of HIF1 α target genes, *Enolase 2* and *Bnip3* (H-K). (L, M) Correlation between relative changes in either *Enolase2* or *Bnip3* and relative ODD-luciferase activities. Two way ANOVA with Bonferroni post-test was used for comparisons in A, B, G, H, I, and J. One way ANOVA with Dunnett post-test was used in the statistical analysis in D while Student t test in was used in F. (n.s.) and (***) indicate non-significant difference and the statistical differences of p<0.001, respectively, with respect to GFP control in hypoxia. (n.s.) indicates non-significant difference with respect to GFP control under normoxia while (ns), (#) and (###) indicate non-significant difference and the statistical differences of p<0.05, and p<0.001, respectively, with respect to GFP control in hypoxia. All experiments were performed as three independent sets and a representative blot or an average of three independent sets was shown in the figure. “N” stands for normoxia and “H” stands for hypoxia.

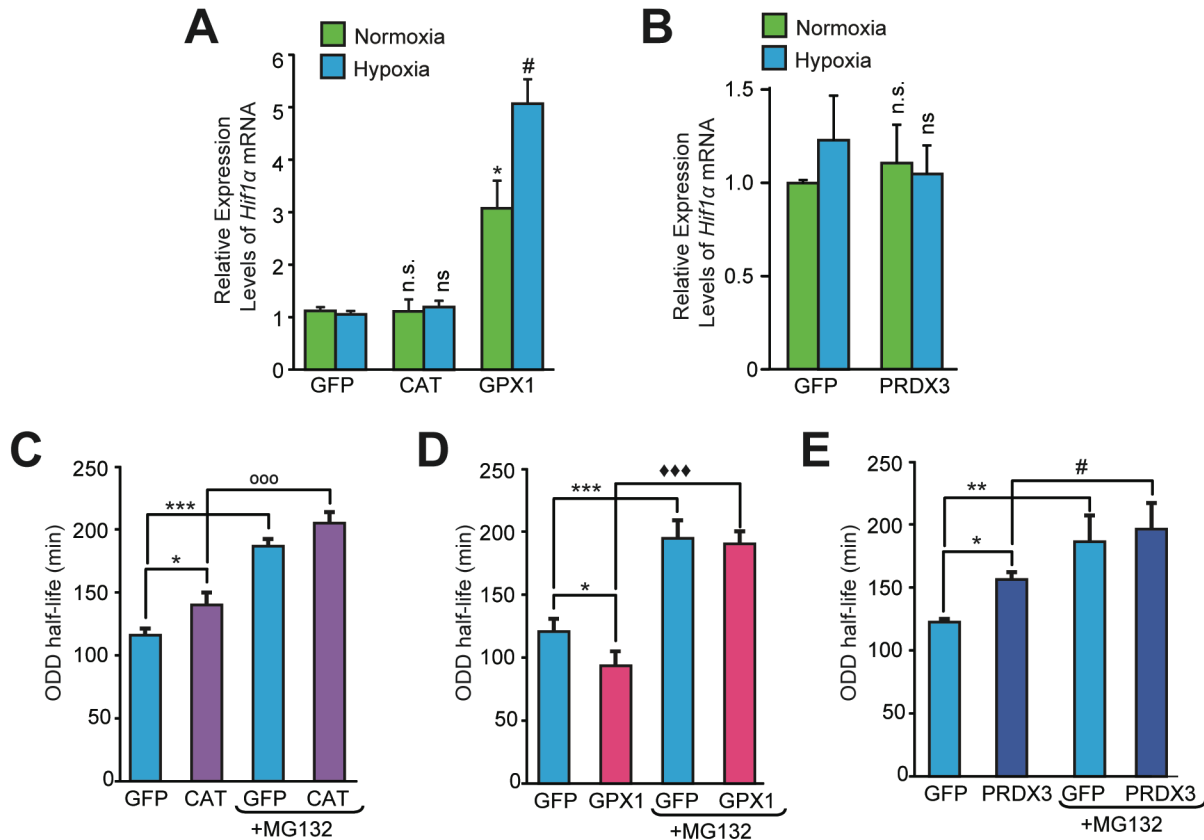


Figure 4. Divergent changes in HIF1 α protein levels induced by antioxidant enzymes capable of scavenging peroxide cannot be attributed to differential changes in HIF1 α mRNA synthesis or stability (A, B) Relative changes in *Hif1 α* mRNA in SH-SY5Y cells in response to forced expression of various antioxidant enzymes. Data were pooled from three independent experiments in the form of mean \pm SD. One way ANOVA with Dunnett's post-test was used for comparing cells expressing catalase or GPX1 with respect to cells expressing GFP and Student's t test was used for comparing cells expressing PRDX3 with respect to GFP. (n.s.) indicates non-significant difference with respect to respective GFP controls under normoxia while (ns) and (#) indicate non-significant difference and the statistical difference of $p < 0.05$, respectively, with respect to GFP control in hypoxia. (C, D, E) Changes in half-life of HIF1 α in SH-SY5Y ODD-Luc cells expressing catalase or GPX1 or PRDX3 with respect to that of respective GFP controls in hypoxia. ODD Half-life was assessed by performing a pulse chase experiment by adding 35 μ M cycloheximide at every twenty minutes for a total of four hours using luciferase activity assay in SH-SY5Y cells expressing these antioxidants pre-treated either with or without 10 μ M MG132. Data were pooled from three independent experiments in the form of mean \pm SD. Two way ANOVA with Bonferroni's post-test was used for statistical analysis. (*), (**), and (***) indicate statistical differences of $p < 0.05$, $p < 0.01$ and $p < 0.001$ with respect to respective GFP controls in hypoxia. (°°°), (♦♦♦) and (#) represent statistical differences of $p < 0.001$ w.r.t CAT, $p < 0.001$ w.r.t GPX1 and $p < 0.05$ w.r.t PRDX3, respectively.

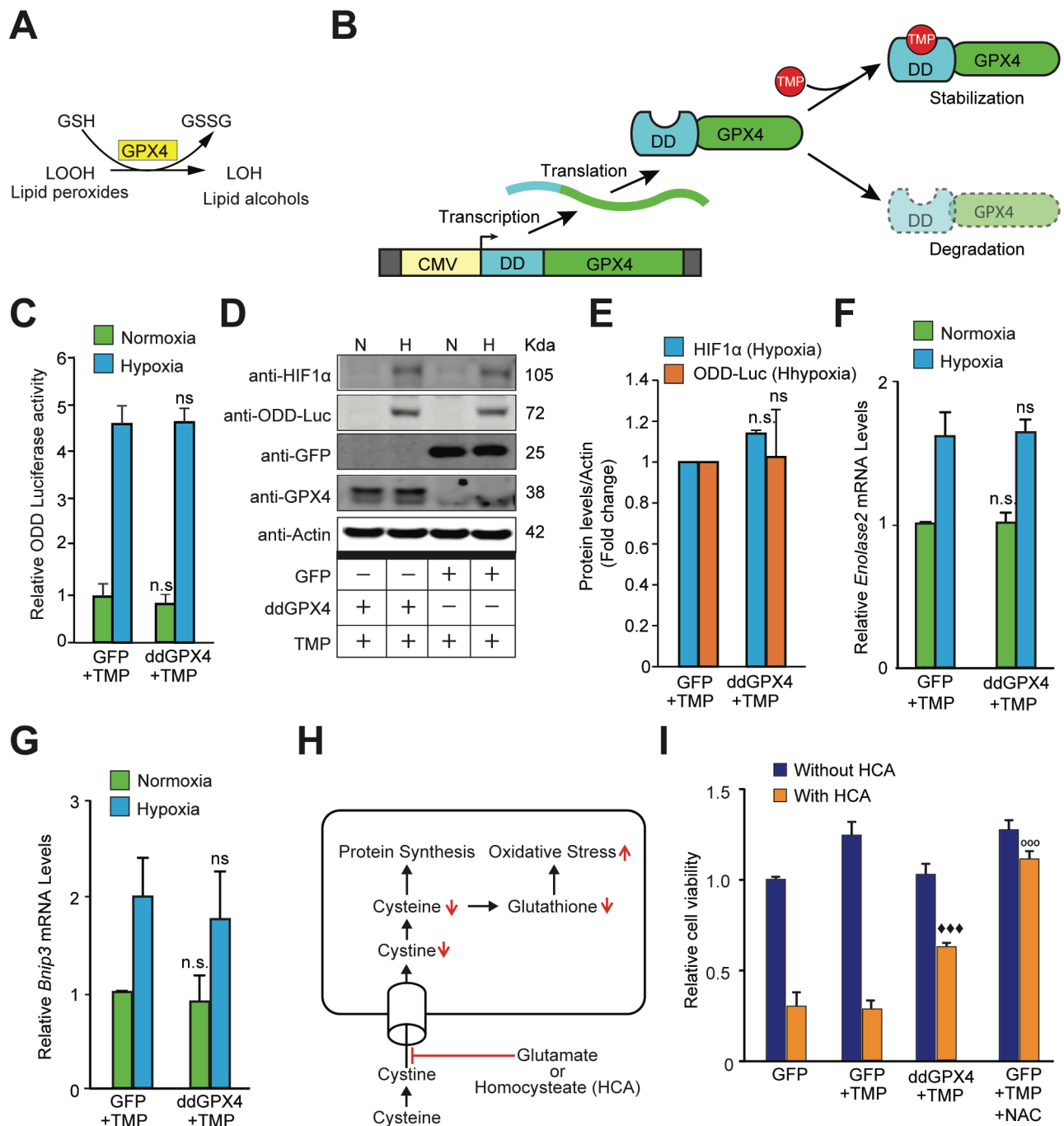


Figure 5. Reactive lipid species do not regulate HIF1α stabilization or its transcriptional activity in hypoxia (A) A schematic diagram showing that GPX4 converts lipid hydroperoxides into lipid alcohols using GSH as a cofactor. **(B)** A schematic showing regulated protein expression of GPX4 fusion containing an optimized destabilization domain when exposed to the antibiotic, Trimethoprim (TMP). Reversible stability of GPX4 protein was conferred by fusing its

coding sequence to a destabilization domain sequence (mutants of *E. Coli* dihydrofolate reductase). Accordingly, GPX4 protein possessing the destabilization domain is degraded resulting in low steady levels of GPX4. Trimethoprim binds to and neutralizes the destabilization domain stabilizing GPX4 protein in a dose dependent manner. **(C-E)** SH-SY5Y ODD-Luc cells were transduced with adenoviral vectors encoding a destabilized form of GPX4 (ddGPX4) or GFP and then exposed to normoxia/hypoxia and were either processed for luciferase activity assay (C) or immunoblotting (D) and densitometric quantitation of three independent western blots of HIF1 α and ODD-Luc protein were shown in (E). 10 μ M TMP was added to ddGPX4 expressing cells after 60h of adenoviral incubation for 12h in order to achieve stabilized GPX4 expression. **(F, G)** Relative changes in mRNA levels of HIF1 α target genes, *Enolase2* and *Bnip3* in SH-SY5Y cells. **(H)** A schematic diagram depicting the glutathione depletion model of oxidative stress. **(I)** PCNs were transduced with GFP/GPX4 for 24h and then treated with 5 mM HCA. Cells were simultaneously treated with 10 μ M TMP. Then, cells were incubated for 24h to induce oxidative stress. Thereafter, viability of cells was measured via the MTT assay. 100 μ M NAC was used as positive control. The final values were pooled as mean \pm S.D. of three independent experiments. Two way ANOVA with Bonferroni's post-test was used for statistical analyses. One way ANOVA with Dunnett's post-test was used for comparing DCF fluorescence and viability. (n.s.), (*) and (***) indicate non-significant difference, and the statistical differences of $p < 0.05$, and $p < 0.001$, respectively, with respect to GFP control under normoxia while (ns), (#) and (###) indicate non-significant difference, and the statistical differences of $p < 0.05$, and $p < 0.001$, respectively, with respect to GFP control in hypoxia. (°°°) indicates statistical difference of $p < 0.001$ with respect to GFP treated with HCA only while (***) indicates statistical difference of $p < 0.001$ with respect to GFP treated with both TMP and HCA.

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Supplemental material

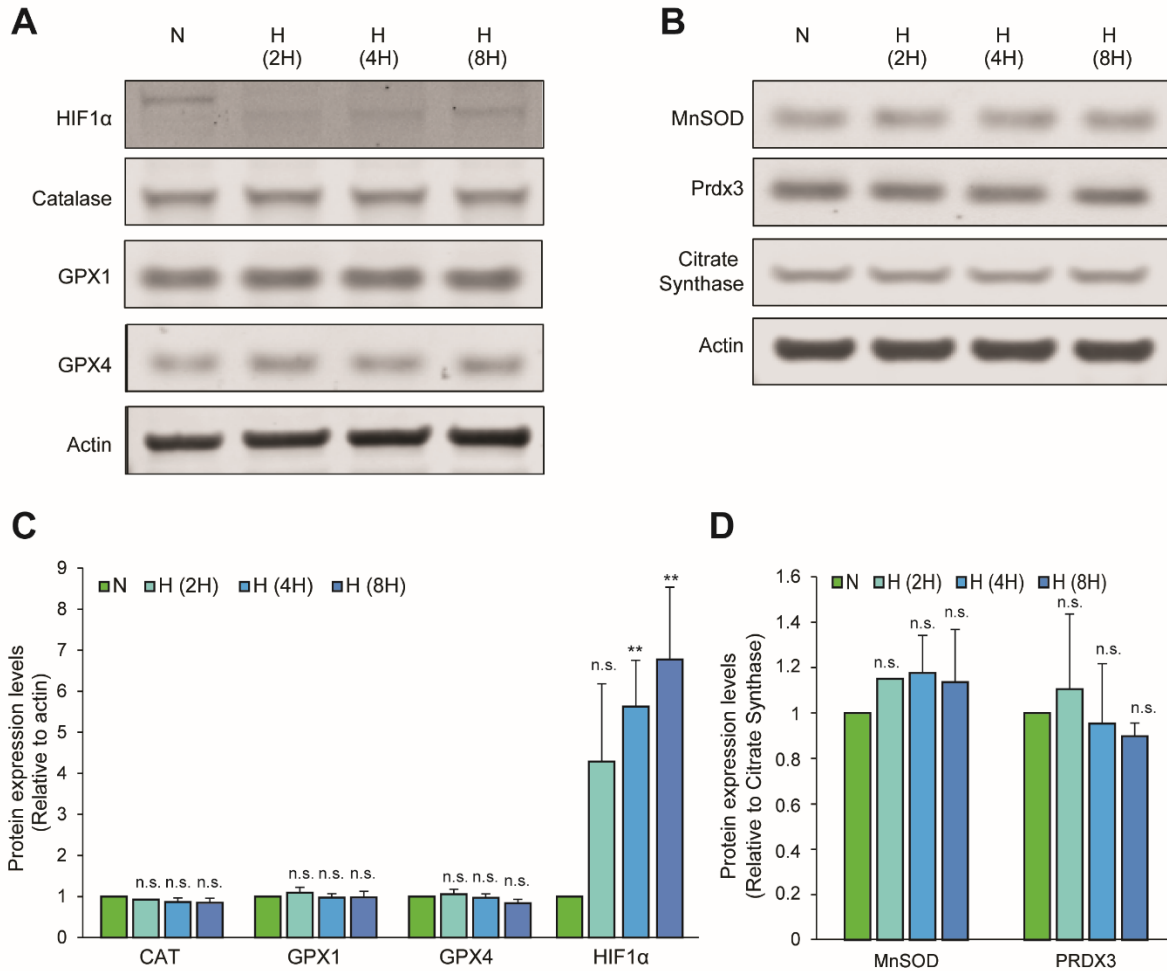


Figure S1. Hypoxic exposure for different time periods does not induce homeostatic increases in antioxidant enzymes Immunoblots of endogenous catalase, GPX1 and GPX4 (**A**) or MnSOD and PRDX3 (**B**) in SH-SY5Y ODD Luc. Cells exposed to either 8h normoxia or different periods of hypoxia (2h, 4h, and 8h). HIF1α immunoblot was used to confirm the hypoxic state of these cells (**C, D**) Densitometric analyses of immunoblots. The protein levels of CAT, GPX1, GPX4, and HIF1α were normalized to actin levels as a loading control while those of MnSOD and PRDX3 were normalized to the mitochondrial protein, citrate synthase. All western blot experiments were performed as three independent sets and a representative blot of each was shown in the figure. One way ANOVA with Dunnett's post-test was used for all comparisons. (n.s.) and (**) indicate non-significant difference, and the statistical difference of $p < 0.001$, respectively, with respect to normoxia control.

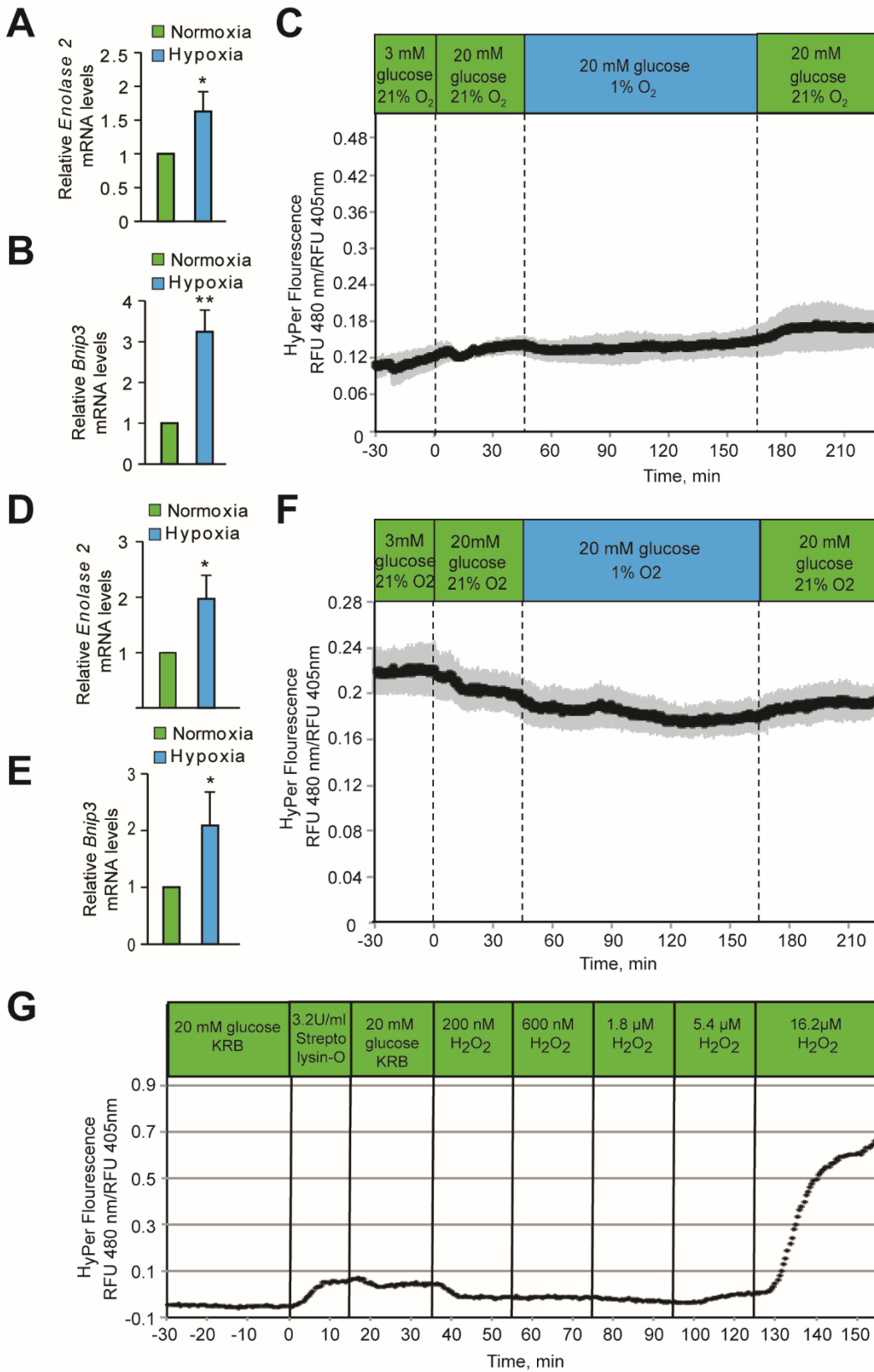


Figure S2. Hypoxia did not increase peroxide levels in SH-SY5Y cells or Hep3B cells. SH-SY5Y cells (**A, B**) or Hep3B cells (**D, E**) were exposed to hypoxia for 2h and then were lysed and processed for mRNA expression analysis of HIF1 α target genes, *Enolase 2* and *Bnip3*. The gene expression data was pooled from three independent experiments in the form of mean \pm SD. The statistical analyses of gene expression data were done using Student's t test (J-K). (**) indicates $p < 0.01$ and (***) indicates $p < 0.001$ with respect to respective normoxia controls. (**C, F**) Hypoxia does not change H₂O₂ levels in either SH-SY5Y cells (**C**) or Hep3B cells (**F**). Glucose stimulation by 20mM glucose was added as reference, and oxygen levels were changed by use of an artificial gas equilibration device placed inline in the flow system. (**G**) Hep3B cells, under normoxic conditions, were treated with bacterial Streptolysin-O to selectively permeabilize the plasma membrane followed by their exposure to increasing concentrations of H₂O₂. Real time measurement of H₂O₂ confirmed the specificity of Hyper signals with respect to H₂O₂. All three experiments (C, F, and G) were carried out separately, but using the same flow culture system. Each Experiment was performed as three independent sets and a representative picture of each is shown.

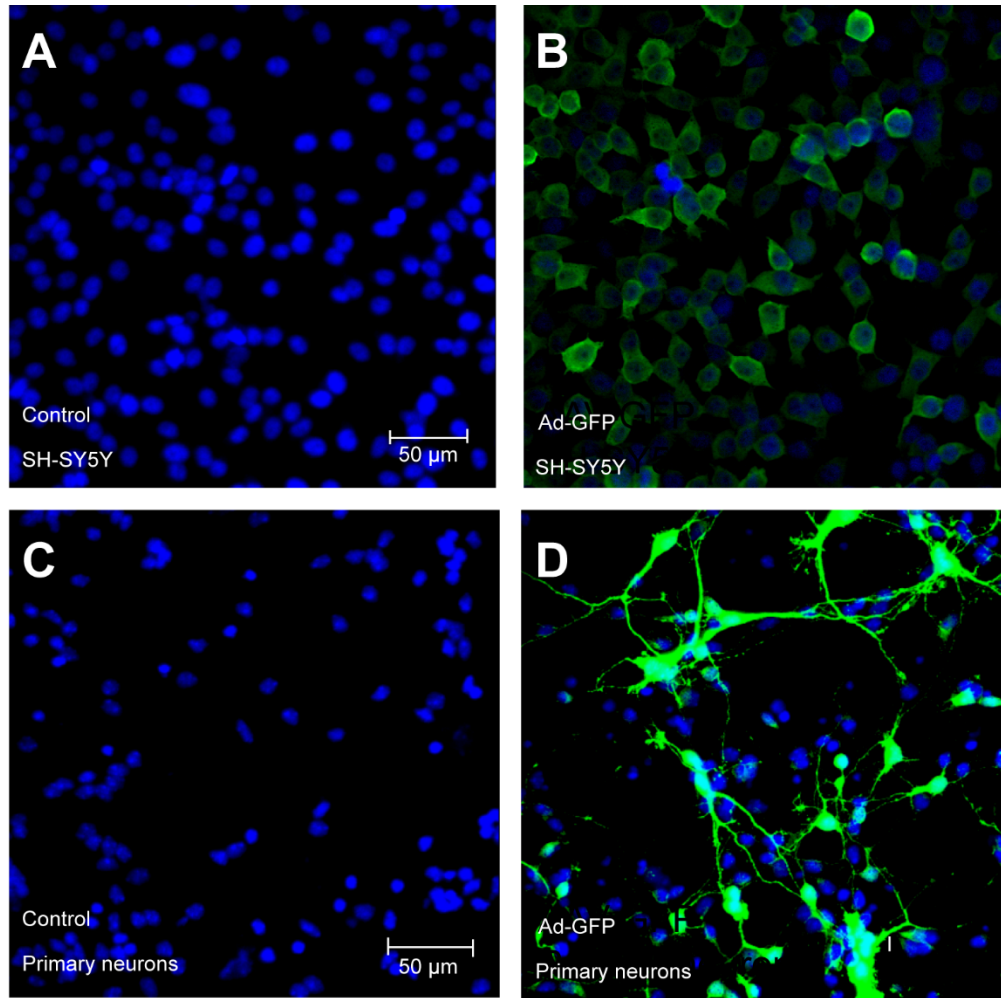


Figure S3. Validation of transgene expression after forced expression of transgenes encoded within adenoviral vectors in SH-SY5Y cells and primary cortical neuronal cultures (PCNs). (A-D) Adenoviruses encoding GFP (Ad-GFP) were used to infect SH-SY5Y cells and PCNs, and expression was examined after 72h (A, B) and 48h (C, D), respectively, to achieve steady-state levels of expression. Cells were immunostained using a GFP antibody to monitor the efficiency of overexpression. Parallel sets without adenoviral transduction were also monitored with GFP antibody and excluded non-specific staining. Experiments were performed as three independent sets and a representative picture of each is shown.

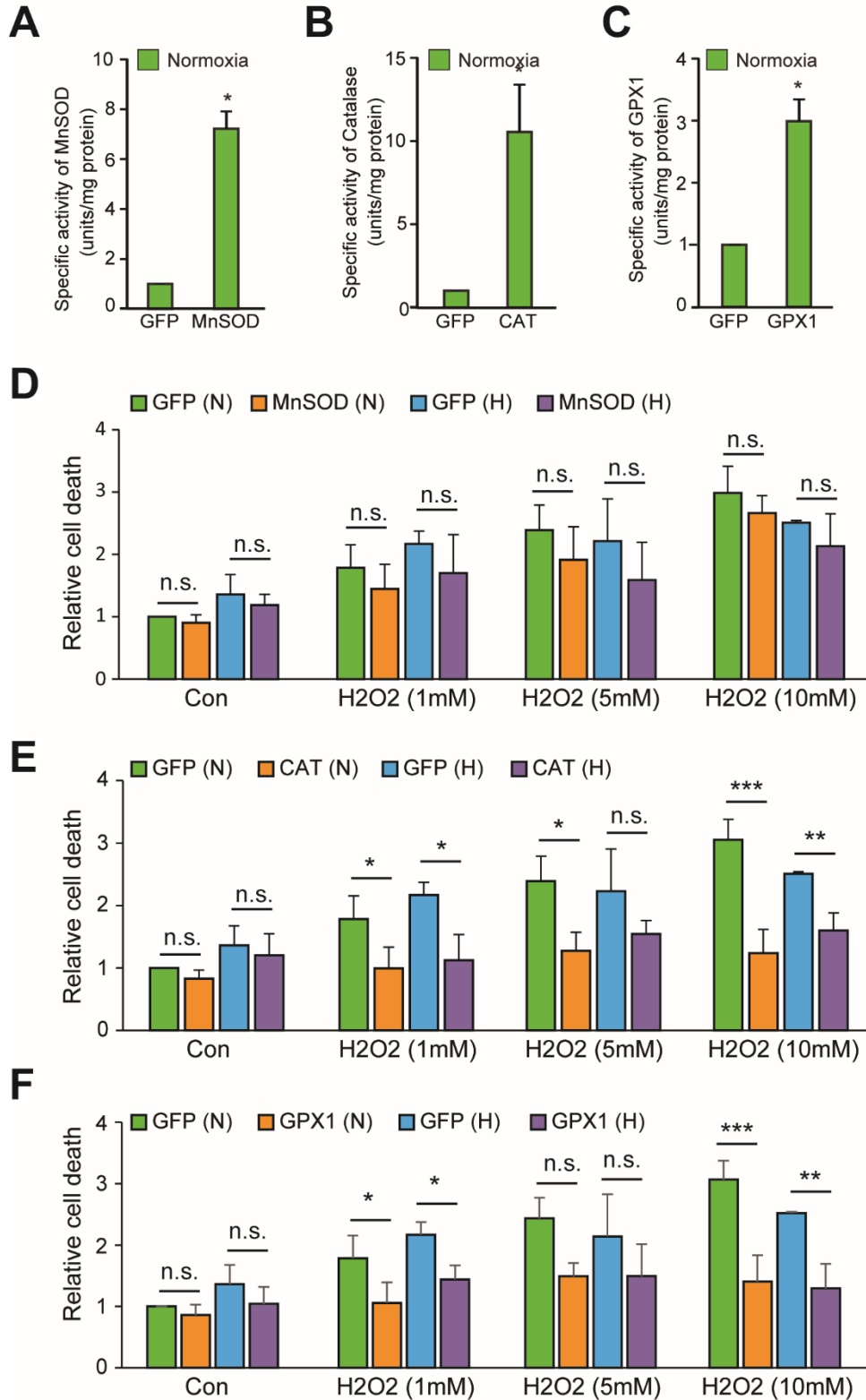


Figure S4. Validation of the enzyme activities of various antioxidants in normoxia and hypoxia. (A-C) SH-SY5Y cells were transduced with adenoviral constructs encoding MnSOD, catalase, GPX1 or a GFP control and incubated for 72h for steady-state expression. Cells were

processed for measurement of specific activities of catalase, MnSOD, and GPX1 enzymes in normoxia in sets overexpressing catalase, MnSOD, and GPX1 adenoviral constructs, respectively. The final values were pooled as mean \pm S.D. of three independent experiments in all cases. The statistical analyses were performed using Student's t test. (*) indicates statistical difference of $p < 0.05$ with respect to respective GFP control. **(D-F)** In order to indirectly assess the enzyme activities of these antioxidant constructs in normoxia as well as hypoxia, SH-SY5Y cells expressing either GFP or antioxidants (MnOSD, Catalase or GPX1) were treated with increasing conc. of H_2O_2 (1mM -10mM) and then were either exposed to normoxia or hypoxia for 4h in parallel. Thereafter, media was carefully removed and 100 μ l of 1xPBS containing 10 μ M Sytox blue dye (ThermoFisher Scientific; Catalog number: S11348) was added in wells and the plate was incubated for 20 min at room temperature in dark before taking the fluorescence reading at Ex/Em: 440/480. The final values were pooled as mean \pm S.D. of three independent experiments in all cases. Two way ANOVA with Bonferroni post-test was used for the statistical analysis. (n.s.), (*) and (***) indicate non-significant difference, and the statistical differences of $p < 0.05$, and $p < 0.001$, respectively, with respect to respective GFP control under normoxia or hypoxia.

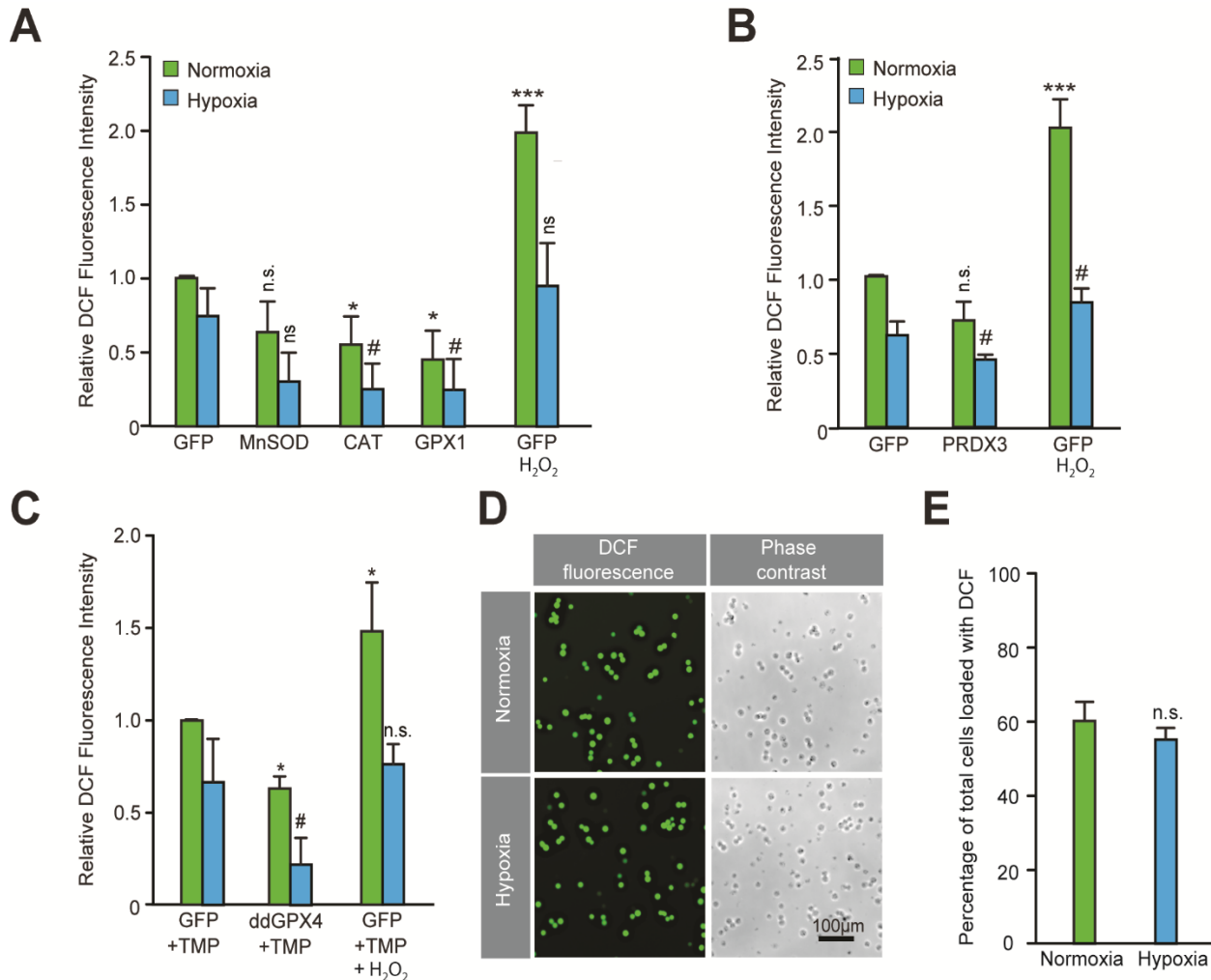


Figure S5. Validation of the functional activity of antioxidants to reduce reactive oxygen species. (A-C) After exposing parallel sets of SH-SY5Y ODD-Luc cells to either normoxia or hypoxia for 4 h, media was removed, cells were washed with 1xPBS, and then 1xPBS with 20 μ M DCFDA was added in each well. Additional parallel sets of cells from both conditions were also treated with 5mM H₂O₂ at the same time and were used as positive controls. Plates from both normoxia and hypoxia were then incubated at room temperature for 30 min in normoxic condition. Thereafter, the fluorescence of DCFDA loaded SH-SY5Y cells were measured through flow cytometry using standard fluorescein wavelengths. Fluorescence values were obtained by multiplying cell counts with mean fluorescence and later converted into relative fold change with respect to cells expressing GFP exposed to either normoxia or hypoxia. The final values were pooled as mean \pm S.D. of three independent experiments in all cases. One way ANOVA with Dunnett's post-test was used for all comparisons and a similar comparison was done in hypoxia separately. (n.s.), (*) and (***) indicate non-significant difference, and the statistical differences of $p < 0.05$, and $p < 0.001$, respectively, with respect to GFP control under normoxia while (ns) and (#) indicate non-significant difference and the statistical differences of $p < 0.05$, respectively, with respect to GFP control in hypoxia. (D, E) DCF loading was equivalent in normoxic and hypoxic cells. SH-SY5Y cells were loaded with DCFDA for 30 min either in normoxia or hypoxia and then exposed to visible light equally to completely oxidize fluorescein.

Illuminated cells incubated under normoxic or hypoxic conditions (from five independent sets) were counted manually to establish whether cells had equal DCFDA loading under both conditions. These values were pooled as mean \pm SD. The statistical analysis was done using Student's t test. (n.s.) indicates non-significant difference with respect to normoxia.

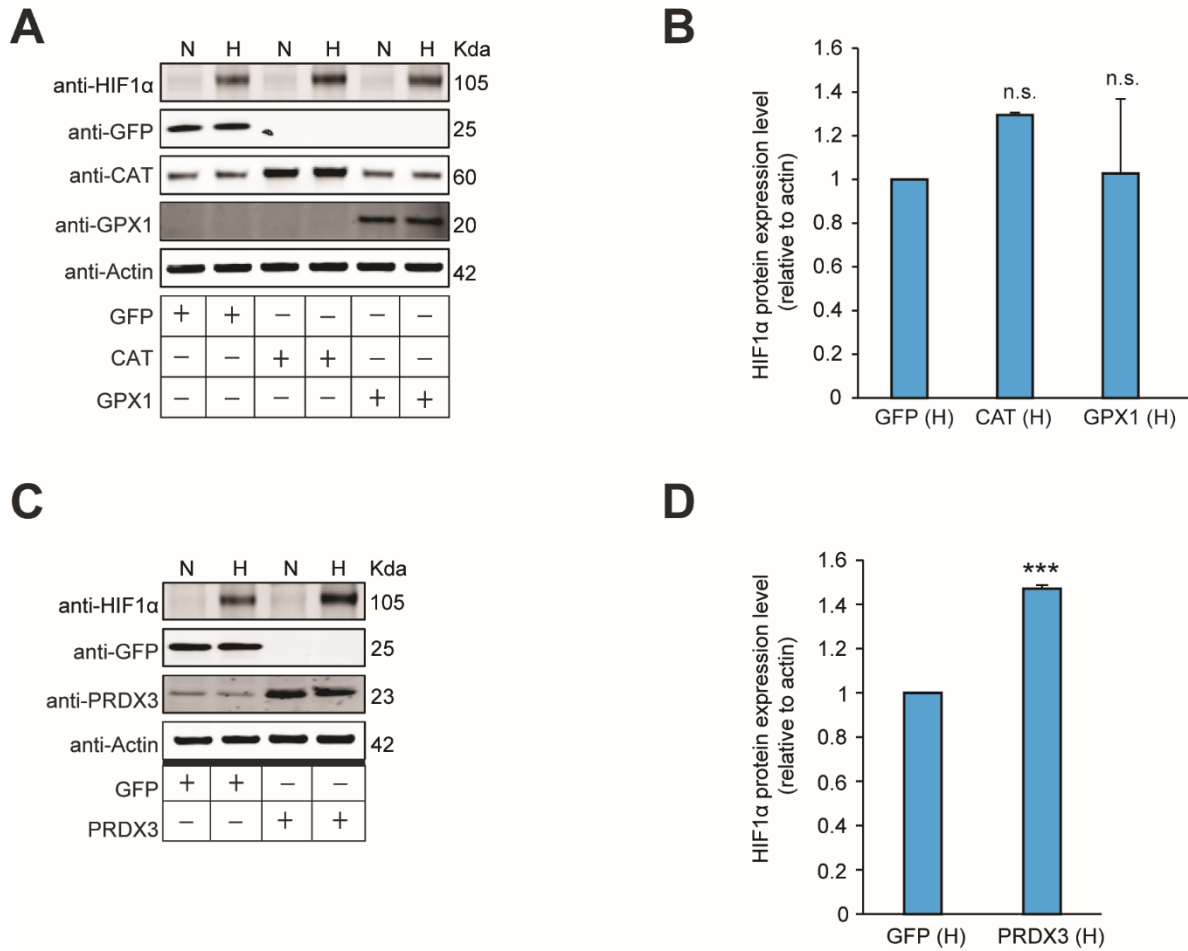


Figure S6. The stabilization of HIF1 α is not oxidant-initiated in hypoxia in primary cortical neurons. Mouse primary neurons were transduced with adenoviral vectors encoding distinct antioxidant enzymes or GFP for 48h and then exposed to normoxia or hypoxia in parallel for 4h. A and C represent immunoblots and B and D represent densitometric analyses of the respective HIF1 α blots. All western blot experiments were performed as three independent sets and a representative blot of each was shown in the figure. The statistical analyses were performed using One way ANOVA with Dunnett post-test in 6(B) and unpaired t test in 6(D). (n.s.) and (***) indicate non-significant difference and the statistical differences of $p < 0.001$, respectively, with respect to GFP control in hypoxia.

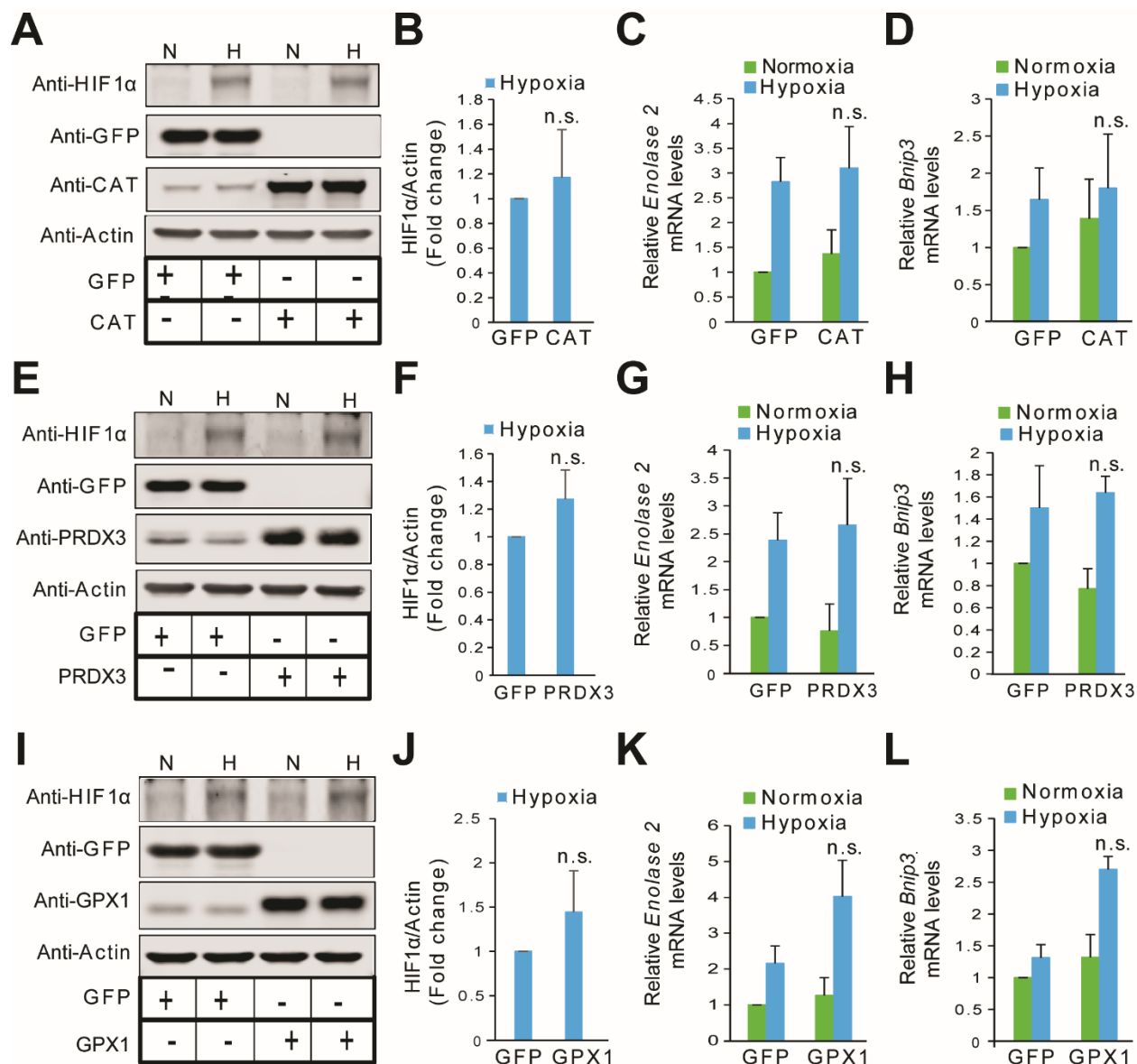


Figure S7. The stabilization of HIF1α is not oxidant-initiated in hypoxia in HeLa cells. HeLa cells were infected with adenoviruses encoding designated antioxidant enzymes and gene expression was assessed after 72h. Cells with forced expression of GFP or designated antioxidant enzymes were exposed to normoxia or hypoxia in parallel experiments and were processed for immunoblotting of HIF1α or mRNA expression analysis of HIF1α target genes, *Enolase 2* and *Bnip3*. **(A, E, I)** Immunoblots showing changes in the protein level of HIF1α in response to catalase (CAT) overexpression (A), peroxiredoxin 3 (PRDX3) overexpression (E) or glutathione peroxidase 1 (GPX1) overexpression (I) under normoxia and hypoxia. **(B, F, J)** Densitometric analysis of changes in the protein level of HIF1α in hypoxia with designated antioxidant enzymes. **(C, G, K)** Changes in mRNA level of HIF1α target gene, *Enolase 2* in respective overexpression conditions in normoxia and hypoxia. **(D, H, L)** Changes in mRNA level of HIF1α target gene, *Bnip3* in respective overexpression conditions in normoxia and hypoxia. The final values were pooled as mean ± S.D. of three independent experiments in all cases.

Student's t test was used to compare the statistical difference between cells expressing antioxidants such as CAT, PRDX3 or GPX1 with that of respective GFP controls under hypoxia in densitometric analysis as well as gene expression analysis. (n.s.) indicates non-significant difference with respect to respective GFP controls under hypoxia. All western blot experiments were performed as three independent sets and a representative blot of each was shown in the figure.

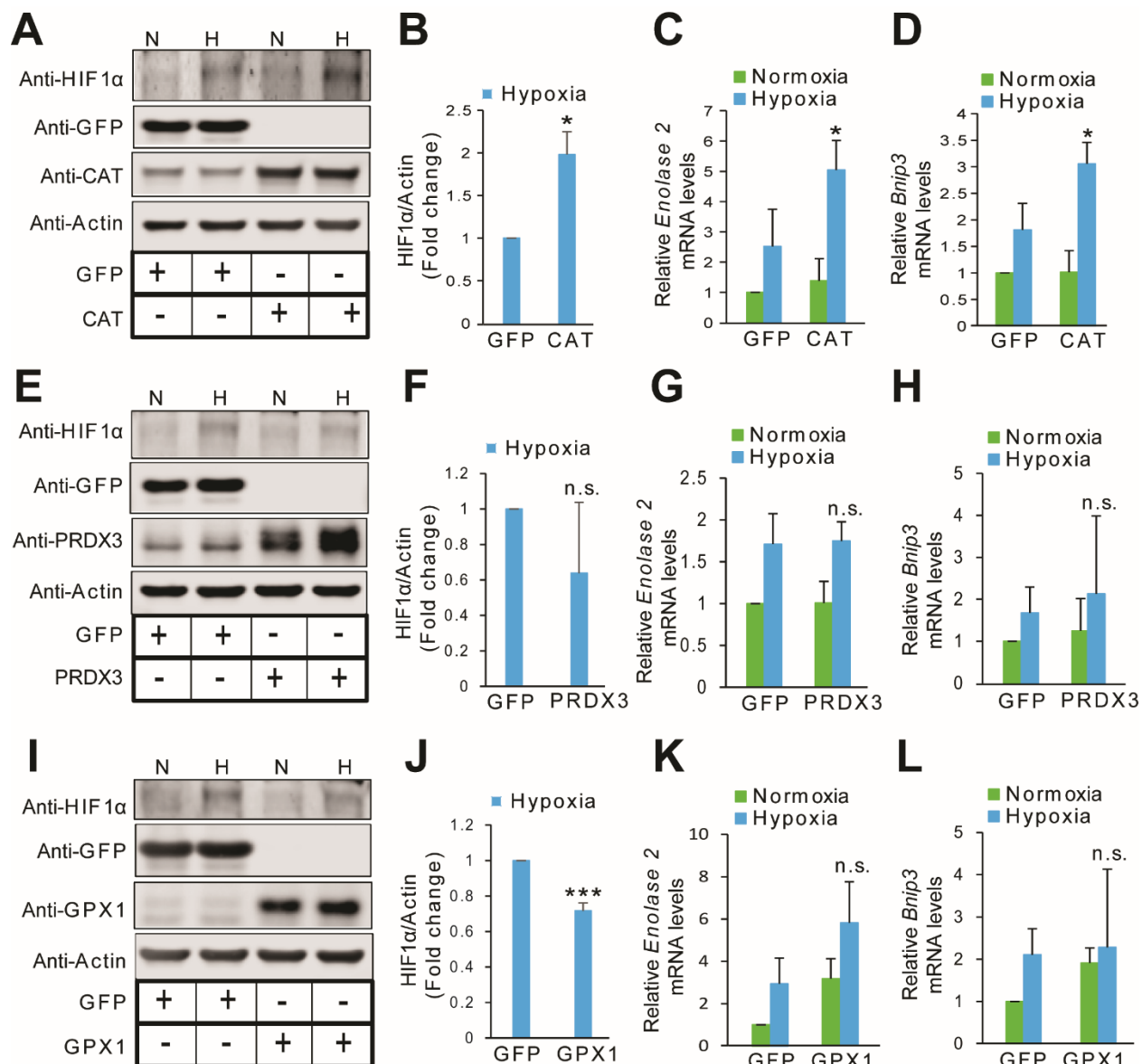


Figure S8. The stabilization of HIF1α is not oxidant-initiated in hypoxia in Hep3B cells. Hep3B cells were infected with adenoviruses encoding distinct antioxidant enzymes and transgenes reached steady state expression after 72h. Transduced cells were then exposed to normoxia or hypoxia in parallel experiments and were processed for immunoblotting to assess protein levels of HIF1α or mRNA analysis for assessing changes in levels of established HIF1α target genes, *Enolase 2* and *Bnip3*. **(A, E, I)** Immunoblots demonstrating changes in the protein level of HIF1α in response to either catalase (CAT) overexpression (A), peroxiredoxin 3 (PRDX3) overexpression, (E) or glutathione peroxidase 1 (GPX1) overexpression (I) under normoxia and hypoxia. **(B, F, J)** Densitometric analysis of changes in the protein level of HIF1α under hypoxia in respective overexpression conditions. **(C, G, K)** Changes in mRNA level of HIF1α target gene, *Enolase 2* in respective overexpression conditions in normoxia and hypoxia. **(D, H, L)** Changes in mRNA level of HIF1α target gene, *Bnip3* in respective overexpression conditions in normoxia and hypoxia. The final values were pooled as mean ± S.D. of three independent experiments in

all cases. Student's t test was used to compare the statistical difference between cells expressing antioxidants such as CAT, PRDX3 or GPX1 with that of respective GFP controls under hypoxia in densitometric analysis as well as gene expression analysis. (n.s.), (*) and (***) indicate non-significant differences, and the statistical differences of $p < 0.05$, and $p < 0.001$, respectively with respect to respective GFP controls under hypoxia. All immunoblot experiments were performed as three independent sets and a representative blot of each was shown in the figure.