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Myc post-transcriptionally induces HIF1 protein and target gene expression in normal and cancer cells

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Abstract

c-Myc is frequently overexpressed in tumors and plays an important role in the regulation of cancer metabolism. Hypoxia-inducible factor-1 (HIF1), the master regulator of the hypoxic response, enhances tumorigenesis and influences metabolism via upregulation of the glycolytic pathway and suppression of mitochondrial respiration. Together, deregulated *Myc* and HIF1 cooperate to lend metabolic advantages to proliferating cancer cells and contribute to the Warburg Effect. Here we show that overexpression of *Myc* significantly stabilizes the alpha subunit of HIF1 (HIF1 α) under normoxic conditions and enhances HIF1 α accumulation under hypoxic conditions in cells. Post-transcriptional regulation of HIF1 α by *Myc* led to the induction of HIF1 α gene targets. Normoxic HIF1 α protein expression was also dependent on *Myc*. Functionally; HIF1 α expression was required for *Myc*-induced anchorage-independent growth and cell proliferation. *Myc*-dependent stabilization of HIF1 α involved either disruption of binding to the VHL complex or post-translational protein modifications. Taken together, our findings uncover a previously uncharacterized regulatory relationship between *Myc* and HIF1 that has important implications for cancer metabolism and development.

INTRODUCTION

Breast cancer is the most common malignancy of women worldwide and targets 13% of women in the United States (NCI). The *c-myc* oncogene, which encodes the transcription factor *c-Myc*, contributes to many forms of cancer and was one of the first genes found to be amplified in breast cancer (1). Studies suggest that at least 15–20% of breast cancers present with significant amplification of *c-myc* and that overexpression of *c-Myc* likely occurs in an even greater number of tumors through other mechanisms (2). Animal model studies have confirmed that constitutive *Myc* expression in the mammary gland is oncogenic in transgenic mice (3). Microarray studies have exposed *Myc* as a weak yet pleiotropic transcription factor, possessing the ability to activate or repress 5–10% of all genes by 1.5-fold or more (4). While these target genes vary greatly depending on the cellular context, commonly targeted processes include the cell cycle, metabolism, and apoptosis (5).

In transformed cells, *Myc* promotes glycolysis by activating glycolytic enzymes (6, 7). Tumor cells often exhibit increased rates of glycolysis even in the presence of adequate oxygen concentrations (the Warburg Effect), although increased rates of glycolysis normally occur under hypoxic conditions (8, 9). HIF1 is the key mediator of the hypoxic response in

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cellular tissues, comprised of a constitutively expressed HIF1 β subunit and an oxygen-responsive HIF1 α subunit (10, 11). In oxygenated tissues, HIF1 α has a very short half-life (10, 12). Rapid turnover is mediated through the hydroxylation of two prolines at amino acids 402 and 564, performed by oxygen-dependent prolyl hydroxylases (13, 14, 15 (reviewed in {Kaelin, 2005 #118}). Prolyl-hydroxylation of HIF1 α leads to ubiquitination by the Von Hippel-Lindau (VHL) E3 ubiquitin ligase and degradation via the 26S proteasome (reviewed in (16, 17)). In a hypoxic environment HIF1 α becomes stabilized (18, 19), although in some instances HIF1 α can be stabilized under normoxia as well (reviewed in (20, 21)). HIF1 α is commonly expressed in cancers, as tumor microenvironments suffer from hypoxia owing to an incomplete vasculature.

Studies have reported HIF1-induced opposition of Myc under hypoxia when Myc is expressed at non-tumorigenic levels. Studies have also revealed the cooperation of HIF1 and deregulated Myc under hypoxia (22–25). However, our data show that Myc regulates HIF1 α expression under both normoxia and hypoxia, and that HIF1 α expression represents an important component of Myc function. Our results demonstrate a novel and complex relationship between Myc, HIF1 α , and tumor development.

MATERIALS AND METHODS

Cell culture, transfection, and retroviral infection

Retroviral-producing PhoeNX cells and breast cancer lines MCF7 and T47D were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. IMEC epithelial cells were cultured in 1:1 DMEM/F12 medium supplemented with 5% FBS and other factors (DiRenzo et al., 2002). Retroviral infection was performed using PhoeNX cells. Briefly, PhoeNX cells were transfected with 7 μ g DNA using Lipofectamine 2000 (Signagen) according to manufacturer's instructions. Virus was harvested from the culture medium two days later and used to infect recipient IMECs. Cells were selected two days later and maintained in 150 μ g/mL hygromycin (Calbiochem) to select for LXSH vectors (LXSH, LXSH-Myc). Hypoxic conditions were induced by culturing cells for two hours in a sealed hypoxia chamber (Billups Rothenberg) after flushing with a mixture of 1% O₂, 5% CO₂, and 94% N₂. Hypoxic conditions were also induced by the addition of CoCl₂ (Sigma) at a concentration of 200 μ M for 3 hours. We refer to the ambient oxygen concentration as normoxic (95% air, 5% CO₂). Although the ambient oxygen concentration is not the physiological normoxic state, this is a frequently used convention in the literature.

Cell counts

Cells were plated at 1×10^4 per well in 6-well plates on Day 0. Cells were maintained as above. Cells were harvested each day for four days and counted using a hemacytometer. At least 100 cells were counted. The experiment was performed in duplicate on two separate occasions. Knockdown cell growth curves were achieved by plating cells at 1×10^4 per well, transfecting with HIF1 α and non-targeting control siRNA 24 hours later (Day 1), and counting cells for three days post-transfection. The experiment was performed in duplicate on two separate occasions.

Soft agar assay

Assays were performed in 6-well plates in duplicate on two separate occasions. The lower layer of agar consisted of 2mL IMEC medium containing 10% FBS and 0.6% Noble Agar (USB). The upper layer consisted of 2mL IMEC medium containing 10% FBS, 0.3% Noble Agar, and 4×10^4 cells. Agar at 50°C was mixed with medium at 37°C, plated, and left to set for 10 min. Plates were fed every other day with 250 μ L IMEC medium containing 10%

FBS. After 10 days, undivided cells and colonies were scored using a reticle, measuring the greatest diameter. 100 cells were counted per well.

Real-Time (RT) PCR

RNA was extracted from log phase cells using Trizol (Invitrogen) according to manufacturer's instructions. RNA was converted into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) and PCR was performed using iQ SYBR Green Supermix and the CFX96 Real-Time PCR Detection System (Bio-Rad). Primers used were: GAPDH GCTCAGACACCATGGGAAGGTGAAG and GCTGATGATCTTGAGGCTGTTGTCATAC; Myc CGAGGAGGAGAACTTCTACCAGC and CGAGAAGCCGCTCCACATACAGTCC; HIF1 α ACAGTATTCCAGCAGACTCAA and CCTACTGCTTGAAAAAGTGAA; HK2 GGATGATTGCCTCGCATCTGC and GGAAGTCTCCGTGTTCTGTCC; ALDOA CCATGCCCTACCAATATCCAGC and GGTGGTAGTCTCGCCATTTGTCC; eNOS GGAGAGATCCACCTCACTGTAGC and CCTTTGCTCTCAATGTCATGC; BGN GGAACATGAACTGCATCGAGATGG and CGATCATCCTGATCTGGTTGTGG; VHL CCTTTGGCTCTTCAGAGATGC and GGTCTTTCTGCACATTTGGGTGG; CUL2 GCAGAAGATTCAACACTACACTTGC and CCTTGCTGTATTCTTCCCAGTACC; RBX1 GCAGCGATGGATGTGGATACC and CCTGTCTGTTTTGAGCCAGC; Elongin B CCACAAGACCACCATCTTCACG and CGTGCTGTTTGACTGGTGAAGC; Elongin C CCTATGGTGGCTGTGAAGGACC and GCAGTTCCAGTGCAATTTTCAGG.

Western blots

Cells were lysed in F-Buffer and cell extracts were normalized for protein content. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibody in 5% milk. Antibodies used include: monoclonal anti-HIF1 α (Novus Biologicals), polyclonal anti-HIF1 α (Novus Biologicals), monoclonal anti-VHL (BD Biosciences), polyclonal anti-VHL (Novus Biologicals), anti-hydroxyproline-564-specific-HIF1 α (kindly provided by R. Freeman), monoclonal anti-c-Myc antibody (C-33; Santa Cruz), polyclonal anti-c-Myc antibody (N262; Santa Cruz), anti- β -tubulin (Santa Cruz), monoclonal anti-CUL2 (Santa Cruz), polyclonal anti-CUL2 (Abcam), polyclonal anti-Rbx1 (Santa Cruz), polyclonal anti-Elongin B (Santa Cruz), monoclonal anti-Elongin C (BD Biosciences), and polyclonal anti-Elongin C (Novus Biologicals). Immunoprecipitations were performed by complexing Protein A/G-Plus Agarose beads with 1 μ g of antibody for one hour and then mixing the beads with cell extract overnight. ImageJ (NCBI) was used to quantitate protein expression levels.

Cycloheximide assay

Cells were plated in 6-well plates and treated with cycloheximide (CHX) 24 hours post-plating. CHX was added at a concentration of 50 μ g/mL to the cells at 20-minute intervals. Hypoxic extracts were made by treating cells with 200 μ M CoCl₂ for 3 hours prior to CHX treatment. Protein extracts were prepared as previously described. HIF1 α and Myc half-lives were calculated after quantitating protein and normalizing to β -tubulin.

siRNA

Experiments involving siRNA were performed by transfecting *HIF1A*, *c-myc*, or non-targeting siRNA (Dharmacon) at 2 μ M in 6-well plates according to manufacturer's instructions. Cells were replated 24 hours following transfection for soft agar analysis. RNA and protein was harvested for analysis 24 or 48 hours following transfection. siRNA growth curve experiments were performed by transfecting *HIF1A* and non-targeting siRNA at 2 μ M in 24-well plates according to manufacturer's instructions.

ON-TARGETplus Human *HIF1A* siRNA: J-004018-07-0010, J-004018-08-0010

ON-TARGETplus Human *c-myc* siRNA: J-003282-23-0005, J-003282-24-0005

ON-TARGETplus Non-targeting siRNA: D-001810-01-05

RESULTS

Myc enhances accumulation of hypoxia regulator HIF1 α

We have previously shown that overexpression Myc in TERT-immortalized human mammary epithelial cells (IMECs) leads to a partial oncogenic transformation with an increased proliferation rate and anchorage-independent growth (26). It is important to note that Myc expression in the transduced IMECs is similar to Myc protein levels found in breast cancer cell lines T47D and MDA-231 and hence represents physiologically relevant expression (26). Whole genome expression analysis revealed that the most significantly enriched Myc-induced cluster corresponded to angiogenesis and vascularization (Supplemental Table; data available on request). Previous studies have linked angiogenesis to Myc in a transgenic model (27, 28). Furthermore, many genes involved in these pathways have been scored as potential direct Myc target genes in microarrays. However, the most prominent regulator of angiogenesis is Hypoxia Inducible Factor 1 (HIF1), which coordinately controls the expression of a large number of genes in the pathway. Therefore, we explored if the strong induction of an angiogenic program is due to activation of HIF1 α instead of a direct effect of Myc.

To assess a potential role for HIF1 in the induction of an angiogenic gene response, we examined the levels of HIF1 α protein in vector and Myc-overexpressing cells under normoxic (95% air, 5% CO₂) or hypoxic conditions (1% O₂, 5% CO₂, 94% N₂). As expected, there were very low levels of HIF1 α in IMECs cultured under normoxic conditions, and the level was induced substantially under hypoxia. Surprisingly, we found that overexpression of Myc enhances the accumulation of HIF1 α under normoxic conditions to a significant level. Moreover, overexpression of Myc exaggerates HIF1 α accumulation under hypoxic conditions (Figure 1A). On average, HIF1 α expression in Myc-overexpressing cells under normoxia is ten times more than in vector IMECs (Figure 1B). We determined whether the increase in protein expression is due to an increase in transcription of the HIF1 α gene by performing quantitative real-time (RT) PCR under normoxic and hypoxic conditions. We found that HIF1 α is not transcriptionally induced by hypoxia, as expected, or by Myc (Figure 1C). Myc transcription is also not affected by hypoxia. These data suggest that Myc-enhanced HIF1 α expression is post-transcriptionally regulated.

We treated the vector control IMEC and IMEC-Myc cells with the proteasome inhibitor MG132 to block protein degradation and assess the total amount of HIF1 α protein being translated under normoxia and hypoxia. As expected, the enhanced level of HIF1 α under hypoxia is an effect of stabilization and not increased translation since protein levels become equal when degradation is inhibited (Figure 1D). We find that, even though normoxic HIF1 α is stabilized significantly by Myc, treatment with MG132 leads to further stabilization indicating that there is some ongoing degradation in IMEC-Myc cells. In contrast, all of the HIF1 α protein appears to be fully stabilized under hypoxia in Myc-overexpressing cells because MG132 treatment gives no further enhancement.

Since HIF1 protein levels are primarily controlled by proline hydroxylation and interaction with the VHL E3 ligase, we tested if the enhanced HIF1 α expression in response to Myc under normoxia is due to the loss of proline hydroxylation. We used an antibody specific to

the prolylhydroxylation site (hydroxy-P564 HIF1 α) to measure the extent of modification relative to HIF1 α protein levels. We confirmed that HIF1 α is prolyl-hydroxylated in normoxic cells, and hydroxylation is suppressed under hypoxia (Figure 1A). To compare HIF1 α modification with or without high levels of Myc, we treated cells with MG132 to prevent turnover of HIF1 α and quantitated the level of prolyl-hydroxylation to total HIF1 α levels. We find that the ratio of prolyl-hydroxylated to total HIF1 α is identical with high and low Myc (Figure 1D), thus the elevated levels of HIF1 α are not due to the suppression of proline hydroxylation. Furthermore, prolyl-hydroxylation is sensitive to reactive oxygen species (ROS), but quenching of ROS with N-acetylcysteine had no effect on the Myc-dependent accumulation of HIF1 α (Supplemental Figure).

We wanted to explore if HIF1 α protein expression is induced by Myc in other cell types. We measured protein levels in *myc*^{-/-} Rat1 fibroblasts before and after stable reconstitution with mouse c-Myc (Figure 1F). As in IMECs, HIF1 α protein levels are enhanced with Myc reconstitution under normoxic conditions, and Myc dramatically exaggerates HIF1 α levels in hypoxia. As expected, there are no changes in HIF1 α mRNA levels due to Myc expression (Figure 1G).

HIF1 induces its target gene expression

We next looked at the expression levels of HIF1 α target genes to determine if there is a functional downstream effect of enhanced HIF1 α by Myc under normoxia in IMECs. We transiently transfected vector and Myc-overexpressing IMECs with siRNAs against *HIF1A*, *c-myc*, or a non-targeting control and achieved depletion at the RNA level (Figure 2A). By RT-PCR, we found that glycolytic enzymes *HK2* and *ALDOA*, nitric oxide synthase *eNOS*, and collagen fibril assembly proteoglycan *BGN* are all transcriptionally upregulated in Myc-overexpressing cells under normoxia (Figure 2B). Of these genes, *HK2* is the only one known to be a direct binding target of both HIF1 α and Myc. Consistent with this, these genes are downregulated in response to HIF1 α depletion. These genes are also all downregulated in response to Myc knockdown, suggesting that there is indeed a functional downstream effect of enhanced HIF1 α expression by Myc.

Myc induces HIF1 expression in breast cancer cells

We also explored whether HIF1 α protein levels are dependent on Myc in human cancer cells. To this end, we examined HIF1 α and Myc in the breast cancer cell lines T47D and MCF7 (Figure 3A). MCF7 cells have 3.5-fold higher Myc protein levels than T47D and have significant constitutive HIF1 α protein under normoxic conditions, whereas T47D cells have virtually undetectable levels (Figure 3B). As in other cell types, Myc protein levels largely correspond to mRNA levels, whereas HIF1 α mRNA levels are equivalent between cell lines (Figure 3C). To test if HIF1 α protein levels are Myc-dependent in MCF7 cells, we used siRNA to deplete native Myc. We found that the loss of Myc led to a corresponding reduction in HIF1 α , suggesting that HIF1 α expression is dependent on Myc in MCF7 cells (Figure 3D).

Myc stabilizes HIF1 α protein

Since we found elevated HIF1 α protein with no change in mRNA levels (Figure 1), we hypothesized that the substantial accumulation of HIF1 α in response to Myc under normoxia and hypoxia could be due to a decrease in its rate of degradation. We treated IMEC and IMEC-Myc cells with cycloheximide (CHX) to block protein synthesis and assessed HIF1 α protein levels at 20 min intervals under both normoxic and hypoxic conditions (Figure 4). As previously described, HIF1 α has an extremely short half-life under normoxia ($t_{1/2}$ <7 min) and is barely detectable in the vector cells. However, we found that overexpression of Myc under normoxic conditions significantly stabilizes HIF1 α protein ($t_{1/2}$ =56 min). This

stabilization can account for most or all of the enhanced HIF1 α protein levels in IMEC-Myc. In contrast, Myc protein turnover is similar between control and Myc-overexpressing cells. Under hypoxia, HIF1 α protein is stabilized ($t_{1/2}$ =42 min) as previously described (11, 29, 30). However, Myc-overexpression further stabilizes HIF1 ($t_{1/2}$ >90 min), which can account for the hyper-induction of HIF1 α in hypoxic IMEC-Myc cells.

Myc enhances accumulation of VHL complex components

We next sought to determine other possible mechanisms that could account for Myc-induced stabilization of HIF1 α protein. Since HIF1 α prolyl-hydroxylation is maintained in IMEC-Myc cells (Figure 1E), we turned our focus to the next step in the normoxic regulation of HIF1 α : the recognition and ubiquitination of prolyl-hydroxylated HIF1 α by the VHL complex. We hypothesized a role for Myc in rendering the VHL complex less functional. We considered that Myc could be regulating expression levels of the components themselves, promoting protein modifications that could impact the formation of the VHL complex or affecting external cofactors that influence function. We first assessed protein levels to determine whether Myc might be downregulating one or more of the VHL components, which would de-stabilize complex formation. Quite unexpectedly, we found that Myc significantly enhances the accumulation of all of the VHL complex components under both normoxic and hypoxic conditions (Figure 5A). Furthermore, except for a two-fold induction of *VHL* RNA, the regulation appears to be entirely post-transcriptional (Figure 5B). As with HIF1 α previously, we wanted to determine whether the expression of these VHL complex components is induced by Myc in other cell types. We again measured protein levels in *myc*^{-/-} Rat1 fibroblasts before and after stable reconstitution with mouse *c-Myc*. As in IMECs, the protein levels of the VHL components are enhanced in response to elevated Myc expression under both normoxic and hypoxic conditions (Figure 5C). Of note, both VHL and Elongin B not only change in abundance but also shift in migration in SDS/PAGE (Figure 5A and 5C), suggesting that there may be Myc-induced changes in protein modification.

HIF1 α exhibits reduced binding to the VHL complex in Myc-overexpressing cells

Since we find that HIF1 α is stabilized even in the presence of upregulated VHL complex components, we hypothesized that the VHL complex is less effective at recognizing and targeting HIF1 α for degradation in Myc-overexpressing cells. To assess the association between the VHL complex and HIF1 α , we performed immunoprecipitations with different components (VHL, CUL2, and Elongin C) and with HIF1 α . Indeed, we find that there is less binding of HIF1 α to VHL complexes in IMEC-Myc cells compared to vector cells under both normoxia and hypoxia (Figure 6A). Normoxic association between HIF1 α and CUL2, Elongin C, and VHL are each reduced to only about 30% of the association seen in vector cells. This reduction may account for a significant portion of the HIF1 α stabilization in IMEC-Myc cells. Furthermore, by performing immunoprecipitations between CUL2, Elongin C, and VHL, we find that there are also apparent changes in VHL complex component associations in IMEC-Myc cells compared to vector cells (Figure 6B). This suggests that, in addition to associating less with HIF1 α , there may be fewer functional VHL E3 ligase complexes formed in Myc-overexpressing cells.

HIF1 α expression is critical for Myc-induced soft agar growth and proliferation

One of the most important questions we wanted to explore is whether or not HIF1 α accumulation contributes to Myc-induced oncogenic transformation. To address this question we performed siRNA depletion experiments and assessed their effects on Myc-induced anchorage independent growth, which is an established hallmark of transformation. We transiently transfected IMEC and IMEC-Myc with siRNAs against *HIF1A*, *c-myc*, or a non-targeting control. Myc depletion served as a positive control because soft agar growth is

Myc-dependent, and loss of Myc expression should ablate the ability of the cells to undergo anchorage-independent growth. We achieved depletion at the protein level for both Myc and HIF1 α (Figure 7A and 7B). Consistent with the Myc-dependence of HIF1 α accumulation, depleting Myc from the IMEC-Myc cells reduced the HIF1 α protein level nearly back to that found in the parental IMECs. We plated transfected cells in soft agar 24 hours post-transfection with siRNA and scored for growth 10 days later. The IMEC-Myc cells transfected with the non-targeting control grew into colonies as expected (Figure 7C). Depletion of Myc predictably led to an almost complete loss of colony growth. Strikingly, the loss of HIF1 α expression was equally as effective in blocking anchorage-independent growth, as HIF1 α depletion almost completely eliminated soft agar colony formation (Figure 7C). Vector control IMECs do not grow in soft agar and are therefore not represented (26). These data show that expression of HIF1 α is a critical component of Myc-induced anchorage independent growth. In support of these data, multiple studies have shown that the knockdown of HIF1 α in tumor cells suppresses tumor growth (31–33).

Beyond anchorage-independent growth, we showed previously that Myc overexpression also enhances the overall proliferation rate of IMECs compared to vector control cells (26). Because of the importance of HIF1 α expression for Myc-induced soft agar growth, we considered that HIF1 α might also influence proliferation rates. We measured IMEC growth rates after transfection with *HIF1A* siRNA and compared them to IMECs transfected with non-targeting siRNA under normoxia (Figure 7D). Depletion of HIF1 α expression greatly reduces the proliferation rate of Myc-overexpressing IMECs, down to the rate of vector control IMECs. *HIF1A* siRNA also had a smaller inhibitory effect on control IMECs. Thus, the enhanced growth rate of IMEC-Myc cells is highly dependent on elevated HIF1 α levels even under normoxia, and the parental cells are also dependent on low basal HIF1 α expression. Although our IMECs are non-transformed, these results are consistent with other studies that found that HIF1 α knockdown significantly reduces the growth rates of glioma cells and prostate cancer cells, respectively (34, 35).

DISCUSSION

In this study we have explored a previously uncharacterized relationship between Myc and HIF1 α expression under both normoxic and hypoxic conditions: Myc-dependent induction of HIF1 protein levels. Previous studies have shown interactions between Myc and HIF1 regulation and function, both synergistic and antagonistic. For example, in cells expressing physiologically normal levels of Myc, HIF1 has been found to functionally oppose Myc activity by disrupting Myc repression of CDKI p21 (36, 37). In addition to antagonizing Myc function through interaction, HIF1 also counters Myc-mediated regulation of mitochondrial biogenesis (4, 24, 38–40). While Myc itself promotes mitochondrial biogenesis and oxidative phosphorylation, HIF1 activates target genes that impair mitochondrial respiration. On the other hand, HIF1 and deregulated Myc cooperate to induce glycolysis in both normoxic and hypoxic tissues (22, 23, 25, 41). Studies suggest that Myc overexpression disrupts the sensitive stoichiometry normally governing HIF1 regulation of Myc. Normoxic cooperation of these transcription factors contributes to the Warburg Effect, a metabolic phenotype of aerobic glycolysis often seen in cancers.

We report that overexpression of Myc induces a dramatic enhancement in the stability of HIF1 α protein under normoxia and also increases stability under hypoxia. A previous study reported that knockdown of Myc in a multiple myeloma cell line decreases HIF1 α expression (42), but we provide the first evidence that overexpression of Myc in non-transformed epithelial and fibroblastic cells single-handedly induces the accumulation of HIF1 α . Furthermore, we show that this induction is entirely post-transcriptional and that it results in the upregulation of HIF1 α target genes. We show that Myc is also required for the

post-transcriptional accumulation of HIF1 α protein in MCF7 breast cancer cells. At the functional level, we show that HIF1 α expression is essential for both Myc-induced anchorage-independent growth and Myc-induced proliferation.

The mechanism of Myc-induced HIF1 α stabilization remains unresolved. Based on measurement of HIF1 α hydroxy-P564 levels after blocking protein turnover, it appears that there is no reduction in hydroxylation with high Myc expression (Figure 1E). The primary effect we observed is that Myc post-transcriptionally upregulates the expression of VHL complex components. Furthermore, we find that HIF1 α association with the VHL complex in cells overexpressing Myc is substantially reduced compared to vector control cells. The decreased interaction between HIF1 α and the VHL complex likely contributes significantly to the enhanced accumulation of HIF1 α protein. This could be due to an unknown modification of HIF1 α or to inactivation of the VHL complex E3 ligase activity. We find evidence of disruption of VHL complex protein stoichiometry and possible post-translational modifications of VHL and Elongin B. Further characterization of changes in the VHL complex in response to high Myc expression will be required to resolve these questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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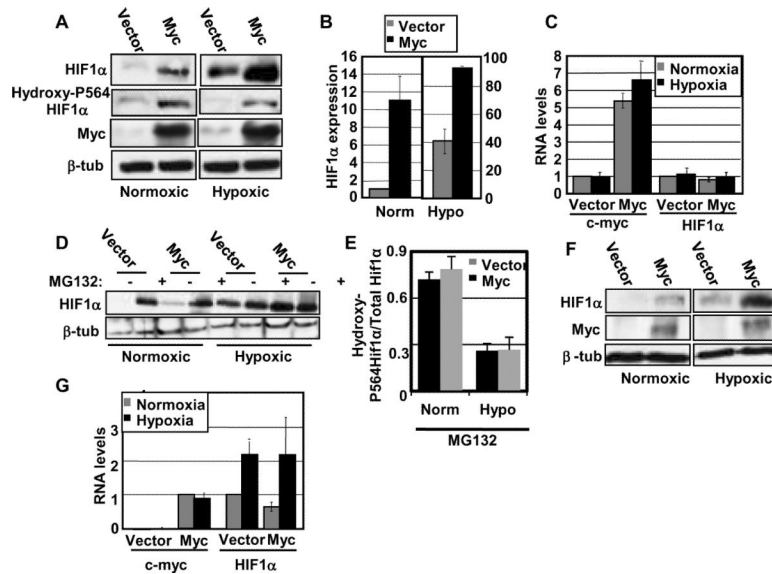


Figure 1. Myc post-transcriptionally enhances accumulation of HIF1 α under both normoxia and hypoxia

A) Western blot analyses of IMECs were performed with equivalent amounts of protein using anti-HIF1 α antibody, anti-hydroxyl P564-specific HIF1 α antibody (OH-P564), anti-c-Myc antibody, and anti- β -tubulin antibody. Hypoxic cell extracts were made after incubating cells for two hours in a hypoxia chamber with 1% oxygen. Normoxic HIF1 α blots are shown at a longer exposure than the hypoxic HIF1 α blots. Norm= Normoxia, Hypo= Hypoxia. B) Average HIF1 α protein expression, normalized to β -tubulin, was quantitated using three independent cell extract sets. Expression levels are normalized to normoxic vector. C) Real-time RT-PCR was performed using RNA harvested from IMECs and primers specific to *HIF1A*, *c-myc*, and *GAPDH* in triplicate. Average *c-myc* and *HIF1A* RNA levels are normalized to *GAPDH* and then normalized to their respective normoxic vectors. D) IMECs were treated with proteasome inhibitor MG132 under normoxic and hypoxic conditions. Western blot analyses were performed with equivalent amounts of protein using anti-HIF1 α and anti- β -tubulin antibodies. E) After treatment with MG132, average hydroxy-P564 HIF1 α protein expression was determined in comparison to total HIF1 α protein expression under normoxia and hypoxia. F) Western blot analyses of *myc*^{-/-} rat fibroblasts (vector or Myc-reconstituted) were performed as described above. G) Real-time RT-PCR was performed using RNA harvested from *myc*^{-/-} rat fibroblasts and primers specific to *HIF1A*, *c-myc*, and *GAPDH* in triplicate. Average *c-myc* and *HIF1A* RNA levels are normalized to *GAPDH*. *HIF1A* is normalized to its normoxic vector. *c-myc* is normalized to normoxic *myc*^{-/-}(Myc) since vector cells express no *c-myc* RNA. Error bars reflect the standard deviation.

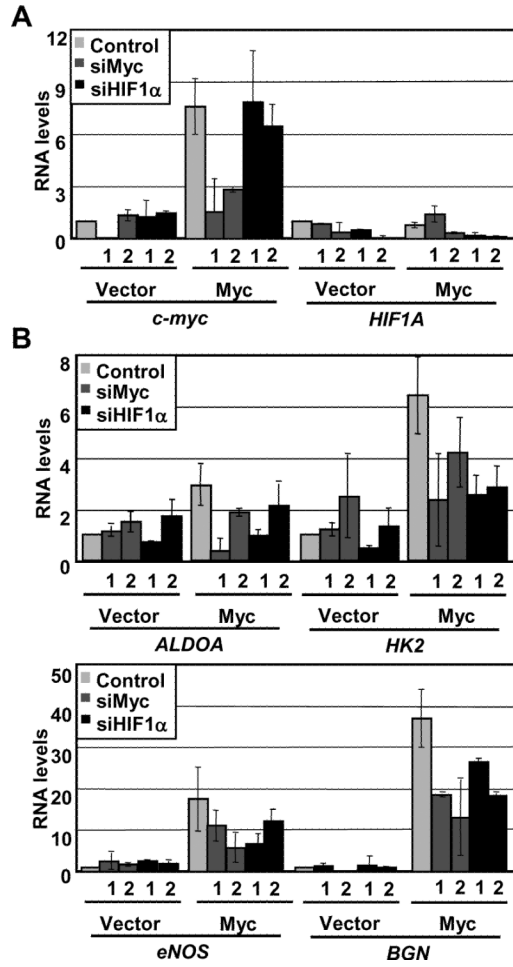


Figure 2. HIF1 α gene targets are transcriptionally upregulated in IMEC-Myc cells
 A) IMECs were transfected with non-targeting siRNA or siRNAs (1 or 2) targeting *HIF1A* or *c-myc*. RNA was isolated 48 hrs post-transfection. Real-time RT-PCR was performed using primers specific to *HIF1A*, *c-myc*, and *GAPDH* in triplicate. Average RNA levels were normalized to *GAPDH* and then normalized to their respective normoxic vectors. B) Real-time RT-PCR was performed using primers specific to *ALDOA*, *HK2*, *eNOS*, *BGN*, and *GAPDH* in triplicate. Average RNA levels were normalized to *GAPDH* and then normalized to their respective normoxic vectors. Error bars reflect the standard deviation.

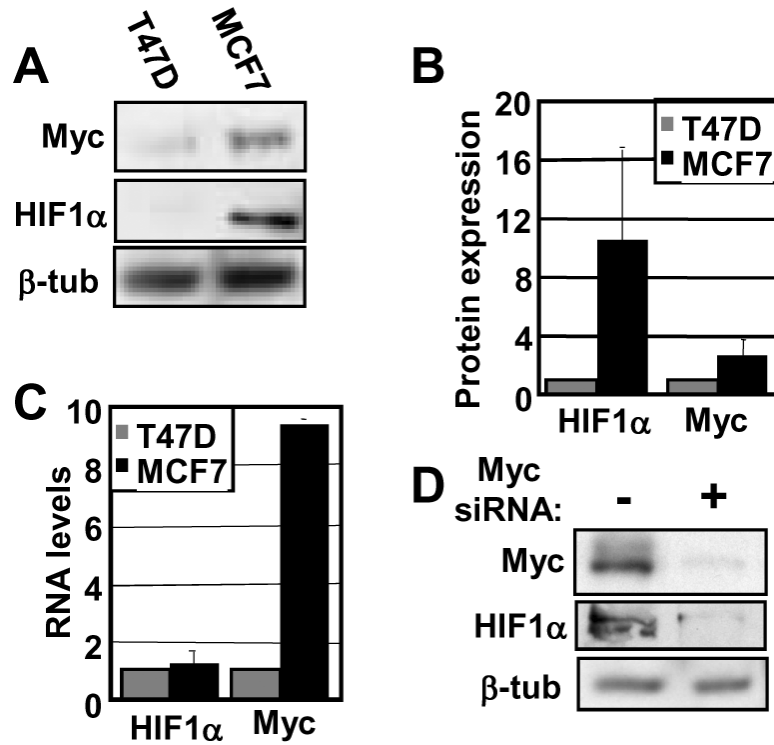


Figure 3. Myc-dependent HIF1 α expression in breast cancer cell lines

A) Western blot analyses of T47D and MCF7 breast cancer cell lines were performed with equivalent amounts of protein using anti-HIF1 α , anti-c-Myc, and anti- β -tubulin antibodies. B) Average HIF1 α and Myc protein expression, normalized to β -tubulin, were quantitated using three independent cell extract sets. Expression levels for HIF1 α and Myc are normalized to T47D. C) Real-time RT-PCR was performed using RNA harvested from T47D and MCF7 cells and primers specific to *HIF1A*, *c-myc*, and *GAPDH*. Average *HIF1A* and *c-myc* RNA levels are normalized to *GAPDH* and then normalized T47D levels. D) MCF7 cells were transfected with siRNA against *c-myc* or non-targeting siRNA. Western blot analyses were performed after 24 hours with equivalent amounts of protein using anti-HIF1 α , anti-c-Myc, and anti- β -tubulin antibodies. Error bars reflect the standard deviation.

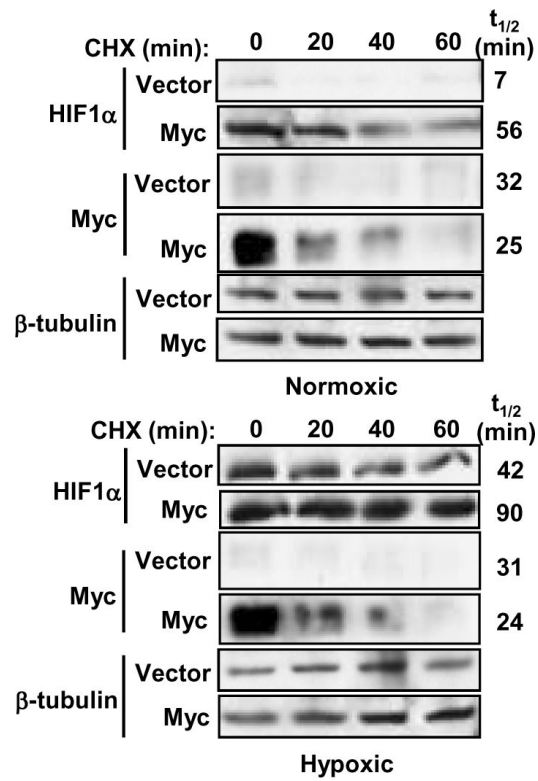


Figure 4. Expression of Myc stabilizes HIF1α protein

IMECs (vector or Myc-overexpressing) were treated with cycloheximide (CHX) for 20, 40, and 60 minutes. Hypoxic extracts were made by treating cells with 200μM CoCl₂ for 3 hours prior to CHX treatment. Western blot analyses were performed with equivalent amounts of protein using anti-HIF1α, anti-c-Myc, and anti-β-tubulin antibodies. Half-lives of HIF1α and Myc were determined after normalization to β-tubulin. Normoxic HIF1α blots are shown at a longer exposure than the hypoxic HIF1α blots.

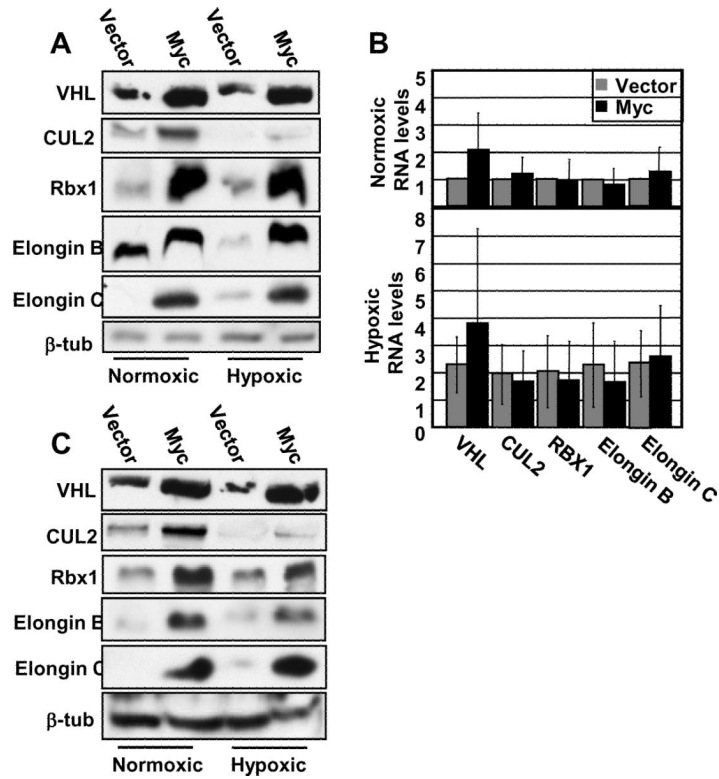


Figure 5. Myc enhances post-transcriptional accumulation of VHL complex components under both normoxia and hypoxia

A) Western blot analyses of IMEC cells were performed with equivalent amounts of protein using anti-VHL, anti-CUL2, anti-RBX1, anti-Elongin B, anti-Elongin C, and anti- β -tubulin antibodies. B) Real-time RT-PCR was performed using RNA harvested from IMECs and primers specific to *VHL*, *CUL2*, *RBX1*, Elongin B (*TCEB2*), Elongin C (*TCEB1*), and *GAPDH* in triplicate. Average RNA levels are normalized to *GAPDH* and then normalized to their respective normoxic vectors. C) Western blot analyses of *myc*^{-/-} rat fibroblasts (+/- reconstituted mouse c-Myc) were performed as described above. Error bars reflect the standard deviation.

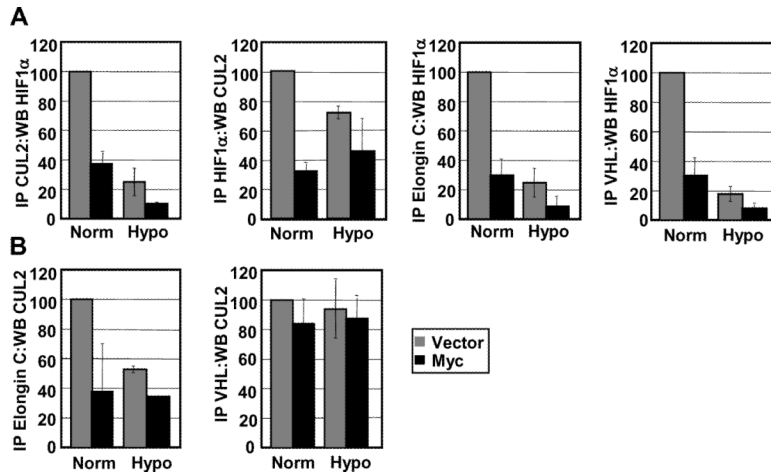


Figure 6. Reduced association of HIF1 α with the VHL complex in Myc-overexpressing cells
 A) Immunoprecipitation and western blot analyses were performed with equivalent amounts of protein from normoxic and hypoxic IMEC extracts. Immunoprecipitations were quantitated using at least two independent cell extract sets. The HIF1 α immunoprecipitation:WB CUL2 was normalized to total CUL2 protein, and all other immunoprecipitations were normalized to total HIF1 α protein. Expression levels were normalized to normoxic vector. B) Quantitated immunoprecipitations between VHL complex components were performed as described above. Immunoprecipitations were normalized to total CUL2 protein. Error bars reflect the standard deviation.

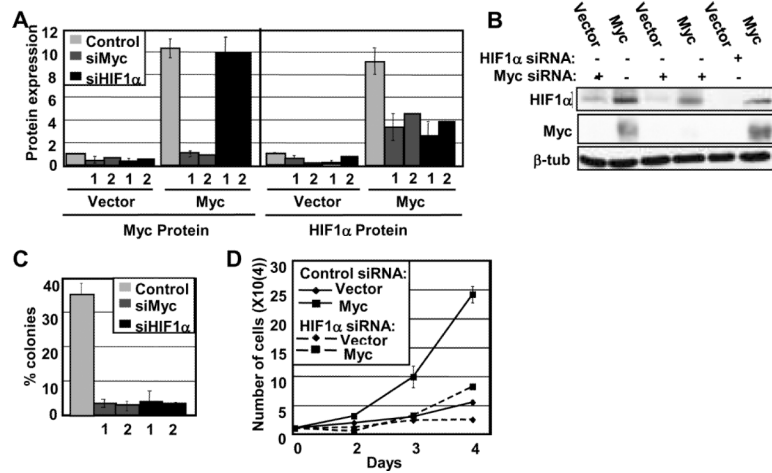


Figure 7. HIF1 α expression is affected by Myc levels and is necessary for anchorage-independent and log phase growth

A, B) IMECs were transfected with non-targeting siRNA or siRNAs (1 or 2) targeting *HIF1A* or *c-myc*. Cell extracts were made 48 hrs post-transfection. Western blot analyses were performed with equivalent amounts of cell extracts using anti-HIF1 α , anti-c-Myc, and anti- β -tubulin antibodies. Average Myc and HIF1 α protein expression, normalized to β -tubulin, were quantitated using three independent cell extract sets. Expression levels for Myc and HIF1 α protein were normalized to their respective vectors. C) IMECs transfected with non-targeting siRNA or siRNAs (1 or 2) targeting *HIF1A* or *c-myc* were plated in soft agar 24 hours post-transfection. The number of colonies per 100 plated Myc-overexpressing cells were counted and measured after 10 days in two independent experiments. Vector cells do not grow in soft agar and are therefore not included in the analysis. D) Cell proliferation rates were measured by counting equivalently plated cells that had been transfected at Day 1 with either *HIF1A* or non-targeting siRNA under normoxia. The graph shows the mean cell number for each day for duplicate experiments. Error bars reflect the standard deviation. Similar results were found for independent siRNAs.