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mAKAP compartmentalizes oxygen-dependent control of HIF-1 α

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Abstract

Induction of the hypoxia inducible transcription factor 1 α (HIF-1 α) pathway occurs during ischemic insult as an adaptation to reduced intracellular oxygen. Enzymes of the protein ubiquitin machinery that signal the destruction or stabilization of HIF-1 α tightly control this transcriptional response. We now show that the A-Kinase Anchoring Protein mAKAP organizes ubiquitin E3 ligases that manage HIF-1 α stability and optimally positions the transcription factor close to its site of action inside the nucleus. Functional experiments in cardiomyocytes demonstrate that depletion of mAKAP or disruption of its targeting to perinuclear regions alters HIF-1 α stability and the transcriptional activation of hypoxia marker genes. Thus we propose that compartmentalization of oxygen sensitive signaling components may influence the fidelity and magnitude of the hypoxic response.

INTRODUCTION

Cellular oxygen levels are maintained within a narrow range (termed normoxia) [1]. A cellular response to reduced oxygen (hypoxia) involves mobilization of the hypoxia inducible transcription factor 1 α (HIF-1 α) pathway [2]. Under normoxic conditions HIF-1 α levels are kept low by ubiquitin mediated proteosomal degradation [3]. During hypoxia the continual destruction of HIF-1 α is halted. This allows the protein to accumulate in the nucleus where it initiates transcription as an adaptive response to reduced oxygen. In the heart induction of HIF-1 α is an early marker of myocardial infarction [4]. Although the molecular mechanisms underlying the destruction or maintenance of HIF-1 α protein are well defined [2], the subcellular organization of factors that regulate these processes have not been investigated. In this report we show that the cardiac A-kinase anchoring protein (mAKAP) organizes components of the protein ubiquitin machinery that signal the death and life of HIF-1 α . During normoxia, mAKAP-mediated clustering of HIF-1 α with negative regulatory factors enhances its degradation. Yet under hypoxic conditions, positive regulatory factors in the mAKAP complex favor the stabilization of HIF-1 α to initiate a transcriptional response.

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RESULTS AND DISCUSSION

Several cardiac AKAPs were screened as potential HIF-1 α scaffolding proteins. Epitope-tagged (GFP) AKAP79, gravin, AKAP18 α and mAKAP were co-expressed with HIF-1 α in HEK293 cells. Immune complexes containing each AKAP were probed for co-purification of V5-tagged HIF-1 α using antibodies against the epitope tag. Only mAKAP precipitated with HIF-1 α as detected by immunoblot (Fig. 1A, top panel, lane 4). Reciprocal experiments detected mAKAP in HIF-1 α immune complexes (Fig. 1B, top panel, lane 2). Control experiments showed that the anchoring protein did not co-purify with another transcription factor, MEF2C (Fig. 1B, top panel, lane 1). mAKAP is a particularly attractive candidate to anchor HIF-1 α , since it is tethered to the perinuclear membrane in cardiomyocytes [5,6]. Sequestering of HIF-1 α at this location could minimize the translocation distance to its site of action in the nucleus.

Ubiquitination of HIF-1 α signals its proteosomal degradation [2]. Thus, it seemed reasonable to establish whether the transcription factor was ubiquitinated in the context of the mAKAP complex. To test this hypothesis HEK293 cells were transfected with plasmids encoding mAKAP and HA-tagged ubiquitin. Ubiquitin was detected in mAKAP immune complexes by immunoblotting (Fig. 1C, top panel). The ubiquitin signal was enhanced upon treatment with the proteasome inhibitor MG132 (Fig. 1C, top panel, lane 2). Apparently mAKAP is one of the ubiquitinated proteins although treatment with a proteasomal inhibitor did not markedly alter its protein levels (Fig. 1C, bottom panel, lane 2). Further experiments established if HIF-1 α was one of the ubiquitinated proteins in the mAKAP complex. Oligonucleotide based RNA interference was used to suppress HIF-1 α expression in HEK293 cells (Fig. 1D & Supplemental Data, Fig. S1). As a consequence, ubiquitin levels were reduced in mAKAP immune complexes isolated from cells where HIF-1 α expression was silenced (Fig. 1D, top panel, lane 2). Collectively, these results suggest that HIF-1 α can be ubiquitinated when bound to mAKAP. A more intriguing implication is that mAKAP may compartmentalize components of the ubiquitination machinery to organize a localized degradation loop for HIF-1 α .

Proteosomal degradation of HIF-1 α under normoxic conditions is triggered by hydroxylation of prolines 402 and 564 [7]. This posttranslational modification event is catalyzed by oxygen-sensitive dioxygenases called PHDs [8]. The PHD2 and PHD3 isoforms are the principal regulators of HIF-1 α levels during normoxia in the heart [9,10]. Accordingly, mAKAP was detected in PHD2 and PHD3 immune complexes isolated from neonatal rat ventricular myocyte (NRVM) extract (Figs. 1E & F, top panels, lanes 2). Control experiments indicated that the PHD1 isoform did not interact with mAKAP (Supplemental Data, Fig. S2). Prolyl hydroxylation of HIF-1 α provides recognition for an E3 ubiquitin ligase complex containing the von Hippel-Lindau (VHL) tumor suppressor protein [11]. VHL is also a component of the mAKAP complex. The anchoring protein was detected in VHL immune complexes isolated from NRVM extracts (Fig. 1G, top panel, lane 2). Reciprocal studies detected VHL in mAKAP immune complexes when both proteins were expressed in HEK293 cells (Fig. 1H, top panel, lane 2). Control experiments confirmed that mAKAP did not bind to HA-tagged PTEN (Fig. 1H, top panel, lane 1 and Supplemental Data, Fig. S3A).

The biochemical data presented thus far suggests that mAKAP assembles the enzymes that signal the degradation of HIF-1 α . To test this notion by an alternate method, we examined whether mAKAP could suppress HIF-1 α transcriptional activity using a luciferase reporter system driven by the VEGF promoter. HIF-1 α transcriptional activity was reduced 59.6 ± 0.1 % (n=3) in mAKAP-transfected cells (Fig. 1J, column 2) when compared cells expressing the vector control (Fig. 1J, column 1, p=0.001). Control experiments showed that expression of another anchoring protein AKAP18 α had no effect on HIF-1 α transcriptional activity (Fig. 1J,

column 3). This lends further support to the notion that mAKAP organizes components of a degradation pathway for HIF-1 α .

Under hypoxic conditions, HIF1 α protein accumulates because PHD activity is neutralized. There are two aspects to this process: reduced oxygen inhibits the PHDs and another ubiquitin E3 ligase targets the enzyme for proteosomal degradation [8,12]. The *seven in absentia* homolog 2 (Siah2) is the E3 ligase that catalyzes this latter step. Thus we postulated that Siah2 and mAKAP might interact. Accordingly, endogenous mAKAP was detected in Siah2 immune complexes isolated from NRVM extracts (Fig. 2A, top panel lane 2). Siah2 self-ubiquitinates and is targeted for proteosomal degradation; consequently many cell based experiments are performed using the catalytically inactive point mutant of the enzyme (H99Y) [13]. Expression of the H99Y Siah2 mutant was used to verify interaction with the anchoring protein in HEK293 cells (Fig. 2B, top panel lane 2 & Supplemental Data, Fig. S3B). Control experiments confirmed mAKAP did not interact with MuRF1, a structurally similar ubiquitin E3 ligase (Fig. 2B, top panel lane 1). If Siah2 neutralizes PHD activity, one would predict that the net effect would be stabilization of the mAKAP-associated pool of HIF-1 α . This view was supported by evidence that more HIF-1 α was detected in mAKAP immune complexes that were isolated from cells expressing wild-type Siah2 than from cells expressing the H99Y mutant (Fig. 2C, top panel). On the basis of our findings, we propose that mAKAP organizes a sub-complex of proteins that regulate the oxygen dependent degradation and activation of HIF-1 α . The presence of Siah2 targets the anchored pool of PHD for ubiquitin dependent degradation (Fig. 2D). This ultimately leads to the stabilization of HIF-1 α (Fig. 2D).

Thus mAKAP is one of a few anchoring proteins that interface with ubiquitin pathways. Interestingly each anchoring protein does so in a different manner. For example, ubiquitination of AKAP79/150 binding partner PSD-95 regulates ion channel surface expression during synaptic plasticity [14] whereas ubiquitin-mediated proteosomal degradation of the mitochondrial anchoring protein AKAP121 effects the oxidative capacity of these organelles [15]. In contrast, mAKAP is not marked for proteosomal degradation but organizes components of the ubiquitination machinery to facilitate the bidirectional control of HIF-1 α stability (Fig 2D).

Three complementary approaches were used to test this hypothesis in heart cells. RNA interference was used to silence the expression of mAKAP in NRVM [16]. Reduced expression of the anchoring protein was confirmed by immunofluorescence staining (Fig. 3A–F). NRVM transfected with the siRNA against mAKAP displayed no mAKAP immunoreactivity compared to control siRNA counterparts (Fig. 3A&D). Changes in HIF-1 α protein levels were assessed by immunoblot in NRVM extracts. HIF-1 α protein was detected in the nuclear fractions of control NRVM exposed to hypoxia (Fig. 3G, top panel, lane 4). Importantly, nuclear accumulation of HIF-1 α was less pronounced in hypoxic NRVM where mAKAP expression had been suppressed (Fig. 3G, top panel, lane 8). Control experiments established that mAKAP protein expression was knocked down by the appropriate siRNA and verified the integrity of the subcellular fractionation procedure using PCAF as a marker for the nuclear fraction (Fig. 3G, middle and bottom panels). To confirm that the decrease in HIF-1 α nuclear translocation altered the hypoxia response, we utilized quantitative PCR to monitor the transcription of two classic HIF-1 α target genes: vascular endothelial growth factor (VEGF) and glucose transporter 1 (Glut-1). Both proteins are recognized as cellular markers for hypoxia [17,18]. VEGF and Glut-1 mRNA levels were elevated 5.9 ± 0.6 ($n=3$, $p=0.001$) and 8.6 ± 0.9 ($n=9$, $p=0.000002$) fold respectively in hypoxic NRVM (Fig. 3H&I). However, knockdown of mAKAP expression blunted the hypoxic upregulation of VEGF by 33% (3.7 ± 0.2 ; $n=3$, $p=0.02$ compared to hypoxic control siRNA, Fig. 3H) and Glut-1 by 25% (6.1 ± 0.4 ; $n=9$, $p=0.02$ compared to hypoxic control siRNA, Fig. 3I). Thus, silencing of mAKAP expression attenuates the HIF-1 α -dependent response to hypoxia in NRVM.

Further support for this concept was provided by competitive displacement of the mAKAP complex from perinuclear membranes in NRVM (Fig. 4A–F). This was achieved by adenoviral expression of the mAKAP 340–1041 fragment. This reagent competes with the native anchoring protein for interaction with nesprin, an outer nuclear membrane protein [19]. Accordingly, mAKAP staining was cytoplasmic and diffuse upon expression of the 340–1041 fragment (Fig. 4D–F). Subcellular fractionation experiments showed that mislocalization of mAKAP impeded the nuclear translocation of HIF-1 α protein during hypoxia (Fig. 4G, top panel, lanes 4 & 8). Control immunoblots confirmed that disruption of mAKAP targeting increased the amount of anchoring protein detected in the cytoplasmic fraction (Fig. 4G, second panel, lanes 5&6). PCAF was used as a nuclear marker and expression of the myc tagged mAKAP 340–1041 fragment was confirmed by immunoblot (Fig. 4G, third & bottom panel, lanes 5 & 6). The functional consequences of mAKAP mislocalization were assessed by measuring changes in the transcriptional activation of the HIF-1 α gene targets, VEGF and Glut-1 (Fig. 4H&I). Under hypoxic conditions, VEGF and Glut-1 mRNA levels were elevated 7.3 ± 0.4 fold ($n=3$, $p=0.0004$, Fig. 4H) and 12.5 ± 1.0 fold ($n=3$, $p=0.00006$, Fig. 4I), respectively. However, competitive displacement of the mAKAP complex blunted transcriptional activation of VEGF and Glut-1 mRNA by 24% and 28% respectively ($n=3$, $p=0.02$ compared to hypoxic control siRNA in Fig. 4H and $n=3$, $p=0.05$ compared to hypoxic control siRNA, in Fig. 4I). Therefore, displacement of mAKAP from perinuclear membranes of myocytes blunts the HIF-1 α -dependent response to hypoxia.

Our final approach attempted to selectively displace HIF-1 α and its regulatory enzymes from mAKAP. Initially, the binding site for HIF-1 α was mapped to the first 340 amino acids on mAKAP (Supplemental Data, Fig. S4). Related experiments demonstrated that the same fragment of the anchoring protein is necessary for interaction with VHL and Siah2 (Supplemental Data, Fig. S4). This region of the anchoring protein was used to specifically displace these enzymes from the anchoring protein in NRVM. Induced adenoviral expression of this mAKAP fragment did not alter the subcellular distribution of the anchoring protein in NRVM (Fig. 5A–F). However, expression of mAKAP 1–340 did reduce the nuclear accumulation of the transcription factor under hypoxic conditions (Fig. 5G, top panel, lanes 4 and 8). Control immunoblots detected PCAF in the nuclear fraction (Fig. 5G, third panel), and confirmed expression of the Myc tagged mAKAP 1–340 fragment (Fig. 5G, bottom panel, lanes 4–8). More importantly, expression of the 1–340 fragment effected hypoxic induction of VEGF and Glut-1 transcription (Fig. 5H&I). VEGF and Glut-1 mRNA levels were upregulated 8.36 ± 1.19 ($n=3$, $p=0.003$, Fig. 5H) and 13.3 ± 0.8 ($n=3$, $p=0.00009$, Fig. 5I) fold respectively in hypoxic NRVM. However, expression of the fragment partially blunted upregulation of VEGF and Glut-1 mRNA levels by 52% and 60% respectively ($n=3$, $p=0.04$ in Fig. 5H and $n=3$, $p=0.05$ in Fig. 5I) in NRVM exposed to hypoxia.

The findings of this report suggest that we have discovered an unanticipated role for mAKAP in cellular oxygen homeostasis. It functions as a scaffolding protein that signals the death and life of HIF-1 α . During normoxia, the mAKAP-mediated clustering of HIF-1 α with regulatory factors such as PHDs and VHL provides a means to enhance the efficiency of protein degradation (Fig. 5J). Yet under hypoxic conditions the recruitment of Siah2 favors the stabilization of HIF-1 α (Fig. 5K). The induction of HIF-1 α target genes protects against ischemic insult in organs vulnerable to injury from oxygen deprivation such as the heart and brain [20]. We now propose that mAKAP, an anchoring protein that is expressed primarily in cardiomyocytes and neurons, is an additional regulatory element that enhances the hypoxic response. It organizes enzymes that constrain HIF-1 α stability and optimally positions the transcription factor close to its site of action inside the nucleus.

Components of the mAKAP complex could also assist in the accumulation of HIF-1 α under pathophysiological conditions where oxygen supply to the heart is limited. This certainly fits

with evidence that HIF-1 α protein levels are elevated upon myocardial infarction and that boosting expression of the transcription factor during pressure overload can prevent the transition from cardiac hypertrophy to heart failure [21]. These findings infer a link between hypoxia and hypertrophic signaling pathways. In fact, mAKAP may provide the link between these pathways since the anchoring protein is up regulated in response to hypertrophic stimuli [5]. Further support for this notion comes from evidence that mAKAP anchors two signaling enzymes that act sequentially to influence cardiomyocyte hypertrophy and HIF-1 α stability. One of these anchored enzymes is the cAMP-responsive guanine nucleotide exchange factor Epac-1 [16]. It is the upstream element in a signaling pathway that modulates the activity of ERK5, a protein kinase known to augment the hypertrophic response [22] and influence HIF-1 α stability [23]. Thus, certain mAKAP complexes may create cellular microenvironments where cAMP signals can feed into O₂ responsive transcriptional activation pathways. This underscores an emerging aspect of AKAP biology: the multiprotein complexes that they maintain not only control second messenger signaling events but also shape broader aspects of cellular regulation.

EXPERIMENTAL PROCEDURES

Antibodies

The following primary antibodies were used for immunoblotting and immunoprecipitation: mouse monoclonal Flag antibody (Sigma, St. Louis, MO), mouse monoclonal HA antibody (Sigma), rabbit polyclonal antibody to GFP (Molecular Probes, Eugene, OR), mouse monoclonal HIF-1 α antibody (Novus Biologicals, Littleton, CO), rabbit polyclonal mAKAP antibody (VO54 and Covance, Princeton, NJ), mouse monoclonal mAKAP antibody (Covance), mouse monoclonal Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA and Upstate, Lake Placid, NJ), mouse monoclonal PCAF antibody (Santa Cruz), rabbit polyclonal PHD2 antibody (Novus Biologicals), rabbit polyclonal PHD3 antibody (Novus Biologicals), mouse monoclonal V5 antibody (Invitrogen), mouse monoclonal Siah2 antibody (Sigma), mouse monoclonal α -tubulin antibody (Sigma), rabbit polyclonal VHL antibody (Santa Cruz) and mouse monoclonal VSV antibody (Sigma).

Plasmid constructs

Plasmids encoding HIF-1 α , MEF2C, PHD1, Siah2 and MuRF1 were constructed using clones from the Mammalian Gene Collection (Open Biosystems, Huntsville, AL) as a template for PCR. The QuikChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce two point mutations (P402A and P564G) in the HIF-1 α construct to abolish oxygen-dependent prolyl hydroxylation [7,24] and a single point mutation (H99Y) in Siah2 to create a catalytically inactive mutant [25].

siRNAs

The custom synthesized mAKAP siRNA (Dharmacon, Lafayette, CO) was based on our previously characterized shRNA [16]. The HIF-1 α siRNA pool is commercially available (Dharmacon), as is the negative control siRNA (Qiagen, Valencia, CA).

Cell culture, transfection and infection

HEK293 cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum. Plasmids were transfected into HEK293 cells using calcium phosphate or Lipofectamine Plus (Invitrogen, Carlsbad, CA). Primary NRVM were prepared as previously described [26]. NRVM were transfected with control or mAKAP siRNA using DharmaFECT1 and analyzed 72 hours later. Adenoviral infection of NRVM was performed as previously described [26].

Immunocytochemistry

NRVM were fixed, permeabilized and incubated with primary and secondary antibodies as previously described [5].

Immunoprecipitations

Immunoprecipitations were performed as previously described [27].

Reporter gene assays

HEK293 cells were plated at a density of 5×10^4 cells/well and transfected with pcDNA3, AKAP18 α or mAKAP along with pVEGF (ATCC, Manassas, VA) and pTK-Renilla (a gift from R.H. Goodman) using *TransIT*-LT1 (Mirus Bio, Madison, WI). After lysing cells in Passive Lysis Buffer (Promega, Madison, WI), the ratio of firefly to Renilla luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

In vivo ubiquitination reactions

HEK293 cells were treated with 40 μ M MG132 (Calbiochem, Gibbstown, NJ) or vehicle (DMSO) for 4 hours at 37°C. Immunoprecipitations were performed as described above.

Hypoxic treatment

NRVM medium was incubated overnight in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with the appropriate gas mix: 5% CO₂/95% N₂ for HIF-1 α protein analysis, 1% O₂/5% CO₂/95% N₂ for HIF-1 α transcriptional analysis. The equilibrated medium was added to cells, which were transferred to the modular incubator chamber. After flushing the chamber again, cells were incubated at 37°C for 4 h for protein analysis and 6 h for transcriptional analysis.

Subcellular fractionation

NRVM were separated into cytosolic fractions and nuclear extracts using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL).

qPCR

Total RNA was extracted from NRVM using the RNeasy (Qiagen) or Absolutely RNA (Stratagene) miniprep kits. RNA was converted to cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). VEGF and Glut-1 mRNA levels were assessed with TaqMan Gene Expression Assays and normalized to 18S.

Statistical analysis

All statistical tests were carried out using one-way ANOVA followed by two-tailed unpaired Student's *t*-tests using Origin (OriginLab Corporation, Northampton, MA). All alpha levels were set to 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. mAKAP assembles components of the HIF-1α degradation pathway

(A) Immunoprecipitated GFP-tagged AKAPs (*indicated*) were screened for V5-tagged HIF-1α binding by immunoblot (*top*). AKAP-GFP immune complexes (*middle*) and V5-HIF-1α inputs (*bottom*) are shown. (B) V5-MEF2C and V5-HIF-1α were screened for mAKAP binding. Co-precipitation of mAKAP was detected by immunoblot (*top*). V5-tagged immune complexes (*middle*) and the mAKAP input (*bottom*) are indicated. (C) The mAKAP complex was probed for ubiquitination from cells treated with the proteasome inhibitor MG132 or vehicle alone (*indicated above each lane*). Incorporation of HA-ubiquitin was detected by immunoblot (*top*). Immunoprecipitated mAKAP was detected by immunoblot (*bottom panel*). (D) Ubiquitination of proteins in the mAKAP complex is reduced upon RNAi

knockdown of HIF-1 α expression. mAKAP immune complexes were isolated from cells exposed to control and HIF-1 α siRNA (*indicated above each lane*). Ubiquitin was detected by immunoblot (*top panel*). mAKAP levels in the immune complexes were detected by a Myc antibody (*middle panel*). All HA-ubiquitinated proteins in cells treated with MG132 were detected by immunoblot (*bottom panel*). **(E)** The prolyl hydroxylase isoform PHD2 was immunoprecipitated and screened for mAKAP binding in NRVM. Co-precipitated mAKAP was detected by immunoblot (*top left blot*). The amount of PHD2 precipitated is indicated (*bottom left blot*). Amount of proteins in Input are shown (*right top and bottom*). **(F)** The endogenous prolyl hydroxylase isoform PHD3 was immunoprecipitated and screened for mAKAP binding in NRVM. Co-precipitated mAKAP was detected by immunoblot (*top left blot*). The amount of PHD3 precipitated is indicated (*bottom left blot*) and protein inputs are shown (*right top and bottom*). **(G)** Endogenous VHL was screened for mAKAP binding in NRVM. Co-precipitated mAKAP was detected by immunoblot (*top left panel*). The amount of VHL immunoprecipitated is shown (*bottom left panel*). Input mAKAP level in both control and VHL immunoprecipitates detected by immunoblot (*top right blot*). **(H)** mAKAP immune complexes from HEK cells were tested for co-precipitation of HA-tagged PTEN and VHL by immunoblot (*indicated above each lane, top panel*). The amount of mAKAP in each immunoprecipitate is indicated (*middle panel*). PTEN and VHL were detected in cell lysate input by immunoblot (*bottom panel*). Molecular weight markers are indicated in figures 1A–I (*all panels*). **(I)** HEK293 cells were transfected with mAKAP, AKAP18 α , or vector alone. In addition both pVEGF, a firefly luciferase reporter vector for HIF-1 α , and pTK-Renilla, a Renilla luciferase expression vector, were transfected. Dual luciferase assays were performed on cell lysates, and firefly luciferase values were normalized to Renilla values. Amalgamated data from three independent experiments is shown. The statistical significance of reporter data in part i was calculated using one-way ANOVA followed by two-tailed Student's t-test. Statistical significance of mAKAP versus vector is $p = 0.001$. Error bars represent S.E.M.

Figure 2. mAKAP coordinates a HIF-1 α activation pathway

(A) Endogenous Siah2 binds to mAKAP in NRVM. Siah2 was immunoprecipitated from NRVM lysates and co-purification of mAKAP was detected by immunoblot (*top left panel*). The amount of Siah2 immunoprecipitated (*bottom panel*) and mAKAP in NRVM lysates (*top, right panel*) are indicated. (B) mAKAP selectively binds to Siah2. Flag-tagged MuRF1 or Flag-tagged Siah2 (indicated above each lane) was co-expressed with mAKAP in HEK293 cells. Flag-tagged proteins in mAKAP immune complexes were detected by immunoblot. The amount of mAKAP in each immunoprecipitate is indicated (middle panel). The levels of MuRF1 and Siah2 in cell lysates are indicated (bottom panel). (C) Siah2 activity stabilizes HIF-1 α that is associated with mAKAP. HEK293 cells were co-transfected with Siah2 or the catalytically inactive Siah2 H99Y mutant and mAKAP. HIF-1 α co-precipitating with mAKAP was detected by immunoblot (top panel). The amount of mAKAP in each immunoprecipitate is indicated (bottom panel). (D) Model showing that PHD becomes ubiquitinated by Siah2 in the mAKAP complex during hypoxia.

Figure 3. RNAi knockdown of mAKAP reduces transcription of HIF-1 α target genes in NRVM (A–F) NRVM were transfected with control (A–C) or mAKAP (D–F) siRNA and incubated for 72 hours. The cells were fixed and stained for mAKAP (A&D) and α -actinin (B&E) as a marker for NRVM. Composite images (C&F) are presented. (G) mAKAP RNAi reduces HIF-1 α nuclear translocation. NRVM were transfected with control or mAKAP siRNA, exposed to normoxic or hypoxic conditions and subjected to subcellular fractionation. Cytoplasmic and nuclear fractions were immunoblotted for HIF-1 α , mAKAP and PCAF (as a marker for the nuclear fraction). (H–I) mAKAP RNAi reduces HIF-1 α transcriptional activity. NRVM were transfected with control or mAKAP siRNA and exposed to normoxic or hypoxic conditions prior to RNA extraction. Quantitative PCR was performed to assess VEGF (H) and

Glut-1 (I) gene expression, which was normalized to 18S. Amalgamated data from at least three independent experiments is shown. The statistical significance of the qPCR data in parts H and I was calculated using one-way ANOVA followed by two-tailed Student's t-test. Statistical significance of mAKAP versus control siRNA is $p=0.03$ for VEGF and $p=0.02$ for Glut-1. Error bars represent S.E.M.

Figure 4. mAKAP mistargeting reduces transcription of HIF-1 α target genes in NRVM (A–F) NRVM were infected with the mAKAP 340–1041 and Tet-OFF adenoviruses and cultured in 50 (A–C) or 0.5 ng/ml (D–F) doxycycline for 48 hours. The cells were fixed and stained for mAKAP (A&D) and Myc (B&E) to detect fragment expression. Composite images (C&F) are presented. (G) The mAKAP 340–1041 fragment reduces HIF-1 α nuclear translocation. NRVM were infected, exposed to normoxic or hypoxic conditions and subjected to subcellular fractionation. Cytoplasmic and nuclear fractions were immunoblotted for HIF-1 α , mAKAP, PCAF (as a marker for the nuclear fraction) and Myc. (H–I) The mAKAP 340–1041 fragment reduces HIF-1 α transcriptional activity. NRVM were infected and exposed to normoxic or hypoxic conditions prior to RNA extraction. Quantitative PCR was performed

to assess VEGF (H) and Glut-1 (I) gene expression, which was normalized to 18S. Amalgamated data from three independent experiments is shown. The statistical significance of the qPCR data in parts H and I was calculated using one-way ANOVA followed by two-tailed Student's t-test. Statistical significance of mAKAP versus control siRNA is $p=0.02$ for VEGF and $p=0.05$ for Glut-1. Error bars represent S.E.M.

Figure 5. HIF-1 α mistargeting reduces transcription of HIF-1 α target genes in NRVM
 (A–F) NRVM were infected with mAKAP 1–340 and Tet-OFF adenoviruses and cultured in 50 (A–C) or 0 ng/ml (D–F) doxycycline for 48 hours. Cells were fixed and stained for mAKAP (A&D) and Myc (B&E) to detect fragment expression. Composite images (C&F) are presented. (G) The mAKAP 1–340 fragment reduces HIF-1 α nuclear translocation. NRVM were infected, exposed to normoxic or hypoxic conditions and then subcellular fractionation. Cytoplasmic and nuclear fractions were immunoblotted for HIF-1 α , mAKAP, PCAF (nuclear fraction marker) and Myc. (H–I) The mAKAP 1–340 fragment reduces HIF-1 α transcriptional activity. NRVM were infected and exposed to normoxic or hypoxic conditions prior to RNA extraction. Quantitative PCR was performed to assess VEGF (H) and Glut-1 (I) gene expression

(normalized to 18S). Amalgamated data from three independent experiments are shown. Statistical significance of the qPCR data in parts H and I was calculated using one-way ANOVA followed by two-tailed Student's t-test. Statistical significance of mAKAP versus control siRNA is $p=0.04$ for VEGF and $p=0.01$ for Glut-1. Error bars represent S.E.M. **(J–K)** Model showing role of mAKAP in regulation of HIF-1 α degradation during normoxia and activation during hypoxia.