INVolVEMENT OF NeT AND hiFi1α IN DISTINCT yEt INTRICATELY LINKED hYPOXIa INDUCED SIGNALLING PATHWAYS
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Running title: Net and HIF1α in hypoxia signaling pathways

The present study compares Net (Negative Ets Transcription factor) and HIF1α (Hypoxia Inducible Factor 1α) regulation by hypoxia. Their protein stabilities are differently regulated by hypoxia, defining three periods in the kinetics: normoxia (high Net levels and low HIF1α levels), early hypoxia (high levels of Net and HIF1α), late hypoxia (degradation of Net and HIF1α). Modulators of PHD (Prolyl Hydroxylase Domain protein) activity induce a mobility shift of Net, similar to HIF1α, suggesting that posttranslational modifications of both factors depend on PHD activity. The three PHDs have different roles in the regulation of Net protein levels – PHD1 and PHD3 are involved in the stabilization of Net, whereas PHD2 controls its degradation in late hypoxia. Net physically interacts with PHD2 in hypoxia, while PHD1 and PHD3 bind to Net in normoxia and hypoxia. Under the same conditions, PHD2 and PHD3 regulate both HIF1α stabilization in early hypoxia and its degradation at late hypoxia, whereas PHD1 is involved in HIF1α degradation in late hypoxia. We describe interconnections between the regulation of both Net and HIF1α at the protein level. Evidence is provided for a direct physical interaction between Net and HIF1α and indirect transcriptional regulation loops that involve the PHDs. Taken together our results indicate that Net and HIF1α are components of distinct signaling pathways that are intricately linked.

Hypoxia is a reduction in the normal level of tissue oxygen tension that occurs in many disease processes including cancer. It arises in solid tumors due to a mismatch between tumor growth and angiogenesis, and is associated with an aggressive phenotype, resistance to radiation therapy and chemotherapy, as well as poor patient prognosis (1,2). The cellular response to hypoxia involves the induction of the Hypoxia Inducible Factor 1α (HIF1α), considered to be the major transcription factor involved in gene regulation by hypoxia (3). In normoxia, HIF1α is hydroxylated by the cellular oxygen “sensors” Prolyl-Hydroxylase Domain proteins (PHD1, PHD2 and PHD3), and degraded by proteasomes [reviews: (4,5)]. The PHDs are not active in hypoxia, resulting in stabilization and activation of transcription by the non-hydroxylated and stabilized form of HIF1α (6,7).

We recently identified a new component of the hypoxic response, the ternary complex factor Net (Elk3) (8). Under basal conditions, Net is a strong repressor of transcription, but it can be converted to an activator by phosphorylation of its activation domain by the growth factor–Ras–mitogen-activated protein kinase pathway (9-12). Net is involved in the regulation of various physiological processes, including cell migration, inflammation, wound healing and angiogenesis (13-15). Loss of Net as a repressor and consequent activation of c-fos expression has been suggested to be a key event in HPV induced carcinogenesis (16). Progression and treatment of cervical as well as other cancers implicate the hypoxic response (1,17). We previously reported that hypoxia enhances Net ubiquitylation, nuclear export, and subsequent proteasomal degradation (8). In a large-scale analysis of RNA expression using microarrays in transformed mouse endothelial cells, we found that the majority of the genes induced in hypoxia require Net and HIF1α, suggesting that the functions of these factors are
closely linked (18). In our current study, we compared Net and HIF1α regulation in response to hypoxia in cells in which Net is a negative regulator (16). These cells (444) are one of the components of a cell-based model of cervical cancer progression (19,20). We demonstrate that the hypoxia induced signaling pathways that involve Net and HIF1α have distinct features and that there are interconnections between Net and HIF1α at various levels. These results suggest that Net and HIF1α cross talk in response to hypoxia and that the functional status of either factor will influence the way the complementary factor orchestrates the physiological outcome.

**Experimental Procedures**

**Cell culture, transfection and hypoxic treatment:** Non-malignant hybrids (called “444”) made between HeLa and normal human fibroblasts (E.J Stanbridge University of California) were maintained in Dulbecco’s Modified Eagles’s medium (DMEM), 1g/L glucose, 10% fetal calf serum (FCS) and 40 ±g/ml gentamycin. Cervical carcinoma HeLa cells were maintained in DMEM, 1g/L glucose, 5% FCS and 40 ±g/ml gentamycin. Human Embryonic Kidney 293 T (HEK293T) cells were maintained in DMEM, 1g/L glucose, 10% FCS, 100 UI/ml penicillin and 100 µg/ml streptomycin. For plasmid transfection experiments, 444 and HeLa cells were transfected with Jet-Pei (Polyplus Transfection). HEK293T were transfected using the calcium phosphate precipitation method. For siRNA transfection experiments, 444 cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as previously described (14,16). The normoxic environment conditions are 19.7% O2, 5% CO2, and 37°C in a ThermoForma incubator. The hypoxia conditions are 1% O2, 5% CO2, and 37°C in a ThermoForma model 3110 incubator (18).

**Chemicals:** Cobalt chloride (CoCl2; Alfa Aesar), iron chloride (FeCl2; Sigma), 2-oxoglutarate (Sigma) and ascorbic acid (Sigma).

**RNA interference and Plasmids:** The small interfering RNAs (siRNAs): human Net, PHD1, PHD2, PHD3, HIF1α siRNA (siGENOME SMARTpool reagent; Dharmacon Inc.); GL2 luciferase control siRNA (Sigma); and ascorbic acid (Sigma).

**Immunoblotting and antibodies:** For preparation of nuclear cell extracts and immunoblotting see Supplemental information. Antibodies and dilutions: anti-mouse Net #2620 (1/1000) for overexpression experiments; anti-rabbit Net #2005 (1/1000) for detection of endogenous levels; rabbit anti-HIF1α (1/500, Santa Cruz Biotechnologies); anti-Flag®M2 (1/2000, Sigma, St-Quentin-Fallavier, France); anti-mouse hemagglutinin HA, anti-TBP (1/1000, IGBMC core facilities), mouse anti-Actin (1/2000, Sigma). For detection of Flag-PHDs in co-IP experiments, mouse-TrueBlot ULTRA HRP-conjugated anti-mouse IgG was used as the secondary antibody (1:2000, Clinisciences). For densitometric quantification TINA2.09 (ISBM; Oxford, UK) software was used.

**Quantitative Real Time PCR:** The quantitative RT-PCR was performed with the LightCycler 480 system (Roche diagnostics) and the SYBR Green I (Roche diagnostics) protocol. Amplification specificity was verified by melting-curve analyses, and the data were quantified with LightCycler software. Oligonucleotides were as follows: RPLPO: 5’-CCGGATATGAGGCAGCAGTT and 5’-GAAGGCTGTGGTGCTGATGG; Net: 5’-AGTCCACTGCTCTCCAGCAT and 5’-GCCAGACGTCATCCAGGATT. The QuantiTect primers for PHD1, PHD2, PHD3 and HIF1α were provided by Qiagen.

**Co-IP:** Co-transfected 444 and HeLa cells were washed trice with ice cold PBS and lysed in immunoprecipitation buffer containing 20mM Tris-HCl (pH7.4), 0.3M KCl, 0.25mM EDTA, 0.125mM EGTA, 0.025% Triton X-100, 1mM PMSF and protease inhibitor cocktail (Roche Diagnostics). Immune complexes were formed by incubation of the lysates overnight at 4°C with anti Flag (M2) agarose. The beads were washed four times with immunoprecipitation buffer, resuspended in non-denaturant Laemmli sample buffer and processed for immunoblotting.

**In vitro translation:** 35S-labeled in vitro translated GHO, GHO(P→A), and Net were prepared using rabbit reticulocyte lysates (TnT Quick kit, Promega). After 45 min of reaction, the translation mixtures were supplemented or not with 100µM FeCl2, 1mM 2-oxoglutarate and 5mM ascorbate for 45 min at 30°C. Reaction
products were analyzed by SDS-PAGE and autoradiography. In vitro shift assays- HeLa and HEK293T cells were transfected with Flag-Net or GHO. 48h after transfection, the cells were treated with 200µM CoCl₂ for 5 hours and then washed twice with ice cold PBS and lysed in Laemmli sample buffer for HEK293T or lysis buffer (50mM Tris-HCl pH8, 150mM NaCl, 0.02% NaN₃, 1% NP40) for HeLa. Total cell extracts were analyzed by immunoblotting.

RESULTS

Hypoxia has different effects on Net and HIF1α protein levels. In order to study the integration of Net in the hypoxia/PHD/HIF1α signaling pathway, we compared hypoxic regulation of Net and HIF1α in nuclear extracts of 444 cells in 1% O₂. As we have shown previously, Net detected with the polyclonal antibody PAb #2005 migrates as a series of bands (Figure 1A) that result from post-translational modification by phosphorylation and possibly other modifications. We confirmed that these bands are downregulated by siRNAs against Net in 444 (data not shown), similar to other cell lines we have studied (8,21). As reported previously, there is an additional non-identified band (n.i.) that is variable and is not downregulated by Net siRNAs. It does not compromise the measurements of Net levels by western blotting. As shown in Figure 1A & 1B, Net protein levels decrease drastically after 12 h of hypoxia. In contrast, HIF1α protein levels follow a bell shape curve, with a transient increase, peaking between 4 and 8 h of hypoxia, followed by a progressive decrease. We observed similar regulation of Net and HIF1α protein levels under hypoxia in total cell extracts (data not shown). To exclude that transcription accounts for the changes, we measured Net and HIF1α mRNA by real-time qRT-PCR. There were non-significant small variations in Net and HIF1α mRNA, which could not account for the observed changes at the protein level (Figure 1C). Our results indicate that there are three time phases in the kinetics: 1) normoxia, high Net levels and low HIF1α levels; 2) early hypoxia (4h to 8h), high levels of Net and HIF1α; 3) late hypoxia (12h to 24h), degradation of Net and HIF1α (Figure 1B).

Modulators of PHD activity induce mobility shifts of Net similar to HIF1α. PHDs require oxygen, iron, ascorbate and 2-oxoglutarate to hydroxylate HIF1α on Pro564 in normoxia (22). Iron-displacing transition metals, such as cobalt, act as strong inhibition of PHDs activity (Figure 2A). In order to investigate possible hydroxylation of Net, we compared the effect of modulating the activity of the PHDs in different systems on the mobility of Net in comparison with GHO [Gal4-HIF1α(531-652)], which contains Pro564 (23-25). As expected from previous reports, 35S-labeled GHO produced by in vitro transcription and translation in rabbit reticulocyte lysate, migrated as a doublet upon SDS-PAGE. Adding iron, ascorbate and 2-oxoglutarate to the reaction induced a shift of migration, converting the slowly migrating non-hydroxylated species to the rapidly migrating hydroxylated form. The shift was not observed with GHO(PA), in which Pro564 is mutated to Ala (Figure 2B). Interestingly, 35S-labeled Net also migrated as a doublet, and the addition of the three cofactors shifted the mobility to the more rapidly migrating form (Figure 2B). In transfected HEK293T cells, the hypoxia mimic, 200µM CoCl₂, decreased the mobility of GHO, as expected from lack of hydroxylation. Similarly, the migration of full length Net was also decreased in HeLa cells. The migration of β-actin remained unchanged, showing that the shift was not due to non-specific effects on migration (Figure 2C). Taken together our data suggest that Net undergoes post-translational modifications that are induced by regulators of PHDs activity.

PHDs are involved in Net regulation in normoxia. In order to investigate whether the PHDs regulate Net protein stability in 444 cells in normoxia, we either downregulated the PHDs with specific siRNA or overexpressed them with expression vectors. The PHDs were efficiently downregulated, as shown by qRT-PCR (Figure 3A). In normoxia, Net protein levels in the nucleus were decreased by downregulation of PHD1 and PHD3, and increased by downregulation of PHD2 (Figures 3B & 3C). When we overexpressed the three PHD isoforms, the opposite effect was observed. PHD1 and PHD3 increased and PHD2 decreased Net protein levels (Figures 3D & 3E). We did not find any significant differences in Net mRNA levels (data not shown), demonstrating
that the three PHDs are involved in the regulation of Net protein stability in normoxia.

PHDs are involved in Net regulation in hypoxia. We then investigated how different levels of the PHDs would affect Net levels in hypoxia. The knockdown of PHD2 (Figures 4A & 4B) globally increased Net levels, and in particular delayed Net downregulation in late hypoxia (12 and 18h), compared to cells transfected with control siRNA targeting luciferase. Overexpression of PHD2 (Figures 4C & 4D) decreased Net levels, which was observed in particular early in hypoxia (4-8h). These results indicate that the loss of Net during hypoxia requires PHD2. Downregulation of PHD1 and to some extent PHD3 (Figures 4A & 4B) globally decreased Net levels, which was particularly evident early in hypoxia (4-8h). Overexpression of PHD1 and PHD3 prevented loss of Net during hypoxia, which was most evident late in hypoxia (12 and 18h). These results indicate that PHD1 and 3 help maintain Net levels during hypoxia. These opposing effects of PHD2 and PHD1 & 3 suggest that their relative levels of expression could fine tune the dynamics of the response to hypoxia, in terms of the levels of Net.

Comparison of HIF1α with Net in terms of their regulation by the PHDs. In order to compare the role of the three PHDs in the regulation of HIF1α in the same experimental conditions as Net, we downregulated and overexpressed the PHD isoforms in hypoxia. Silencing PHD2 and PHD3 significantly increased HIF1α protein level throughout the time course (Figures 5A & 5B). PHD1 downregulation had no significant effects on HIF1α protein levels in normoxia and early hypoxia (up to 8h; 8h was not statistically significant), but delayed degradation of HIF1α in late hypoxia (12-18h, Figures 5A & 5B). Overexpression of PHD2 and PHD3 strongly inhibited HIF1α levels throughout the time course, which was the opposite of downregulation (Figures 5C & 5D). Overexpression of PHD1 decreased HIF1α induction early in hypoxia (4h), a time at which downregulation had no effect. This would be expected from an increased activity of PHD1 due to overexpression. PHD1 overexpression also had an effect at 12h (Figure 5D), which is apparently paradoxical since downregulation had the same effect. This suggests that there are complex regulatory mechanisms that involve factors other than PHD1 expression levels. In contrast to Net regulation, both PHD2 and PHD3 play similar role in all three time-phases of hypoxia, whereas PHD1 appears to be mainly involved in the late phase. Taken together, our results show that both Net and HIF1α are regulated by PHDs, but in different manners.

Crosstalk between Net and HIF1α at the protein level. In order to investigate crosstalk between Net and HIF1α, we analysed their reciprocal protein levels after inhibition by siRNA in normoxia (0h), and early (6h) and late (12h and 24h) hypoxia. We found that downregulation of Net impaired stabilization of HIF1α in early hypoxia and its degradation in late hypoxia, compared to the control (Figure 6A). Downregulation of HIF1α inhibited downregulation of Net in late hypoxia (Figure 6C). Overexpression of Net resulted in higher and prolonged induction of HIF1α (Figure 6E). There were no significant changes at the mRNA level, as shown by qRT-PCR (right panels, Figures 6B, 6D & 6F). These results indicate that Net is required for rapid induction of HIF1α by hypoxia, whereas HIF1α is necessary for Net degradation in hypoxia.

Net and HIF1α regulate PHD expression in hypoxia. We investigated whether Net and HIF1α knock down would affect PHDs expression at the mRNA level in normoxia (0h), and early (6h) and late (12h and 24h) hypoxia. The specific siRNA were efficient, since they decreased Net and HIF1α mRNA levels by at least 80% (Figures 7A & 7E). Hypoxia stimulated the expression of PHD2 2-3 fold and PHD3 about 6 fold, as expected (Figures 7C, 7D, 7G & 7H), but did not affect PHD1 levels (Figures 7B & 7F). Net downregulation had no significant effect on PHD1 and PHD2 levels in normoxia and hypoxia (Figures 7B & 7C), but decreased hypoxic induction of PHD3 (Figure 7D). HIF1α downregulation inhibited hypoxic induction of both PHD2 and PHD3 mRNA levels (Figures 7G & 7H), but had no significant effect on PHD1 levels (Figure 7F). These results show that the Net and HIF1α regulate different PHDs, Net regulates PHD3 whereas HIF1α regulates both PHD2 and PHD3. In addition, the subsequent effects of the PHDs on the levels of Net and HIF1α suggest that transcriptional regulation could contribute to the cross talk between Net and HIF1α at the protein level.

Net physically interacts with the PHDs and HIF1α. We also investigated physical
interactions between Net and the PHDs. 444 cells were co-transfected with Net and one of the three isoforms of Flagged-PHDs, or with the empty vector pcDNA3 as a control. The PHDs were immunoprecipitated with Flag antibody coupled beads, and co-precipitated Net was detected by western blotting. We found that, in normoxia, Net specifically interacted with PHD1 and PHD3 but not detectably with PHD2 (Figures 8A & 8C). Similar results were obtained with co-expressed Net and the three PHDs in HeLa cells (data not shown). Interestingly, under hypoxic mimic conditions (CoCl2), Net interacted with PHD2 as well as with PHD1 and PHD3 (Figure 8B & 8C). We also investigated whether Net interacts with HIF1α. We co-expressed HIF1α and FlagNet in HeLa cells and immunoprecipitated FlagNet using Flag (M2) antibody coupled beads. HIF1α specifically co-immunoprecipitated with FlagNet (Figure 8D), indicating that Net physically interacts with HIF1α under these conditions. Taken together these data indicate that the PHDs, Net and HIF1α can interact at the protein level, and that the complexes that form depend upon hypoxia and the presence of HIF1α.

**DISCUSSION**

In this study, we compared the hypoxia/PHDs/Net and hypoxia/PHDs/ HIF1α pathways in human non-tumorigenic HPV positive 444 cells. These cells were used because Net repression of the c-fos oncogene appears to account for their non-transforming properties (16). Hypoxia is a mechanism by which Net repression can be relieved (8), indicating that the 444 cell line is a good model to investigate the consequences of hypoxia induced loss of Net on transformation. In addition, there are related transformed (HeLa, CGL3) and non-transformed (IMR90 fibroblasts) cells (19,20), which could be used to study the effects of transformation on Net’s role in hypoxia. We show that hypoxia regulates Net and HIF1α in 444 cells, and that there are similarities and differences in this regulation. They exhibit similar mobility shifts in response to modulators of PHD activity. However the three PHDs regulate Net and HIF1α protein stability in different ways, suggesting that the hypoxia-Net and −HIF1α pathways are distinct. However, the two pathways are interlinked at the level of regulation of PHD expression and protein-protein interactions (see Figure 9 for a schematic representation). These links could account for our previous observations, that Net and HIF1α share a large number of target genes (18).

Our results show that Net and HIF1α are differently regulated during the time course of hypoxia, suggesting that they have different roles in the hypoxic response. Net levels are high in normoxia and early hypoxia, whereas HIF1α is induced in early hypoxia. Both factors are degraded in late hypoxia. HIF1α induction is required for the cellular response to hypoxia, and its degradation in late hypoxia protects cells against necrotic cell death and adapts them to chronic hypoxia (26). The intricate link between Net and HIF1α suggests that Net modulates these functions of HIF1α.

The PHDs, are known to hydroxylate HIF1α on specific proline residues and thus regulate its stability (7,25), which causes a characteristic shift in mobility on SDS-PAGE (23,24). Net undergoes similar mobility shifts using in vitro and in vivo assays, indicating that it may also be hydroxylated. The multiple Net bands in SDS-PAGE might correspond to different posttranslational modifications (21,27), including hydroxylation. PHDs hydroxylate a number of proteins. PHD1 is involved in proline hydroxylation of RbpI, the large subunit of RNA polymerase II (28). The kinase activity of IKKβ may be inhibited by hydroxylation by PHD1 and PHD2 (29). The stability of ATF4 (30) and myogenin (31) are regulated by PHD3. However it has not been proven directly that either protein is hydroxylated. We did not detect hydroxylated prolines in Net by mass-spectroscopy, but these studies are not conclusive and are hampered by the proline rich nature of the peptide (data not shown, in collaboration with Prof. A van Dorsselaer).

We have shown that the three PHDs affect Net in different ways (Figure 9). PHD1 and PHD3 contribute to stabilization, whereas PHD2 is involved in Net degradation. In addition, we demonstrate that Net physically interacts with PHD2 in hypoxia, whereas PHD1 and PHD3 bind to Net in normoxia and hypoxia. In contrast, PHD2 and PHD3 regulate HIF1α stabilization in early and late hypoxia, whereas PHD1 appears to be involved in HIF1α degradation in late hypoxia. HIF1α has been reported to be mainly regulated by PHD2, although some studies suggest that all three
isoforms can hydroxylate HIF1α with different efficiency (32). Other proteins are also differentially regulated by the PHDs. PHD3 binds to myogenin and increases its stability (31), and also induces oxygen dependent degradation of ATF4 (30). PHD1 and PHD2 coimmunoprecipitate with Rpb1 in response to oxidative stress, but PHD1 is necessary for Rpb1 hydroxylation and consequent degradation, whilst PHD2 has an inhibitory effect on this process (28).

We present evidence that the Net and HIF1α pathways are interconnected at several levels. Changes in the initial protein levels of Net influence hypoxic regulation of HIF1α, suggesting that Net acts as a "rheostat", which modulates the HIF1α mediated hypoxic response in the cell. Induction of PHD2 and PHD3 in hypoxia partially counterbalances the diminution of oxygen, leading to increased hydroxylation and consequently proteasomal degradation of HIF1α (33). Net inhibition downregulates PHD3 in hypoxia and may thereby delay degradation of HIF1α. Similarly, HIF1α inhibition prevents the hypoxic induction of PHD2, which appears to be involved in Net downregulation in late hypoxia. This could explain why HIF1α inhibition stabilizes Net in late hypoxia. However, the crosstalk between Net and HIF1α might also be a result of their physical interaction.

We found that HIF1α downregulation inhibits the hypoxic induction of PHD2 and PHD3 mRNA levels (Figure 9), as expected from previous studies (33-35). Net downregulation decreased PHD3 mRNA induction in hypoxia, suggesting that Net is a positive transcriptional regulator of its expression. In contrast, Net represses PHD2 and PHD3 expression in transformed mouse skin endothelial cells (SEND) (18), indicating that Net’s role in the hypoxic response is cell-type specific.

Our results show that Net physically interacts with PHD1 and PHD3 in normoxia and in hypoxia mimic (cobalt chloride; Figure 9). These results suggest that Net could be a substrate for both enzymes. Interestingly, the interaction of PHD3 with myogenin increases its stability by preventing VHL-mediated degradation (31), which indicates that Net stabilization by PHDs could be also mediated by hydroxylation independent mechanism. Interestingly, we found that PHD2 binds to Net only in hypoxic mimic conditions, which could be related to its involvement in the degradation of Net in late hypoxia. Moreover, we found that Net and HIF1α physically interact. This interaction could involve the PHDs, perhaps through the formation of a multi-protein complex. An interesting possibility is that HIF1α stabilization in hypoxia results in the recruitment of PHD2, which is involved in the degradation of Net in late hypoxia.

In summary, this study demonstrates that Net and HIF1α form different signaling pathways, which are intricately linked at several levels. Thus, we have added important insights into the understanding of the mechanism of hypoxic regulation of Net and its integration into HIF1α signaling. Pharmacological modulation of Net and HIF1α and the investigation of the effect on cell physiology will further increase our understanding of the complex interplay between both transcription factors in the regulation of cellular adaptation to hypoxia.

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**FOOTNOTES**

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The abbreviations used are: HIF1α, hypoxia inducible factor 1 alpha; PHD, prolyl-hydroxylase domain proteins; SEND, skin endothelial cells; ATF4, activating transcriptional factor 4.

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**FIGURE LEGENDS**

Fig. 1. Hypoxia has different effects on Net and HIF1α protein levels in 444 cells. Net and HIF1α protein levels in nuclear extracts from cells incubated in hypoxia (1% O2) for 0, 4, 6, 8, 12, 18 and 24 hours. (A) Representative WB for hypoxic regulation of Net and HIF1α in 444 cells. (B) Densitometric quantification of relative mean levels of Net and HIF1α protein normalized to TBP (n=4; *p<0.05 for Net, #p<0.05 for HIF1α; in comparison to time point 0, Student’s t-test). Three time phases in the kinetics are indicated, normoxia, and early and late hypoxia. (C) Hypoxia does not affect Net and HIF1α mRNA levels in 444 cells incubated for 0, 6, 12 and 24 hours at 1% O2. Representative graph of Net and HIF1α mRNA normalized to the RPLPO (n=3).

Fig. 2. Modulators of PHD activity induce mobility shift of Net, similar to HIF1α (A) Schematic illustration of the effect of FeCl2, 2-oxoglutarate, ascorbate or CoCl2 on hydroxylation state of a protein hydroxylated by a prolyl-4-hydroxylase. (B) 35S-labeled GAL4-HIF1α-(531–652) or GAL4-HIF1α-(531–652)-P564A or Net were in vitro transcribed and translated in reticulocytes in absence or presence of PHD cofactors (100µM FeCl2, 1mM 2-oxoglutarate and 5mM ascorbate). The lysates were subjected to SDS-PAGE and autoradiography. (C) HeLa and HEK293T were respectively transfected with Net or GHO and treated with or without 200µM CoCl2 for 5 hours.

Fig. 3. In normoxia, the three PHDs regulate Net in different ways. (A-C) Knock down of PHDs with siRNA affects Net protein levels in the nucleus. 444 cells were transfected with anti-PHD1, PHD2, PHD3 or luciferase (control) siRNA. Downregulation of PHD mRNA levels estimated by qRT-PCR (A). (B) Representative WB. (C) Densitometric quantification of relative mean levels of Net protein normalized to TBP (n=3; *p<0.05, Student’s t-test). (D, E) PHD overexpression affects Net protein levels in the nucleus. 444 cells were transfected with an expression vector for Flag-PHD1, Flag-PHD2, Flag-PHD3 or the empty pcDNA3 vector. (D) Representative WB. (E) Densitometric
quantification of relative mean levels of Net protein normalized to TBP (n=3; *p<0.05, Student’s t-test).

Fig. 4. In hypoxia, the PHDs regulate Net in different ways. (A, B) Knock down of PHDs with siRNA affects Net protein levels and the kinetics of downregulation of Net protein in hypoxia. 444 cells were transfected with anti-PHD1, PHD2, PHD3 or luciferase (control) siRNA and 48 h later incubated for 0, 4, 6, 8, 12, 18 and 24 hours in hypoxia (1% O₂). (A) Representative WBs. (B) Densitometric quantification of relative mean levels of Net protein normalized to TBP (n=3; *p<0.05 in comparison to time point 0, #p<0.05 in comparison to the respective time point of the control, Student’s t-test). (C, D) PHD overexpression affects Net protein levels and the kinetics of Net protein downregulation in hypoxia. 444 cells were transfected with an expression vector for PHD1, PHD2, PHD3 or the empty pcDNA3 vector and 48 h later subjected to 1% O₂ hypoxia for 0, 4, 6, 8, 12, 18 and 24 hours. (C) Representative WBs. (D) Densitometric quantification of relative mean levels of Net protein normalized to TBP (n=3; *p<0.05 in comparison to time point 0, #p<0.05 in comparison to the respective time point of the control, Student’s t-test).

Fig. 5. PHDs are differently involved in HIF1α regulation in hypoxia. (A, B) Knock down of PHDs with siRNA affects HIF1α protein levels and the kinetics of induction and downregulation of HIF1α protein in hypoxia. 444 cells were transfected with anti-PHD1, PHD2, PHD3 or luciferase (control) siRNA and 48 h later incubated for 0, 4, 6, 8, 12, 18 and 24 hours in hypoxia (1% O₂). (A) Representative WB. (B) Densitometric quantification of relative mean levels of HIF1α protein normalized to TBP (n=3; *p<0.05 in comparison to time point 0, #p<0.05 in comparison to the respective time point of the control, Student’s t-test). (C, D) PHD overexpression affects HIF1α protein levels and the kinetics of induction and downregulation of HIF1α protein in hypoxia. 444 cells were transfected with an expression vector for PHD1, PHD2, PHD3 or empty pcDNA3 vector and 48 h later subjected to 1% O₂ hypoxia for 0, 4, 6, 8, 12, 18 and 24 hours. (C) Representative western blots. (D) Densitometric quantification of relative mean levels of HIF1α protein normalized to TBP (n=3; *p<0.05 in comparison to time point 0, #p<0.05 in comparison to the respective time point of the control, Student’s t-test).

Fig. 6. Cross-linked regulation of the protein levels of Net and HIF1α. (A, B) Net knock down with siRNA affects induction and downregulation of HIF1α protein in hypoxia. 444 cells were transfected with siRNA against human Net or luciferase and incubated in 1% O₂ hypoxia for 0, 6, 12 and 24 hours. (A) Densitometric quantification of relative mean levels of HIF1α protein normalized to TBP (n=3). (B) Downregulation of Net does not affect HIF1α mRNA levels. Representative graph of HIF1α mRNA normalized to the RPLPO (n=2). (C, D) HIF1α knock down with siRNA prevents downregulation of Net protein in hypoxia. 444 cells were transfected with siRNA against human HIF1α or luciferase and incubated in 1% O₂ hypoxia for 0, 6, 12 and 24 hours. (C) Densitometric quantification of relative mean levels of Net protein normalized to TBP (n=3). (D) Downregulation of HIF1α does not affect Net mRNA levels. Representative graph of HIF1α mRNA normalized to the RPLPO (n=2). (E, F) Net overexpression enhances hypoxia-induced stabilization of HIF1α protein. 444 cells were transfected with an expression vector for human Net or empty vector and incubated in 1% O₂ hypoxia for 0, 6, 12 and 24 hours. (E) Representative graph of a densitometric quantification of relative mean level of Net and HIF1α protein normalized to TBP (n=3). (F) Overexpression of Net does not affect HIF1α mRNA levels. Representative graph of HIF1α mRNA normalized to the RPLPO (n=2).

Fig. 7. Net and HIF1α are involved in the regulation of PHDs mRNA levels. (A-D) 444 cells were transfected with siRNA against human Net and 48 h later incubated in 1% O₂ hypoxia for 0, 6, 12 and 24 hours. Representative graphs of mRNA levels of (A) Net, (B) PHD1, (C) PHD2 and (D) PHD3 analyzed by qRT-PCR normalized to the RPLPO (n=2). (E-H) 444 cells were transfected with siRNA against human HIF1α and 48 h later incubated in 1% O₂ hypoxia for 0, 6, 12 and 24 hours.
Representative graphs of mRNA levels of (E) HIF1α, (F) PHD1, (G) PHD2 and (H) PHD3 analysed by qRT-PCR normalized to the RPLPO (n=2).

**Fig. 8.** Interaction of Net with PHDs and HIF1α. (A, B) Co-IP of Flag-PHDs and Net in normoxia (A) and hypoxia mimic (B). 444 cells were co-transfected with plasmids overexpressing Net and one of the three isoforms of Flag-PHDs or the empty pcDNA3 vector as a control. 48 h after the transfection the cells were directly lysed (A) or treated for 8 h with 500 µM cobalt chloride (CoCl2) and a proteasome inhibitor (B) and Co-IPed with anti-Flag (M2) agarose beads. (C) Densitometric quantification of Net protein levels in normoxia and hypoxia mimic (CoCl2 treatment). The represented values are means of the quantification of 3 independent experiments for each condition (*p<0,05). (D) Co-IP of FlagNet and HIF1α. HeLa cells were transfected with plasmids overexpressing HIF1α and Flag-Net or the empty vector as a control. IPs were performed with the prepared total cell lysates using anti-Flag (M2) agarose beads.

**Fig. 9.** Schematic representation of salient features of the hypoxia signaling pathways that involve Net and HIF1α. The response to 1% O2 can be divided into normoxia (0h, low HIF1α, high Net), early hypoxia (4-12h; high Net and HIF1α) and late hypoxia (12-24h; low Net and HIF1α). In normoxia, PHD1/3 bind to and stabilise Net, whereas PHD2/3 degrade HIF1α. In early hypoxia, Net binds to PHD2 and HIF1α, in addition to PHD1/3, which is represented as one complex for simplicity. Net stabilizes HIF1α and regulates the expression of PHD3. HIF1α regulates the expression of PHD2/3. In late hypoxia PHD2 and HIF1α degrade Net whereas PHD1/2/3 degrade HIF1α. The terms “stabilize” and “degrade” imply that the named proteins participate in these processes, probably with other components that have not been identified in this study.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 9

PHD1/3 bind Net
PHD1/3 stabilise Net
PHD2/3 degrade HIF1α

PHD1/2/3 bind Net
HIF1α binds Net
Net stabilises HIF1α
Net regulates PHD3
HIF1α regulates PHD2/3

PHD2/HIF1α degrade Net
PHD1/2/3/Net degrade HIF1α
Involvement of Net and HIF1alpha in distinct yet intricately linked hypoxia induced signalling pathways
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