



LUND UNIVERSITY

Differential regulation of HIF-1 α and HIF-2 α in neuroblastoma: Estrogen-related receptor alpha (ERR α) regulates HIF2A transcription and correlates to poor outcome.

Hamidian, Arash; von Stedingk, Kristoffer; Thorén, Matilda; Mohlin, Sofie; Pålman, Sven

Published in:
Biochemical and Biophysical Research Communications

DOI:
[10.1016/j.bbrc.2015.04.083](https://doi.org/10.1016/j.bbrc.2015.04.083)

2015

[Link to publication](#)

Citation for published version (APA):
Hamidian, A., von Stedingk, K., Thorén, M., Mohlin, S., & Pålman, S. (2015). Differential regulation of HIF-1 α and HIF-2 α in neuroblastoma: Estrogen-related receptor alpha (ERR α) regulates HIF2A transcription and correlates to poor outcome. *Biochemical and Biophysical Research Communications*, 461(3), 560-567. <https://doi.org/10.1016/j.bbrc.2015.04.083>

Total number of authors:
5

General rights

Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc



Differential regulation of HIF-1 α and HIF-2 α in neuroblastoma: Estrogen-related receptor alpha (ERR α) regulates *HIF2A* transcription and correlates to poor outcome



Arash Hamidian, Kristoffer von Stedingk, Matilda Munksgaard Thorén, Sofie Mohlin¹,
Sven Pålman^{*,1}

Lund University, Translational Cancer Research, Lund University Cancer Center at Medicon Village, Building 406, SE-223 81 Lund, Sweden

ARTICLE INFO

Article history:

Received 14 April 2015

Available online 23 April 2015

Keywords:

Hypoxia

Transcription

HIF-2 α

ERR α

Neuroblastoma

ABSTRACT

Hypoxia-inducible factors (HIFs) are differentially regulated in tumor cells. While the current paradigm supports post-translational regulation of the HIF- α subunits, we recently showed that hypoxic HIF-2 α is also transcriptionally regulated via insulin-like growth factor (IGF)-II in the childhood tumor neuroblastoma. Here, we demonstrate that transcriptional regulation of HIF-2 α seems to be restricted to neural cell-derived tumors, while HIF-1 α is canonically regulated at the post-translational level uniformly across different tumor forms. Enhanced expression of *HIF2A* mRNA at hypoxia is due to *de novo* transcription rather than increased mRNA stability, and chemical stabilization of the HIF- α proteins at oxygen-rich conditions unexpectedly leads to increased *HIF2A* transcription. The enhanced *HIF2A* levels do not seem to be dependent on active HIF-1. Using a transcriptome array approach, we identified members of the Peroxisome proliferator-activated receptor gamma coactivator (PGC)/Estrogen-related receptor (ERR) complex families as potential regulators of *HIF2A*. Knockdown or inhibition of one of the members, ERR α , leads to decreased expression of *HIF2A*, and high expression of the ERR α gene *ESRRA* correlates with poor overall and progression-free survival in a clinical neuroblastoma material consisting of 88 tumors. Thus, targeting of ERR α and pathways regulating transcriptional HIF-2 α are promising therapeutic avenues in neuroblastoma.

© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Solid tumors frequently develop regions of hypoxia – low oxygen tensions – as highly proliferating tumor cells tend to outgrow the formation of new blood vessels within the tumor. Transformed and normal cells mainly adapt to hypoxic surroundings via stabilization of the oxygen sensitive α -subunits of the hypoxia inducible transcription factors (HIFs). Expression of HIF-1 α and HIF-2 α , as well as overall tumor hypoxia, correlates with metastasis, drug resistance and poor outcome in a number of different tumor forms, including neuroblastoma [1–7]. Although HIF-1 α and HIF-2 α have high sequence homology and share a large proportion of target genes, accumulating evidence shows that the regulation, and

action, of these two proteins significantly differ in tumor cells [2,3,5,6,8–10].

The childhood tumor neuroblastoma is believed to originate from dividing precursor cells of the sympathoadrenal cell lineage during formation of the sympathetic nervous system (SNS). We have previously shown that HIF-1 α and HIF-2 α proteins are differentially expressed in neuroblastoma cells, with acute and transient stabilization of HIF-1 α , and prolonged expression of HIF-2 α during hypoxic conditions [2]. Presence of tumor cells with intense HIF-2 α staining correlates with aggressive and metastatic disease, whereas HIF-1 α expression rather correlates to favorable disease in neuroblastoma [2,6]. In addition, HIF-2 α is expressed in peri-vascular niches in both neuroblastoma and glioblastoma [5,11], whereas HIF-1 α expression mainly is restricted to hypoxic regions [11]. We have recently shown that HIF-2 α is regulated at the transcriptional level by insulin-like growth factor (IGF)-II in neuroblastoma, and that these two proteins are co-expressed in embryonic neuroblasts of the developing human SNS [10].

* Corresponding author.

E-mail address: sven.pahlman@med.lu.se (S. Pålman).

¹ Co-senior authors.

Here, we show that hypoxia-induced expression of *HIF2A* mRNA mimics protein expression patterns in a time-dependent manner with increased levels over time and a rate shift around 48 h after the hypoxic onset. While *HIF1A* mRNA levels decrease under prolonged hypoxia (at 1% O₂) uniformly in a number of cultured tumor cells (neuroblastoma, breast cancer, prostate cancer, renal cell carcinoma (RCC), and non-small cell lung carcinoma (NSCLC)), increased *HIF2A* expression is only observed in neuroblastoma cells. The observed changes in *HIF* mRNA at hypoxia are not due to alterations in overall mRNA stability. In addition, chemical stabilization of the HIF proteins at normoxia (21% O₂) leads to increased *HIF2A*, and decreased *HIF1A* mRNA levels, in conformity with results observed when cells are cultured at hypoxia. Using a quantitative real time (qRT)-PCR based array, we identified members of the Peroxisome proliferator-activated receptor gamma coactivator (PGC)/Estrogen-related receptor (ERR) complex families as potential regulators of *HIF2A*. Knockdown or inhibition of specifically one member, *ERRα*, downregulates *HIF2A* mRNA levels in normoxic and hypoxic neuroblastoma cells. Supporting a role for *ERRα* in tumors with an HIF-2α-driven aggressive phenotype, high expression of the gene encoding *ERRα*, *ESRRA*, correlates with poor overall and progression-free survival in a clinical neuroblastoma data set consisting of 88 tumors.

2. Material and methods

2.1. Cells and reagents

Human neuroblastoma cell lines SK-N-BE(2)c and KCN-69n (kind gifts from Drs. June Biedler, Memorial Sloan Kettering Cancer Institute and Robert Ross, Fordham University), breast cancer cell line MCF7 (ATCC), prostate cancer cell line DU 145 (ATCC), renal cancer cell adenocarcinoma cell line 786-0 (ATCC), and non-small cell lung cancer cell line A549 (ATCC) were routinely grown as monolayers in Minimal Essential (SK-N-BE(2)c, KCN-69n, MCF7), RPMI-1640 (DU 145), F-12 Kaighn's modification (A549), or Dulbecco's Modified Eagle's (786-0) medium supplemented with fetal bovine serum and antibiotics. For culturing of MCF7 cells, growth medium was supplemented with sodium pyruvate, essential amino acids and insulin. Hypoxia was generated in an InvivoO2 hypoxia workstation (Ruskin Technologies) or a Whitley H35 Hypoxystation (Don Whitley Scientific). Cells were treated with Actinomycin D (5 μg/ml, Sigma–Aldrich), 2,2'-dipyridyl (DIP) (200 μM, Sigma–Aldrich), or XCT790 (1–5 μM, Sigma–Aldrich).

2.2. Transfection

Transfections were carried out in serum- and penicillin-free OPTI-MEM medium (Gibco) using Lipofectamine 2000 (Invitrogen) as transfection reagent. For HIF knockdown studies, cells were transfected with siRNA duplexes (50 nM, Ambion) targeting *HIF-1α* or *HIF-2α* respectively, and as a control, the inverted or scrambled *HIF-1α* sequence was used. Sequences as previously described [12]. For *ESRRA* knockdown studies, siRNA against *ESRRA* (s4830; Ambion) or a non-targeting control siRNA (siC #2, Ambion) was used at final concentrations of 5 nM.

2.3. Western blotting

Cells were lysed in RIPA supplemented with Complete Protease Inhibitor (Roche Diagnostics). Proteins were separated by SDS/PAGE and transferred to Hybond-C-Extra Nitrocellulose

membranes (Amersham). Antibodies are listed in [Supplementary Table S1](#).

2.4. Quantitative real-time PCR

Total RNA was extracted manually using the QIAshredder and RNeasy Mini Kits (Qiagen) according to the manufacturer's recommendation or automatically using Arrow (DiaSorin) with Arrow RNA (Tissue Kit -DNA Free) Kit (DiaSorin). cDNA synthesis and quantitative PCR analysis was performed as described [10]. Expression levels of reference genes (*YWHAZ*, *SDHA* and *UBC* for SK-N-BE(2)c, KCN-69n, 786-0 and MCF7 cells; *GAPDH* for DU 145 cells; and *UBC*, *TBP* and *HPRT1* for A549 cells) were used to normalize gene-of-interest expression [13]. Primer sequences are listed in [Supplementary Table S2](#).

2.5. Expressed transcription factor knockdown transcriptome PCR array

The expressed transcription factor knockdown transcriptome PCR array (Sabiosciences) was used to simultaneously investigate a total of 270 known transcription factors. SYBR Green quantitative-PCR analysis was performed in a 7300 Real-Time PCR system (Applied Biosystems), to quantify expression of *HIF2A* and *HIF1A* using *GAPDH* as a reference gene. Fold changes in expression as a result of each siRNA treatment relative to negative siRNA control were calculated and normalized to *GAPDH* in accordance to manufacturer's instructions.

2.6. Statistical analyses

All values are reported as mean ± SEM from at least three independent experiments, unless otherwise stated. Two-sided student's unpaired t test was used for statistical analyses and three levels of significance were used, *p < 0.05, **p < 0.01, ***p < 0.001. No asterisk (*) or n.s. indicates no significance. A publicly available data set containing 88 neuroblastomas [14] was acquired from R2: microarray analysis and visualization platform (<http://r2.amc.nl>). Kaplan–Meier and logrank survival analyses were performed using R statistical language and the survival package [15]. Bonferroni correction for multiple testing was employed to account for survival group optimization.

3. Results

3.1. Hypoxia-induced transcriptional regulation of *HIF2A* is restricted to neural-derived tumors

HIF proteins are differentially regulated in neuroblastoma with *HIF-1α* present mainly at acute phases of hypoxia (4–24 h) after which protein levels rapidly decline. *HIF-2α*, on the other hand, is predominantly expressed at prolonged phases of hypoxia (48–72 h) [2]. Here, we show that mRNA expression patterns of *HIF2A* mimic *HIF-2α* protein expression in neuroblastoma cells with continuous increase over time (Fig. 1A and [2]). *HIF1A* mRNA levels on the contrary decrease over time, despite stabilization of the protein at acute hypoxia (Fig. 1B). To verify transcriptional activity of *HIF-2α* even after prolonged exposure to hypoxia, we analyzed the expression of well-established [2] and strongly HIF-2-driven target genes in neuroblastoma. Indeed, *VEGFA* and *DEC1* mRNA expression closely follows *HIF2A* expression, with highest expression at later time points (Fig. 1C–D).

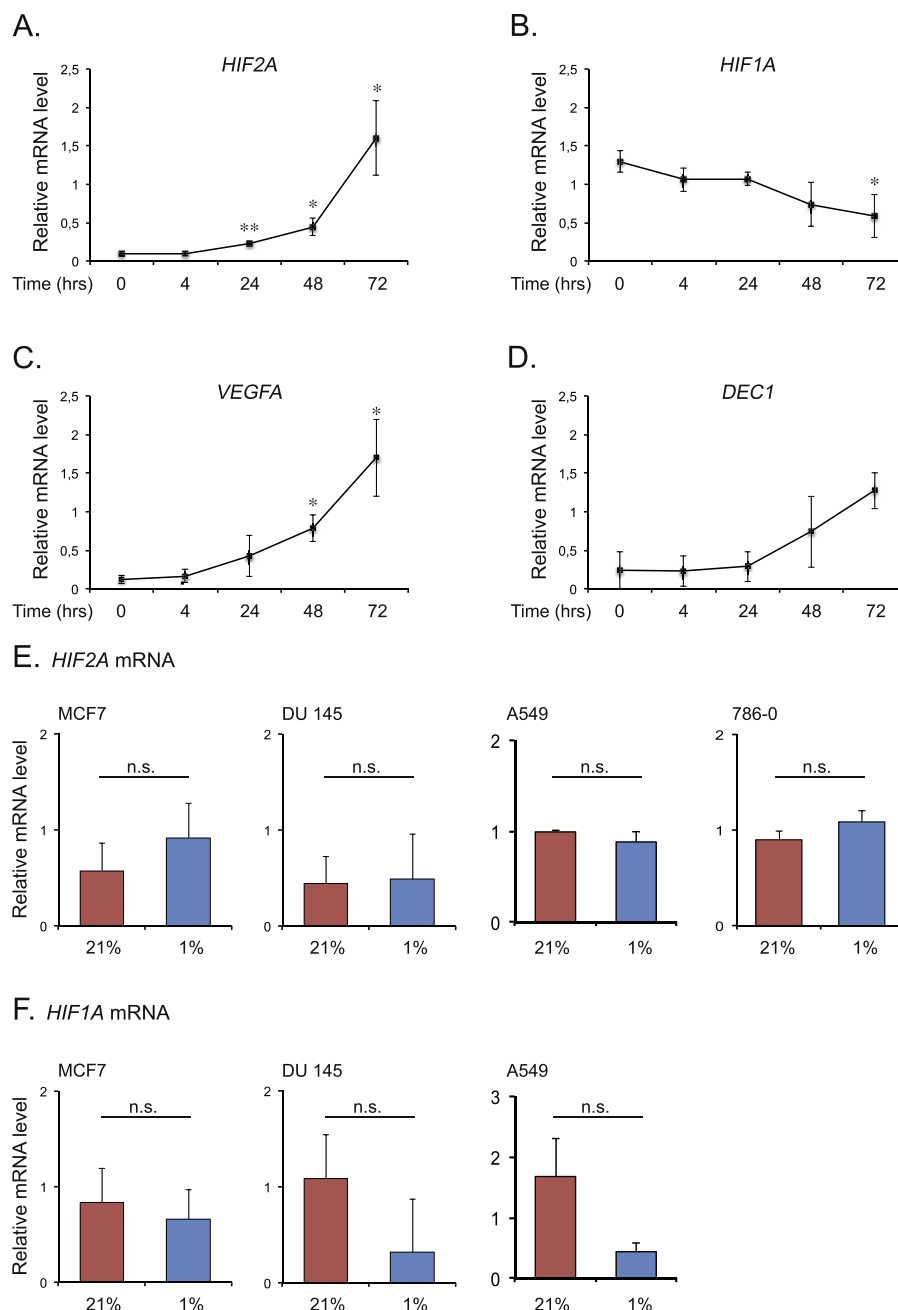


Fig. 1. HIF2A, but not HIF1A, mRNA is elevated in neuroblastoma cells at hypoxia (A–B) HIF2A (A) and HIF1A (B) mRNA was measured in neuroblastoma SK-N-BE(2)c cells cultured at hypoxia (1% O₂). (C–D) VEGFA (C) and DEC1 (D) mRNA expression was measured as down stream targets of HIF-2. (E–F) HIF2A (E) and HIF1A (F) mRNA expression in MCF7 breast cancer, DU 145 prostate cancer, A549 non-small cell lung cancer and 786-0 renal clear cell carcinoma cells cultured at 21 or 1% O₂ for 48 (MCF7, DU 145, 786-0) or 72 (A549) hours. Relative mRNA expression was measured by qRT-PCR and data are presented as mean \pm SEM from two (A549) or three independent experiments. Statistical significance was calculated using student's t test, * p < 0.05, ** p < 0.01. No asterisk (*) indicates no significance.

We sought to establish if the observed increase in HIF2A mRNA expression in hypoxic neuroblastoma cells was a general tumor cell phenomenon. In glioblastoma, another neural cell-derived tumor with an apparent dependency on HIF-2 α [5,16,17], it has been suggested that HIF2A transcription is induced at hypoxia, in particular in glioblastoma stem cell-enriched primary cultures [5,18]. On the other hand, long-term hypoxic culturing of breast cancer, prostate cancer, renal cell carcinoma, and non-small cell lung carcinoma cell lines did not result in any significant effects on HIF2A mRNA (Fig. 1E). Transcription of HIF1A, however, is unchanged or even decreased uniformly in all tumor forms investigated (Fig. 1B,F and [5]).

3.2. Differential changes in HIF mRNA levels are not due to mRNA stability

To address if the increase in HIF2A mRNA levels at hypoxia was due to altered mRNA stability or *de novo* transcription, we treated neuroblastoma cells with actinomycin D, an inhibitor of RNA synthesis [19,20] for 2, 4, and 6 h after an initial priming of cells for 72 h at normoxia or hypoxia. There was no marked difference in either HIF2A or HIF1A mRNA levels between cells cultured at normoxia or hypoxia when RNA synthesis was blocked (Fig. 2). Thus, the overall actinomycin D-induced decrease in both HIF2A and HIF1A mRNA rather supports active transcription of the HIF genes. If the

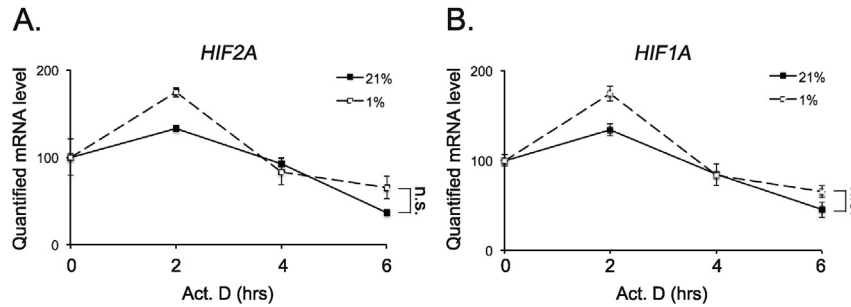


Fig. 2. Altered *HIF* mRNA expression at prolonged hypoxia is not due to changes in mRNA stability. SK-N-BE(2)c cells were cultured at 21% or 1% O₂ for 72 h. Post-72 h, cells were harvested at the indicated time points following actinomycin D treatment. Relative mRNA expression was measured by qRT-PCR and data are presented as mean \pm SEM from three independent experiments; n.s. – not significant.

elevation of *HIF2A* mRNA in hypoxic neuroblastoma cells would be explained by increased mRNA stability, the difference in mRNA levels between actinomycin D treated normoxic and hypoxic cells would be substantial.

3.3. *HIF* protein stabilization leads to induced *HIF2A* mRNA expression

Cells exposed to hypoxia initiate a switch in their transcriptional program, mainly mediated via HIF-1 and HIF-2. In order to determine the effects on HIF mRNA expression solely by stabilization of the HIFs and not by activating the complete hypoxic machinery, we treated two neuroblastoma cell lines with 2,2'-dipyridyl (DIP), an iron chelator, at normoxia. This leads to inhibition of the iron-dependent hydroxylation and subsequent degradation of the HIF alpha subunits and instead, HIF-1 α and HIF-2 α are stabilized (Fig. 3A–B and reviewed in Ref. [21]). Surprisingly, also *HIF2A* mRNA expression is induced by DIP treatment (Fig. 3C), whereas *HIF1A* mRNA levels decrease (Fig. 3D), mimicking the hypoxic response (cf. Fig. 1A–B).

Considering that *HIF2A* and *HIF1A* mRNA levels consistently display opposing expression patterns at hypoxia (Figs. 1A–B and 3C–D), we knocked down these two transcription factors individually and looked at their mRNA expression. Indeed, knockdown of *HIF1A* lead to increased transcription of *HIF2A*, whereas knockdown of *HIF2A* increased the transcription of *HIF1A* (Fig. 3E).

3.4. Transcription of *HIF2A* is regulated by *ERR α*

To further investigate the possible interregulation between *HIF2A* and *HIF1A*, we used a commercially available quantitative real time (qRT)-PCR based array, where 270 different transcription factors (including HIF-1 α and HIF-2 α) have been knocked down using RNAi techniques (Expressed transcription factor knockdown transcriptome PCR array, Sabiosciences). When analyzing the effects on *HIF* mRNA after transcription factor knockdown, we could conclude that *HIF2A* transcription seemed to be more complex than that of *HIF1A* (ten vs. two hits, fold change cut-off at \pm 4) (Table 1). However, neither *HIF1A* nor *HIF2A* were identified as hits for potential regulation of each other (Table 1).

Instead, we looked at the list of potential regulators of *HIF2A* transcription and identified coactivator PGC-1 β . Coactivators constitute a complex array of factors on which transcriptional regulation by gene- and cell-specific DNA-binding transcription factors depend upon. Coactivators of the PGC-1 family, PGC-1 α and PGC-1 β in particular, respond to multiple signals and enhance the transcriptional activity of key transcription factors involved in mitochondrial biogenesis, fatty acid utilization, electron transport chain assembly and angiogenesis [22]. Coactivation of *ERR α* by

PGC-1 α/β has been shown to play a role in the angiogenic and proliferative processes in tumors. More specifically, all members of the ERR family have been reported to physically interact with the HIFs in breast cancer, and the specific interaction between *ERR α* /PGC-1 α is known to drive *HIF2A* transcription in skeletal muscle [22–24]. Since HIF-2 α and HIF-1 α are well-established inducers of VEGF-A and other pro-angiogenic factors, the PGC and ERR family members as potential regulators of *HIF2A* transcription were of particular interest. We could not establish a link between *HIF2A* transcription and PGC-1 α , PGC-1 β or *ERR γ* based on knockdown studies in neuroblastoma cells. However, RNAi-mediated knockdown of *ERR α* resulted in downregulated *HIF2A* mRNA levels both at normoxia (Fig. 4A) and hypoxia (Fig. 4B). To further validate the possible effects of *ERR α* on *HIF2A* transcription, we treated neuroblastoma cells with the *ERR α* inverse agonist XCT790. Expression of *HIF2A* mRNA decreased in an inhibitor concentration-dependent fashion (Fig. 4C). Of note, *ESRRA* mRNA levels were not affected by hypoxia (Fig. 4C).

3.5. Expression of *ESRRA* correlates to disease stage and poor prognosis in neuroblastoma

Having identified *ERR α* as a regulator of *HIF2A* transcription, we sought to pinpoint if *ERR α* expression levels carried any prognostic value in neuroblastoma. Using a publically available data set consisting of 88 tumors [14], we could show that high expression of *ESRRA* significantly correlates to poor overall and progression-free survival in neuroblastoma (bonf. p-values equal 0.014 and 0.00044, respectively) (Fig. 4D–E).

4. Discussion

We have recently established that HIF-2 α is regulated at the transcriptional level in neuroblastoma cells, likely in addition to the canonical post-translational regulation that occurs in normal and tumor cells [10]. Here we establish that transcriptional regulation of *HIF2A* is not a general tumor cell phenomenon, but rather appears to be restricted to neurally derived tumors such as neuroblastoma and glioblastoma. In addition, these tumors share the characteristics of restricted areas with a pseudo-hypoxic phenotype with HIF-2 α positive tumor cells located in peri-vascular niches in tumor specimens. High HIF-2 α levels in neuroblastoma and glioblastoma correlate with aggressive, disseminating disease and as knockdown leads to impaired tumor growth *in vivo* in both these tumor forms [2,5,11], *HIF2A* qualify as an oncogene. Interestingly, gain-of-function mutations have been reported in two neuroblastoma-related tumor forms, paraganglioma and pheochromocytoma [25–30], further supporting an oncogenic role of *HIF2A* in neural cell-derived tumors. These results highlight the importance of

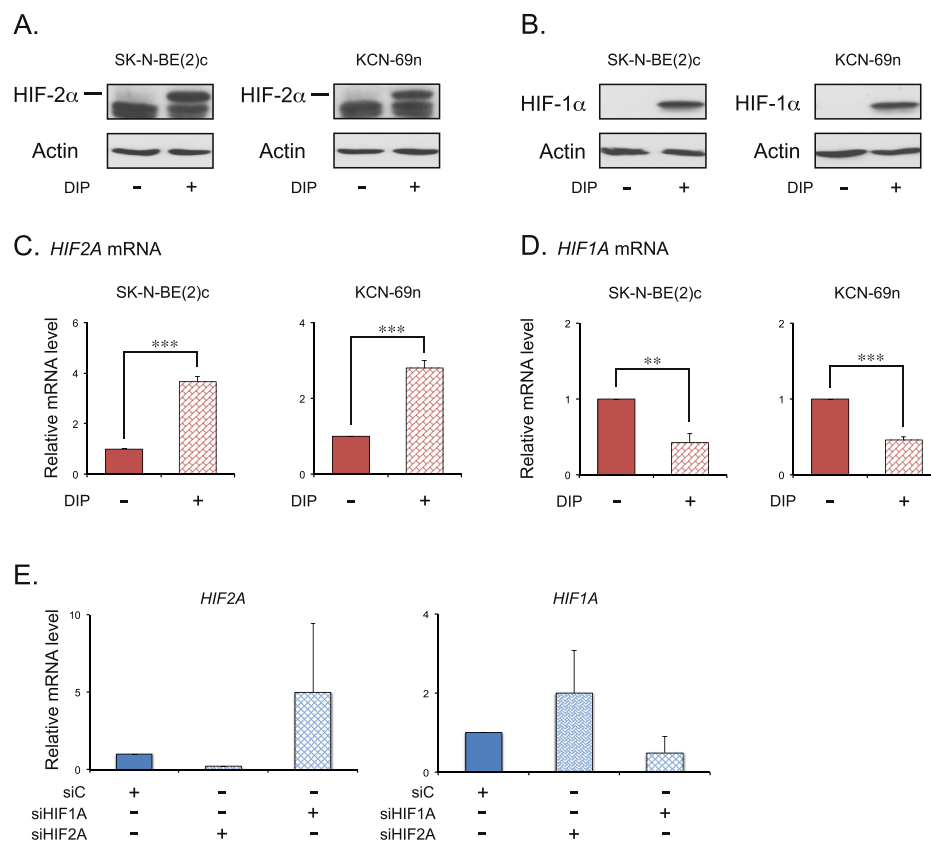


Fig. 3. *HIF1A* and *HIF2A* show opposing mRNA expression patterns upon stabilization of HIF protein and knockdown of HIF mRNA. (A–B) SK-N-BE(2)c and KCN-69n cells were treated with 2'2 dipyridyl (DIP) for 4 h at 21% O₂ and protein expression was determined by western blot. Actin was used as loading control. (C–D) Cells were treated as in (A–B) and *HIF2A* (C) and *HIF1A* (D) mRNA was quantified. (E) Knockdown of *HIF2A* and *HIF1A* using siRNAs in SK-N-BE(2)c cells, followed by 72 h at 1% O₂. As an internal control (siC) a scrambled HIF-1α sequence was used. Quantification of mRNA levels was performed using qRT-PCR. Data are presented as mean ± SEM from three independent experiments. Statistical significance was calculated using student's t test, **p < 0.01, ***p < 0.001. No asterisk (*) indicates no significance.

Table 1
Genes altering the expression of HIF1A and HIF2A upon knock-down.

Gene symbol	Description	Possible effect	Fold change
HIF1A	Hypoxia inducible factor 1, alpha subunit	Internal control for <i>HIF1A</i>	−9,0
RUNX1	Runt-related transcription factor 1	Negative regulator candidate of <i>HIF1A</i>	+4,6
HDAC1	Histone deacetylase 1	Positive regulator candidate of <i>HIF1A</i>	−5,3
EPAS1	Endothelial PAS domain protein 1	Internal Control for <i>HIF2A</i>	−24,4
PRMT1	Protein arginine methyltransferase 1	Negative regulator candidate of <i>HIF2A</i>	+13,9
PRMT5	Protein arginine methyltransferase 5	Negative regulator candidate of <i>HIF2A</i>	+5,2
PPARGC1B	Peroxisome proliferator-activated receptor gamma, coactivator 1 beta	Negative regulator candidate of <i>HIF2A</i>	+5,2
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin	Negative regulator candidate of <i>HIF2A</i>	+7,1
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin	Negative regulator candidate of <i>HIF2A</i>	+6,0
TCEAL1	Transcription elongation factor A	Negative regulator candidate of <i>HIF2A</i>	+5,2
ZKSCAN1	Zink finger with KRAP and SCAN domains 1	Negative regulator candidate of <i>HIF2A</i>	+4,9
YY1	YY1 transcription factor	Negative regulator candidate of <i>HIF2A</i>	+4,8
SMAD1	SMAD family member 1	Negative regulator candidate of <i>HIF2A</i>	+4,7
HDAC1	Histone deacetylase 1	Positive regulator candidate of <i>HIF2A</i>	−5,1

Screening of *HIF1A* and *HIF2A* gene expression modulators using transcription factor knockdown transcriptome PCR arrays. Quantification of *HIF1A* and *HIF2A* gene expression levels using qRT-PCR are expressed as fold changes based on Ct calculations using *GAPDH* as a reference gene. Non-target siRNA treated sample wells served as negative internal control. Data presented represent one experiment.

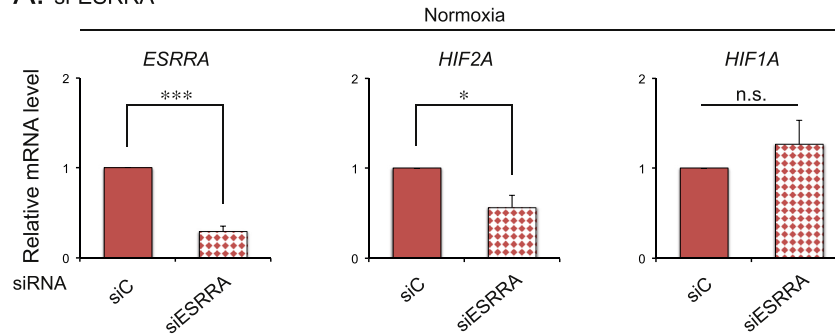
understanding and exploring the transcriptional regulation of HIF-2α in order to unravel novel therapeutic strategies for HIF-2α driven aggressive tumor phenotypes.

There was no difference in the overall HIF mRNA stability between normoxic and hypoxic neuroblastoma cells, indicating that the observed increase in *HIF2A* mRNA is explained by *de novo* transcription. This result is supported by previous findings by Lin et al., showing that HIF mRNA expression is insensitive to changes

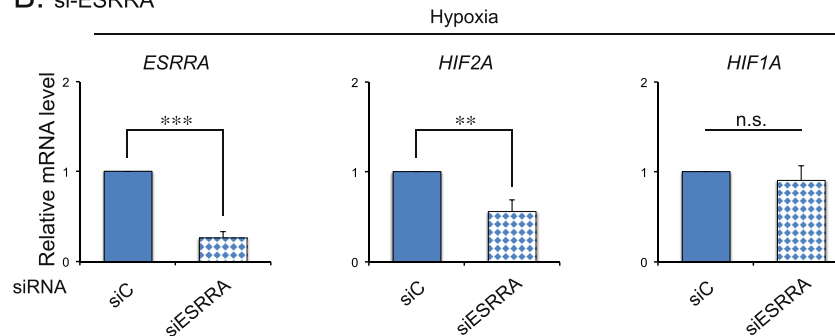
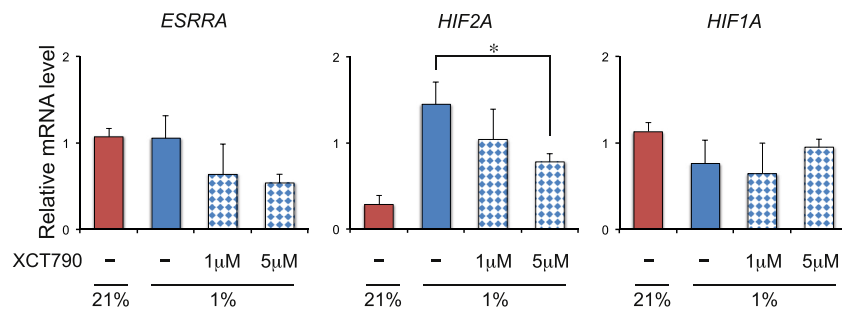
in mRNA stability [31]. In addition, these results support the notion that induced expression of the HIF-2α protein at hypoxia to a large part can be explained by enhanced *HIF2A* transcription and not solely by protein stabilization.

Despite an acute stabilization of the HIF-1α protein, *HIF1A* mRNA expression decreases in hypoxic neuroblastoma cells. Since the effects on *HIF1A* mRNA is not explained by decreased mRNA stability, it is tempting to speculate that the degradation of

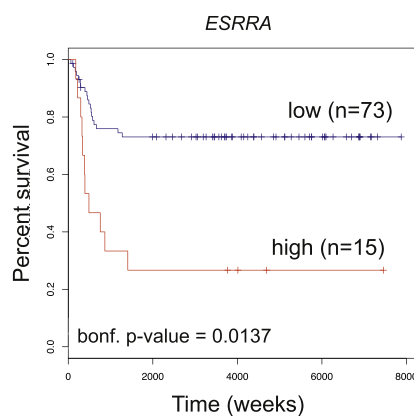
A. si-ESRRA



B. si-ESRRA

C. ERR α inverse agonist

D. Overall survival (OS)



E. Progression-free survival (PFS)

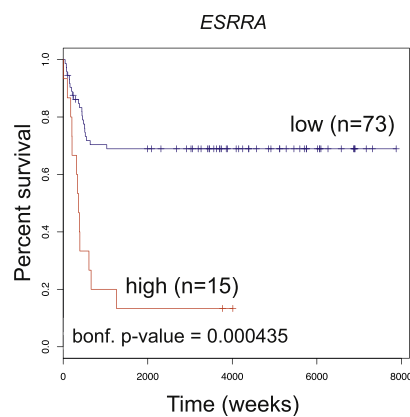


Fig. 4. Transcription of *HIF2A* depends on *ERR α* activity. (A–B) Knockdown of *ESRRA* in SK-N-BE(2)c cells using siRNA, followed by 72 h of normoxia (21% O₂, A) or hypoxia (1% O₂, B). (C) Treatment with *ERR α* inverse agonist XCT-790 for 72 h at hypoxia with indicated concentrations of XCT-790. Normoxic cells were used as an internal control for *HIF2A* expression. Relative mRNA expression was measured by qRT-PCR and data are presented as mean \pm SEM from three independent experiments. Statistical significance was calculated using student's t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. No asterisk (*) or n.s. indicates no significance. (D–E) High *ESRRA* mRNA expression in a clinical neuroblastoma material consisting of 88 tumors correlate with poor overall (D) and progression free (E) survival. Bonferroni corrected logrank p-values are provided.

synthesized *HIF1A* mRNA at hypoxia is enhanced. In addition, decreased *HIF1A* mRNA expression patterns are repeated in a number of different tumor forms that all stabilize the HIF-1 α protein at hypoxia, confirming an exclusive post-translational regulatory mechanism of HIF-1 α in tumor cells.

Unexpectedly, stabilization of the HIF- α proteins at normoxic conditions (by inhibiting the iron-dependent hydroxylation of the α subunits), leads to increased *HIF2A*, and decreased *HIF1A*, mRNA levels, mimicking the response observed when neuroblastoma cells are cultured at hypoxia. This might suggest an autocrine-/paracrine regulatory loop of the HIF transcription factors. Indeed, knockdown of HIF-1 α enhanced expression of *HIF2A* and vice versa, indicating either direct or indirect interplay between these two proteins to at least some extent. However, when analyzing the effect on HIF mRNA levels after knockdown of 270 different transcription factors (including HIF-1 α and HIF-2 α), neither *HIF1A* nor *HIF2A* were identified as hits for possible regulation of each other. Considering the somewhat modest effect seen after HIF knockdown using siRNAs (Fig. 3E), and the crude cut-off (>4-fold change in expression levels) used for the transcriptome array, the effects that HIF-1 α and HIF-2 α might have on each other would probably be too small for detection by this methodological approach.

Of the potential regulators that were identified, PGC-1 β was of particular interest due to the known role of the PGC-1/ERR family members in angiogenesis [32], and regulation of HIF expression in skeletal muscle [24]. The consequent downregulation of *HIF2A* mRNA expression after knockdown or inhibition of ERR α , and the strong correlation between high expression of *ESRRA* and poor overall and progression-free survival in neuroblastoma strongly suggest that ERR α plays a role in aggressive neuroblastoma behavior. Interestingly, *ESRRA* was recently identified as one of the most strongly expression-correlated regulators in an integrative analysis of 111 reference human epigenomes [33]. When each regulator was linked to the cell/tissue type that they most highly correlated with, *ESRRA* was found to have a regulatory relationship exclusively with the fetal adrenal gland [33], the organ in which more than half of all neuroblastomas arise. Hence, ERR α appears highly relevant in neuroblastoma and serves as a potential therapeutic target for aggressive tumor growth in patients.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by the Swedish Cancer Society, the Children's Cancer Foundation of Sweden, the Swedish Research Council, Fru Berta Kamprads stiftelse, the SSF Strategic Center for Translational Cancer Research – CREATE Health, VINNOVA, BiOCARE, a Strategic Research Program at Lund University, Hans von Kantzows Stiftelse, Gunnar Nilsson's Cancer Foundation and the research funds of Malmö University Hospital.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2015.04.083>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2015.04.083>.

References

- [1] A. Giatromanolaki, M.I. Koukourakis, E. Sivridis, et al., Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival, *Br. J. Cancer* 85 (2001) 881–890.
- [2] L. Holmquist-Mengelbier, E. Fredlund, T. Löfstedt, et al., Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype, *Cancer Cell* 10 (2006) 413–423.
- [3] K. Helczynska, A.M. Larsson, L. Holmquist Mengelbier, et al., Hypoxia-inducible factor-2alpha correlates to distant recurrence and poor outcome in invasive breast cancer, *Cancer Res.* 68 (2008) 9212–9220.
- [4] A. Kronblad, K. Jirstrom, L. Ryden, et al., Hypoxia inducible factor-1alpha is a prognostic marker in premenopausal patients with intermediate to highly differentiated breast cancer but not a predictive marker for tamoxifen response, *Int. J. Cancer. J. Int. du Cancer* 118 (2006) 2609–2616.
- [5] Z. Li, S. Bao, Q. Wu, et al., Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells, *Cancer Cell* 15 (2009) 501–513.
- [6] R. Noguera, E. Fredlund, M. Piqueras, et al., HIF-1alpha and HIF-2alpha are differentially regulated in vivo in neuroblastoma: high HIF-1alpha correlates negatively to advanced clinical stage and tumor vascularization, *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 15 (2009) 7130–7136.
- [7] M. Schindl, S.F. Schoppmann, H. Samonigg, et al., Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer, *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 8 (2002) 1831–1837.
- [8] J.M. Heddleston, Z. Li, R.E. McLendon, et al., The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype, *Cell. Cycle* 8 (2009) 3274–3284.
- [9] W.Y. Kim, S. Perera, B. Zhou, et al., HIF2alpha cooperates with RAS to promote lung tumorigenesis in mice, *J. Clin. Investigation* 119 (2009) 2160–2170.
- [10] S. Mohlin, A. Hamidian, S. Pahlman, HIF2A and IGF2 expression correlates in human neuroblastoma cells and normal immature sympathetic neuroblasts, *Neoplasia* 15 (2013) 328–334.
- [11] A. Pietras, D. Gisselsson, I. Øra, et al., High levels of HIF-2alpha highlight an immature neural crest-like neuroblastoma cell cohort located in a perivascular niche, *J. Pathology* 214 (2008) 482–488.
- [12] H.M. Sowter, R.R. Raval, J.W. Moore, et al., Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia, *Cancer Res.* 63 (2003) 6130–6134.
- [13] J. Vandesompele, K. De Preter, F. Pattyn, et al., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, *Genome Biol.* 3 (2002) 1–12. RESEARCH0034.
- [14] J.J. Molenaar, J. Koster, D.A. Zwijsenburg, et al., Sequencing of neuroblastoma identifies chromothripsis and defects in neuritogenesis genes, *Nature* 483 (2012) 589–593.
- [15] T. Therneau, A Package for Survival Analysis in S, R Package Version 2.37-7, 2013. <http://CRAN.R-project.org/package=survival>.
- [16] A. Pietras, A.M. Katz, E.J. Ekström, et al., Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth, *Cell. Stem Cell.* 14 (2014) 357–369.
- [17] L.K. Mathew, N. Skuli, V. Mucuj, et al., miR-218 opposes a critical RTK-HIF pathway in mesenchymal glioblastoma, *Proc. Natl. Acad. Sci. U. S. A.* 111 (2014) 291–296.
- [18] W. Yang, J. Wei, T. Guo, et al., Knockdown of miR-210 decreases hypoxic glioma stem cells stemness and radioresistance, *Exp. Cell Res.* 326 (2014) 22–35.
- [19] H.M. Sobell, Actinomycin and DNA transcription, *Proc. Natl. Acad. Sci. U. S. A.* 82 (1985) 5328–5331.
- [20] O. Bensaud, Inhibiting eukaryotic transcription: which compound to choose? how to evaluate its activity? *Transcription* 2 (2011) 103–108.
- [21] E.A. Cho, H.K. Song, S.H. Lee, et al., Differential in vitro and cellular effects of iron chelators for hypoxia inducible factor hydroxylases, *J. Cell. Biochem.* 114 (2013) 864–873.
- [22] G.C. Rowe, A. Jiang, Z. Arany, PGC-1 coactivators in cardiac development and disease, *Circulation Res.* 107 (2010) 825–838.
- [23] A. Ao, H. Wang, S. Kamarajugadda, et al., Involvement of estrogen-related receptors in transcriptional response to hypoxia and growth of solid tumors, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 7821–7826.
- [24] K.A. Rasbach, R.K. Gupta, J.L. Ruas, et al., PGC-1alpha regulates a HIF2alpha-dependent switch in skeletal muscle fiber types, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 21866–21871.
- [25] Z. Zhuang, C. Yang, F. Lorenzo, et al., Somatic HIF2A gain-of-function mutations in paraganglioma with polycythemia, *N. Engl. J. Med.* 367 (2012) 922–930.
- [26] K. Pacak, I. Jochmanova, T. Prodanov, et al., New syndrome of paraganglioma and somatostatinoma associated with polycythemia, *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 31 (2013) 1690–1698.
- [27] C. Yang, M.G. Sun, J. Matro, et al., Novel HIF2A mutations disrupt oxygen sensing, leading to polycythemia, paragangliomas, and somatostatinomas, *Blood* 121 (2013) 2563–2566.

- [28] F.R. Lorenzo, C. Yang, M. Ng Tang Fui, et al., A novel EPAS1/HIF2A germline mutation in a congenital polycythemia with paraganglioma, *J. Mol. Med.* 91 (2013) 507–512.
- [29] I. Comino-Mendez, A.A. de Cubas, C. Bernal, et al., Tumoral EPAS1 (HIF2A) mutations explain sporadic pheochromocytoma and paraganglioma in the absence of erythrocytosis, *Hum. Mol. Genet.* 22 (2013) 2169–2176.
- [30] A. Buffet, S. Smati, L. Mansuy, et al., Mosaicism in HIF2A-related polycythemia-paraganglioma syndrome, *J. Clin. Endocrinol. metabolism* 99 (2014) E369–E373.
- [31] Q. Lin, X. Cong, Z. Yun, Differential hypoxic regulation of hypoxia-inducible factors 1alpha and 2alpha, *Mol. Cancer Res. MCR* 9 (2011) 757–765.
- [32] Z. Arany, S.Y. Foo, Y. Ma, et al., HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha, *Nature* 451 (2008) 1008–1012.
- [33] C. Roadmap Epigenomics, A. Kundaje, W. Meuleman, et al., Integrative analysis of 111 reference human epigenomes, *Nature* 518 (2015) 317–330.