The chromatin remodeler ISWI regulates the cellular response to hypoxia: role of FIH

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ABSTRACT The hypoxia-inducible factor (HIF) is a master regulator of the cellular response to hypoxia. Its levels and activity are controlled by dioxygenases called prolyl-hydroxylases and factor inhibiting HIF (FIH). To activate genes, HIF has to access sequences in DNA that are integrated in chromatin. It is known that the chromatin-remodeling complex switch/sucrose nonfermentable (SWI/SNF) is essential for HIF activity. However, no additional information exists about the role of other chromatin-remodeling enzymes in hypoxia. Here we describe the role of imitation switch (ISWI) in the cellular response to hypoxia. We find that unlike SWI/SNF, ISWI depletion enhances HIF activity without altering its levels. Furthermore, ISWI knockdown only alters a subset of HIF target genes. Mechanistically, we find that ISWI is required for full expression of HIF mRNA and protein levels by changing RNA polymerase II loading to the FIH promoter. Of interest, exogenous FIH can rescue the ISWI-mediated up-regulation of CA9 but not BNIP3, suggesting that FIH-independent mechanisms are also involved. Of importance, ISWI depletion alters the cellular response to hypoxia by reducing autophagy and increasing apoptosis. These results demonstrate a novel role for ISWI as a survival factor during the cellular response to hypoxia.

INTRODUCTION

Oxygen is a vital requirement for survival of most multicellular organisms. However, perturbations to the supply of oxygen lead to the initiation of a complex set of events, including altering the cell metabolic state and the cell cycle profile. The majority of these events are controlled by a specific transcriptional response aimed at restoring the supply of oxygen (Rocha, 2007).

Central to the transcriptional response to hypoxia is the transcription factor hypoxia-inducible factor (HIF). HIF consists of a dimer between an oxygen-labile α subunit and a constitutively expressed β subunit, HIF1-β/aryl hydrocarbon nuclear translocator. There are three HIF-α proteins (1, 2, and 3) (Hirota and Semenza, 2005). The HIF-α protein levels are regulated by a class of Fe(II)- and 2-oxoglutarate–dependent dioxygenases called prolyl-hydroxylases (PHDs), of which there are four, although only PHD1, 2, and 3 have been shown to regulate HIF-α subunits in a nonredundant manner (Appelhoff et al., 2004). During hypoxia these enzymes catalyze hydroxylation of proline residues (Pro-402 and Pro-564 in human HIF-1α oxygen-dependent degradation domain), promoting interaction with the von Hippel–Lindau ubiquitin ligase complex, which is followed by ubiquitination and proteasomal degradation of HIF-α (Appelhoff et al., 2004; Acker et al., 2006).

Another regulator of the HIF pathway is factor inhibiting HIF (FIH), an asparaginyl hydroxylase that mediates an oxygen-dependent hydroxylation event in the C-terminal activation domain of HIF-α that inhibits HIF’s interaction with p300/CREB, a coactivator, and histone acetyl transferase (Mahon et al., 2001). It has also been demonstrated that FIH and PHD hydroxylation induce a differential response from HIF target genes, indicating that these sensors act to fine tune the hypoxia response (Kasper et al., 2005; Dayan et al., 2006).

Despite significant advances in our understanding of HIF transcriptional regulation, very little is known about the role of chromatin and chromatin remodeling in the hypoxia response. To induce transcription of target genes, the chromatin surrounding the enhancer regions and promoters must be conducive to HIF binding.
Modulation of chromatin can occur in several different ways, including histone tail modifications (Shilatifard, 2006), incorporation of histone variants (Jin et al., 2005), and nucleosome repositioning (Varga-Weisz and Becker, 2006).

We previously investigated whether ATP-dependent remodeling plays a role in the hypoxia response, demonstrating that switch/sucrose nonfermentable (SWI/SNF) is required for HIF-1α mRNA (Kenneth et al., 2009). However, nothing is known about how specific this response is to SWI/SNF or whether any other chromatin-remodeling enzymes play a part in the cellular response to hypoxia.

A SWI/SNF-related chromatin-remodeling enzyme, imitation switch (ISWI), belongs to a family of SNF2 helicases that are highly conserved throughout evolution (Flaus et al., 2006). The catalytic center of ISWI contains either hSNF2h or hSNF2l as the ATP-dependent motor of nucleosome remodeling (Okabe et al., 1992; Aihara et al., 1998). These proteins are found in combination with multiple regulatory subunits, forming distinct complexes, with diverse and yet to be well defined roles within the cell (Corona and Tamkun, 2004). Dissecting the contribution of ISWI to any given individual cellular processes is complex, but there has been some progress. For example, ISWI has been shown to be involved in chromatin assembly and nucleosome spacing (Collins et al., 2002; Fryodorov et al., 2004; Hanai et al., 2008), replication (Bozhenok et al., 2002), and transcriptional repression and activation (LeRoy et al., 1998). However, there has been no study of the role of ISWI in regulating the cellular hypoxia response.

In this article we analyze the role of ISWI in the control of HIF-mediated transcription and how ISWI modulates the cellular response to hypoxia. We find that ISWI negatively regulates HIF transcriptional activity. Mechanistically, ISWI directly controls the FIH gene by modulating RNA polymerase II loading. Of interest, we find that ISWI controls a subset of HIF-dependent genes by changing RNA polymerase recruitment and mRNA stability. These results indicate that ISWI plays a direct role in the modulation of the cellular response to hypoxia.

RESULTS

ISWI depletion results in an increase in HIF luciferase reporter activity

We previously showed that components of the SWI/SNF chromatin-remodeling complex are important for the levels of HIF-1α, with the subunit BAF57 being responsible for the recruitment of NF-κB and RNA polymerase II to the HIF-1α promoter (Kenneth et al., 2009). However, there are no studies analyzing how other chromatin-remodeling complexes function in hypoxia. To address this question, we chose to investigate the function of the important and evolutionarily conserved chromatin-remodeling family ISWI in the regulation of the cellular response to hypoxia.

We generated stable cell lines in U2OS and HeLa backgrounds expressing a luciferase reporter construct possessing three copies of the hypoxia-responsive element (HRE) consensus binding site. Cells were depleted of HIF-1α and the catalytic subunits of ISWI (hSNF2h and hSNF2l) using small interfering oligonucleotides (small interfering RNAs [siRNAs]) and exposed to desferoxamine (DFX) or 1% O2 (HPX) for 24 h prior to harvest and luciferase activity measured (Figure 1, A–D).

As predicted, when HIF-1α was depleted, luciferase activity was abolished even in the presence of DFX or hypoxia. Of interest, and in contrast with SWI/SNF (Kenneth et al., 2009), ISWI depletion resulted in a significant increase in luciferase reporter activity (Figure 1, A–D). In addition, we used a U2OS cell line expressing the PHD2 promoter fused to luciferase (Metzen et al., 2005), a hypoxia-responsive gene involved in the negative feedback regulation of the hypoxia response. This reporter also displayed a significant increase upon DFX treatment when ISWI was depleted (Figure 1E).

Chromatin remodelers are important for transcription, and given that ISWI forms the catalytic center of a wide variety of chromatin-remodeling complexes, we wanted to determine whether ISWI depletion resulted in a general derepression of transcription. To investigate this possibility, we used a U2OS stable cell line expressing a luciferase NF-κB reporter, depleted either RelA or ISWI, and treated with a NF-κB inducing stimulus, tumor necrosis factor-α (TNF-α). Of importance, when ISWI was depleted there was no increase in luciferase activity in response to TNF-α treatment (Figure 1F), suggesting specificity to the hypoxia response. Western blot analysis for HIF-1α and hSNF2h in the presence or absence of DFX demonstrated that the siRNA-mediated knockdown of these proteins was effective (Figure 1G).

ISWI depletion modulates a subset of HIF-dependent targets in hypoxia

Because our initial analysis was based on reporter gene assays, we next determined whether ISWI also changed the levels of endogenous HIF targets and whether this was a generalized effect. For this purpose we chose a panel of HIF targets involved in different cellular processes. First, we validated the knockdown of ISWI (Figure 2A). Using quantitative-PCR (qPCR), our analysis demonstrated that ISWI represses CA9, BNIP3, and PHD2 (Figure 2, B–D), whereas it does not alter the levels of DEC1 (Figure 2E) and other genes such as PHD3 and Glut1 (unpublished data). Of interest, the levels of Glut3 are reduced in the absence of ISWI (Figure 2F). To investigate whether the increased levels of CA9 and BNIP3 observed when ISWI was depleted were dependent on HIF, we performed codepletion experiments of ISWI and HIF-1α (Figure 2, G and H). This analysis demonstrated that in the absence of HIF-1α, ISWI depletion is not sufficient to increase CA9 or BNIP3 levels (Figure 2, G and H). These results suggest that ISWI has a specific role in the modulation of HIF-dependent genes and does not alter global HIF transcription, further demonstrating specificity of ISWI in the control of HIF activity.

ISWI depletion does not alter HIF mRNA or protein levels

SWI/SNF regulates HIF activity by directly controlling HIF-1α mRNA levels (Kenneth et al., 2009). To investigate whether ISWI was also modulating HIF activity by altering HIF levels in the cells, we analyzed HIF levels in the presence or absence of ISWI (Figure 3). qPCR confirmed that the levels of HIF-1α mRNA were unaltered by ISWI depletion in both U2OS and HeLa cells (Figure 3, A and B). Furthermore, ISWI depletion had no effect in the levels of HIF-1β or HIF-2α mRNA (Figure 3, C and D). This result was also evident at the protein level, as HIF-1α and HIF-1β remained unchanged by ISWI knockdown (Figure 3, E and F). Taken together, these results demonstrate that ISWI does not act in the same manner as SWI/SNF, suggesting an alternative mechanism for the modulation of HIF activity. Of interest, in U2OS cells, hypoxia induces HIF-2α mRNA, and HIF-1α depletion induces enhanced levels of HIF-2α in both cell lines tested. Although nothing is known about the mechanism behind this observation, similar results demonstrating the antagonism between HIF-1α and HIF-2α were described (Gordan et al., 2007).

ISWI depletion reduces FIH mRNA

An important regulator of HIF-1α activity without changing HIF levels is FIH (Stolze et al., 2004). FIH hydroxylates N803 in the transcriptional domain of HIF-1α, preventing its interaction with CBP/p300
and modulating the expression of a subset of HIF target genes (Mahon et al., 2001; Dayan et al., 2006).

We then investigated whether ISWI depletion changed FIH levels. We found that ISWI controls FIH mRNA, as loss of ISWI reduced FIH mRNA levels in the absence of hypoxia (Figure 4A), with similar results also observed in HeLa cells (Figure 4B). FIH protein levels were also significantly reduced when ISWI was depleted both in normoxia and following hypoxia (Figure 4C). To confirm the specificity of the siRNA, a variety of siRNA oligonucleotides were used against ISWI. It was found that in all cases in which ISWI was depleted, indicated by loss of hSNF2h, FIH protein levels were also reduced (Figure 4D). Furthermore, upon hSNF2h overexpression, we could also detect a slight increase in the protein levels of FIH (Figure 4E). These results suggest that ISWI regulates the FIH gene to allow for transcription to occur.

**ISWI controls RNA polymerase loading to the FIH gene**

Given that we observed a reduction in FIH mRNA in the absence of ISWI, we postulated that this was due to an impairment of the transcriptional machinery to transcribe the FIH gene. To investigate this, chromatin immunoprecipitation was used to measure the amount of RNA polymerase II (Pol II) present at the FIH gene. We found that in
of dimethylated and trimethylated K36 on histone H3 present at the FIH gene. ISWI depletion reduced the levels of dimethylated H3K36 (Figure 5D), a modification that restricts polycomb-mediated silencing (Yuan et al., 2011) but had no significant effect in the levels of trimethylated H3K36 (Figure 5E), a modification associated with actively transcribed genes (Nimura et al., 2009). These results demonstrate that ISWI depletion does not alter epigenetic markers globally.

ISWI depletion alters FIH protein levels and HIF target gene expression

The protein levels of BNIP3 and CA9, well-known HIF-1 target genes, induced by hypoxia and dependent on HIF-1α for hypoxic induction, are up-regulated by ISWI depletion, suggesting that HIF-1α is more active (Figure 6A). Of importance, ISWI-mediated effects on both CA9 and BNIP3 depend on HIF-1α, as codepletion of ISWI and HIF-1α results in a total loss of induction (Figure 6B). CA9 is an FIH-inhibited gene (Dayan et al., 2006), as shown by its derepression with FIH depletion (Figure 6C). However, there is no additive effect when FIH and ISWI are codepleted, suggesting that these proteins operate in the same pathway. On the other hand, BNIP3, which is regarded as an FIH-independent gene (Wenger et al., 2005), is not altered by FIH depletion but is increased by ISWI knockdown (Figure 6C).

To firmly demonstrate that the increase in CA9 expression was in fact due to the reduction of FIH, we performed rescue experiments with exogenous FIH. As expected, the increase in CA9 disappeared with FIH overexpression, whereas the increase in BNIP3 was still present. These results indicate that the CA9 derepression observed following ISWI depletion depends on FIH, whereas that of BNIP3 is not (Figure 6D).

Using siRNA-mediated depletion of BNIP3, we demonstrated that BNIP3 is present as a doublet in U2OS cells (Figure 6F). This running pattern for BNIP3 was previously observed by others (Tracy et al., 2007).

ISWI depletion alters CA9 and BNIP3 mRNA transcription and stability

Our results suggest that ISWI modulates CA9 and BNIP3 expression by altering the levels of mRNA for these genes (Figures 2 and 6). To determine whether this was due to increased transcription, we

the absence of ISWI, there was a 40% reduction in Pol II at the promoter-proximal region of FIH (Figure 5A). Of interest, the levels of acetylated H3 on the FIH gene were increased by ISWI depletion, suggesting that ISWI is involved in the recruitment of histone deacetylases (Figure 5B). We could not detect any difference in the levels of total histone H3 at the FIH promoter (Figure 5C), indicating that ISWI depletion did not increase the number of nucleosomes present at the FIH promoter. In addition, we investigated the levels

FIGURE 2: ISWI depletion modulates a subset of HIF-dependent targets in hypoxia. U2OS cells were exposed to 0, 4, 8, and 24 h of hypoxia, mRNA collected, and reverse transcribed, and quantitative real-time PCR was performed for the indicated genes: (A) ISWI, (B) CA9, (C) BNIP3, (D) PHD2, (E) DEC1, (F) Glut3. U2OS cells were depleted of ISWI, HIF-1α, or a combination of ISWI and HIF-1α, using siRNA oligonucleotides prior to exposure to 1% O2 for 24 h. mRNA was collected and reverse transcribed and quantitative real-time PCR was performed for the indicated genes: (G) BNIP3, (H) CA9. For all experiments samples were normalized to actin and to 24-h NT control. Bars represent the average of at least three independent biological replicates, and error bars represent the SE. The p values were calculated using Student’s t test (*p < 0.05; **p < 0.01; ***p < 0.001).
We observed a significant increase in RNA polymerase II present at these promoters. Furthermore, levels of acetylated H3 were also higher when ISWI was depleted (Figure 7, C and D).

Because mRNA levels can also be controlled by changes in its stability, we investigated whether ISWI depletion changed the half-life of CA9 or BNIP3. For this purpose, we performed actinomycin D chase experiments and measured the mRNA levels for these two genes (Figure 7, E and F). Of interest, both CA9 and BNIP3 have more stable mRNAs when ISWI is depleted, although this is only statistically significant for BNIP3 (Figure 7F). These results suggest that ISWI modulates both transcription and mRNA stability of these genes.

**ISWI depletion alters cell proliferation, autophagy, and apoptosis markers**

To address the biological role of ISWI in the hypoxia response, we analyzed several of the known hypoxia-induced cellular responses in the presence or absence of ISWI.

We first analyzed the effect of ISWI depletion on cellular proliferation both in normoxia and hypoxia by performing cell counting experiments. Under both conditions, ISWI depletion reduces cellular proliferation (Figure 8A). Hypoxia induces a reduction in cell proliferation in these cells (Kenneth et al., 2009); however, ISWI depletion results in a further reduction in cell numbers (Figure 8A). Of importance, when we analyzed cell viability by trypan blue staining, we observed that only in hypoxia did ISWI depletion induce a loss in viability (Figure 8B).

One of the major hypoxia-induced cellular responses is autophagy (Bellot et al., 2009). Autophagy can be seen either as a cell survival or a cell death mechanism. It is characterized by the presence of autophagosomes, which incorporate a cleaved light chain 3 (LC3) protein, LC3-II (Kabeya et al., 2000). In the absence of ISWI, the levels of LC3-II were reduced (Figure 8C) as assessed by Western blot. Similarly, when we investigated LC3-II foci by microscopy, we could detect that ISWI depletion resulted in lower number of foci if the GFP-LC3 cells exposed to hypoxia (Figure 8D). An additional marker for autophagy is the loss of p62/SQSTM1 (Jaakkola and Pursiheimo, 2009; Pursiheimo et al., 2009). Whereas in control cells treated with hypoxia we observed a reduction in the levels of p62, in the absence of ISWI the remaining p62 levels were higher (Figure 8C). These results are in agreement with the GFP-LC3 imaging (Figure 8D) and LC3 cleavage analysis (Figure 8C).
Given that we observed a reduction in cell viability in hypoxia when ISWI was depleted, we analyzed cell extracts for markers of apoptosis. Autophagy and apoptosis are in balance with each other, and many different stimuli activate both of these pathways simultaneously (Levine et al., 2008). In agreement with a loss in autophagy markers, we observed a loss of pro–caspase-3 and increased cleaved poly(ADP-ribose)polymerase (PARP), typical indicators of apoptosis. We could also detect an increase in active caspase-3 and caspase-7, poly(ADP-ribose)polymerase (PARP), typical indicators of apoptosis.

**DISCUSSION**

It is well established that hypoxia induces a coordinated transcriptional program that allows cells to survive and adapt to this harsh condition (Rocha, 2007). This program is mediated by a family of transcription factors called HIFs (Kenneth and Rocha, 2008). However, for transcription to occur, the chromatin has to be in a state permissive for transcription factor binding to target sequences.

Chromatin has a highly complex structure with multiple protein complexes coordinating the access to the underlying DNA (Varga-Weisz and Becker, 2006). The basic unit of chromatin is the nucleosome, consisting of approximately 147 base pairs of DNA wrapped around an octamer of histone proteins (Flaus et al., 2006). It is also a highly dynamic structure, with several enzymatic activities contributing to it (Flaus et al., 2006; Varga-Weisz and Becker, 2006). As such, several classes of enzymes can promote the modification of histone tails, changing the dynamics between protein and DNA association (Shilatifard, 2006).

Other enzymes use the energy of ATP to move or displace nucleosomes from the DNA. These are called ATP-dependent chromatin-remodeling enzymes (Flaus et al., 2006; Varga-Weisz and Becker, 2006).

There is almost no information regarding the structure of chromatin in hypoxia. Our previous work demonstrated the crucial role of an ATP-dependent chromatin-remodeling complex, SWI/SNF, in the control of HIF (Kenneth et al., 2009). However, no other enzymes have been investigated in the context of the hypoxia response. Here we demonstrate the involvement of another chromatin-remodeling enzyme, ISWI, in the cellular response to hypoxia. We show that ISWI controls the levels of FIH transcription and mRNA, leading to specific alterations in the hypoxia-induced genes. Of importance, we find that ISWI controls one of the main cellular responses to hypoxia, autophagy.

ISWI has been studied mainly in vitro, as well as in the model organism yeast. However, it is highly conserved in evolution (Flaus et al., 2006). Biochemically, its role seems to be to maintain an evenly spaced number of nucleosomes (Racki and Narlikar, 2008) and, as such, it has mostly transcriptional repressive activities (Racki and Narlikar, 2008). ISWI is part of several complexes in the cell, associating with a number of accessory partners that convey specificity and targeting of functions. These include ATP-utilizing chromatin remodeling and assembly factor (ACF), chromatin remodeling and assembly complex (CHRAC), and remodeling and spacing factor (RSF; Collins et al., 2002; Fyodorov et al., 2004; Hanai et al., 2008). However, none of these has been studied in the context of hypoxia.

Our data demonstrate that ISWI is required for FIH mRNA maintenance (Figure 4). It is required for RNA polymerase loading to the FIH, but its depletion also results in changes to the levels of acetylated histone H3 and dimethylated H3K36; however, no changes were observed in the levels of total H3 or trimethylated H3K36 (Figure 4). Changes in acetylated H3 were expected since ISWI is known to associate with histone deacetylases and be required for deacetylation of histones (Alenghat et al., 2006). Dimethylated and trimethylated

**FIGURE 4:** ISWI depletion reduces FIH mRNA and protein. (A, B) FIH (black) and ISWI (white) mRNA after ISWI depletion by siRNA oligonucleotides in U2OS (A) and HeLa (B) cell lines. For all experiments samples were normalized to actin and to NT control. Bars represent the average of at least three independent biological replicates, and error bars represent the SE. The p values were calculated using Student’s t test (*p < 0.05; **p < 0.01; ***p < 0.001). (C) Western blot depicting hSNF2h, HIF-1α, FIH, and PCNA as a loading control in U2OS cells. (D) Western blot depicting the effect of multiple siRNA oligonucleotides directed against ISWI (1–8) in U2OS cells. (E) Western Blot depicting the effect of hSNF2h overexpression on the levels of FIH in U2OS cells.
This suggests that FIH function is more important in conditions of pathological deregulation of oxygen availability and is not so relevant for developmental hypoxia.

Given the many functions of ISWI, it is not surprising that it is an essential gene, required for development of most organisms (Brown et al., 2007). However, whether ISWI regulates metabolism in adult tissues has not been investigated. In T cells, ISWI was shown to specifically regulate certain cytokine production, increasing interleukin-2 (IL-2) and reducing IL-3 (Precht et al., 2010), two important cytokines in the control of autoimmune diseases and hematopoietic proliferation, respectively (Precht et al., 2010). Further investigation is therefore required to determine whether ISWI modulates metabolism in adult tissues.

We focused on the regulation of two major HIF genes, CA9 and BNIP3, by ISWI. We found that in the absence of ISWI both genes were elevated at the mRNA and protein levels. Furthermore, ISWI depletion induced not only increased markers of active transcription at the promoters of these genes, but also increased mRNA half-life (Figure 2, 6, and 7). Although it is known that ISWI can activate and repress the transcription of genes, a connection with mRNA degradation had not been found before. However, studies in yeast revealed that ISWI suppresses antisense transcripts and the transcription of noncoding RNAs (Whitehouse et al., 2007; Yadon et al., 2010). More recently, in Drosophila, ISWI was shown to be required for the organization of nucleoplasmic omega speckles, which are essential for RNA processing and maturation (Onorati et al., 2011). Our data link ISWI to mRNA stability in mammalian cells (Figure 7). Whether ISWI alters the process of mRNA maturation or decay or even alters the expression of noncoding RNAs such as microRNAs in mammalian cells is unknown and therefore requires further investigation.

Despite altering the levels of CA9 and BNIP3, ISWI depletion does not alter HIF activity globally. There are some genes that are not changed, such as DEC1, and others, such as Glut3, whose expression decreases when ISWI is depleted. Neither DEC1 nor Glut3 has been reported to depend on FIH for regulation (Dayan et al., 2006). Furthermore, genes that are activated by ISWI inhibition are usually indirect (Fazzio et al., 2001). Additional research is needed to determine the mechanism of ISWI function in vivo.

Functionally, we find that ISWI depletion results in reduced autophagy markers following hypoxia. We also found changes in the levels of apoptosis and proliferation. ISWI has been shown to be required for correct DNA replication (Varga-Weisz and Becker, 2006), in accordance with our results demonstrating a lowering of proliferation (Figure 8). However, there is no published work on the role of ISWI in the process of autophagy. Our data suggest that ISWI is

H3K36s are markers of active transcription and serve to restrict polycomb-mediated repression of genes (Schmitges et al., 2011). In contrast to trimethylated H3K36, which remained unchanged, the levels of dimethylated H3K36 were reduced. We do not know the mechanism behind this change. It is possible that ISWI is important for the recruitment of a methyltransferase to this promoter such as SET domain transferases (Krajewski and Vassiliev, 2010). Further investigation would determine whether this is the case.

Recently, the phenotype of the FIH-null mouse was described. Surprisingly, FIH-deleted mice have no phenotypes associated with increased HIF or hypoxia responses (Zhang et al., 2010). Instead, these mice have metabolic changes, including increased metabolic rates and resistance to damage from a high-fat diet (Zhang et al., 2010). This suggests that FIH function is more important in conditions of pathological deregulation of oxygen availability and is not so relevant for developmental hypoxia.
required for autophagy in hypoxia to the detriment of apoptosis. This suggests that ISWI is a survival factor in hypoxia. The survival function of ISWI has been described in the context of ovarian cancer, in which hSNF2h and RSF1 are required for cancer growth and survival (Sheu et al., 2008).

In summary, we found that the hypoxia response involves another chromatin remodeler complex, ISWI. ISWI acts as a specificity determinant for HIF targets and cell fate decisions. Our findings suggest that chromatin structure is altered specifically in hypoxia to allow for cells to respond and adapt properly to changes in oxygen availability and that ISWI in particular acts as a survival mechanism for cells in hypoxia.

MATERIALS AND METHODS

Cells
U2OS osteosarcoma and HeLa cervical cancer cell lines were obtained from the European Collection of Cell Cultures and grown in DMEM (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA), 50 U/ml penicillin (Lonza), and 50 μg/ml streptomycin (Lonza) for no more than 30 passages at 37°C and 5% CO₂. Stable cell lines U2OS-HRE-luciferase, HeLa-HRE-luciferase, and U2OS-PHD2-luciferase were generated by cotransfection of 3xHRE-luciferase construct (a kind gift from G. Melilo, National Cancer Institute, National Institutes of Health, Bethesda, MD) and PHD2-promoter luciferase (a kind gift from E. Metzen, University of Duisburg-Essen, Essen, Germany) with a puromycin-resistant construct. Stable clones were selected with 1.0 μg/ml and maintained in 0.5 μg/ml puromycin.

Plasmids and chemicals
pCDNA3-FIH was a kind gift from E. Metzen, and CMV-hSNF2h was obtained from OriGene (Rockville, MD). Desferroxamine and actinomycin D were obtained from Sigma-Aldrich (St. Louis, MO).

siRNA transfection
siRNA duplex oligonucleotides were synthesized by MWG (High Point, NC) and transfected using Interferin (Polyplus, Illkirch, France) per the manufacturer’s instructions.

siRNA sequences are as follows: control, AAC AGU CGC GGU UGC GAC UGG (van Uden et al., 2008); HIF-1, CGU AUG ACC AGC AAC UUG A (van Uden et al., 2008); ISWI (4), AGA UGA GAA GCA GAA CUU ATT; 1, CAU CUC CAC UGA ACA UGA ATT; 2, GAU UGU CAU GGA CUU CUU ATT; 3, AGA AGA CUG CAG AGA UGA ATT; 5,
Hypoxia inductions
Cells were incubated at 1% O₂ in an Invivo 300 hypoxia workstation (Ruskinn, Pencoed, Bridgend, United Kingdom). Cells were lysed for protein extracts, with RNA extraction in the workstation to avoid reoxygenation. Cells were lysed for protein using a NP-40 lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40 [Calbiochem, La Jolla, CA], 2 mM EDTA, 250 M Na₃VO₄, 1 mM dithiothreitol, 10 mM NaF, and phosphatase inhibitors, 1 tablet/10 ml [Roche Complete; Roche, Indianapolis, IN]) and for RNA using PeqGOLD Total RNA Kit (Peqlab, Erlangen, Germany) as per manufacturer’s instructions. For hypoxia-mimetic conditions, cells were treated with 200 μM desferroxamine for 24 h prior to harvest using passive lysis buffer (Promega, Madison, WI).

qPCR and PCR sequences
cDNA was made using QuantiTect Rev. Transcription Kit (Qiagen, Valencia, CA) diluted 1:30 and analyzed using a p5000 rt-PCR machine (Stratagene, Santa Clara, CA) and Brilliant II SYBR Green QPCR Low ROX Master Mix (600830, Stratagene).

PCR primers are as follows:

- **HIF-1α**: For, CAT AAA GTC TGC AAC ATG GAA GGT; and Rev, ATT TGA TGG GTG AGG AAT GGG TT
- **HIF-1β**: For, CAA GCC CCT TGA GAA GTC AG; and Rev, GAG GGG CTA GGC CAC TAT TC
- **HIF-2α**: For, GCG CTA GAC TCC GAG AAC AT; and Rev, TGG CCA CTT ACT ACC TGA CCC TT
- **β-Actin**: For, GTG GGA GTG GGT GGA GGC; and Rev, TCA ACT GGT CTC AAG TCA GTG
- **CA9**: For, CTT TGC CAG AGT TGA CGA GG; and Rev, CAG CAA CTG CTC ATA GGC AC
- **hSNF2h**: For, TAC AAA CAA CTG CAT G; and Rev, CAG CAA CTG CTC ATA GGC AC
- **BNIP3**: For, GCC CAC CTC GCT CGC AGA CAC; and Rev, CAA TCC GAC GCT GAA AGC
- **PHD2**: For, GAA AGC CAT GTC GAG G; and Rev, TGG GGA GAA AAG TCT TTT CCA
- **FIH**: For, TTC GGA GGG AAT AAG ATG; and Rev, CTG CTC ATT GTC AGG AAG
- **BNIP3**: For, GCC CAC CTC GCT CGC AGA CAC; and Rev, CAA TCC GAT GGC CAG CAA ATG AGA
- **PHD2**: For, GAA AGC CAT GTC GAG G; and Rev, TGG GGA GAA AAG TCT TTT CCA
- **Glut3**: For, CAA TGC TCC GTA GGA GAT CAT AA; and Rev, AAA GCG GTT GAC GAA GAG T

FIGURE 7: ISWI controls transcription and mRNA stability of BNIP3 and CA9 genes in hypoxia. U2OS cells were depleted of ISWI using siRNA oligonucleotides and treated with 1% O₂ for 24 h. Chromatin immunoprecipitation was performed using RNA polymerase II (Pol II) (A, B) or acetylated histone H3 (AcH3) (C, D) for the BNIP3 (A, C) and CA9 promoters (B, D). Antibodies and enrichment measured by real-time quantitative PCR, normalizing to input and control siRNA. (E, F) U2OS cells were depleted of ISWI by siRNA with a nontargeting siRNA as a control, exposed to hypoxia (1% O₂ for 24 h and treated with 10 μg/ml actinomycin D for the final (0, 4, or 8) hours of the hypoxia treatment. mRNA was extracted and reverse transcribed, and qPCR was performed for CA9 (E) and BNIP3 (F). All samples were normalized to actin and then to 0 h of actinomycin treatment for both NT and ISWI siRNAs. Data points represent the average of at least three independent biological replicates. Bars represent the average of at least three independent biological replicates, and error bars represent the SE. The p values were calculated using Student’s t test (*p < 0.05; **p < 0.01; ***p < 0.001).
Antibodies

Antibodies used were as follows: HIF-1α (MAB1536; R&D Systems, Minneapolis, MN), HIF-2α (PA1-16510; Thermo Scientific, Waltham, MA), acetyl-H3 (06-599; Millipore, Billerica, MA), di-methyl-H3 K36 (07-369; Millipore), tri-methyl H3 K36 (9763; Cell Signaling, Beaver, MA), Total-H3 (Ab1791; Abcam, Cambridge, MA), β-actin (A5441; Sigma-Aldrich), PHD2 (ab4561; Abcam), CA9 (NB100-417; Novus Biologicals, Littleton, CO), polymerase II CTD (sc-47701; Santa Cruz Biotechnology, Santa Cruz, CA), hSNF2h (A301-017A; Bethyl Laboratories, Montgomery, AL), FIH (NB100-428; Novus Biologicals), HIF-1β (3718; Cell Signaling), proliferating cell nuclear antigen (PCNA; P8825; Sigma-Aldrich), BNIP3 (ab10433; Abcam), LC3-II (5F10; NanoTools, Teningen, Germany), p62/STQM (610832; BD Transduction Labs, Lexington, KY), cleaved caspase-7 (9491; Cell Signaling), caspase-3 (9662; Cell Signaling), cleaved caspase-3 (9661; Cell Signaling), and cleaved PARP (9541; Cell Signaling).

Fluorescence microscopy

For immunofluorescence, cells were grown on coverslips and treated as indicated prior to fixation by incubation in 3.7% formaldehyde/PBS (pH 6.8) for 15 min. Cells were permeabilized in PBS–0.1% Triton X-100 for 15 min and then blocked in PBS–0.05% Tween 20 supplemented with 1% normal donkey serum for 30 min. Cells were analyzed and images were acquired using a DeltaVision microscope (Applied Precision, Issaquah, WA). Images were deconvolved and analyzed using OMERO client software (Open Microscopy Environment, http://www.openmicroscopy.org/site).

Chromatin immunoprecipitation

Proteins were cross-linked with formaldehyde for 10 min. Glycine 0.125 M was added, and cells were washed with phosphate-buffered saline. Cells were lysed with lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotonin), followed by sonication and centrifugation. The supernatant was precleared with sheared salmon sperm DNA and protein G-Sepharose beads (Sigma-Aldrich). The supernatant was incubated with specific antibodies overnight and then with protein G-Sepharose beads for 1 h. After an extensive wash step, the complexes were eluted with buffer (100 mM NaHCO₃, 1% SDS) and incubated with proteinase K. DNA was purified using QIAquick PCR Purification Kit (Qiagen). PCR was performed using the following primers: FIH: For, AGA CTC GGG CAC ATG GGG CA; and Rev, CCC ACC GGA ACC GGA AGC TC. CA9: For, GAC AAA CCT GTG AGA CTT TGG CTC C; and Rev, AGT GAC AGC AGC AGT TGC ACA GTG. BNIP3: For, ACC ATC CGT TCA CGC AGC GG; and Rev, GCT GCC ACC AGG TCC CTC CT.
ISWI regulates FIH levels

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