Sprouty2 Protein Regulates Hypoxia-inducible Factor-α (HIFα) Protein Levels and Transcription of HIFα-responsive Genes*

Received for publication, January 8, 2016, and in revised form, May 27, 2016. Published, JBC Papers in Press, June 8, 2016, DOI 10.1074/jbc.M116.714139

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The α-subunits of hypoxia-inducible factors (HIF1α and HIF2α) promote transcription of genes that regulate glycolysis and cell survival and growth. Sprouty2 (Spry2) is a modulator of receptor tyrosine kinase signaling and inhibits cell proliferation by a number of different mechanisms. Because of the seemingly opposite actions of HIFα subunits and Spry2 on cellular processes, we investigated whether Spry2 regulates the levels of HIFα and HIFα protein levels. In cell lines from different types of tumors in which the decreased protein levels of Spry2 have been associated with poor prognosis, silencing of Spry2 elevated HIFα protein levels. Increases in HIF1α and HIF2α protein levels due to silencing of Spry2 also up-regulated HIFα target genes. Using HIF1α as a prototype, we show that Spry2 decreases HIF1α stability and enhances the ubiquitylation of HIF1α by a von Hippel-Lindau protein (pVHL)-dependent mechanism. Spry2 also exists in a complex with HIF1α. Because Spry2 can also associate with pVHL, using a mutant form of Spry2 (3P/3A-Spry2) that binds HIF1α, but not pVHL, we show that WT-Spry2, but not the 3P/3A-Spry2 decreases HIF1α protein levels. In accordance, expression of WT-Spry2, but not 3P/3A-Spry2 results in a decrease in HIF1α-sensitive glucose uptake. Together our data suggest that Spry2 acts as a scaffold to bring more pVHL/associated E3 ligase in proximity of HIF1α and increase its ubiquitylation and degradation. This represents a novel action for Spry2 in modulating biological processes regulated by HIFα subunits.

The four Sprouty proteins (Spry1 to Spry4), which are products of different genes, regulate downstream signaling from certain receptor tyrosine kinases and therefore play a major role in development (1–5). Because cell proliferation processes in normal development and tumor growth overlap, some of the Spry2 proteins, such as Spry1, Spry2, and Spry4, also regulate tumor growth (6–16). Among the four isoforms, Spry2 is ubiquitously expressed and well studied in cancer. In cancers of the liver, lung, breast, and prostate, Spry2 levels are decreased (6–11, 17–19). The decrease in Spry2 levels in these cancers has been correlated to poor patient prognosis and shorter survival of patients implicating Spry2 as a tumor suppressor. Therefore, a number of studies have investigated mechanisms that regulate the expression of Spry2, as well as how Spry2 modulates signaling via receptor tyrosine kinases (20–25). At the post-translational level, Spry2 has been shown to be ubiquitylated and targeted for proteosomal degradation by c-Cbl (26, 27), Siah2 (28), Ned4-1 (29), and pVHL with its associated E3 ligase (30). Interestingly, in some patient-derived hepatocellular carcinomas, when Spry2 levels were decreased, the levels of Ned4-1 were elevated (8).

It is now well established that as tumors proliferate rapidly prior to angiogenesis, the oxygen from the nearby vasculature cannot diffuse throughout the entire tumor resulting in the formation of a hypoxic environment (31). Cells adapt to the hypoxic environment by altering a variety of cellular processes that are heralded by an increase in the levels of the transcription factors, hypoxia-inducible factors (HIFs) (32–36). HIFs are heterodimers composed of one of three α-subunits (HIF1α, HIF2α, and HIF3α) and a β-subunit (HIF1β). The role of HIF3α in hypoxic gene regulation is not well understood, but a splice variant of HIF3α may function as an inhibitor of gene transcription during hypoxia (37, 38). In normoxia, HIF1α and HIF2α are degraded by a well characterized proteosomal mechanism (39, 40). Prolyl hydroxylase domain proteins (PHDs) bind to the HIFα subunits and hydroxylate two proline residues (41, 42). These hydroxy-prolyl residues serve as the docking site for pVHL and its associated E3 ubiquitin ligase resulting in the ubiquitylation and subsequent proteosomal degradation of HIFα subunits (43–46). Because the hydroxylation reaction requires oxygen, in hypoxia the activities of the PHDs are attenuated, and therefore, the hydroxylation, ubiquitylation, and degradation of HIFα subunits are inhibited. This permits the accumulation of HIFα protein levels and consequently enhances their ability to regulate transcription of genes, such as those that regulate proliferation, angiogenesis, drug metabolism, and glycolysis to promote tumor survival and growth (47–49).
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Because of the anti-proliferative and tumor suppressor actions of Spry2, we investigated whether Spry2 alters the levels of HIFα subunits and/or their ability to alter transcription of their target genes. Herein, we report the novel ability of endogenous Spry2 to promote the degradation of HIF1α and HIF2α resulting in a decrease in the expression of the genes that the two HIFs regulate. With HIF1α as the prototype of the two HIFα proteins (HIF1α and HIF2α), we demonstrate that Spry2 decreases HIF1α protein stability and enhances the ubiquitylation of HIF1α. Using different approaches, we also show that Spry2 exists in a complex with HIF1α. Because we previously reported that Spry2 is capable of binding to pVHL (30), we hypothesized that Spry2, by binding with HIF1α, increases the amount of pVHL in the vicinity of HIF1α and promotes its degradation through a pVHL-dependent mechanism. Indeed, our studies show that when pVHL is silenced, Spry2 cannot alter HIF1α protein levels. Additionally, a mutant form of Spry2 that does not bind to pVHL also does not decrease HIF1α protein levels. In agreement, wild-type (WT) Spry2 inhibits HIF1α-sensitive glucose uptake, whereas the mutant form of Spry2, which does not associate with pVHL, does not alter glucose uptake.

Results

Endogenous Spry2 Regulates HIF1α and HIF2α Protein Levels—Because previous studies have suggested that Spry2 plays a critical role in the regulation of hepatocellular, prostate, breast, and lung carcinomas, we utilized HuH7 cells that are derived from a human hepatocellular carcinoma (6–11, 17–19). In these cells, the siRNA-mediated silencing of endogenous Spry2 resulted in a 2.5-fold elevation of endogenous HIF1α protein levels (Fig. 1A). As established by numerous studies, HIF1α levels are not detectable in normoxia. However, once the cells are exposed to hypoxia, HIF1α protein levels are elevated (Fig. 1A). It is also important to note that as reported in our earlier report (30), Spry2 levels are also elevated by hypoxia (Fig. 1A). The increase in HIF1α protein levels upon silencing of endogenous Spry2 is not accompanied by a change in HIF1α mRNA levels (Fig. 1B). Like HIF1α, endogenous HIF2α protein levels in HuH7 cells were also increased by hypoxia and further elevated by 2-fold upon silencing of Spry2 (Fig. 1C) without any changes in HIF2α mRNA levels (Fig. 1D). A second Spry2 siRNA targeting a different sequence in the mRNA also elevated HIF1α and HIF2α protein levels in HuH7 cells (Fig. 2A). Notably, Spry2 is modified by phosphorylation and migrates as multiple bands (50), which depending on length of exposure of the blots can be observed as a single band or a doublet (cf. Figs. 1, A and B, and 2A). Together, these findings suggest that Spry2 alters HIF1α and HIF2α protein levels post-transcriptionally.

The generality of our findings is exemplified by the observations that silencing of Spry2 with two different siRNAs results in an elevation of HIF1α protein levels in the following panel of cell lines derived from tumors in which Spry2 plays a crucial role: breast cancer-derived cell lines (MCF-7 and MDA-MB-231); lung cancer cell line (A549); and another hepatocellular carcinoma cell line (Hep3B) (Fig. 2B). Different cell types express different amounts of Spry2 and different amounts of post-translationally modified Spry2. As mentioned above, Spry2 migrates as a doublet representing its phosphorylated and dephosphorylated forms (29, 50), and thus, it would appear that in A549 cells a larger amount of Spry2 is phosphorylated, whereas in Hep3B cells a larger amount of dephosphorylated Spry2. Moreover, Western blots probed for HIF1α (A) and HIF2α (B) images from same blot (C), Spry2, and actin (loading control) are shown. Graphs in lower panels show mean ± S.E. of densitometric analysis of HIF1α (A) or HIF2α (B) normalized to actin from three (A) or four (C) independent experiments. Transient levels of HIF1α (B), HIF2α (C), and Spry2 were monitored using qRT-PCR, and relative levels were calculated as described under “Experimental Procedures.” Graphs are mean ± S.E. from three independent experiments. Statistical significance was assessed using unpaired Student’s t tests (A and C); n.s., not significant.

Silencing of Spry2 increases HIF1α and HIF2α protein amounts without altering their mRNA levels. HuH7 cells transfected with control (Cntrl) siRNA or siRNA targeting Spry2 were cultured in normoxia (N) (21% O2) or hypoxia (H) (3% O2) for 8 h (A and B) or 24 h (C and D). Representative Western blots probed for HIF1α (A), HIF2α (B) images from same blot (C), Spry2, and actin (loading control) are shown. Graphs in lower panels show mean ± S.E. of densitometric analysis of HIF1α (A) or HIF2α (B) normalized to actin from three (A) or four (C) independent experiments. Transient levels of HIF1α, HIF2α, and Spry2 were monitored using qRT-PCR, and relative levels were calculated as described under "Experimental Procedures." Graphs are mean ± S.E. from three independent experiments. Statistical significance was assessed using unpaired Student’s t tests (A and C); n.s., not significant.

FIGURE 1. Silencing of Spry2 increases HIF1α and HIF2α protein amounts without altering their mRNA levels. HuH7 cells transfected with control (Cntrl) siRNA or siRNA targeting Spry2 were cultured in normoxia (N) (21% O2) or hypoxia (H) (3% O2) for 8 h (A and B) or 24 h (C and D). Representative Western blots probed for HIF1α (A), HIF2α (B) images from same blot (C), Spry2, and actin (loading control) are shown. Graphs in lower panels show mean ± S.E. of densitometric analysis of HIF1α (A) or HIF2α (B) normalized to actin from three (A) or four (C) independent experiments. Transient levels of HIF1α, HIF2α, and Spry2 were monitored using qRT-PCR, and relative levels were calculated as described under "Experimental Procedures." Graphs are mean ± S.E. from three independent experiments. Statistical significance was assessed using unpaired Student’s t tests (A and C); n.s., not significant.
PCR. As examples of HIF1α-regulated genes, we monitored the mRNA levels of the glucose transporter GLUT1 and the glycolytic enzyme phosphoglycerate kinase 1 (PGK1). Consistent with the changes in protein levels of HIF1α, Spry2 silencing resulted in a significant elevation of GLUT1 and PGK1 mRNA levels (Fig. 3, A and B). A representative blot probed for HIF1α, Spry2, and actin and captured with the Bio-Rad ChemiDoc XRS+ instrument is shown. Graph shows mean ± S.E. of densitometric analysis of HIF1α normalized to actin from three independent experiments.

As a second approach to monitor the ability of Spry2 to modulate HIF1α-elicited transcription, we transfected HuH7 cells with a luciferase reporter plasmid composed of the phosphofructose kinase (PFK) promoter fused to a luciferase reporter gene (PFK-Luc) and a Renilla luciferase plasmid as a control for transfection efficiency. Transcription of the PFK gene and the PFK promoter-Luc construct is regulated by HIF1α but not by HIF2α (58). In these cells, the ability of HIF1α to regulate transcription, as measured by luciferase activity, was elevated 2.5-fold by hypoxia (Fig. 3C). Moreover, silencing of Spry2 further elevated luciferase activity by 63% (Fig. 3C). Silencing of HIF1α, but not HIF2α, abolished the ability of Spry2 targeting siRNA to enhance luciferase activity (Fig. 3C) demonstrating that silencing of Spry2 enhances the transcription of PFK-Luc via changes in HIF1α levels. These data (Fig. 3, A–C) also show that, as reported by others (54, 55, 58, 59), the expressions of GLUT1, PGK1, and PFK-Luc are regulated by HIF1α and not HIF2α.

Recently, erythropoietin (EPO) was identified as an HIF2α-responsive gene (61, 62). Therefore, to determine whether the enhanced HIF2α levels observed upon silencing of Spry2 (Fig.

**FIGURE 2.** Two siRNAs against Spry2 enhance HIF1α protein levels in a variety of cell lines, and ectopic expression of Spry2 decreases HIF1α protein levels. A, HuH7 cells transfected with control (Cntrl) siRNA or a different siRNA targeting Spry2 were cultured in hypoxia (H) (3% O2). Western blots probed for HIF1α, HIF2α, Spry2, and actin (loading control) are shown. B, MCF7, MDA-MB-231, A549, and Hep3B cells transfected with control (–) or one of two different Spry2 siRNAs (1 and 2) were cultured in hypoxia (3% O2). A representative blot probed for HIF1α, Spry2, and actin and captured with the Bio-Rad ChemiDoc XR5+ instrument is shown. Graph shows mean ± S.E. of densitometric analysis of HIF1α normalized to actin from three independent experiments. C, control or HA-Spry2-expressing HeLa cells were cultured in normoxia (N) (21% O2) or hypoxia (H) (3% O2). Representative blot probed for HIF1α, HA-Spry2, and ERK1/2 (loading) is shown. Graph below shows mean ± S.E. of densitometric analysis of HIF1α normalized to actin from four independent experiments. Statistical significance was assessed by unpaired Student’s t test (B and C).
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Sprouty2 Regulates the Stability and Ubiquitylation of HIF1α—The lack of any changes in mRNA levels of HIF1α or HIF2α when expression of Spry2 is silenced in the face of a 2–2.5-fold increase in the levels of the two proteins (Fig. 1) would suggest that Spry2 regulates HIF1α and HIF2α post-translationally. The post-translational regulation of HIF1α and HIF2α involves very similar mechanisms consisting of hydroxylation of two prolyl residues that serve as the binding site for von Hippel-Lindau protein (pVHL) and associated E3 ligase (39, 40). Therefore, using HIF1α as a prototypic member, we first examined whether Spry2 altered the stability of the HIF1α protein. Essentially, HuH7 cells transfected with control siRNA or siRNA against Spry2 were treated with the protein translation inhibitor cycloheximide, and the levels of HIF1α were monitored at different times. As shown in Fig. 4A, silencing of Spry2 increased the half-life of HIF1α from 4.3 to 7.1 min. The major
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Sprouty2 Exists in a Complex with HIF1α and Regulates HIF1α through pVHL-dependent Mechanism—To identify the mechanism by which Spry2 regulates HIF1α ubiquitylation and degradation, we determined whether endogenous Spry2 and HIF1α exist in the same complex. In hypoxic HuH7 cells treated with the proteosomal inhibitor MG132, immunoprecipitates of Spry2 contained endogenous HIF1α (Fig. 5A). Additionally, by immunocytochemistry, we also observed the co-localization of Spry2 and HIF1α, and this co-localization was markedly decreased upon silencing of either HIF1α or Spry2 (Fig. 5B).

The co-localization of Spry2 and HIF1α within intact cells was further confirmed using the proximity ligation assay (PLA) that permits detection of interacting proteins within intact cells (64). As shown in Fig. 5C, the PLA approach also showed that Spry2 and HIF1α are in close proximity, and the PLA signal is markedly diminished when HIF1α is silenced. Interestingly, the data in Fig. 5, B and C, show that Spry2 and HIF1α interact in the nucleus. Although the localization of Spry2 in cytoplasm and membrane ruffles has been previously reported (65, 66), this is the first demonstration of Spry2 being present in the nucleus. Overall, using three different approaches, the data in Fig. 5 demonstrate that endogenous Spry2 and HIF1α exist in a complex.

Previous work from our laboratory has shown that Spry2 is also hydroxylated by PHDs and can bind to pVHL (30). With this in mind and the data demonstrating the existence of endogenous Spry2 in a complex with HIF1α (Fig. 5), we postulated that Spry2 by associating with HIF1α brings more pVHL in proximity of HIF1α, and therefore, it enhances the degradation of HIF1α. To investigate the involvement of pVHL in the ability of Spry2 to regulate HIF1α, HuH7 cells were transfected with two different siRNAs targeting pVHL and the effect of Spry2 silencing on HIF1α protein was monitored. Indeed, as shown previously (Fig. 1A), silencing of Spry2 resulted in a significant increase in HIF1α protein levels without altering pVHL protein levels (Fig. 6A). However, once pVHL is silenced...
with either of the two pVHL targeting siRNAs, the silencing of Spry2 no longer affected HIF1α/HIF2α protein levels (Fig. 6A). These data suggest that Spry2 regulates HIF1α/HIF2α in a pVHL-dependent mechanism.

To further investigate the contribution of pVHL in the ability of Spry2 to regulate HIF1α, we utilized a mutant form of Spry2 that cannot bind pVHL. Previously, using proteomic analyses we showed that Pro-18, Pro-144, and Pro-160 on Spry2 are hydroxylated (30). The substitution of these three Pro residues to Ala abolished the ability of Spry2 to bind pVHL (30). Here, we have designated this 3Pro3Ala substituted form of Spry2 as “3P/3A-Spry2.”

To determine whether the 3P/3A-Spry2 could alter HIF1α protein levels, HEK293T cells were transfected with empty vector, wild-type (WT)-Spry2, or 3P/3A-Spry2, and HIF1α levels were monitored. As expected and shown for HeLa cells (Fig. 2C), the expression of WT-Spry2 resulted in a 50% reduction in HIF1α protein levels compared with empty vector transfected cells (Fig. 6B). Intriguingly, the expression of 3P/3A-Spry2, which cannot bind pVHL, had no significant effect on HIF1α protein levels compared with vector-transfected cells (Fig. 6B). Similar results were observed in HeLa cells stably expressing no Spry2, WT-Spry2, or 3P/3A-Spry2 (Fig. 6C). Once again, HeLa cells expressing WT-Spry2 had a 50% reduction in HIF1α protein levels, although the expression of 3P/3A-Spry2 had no significant effect on HIF1α levels when compared with cells not expressing Spry2 (Fig. 6C). These data provide further evidence that Spry2 regulates HIF1α in a pVHL-dependent manner because when Spry2 cannot bind pVHL, it cannot alter HIF1α protein levels.

To determine whether the ubiquitylation of HIF1α was altered when WT-Spry2 or 3P/3A-Spry2 were overexpressed in HEK293T cells, we transfected these two forms of Spry2 together with FLAG-ubiquitin and HIF1α and monitored HIF1α ubiquitylation after immunoprecipitating HIF1α. As shown in Fig. 6D, ubiquitylation of HIF1α in cells expressing WT-Spry2 was approximately twice as high as that in cells expressing 3P/3A-Spry2.
We reasoned that if Spry2 was bringing more pVHL in proximity to HIF1α/H9251, then in immunoprecipitates of pVHL the amount of HIF1α/H9251 co-immunoprecipitating should be diminished when Spry2 is silenced by siRNA. Indeed, when Spry2 was silenced in HuH7 cells, immunoprecipitates of pVHL contained 50% less HIF1α/H9251 (Fig. 6E). Notably, the protein levels of PHD1, PHD2, PHD3, and pVHL were not significantly altered by the expression of WT-Spry2 or 3P/3A-Spry2 compared with empty vector-transfected cells (Fig. 7A).

3P/3A-Spray2 Can Associate with HIF1α and Inhibit the Phosphorylation of AKT—To address the possibility that the mutation of the three proline residues in 3P/3A-Spray2 abrogated its ability to associate with HIF1α and therefore abolished its ability to alter HIF1α protein levels, we investigated whether the 3P/3A-Spray2 interacted with HIF1α. HEK293T cells were transfected with WT-Spry2 and 3P/3A-Spry2 and placed in hypoxia. The WCL indicates that equal amounts of the two Spry2 constructs were transfected into the cells (Fig. 7B). Because both WT-Spry2 and 3P/3A-Spry2 co-immunoprecipitated with HIF1α (Fig. 7B), the substitution of the three prolines on Spry2 with alanines does not alter the ability of Spry2 to associate with HIF1α.

Spry2 has been shown to inhibit the downstream signaling processes such as phosphorylation and activation of AKT (22, 67, 68). Therefore, we also determined whether the 3P/3A-Spray2 retained its ability to modulate downstream signaling processes.
such as phosphorylation of AKT. Using the HEK293T cell lysate from experiments described in Fig. 6B, the levels of phosphorylation of AKT (pAKT) on Ser-473 were monitored. As expected from our previous findings (69), the expression of WT-Spry2 reduced pAKT content by more than 50%. Likewise, 3P/3A-Spry2 also reduced pAKT levels to a similar extent (Fig. 7C). Thus, 3P/3A-Spry2 retains its ability to inhibit the phosphorylation of AKT. Taken together, these data indicate that 3P/3A-Spry2 only lacks the ability to bind to pVHL (30) and to alter HIF1α/HIF1β levels (Fig. 6B and C) but retains its other functions such as binding to HIF1α and inhibiting AKT phosphorylation (Fig. 7B and C).

WT-Spry2, but Not 3P/3A-Spry2, Regulates HIF1α-sensitive Glucose Uptake—Because 3P/3A-Spry2 does not alter HIF1α protein levels, we reasoned that a biological function that is regulated by HIF1α would be susceptible to modulation by WT-Spry2 but not 3P/3A-Spry2. In this context, it is well established that HIF1α regulates the expression of certain glucose transporters, such as GLUT1 and GLUT3, and the increased expression of these glucose transporters enhances glucose uptake into cells (59, 70). Therefore, in HuH7 and HeLa cells, we monitored HIF1α-sensitive glucose uptake after transfecting either WT- or 3P/3A-Spry2. As shown in Fig. 8A, 2-deoxy-
β-[3H]glucose uptake in HeLa cells exposed to hypoxia was linear over time, and silencing of HIF1α decreases 2-deoxy-β-[3H]glucose uptake by 31%. Because HIF1α does not regulate all isoforms of glucose transporters, silencing of HIF1α only diminishes that portion of the glucose transport that is HIF1α-dependent; in the case of HeLa cells, this is 30% of the total glucose uptake in hypoxia. Here, we refer to this as the HIF1α-sensitive glucose uptake. Moreover, the addition of unlabeled 2-deoxy-β-glucose markedly inhibited 2-deoxy-β-[3H]glucose uptake in the presence and absence of HIF1α demonstrating the specificity of glucose uptake via glucose transporters (Fig. 8A).

For the experimental data presented in Fig. 8, B and C, by monitoring 2-deoxy-β-[3H]glucose uptake with or without HIF1α silencing, we determined the amount of HIF1α-sensitive glucose uptake in cells transfected with vector, WT-Spry2, or 3P/3A-Spry2. In HeLa cells, the HIF1α-sensitive portion of glucose uptake was significantly inhibited (61%) by WT-Spry2, but it was not affected by 3P/3A-Spry2 (Fig. 8B). Similarly, in HuH7 cells, WT-Spry2 reduced HIF1α-sensitive 2-deoxy-β-[3H]glucose uptake by 73%, whereas 3P/3A-Spry2 did not affect it (Fig. 8C). Additionally, in HuH7 cells, total glucose uptake was elevated by ∼3.5-fold upon exposure to hypoxia, and interestingly, the transfection of WT-Spry2 or 3P/3A Spry2 did not alter glucose uptake in cells incubated in normoxia in which HIF1α protein levels would be much lower (data not shown). These findings are consistent with the observations that WT-Spry2, but not 3P/3A-Spry2, regulates HIF1α protein content in cells (Fig. 6, B and C).

Discussion

Sprouty proteins, especially Spry2, working via a number of different mechanisms, have been shown to modulate signaling downstream of receptor tyrosine kinases to inhibit cell proliferation and migration (20, 21, 71, 72). Consistent with these findings, several studies have shown that Spry2 levels are decreased in different forms of cancer, including those of the breast, liver, prostate, and lung, suggesting a potential role for Spry2 as a tumor suppressor (6–11, 17–19). Because decreased protein levels of Spry2 in hepatocellular carcinomas have been associated with poor patient survival and prognosis, it has been suggested that Spry2 levels could be utilized as a prognostic marker (73). However, the role of HIFα subunits, in particular HIF1α, is now well established in promoting tumor survival, growth, and metastasis. Specifically, HIF1α by augmenting the transcription of genes encoding for glucose transporters, GLUT1 and GLUT3, as well as several glycolytic enzymes, provides tumors with a growth advantage in hypoxia (54–60). Hence, although Spry2 may have tumor suppressor functions, HIFα subunits, especially HIF1α, act as tumor promoters (47–49). In this study, using HIF1α as a prototype of HIFα subunits, we have uncovered a novel mechanism for regulation of HIFα protein levels by Spry2. Our findings, for the first time, demonstrate that endogenous Spry2 regulates HIF1α protein levels in a variety of different cell types derived from breast (MCF-7 and MD-MB-231), liver (HuH7 and Hep3B), and lung (A549) cancer in which Spry2 (Fig. 2B) plays a critical role. Additionally, silencing of Spry2 in HEK293T (Fig. 4B) or its ectopic expression in these cells and HeLa cells regulates HIF1α protein levels (Figs. 2C and 6, B and C). These observations establish the generality of our findings. The silencing of endogenous Spry2 elevates HIF1α and HIF2α protein levels (Fig. 1, A and C) and augments their ability to regulate their respective target genes GLUT1, PGK1, and PFK for HIF1α and EPO for HIF2α (Fig. 3, A–D). Consistently, the overexpression of Spry2 (Figs. 2C and 6B) or other Spry isoforms, Spry1, Spry3, and Spry4 (data not shown), decrease HIF1α levels demonstrating the ability of different Spry isoforms to modulate HIF1α levels. Hence, the findings described in this study are also applicable to the other Spry isoforms.

It is well established that pVHL and associated E3 ligase degrades HIF1α and HIF2α under normoxic conditions via a process that involves their hydroxylation by PHDs (41, 42). In hypoxia, the lack of free molecular oxygen reduces, but does not completely inhibit, activities of the PHDs (74, 75). This results in a reduction of pVHL/associated E3 ligase binding to HIFα subunits, and therefore, a decrease in their ubiquitylation and degradation in hypoxia (39, 40). Herein, we demonstrate that Spry2 decreases the amount of HIF1α by increasing its ubiquitylation and degradation even in hypoxic conditions suggesting that Spry2 enhances the proteosomal degradation of HIF1α (Fig. 4). This process requires pVHL because silencing of pVHL abrogated the ability of Spry2 to regulate HIF1α (Fig. 6A).

Furthermore, for the reasons described below, our data suggest that Spry2, which we have previously shown to bind pVHL (30), exists in a complex with HIF1α and thereby brings more pVHL in the proximity of HIF1α to permit its ubiquitylation and degradation. First, using three different approaches, we demonstrate that Spry2 and HIF1α exist in a complex (Fig. 5). Second, the silencing of pVHL expression abrogates the increase in HIF1α levels when Spry2 is silenced, and the expression of WT-Spry2, but not 3P/3A-Spry2 that does not bind pVHL (30), decreases HIF1α levels (Fig. 6, A–C). Notably, 3P/3A-Spry2 is as effective as WT-Spry2 in attenuating AKT phosphorylation (Fig. 7C) and with associated HIF1α (Fig. 7B). Thus, 3P/3A-Spry2 does not lose all of its biological functions. Third, ubiquitylation of HIF1α when WT-Spry2 is overexpressed is enhanced 2-fold as compared to when 3P/3A-Spry2 is overexpressed (Fig. 6D), and immunoprecipitates of pVHL from HuH7 cells in which Spry2 has been silenced contain ∼50% less HIF1α (Fig. 6E). All of this evidence strongly supports the notion that Spry2, by interacting with both pVHL and HIF1α, brings more pVHL in proximity of HIF1α and thereby enhances HIF1α ubiquitylation and degradation. The latter is supported by our data that show silencing of Spry2 diminishes the ubiquitylation of HIF1α (Fig. 4B). Consistent with our proposal that Spry2 acts as a scaffold to bring more pVHL/associated E3 ligase in proximity of HIF1α, unlike WT-Spry2, the 3P/3A-Spry2 that does not bind pVHL also does not inhibit HIF1α-sensitive glucose transport in HeLa and HuH7 cells (Fig. 8). The paradigm we propose fits well with the function of Spry2 as a scaffolding protein, which by interacting with other proteins regulates different signaling pathways and biological functions (20–25).

One of the interesting and novel observations by immunocytochemistry and PLA (Fig. 5, B and C) from our studies is the
Spry2 also inhibits HIF1α transcription of HIF1α could propose that in the studies of Liu expression of Spry2 (90). Hence, in light of our findings, one and ERK1/2 activation. However, miR-21 also decreases the associated decrease in Spry2 levels, which was not monitored in that conditions as those in Fig. 1, silencing of Spry2 in HuH7 cells did not alter ERK1/2 phosphorylation status suggesting that ERK1/2 do not contribute to the elevation of HIF1α levels suggesting AKT activity has no role in the ability of Spry2 to inhibit ERK1/2 phosphorylation. One study by Liu et al. (84) demonstrated that microRNA-21 (miR-21) elevated HIF1α levels due to modest increases in AKT and ERK1/2 activation. However, miR-21 also decreases the expression of Spry2 (90). Hence, in light of our findings, one could propose that in the studies of Liu et al. (84) miR21-mediated decrease in Spry2 levels, which was not monitored in that study, contributes to the elevation in HIF1α protein levels. Our findings that silencing of endogenous Spry2 elevates the transcription of HIF1α-responsive genes (Fig. 3, A–C) and that Spry2 also inhibits HIF1α-sensitive glucose uptake (Fig. 8) implicate Spry2 as a regulator of glycolysis. In this context, Wang et al. (91) showed that expression of a dominant negative Spry2 with AKT resulted in increased membrane levels of GLUT1 and GLUT4 as well as an increase in the glycolysis master regulator pyruvate kinase M2 (PKM2). However, that study did not delineate the mechanism by which Spry2 up-regulated PKM2. Given our findings in this report and the fact that PKM2 transcription and expression are elevated by HIF1α (92), it is tempting to speculate that the up-regulation of PKM2, GLUT1, and GLUT4 by dominant negative Spry2 is due to an elevation of HIF1α levels because dominant negative Spry2 would ablate the ability of endogenous Spry2 to decrease HIF1α levels.

In summary, we showed that Spry2 acts as a scaffold that complexes with HIF1α and pVHL elevating the local concentrations of pVHL to ubiquitylate HIF1α in hypoxia and target it for degradation. The regulation of HIF1α levels by Spry2 also translates into regulation of HIF1α-responsive genes and glucose uptake. Thus, Spry2 also inhibits the expression of glycolytic genes and HIF1α-sensitive glucose uptake. Because glucose uptake and glycolysis are critical in providing survival advantage and promotion of cancer, our data suggest Spry2, in part, may exert its “tumor suppressor” actions by regulating HIF1α protein levels and gene transcription. As a corollary, when Spry2 is decreased or lost in certain forms of cancer, the transcription of HIF1α-regulated genes would be elevated and further promote the progression of the cancer. Overall, these findings reveal a novel mode of action for Spry2 in regulating cellular signaling and biological functions such as glucose uptake.

**Experimental Procedures**

**Chemicals and Reagents—**Cycloheximide and N-ethylmaleimide were purchased from Sigma. MG132 was purchased from Selleck Chem (Houston, TX). All primers, probes, and siRNAs were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The sequences of the various siRNAs used are described in supplemental Table 1A. Antibodies used for different applications and their dilutions are listed in supplemental Table 1B.

**DNA Constructs—**The cloning of human Spry2 cDNA into the pHM6-HA-vector is described elsewhere (93). pHM6-HA-3P/3A-Spry2 was generated using site-directed mutagenesis of the wild-type pHM6-HA-Spry2 to mutate prolines 18, 144, and 160 (30). The cDNA of HIF1α was purchased from Origene and subcloned into pcDNA3 vector using HindIII and NotI restriction sites. pRG-TK was kindly provided by Dr. Neil Clipstone, Loyola University Chicago. pGL2-Pfkfb3/−3566 was kindly provided by Ramon Bartrons, University of Barcelona. PCMVF-3×-FLAG-ubiquitin was kindly provided by Dr. Adrian Marchese, Medical College of Wisconsin. The cloning of FLAG-Spry1 was described previously (29). FLAG-Spry1, FLAG-Spry3, and FLAG-Spry4 were generous gifts from Dr. Graeme Guy, Institute of Molecular and Cell Biology, Singapore. All constructs were verified by sequencing.

**Cell Culture—**HuH7 and Hep3B cells were kindly provided by Dr. Basabi Rana, University of Illinois at Chicago. HuH7 cells were cultured in DMEM/nutrient mixture F-12 50:50 supplemented with HEPES (10 mM), 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Hep3B cells were cultured...
in MEM supplemented with HEPES (10 mM), sodium pyruvate (1 mM), MEM non-essential amino acid solution (1X), 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). HEK293T cells were kindly provided by Dr. Jody Martin, Loyola University, Chicago. HeLa cells were kindly gifted by the late Dr. Jill Lahti, St. Jude Children’s Research Hospital, Memphis, TN. HEK293T and HeLa cells were cultured in high glucose containing DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). MDA-MB-231 and MCF7 cells were kindly provided by Dr. Ajay Rana, University of Illinois at Chicago or Dr. Paula McKeown-Longo, Albany Medical College. MDA-MB-231 and MCF7 cells were cultured in F-12K supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained under normoxic conditions (21% O2) in a water-jacket CO2 incubator (Thermo Fisher Scientific, Waltham, MA) at 37 °C, 5% CO2. Cells were maintained under hypoxic conditions (3% O2) in a Coy Hypoxic Chamber (Grass Lake, MI) at 37 °C, 5% CO2. Media used for hypoxic experiments were pre-equilibrated under hypoxia for 16 h.

Silencing of Endogenous Spry2—Cells (150,000–250,000/35-mm dish) were plated, and the next day they were transfected using Trans-IT TKO (Mirus Bio LLC, Madison, WI) transfection reagent with 20 nm 27-mer (control) mutant Spry2 siRNA containing three ribonucleotide substitutions of the Spry2 siRNA or Spry2 siRNA duplex following the manufacturer’s protocol. The cells were placed in hypoxia the next day, and the media were changed to pre-equilibrated hypoxic media. Cell lysates were collected after either 8 h (for HIF1α studies) or 24 h (for HIF2α studies).

Isolating RNA, cDNA Synthesis, and qRT-PCR—HuH7 cells were plated (250,000/35-mm dish or 80,000/12-well plate) and transfected the following day with control (40 nM), Spry2 (20 nM), or Spry2 + HIF1α, or Spry2 + HIF2α (as described above under “Isolating RNA, cDNA Synthesis, and qRT-PCR”) and 1 μg of pGL2-Pfkfb3/−3566 plus 10 ng of pRG-TK and incubated in normoxia or hypoxia (3% O2) for 24 h. The cells were lysed in Passive Lysis Buffer (Promega, Madison, WI). Luciferase assays were performed using the Promega Dual-Luciferase kit and read on a PHERAStar FS plate reader (BMG Labtech, Cary, NC).

Stability of HIF1α—HuH7 cells (250,000/35-mm dish) were plated and the following day transfected with 20 nM each of control or Spry2 siRNAs as stated above. The cells were placed in hypoxia, and 8 h later cycloheximide (200 μM) was added. The cells were lysed after 0, 5, or 10 min in Laemmli sample buffer. HIF1α, Spry2, ERK1/2, and actin levels were monitored using Western blotting.

Ubiquitylation Assays—HEK293T cells (300,000/60-mm dish) were plated and the following day transfected with 1.25 μg of pJX40-FLAG-ubiquitin and 625 ng of pcDNA3-HIF1α along with either control or Spry2 siRNAs or WT-Spry2 or 3P/3A-Spry2. The following day the cells were placed in hypoxia for 12 h, and 25 μM MG132 was added for an additional 4 h for silencing of Spry2 experiments or 2 h for overexpression experiments. The cells were lysed in denaturing lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% SDS, 1% Nonidet P-40, and 0.5% sodium deoxycholate with the following protease inhibitors: 50 mM NaF, 5 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 150 mM aprotinin, 1.5 mM pepstatin A, 25 μM MG132, 4.7 mM leupeptin, 6.6 mM benzamidine, 8.3 mM sodium pyrophosphate, 5 mM N-ethylmaleimide, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). The samples were sonicated twice for 10 s on ice. Lysate (100 μg) was diluted 1:10 with lysis buffer without SDS and incubated with 3 μg of anti-FLAG-M2, anti-HIF1α, or mouse IgG for 1 h. Protein G beads (Roche Applied Science) (20 μl slurry) were incubated with the lysate for 2 h. The beads were washed three times with lysis buffer with 0.1% SDS. Heating in Laemmli sample buffer eluted the bound proteins, which were then separated by SDS-PAGE, and HIF1α and FLAG-ubiquitin were detected by Western blotting analysis.
Sprouty2 Regulates Hypoxia-inducible Factor-α

Co-immunoprecipitation (Co-IP) of Endogenous Spry2 and HIF1α—HuH7 cells (800,000/60-mm dish) were plated and the following day exposed to hypoxia for 8 h with the proteasomal inhibitor MG132 (25 μM) treatment during the last 4 h. Cells were washed twice with ice-cold PBS, lysed in lysis buffer (as described above except with 1% Triton X-100 instead of SDS, Nonidet P-40, and sodium deoxycholate), and rotated for 30 min at 4 °C. Lysates were centrifuged at 15,000 × g for 15 min, and 500 μg of protein was rotated with 3 μg of anti-N-terminal Spry2 antibody overnight at 4 °C. The next day the lysates were incubated rotating with 30 μl of a slurry of protein G beads (Roche Applied Science) for 2 h at 4 °C. The beads were washed three times in lysis buffer and then heated to 95 °C for 10 min in Laemmli buffer to collect bound proteins, which were analyzed for HIF1α and Spry2 by Western blotting.

ICC and PLA—HuH7 cells were plated in an 8-well chamber slide and the following day transfected with 20 nM each of control or Spry2 siRNAs (ICC only) or control (for HIF1α) or HIF1α siRNAs (ICC and PLA) as stated above. The next day cells were placed in hypoxia for 8 h and then fixed with 3.7% formaldehyde in Pipes, pH 6.8, for 10 min at room temperature, washed, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 10% normal goat serum for 1 h at room temperature (ICC) or the PLA kit (Sigma) blocking solution for 30 min at 37 °C (PLA). Rabbit Spry2 (Rockland, Limerick, PA) (1:400 dilution) and mouse HIF1α (BD Biosciences) (1:50 dilution) were incubated overnight at 4 °C. For ICC, the secondary antibodies of goat anti-rabbit conjugated with Alexa Fluor 488 or goat anti-mouse conjugated to Alexa Fluor 594 (ThermoFisher, Grand Island, NY) (1:500 dilution) were incubated on the slide for 1 h. For PLA (Sigma), the manufacturer’s protocol was followed. Confocal images were obtained with a TCS SP5 laser scanning confocal microscope (Leica, Exton, PA) equipped with DMI6000 inverted microscope with blue diode (405 nm), argon (458/476/496/514 nm), diode pumped solid state (561 nm), and HeNe (633 nm) lasers and a ×63 HCX PL APO A/Blue (numerical aperture, 1.4 oil immersion) objective lens. The software used to capture the images was Leica Application Suite Advanced Fluorescence. For ICC, five fields were taken for each condition. For PLA, 10–15 fields were taken of each condition, and PLA puncta were quantified using ImageJ64 software (National Institutes of Health) using the “analyze particles” function. The ×3 zoomed insets were generated using “Zoom in images and stacks” macro by Gilles Carpentier.

Co-IP of HIF1α with pVHL—HuH7 cells (650,000/60-mm dish) were plated and the next day transfected with 20 nM control or Spry2 siRNAs as stated above. The following day, the cells were exposed to hypoxia for 8 h with proteasomal inhibitor MG132 (10 μM) treatment during the last 4 h. Immunoprecipitation of pVHL was performed using the buffer described under “Ubiquitylation Assays” except the buffer contained 0.1% SDS instead of 1% SDS. The samples were centrifuged at 15,000 rpm for 15 min, and 250 μg of protein was rotated with 3 μg of anti-pVHL (BD Biosciences)-bound bead slurry (15 μl/IP) (Roche Applied Science) overnight at 4 °C. The next day the beads were washed three times with the buffer diluted 1:1 with PBS. The bound proteins were eluted in Laemmli buffer and analyzed using Western blotting for HIF1α and pVHL.

Co-IP of WT-Spry2 and 3P/3A Spry2 with HIF1α—HEK293T cells (500,000/60-mm dish) were plated, and the next day the cells were transfected with 750 ng of pHM6-vector, pHM6-WT-HA-Spry2, or pHM6–3P/3A-HA-Spry2 along with 750 ng of pcDNA3-HIF1α with TransIT-2020 (Mirus Bio LLC, Madison, WI) following the manufacturer’s protocol. The next day the cells were incubated in hypoxia for 16 h. Immunoprecipitation protocol was modified from the two-step lysis method in Ref. 94. Protein G bead slurry (Roche Applied Science) was incubated overnight at 4 °C with 2 μg of antibody (anti-HIF1α, Novus Biologicals, Littleton, CO) or mouse IgG (Sigma) in PBS with 0.5% CHAPS and 5% BSA. The next day the cells were lysed in 500 μl of Triton X-100 hypotonic buffer (94) with the addition of protease and phosphatase inhibitors stated above. The samples were sonicated twice at 15% power for 10 s. NaCl was added to each tube to a final concentration of 420 mM, and the samples were incubated on ice for 1 h. The samples were sonicated again as stated previously and centrifuged at 10,000 × g for 10 min. The supernatant (150 μg) was incubated with antibody-bound beads (either mouse IgG or HIF1α) for 2 h. The samples were washed three times in the hypotonic buffer with 150 mM NaCl. The final wash was removed, and the bound proteins were eluted off the beads by heating in Laemmli sample buffer.

pVHL Silencing—HuH7 cells were plated (250,000/35-mm dish) and transfected with 20 nM each of control or Spry2 siRNAs along with 40 nM each of control or one of two pVHL siRNAs (pVHL siRNA1 or siRNA4). The next day the cells were incubated in hypoxia for 8 h. The cells were lysed in Laemmli sample buffer, and the levels of HIF1α, Spry2, actin, and pVHL were monitored using Western blotting.

Expression of Wild-type (WT) Spry2 and Spry2 P18A/P144A/P160A (3P/3A) Mutant Spry2—HEK293T cells were plated (250,000/35-mm dish) and transfected the next day using TransIT-2020 transfection reagent (Mirus Bio LLC, Madison WI) with 250 ng of pHM6 vector, pHM6–WT-HA-Spry2, or pHM6–3P/3A-HA-Spry2 following the manufacturer’s protocol. After 24 h, the cells were placed in hypoxia for 16 h. The cells were lysed in Laemmli sample buffer and analyzed for HIF1α, HA-Spry2, actin, ERK1/2, pAKT Ser-473, PHD1, PHD2, PHD3, and pVHL levels using Western blotting.

Glucose Uptake Assays—HeLa or HuH7 cells (40,000/well of 24-well plate) were plated and the following day transfected with 20 nM each of control or HIF1α siRNAs and 200 ng each of either pHM6-vector, pHM6–WT-HA-Spry2, or pHM6–3P/3A-HA-Spry2 using TransIT-HeLaMONSTER (Mirus) (HeLa) or TransIT-X2 (Mirus) (HuH7). Quadruplicate sets of wells for each condition were transfected. The next day the cells were placed in the hypoxic chamber, and the media were changed to pre-equilibrated hypoxic media. After 24 h, the cells were washed with Krebs-Ringer HEPES Buffer (KRH) (modified from Cold Spring Harbor Protocols) and then incubated in KRH with 100 μM 2-deoxy-D-[3H]glucose (0.5 μCi/well) for 10 min. One set of duplicate wells was incubated with an additional 20 mM 2-deoxy-D-glucose to compete with 2-deoxy-D-[3H]glucose and determine specific glucose uptake. The cells
were then washed three times in ice-cold KRH and lysed in 0.5 M NaOH with 0.1% Triton X-100. An aliquot (10 μl) of the lysate from each sample was used for protein determination. Another aliquot of the lysate (140 μl) was transferred to a scintillation vial. Each well was washed once with 300 μl of scintillation fluid. The vials were counted on a Beckman Coulter scintillation counter. Data were calculated to represent picomoles of 2-deoxy-D-[3H]glucose uptake/μg of total protein. To determine the HIF1α-specific glucose uptake, the average of the HIF1α siRNA-transfected samples was subtracted from the control siRNA-transfected samples.

Statistical Analysis—All data are represented as means ± S.E. of the mean. Data were analyzed as indicated in the figure legends with two-tailed unpaired t tests, one-way analysis of variance (ANOVA), or two-way ANOVA; p < 0.05 was considered significant. n.s. indicates not significant. All statistical tests were calculated using Prism 6 (GraphPad, La Jolla, CA).

Author Contributions—K. C. H. planned and designed the experiments, collected and interpreted data, and helped write the manuscript. T. B. P. planned and designed the experiments, interpreted data, helped write the manuscript.

Acknowledgment—We thank Dr. Xianlong Gao, Dept. of Cell and Molecular Physiology, Stritch School of Medicine, Loyola University at Chicago, for helpful discussions and suggestions during the progress of this work.

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