DJ-1/PARK7 is an important mediator of hypoxia-induced cellular responses

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In cancer, DJ-1/PARK7 acts as an oncogene that drives Akt-mediated cell survival. Although amplification of DJ-1 has been described in several types of tumors, the mechanistic basis of DJ-1's oncogenic effect remains incompletely understood. A tumor's ability to adapt to hypoxia is absolutely critical for its survival and progression, and this adaptation is largely mediated by the transcription factor HIF1. The stabilization of HIF1 subunits during hypoxia is at least partly dependent on the PI3K/Akt/mTOR pathway. We hypothesized that DJ-1, a positive regulator of Akt when over-expressed, might be involved in regulating HIF1 transcriptional activity under hypoxic conditions. Our results show that loss of DJ-1 in human cell lines and transformed mouse fibroblasts decreases the transcription of a variety of HIF1-responsive genes during hypoxia. Moreover, DJ-1 expression is critical for the Akt and mTOR activities that sustain HIF1 stability. Surprisingly, DJ-1 also regulates the activity of the metabolic sensor AMPK, especially during hypoxia. Finally, DJ-1 appears to protect cells against hypoxia-induced cell death and is required for their adaptation to severe hypoxic stress. Our work positions DJ-1 as an upstream activator of HIF1 function in cancer cells and establishes that DJ-1's oncogenic activity stems from its ability to increase a cell's resistance to hypoxic stress through DJ-1's regulatory effects on mTOR and AMPK. The discovery of these functions of DJ-1 strengthens the case for the development of therapeutics that target DJ-1 activity in cancer cells.

mTOR | AMPK | apoptosis

The microenvironment surrounding a tumor is characterized by regions of fluctuating or chronic hypoxia and nutrient depletion. When tumor cells are exposed to hypoxia, the hypoxia-inducible factor-1 (HIF1) is stabilized. HIF1 is a heterodimer of an oxygen-insensitive HIF1-β subunit and an oxygen-sensitive HIF1-α subunit, whose activation is a multistep process involving HIF1-α stabilization and activation that largely depend on its binding to the von Hippel-Lindau (VHL) protein. When HIF1 is activated, this transcription factor promotes the transcription of many genes, including angiogenic factors, glucose transporters, and glycolytic enzymes (1). Induction of these genes in turn leads to altered cellular metabolism and stimulation of revascularization, which provides the additional blood vessels needed to sustain cellular proliferation. This adaptation by the tumor to growth during hypoxic stress contributes to malignant progression and correlates with a poor clinical outcome in several types of cancers (2, 3). Dissecting the molecular mechanisms that underlie the response of tumor cells to hypoxic stress is therefore of considerable relevance to cancer biology.

The "mammalian target of rapamycin" (mTOR) is a serine/threonine kinase that influences many aspects of cellular physiology, including cell size regulation, cytoskeletal organization, autophagy, mRNA transcription, and protein translation (4). One mechanism by which mTOR controls translation is by directly phosphorylating the key translation regulators p70 ribosomal S6 kinase (p70-S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) (5, 6). Although most translation is down-regulated during hypoxia, mRNAs crucial for surviving under hypoxic conditions, such as those encoding HIF1α/β and vascular endothelial growth factor (VEGF), continue to be translated. Another important function of mTOR is to mediate intracellular signaling downstream of phosphoinositide 3-kinase (PI3K) and Akt. These functions of mTOR may be linked, as PC-3 cells subjected to hypoxia demonstrate increased phosphorylation (and thus activation) of Akt at serine 473 (7). Several other cell types have also been shown to activate the PI3K/Akt pathway in response to hypoxia (8, 9), and interference with Akt reduces the accumulation of HIF1 that occurs under hypoxic condition (10). Taken together, these findings suggest that stimulation of the PI3K/Akt/mTOR pathway leads to up-regulation of HIF1 expression at the level of translation, and that hypoxia should be activating a factor that impinges on the PI3K/Akt/mTOR pathway and promotes resistance to hypoxia.

DJ-1 (CAP1/RS/PARK7) is a molecule that occupies a pivotal position in cellular biology because a loss or gain of its function drives abnormal cellular responses leading either to cell death (in neurodegenerative disease) or unregulated cell survival (in cancer), respectively. With respect to neurodegenerative disease, deletions and loss-of-function mutations in the human PARK7 gene have been associated with autosomal recessive, early onset Parkinson's disease (11–14). With respect to cancer, DJ-1 was first described as an oncogene that cooperates with H-Ras and transforms cells by increasing cell proliferation and resistance to cell cycle arrest (15, 16). DJ-1 is also expressed in many advanced stage ovarian carcinomas (17) and in primary breast and lung cancers where DJ-1 immunoreactivity positively correlates with phosphorylated Akt and poor prognosis (16). In prostate cancers, DJ-1 binds to androgen receptors and is involved in the control of cell death (18).

Over-expression and mRNA knockdown experiments have suggested that DJ-1 promotes cell survival by enhancing Akt phosphorylation and thus inhibition of PTEN function (16). There is also an abundance of literature supporting the notion that DJ-1 promotes cell survival by protecting cells from oxidative stress. DJ-1 over-expression confers resistance to oxidative stress, and a decrease in DJ-1 because of either RNA interference (RNAi) (19) or knockout of the DJ-1 gene enhances cytotoxicity mediated by hydrogen peroxide (H_2O_2) and MPTP (11, 16). DJ-1 is, thus, considered to be an anti-oxidant protein, consistent with its ability to quench reactive oxygen species and to acquire a more acidic isoelectric point following oxidative stress (20, 21).

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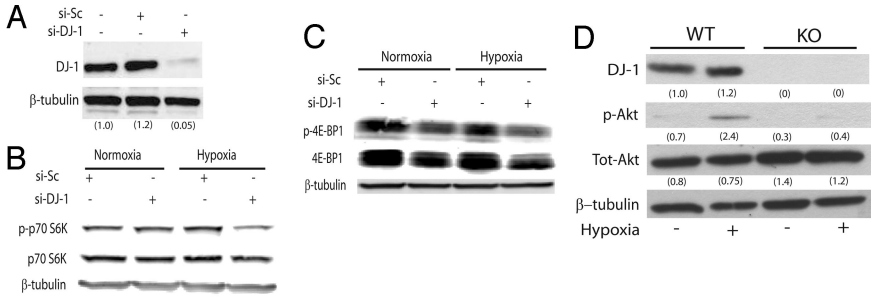

Fig. 1. DJ-1 is required for mTOR activity during normoxia and hypoxia. (*A*) Knockdown confirmation. U2OS cells were transfected with control scrambled siRNA (si-Sc) or DJ-1 siRNA (siDJ-1) and lysates were analyzed 48 h later by Western blotting to detect DJ-1 protein. β-tubulin, loading control. Levels of DJ-1 are normalized against β-tubulin and expressed as fold change relative to base expression determined using control siRNA. (*B* and *C*) Reduced p70-S6K and 4E-BP1 phosphorylation. U2OS cells were transfected with either control or DJ-1 siRNA and treated 48 h later with normoxia (20% O_2) or hypoxia (0.2% O_2) for 8 h. Levels of total and phosphorylated (p-) p70-S6K (*B*) and 4E-BP1 (*C*) were determined by Western blotting. (*D*) Altered Akt phosphorylation. WT and DJ-$1^{-/-}$ MEFs were cultured for 8 h under normoxia or hypoxia and lysates were assayed by Western blotting to detect DJ-1 and Akt phosphorylation. Levels of DJ-1 are normalized against β-tubulin and expressed as fold change relative to base expression determined using WT MEFs under normoxia. Levels of p-Akt, total-Akt are expressed as fold change relative to tubulin. For all Figures, results shown are representative of at least three independent experiments.

All of the above factors led us to consider whether a tumor's resistance to hypoxia could result from DJ-1's oncogenic activity, and whether that activity was due to DJ-1's effect on the PI3K/Akt/mTOR pathway and HIF1 induction. We therefore examined the role of DJ-1 during hypoxia.

Results

DJ-1 Is Required for mTOR Activity During Normoxia and Hypoxia. To assess whether DJ-1 modulates mTOR activity, we performed DJ-1 knockdown experiments in the human osteosarcoma cell line U2OS. Transfection of U2OS cells with siRNA directed against DJ-1 (siDJ-1) achieved >80% efficiency in reducing endogenous DJ-1 protein levels (Fig. 1*A*). Interestingly, knockdown of DJ-1 also led to significantly reduced phosphorylation of the two best-characterized direct substrates of mTOR, p70-S6K (Thr-389, the major rapamycin-sensitive site), and 4E-BP1 (Thr-70, one of several rapamycin-sensitive sites) under both normoxia and hypoxia (Fig. 1*B* and *C*) (5, 22). Quantitation revealed that p70-S6K phosphorylation in hypoxic U2OS cells was decreased by 50% upon treatment with siDJ-1 [see supporting information (SI) Fig. S1*A*]. In addition, siDJ-1 treatment caused a marked reduction in 4E-BP1 phosphorylation in both normoxic and hypoxic U2OS cells, where as only a slight decrease in total 4EBP1 levels were observed at normoxia (Fig. S1*B*).

The serine/threonine kinase Akt is an upstream positive regulator of mTOR (23). When we subjected transformed MEFs derived from DJ-$1^{+/+}$ (wild type; WT) or DJ-$1^{-/-}$ (knock-out; KO) mice (11) to hypoxia, we observed decreased phosphorylation of Akt in hypoxic cells in the absence of DJ-1 (Fig. 1*D*). Our data demonstrate that Akt-mediated regulation of mTOR is at least partly dependent on DJ-1 expression, and support the hypothesis that the oncogenic properties of DJ-1 are likely due to its effects on the PI3K/Akt/mTOR cell survival pathway.

DJ-1 Is Essential for HIF1 Target Gene Expression. The increased stability of the HIF1 protein that occurs during hypoxia is known to be regulated in part by the mTOR pathway (24). Because of DJ-1's effects on the mTOR substrates p70-S6K and 4E-BP1 (Fig. S1), we hypothesized that a lack of DJ-1 might also modify the expression of HIF1. To test this, U2OS cells were cultured for 8 h under hypoxia with or without DJ-1 knockdown, and levels of HIF1α in these cells were evaluated by Western-blot. After treatment, we observed a significant decrease in HIF1α expression in DJ-1 knock downed cells (30% as compared with si-Sc cells), especially the hypoxia inducible form, meaning that DJ-1 is necessary for the full HIF1α induction (Fig. 2*A*). We confirmed this data in WT and DJ-$1^{-/-}$ MEFs that show the same pattern of down-regulation of HIF1α induced-form in absence of DJ-1 after hypoxia exposure (Fig. S2). To examine whether positive regulation of HIF1 by DJ-1 directly affects its transcriptional activity, expression of several HIF1 target genes during hypoxia was tested. To assess the influence of DJ-1 on HIF1 transcriptional activity, we subjected WT and DJ-$1^{-/-}$ MEFs to hypoxia for 4 or 8 h, and assayed VEGF mRNA expression using quantitative real-time RT-PCR. We observed that DJ-1 deficiency led to a decrease in VEGF levels as soon as 4 h after initiation of hypoxia (Fig. 2*B*). To confirm that endogenous DJ-1 is necessary for complete HIF1 activation, U2OS cells transiently expressing siDJ-1 or control siRNA were cultured for 8 h or overnight under hypoxia and mRNA levels of several HIF1 target genes were determined by quantitative real-time RT-PCR. Knockdown of DJ-1 in U2OS cells decreased the mRNA expression of two major angiogenic factors, VEGFα and angiopoietin-2 (Ang-2), by 4-fold and 2-fold, respectively, compared with controls (Figs. 2*C* and S3*A*). We next investigated whether DJ-1 regulates apoptotic (BNIP3), metastatic (PAI-1), and/or various metabolic (HK1, LDH) HIF1 targets. Knockdown of DJ-1 reduced the hypoxia-induced expression of these genes by at least 2-fold (Figs. 2*D* and *E* and S3 *B* and *C*), with a dramatic 12-fold reduction observed for PAI-1 (Fig. 2*D*). Thus, WT levels of endogenous DJ-1 are critical for HIF1 transcriptional activity.

DJ-1 Influences AMPK Expression and Autophagy During Hypoxia. Because DJ-1 was required for the induction of genes necessary to adapt to hypoxic stress, we next analyzed whether DJ-1 also influenced cellular metabolism during hypoxia. The 5′-AMP-activated protein kinase (AMPK) is a metabolic sensor and important for the adaptive responses of energetically stressed cells in the hypoxic and glucose-deprived microenvironments common in solid tumors (25). AMPK is sensitive to hypoxic stress, and the induction of this molecule in hypoxic MEFs shows kinetics that parallel those of HIF1 protein expression (26). This rapid co-induction of AMPK activity and HIF1 stabilization suggests that the AMPK and HIF1 systems operate in parallel during hypoxia. The hypothesis is that they may act in a concerted response to this stress. To determine whether DJ-1 modulates AMPK expression and activation, we investigated AMPK phosphorylation in hypoxic U2OS cells with or without DJ-1 knockdown. We found that phosphorylation of AMPK on Thr-172 (p-AMPK), as well as total AMPK levels, were reduced

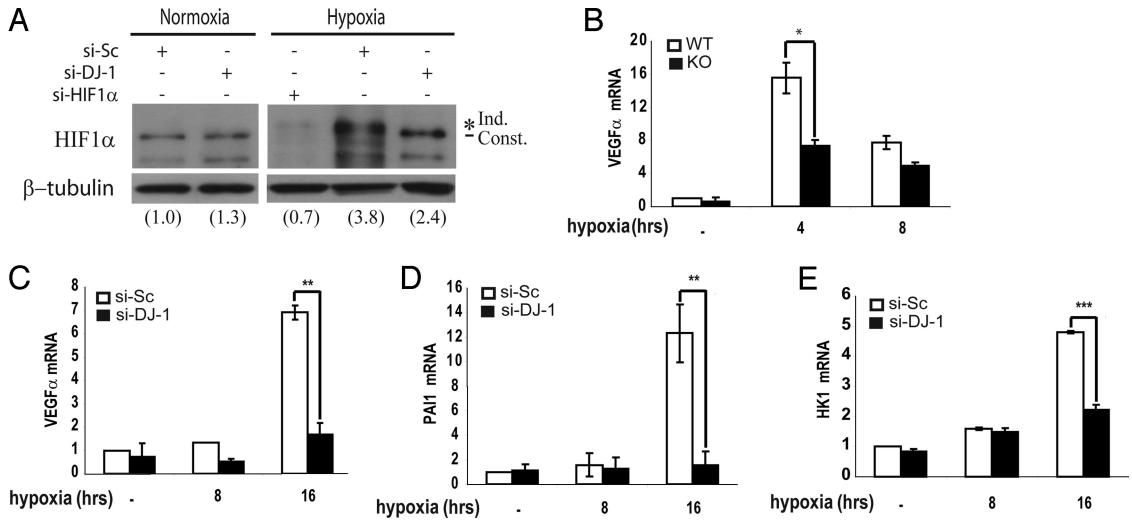

Fig. 2. DJ-1 is essential for HIF1 target gene expression. U2OS cells were transfected with control (si-Sc), DJ-1 siRNA (siDJ-1) or HIF1α siRNA (siHIF1α) and treated 48 h later with normoxia or hypoxia for 8 h. (*A*) Levels of HIF1α were determined in lysates by Western blotting. Levels of HIF1α are normalized against β-tubulin and expressed as fold change relative to base expression determined using control siRNA under normoxia. * and − symbols are representative of induced and constitutive form of HIF1α respectively. DJ-1 WT and KO MEFs (*B*) or U2OS cells (*C–E*) transfected with control (si-Sc) or DJ-1 siRNA (siDJ-1) and treated 48 h later with normoxia or hypoxia, were cultured for the indicated times under 0.2% O_2 and transcription levels of the target genes VEGFα (*B* and *C*), PAI-1 (*D*) and HK1 (*E*) were determined by quantitative real-time RT-PCR analysis. Data shown are the mean fold induction ± SD of mRNA compared to base expression determined using WT MEFs or control siRNA under normoxia. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (Student *t* test) and results shown are representative of at least three independent experiments.

by ≈40% in normoxic U2OS cells lacking DJ-1 (Fig. 3*A*). After 8 h of hypoxia, p-AMPK was increased in U2OS cells expressing DJ-1 but not in cells subjected to DJ-1 knockdown (Fig. 3*B*), this data were confirmed using WT and DJ-$1^{-/-}$ MEFs (data not shown).

Energy depletion within a cell can be defined as an increase in the AMP:ATP ratio. When we examined ATP production in WT and DJ-$1^{-/-}$ MEFs, we found a significant reduction (20%) in the relative amount of total ATP present in hypoxic DJ-$1^{-/-}$ MEFs compared with hypoxic WT MEFs (Fig. 3*C*). Therefore, although ATP was depleted in both WT and DJ-$1^{-/-}$ MEFs in response to hypoxia (as expected), the rate of depletion was markedly greater in the absence of DJ-1. Importantly, both WT and DJ-$1^{-/-}$ MEFs remained viable under the hypoxia conditions used for this experiment (data not shown). In general, an increase in the AMP:ATP ratio within a cell potently induces AMPK phosphorylation. Hence, better AMPK activation might have been expected in siDJ-1-treated U2OS cells compared to controls, which was not the case

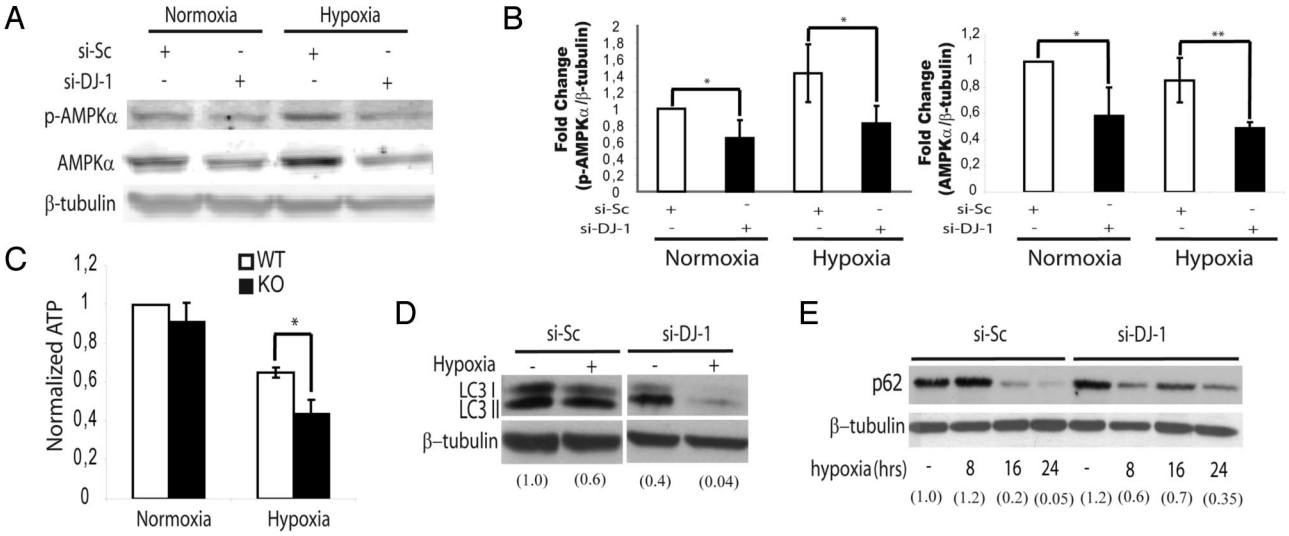

Fig. 3. DJ-1 influences AMPK expression and autophagy during hypoxia. (*A*) Decreased AMPK phosphorylation. U2OS cells were transfected with control (si-Sc) or DJ-1 (siDJ-1) siRNA and treated 48 h later with normoxia or hypoxia for 8 h. Levels of total AMPK and p-AMPK were determined in lysates by Western blotting. (*B*) Quantitation of *A*. Levels of p-AMPK (*Left*) and total AMPK (*Right*) are normalized against β-tubulin and expressed as mean fold induction ± SD compared to base expression determined using control siRNA under normoxia. (*C*) Reduced ATP. WT and DJ-$1^{-/-}$ MEFs were exposed to normoxia or hypoxia for 8 h and total cellular ATP levels were measured in whole cell lysates. Data shown are normalized to the level of total ATP in normoxic WT cells. (*D*) Impaired LC3 processing. U2OS cells treated as in *A* were treated with normoxia or hypoxia for 8 h and analyzed by Western blotting for levels of LC3-I and LC3-II. (*E*) Impaired p62 turnover. U2OS cells were treated as in *A* and subjected to normoxia or hypoxia for the indicated times. Lysates were analyzed by Western blotting for levels of p62. Levels of LC3I (*D*) and p62 (*E*) are normalized against β-tubulin and expressed as fold change relative to base expression determined using control siRNA under normoxia.

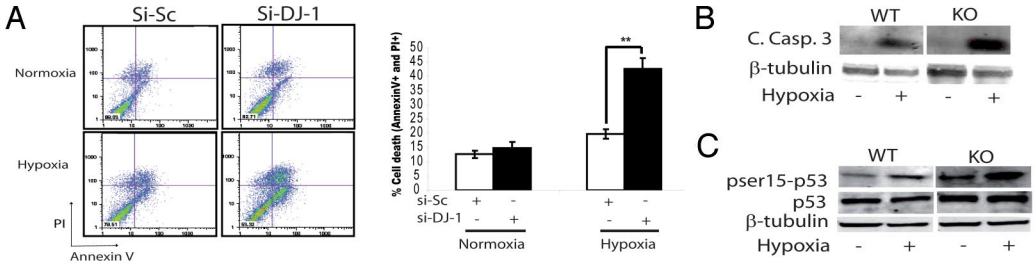

Fig. 4. DJ-1 protects cells from hypoxia-induced apoptosis. (*A*) Increased apoptosis. U2OS cells were transfected with control or DJ-1 siRNA and treated 24 h later with normoxia or hypoxia for 48 h. Floating and attached cells were harvested and the number of annexinV/PI positive cells was measured by flow cytometry. Quadrant (left) and graphical (right) representations of these data are shown. (*B*) Increased caspase3 cleavage. WT and DJ-1$^{-/-}$ MEFs were cultured for 8 h under normoxia or hypoxia and lysates were assayed by Western blotting to detect cleaved caspase3. (*C*) Altered p53 phosphorylation. WT and DJ-1$^{-/-}$ MEFs treated as in (*B*) were analyzed by Western blotting to detect p53 phosphorylation.

(Fig. 3*B*). Thus, AMPK activity is altered in absence of DJ-1, indicative of an inefficient cellular response to hypoxic stress. This inefficiency in AMPK phosphorylation in the absence of DJ-1 may compromise cellular survival.

Hypoxia can activate the autophagic pathway in human cancer cell lines (27). To determine whether DJ-1 expression influences hypoxia-induced autophagy, we analyzed the processing of the microtubule-associated protein light chain 3 (LC3-I, the mammalian homologue of the yeast Atg8 protein). During autophagy, LC3-I undergoes cleavage and lipidation to yield LC3-II, and quantitation of LC3-I and LC3-II levels in a culture gives a good measurement of the degree of autophagy (28). We subjected U2OS cells expressing either siDJ-1 or control siRNA to hypoxia and measured LC3-I and LC3-II levels in these cells. Surprisingly, LC3 processing was enhanced in DJ-1 knockdown cells compared with control cells at normoxia. After 8 h of hypoxia, LC3 processing increased in control cells as expected, whereas both LC3-I and LC3-II were missing in DJ-1 knockdown cells (Fig. 3*D*). Thus, in cells lacking DJ-1, early stages of autophagy occur spontaneously without any treatment, and there appears to be a defect in the stability of LC3 as fast clearance of LC3-I and LC3-II is observed after hypoxia exposure. However, the question remains whether the autophagic process is fully accomplished after DJ-1 knockdown.

Sequestosome 1 (SQSTM1/p62) is a multifunctional signaling adaptor protein that controls a variety of cellular functions, including the recognition of protein aggregates for autophagic clearance. Moreover, p62 interaction with LC3 is required for the degradation of polyubiquitylated protein aggregates by autophagy (29). Proper turnover of p62 by autophagy is required to avoid excessive aggregate formation (30), and hypoxia is an activator of autophagy-associated p62 clearance (31). We decided to investigate whether DJ-1 plays a role in p62 turnover. U2OS cells transfected with either siDJ-1 or control siRNA were exposed to hypoxia for 24 h and levels of p62 in these cells were evaluated by Western blot. Cells expressing control siRNA showed a dramatic reduction in p62 in response to 16 h of hypoxia, but significant levels of p62 persisted in hypoxic siDJ-1-expressing U2OS cells (Fig. 3*E*). These results indicate that WT levels of DJ-1 expression are required for proper turnover of p62 by hypoxia-induced autophagy. Thus, we can conclude that without DJ-1 autophagy in response to lowered oxygen availability is partly impaired as it is not accompanied by p62 down-regulation.

DJ-1 Protects Cells from Hypoxia-Induced Apoptosis. Hypoxia confers protection against apoptosis via PI3K/Akt pathway in cancer cells (32). We therefore investigated whether DJ-1 inhibition affected the ability of U2OS cells to survive in an oxygen-deprived environment. U2OS cells expressing either siDJ-1 or control siRNA were subjected to hypoxia for 48 h and apoptosis was assessed by annexinV-PI staining and flow cytometry. We found that cells expressing normal levels of DJ-1 had a survival advantage over DJ-1 knockdown cells such that the latter displayed double the rate of hypoxia-induced cell death (Fig. 4*A*). We also assayed caspase3 cleavage in hypoxic WT and DJ-1$^{-/-}$ MEFs and again saw a protective effect mediated by DJ-1 expression, in that DJ-1$^{-/-}$ MEFs showed significantly more caspase3 cleavage (20-fold increase, hypoxia vs. normoxia) than did WT MEFs (7-fold increase, hypoxia vs. normoxia) (Figs. 4*B* and S4*A*). These results suggest that, under hypoxia, DJ-1 expression prevents caspase3 cleavage and allows a cell to resist apoptosis.

There is a great deal of evidence suggesting that severe hypoxia leads to an accumulation of p53 in cells, and that this excess p53 may promote HIF1 degradation by facilitating the formation of a tertiary complex between HIF1, p53 and Mdm2 (33, 34). The induction of p53 during severe hypoxia results in up-regulation of PUMA and Bad, which then initiate the intrinsic apoptotic program (35). To determine whether DJ-1 had any effect on p53, we analyzed p53 expression levels in WT and DJ-1$^{-/-}$ MEFs during hypoxia. In DJ-1$^{-/-}$ MEFs, hypoxia treatment strongly increased the phosphorylation of p53 at Ser-15, one of several phosphorylation sites that regulate p53 transcriptional activity (Figs. 4*C* and S4*B*). Total p53 was not increased. In contrast, p53 phosphorylation was not that elevated in hypoxic WT MEFs (Fig. 4*C*). Taken together, our results indicate that DJ-1 regulates p53 induction and that DJ-1 depletion renders cells more susceptible to hypoxia-induced death. It is thus possible that DJ-1 acts as a negative regulator of p53-dependent pro-apoptotic gene expression programs that are activated under hypoxia.

Discussion

It is now well established that Akt and HIF1 are both involved in processes, such as angiogenesis, glucose uptake, and glycolysis that support tumor growth (36). Much effort has been expended on elucidating whether the PI3K/Akt/mTOR pathway is the key activator of HIF1 that enables tumor cells to adapt to hypoxic conditions. The current literature strongly suggests that Akt-mediated activation of HIF1 under hypoxia is not universal but depends on the cell types and experimental systems used to examine this event. Several studies have identified PI3K and Akt as important elements in the induction of HIF1 protein and activity during hypoxia. For example, PI3K, Akt, and HIF1 are all required for the increased VEGF expression associated with hypoxia, and this VEGF induction is negatively regulated by PTEN (8). The PI3K/Akt/PTEN pathway also regulates HIF1 activity through inhibition of FOXO3A (37, 38), and impairment of PI3K activity prevents hypoxia-induced, NF-κB-dependent activation of HIF1 (39). However, other studies have shown that forced activation of the PI3K/Akt pathway does not affect HIF1

transcriptional activity under normoxic or hypoxic conditions, and HIF1 and Akt can independently promote tumor growth and angiogenesis *in vivo* (40). Because DJ-1 is a potent activator of the PI3K/Akt pathway in oncogenic situations, we have investigated whether DJ-1 affects HIF1 protein levels and its transcriptional activity. We have demonstrated, in two different cell types (U2OS cells and transformed MEFs), that a lack of DJ-1 alters the expression of the HIF1α-induced form and impairs the transcription of many classical HIF1 target genes involved in angiogenesis, glucose metabolism, and apoptosis. As HIF1 transactivates many genes known to be affected by Akt, we had expected that DJ-1 would modulate some of them. However, it was surprising that the expression of all HIF1 target genes examined was DJ-1 dependent.

The rapamycin-sensitive functions of mTOR are not essential for the initiation of HIF1 induction in hypoxic cells but are needed for maximal HIF1 protein expression as well as for optimal HIF1-dependent gene expression under hypoxic conditions. We therefore examined whether DJ-1 could modulate the activity of the mTOR pathway during hypoxia. Our data revealed that knockdown of DJ-1 altered the phosphorylation status of the mTOR targets p70-S6K and 4E-BP1. These findings are in line with *in vivo* data showing that Akt-dependent induction of HIF1 activity is entirely mTOR-dependent (41). Although this notion remains somewhat controversial, our results support a scenario in which hypoxia-induced activation of HIF1 requires optimal DJ-1 expression, and DJ-1 acts through Akt and the mTOR pathway.

Our study has also revealed that, under hypoxic conditions, the metabolic sensor AMPK is rapidly activated in DJ-1-expressing cells but not in DJ-1 knockdown cells. These data indicate that DJ-1 is part of the concerted and complex cellular response to hypoxia that involves AMPK and HIF1, and that the effector functions of these molecules are highly dependent on DJ-1 expression. DJ-1 also appears to play a role in autophagy, a process thought to maintain sufficient ATP during periods of energy limitation and to ensure the functionality of vital cellular processes such as transcription and protein synthesis under conditions of limited oxygen availability. The lack of correct LC3 processing and p62 clearance in DJ-1 knockdown cells suggests a positive role for DJ-1 in the step by step process of the hypoxic autophagy. Lastly, our flow cytometric analyses showed that, although hypoxia had no effect on cells expressing DJ-1, knockdown of DJ-1 expression significantly sensitized hypoxic cells to apoptosis. These data were consistent with the increased caspase3 cleavage and p53 Ser-15 phosphorylation we observed in DJ-1$^{-/-}$ MEFs. Moreover, our findings point to an association between loss of DJ-1 function and increased p53-mediated apoptosis under hypoxia.

Interestingly, Pisani *et al.* (42), have demonstrated that DJ-1-deficient dopaminergic neurons show enhanced sensitivity to energy metabolism impairment when they assess the responses of dopaminergic cells to combined oxygen and glucose deprivation. This appears of particular interest because other authors reported that even an early stage of Parkinson's disease causes a subnormal response to hypoxia (43), and DJ-1, whose gene mutations lead to an inherited form of early-onset parkinsonism, is mainly involved in dopaminergic neuronal function in this pathology (11). Therefore, it would make sense to consider that DJ-1 may serve to protect not only cancer cells but also neuronal cells from hypoxia induced cell death. Hence, our work may provide an opportunity to gain further insights into DJ-1 function in the altered neuronal cell response to hypoxia occurring during the early onset Parkinson's disease. In this line, Aleyasin *et al.* (44), already demonstrated that DJ-1 is central to the ability of the brain to respond to stroke (interruption of blood supply due to ischemia or hemorrhagy) as its deficiency sensitizes brains to ischemic damage *in vivo*, whereas DJ-1 expression does the converse. These data support the hypothesis that Parkinson's disease patients with DJ-1 deficiency could be more sensitive to other neuropathologies such as stroke.

In conclusion, our results provide a more comprehensive picture of the role of DJ-1 in tumor progression, and suggest that DJ-1's oncogenic properties are due to its ability to increase HIF1 transcriptional activity and thus protect cells from hypoxia-induced apoptosis. Furthermore, we have shown that this effect involves links between DJ-1, Akt activation, mTOR activation, HIF1 activation and inhibition of p53-mediated apoptosis. Given that most cancer cells lose the ability to undergo hypoxia-induced cell death, our work implies that the pathological elevations of DJ-1 found in breast cancer and lung cancer cells maintain tumor cell survival under hypoxic conditions. However, it remains possible that the ability of DJ-1 to modulate HIF1 activity also relies on its capacity to interact with other factors (such as VHL) that influence HIF1 (45). Further definition of the pathways activated during the cellular response to hypoxia will help us to better understand the oncogenic role of DJ-1.

Materials and Methods

Flow Cytometric Snalysis. Cell death assays were performed using Annexin V-APC and PI staining (BD PharMingen). Data were analyzed using a FACS-Calibur (BD Biosciences), CellQuest Pro (BD Biosciences) and FlowJo 7.1.1 software (Tree Star).

Measurement of ATP Production. Quantitation of the ATP present in WT and DJ-1$^{-/-}$ MEFs was carried out using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Results were first normalized against cell viability as determined by the Cell Titer96 Aqueous One Solution Cell Proliferation Assay (Promega), and then expressed relative to ATP production in normoxic WT MEFs.

Statistical Analysis. Data were analyzed by the Student *t* test. Data are presented as mean $\pm$ SD, and values of $P < 0.05$ were considered statistically significant.

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