Negative feedback control of HIF-1 through REDD1-regulated ROS suppresses tumorigenesis

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The HIF family of hypoxia-inducible transcription factors are key mediators of the physiologic response to hypoxia, whose dysregulation promotes tumorigenesis. One important HIF-1 effector is the REDD1 protein, which is induced by HIF-1 and which functions as an essential regulator of TOR complex 1 (TORC1) activity in *Drosophila* and mammalian cells. Here we demonstrate a negative feedback loop for regulation of HIF-1 by REDD1, which plays a key role in tumor suppression. Genetic loss of *REDD1* dramatically increases HIF-1 levels and HIF-regulated target gene expression in vitro and confers tumorigenicity in vivo. Increased HIF-1 in *REDD1*$^{-/-}$ cells induces a shift to glycolytic metabolism and provides a growth advantage under hypoxic conditions, and HIF-1 knockdown abrogates this advantage and suppresses tumorigenesis. Surprisingly, however, HIF-1 up-regulation in *REDD1*$^{-/-}$ cells is largely independent of mTORC1 activity. Instead, loss of REDD1 induces HIF-1 stabilization and tumorigenesis through a reactive oxygen species (ROS) -dependent mechanism. *REDD1*$^{-/-}$ cells demonstrate a substantial elevation of mitochondrial ROS, and antioxidant treatment is sufficient to normalize HIF-1 levels and inhibit REDD1-dependent tumor formation. REDD1 likely functions as a direct regulator of mitochondrial metabolism, as endogenous REDD1 localizes to the mitochondria, and this localization is required for REDD1 to reduce ROS production. Finally, human primary breast cancers that have silenced REDD1 exhibit evidence of HIF activation. Together, these findings uncover a specific genetic mechanism for HIF induction through loss of REDD1. Furthermore, they define REDD1 as a key metabolic regulator that suppresses tumorigenesis through distinct effects on mTORC1 activity and mitochondrial function.

hypoxia | mTOR | mitochondria | breast cancer | tuberous sclerosis

Control of cellular metabolism plays an important role in human tumorigenesis. Nascent tumor cells must survive a variety of environmental stresses, including hypoxia and energy stress, to allow tumor progression (1, 2). A key mediator of these metabolic adaptations is the hypoxia-inducible factor HIF. HIF is a heterodimeric transcription factor whose activity is induced in response to hypoxia and which regulates genes that mediate a variety of hypoxia-adaptive functions including the shift to glycolytic metabolism, enhancement of angiogenesis, and suppression of oxidative phosphorylation (3–5). The HIF family includes three HIFα subunits (HIF-1α, HIF-2α, and HIF-3α) and a common HIF-1β subunit (also known as ARNT). The key role of HIF in human tumorigenesis is underscored by von Hippel–Lindau (VHL) tumor suppressor syndrome, which results from germline mutations in *VHL*, a gene encoding a subunit of a ubiquitin ligase complex which targets HIFα subunits for oxygen-dependent degradation (6). Other pathways may contribute to HIF dysregulation in different cancer settings, as recent work has demonstrated an important role for aberrant HIF up-regulation in promoting tumorigenesis in prostate and other cancers downstream of the PI3K-mammalian TOR complex 1 (mTORC1) pathway (7).

An additional mechanism for HIF regulation is through reactive oxygen species (ROS). Hypoxia is known to induce a burst of mitochondrial ROS which has been demonstrated by multiple groups to contribute to HIFα stabilization under hypoxic conditions (8–10). The precise mechanisms of this effect are being intensively investigated (11). A specific role for ROS in promoting HIFα stabilization in cancer is suggested by work demonstrating that the ability of antioxidants to suppress tumorigenesis in some model systems is mediated through their ability to inhibit HIF (12). As noted above, HIF itself has been shown to suppress oxidative phosphorylation and ROS production (3, 4), suggesting the possibility of a negative feedback loop for HIF regulation by ROS. Nevertheless, a specific genetic mechanism for such a ROS-HIF pathway in human cancer has not been demonstrated.

REDD1 (also known as RTP801, DDIT4, and Dig1) was initially identified as a hypoxia-regulated HIF-1 target gene involved in regulation of cell survival (13). Subsequent genetic studies demonstrated a major function of REDD1 and its orthologs as a hypoxia-induced regulator of TORC1 activity both in *Drosophila* and mammalian cells (14, 15). Thus, in *REDD1*$^{-/-}$ cells, mTORC1 activity is not appropriately suppressed in response to hypoxia (14, 16). The mechanism of this effect involves the ability of hypoxia-induced REDD1 to activate the tuberous sclerosis tumor suppressor complex TSC1/2, an upstream inhibitor of mTORC1, by titrating away inhibitory 14-3-3 proteins from TSC2 (16). Multiple studies have also implicated REDD1 in regulation of ROS (13, 17), yet the role and significance of REDD1-regulated ROS has not been clear.

A growing body of data links REDD1 to tumor suppression. First, REDD1 has been demonstrated to contribute to apoptotic cell death in multiple contexts, arguing for a potential role for REDD1 in tumor cell survival (13, 18). Second, REDD1 has been implicated as a possible contributor to the tumor-suppressive effect of Foxo transcription factors as both a direct (19) and indirect (18) Foxo transcriptional target. Third, genetic ablation of REDD1 potentiates proliferation and anchorage-independent growth selectively under hypoxic conditions, and potently enhances tumorigenic growth in a model system (16). Finally, down-regulation of REDD1 expression is observed in a subset of human cancers (16). It is unknown, however, which functional properties of REDD1 contribute to its potential role in tumor suppression. Using a genetic model, we have uncovered a specific mTORC1-independent mechanism for REDD1-mediated tumor

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suppression. These studies demonstrate that REDD1 inactivation induces ROS dysregulation and consequent HIF-1α induction that promotes tumorigenesis.

Results

Up-Regulation of HIF-1α Protein and Increased Glycolytic Metabolism in *REDD1*$^{-/-}$ Cells. REDD1 deletion dramatically enhances tumorigenesis through an unknown mechanism in immortalized mouse embryonic fibroblasts (MEFs) expressing activated (myristoylated) AKT (myr-AKT MEFs) (16). We first addressed the requirement for AKT in this process by testing the tumorigenicity of *REDD1*$^{-/-}$ or wild-type immortalized MEFs expressing a control vector (vector MEFs) (Fig. 1*A*). As expected, wild-type vector MEFs never formed tumors, whereas the matched *REDD1*$^{-/-}$ vector MEFs formed large, rapidly growing tumors in 100% of mice (Fig. 1*A*). Thus, loss of REDD1 in this context induces a hypoxia-dependent increase in proliferation and anchorage-independent growth in vitro (16), and is sufficient to confer tumorigenicity in vivo. Treatment of *REDD1*$^{-/-}$ tumors with rapamycin, a potent mTORC1 inhibitor, substantially reduces tumor growth in this model, consistent with the view that REDD1 loss mediates its effect in vivo at least in part through dysregulation of mTORC1 activity (Fig. S1*A*) (20).

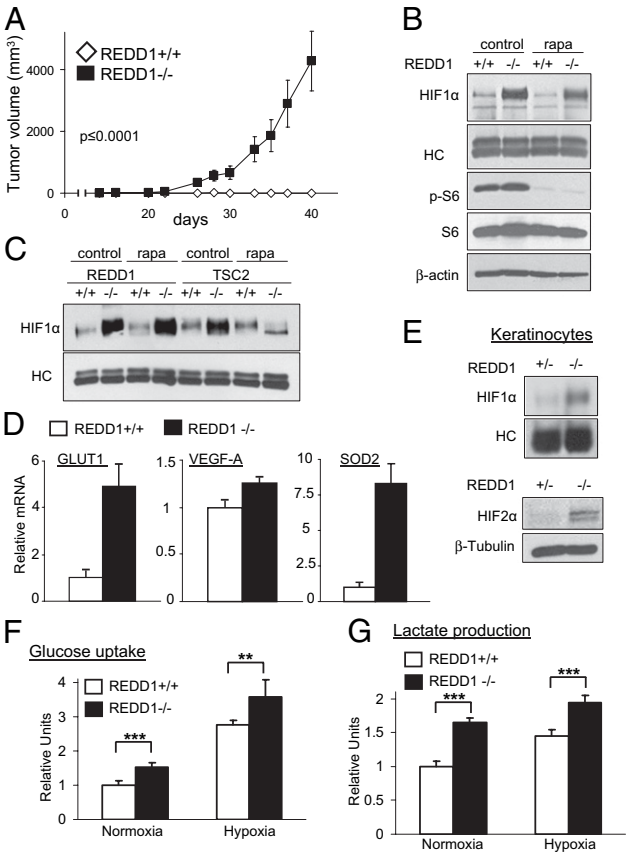

Fig. 1. REDD1 loss confers tumorigenicity and activates a HIF transcriptional program. (*A*) *REDD1*$^{-/-}$ cells are tumorigenic. Immortalized MEFs were transduced with a control retroviral vector, and then injected (4×10^6 cells) into the flanks of nude mice (n = 6 per genotype). P value by repeated-measures ANOVA. (*B*) Relative overexpression of HIF-1α protein in tumorigenic *REDD1*$^{-/-}$ MEFs is not affected by rapamycin (50 nM, 24 h). (*Upper*) HIF-1α was detected by immunoprecipitation (IP)/western analysis under normoxia. Immunoglobulin heavy chain (HC) is shown as a loading control. (*Lower*) Western analysis for phosphorylated S6 (P-S6, S235/236) indicates mTORC1 activity. (*C*) Rapamycin (50 nM, 24 h) abolishes elevated HIF-1α expression in *TSC2*$^{-/-}$ MEFs. Cells were treated under hypoxia (1% O_2) before IP/western analysis. (*D*) Induction of HIF-1α target genes in *REDD1*$^{-/-}$ MEFs described in *A*, assessed by quantitative real-time RT-PCR (qRT-PCR). (*E*) Increased HIF-1α and HIF-2α in *REDD1*$^{-/-}$ immortalized primary keratinocytes exposed to hypoxia (1% O_2, 4 h), followed by IP/Western (*Upper*) or Western (*Lower*) analysis. (*F*) Increased glucose uptake in *REDD1*$^{-/-}$ MEFs. Cells were cultured for 72 h in normoxia or hypoxia (1% O_2), followed by direct measurement of residual glucose in the medium (***P < 0.001; **P < 0.01 by Student's t test). (*G*) Increased lactate production in *REDD1*$^{-/-}$ MEFs cultured as in *F* (***P < 0.001).

Expression profiling of the respective wild-type and highly tumorigenic *REDD1*$^{-/-}$ MEF populations, followed by gene set enrichment analysis (21), identified a prominent gene expression signature that was associated with glycolysis. In particular, many genes directly regulated by HIF-1α were up-regulated in tumorigenic *REDD1*$^{-/-}$ cells compared with matched wild-type cells (Fig. S1*B*). Consistent with this finding, HIF-1α protein levels were dramatically elevated in these *REDD1*$^{-/-}$ cells versus the matched wild-type cells, under both normoxia and hypoxia (Fig. 1 *B* and *C*). Of note, levels of HIF-2α mRNA were more than 50-fold lower than those of HIF-1α in these cells (Fig. S1*C*). HIF-1α dysregulation in tumorigenic MEFs was associated with substantial up-regulation of multiple direct HIF-1α target genes (Fig. 1*D*). Furthermore, HIF dysregulation induced by REDD1 also occurred in epithelial cells, as *REDD1*$^{-/-}$ primary immortalized murine keratinocytes exhibited up-regulation of endogenous HIF-1α and HIF-2α compared with their wild-type counterparts (Fig. 1*E*).

We initially hypothesized that elevated HIF-1α levels in tumorigenic *REDD1*$^{-/-}$ MEFs might be attributed to mTORC1 dysregulation. Surprisingly, however, rapamycin did not affect the relative overexpression of HIF-1α in the absence of REDD1, even though it suppressed mTORC1 activity and modestly down-regulated HIF-1α in both *REDD1*$^{-/-}$ and wild-type cells (Fig. 1*B*). Additionally, rapamycin had little or no effect on the mRNA for HIF-1α in tumorigenic *REDD1*$^{-/-}$ cells (Fig. S1*D*). Of note, the situation was quite different in *TSC2*$^{-/-}$ cells, which exhibit increased mTORC1 activity and elevated HIF-1α protein and mRNA levels that can be fully suppressed to baseline (wild-type) levels by the addition of rapamycin (Fig. 1*C* and Fig. S1 *D* and *E*). Thus, unlike in *TSC2*$^{-/-}$ cells, mTORC1 activity does not account for the dysregulated expression of HIF-1α in tumorigenic *REDD1*$^{-/-}$ cells.

Coincident with HIF-1α up-regulation, glucose uptake was substantially increased in tumorigenic *REDD1*$^{-/-}$ cells compared with the matched nontumorigenic wild-type cells, under both hypoxic and normoxic conditions (Fig. 1*F* and Fig. S1*F*). Additionally, *REDD1*$^{-/-}$ cells exhibited a marked increase in lactate production under both normoxic and hypoxic conditions (Fig. 1*G*). Together, these findings demonstrate that increased HIF-1α levels and a shift to glycolytic metabolism accompany tumorigenesis resulting from REDD1 loss.

Requirement for HIF-1α in Murine and Human Tumorigenesis Induced by REDD1 Loss. These data prompted us to test directly whether HIF-1α is an important contributor to tumorigenesis resulting from REDD1 loss. Therefore, we inhibited HIF-1α expression in tumorigenic *REDD1*$^{-/-}$ cells by stable expression of a HIF-1α-directed lentiviral shRNA construct, and compared the effects with expression of a control lentivirus. This approach markedly reduced endogenous HIF-1α protein levels (to approximately the level observed in wild-type cells) (Fig. 2*A*), and also decreased endogenous HIF-1α mRNA and target gene expression in these cells (Fig. S2). Under normoxic conditions, HIF-1α-ablated cells did not show significant differences in cell proliferation or survival in vitro, but under hypoxia the proliferation of these cells was significantly impaired compared with control cells (Fig. 2*B*). To test the contribution of HIF-1α to tumorigenesis in vivo, we injected either the HIF-1α shRNA-expressing or control cells into immunodeficient mice. Inhibition of HIF-1α in this setting substantially suppressed tumorigenesis and was associated with down-regulation of HIF-1α target genes in vivo (Fig. 2 *C*–*E*).

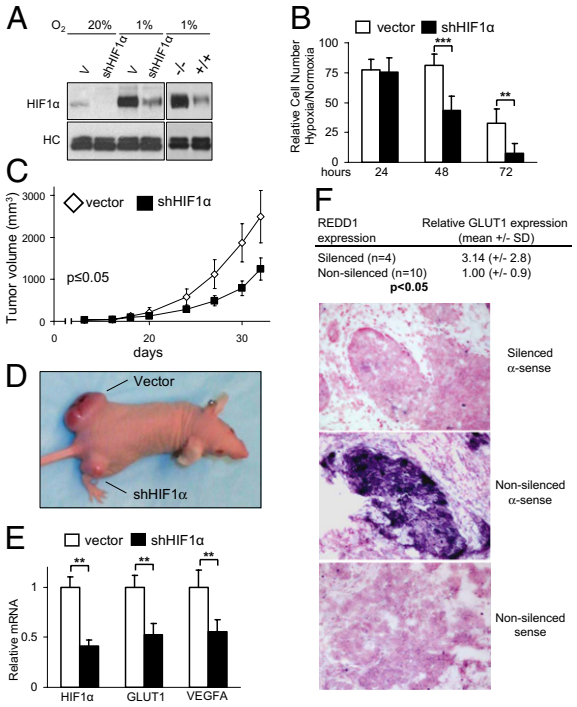

Fig. 2. HIF-1α drives tumorigenesis induced by loss of REDD1. (*A*) Knockdown of HIF-1α in tumorigenic *REDD1*$^{-/-}$ MEFs by lentiviral HIF-1α-directed shRNA (shHIF-1α) or control vector (V), assessed by IP/Western analysis. Right lanes: HIF-1α levels postknockdown approximate those in wild-type cells (compare lanes 3 and 4, 5 and 6). Ig heavy chain (HC) serves as a loading control. (*B*) HIF-1α knockdown decreases proliferation of *REDD1*$^{-/-}$ cells under hypoxia. Equal numbers of the indicated *REDD1*$^{-/-}$ MEFs were plated in normoxia or hypoxia (1% O_2), and cell numbers were determined at the times shown (***$P < 0.001$; **$P < 0.01$). (*C*) HIF-1α knockdown inhibits tumorigenesis of *REDD1*$^{-/-}$ cells. Vector or HIF-1α knockdown (shHIF-1α) *REDD1*$^{-/-}$ MEFs (4×10^6 cells) were injected into opposite flanks of nude mice ($n = 6$). *P* value by repeated-measures ANOVA. (*D*) Representative photograph, day 30. (*E*) Down-regulation of HIF-1α and its target genes by HIF-1α knockdown in *REDD1*$^{-/-}$ tumors. Equal microgram amounts of RNA from tumors generated in *C* were pooled for analysis by qRT-PCR (**$P < 0.01$). (*F*) Up-regulation of Glut-1 in primary human tumors that have silenced REDD1. (*Upper*) Frozen primary microdissected breast cancer specimens were analyzed for REDD1 and Glut-1 mRNA by qRT-PCR, and the mean Glut-1 expression was compared in tumors with silenced versus nonsilenced REDD1 (see text and *Methods*). *P* value by Student's *t* test. (*Lower*) RNA in situ hybridization for REDD1 (α-sense probe, blue staining) in representative breast tumors. Sense probe is shown as a specificity control.

Thus, a HIF-1α-dependent program is an essential driving factor in tumorigenesis resulting from REDD1 loss.

To determine whether these observations were relevant in human tumorigenesis, we examined the relationship between REDD1 and HIF target gene expression in primary human breast carcinomas. We previously demonstrated that a subset of breast carcinomas exhibit silencing of REDD1 expression compared with normal epithelia (16). We therefore asked whether expression of the HIF effector gene Glut-1 was up-regulated in breast carcinomas that had silenced REDD1 compared with those with nonsilenced REDD1. To avoid confounding effects of stromal and other cells, we examined a cohort in which malignant cells from the primary tumor were isolated using laser-capture microdissection (22), and then confirmed REDD1 expression by RNA in situ hybridization in the original tumor specimens (Fig. 2*F*). As predicted by our mouse model, tumors with silenced REDD1 exhibited a statistically significant elevation of Glut-1 expression compared with tumors with nonsilenced REDD1 (Fig. 2*F*). These data support the relevance of REDD1-dependent regulation of HIF activity in human tumorigenesis.

REDD1 Regulation of HIF-1α Stability. The above data imply the existence of a negative feedback pathway whereby REDD1, which is induced by HIF-1α, functions to control HIF levels through an mTORC1-independent pathway and thereby suppress tumorigenic growth. To elucidate this pathway in detail, we first demonstrated that HIF-1α dysregulation was a specific consequence of REDD1 loss, as reconstitution of REDD1 into these *REDD1*$^{-/-}$ MEFs substantially reversed high-level expression of HIF-1α target genes and proteins (Fig. 3*A* and Fig. S3).

We then focused on potential translational or posttranslational mechanisms for HIF-1α dysregulation in *REDD1*$^{-/-}$ cells, because the small increase in HIF-1α mRNA did not account for the large difference in protein expression in these cells. By pulse labeling, we demonstrated that there is essentially no difference in the synthesis rate of HIF-1α in the absence of REDD1 (Fig. 3*B*). To test the possibility that HIF-1α was stabilized preferentially in *REDD1*$^{-/-}$ cells, we first treated *REDD1*$^{-/-}$ or matched wild-type cells with a proteasome inhibitor, MG132. Because HIF-1α is normally degraded under normoxic conditions by a proteasome-dependent mechanism, we predicted that proteasome inhibition would induce a more modest effect on HIF-1α in *REDD1*$^{-/-}$ cells if HIF-1α were already stabilized. Indeed, proteasome inhibition induced an ≈10-fold increase in HIF-1α in wild-type, whereas only inducing a 2-fold increase in matched *REDD1*$^{-/-}$ cells (Fig. 3*C*). Similarly, treatment of matched *REDD1*$^{-/-}$ and wild-type cells with deferoxamine (DFO), an iron chelator that induces potent HIF stabilization, had a larger effect in wild-type cells (Fig. 3*D*). As a result, DFO treatment resulted in nearly equal HIF-1α levels in *REDD1*$^{-/-}$ and wild-type cells. Finally, performing a time course following treatment of cells with cycloheximide to block protein

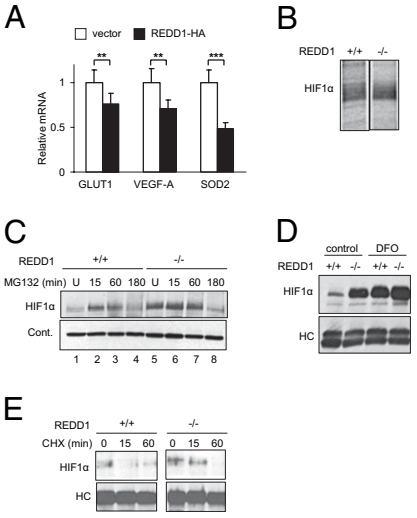

Fig. 3. REDD1 regulates HIF-1α stability. (*A*) REDD1 reconstitution down-regulates HIF-1α target genes. Tumorigenic *REDD1*$^{-/-}$ MEFs were infected with a retrovirus expressing REDD1 or a control vector, and then exposed to 1% O_2 (4 h); RNA analysis was carried out by qRT-PCR (***$P < 0.001$; **$P < 0.01$). (*B*) HIF-1α synthesis is not affected in *REDD1*$^{-/-}$ cells. Wild-type or matched tumorigenic *REDD1*$^{-/-}$ MEFs were pulse-labeled with ^{35}S (1 h, 1% O_2) followed by IP for HIF-1α. (*C*) Proteasome inhibition demonstrates increased baseline HIF-1α stability in *REDD1*$^{-/-}$ cells (compare lanes 1 and 2, 5 and 6). Cells were untreated (U) or treated with MG132 (100 μM) followed by IP/Western analysis. Loading control (cont.) is total AKT. (*D*) Deferoxamine (DFO) treatment (150 μM, 4 h) preferentially affects unstable HIF-1α in wild-type versus *REDD1*$^{-/-}$ cells. (*E*) HIF-1α half-life is prolonged in *REDD1*$^{-/-}$ cells. Cells were treated with cycloheximide (CHX; 10 μg/mL) under normoxia for the indicated times before IP/Western analysis.

synthesis revealed a prolonged half-life of HIF-1α in *REDD1*$^{-/-}$ versus wild-type cells (Fig. 3*E*). Taken together, these data demonstrate that loss of REDD1 is associated with preferential stabilization of HIF-1α.

Requirement for Mitochondrial REDD1 in ROS Regulation. In our initial gene expression profiling analysis of wild-type and highly tumorigenic *REDD1*$^{-/-}$ MEF populations, several of the most prominent signatures identified were those associated with changes in mitochondrial function and ROS production (Fig. S4*A*). Given that ROS are established mediators of HIF-1α stabilization, we tested ROS and their contribution to HIF-1α stabilization in *REDD1*$^{-/-}$ cells. We first isolated mitochondria from tumorigenic *REDD1*$^{-/-}$ or matched wild-type MEFs (Fig. S4*B*), and then examined mitochondrial ROS production (Fig. 4*A*). A significant and reproducible increase in multiple ROS species, including superoxide (O_2^-) and peroxide (H_2O_2), was evident in mitochondrial preparations from *REDD1*$^{-/-}$ cells (Fig. 4*A*). As a control for the ROS measurement, we treated mitochondria with MnTMPyP, a superoxide dismutase mimetic. This agent decreased the measured superoxide levels of these mitochondria in a dose-dependent fashion, as predicted (Fig. S4*C*). Increased ROS were also detected in *REDD1*$^{-/-}$ whole cells compared with their matched wild-type counterparts (Fig. 4*B*). To further establish whether these effects on mitochondrial ROS production were linked specifically to REDD1, we demonstrated that retroviral reconstitution of REDD1 into *REDD1*$^{-/-}$ cells significantly decreased cellular ROS (Fig. 4*C*).

Multiple protein sequence analysis algorithms predict that REDD1 is mitochondrial-localized (23). Indeed, upon cellular fractionation, we found that a significant portion of endogenous REDD1 (>10%) is localized to the mitochondria (Fig. 4*D*). This finding was corroborated by immunofluorescent staining and confocal microscopy, which demonstrated colocalization of REDD1 protein and mitochondria (Fig. 4*E*). To determine whether REDD1 was specifically required within the mitochondria for ROS regulation, we sought to identify amino acid motifs that might be involved in REDD1 localization. One such phylogenetically conserved motif is a polylysine (KKK) sequence at the C terminus (Fig. S5). Mutation of these lysine residues to arginine (K3R3 mutant) did not affect REDD1 stability but significantly compromised REDD1 mitochondrial localization (Fig. 4*F*). Furthermore, reconstitution of this mutant into *REDD1*$^{-/-}$ cells failed to normalize mitochondrial ROS levels compared with wild-type REDD1 (Fig. 4*G*). Finally, reconstituted wild-type REDD1 was sufficient to down-regulate expression of HIF target genes in these cells, whereas the mislocalized mutant lacked this ability (Fig. 4*H*). These findings suggest that REDD1 is a regulator of mitochondrial ROS and HIF activity through its localization to the mitochondria.

HIF-1α Stabilization and Tumor Promotion Through REDD1-Regulated ROS. We then tested directly whether ROS contributed to HIF-1α stabilization in *REDD1*$^{-/-}$ cells. Consistent with this hypothesis,

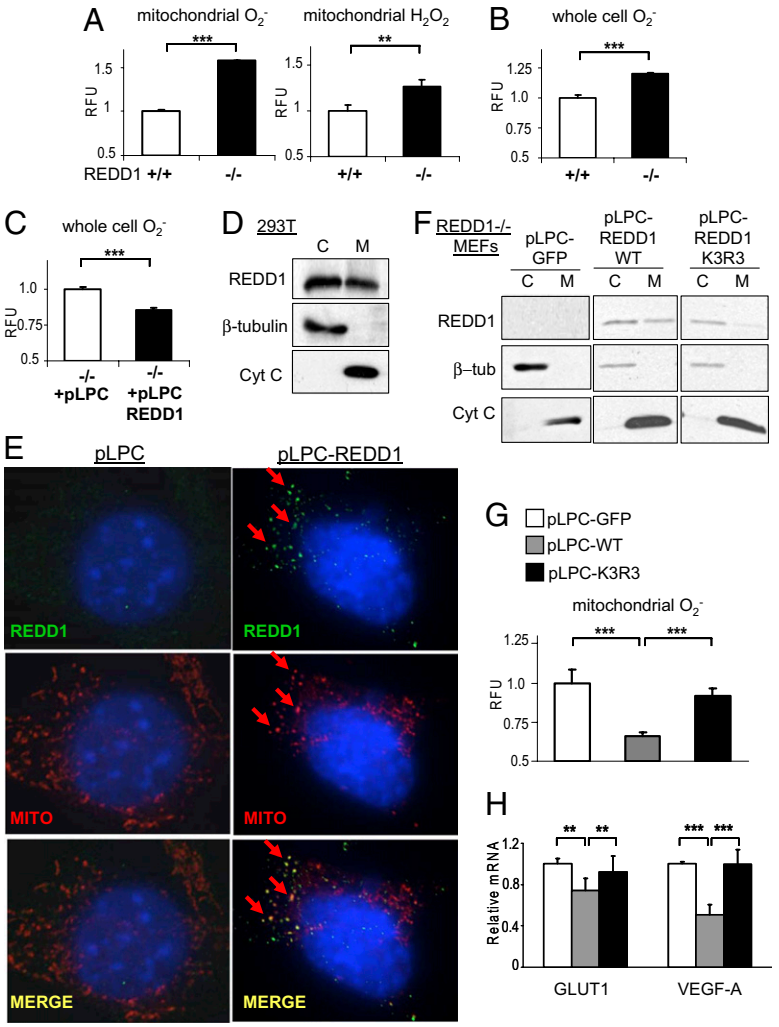

Fig. 4. Increased mitochondrial ROS induced by loss of REDD1. (*A*) Increased mitochondrial ROS in *REDD1*$^{-/-}$ cells. Mitochondria were isolated from matched wild-type or tumorigenic *REDD1*$^{-/-}$ cells, and ROS were measured by relative fluorescence units (RFU) after staining for superoxide (O_2^-) with dihydroethidium (DHE; *Left*) or for peroxide (H_2O_2) with Amplex Red (*Right*) as described in *Methods* (***$P < 0.001$; **$P < 0.01$). (*B*) Increased ROS (DHE) in whole *REDD1*$^{-/-}$ versus wild-type cells (***$P < 0.001$). (*C*) Retroviral REDD1 reconstitution reduces ROS (DHE) in *REDD1*$^{-/-}$ cells (***$P < 0.001$). (*D*) Endogenous REDD1 is a mitochondrial protein. Cellular fractionation of 293T cells was carried out before Western analysis for endogenous REDD1 in the cytosol (C) and mitochondria (M). (*E*) Confocal imaging demonstrates colocalization of REDD1 and mitochondria. *REDD1*$^{-/-}$ MEFs were reconstituted with control retroviral vector (pLPC) or REDD1, and then costained with anti-REDD1 antibody and MitoTracker Red (MITO). Arrows show colocalization. Nuclei were stained with Hoechst dye. (*F*) Reconstituted wild-type (WT) REDD1, but not the K3R3 mutant, exhibits physiological mitochondrial (M) localization. (*G*) Mitochondrial localization is required for REDD1-mediated suppression of ROS. Mitochondrial preparations shown in *F* were used for measurement of ROS (DHE) (***$P < 0.001$). (*H*) HIF-1α target gene suppression by REDD1 is associated with ROS suppression and mitochondrial localization. Relative mRNA expression was measured by qRT-PCR in triplicate samples (**$P < 0.01$; ***$P < 0.001$). Legend as shown in *G*.

treatment with ascorbate, which functions to reduce the catalytic iron of the HIF prolyl hydroxylase, dramatically lowered HIF-1α levels and abolished the difference between *REDD1*$^{-/-}$ and wild-type cells (Fig. 5*A*). A similar effect on HIF-1α levels was observed following treatment with N-acetyl cysteine, a thiol-containing antioxidant that increases intracellular glutathione synthesis (Fig. S6*A*). Importantly, antioxidant treatment had little effect on HIF-1α levels in *TSC2*$^{-/-}$ cells, and no effect on mTORC1 activity in wild-type and *REDD1*$^{-/-}$ cells (Fig. S6 *A* and *B*). These data support our finding that HIF-1α dysregulation in *REDD1*$^{-/-}$ cells is mTORC1-independent and is mediated instead by elevated ROS.

Given these findings, we lastly wished to test directly whether REDD1-regulated ROS were important for its effects on tumorigenesis. Consistent with this hypothesis, antioxidant (ascorbate) treatment led to a decrease in cellular proliferation in *REDD1*$^{-/-}$ cells relative to wild-type cells under hypoxia (Fig. 5*B*). As expected, this antioxidant treatment significantly decreased mitochondrial ROS in *REDD1*$^{-/-}$ cells (Fig. 5*C*). Most significantly, antioxidant treatment resulted in a significant attenuation of tumor growth in animals injected with *REDD1*$^{-/-}$ cells compared with controls (Fig. 5*D*). Of note, the magnitude of the antioxidant effect in vivo was similar to that of HIF-1α knockdown in *REDD1*$^{-/-}$ cells by RNA interference (Fig. 2*C*). Taken together, these findings demonstrate that REDD1 mediates a negative feedback pathway to HIF-1α through regulation of ROS that contributes to tumor suppression in vivo (Fig. 5*E*).

Discussion

Dysregulation of HIF is increasingly recognized as a hallmark event in many human cancers (7). Here we demonstrate a genetic mechanism whereby loss of the HIF-1 transcriptional target gene REDD1 disrupts a negative feedback pathway, leading to increased stability and function of HIF-1α and promoting tumorigenesis. Consequently, *REDD1*$^{-/-}$ cells show an increase in HIF-1 target gene expression and glycolytic metabolism under both normoxic and hypoxic conditions. Immortalized *REDD1*$^{-/-}$ cells are highly tumorigenic compared with their wild-type counterparts, and we show directly that elevated HIF-1 activity contributes significantly to this phenotype. We provide evidence that this REDD1-HIF feedback pathway is operative not only in MEFs but also in other cells types and in human tumors as well. *REDD1*$^{-/-}$ epithelial cells show dysregulation of both HIF-1α and HIF-2α (Fig. 1*E*). Furthermore, human primary breast tumors that exhibit REDD1 down-regulation express significantly higher levels of the HIF effector gene Glut-1 compared with tumors that have not silenced REDD1 (Fig. 2*F*).

REDD1 is well-known to play an essential role in suppression of mTORC1 activity in response to hypoxia, both in *Drosophila* and mammalian cells. The existence of feedback pathways induced by mTORC1 suppression, leading to activation of both PI3K and MAPK signaling, may explain the contribution of increased REDD1 expression to transformation reported in some studies (24). In contrast, we find that a key pathway contributing to tumorigenesis following REDD1 loss is mTORC1-independent. We show that loss of REDD1 is associated with an elevation of mitochondrial ROS that drives HIF-1α stabilization. Consistent with the view that regulation of ROS by REDD1 is distinct from its ability to inhibit mTORC1 activity, we find that blocking mTORC1 activity has no effect on the relative expression of HIF-1α in *REDD1*$^{-/-}$ versus wild-type cells. Conversely, antioxidant treatment that restores normal levels of HIF-1α in *REDD1*$^{-/-}$ cells has no effect on mTORC1 activity. These findings are in contrast to *TSC2*$^{-/-}$ cells, which we and others find exhibit HIF-1α dysregulation that is independent of HIF stabilization and can be reversed by rapamycin treatment (25). The distinct mTORC1-dependent and -independent functions of REDD1 may be explained by our finding that mitochondrial localization is essential for the ability of REDD1 to regulate

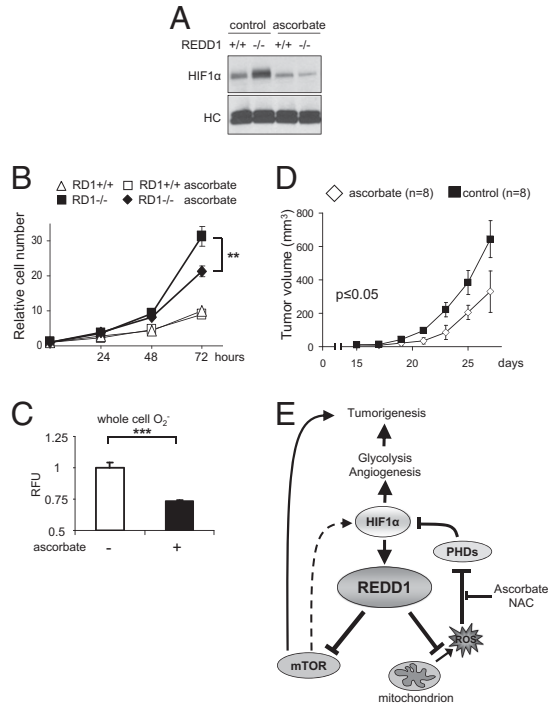

Fig. 5. Antioxidant treatment blocks HIF-1α and tumorigenesis induced by REDD1 loss. (*A*) Antioxidant treatment (ascorbate, 100 μM, 4 h) normalizes HIF-1α levels in tumorigenic *REDD1*$^{-/-}$ MEFs. (*B*) Antioxidant treatment (ascorbate, 100 μM) selectively inhibits proliferation of *REDD1*$^{-/-}$ MEFs under hypoxia (1% O_2). Note little or no effect of ascorbate in wild-type cells (**$P = 0.01$). (*C*) Antioxidant treatment (ascorbate, 100 μM, 4 h) inhibits mitochondrial ROS, measured by relative fluorescence units (RFU) after staining with DHE (***$P < 0.001$). (*D*) Antioxidant treatment inhibits tumorigenic growth of *REDD1*$^{-/-}$ cells in vivo. Tumorigenic *REDD1*$^{-/-}$ MEFs (4×10^6 cells) were injected into nude mice treated with ascorbate (5 g/L) in drinking water. *P* value by repeated-measures ANOVA. (*E*) Schematic diagram of proposed roles for REDD1 in mTORC1-dependent and mitochondrial ROS-dependent tumor suppression. REDD1 loss induces mTORC1 dysregulation under hypoxia, which promotes tumorigenesis. REDD1 also plays a distinct role in mitochondria to control ROS production, as REDD1 loss induces mitochondrial ROS, which promotes HIF-1α stabilization and HIF-dependent metabolic adaptations that drive tumorigenesis.

ROS (Fig. 4*G*). In contrast, the cellular compartment in which REDD1 resides for mTORC1 regulation is likely to be a triton-insoluble membrane fraction which contains the majority of cellular Rheb, the target of REDD1-TSC1/2 activity (16). Although the specific mechanism of REDD1 function in the mitochondria awaits further studies, REDD1 has been reported to interact with mitochondrial proteins in vitro (26), suggesting the possibility of a direct effect of REDD1 on electron transport.

Our finding that REDD1 is a regulator of mitochondrial ROS is in keeping with previous work demonstrating that HIF transcriptional targets play an important role in controlling mitochondrial function (3, 27). Multiple direct HIF-1 target genes have recently been implicated in the regulation of mitochondrial function, including pyruvate dehydrogenase kinase (PDK1), the LON protease, and the COX4-2 subunit, with the net effect in each case being down-regulation of mitochondrial ROS production (3, 28). We provide strong evidence that REDD1, like these proteins, is a HIF-1 transcriptional target that plays an essential role in regulating mitochondrial function and consequently limiting ROS production.

The contribution of ROS to tumorigenesis has long been suspected, and until recently this effect has been attributed to ROS-induced DNA damage (29). Emerging data, however, point to a role for increased ROS in promoting tumorigenesis through

dysregulation of HIF-1 (12). Yet it has not been well-established which specific genetic mechanisms might be most relevant in triggering the ROS-HIF pathway in tumor cells. Presumably, disruption of many proteins involved in ROS generation (e.g., COX subunits) would have a detrimental effect on tumor cell metabolism and therefore not be selected during tumorigenesis. In contrast, REDD1 is not required for normal embryonic development or cellular homeostasis under unstressed conditions (30). Therefore, silencing of REDD1 in nascent tumor cells may not be immediately detrimental, but instead may induce ROS dysregulation that drives HIF-1α stabilization while at the same time disabling the REDD1-TSC1/2 pathway that suppresses mTORC1 under hypoxic conditions. The net result of REDD1 down-regulation may therefore be increased tumor cell adaptation to hypoxic conditions, as well as increased cellular translation and cell growth potential (Fig. 5*D*). These combined effects may well explain the dramatic induction of tumorigenesis that we observe in the context of genetic loss of REDD1.

Methods

Cells, Cell Culture, and Reagents. The *REDD1*$^{-/-}$ allele was generated as previously described (30). Primary MEFs were immortalized by retroviral transduction of SV40 large T-antigen unless indicated otherwise, and were maintained in DMEM/10% FCS/Pen/Strep, except where noted. Reagents and antibodies are listed in *SI Methods*.

qRT-PCR and Breast Tumor Analysis. Total RNA extraction, cDNA synthesis, and qRT-PCR were carried out as described previously (31). For primary human breast carcinomas, laser-capture microdissection, qRT-PCR, and analysis of REDD1 expression in tumor and matched normal epithelia were performed as previously described (16). Primer sequences are listed in *Table S1*. See *SI Methods* for details.

Lentiviral shRNA. HIF-1α shRNA plasmids were obtained from the RNAi Consortium shRNA Library generated at the Broad Institute (32). Lentiviral stocks were generated and infections were performed (two rounds) as described previously (33).

Cellular Fractionation. To isolate mitochondrial proteins, cellular fractionation was performed as described previously (34). See *SI Methods* for details.

Measurement of ROS. For detection of the superoxide anion, DHE (dihydroethidium; Invitrogen) was added (5 μM) and evaluated in a SpectraMax M5 fluorescent microplate reader (Molecular Devices). To assess mitochondrial hydrogen peroxide, an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was used according to the manufacturer's protocol. ROS measurement in whole cells was performed using DHE or CM-H_2DCFDA [5-(and 6-)chloromethyl-2,7-dichlorodihydro-fluorescein diacetate, acetyl ester; Invitrogen] in full medium at final concentrations of 5 and 3 μM for 15 and 30 min in the dark at 37 °C, respectively. See *SI Methods* for details.

Tumorigenesis Assays. All animal studies were conducted according to protocols approved by the accredited MGH Subcommittee on Research Animal Care. For the ascorbate treatment experiment, mice were given drinking water containing 5 g/L ascorbate, which was changed weekly. Additional details are provided in *SI Methods*.

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