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REDD1, an inhibitor of mTOR signalling, is regulated by the CUL4A–DDB1 ubiquitin ligase

Samiksha Katiyar[1], Enbo Liu[1], Christine A. Knutzen[1], Elizabeth S. Lang[1], Christian R. Lombardo[2], Sabita Sankar[2], Julia I. Toth[1], Matthew D. Petroski[1], Ze'ev Ronai[1] & Gary G. Chiang[1+]

[1]Signal Transduction Program, Burnham Institute for Medical Research, La Jolla, California, USA and [2]Celgene, San Diego, California, USA

The cellular response to hypoxia involves several signalling pathways that mediate adaptation and survival. *REDD1* (regulated in development and DNA damage responses 1), a hypoxia-inducible factor-1 target gene, has a crucial role in inhibiting mammalian target of rapamycin complex 1 (mTORC1) signalling during hypoxic stress. However, little is known about the signalling pathways and post-translational modifications that regulate REDD1 function. Here, we show that REDD1 is subject to ubiquitin-mediated degradation mediated by the CUL4A–DDB1–ROC1–β-TRCP E3 ligase complex and through the activity of glycogen synthase kinase 3β. Furthermore, REDD1 degradation is crucially required for the restoration of mTOR signalling as cells recover from hypoxic stress. Our findings define a mechanism underlying REDD1 degradation and its importance for regulating mTOR signalling.

Keywords: REDD1; mTOR; hypoxia; ubiquitin; CUL4A

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INTRODUCTION

REDD1 (regulated in development and DNA damage responses 1) is a recently described component of the stress response. *REDD1* was first identified as a gene induced by hypoxia and DNA damage (Ellisen *et al*, 2002; Shoshani *et al*, 2002). Other environmental stresses such as energy stress, glucocorticoid treatment and reactive oxygen species have also been reported to induce *REDD1* transcription (Lin *et al*, 2005; Sofer *et al*, 2005). The *REDD1* gene is present in human, mouse and *Drosophila*, and is ubiquitously expressed in most adult tissues, whereas the expression of a related gene, *REDD2*, is highly restricted (Ellisen *et al*, 2002; Shoshani *et al*, 2002; Reiling & Hafen, 2004; Sofer *et al*, 2005). The 232-amino-acid REDD1 gene product lacks any identifiable enzymatic activity or functional domains. Initial studies into the function of REDD1 suggested either a pro- or an anti-apoptotic role during the stress response, depending on the cell context (Ellisen *et al*, 2002; Shoshani *et al*, 2002). A significant advancement in the understanding of REDD1 function came with the discovery that it is a crucial regulator of the target of rapamycin (TOR) signalling pathway during hypoxic stress (Brugarolas *et al*, 2004; Reiling & Hafen, 2004).

The mammalian target of rapamycin complex 1 (mTORC1) orchestrates cell growth in response to several environmental inputs such as growth factor and nutrient availability, as well as hypoxia and energy stress (Chiang & Abraham, 2007). mTORC1 consists of the mammalian target of rapamycin (mTOR) and the mTOR-interacting proteins, Raptor and mammalian lethal with Sec-thirteen-8 (mLST8). The two best-characterized mTORC1 substrates—eukaryotic translational initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1)—are components of the translational control machinery. Phosphorylation of these two substrates by mTOR promotes cap-dependent translation and ribosomal biogenesis, respectively. Hypoxia had previously been shown to inhibit mTORC1-dependent phosphorylation of S6K1 and 4E-BP1 dominantly over other mTORC-activating inputs such as growth factors and nutrients (Arsham *et al*, 2003). This effect was subsequently shown to require REDD1 expression and was dependent on tuberous sclerosis complex (TSC) function (Brugarolas *et al*, 2004; Reiling & Hafen, 2004). Recently, a molecular mechanism responsible for the REDD1-dependent inhibition of mTORC1 through 14-3-3 proteins has been described (DeYoung *et al*, 2008).

Here, we report that REDD1 is rapidly degraded by the ubiquitin–proteasome system mediated by the CUL4A (Cullin 4A)–DDB1 (DNA damage-binding protein 1)–ROC1 (regulator of cullins 1) ubiquitin ligase through glycogen synthase kinase 3 (GSK3)-β phosphorylation and β-transducin repeat-containing protein (β-TRCP) activity. Thus, the CUL4A–DDB1–ROC1–β-TRCP ubiquitin ligase complex acts to regulate mTOR signalling by modulating the stability of REDD1. The ubiquitin-mediated regulation of REDD1 described here highlights an emerging theme of ubiquitin-mediated regulation of mTORC1 signalling, as recent studies have identified other nodes in the

[1]Signal Transduction Program, Burnham Institute for Medical Research, 10901 North Torrey, Pines Road, La Jolla, California 92037, USA
[2]Celgene, 4550 Towne Center Court, San Diego, California 92121, USA
[+]Corresponding author. Tel: +1 858 795 5235; Fax: +1 858 713 6274; E-mail: gchiang@burnham.org

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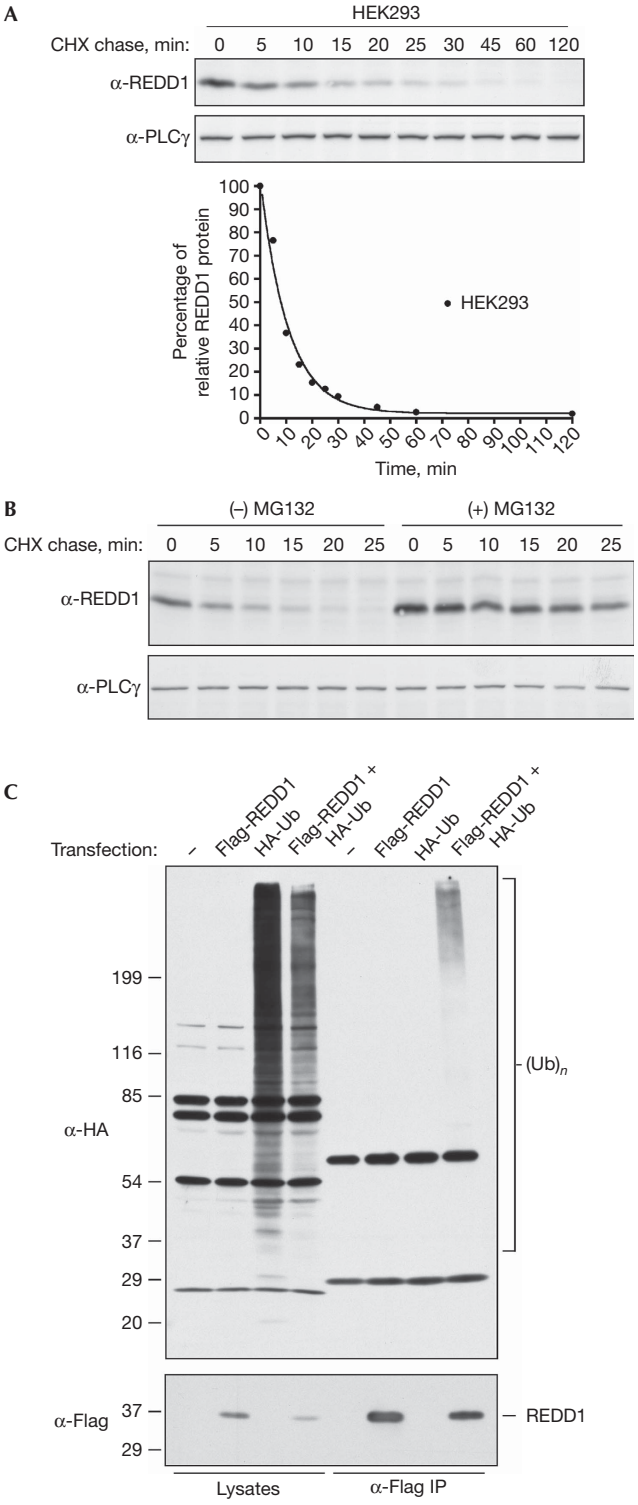

Fig 1 | Stability of REDD1 is regulated by ubiquitin-mediated proteolysis. (**A**) REDD1 expression was induced in HEK293 cells by exposure to 1% O_2 for 18 h. CHX was added (10 μg/ml) and cells were harvested in 20% O_2 (normoxia) at the indicated time points. Top panel, immunoblot of REDD1 and PLCγ (loading control). Bottom panel, densitometric quantitation of REDD1 levels, normalized to PLCγ levels. (**B**) REDD1 expression was induced in HEK293 cells by exposure to 1% O_2 for 18 h. MG132 (10 μM) was added for 30 min, prior to CHX addition and harvesting of cells in 20% O_2 at the indicated time points. Whole cell lysates immunoblotted for REDD1 and PLCγ. (**C**) HEK293 cells were co-transfected with vector (–), FLAG-tagged REDD1 alone, HA-tagged ubiquitin (HA-Ub) alone, or both as indicated. Cells were treated with MG132 for 2 h and harvested. Whole cell lysates and α-FLAG immunoprecipitates were immunoblotted with α-FLAG or α-HA antibodies. CHX, cycloheximide; HA, haemagglutinin; HEK, human embryonic kidney; PLCγ, phospholipase Cγ; REDD1, regulated in development and DNA damage responses 1.

mTORC1 network, such as TSC2 and mTOR itself, as crucial targets of the ubiquitin–proteasome system (Hu *et al*, 2008; Mao *et al*, 2008).

RESULTS AND DISCUSSION

Ubiquitin-mediated degradation of REDD1

Although the detailed molecular mechanisms underlying mTOR inhibition by REDD1 are now emerging, little is known about the signalling pathways and post-translational modifications that regulate REDD1. We examined endogenous REDD1 stability in cells following its induction by hypoxia. After treatment of HEK (human embryonic kidney)-293 cells with cycloheximide (CHX), endogenous REDD1 levels decreased rapidly within minutes, with a calculated half-life of 5–7 min (Fig 1A). Similar results were observed in HCT-116 (human colorectal carcinoma cell line), MCF-7 (human breast adenocarcinoma cell line) and U2OS (human osteosarcoma cell line) cells (supplementary Fig S1 online; data not shown). Furthermore, REDD1 stability did not differ under hypoxia (1% O_2), indicating that the mechanisms that control REDD1 stability are independent of cellular oxygen tension (supplementary Fig S1 online).

Recently, the rapid degradation of REDD1 was also described in mouse embryonic fibroblasts, although the underlying mechanism was not elucidated (Kimball *et al*, 2008). We hypothesized that the rapid turnover of endogenous REDD1 observed after CHX treatment was due to proteasome-mediated degradation. Indeed, in cells treated with the proteasome inhibitor MG132, the half-life of REDD1 was increased 10-fold (Fig 1B and supplementary Fig S1 online), showing that REDD1 degradation proceeds through the proteasome. Next, we examined the ubiquitination status of REDD1 in intact cells. Co-transfection of FLAG-epitope-tagged REDD1 with haemagglutinin (HA)-epitope-tagged ubiquitin (HA-Ub) resulted in a stereotypic smear indicative of poly-ubiquitination in the REDD1 immunoprecipitates (Fig 1C). These findings show that REDD1 degradation is regulated by ubiquitin-dependent proteasomal degradation.

Phosphorylation of REDD1 regulates protein stability

Treatment of cells with MG132 led to the accumulation of a shifted, faster migrating form of REDD1, which was particularly evident when REDD1 samples were resolved on high percentage (12.5%) SDS–polyacrylamide gel electrophoresis (PAGE) gels. We hypothesized that this faster migrating form of REDD1 was due to phosphorylation. Although phosphorylation usually results in retarded protein migration during SDS–PAGE, there are some exceptions, such as mini-chromosome maintenance protein 2

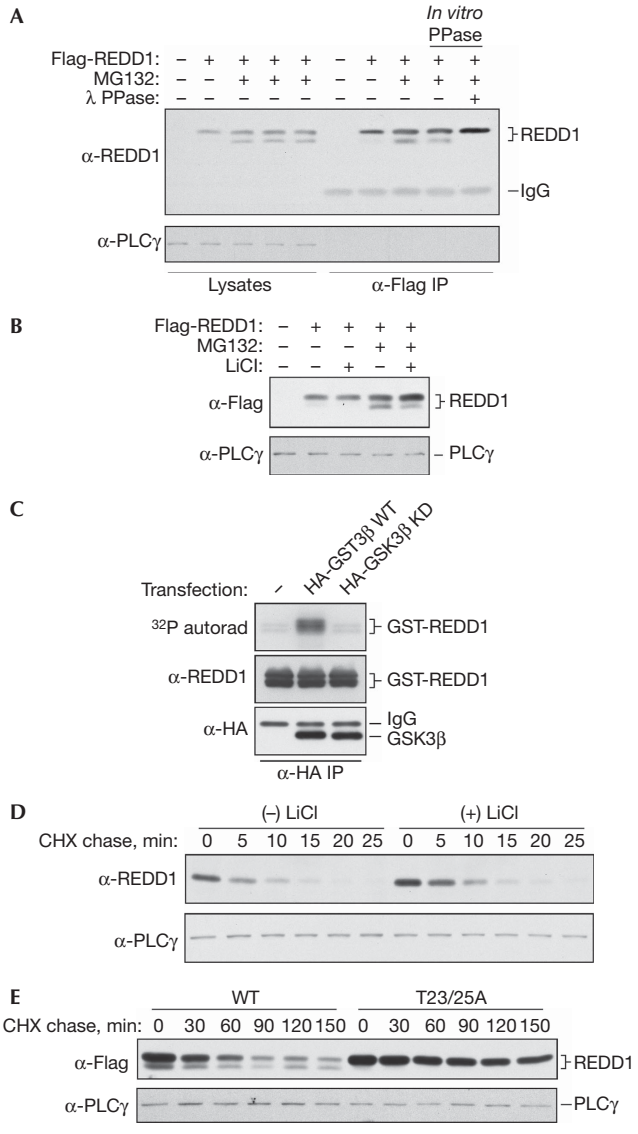

Fig 2 | GSK3β-dependent phosphorylation of REDD1 regulates stability. (**A**) HEK293 cells were transfected with FLAG-REDD1 and treated with MG132 for 2 h. FLAG immunoprecipitates were incubated with buffer alone (lane 3) or λ phosphatase (lane 4) for 30 min at 30 °C. Whole cell lysates and immunoprecipitates were blotted with α-FLAG and α-PLCγ antibodies. (**B**) HEK293 cells were transfected with FLAG-REDD1. Where indicated, cells were treated with 20 mM LiCl for 2 h and for an additional 2 h with MG132. Whole cell lysates and α-FLAG immunoprecipitates were immunoblotted with α-FLAG antibodies. (**C**) HEK293 cells were transfected with HA-GSKβ wild type (WT) or kinase-dead (KD). α-HA immunoprecipitates were incubated with purified, full-length GST-REDD1 and [γ^{32}P]-ATP for the *in vitro* kinase reaction. Samples were exposed for autoradiography (top) or immunoblotted with α-REDD1 or α-HA antibodies. (**D**) HEK293 cells were placed in 1% O_2 for 18 h to induce REDD1 expression. Where indicated, 20 mM LiCl was added to the cells for 4 h. CHX was added and cells were harvested at the indicated time points. Lysates were immunoblotted for REDD1 and PLCγ. (**E**) HEK293 cells were transfected with FLAG-REDD1 WT or T23/25A double mutant. CHX was added and cells were harvested at the indicated time points. Whole cell lysates were immunoblotted with α-FLAG or α-PLCγ antibodies. GSK3β, glycogen synthase kinase-3β; HA, haemagglutinin; HEK, human embryonic kidney; PLCγ, phospholipase Cγ; REDD1, regulated in development and DNA damage responses 1.

(MCM2), which migrate faster when phosphorylated (Tsuji *et al*, 2006). To test this hypothesis, α-FLAG immunoprecipitates from transfected, MG132-exposed HEK293 cells were treated with λ phosphatase. Following phosphatase treatment, the shifted form of REDD1 disappeared, indicating that the ectopically expressed REDD1 is phosphorylated in cells (Fig 2A). A similar mobility shift in endogenous REDD1 from cells treated with MG132, which was abrogated by phosphatase treatment, was also observed (supplementary Fig S2 online).

Next, we applied a proteomic approach to identify REDD1-phosphorylation sites (see supplementary information online). Among the most prominent phosphorylation sites identified were Ser 19, Thr 23, Thr 25 and Ser 121 (supplementary Fig S2 online). Both Thr 23 and Thr 25 lie in an S/T-P motif, a preferred target of proline-directed kinases, and Ser 121 is in an S/T-Q motif, which is a consensus sequence for the DNA damage kinases, ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR). Interestingly, Thr 23 and Thr 25 fit the consensus sequence for unprimed GSK3β phosphorylation, whereas Ser 19, based on its position relative to Thr 23, conforms to the S/T(–4) spacing seen in primed GSK3β substrates (Cohen & Frame, 2001). To examine the possibility that GSK3β might contribute to REDD1 phosphorylation, the effect of the GSK3β inhibitor, lithium chloride (LiCl), on REDD1 mobility was examined (Fig 2B). LiCl treatment reduced the amount of mobility-shifted REDD1 compared with untreated cells. Furthermore, wild-type GSK3β, but not kinase-dead GSK3β, phosphorylated a GST–REDD1 fusion protein *in vitro* (Fig 2C). Taken together, these data indicate that REDD1 undergoes GSK3β-dependent phosphorylation. On the basis of this evidence, degradation of REDD1 might be mediated by GSK3β-dependent phosphorylation. Indeed this was found to be the case as REDD1 stability was increased in the presence of LiCl (Fig 2D; supplementary Fig S2 online).

To examine the contribution of the sites identified by mass spectrometry towards REDD1 phosphorylation and stability, phosphorylation site mutants—S19A, T23/25A double mutant and S121A—were generated by site-directed mutagenesis and the SDS–PAGE migration of each of these mutants following MG132 treatment was examined. The S19A mutant showed a reduced amount of the faster-migrating band and the T23/25A double mutant completely lacked the faster-migrating band (supplementary Fig S2 online). By contrast, the S121A mutant migrated similarly to wild-type REDD1, implying that phosphorylation of this site does not contribute to the mobility shift of REDD1. The stability of the various REDD1 phosphorylation-site mutants was then tested. In contrast to the approximately 30 min half-life of ectopically expressed wild-type REDD1, the T23/25A mutant showed a four- to fivefold increase in half-life (Fig 2E; supplementary Fig S2 online), suggesting that the phosphorylation of these sites affects REDD1 stability. Point mutation of Ser 19 or

Thr 23 also increased REDD1 stability, compared with wild-type (supplementary Fig S2 online), suggesting that phosphorylation at these sites is important. The stability of S121A was comparable to that of wild-type REDD1 (supplementary Fig S2 online). These findings indicate that phosphorylation of REDD1 at Thr 25, Thr 23 and Ser 19 mediates REDD1 degradation.

Identification of the E3 ligase that degrades REDD1

To identify the E3 ligase responsible for REDD1 degradation, we took a candidate approach to test the interaction of REDD1 with various E3 ligase components—CUL1, 2, 3, 4A, 4B and 7. In initial co-expression experiments, a strong interaction between REDD1 and CUL4A, weaker interactions with CUL1 and CUL7, and no detectable interactions with CUL2, 3 or 4B were observed. In subsequent co-expression experiments, REDD1 was found to associate with DDB1, another component of the CUL4A–DDB1–ROC1 E3 ligase complex (data not shown). Because we found that GSK3β-dependent phosphorylation of REDD1 regulates its stability, and recent evidence has shown that the CUL4A–DDB1–ROC1 ubiquitin ligase utilizes WD40-repeat proteins as substrate adaptors (Hu *et al*, 2008), we hypothesized that REDD1 degradation could be mediated by β-TRCP, a WD40-repeat protein known to target proteins phosphorylated by GSK3β for degradation. In co-expression experiments, β-TRCP associated with REDD1, as well as with DDB1 and CUL4A (supplementary Fig S3 online). Significantly, when CUL4A, DDB1, β-TRCP and REDD1 were co-expressed, all four proteins existed in a complex (Fig 3A). In addition, endogenous REDD1 also associated with DDB1 and β-TRCP (Fig 3B). To assess further the contribution of CUL4A to REDD1 stability, short interfering RNA (siRNA) was used to knock down CUL4A expression. In CUL4A siRNA-transfected cells, REDD1 stability was increased compared with REDD1 stability in control (luciferase) siRNA-transfected cells (Fig 3C). As downregulation of CUL4A increases REDD1 stability, overexpression of CUL4A is predicted to reduce REDD1 stability. As the already short (≈5 min) half-life of endogenous REDD1 made this prediction difficult to observe, we co-expressed both REDD1 and CUL4A, taking advantage of the longer half-life of the ectopically expressed REDD1. As predicted, REDD1 stability was noticeably lower when expressed with CUL4A than when expressed alone (supplementary Fig S3 online). Furthermore, consistent with the CUL4A siRNA experiment, knockdown of DDB1 or β-TRCP also increased the half-life of REDD1 (Fig 3D,E; supplementary Fig S3 online). As β-TRCP and DDB1 are subunits of other E3 ligases (CUL1-based E3 ligases and the recently described CUL4B–DDB1–Raptor complex; Kitagawa *et al*, 1999; Ghosh *et al*, 2008), and because we found weak interactions with CUL1 and CUL7, it was formally possible that other E3 ligases could participate in REDD1 degradation. To address this possibility, CUL1, CUL7 or Raptor expression was knocked down by siRNA; however, no stabilization of REDD1 was observed (supplementary Fig S4 online). Taken together, these data indicate that the CUL4A–DDB1–ROC1–β-TRCP complex is a major E3 ligase that regulates REDD1 stability.

REDD1 degradation and recovery of mTOR signalling

Next, we sought to understand the biological importance of the rapid degradation of REDD1. As induction of REDD1 expression by hypoxia or by ectopic overexpression blocks phosphorylation

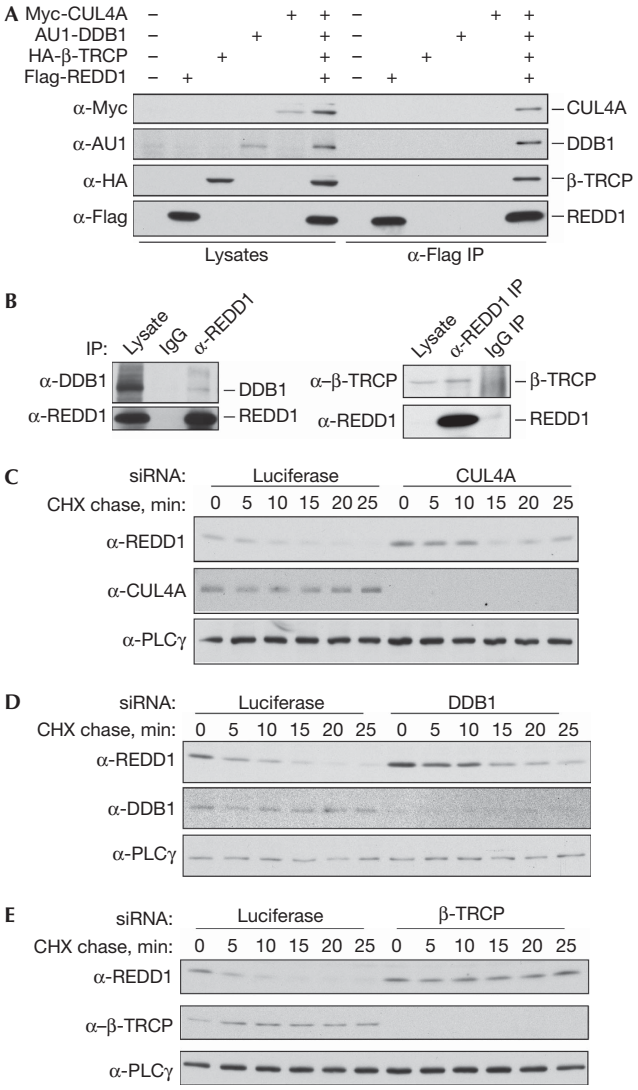

Fig 3 | Physical and functional interaction of REDD1 with the CUL4A–DDB1 E3 ligase. (**A**) HEK293 cells were transfected with Myc-CUL4A, AU1-DDB1, HA-β-TRCP and FLAG-REDD1 either alone or in combination. Cells were treated with MG132, and α-FLAG immunoprecipitates (IP) from whole cell lysates were immunoblotted with α-Myc, α-AU1, α-HA or α-FLAG antibodies. (**B**) MCF-7 cells were placed in 1% O_2 for 24 h to induce REDD1 expression. Cells were treated with MG132, and rabbit IgG or α-REDD1 immunoprecipitates were immunoblotted with α-REDD1 and α-DDB1 or α–β-TRCP antibodies as indicated. (**C**,**D**,**E**) MCF-7 cells were transfected with siRNAs against control (luciferase), (**C**) CUL4A, (**D**) DDB1 or (**E**) β-TRCP. 24 h post-transfection, cells were incubated an additional 24 h in 1% O_2. CHX was added, and cells were harvested at the indicated time points. Whole cell lysates were immunoblotted with the indicated antibodies (α-REDD1, α-PLCγ, α-CUL4A, α-DDB1 and α–β-TRCP). β-TRCP, beta-transducin repeat-containing protein; CUL4A, cullin 4A; DDB1, DNA damage-binding protein 1; HA, haemagglutinin; HEK, human embryonic kidney; MCF-7, human breast adenocarcinoma cell line; PLCγ, phospholipase Cγ; REDD1, regulated in development and DNA damage responses 1; siRNA, small interfering RNA.

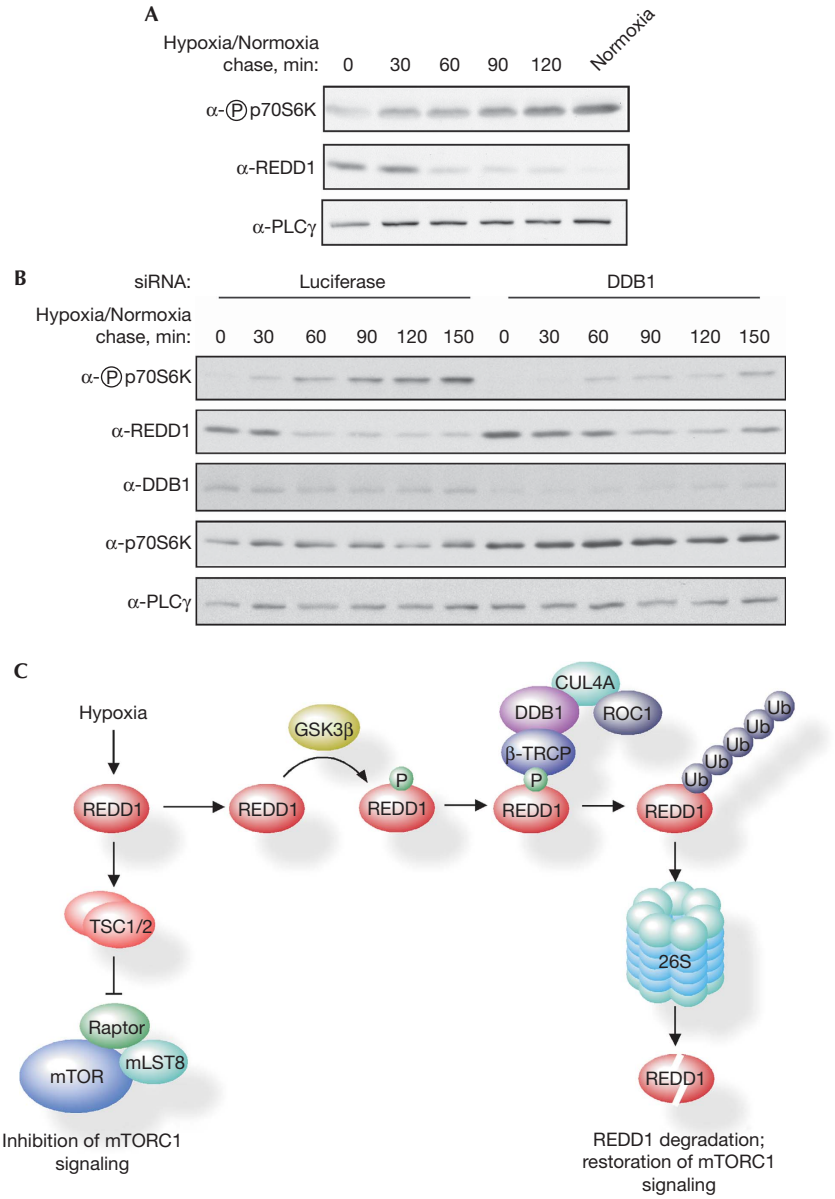

Fig 4 | Recovery of mTOR signalling by degradation of REDD1. (**A**) MCF-7 cells were exposed to 1% O_2 for 18 h to induce REDD1 expression. Cells were placed in 20% O_2 (normoxia) and harvested at the indicated time points. Whole cell lysates were immunoblotted with α-phospho-p70S6K (T389), α-REDD1 or α-PLCγ antibodies. (**B**) MCF-7 cells were transfected with control (luciferase) or DDB1-specific siRNAs. 24 h post-transfection, cells were incubated an additional 24 h in 1% O_2. Cells were placed in normoxia and harvested at the indicated time points. Whole cell lysates were immunoblotted with α-phospho-p70S6K (T389), α-REDD1, α-DDB1, α-p70S6K (total) and α-PLCγ antibodies. (**C**) Model for the regulation of REDD1 and mTOR signalling by ubiquitin-mediated degradation. Under hypoxia, REDD1 is upregulated and inhibits mTOR signalling through TSC. Phosphorylation of REDD1 by GSK3β triggers the recruitment of the CUL4A–DDB1–ROC1–β-TRCP E3 ligase complex, and results in REDD1 ubiquitination and degradation by the proteasome. Degradation of REDD1 alleviates mTOR inhibition. β-TRCP, beta-transducin repeat-containing protein; CUL4A, cullin 4A; DDB1, DNA damage-binding protein 1; GSK3β, glycogen synthase kinase-3β; HA, haemagglutinin; HEK, human embryonic kidney; MCF-7, human breast adenocarcinoma cell line; mTOR, mammalian target of rapamycin; p70S6K, ribosomal S6 kinase 1; PLCγ, phospholipase Cγ; REDD1, regulated in development and DNA damage responses 1; ROC1, regulator of cullins 1; siRNA, small interfering RNA; TSC, tuberous sclerosis complex.

of mTOR targets such as S6K1 or 4E-BP1 (Brugarolas *et al*, 2004), one possible function for the rapid degradation kinetics of REDD1 is that it would allow cells to quickly restore mTOR signalling once the stress conditions—for example, hypoxia—are alleviated. Consistent with this idea, transition of cells from hypoxia to normoxia results in the re-establishment of mTOR signalling concomitant with REDD1 degradation (Fig 4A). If REDD1 degradation is accelerated during the recovery of cells from

hypoxia, then a faster recovery of mTOR signalling should be observed. Indeed, treatment of cells with CHX increased the recovery rate of mTOR signalling once cells were removed from hypoxia (supplementary Fig S5 online). Conversely, if REDD1 is stabilized, then recovery of mTOR signalling should be delayed. In agreement with this prediction, treatment of cells with MG132 delayed the recovery of mTOR signalling (supplementary Fig S5 online). Knockdown of DDB1 or β-TRCP by siRNA also results in a similar delay in mTOR-signalling recovery when cells are shifted to normoxia, an effect consistent with the stabilization of REDD1 (Fig 4B; supplementary Fig S5 online). Taken together, these data indicate that restoration of mTOR signalling during the recovery of cells from hypoxic stress is crucially dependent on REDD1 degradation by the CUL4A–DDB1–ROC1–β-TRCP E3 ligase complex.

In this study we examined the stability of REDD1, a crucial inhibitor of mTOR signalling during hypoxic stress. We have shown that the rapid degradation of REDD1 is mediated by the CUL4A–DDB1–ROC1–β-TRCP E3 ligase complex and is regulated by REDD1 phosphorylation at Thr 25, Thr 23 and Ser 19 through the activity of GSK3β. Although the hypoxia–normoxia shift paradigm using established cell lines adapted to ambient oxygen might have some limitations in mimicking events *in vivo*, we believe that the degradation of REDD1 might be an important physiological mechanism by which mTOR signalling can be rapidly restored as cells recover from hypoxic stress (Fig 4C).

The inhibitory effect of REDD1 on mTOR signalling suggests that it might function in a manner similar to a tumour suppressor, as the phosphoinositide-3 kinase (PI(3)K)–AKT–mTOR axis is frequently deregulated in cancer. In agreement with this hypothesis, decreased REDD1 messenger RNA (mRNA) expression has been observed in primary breast tumours (DeYoung *et al*, 2008). Furthermore, transformed *REDD1*$^{-/-}$ mouse embryonic fibroblasts formed larger tumours with a decreased latency in nude mice than their *REDD1*$^{+/+}$ counterparts, indicating that the loss of REDD1 can potentiate tumour growth (DeYoung *et al*, 2008). On the basis of our results, we postulate that increased proteolysis of REDD1 could be an additional mechanism by which mTOR signalling is upregulated during tumorigenesis. It is noteworthy that amplification and overexpression of CUL4A has been observed in primary breast tumours and in hepatocellular carcinomas (Chen *et al*, 1998; Yasui *et al*, 2002). In addition, increased β-TRCP mRNA and protein expression has also been found in primary colorectal and pancreatic cancers (Ougolkov *et al*, 2004; Muerkoster *et al*, 2005). Future studies will define whether dysregulation of REDD1 degradation is a common event resulting in elevated mTOR signalling during tumour development.

METHODS

Cell culture, DNA constructs, siRNA and transfections. HEK293, MCF-7 and U2OS cells were grown in high-glucose Dulbecco's modified Eagle's medium (Cellgro, Manassas, VA, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA). HCT-116 cells were grown in McCoy's modified 5A media (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum. For hypoxia experiments, cells were incubated in 1% O_2 in an Invivo$_2$ 400 hypoxia workstation (Ruskinn Technology, Pencoed, UK).

The REDD1 complimentary DNA (cDNA) was generated by reverse transcription PCR (RT–PCR) using mRNA from hypoxic HEK293 cells. The Flag epitope tag or HA epitope tag was introduced at the amino terminus by PCR. The Flag-β-TRCP construct was provided by Dr Wenyi Wei (Harvard Medical School, Boston, MA, USA). The pcDNA3–HA-β-TRCP construct was produced as described previously (Fuchs *et al*, 1999). The pcDNA3–Myc-CUL4A and pcDNA3–T7-DDB1 constructs were provided by Dr Xiaohua Wu (Scripps Research Institute, La Jolla, CA, USA). The T7-epitope tag in the pcDNA3–T7-DDB1 construct was replaced with a HA-epitope tag by PCR. The pcDNA3–GSK3β wild-type and kinase-dead (K85A) plasmids were obtained from Addgene (Cambridge, MA, USA). Mutant REDD1 cDNAs were generated with the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All cDNAs were confirmed by sequencing. The sources and sequences of siRNAs used are listed in the supplementary information online

Expression plasmids were transfected into cells using the calcium phosphate method. Cells were used in experiments 24–48 h post-transfection. siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen) at a final concentration of 100 nM.

Antibodies, reagents and immunoblotting. The α-REDD1 antibody was obtained from Proteintech (Chicago, IL, USA). The α-Myc (9E10) and α-AU1 antibodies were purchased from Covance (Berkeley, CA, USA). The α-Flag M2 antibody, α-Flag M2 agarose, α-HA agarose, protein A agarose and CHX were purchased from Sigma (St Louis, MO, USA). The α-PLCγ antibody was described previously (Ting *et al*, 1992). The rabbit anti-mouse IgG was purchased from Pierce Biotechnology (Rockford, IL, USA). The α-CUL4A, α-CUL7 and α-Raptor antibodies were purchased from Bethyl (Montgomery, TX, USA). The α-DDB1 and α-CUL1 antibodies were purchased from Invitrogen. The α-phospho-p70S6K (T389), α-phospho-S6 (S240/244) and α-ribosomal protein S6 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The α-p70S6K antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MG132 was purchased from Calbiochem (San Diego, CA, USA).

Immunoprecipitations and immunoblotting were carried out as described previously (Chiang & Abraham, 2005).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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