Protein synthesis involves the translation of ribonucleic acid information into proteins, the building blocks of life. The initial step of protein synthesis is the binding of the eukaryotic translation initiation factor 4E (eIF4E) to the 7-methylguanosine (m7-GpppG) cap of messenger RNAs. Low oxygen tension (hypoxia) represses cap-mediated translation by sequestering eIF4E through mammalian target of rapamycin (mTOR)-dependent mechanisms. Although the internal ribosome entry site is an alternative translation initiation mechanism, this pathway alone cannot account for the translational capacity of hypoxic cells. This raises a fundamental question in biology as to how proteins are synthesized in periods of oxygen scarcity and eIF4E inhibition. Here we describe an oxygen-regulated translation initiation complex that mediates selective cap-dependent protein synthesis. We show that hypoxia stimulates the formation of a complex that includes the oxygen-regulated hypoxia-inducible factor 2a (HIF-2a), the RNA-binding protein RBM4 and the cap-binding eIF4E2, an eIF4E homologue. Photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) analysis identified an RNA hypoxia response element (rHRE) that recruits this complex to a wide array of mRNAs, including that encoding the epidermal growth factor receptor. Once assembled at the rHRE, the HIF-2a–RBM4–eIF4E2 complex captures the 5’ cap and targets mRNAs to polysomes for active translation, thereby evading hypoxia-induced repression of protein synthesis. These findings demonstrate that cells have evolved a program by which oxygen tension switches the basic translation initiation machinery.

Here we describe an oxygen-regulated mechanism that mediates selective cap-dependent translation during hypoxia and eIF4E inactivation (Supplementary Fig. 1). We began our investigation into this alternative mechanism of protein synthesis by examining the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase that has a critical function in cell proliferation, tissue development and cancer. Hypoxia activates the translation of EGFR mRNA through HIF-2a (ref. 12), a member of the HIF family of transcription factors involved in maintaining cellular oxygen homeostasis. We speculated that HIF-2a might orchestrate a gene program that enabled the translation of EGFR mRNA during periods of eIF4E inactivation. We treated cells with transcription inhibitors to preclude activation of HIF-2a target genes during hypoxia, and found that hypoxia caused the accumulation of EGFR protein in a HIF-2a-dependent manner even in transcription-incompetent glioma or primary cultures of renal epithelial cells (Fig. 1a and Supplementary Fig. 2). The EGFR mRNA was captured by polysomes and translated de novo in hypoxic cells treated with actinomycin D (Fig. 1b, and Supplementary Figs 3 and 4). Silencing of HIF-2a abolished the association of EGFR mRNA with polysomes and prevented its de novo synthesis (Fig. 1b, and Supplementary Figs 3 and 4). In contrast, silencing of HIF-1α, a paralogue of HIF-2a, did not prevent the hypoxic induction of EGFR translation and capture by polysomes...
(Fig. 1a, b, and Supplementary Figs 3 and 4). In addition, ablation of HIF-1α, a protein required for HIF transcriptional activity, had no effect on hypoxia-inducible translation of the EGFR mRNA (Fig. 1a, b, and Supplementary Fig. 3). HIF-2α, but not HIF-1α, was observed in polysome fractions of hypoxic cells, suggesting its direct involvement in the translational machinery (Fig. 1c and Supplementary Fig. 5). RNA immunoprecipitation revealed that HIF-2α associates with the 3′ untranslated region (UTR) of EGFR mRNA between nucleotides 4295 and 4861 (Fig. 1d, Supplementary Fig. 6 and Supplementary Table 1). This segment was both necessary and sufficient to enhance the translation of a luciferase reporter during hypoxia in a HIF-2α-dependent manner even in the absence of transcription (Fig. 1e and Supplementary Fig. 7). Silencing HIF-2α, but not HIF-1α, considerably decreased the rate of global hypoxic translation, highlighting its participation in hypoxic protein synthesis beyond EGFR translation (Fig. 1f and Supplementary Fig. 8).

HIF-2α does not contain a classical RNA recognition motif; we therefore searched for potential interacting partners that could bind the EGFR 3′ UTR. Immunoprecipitation revealed a band of 40 kDa specifically associated with HIF-2α that was identified as RNA-binding motif protein 4 (RBM4; Supplementary Fig. 9), a protein involved in translation control 18,19. Co-immunoprecipitation revealed that endogenous RBM4 interacted with the amino-terminal region of HIF-2α but not with HIF-1α during hypoxia (Fig. 2a and Supplementary Fig. 10). Furthermore, RBM4 assembled with the EGFR 3′ UTR in vivo and in vitro independently of oxygen tension (Fig. 2b and Supplementary Fig. 11). Silencing experiments revealed that RBM4 is essential for HIF-2α recruitment to the EGFR 3′ UTR (Fig. 2b and Supplementary Fig. 11a), and the hypoxic induction of EGFR protein (Supplementary Fig. 12) and the ability of the 4295–4861 EGFR 3′ UTR segment to induce hypoxia-dependent translation of a reporter construct (Fig. 2c). In addition, depletion of RBM4 caused decreased hypoxic cellular translation to levels similar to those observed in HIF-2α-incompetent cells (Fig. 2d and Supplementary Fig. 13). Consistent with the RNA immunoprecipitation, multiple PAR-CLIP sequenced reads for HIF-2α–RBM4 and RBM4 concentrated at the same site within the EGFR 3′ UTR 4295–4861 fragment that confers hypoxic translation on a reporter protein but not elsewhere in the transcript (Supplementary Figs 14 and 15). The crosslink sites were near a CGG trinucleotide, a feature of RBM4-binding motifs20,21. Mutation of this CGG motif, but not that of another CGG sequence, was sufficient to

Figure 2 | RBM4 recruits HIF-2α to the 3′ UTR for hypoxic translation.

a, Co-immunoprecipitation of HIF-2α in normoxia (21% O2, left) and hypoxia (1% O2, right). WCL, whole cell lysate. b, RNA immunoprecipitation of HIF-2α and RBM4 in HIF-2α or RBM4 knockout cells. IN, input; nt, nucleotides; RN, RNase-treated. c, Effect of silencing HIF-2α or RBM4 on the hypoxic expression of a luciferase reporter fused to the 4295–4861 segment of the EGFR 3′ UTR. d, Global translation rates in normoxic or hypoxic HIF-2α and/or RBM4 knockout cells. e, Expression of a luciferase reporter containing a CGG→AAA mutation near RBM4 crosslinking sites, or in an unrelated upstream region (uCGG). Results in c and e are means and s.e.m. (n = 3). Significances of fold changes (Student’s t-test) are shown. Experiments were performed in U87MG glioblastoma.

Figure 3 | HIF-2α–RBM4 recruits the m7-GTP cap by means of an interaction with eIF4E2.

a, Dual luciferase assays in cells transfected with reporter constructs containing the 5′ UTR of actin or lamin a/c with or without a 3′ rHRE. Significances of fold changes (Student’s t-test) are shown. Results are means and s.e.m. (n = 3). DMOG, dimethylsulfoxide. b, Co-immunoprecipitation of HIF-2α and RBM4 in hypoxia (1% O2). c, Capture assays using m7-GTP beads in hypoxic cell lysates depleted in eIF4E (top) or eIF4E2 (bottom). GTP, proteins dislodged from the beads by GTP; m7-GTP, proteins bound to m7-GTP beads after GTP wash. d, Western blot of total EGFR, PDGFRα, IGF1R, HIF-2α, eIF4E and eIF4E2 levels in eIF4E or eIF4E2 knockdown cells. The lower (darker) panels display mRNA levels. e, Polysomal distribution of mRNA coding for HIF-2α–RBM4 targets in hypoxic eIF4E2 knockdown cells. XAF1, XIAP-associated factor 1. Experiments were performed in U87MG glioblastoma.

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disrupt the secondary structure and to abolish hypoxia-inducible translation of a reporter construct (Fig. 2e, and Supplementary Figs 16 and 17). In addition, shorter segments of the EGFR 3′ UTR that altered the secondary structure were unresponsive to hypoxia in luciferase assays (Supplementary Figs 18 and 19). Consistent with the 35S-labelling experiments, a wide array of mRNAs interacted with the HIF-2α–RB M4 complex (Supplementary Fig. 20a, Supplementary Information and Supplementary Table 2). Similarly to EGFR mRNA, multiple sequence reads for HIF-2α–RB M4 and RB M4 concentrated at the same site near CG(G) nucleotides in most candidates (Supplementary Fig. 20b, c). Several PAR-CLIP candidates were validated for HIF-2α-dependent hypoxic induction (Supplementary Figs 20c and 21). We suggest that RB M4 binds to specific regions in the 3′ UTR of mRNAs to recruit HIF-2α and induce hypoxic translation. These RNA sequences are referred to as rHREs.

A key characteristic of the EGFR rHRE is that it confers hypoxia-inducible translation to several unrelated 5′ UTRs that are otherwise unable to initiate translation during hypoxia (Fig. 3a). We therefore suspected that the rHRE might exploit the cap, because it is a common feature used by the 5′ UTRs of mRNAs to initiate protein synthesis. The RB M4–HIF-2α complex of hypoxic cells was captured by m7GTP beads (Supplementary Fig. 22). Immunoprecipitation experiments showed that HIF-2α–RB M4 specifically associates with the cap-binding protein eIF4E2, a homologue of eIF4E (Fig. 3b and Supplementary Fig. 23a, b). We therefore reasoned that eIF4E2 is recruited by HIF-2α–RB M4 to activate selective cap-dependent translation of rHRE-containing mRNAs during hypoxia and inhibition of eIF4E by 4E-binding protein (4EBP)22,23. Immunoprecipitation revealed that 4EBP has more affinity for eIF4E than for eIF4E2, which is consistent with previously published results (Supplementary Fig. 23c)24,25. Silencing of eIF4E2, but not that of eIF4E, prevented the binding of HIF-2α–RB M4 from hypoxic cells to m7GTP beads (Fig. 3c). In addition, ablation of eIF4E2 prevented the hypoxic induction of multiple proteins identified by PAR-CLIP, including EGFR, whereas silencing of eIF4E had no discernible effect (Fig. 3d and Supplementary Figs 20b and 24). Ablation of eIF4E2 prevented the capture of rHRE-containing mRNAs by polysomes (Fig. 3e, and Supplementary Figs 25 and 26). The HIF-2α–RB M4–eIF4E2 complex also recruited the RNA helicase eIF4A, a fundamental component of translation initiation26 (Fig. 3b and Supplementary Fig. 23b). Taken together, these results demonstrate that eIF4E2 is a member of a hypoxic translation initiation complex that mediates selective cap-dependent protein synthesis independently of eIF4E.

Figure 3d shows that eIF4E might be involved in the translation of mRNAs coding for EGFR, platelet-derived growth factor receptor α (PDGFRα) and insulin-like growth factor 1 receptor (IGF1R) during normoxia. This raises the intriguing possibility that the cap-dependent translational machinery switches from eIF4E to eIF4E2 as a function of oxygen tension. This oxygen-dependent switch was clearly observed between eIF4E and eIF4E2 in polysomes: eIF4E participation in the translational machinery was essentially limited to normoxia, and eIF4E2 participation to hypoxia (Fig. 4a, and Supplementary Figs 27 and 28). Ablation of HIF-2α abolished the hypoxic shift of eIF4E2 to polysomes, attesting to the role of HIF-2α as the oxygen-regulated subunit of the eIF4E2–RB M4–HIF-2α complex (Fig. 4a, and Supplementary Fig. 27c). In contrast, treatment with rapamycin, an inhibitor of mTOR and eIF4E, prevented the accumulation of eIF4E in normoxic polysomes but had no effect on eIF4E2 (Supplementary Fig. 27e). Treatment with rapamycin, or silencing of eIF4E, significantly decreased the expression of rHRE-containing reporter mRNAs in normoxia but had no effect in hypoxia (Fig. 4b and Supplementary Fig. 29). Silencing any of the participants of the HIF-2α–RB M4–eIF4E2 cap-binding complex prevented the translation of rHRE-containing mRNAs in hypoxia but not in normoxia. Silencing of eIF4E decreased the rate of cellular protein synthesis in normoxia but had no effect in hypoxia (Fig. 4c and Supplementary Fig. 30). In stark contrast, depletion of eIF4E2 considerably limited the global rate of hypoxic translation without affecting protein synthesis in cells maintained in normoxia (Fig. 4c and Supplementary Fig. 30). These results demonstrate that oxygen tension regulates the cap-dependent protein synthesis machinery by switching from eIF4E- to eIF4E2-dependent translation in a HIF-2α-dependent manner (Supplementary Fig. 1).

Here we have identified a selective cap-dependent translation initiation mechanism that operates independently of eIF4E and that targets mRNAs for protein synthesis during hypoxia. The results suggest that the HIF-2α–RB M4–eIF4E2 complex is extensively involved in coordinating the translation response to low oxygen availability and is therefore essential in cellular oxygen homeostasis. This complex probably recruits functional homologues of the canonical eIF4E-dependent pathway, as well as distinct components, to initiate hypoxic protein synthesis. This process is regulated by the oxygen-sensing machinery first identified as the main regulator of the transcriptional response to hypoxia13–16. A human population that recently migrated to the Tibetan highlands contains a point mutation in the gene encoding HIF-2α (EPAS1), further emphasizing the evolutionary role of HIF-2α in the adaptation to high altitude and low oxygen tension27. The target mRNAs code for proteins such as EGFR, PDGFRα and IGF1R that are implicated in the adaptive response to hypoxia as well as a wide variety of biological processes including development and cancer. The role of these receptor tyrosine kinases in human malignancy.

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**Figure 4** | An oxygen-regulated switch from eIF4E- to eIF4E2-dependent protein synthesis. **a.** eIF4E and eIF4E2 polysome association in normoxia and hypoxia. **b.** Dual luciferase assays in normoxic (left) and hypoxic (right) cells transfected with constructs containing actin or EGFR 5′ UTRs and EGF or rHREs. Assays were performed on cells treated with rapamycin (rapa.), an inhibitor of mTOR and eIF4E (4E), and in knockdown cells of eIF4E, eIF4E2 (4E2), HIF-2α (H2α) or RB M4 and in a eIF4E–HIF-2α double knockdown. Significances of fold changes (Student’s t-test) are shown, P, parental. Results are means and s.e.m. (n = 3). **c.** Global translation rates in normoxic or hypoxic eIF4E or eIF4E2 knockdown cells. Experiments performed in U87MG glioblastoma.

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1. HIF-2α-mediated selective cap-dependent translation in hypoxia...
is particularly well documented and they are at the centre of targeted therapy\textsuperscript{11,28}. EGFR is often overproduced by tumours that harbour a wild-type EGFR gene, suggesting that cancer cells hijack the eIF4E2 pathway for their proliferative advantage\textsuperscript{29,30}. The results shown here provide the foundation for further investigation of the adaptive properties of the basic protein synthesis machinery in response to environmental conditions.

**METHODS SUMMARY**

With the exception of the human renal proximal tubular epithelial cells (a gift from C. Kennedy), cell lines were obtained from the American Type Culture Collection and propagated as suggested and maintained in epithelial cell medium (ScienCell). Hypoxia was induced by incubation for 24 h at 37 °C in a 1% O\textsubscript{2}, 5% CO\textsubscript{2} and N\textsubscript{2}-balanced atmosphere unless otherwise indicated. Polyosme analysis was performed as described previously\textsuperscript{12}, with the addition that proteins were isolated by precipitation with trichloroacetic acid and analysed by western blotting. Generation of luciferase constructs, RNA isolation, polymerase chain reaction and polyosme analysis are outlined in Supplementary Methods. All short interfering RNA, short hairpin RNA and adenoviral infections were performed with commercially available products and are further described in Supplementary Methods. PAR-CLIP analysis was performed as described previously\textsuperscript{31} and is further outlined in Supplementary Methods. Statistical analyses were performed with paired two-tailed Student’s t-tests.

The protocols for the following are described in detail in Supplementary Methods: western blotting, radioisotope labelling, RNA immunoprecipitation and cap-binding assays.

**Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.**

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METHODS

Cell culture and reagents. Human renal proximal tubular epithelial cells were maintained in epithelial cell medium (ScienCell). All other cell lines were obtained from the American Type Culture Collection and propagated as suggested. Cells were incubated at 37°C in a 5% CO₂ environment. Hypoxia was induced by incubation for 24 h at 37°C in a 1% O₂, 5% CO₂, and N₂-balanced atmosphere unless otherwise indicated. Heat shock was induced by incubating cells at 42°C for 30 min. Dimethylthiazolyl [35S]-Met incorporation. Protein synthesis by [35S]-Met incorporation. (WCL) is defined as 5% of the input used for immunoprecipitations.

Western blot analysis. Western blot analysis was performed using standard techniques. Monoclonal antibodies were used to detect EGFR (Ab-12; LabVision), green fluorescent protein (GFP; Roche), HIF-1α (Novus) and HIF-1β (Novus). Polyclonal antibodies were used to detect HIF-2α (Novus), actin (Sigma), L26 (Abcam), L5 (Abcam), S13 (Abcam), PDGFRα (Assay Biotech), IGFIR (Cell Signaling), RBM4 (ProteinTech Group), 4EBP1 (Cell Signaling), 4EBP1-P (Cell Signaling), AKT (Cell Signaling), AKT-P (Cell Signaling), EGFR-P (Cell Signaling), S6-P (Cell Signaling), eIF4E (Santa Cruz), eIF4E2 (Genetex) and eIF4G1 (Novus). Primary antibody against eIF4A1 was created in the laboratory of N. Sonenberg. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse (Amersham Biosciences) or anti-rabbit (Jackson ImmunoResearch). Bands were detected by enhanced chemiluminescence (Pierce). Whole cell lysate (WCL) is defined as 5% of the input used for immunoprecipitations.

Protein synthesis by [35S]-Met incorporation. Cells were grown for 48 h in 10-cm plates. Serum-free conditions supplemented with 1% insulin-transferrin-selenium (Invitrogen) were used when cells were incubated under hypoxia to detect stronger de novo RNA immunoprecipitation. Formaldehyde (1%) was added to cells for 30 min at 21°C. Glycine (200 mM) was added for 5 min to stop the reaction, followed by two washes with cold PBS. Cells were lysed in 1 ml of modified RIPA. RNase inhibitor (40 U ml⁻¹; Ambion) was added to modified RIPA just before use. Samples were sonicated at 50% amplitude for two cycles of 30 s (2 s on, 2 s off) with a 1-min pause between cycles. RNase treatment (12 μl of 20 mg ml⁻¹ DNase I, 25 mM MgCl₂, 5 mM CaCl₂) was performed for 30 min at 37°C. The reaction was stopped by adding 20 mM EDTA. For RNase-treated control samples, 5 μl of a 10 mg ml⁻¹ RNase A solution was added for 30 min at 37°C (RNase A; Fermentas). Samples were pre-cleared by using 10 μl of Dynabeads (Invitrogen) for 15 min at 4°C. Beads were removed by using a magnetic stand (Promega). Immunoprecipitation was performed at 2 μg ml⁻¹ primary antibody overnight at 4°C. Samples were centrifuged for 15 min at 12,000 g and 4°C. The supernatant was incubated with 20 μl of Dynabeads equilibrated for 1 h in 2% BSA at 4°C. Beads were recovered and washed five times with modified RIPA and eluted with 20 μl of 0.1 M glycine (pH 3.0). Bound proteins were removed by adding 200 μl NaCl and 20 μg of Proteinase K to the supernatant and incubating for 1 h at 42°C. Crosslinking was reversed by incubation overnight at 65°C. RNA extraction and RT-PCR analysis were performed to identify interacting RNA segments. Inputs were 2% of the sample. Primers are listed in Supplementary Table 1.

Adenoviral infections. Adenoviruses encoding GFP, HIF-1α and HIF-2α were generated and used as described previously. Analysis of cap-binding proteins. Cells on two 150-mm plates were washed with PBS and lysed in 1 ml of lysis buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 25 mM MgCl₂, 0.5% Nonidet P40, plus standard protease and phosphatase inhibitors). Extracts were clarified by centrifugation for 10 min at 10,000 g and 4°C. Supernatants were pre-cleared for 10 min with 30 μl of Sepharose 4B beads (Sigma) at 4°C. Beads were removed by centrifugation for 30 s at 500 g; supernatants were incubated for 1 h with 50 μl of 7-methyl GTP-Sepharose 4B beads (GE Healthcare) at 4°C. Pelleted beads were washed four times with 0.5 ml of lysis buffer and resuspended for 1 h in 0.6 ml of lysis buffer containing 1 mM GTP at 4°C. After four final washes with lysis buffer, the beads were resuspended in sample buffer and boiled for 1 min. Concentrated GTP wash, m⁷GTP-bound proteins and 5% input taken just before m⁷GTP beads were added were subjected to SDS–PAGE.

Statistical analysis. P values associated with all comparisons were based on paired two-tailed Student’s t-tests. Results are shown as means and s.e.m. (n = 3).

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