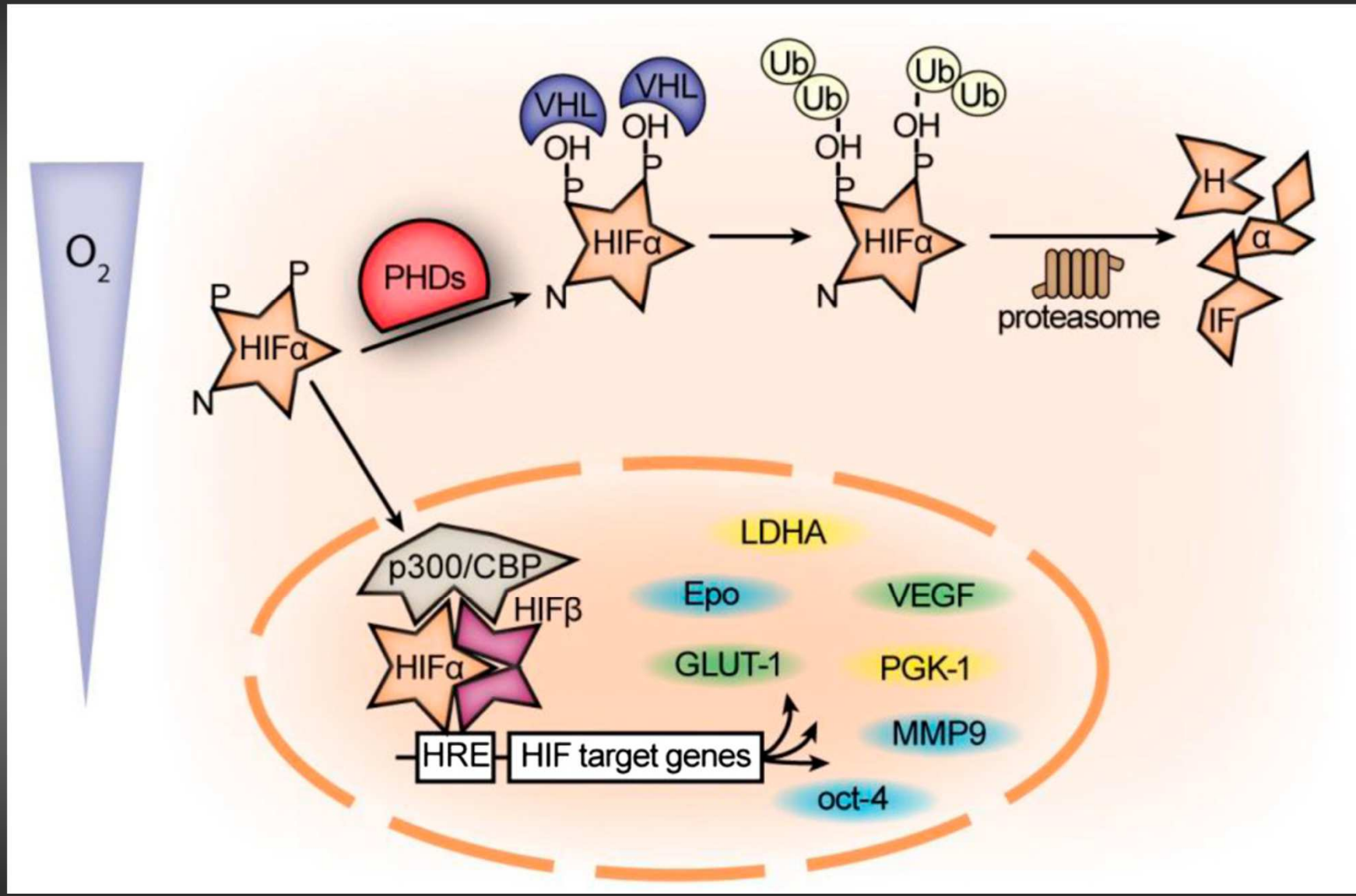
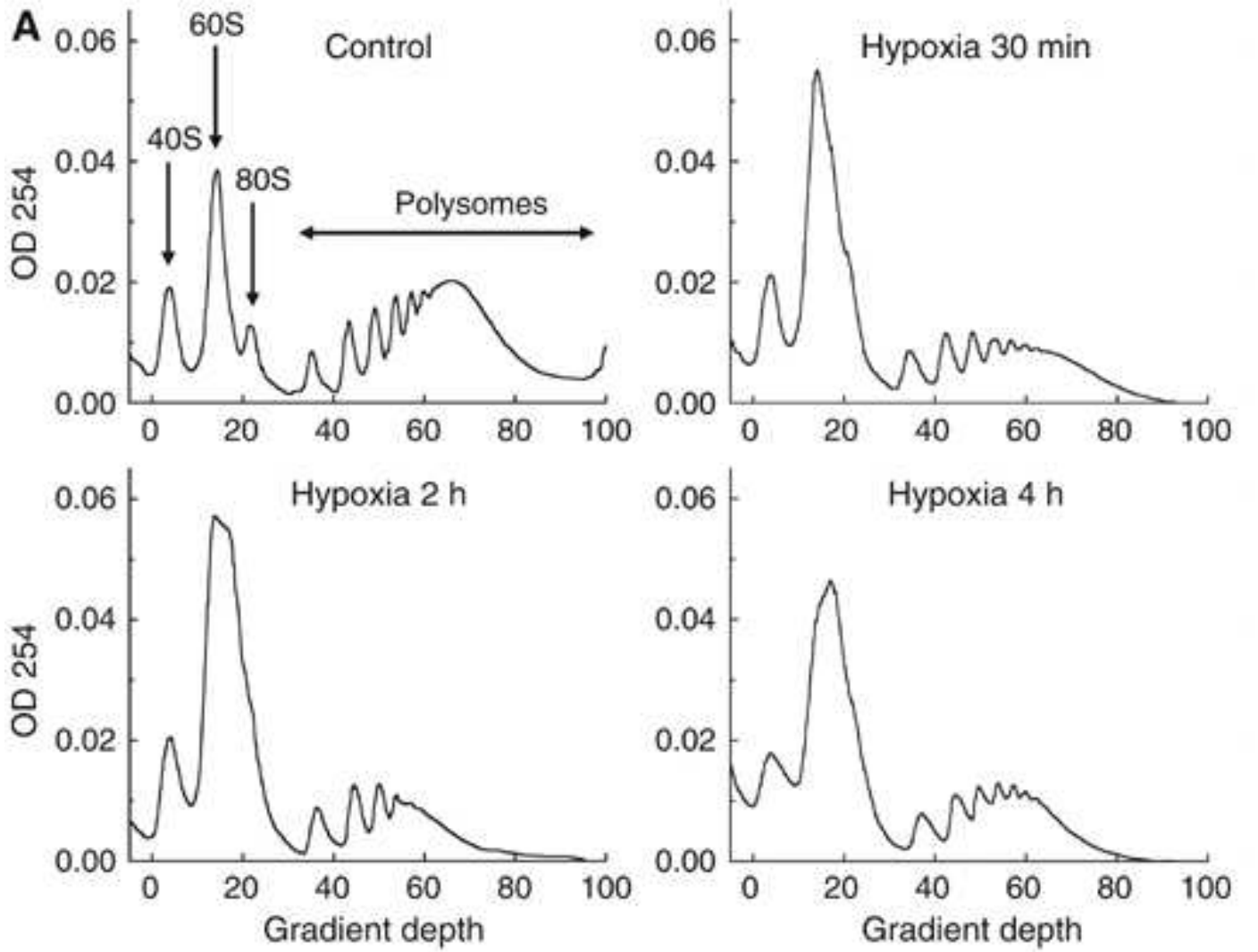

O2
HIFα
P
P
N
PHDs
VHL
VHL
OH
OH
P
P
HIFα
N
Ub
Ub
Ub
Ub
OH
OH
P
P
HIFα
N
proteasome
H
α
IF
p300/CBP
HIFβ
HIFα
HRE
HIF target genes
LDHA
Epo
VEGF
GLUT-1
PGK-1
MMP9
oct-4

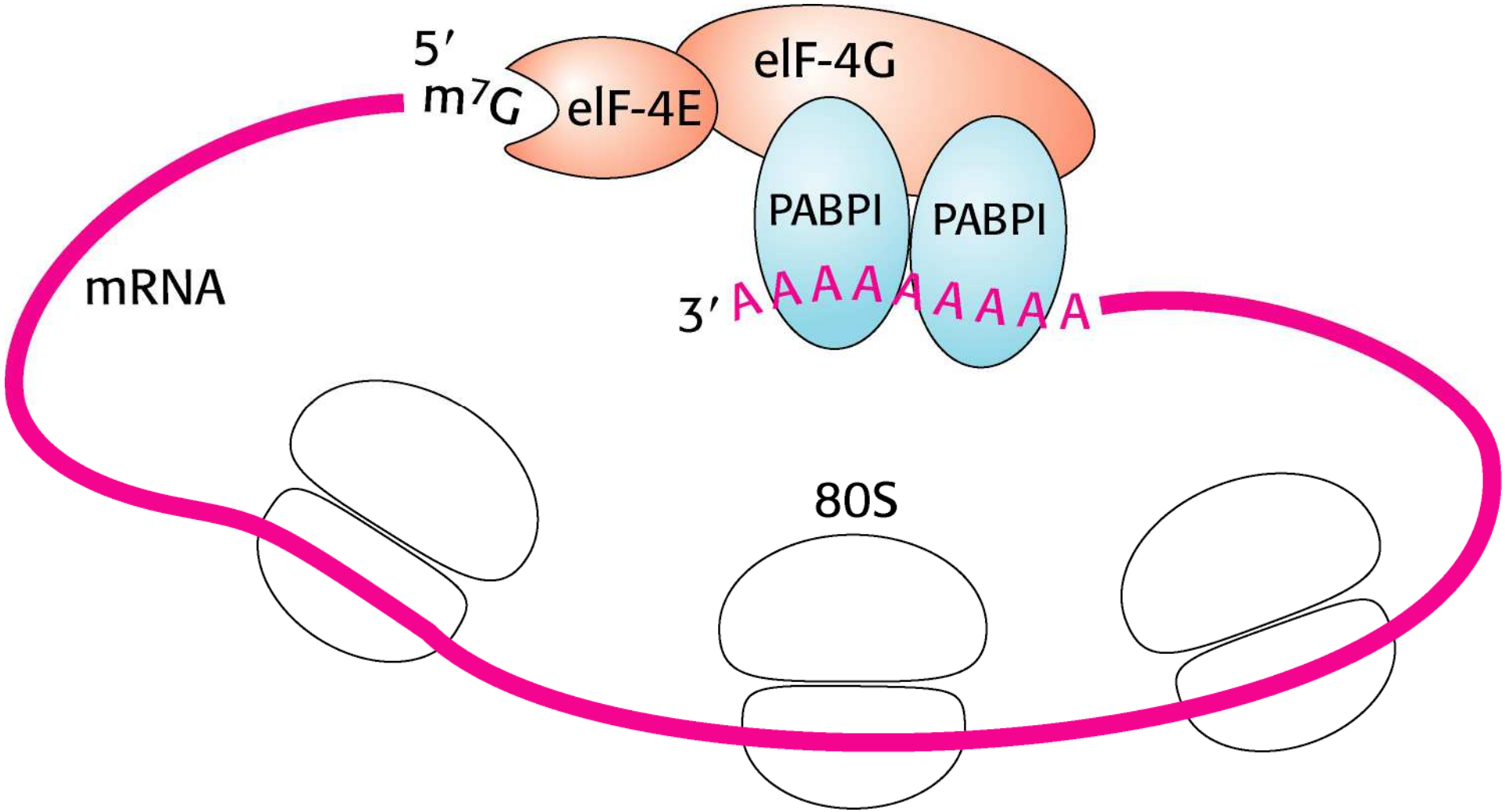
An oxygen-regulated switch in the protein synthesis machinery

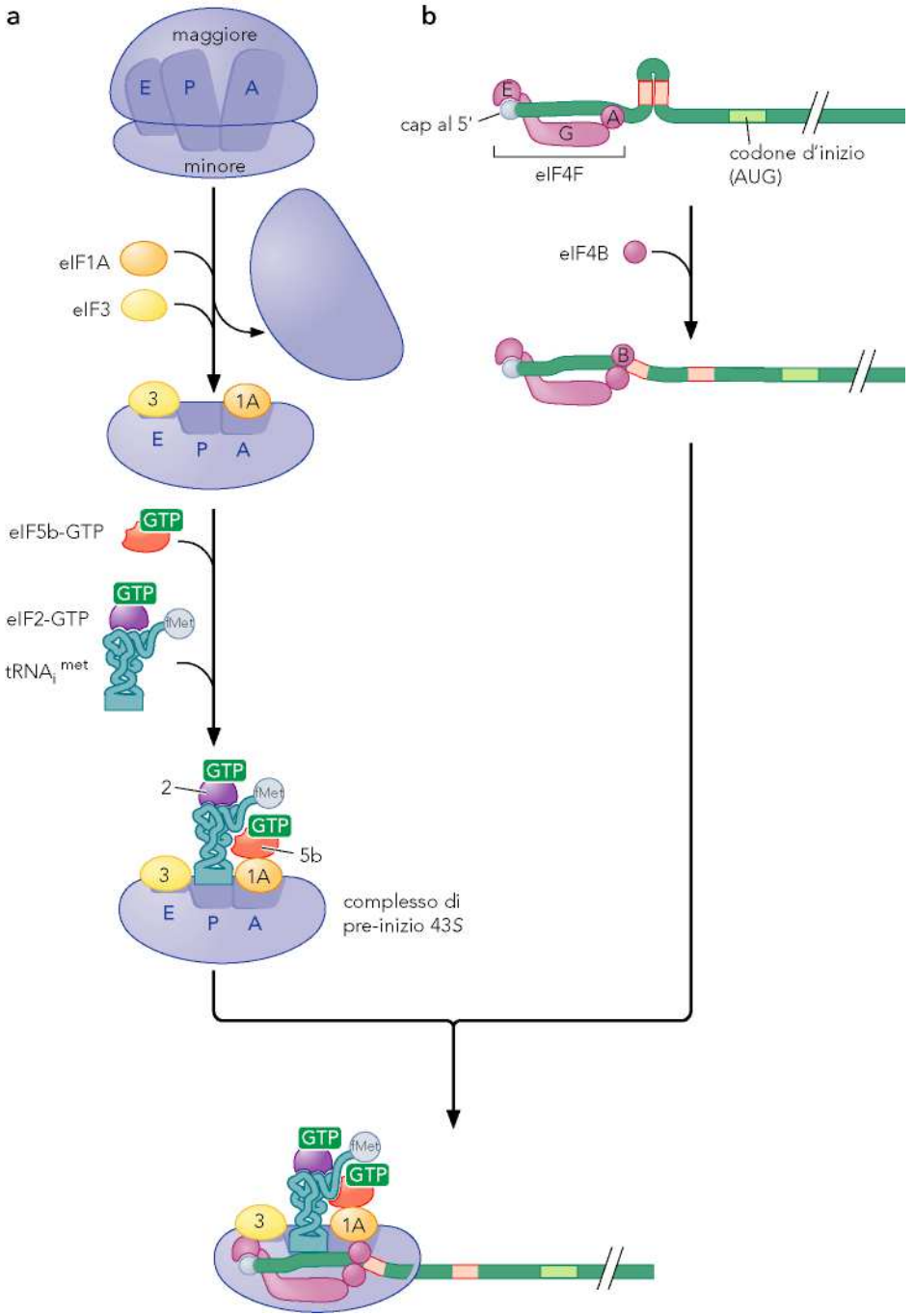
Hypoxia inhibits mRNA translation



- The initial step of protein synthesis is the binding of the eukaryotic translation initiation factor 4E (eIF4E) to the 7-methylguanosine (m7-GpppG) 5’ cap of messenger RNAs

.

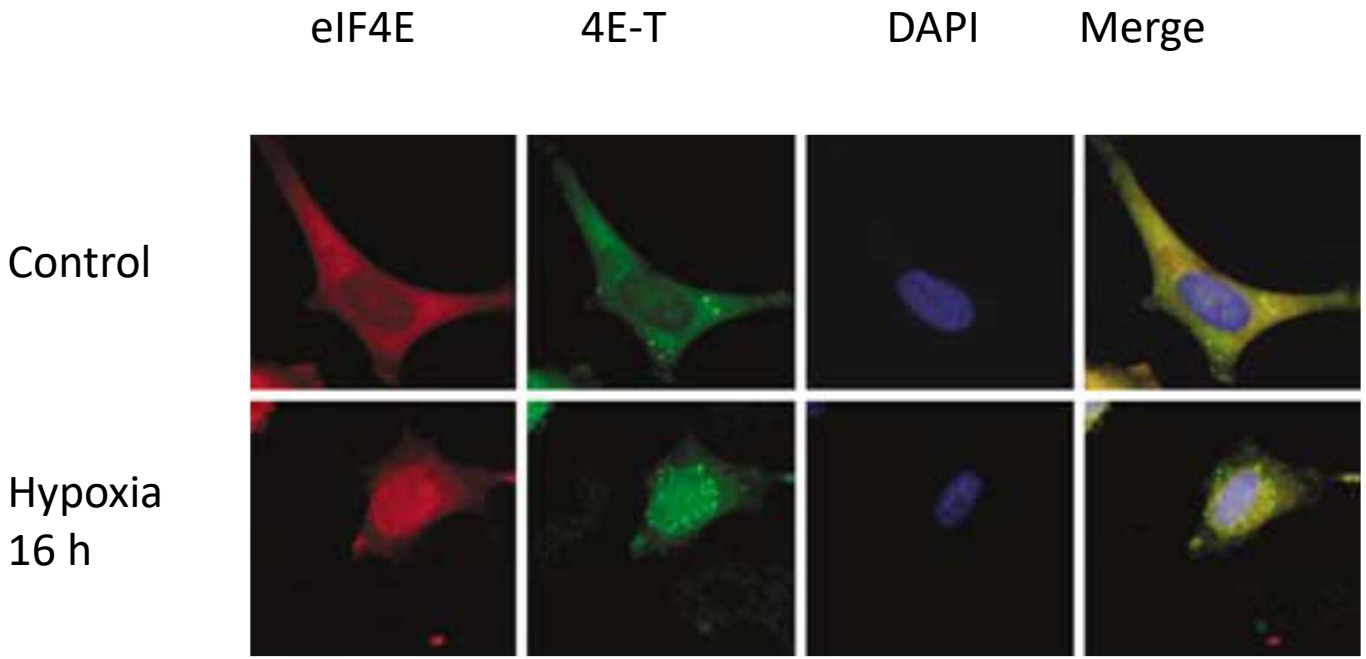




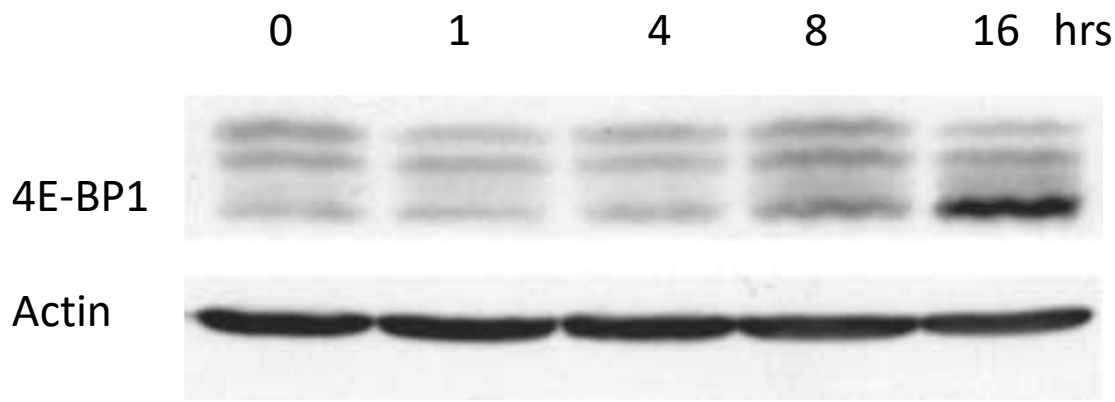
Low oxygen tension (hypoxia) represses cap-mediated translation by sequestering eIF4E.

.

eIF4E relocalizes during hypoxia

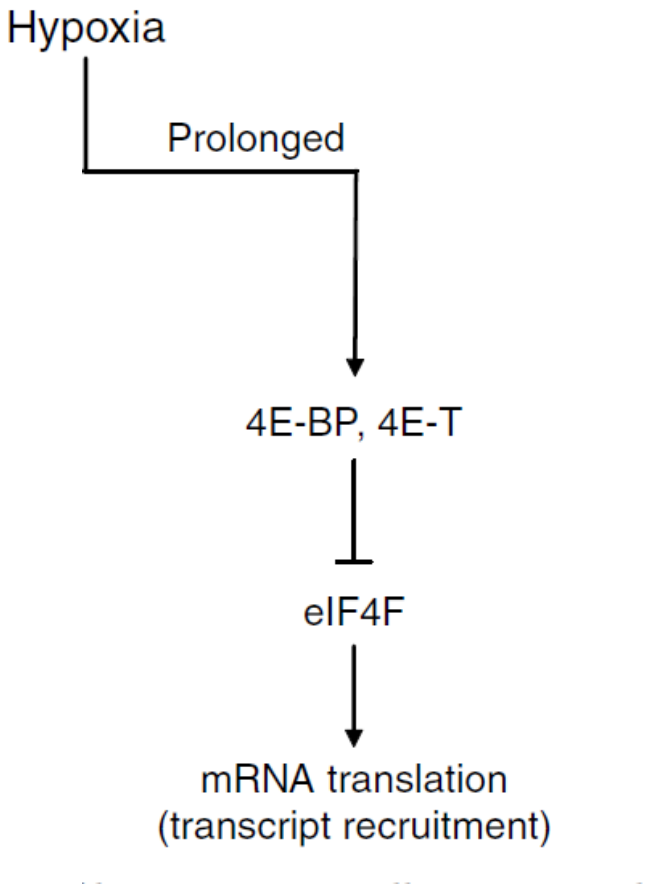


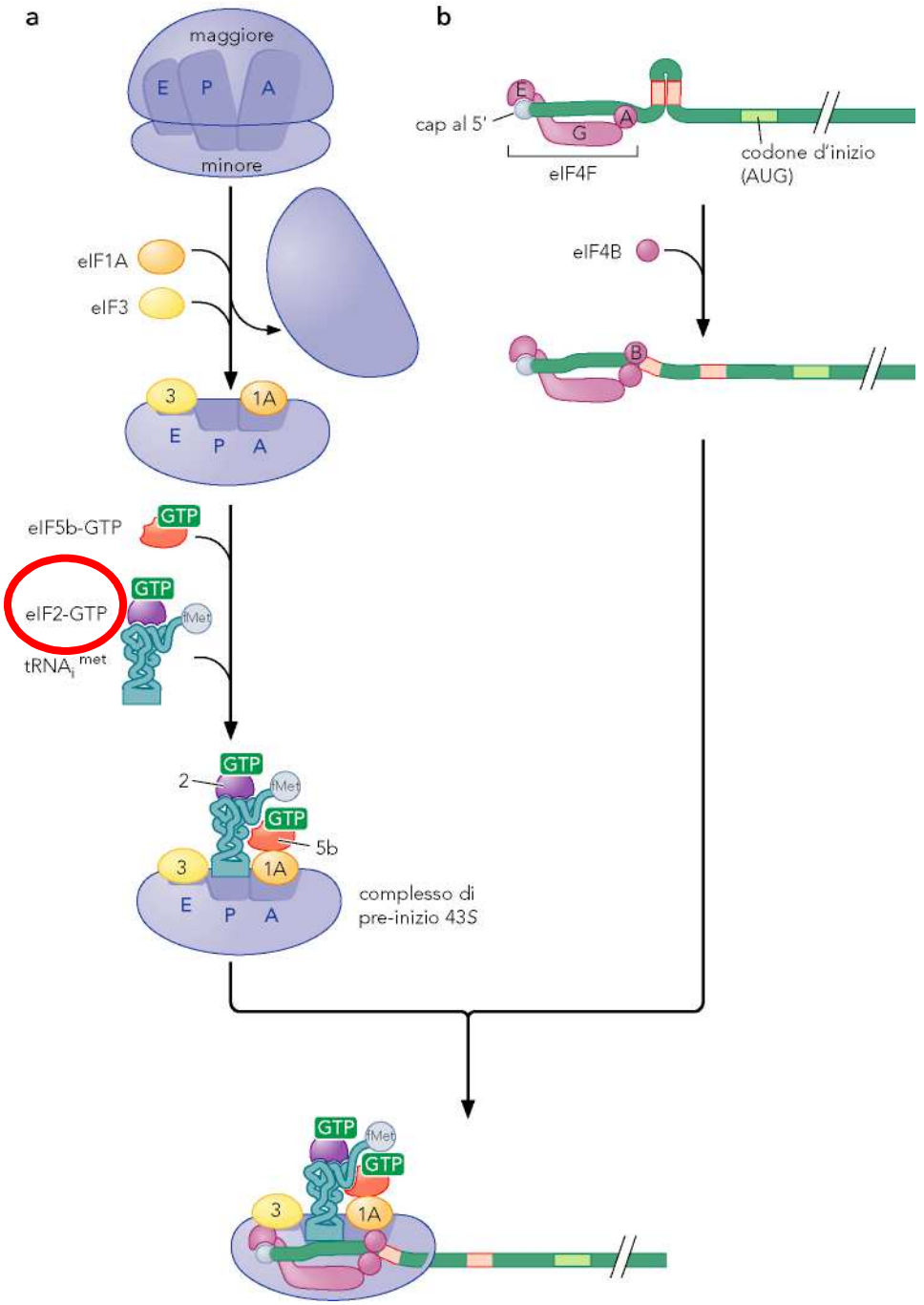
The shuttling protein 4E-T is a known regulator of eIF4E localization and is capable of binding and transporting it to the cell nucleus
Correlation with the gradual dephosphorylation of 4E-T



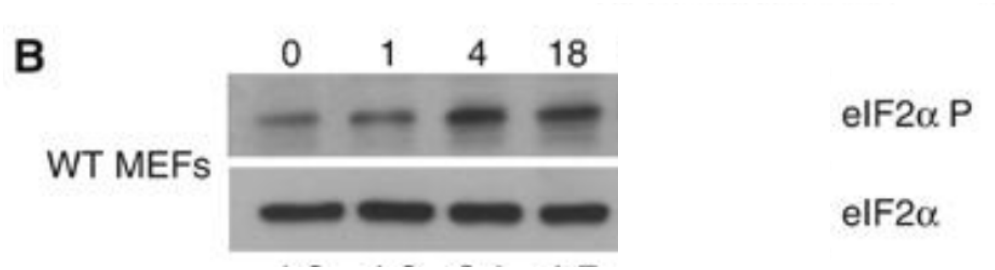
4E-BP1 an inactive complex shows both a small induction at 8 h and a strong dephosphorylation after 16 h of hypoxia

effects of hypoxia on mRNA translation



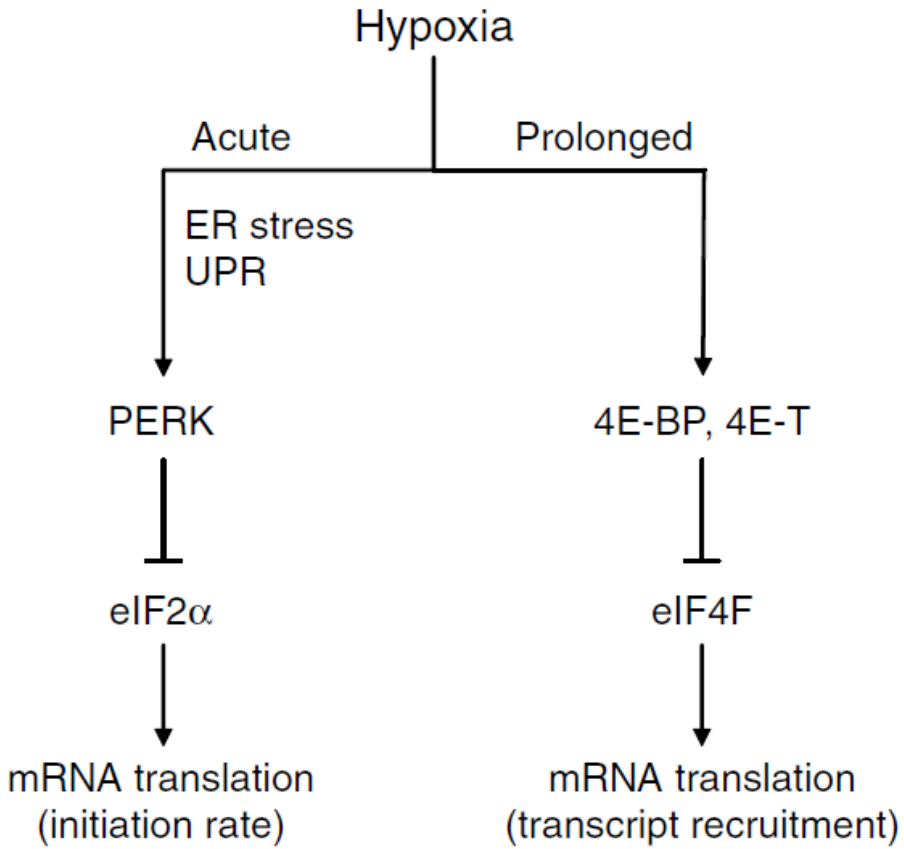


Inhibition of translation during acute hypoxia is mediated by eIF2α phosphorilation



mouse embryo fibroblasts (MEFs)

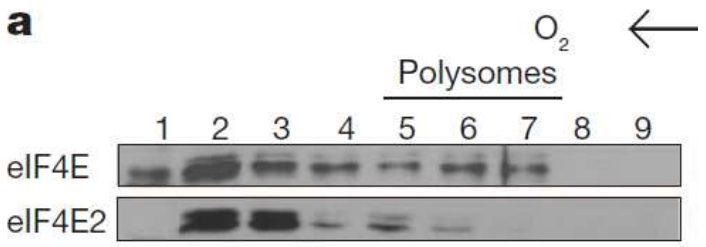
effects of hypoxia on mRNA translation



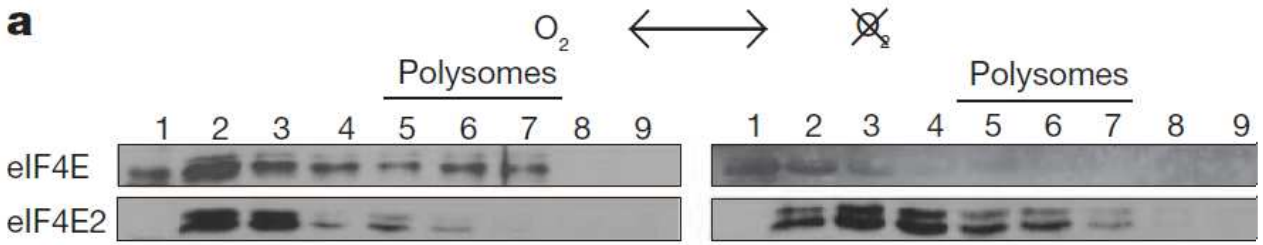
Acute hypoxia causes transient eIF2a phosphorylation due to PERK activation as a part of the UPR. This results in inhibition of the rate of translation initiation.
Following prolonged hypoxic conditions, activation of 4E-BP and 4E-T causes disruption of eIF4F, which inhibits the recruitment of mRNA to polysomes.
Both molecular mechanisms affect specific mRNAs to varying degrees, resulting in differential gene expression.

- A fundamental question in biology is as to how proteins are synthesized in periods of oxygen scarcity and eIF4E inhibition.

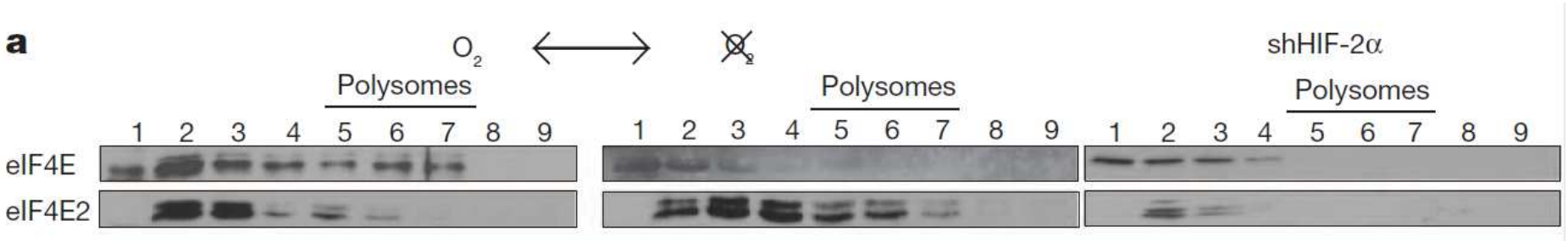
An oxygen-regulated switch from eIF4E- to eIF4E2-dependent protein synthesis.



An oxygen-regulated switch from eIF4E- to eIF4E2-dependent protein synthesis.



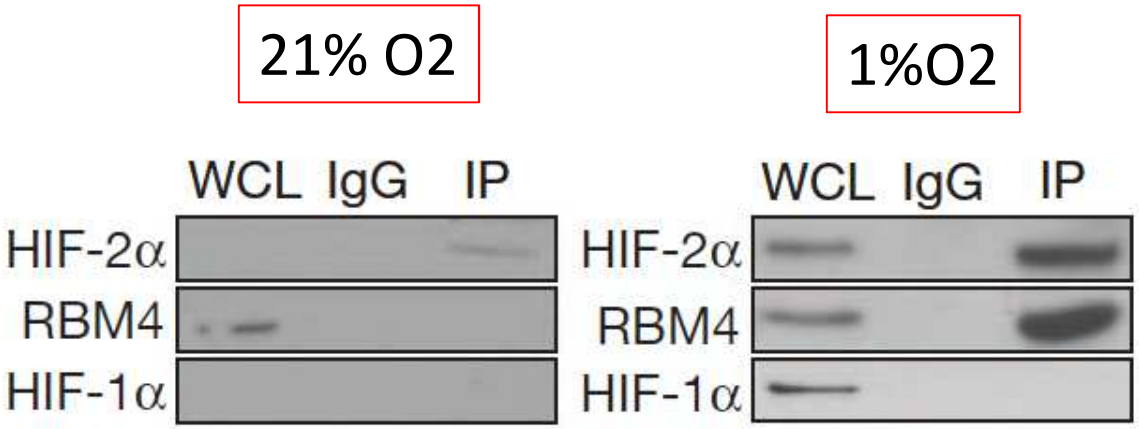
eIF4E and eIF4E2 polysome association in normoxia and hypoxia



hypoxia stimulates the switch from the cap-bindingeIF4E to to eIF4E2 homologue dependent from the oxygen-regulated hypoxia-inducible factor 2a (HIF-2a)

RNA-binding protein RBM4 recruits HIF-2ain hypoxia

Co-immunoprecipitation of HIF-2a



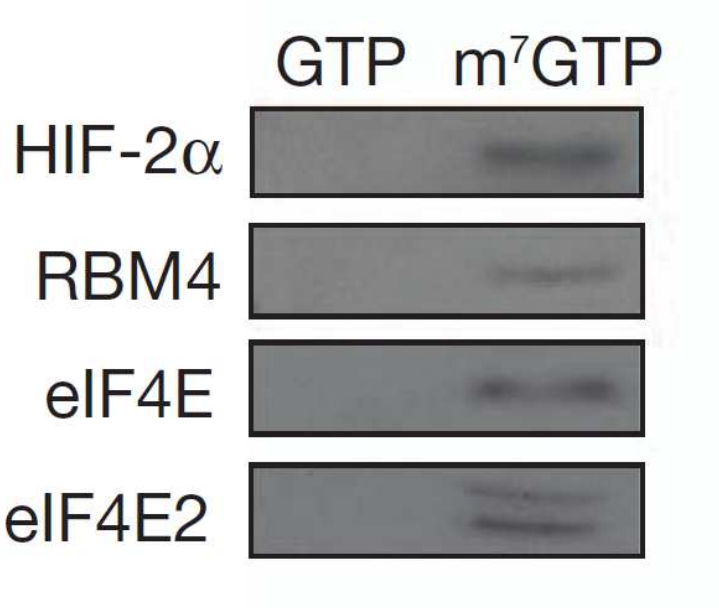
Co-immunoprecipitation of HIF-2a with RBM4 in hypoxia (right)

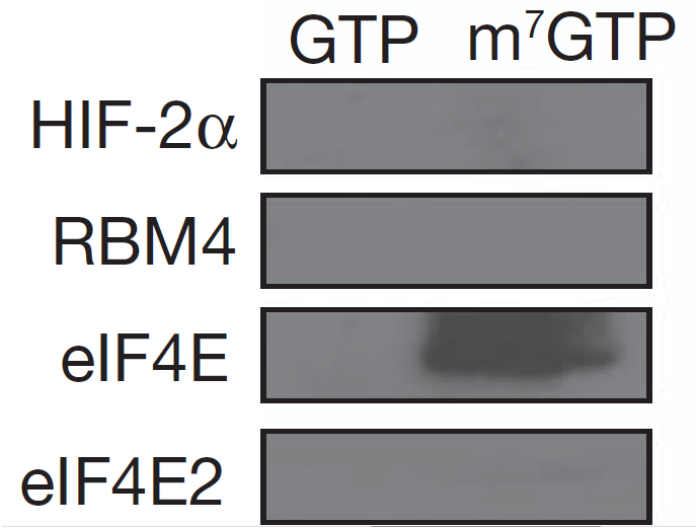
WCL, whole cell lysate

RNA-binding protein RBM4
oxygen-regulated hypoxia-inducible factor 2a (HIF-2a)

HIF-2a–RBM4 recruits the m7-GTP cap by means of an interaction with eIF4E2

Capture assays using m7-GTP beads in hypoxic cell lysates



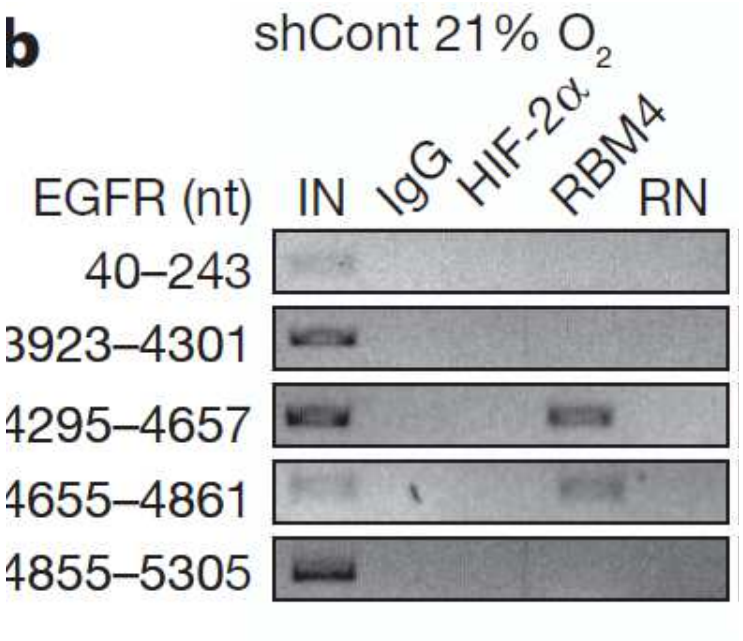


depleted in eIF4E2

GTP, proteins dislodged from the beads by GTP; m7GTP, proteins bound to m7-GTP beads after GTP wash

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

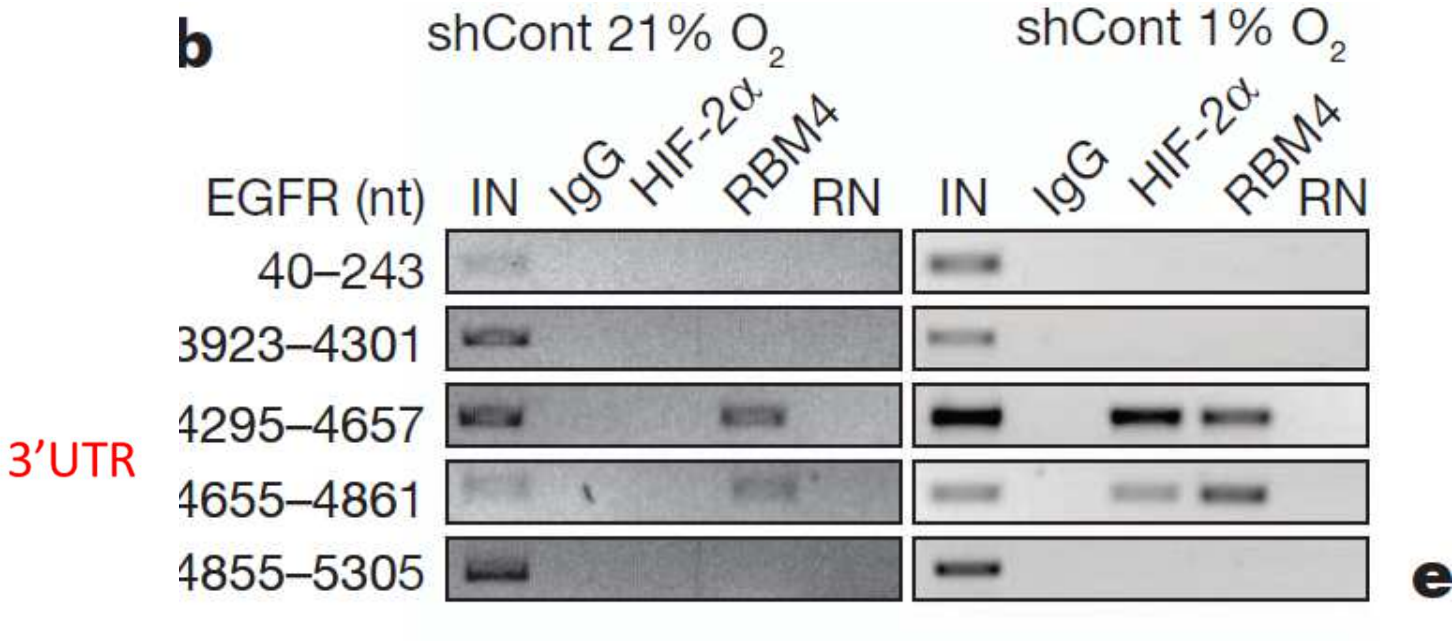
RBM4 recruits HIF-2a to the 3’UTR for hypoxic translation



3’UTR

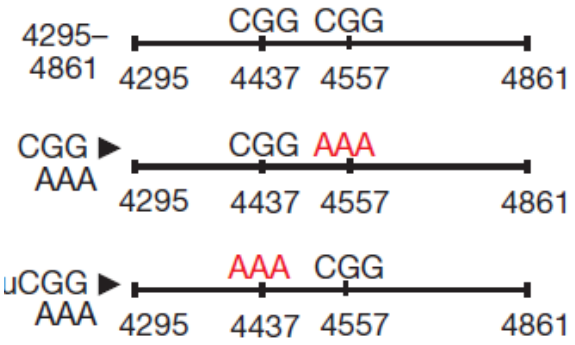
RNA immunoprecipitation of HIF-2a and RBM4
IN, input; nt, nucleotides; RN, RNase-treated

RBM4 recruits HIF-2a to the 3'UTR for hypoxic translation

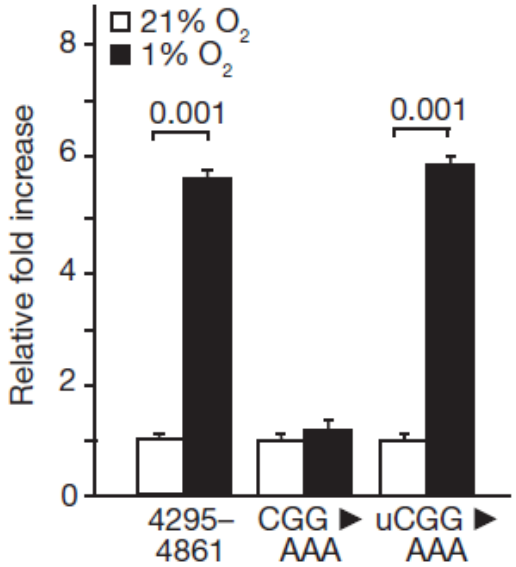


RNA immunoprecipitation of HIF-2a and RBM4 in HIF-2a or RBM4 knockdown cells.
IN, input; nt, nucleotides; RN, RNase-treated

Expression of CGGRAAA mutation near RBM4 crosslinking sites or in an unrelated upstream region (uCGG)

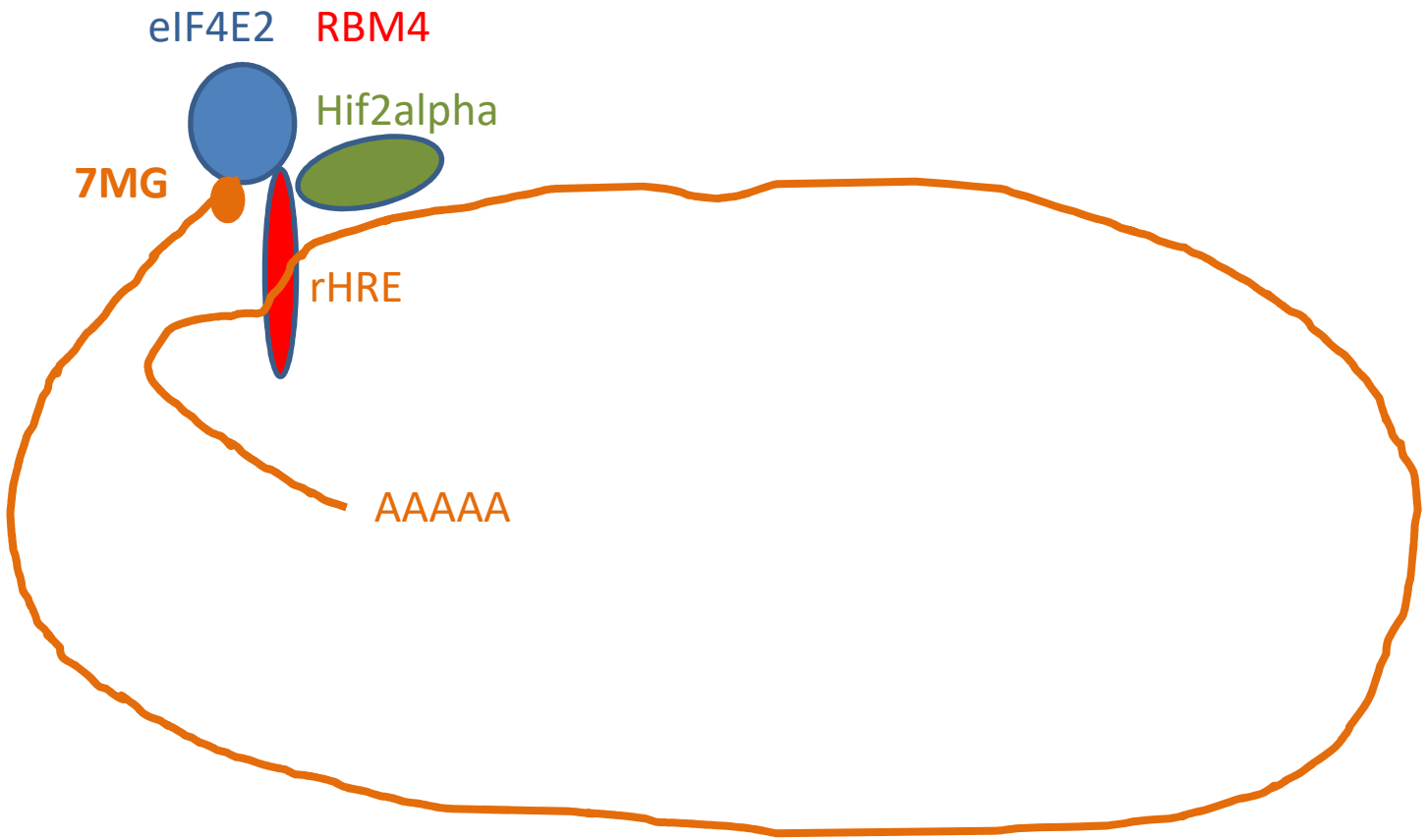


RNA hypoxia response element (rHRE)

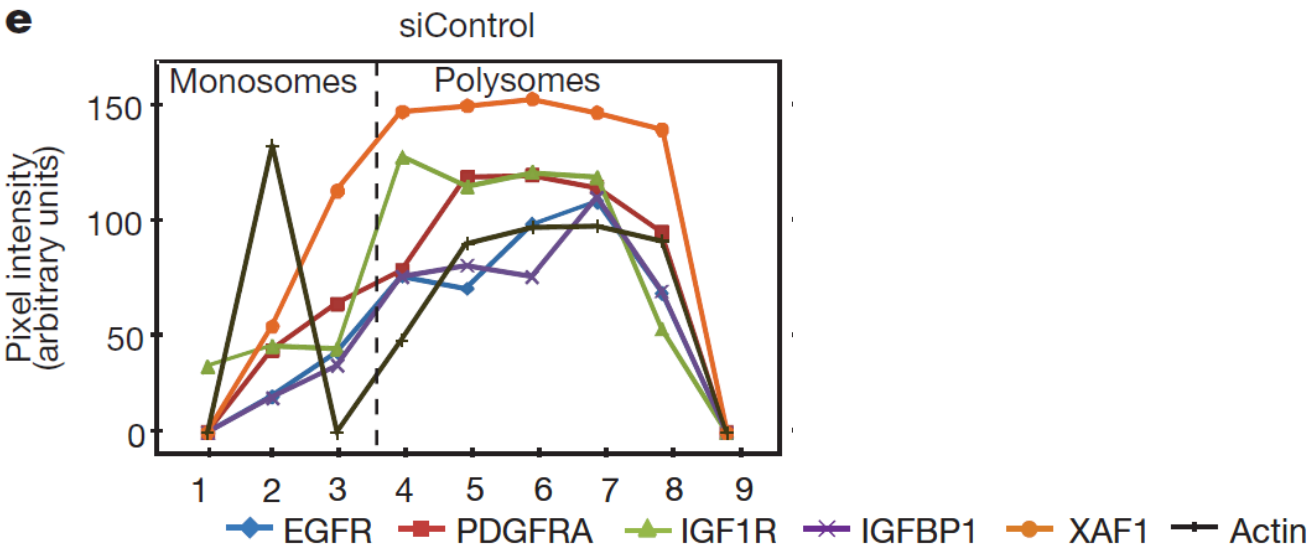


- Ribonucleoside-enhanced crosslinking and immunoprecipitation analysis identified an RNA hypoxia response element (rHRE) that recruits this complex to a wide array of mRNAs, including that encoding the epidermal growth factor receptor.

Complesso Quaternario: mRNA, Fattore alternativo che riconosce il Cap (eiF4E2), Fattore secondo che risponde all'ipossia (hif2alpha) e proteina che lega RNA RBM4

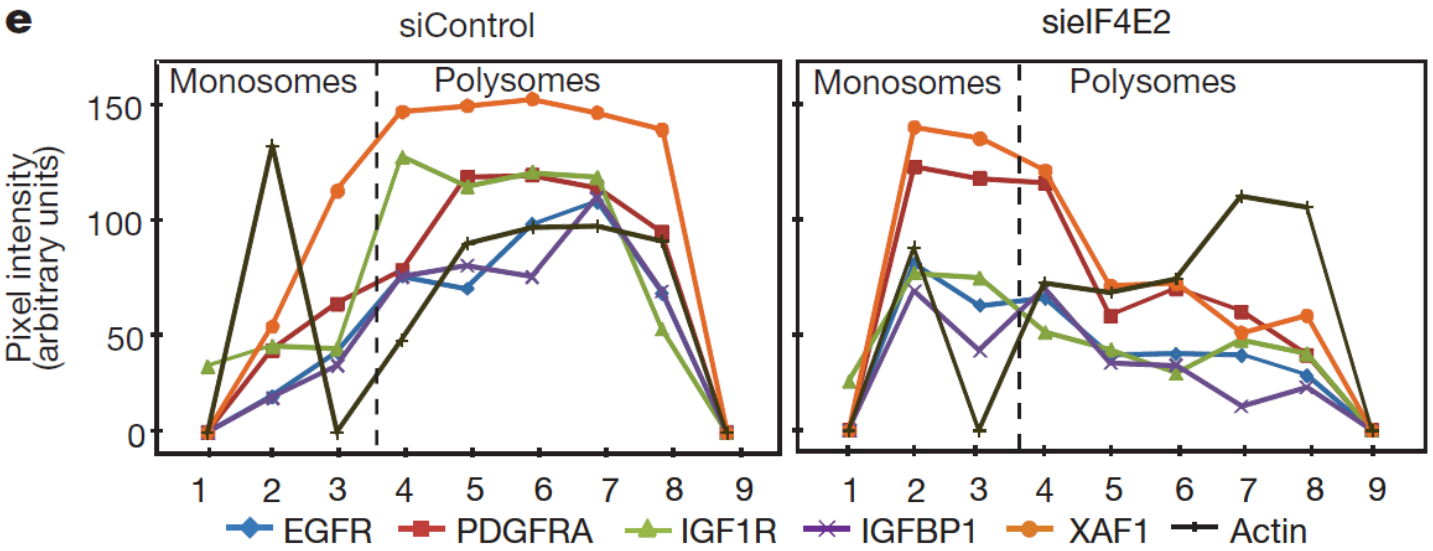


Polysomal distribution of mRNA coding for HIF-2a–RBM4 targets in hypoxic eIF4E2 knockdown cells



the HIF-2a–RBM4–eIF4E2 complex captures the 5’ cap and targets mRNAs to polysomes for active translation

Polysomal distribution of mRNA coding for HIF-2a–RBM4 targets in hypoxic eIF4E2 knockdown cells



the HIF-2a–RBM4–eIF4E2 complex captures the 5’ cap and targets mRNAs to polysomes for active translation

- 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.

- Here we describe an oxygen-regulated translation initiation complex that mediates selective cap-dependent protein synthesis.

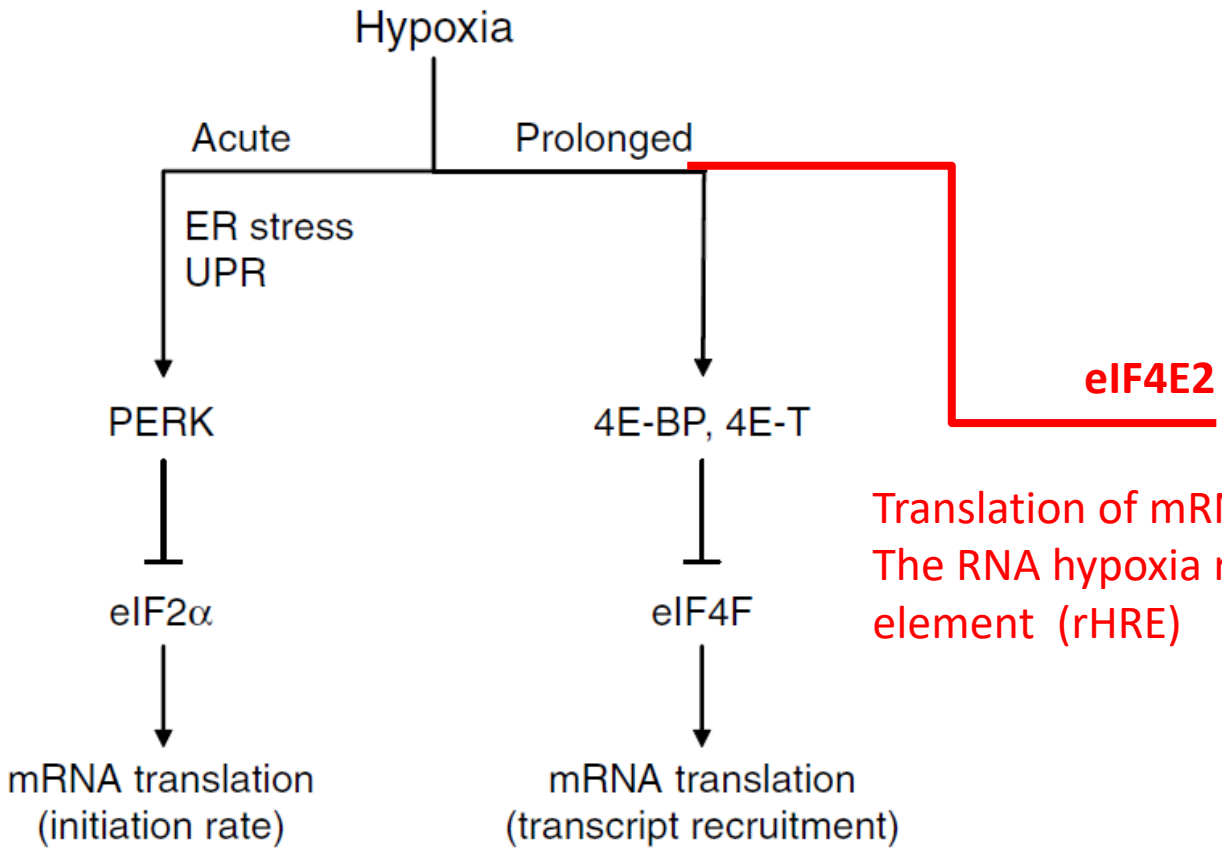
- We show that hypoxia stimulates the formation of a complex that includes the oxygen-regulated hypoxia-inducible factor 2a (HIF-2a), the RNA-binding protein RBM4 and the cap-binding eIF4E2, an eIF4E homologue.

- Ribonucleoside-enhanced crosslinking and immunoprecipitation 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.

effects of hypoxia on mRNA translation



Translation of mRNA containing
The RNA hypoxia response
element (rHRE)

The role of EGFR in hypoxia

Irving Donadon
Università degli Studi di Ferrara

17/05/2017

1 – Hypoxia-induced effects

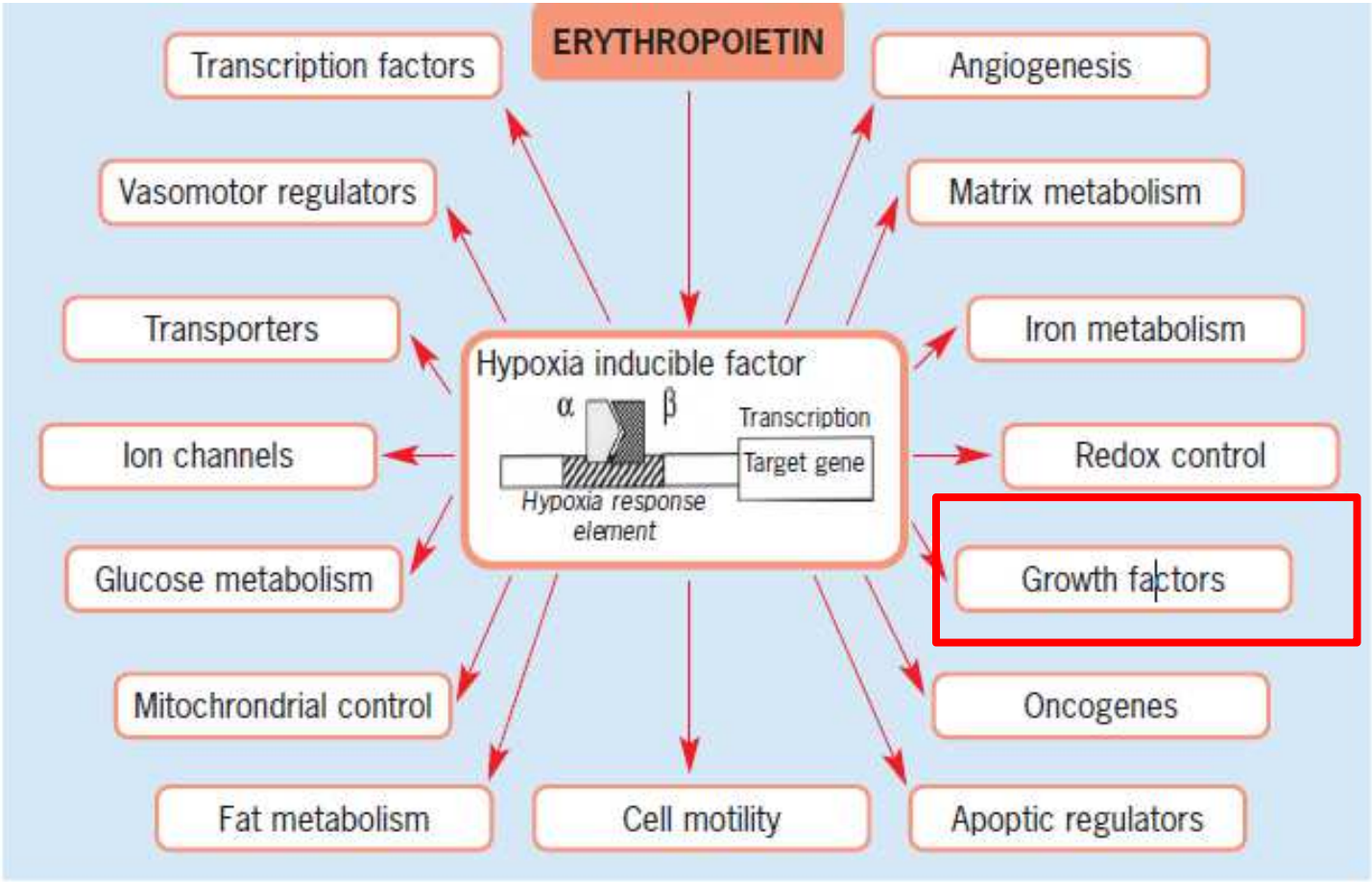


Fig 1. The hypoxia-inducible factor (HIF) transcriptional cascade directly regulates genes with key functions in a broad range of processes. The complex binds in a sequence-specific manner to control elements in DNA, termed hypoxia-response elements, at target gene loci.

2 – EGFR (epidermal growth factor receptor)

- Growth factor receptor;

- induces cell differentiation and proliferation;

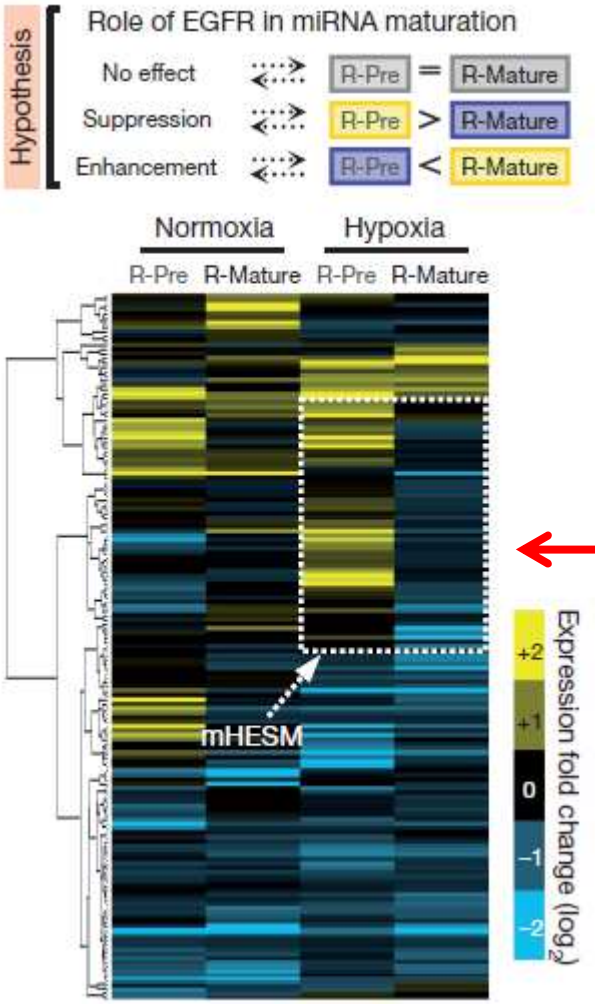
- **tyrosine kinase** → phosphorylation of intracellular substrates → leads to cell growth, DNA synthesis and expression of oncogenes.

=> EGFR is thought to be involved into the development of cancer, as the EGFR gene is often amplified, and/or mutated in cancer cells.

Hypoxia is known to **upregulate EGFR**.

=> EGFR upregulation compromises miRNA maturation.

3 - EGFR role in miRNA maturation

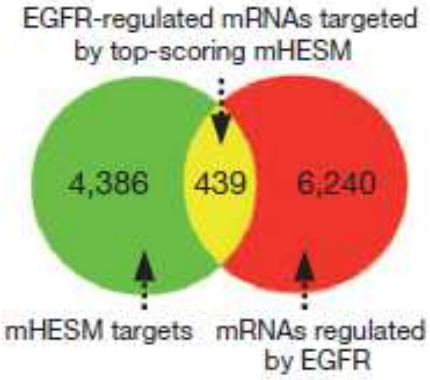


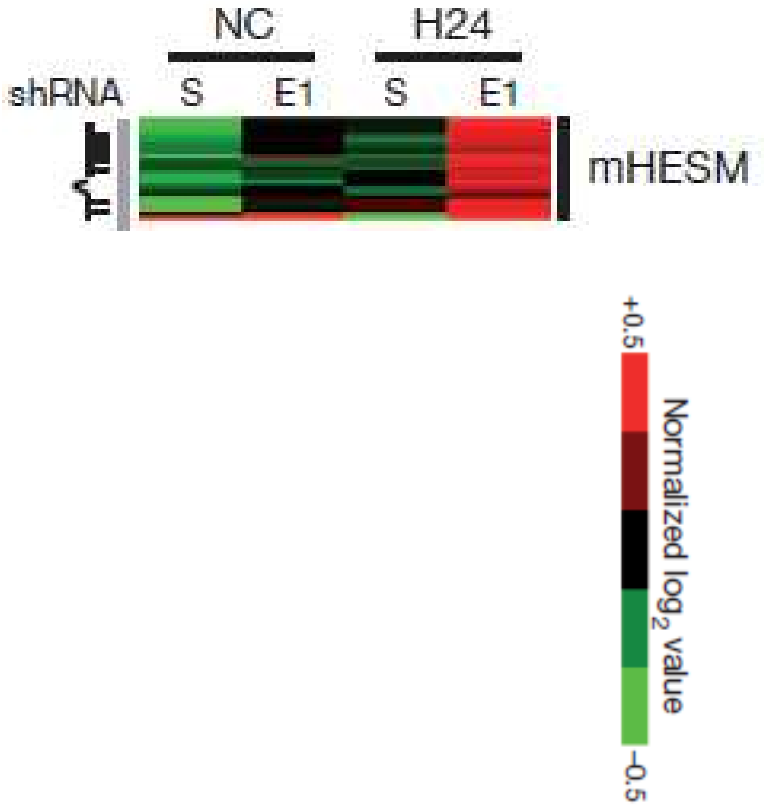
hierarchical clustering analysis

Identification of a distinct cluster of miRNA affected by EGFR under hypoxia (**mHESM**).

4 - mHESM targets

S = Scrambled control
E = EGFR shRNA

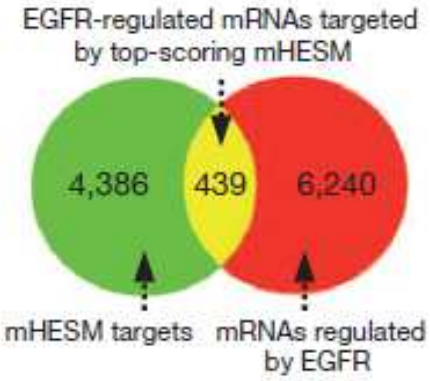




Under hypoxia, silencing of EGFR is related to mHESM maturation.

4 - mHESM targets

S = Scrambled control
E = EGFR shRNA

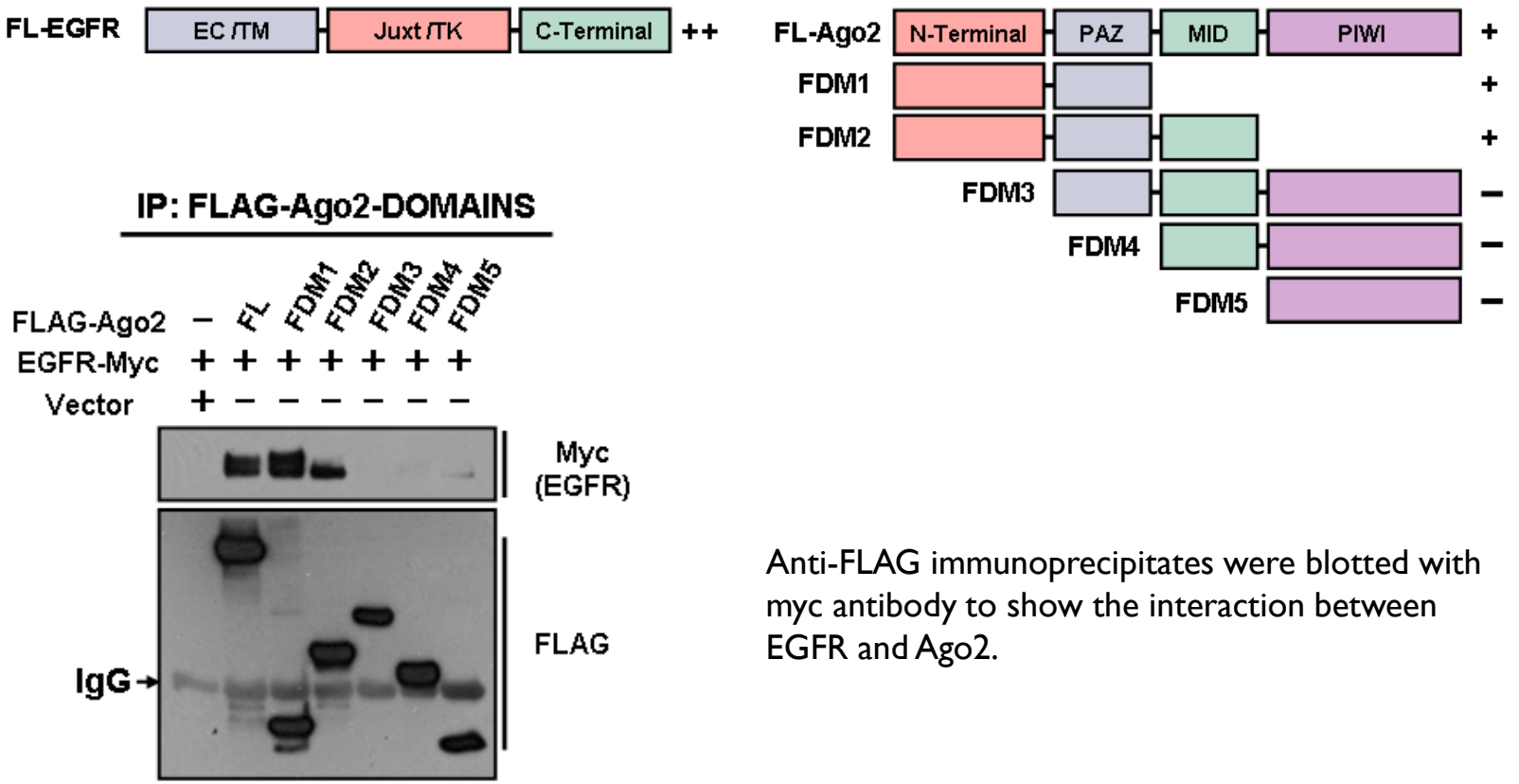


In response to hypoxia, EGFR reduces the production of mHESM enhancing the expression of corresponding mRNA targets.

AE = average expression

How does EGFR compromise miRNA maturation?

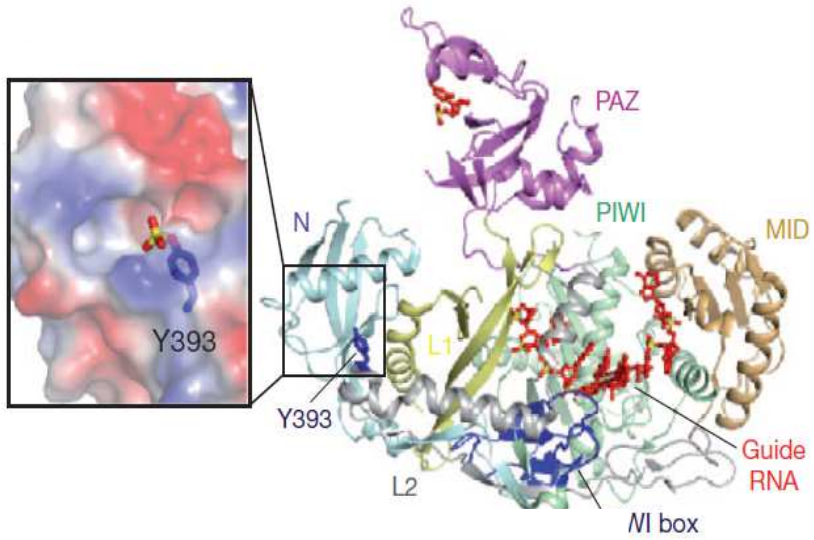
5 - EGFR-AGO2 interaction



Anti-FLAG immunoprecipitates were blotted with myc antibody to show the interaction between EGFR and Ago2.

Under hypoxia, EGFR interacts with the **N-terminal** region of AGO2.

7 – Highly conserved Tyr in AGO2



393

D P Y V R E F G	Hs_AGO2
D P Y I Q E F G	Hs_AGO1
D P Y L K E F G	Hs_AGO4
D N Y A G E F G	At_AGO1
D S Y V Q E F G	Dm_AGO1
D T Y L T Q Y G	Sp_AGO1
D P F V Q E F Q	Hs_AGO3
D O F A H E F G	Ce_ALG1
E K E E S S A P	Kp_AGO1
S L T L G K F K	Nc_QDE2

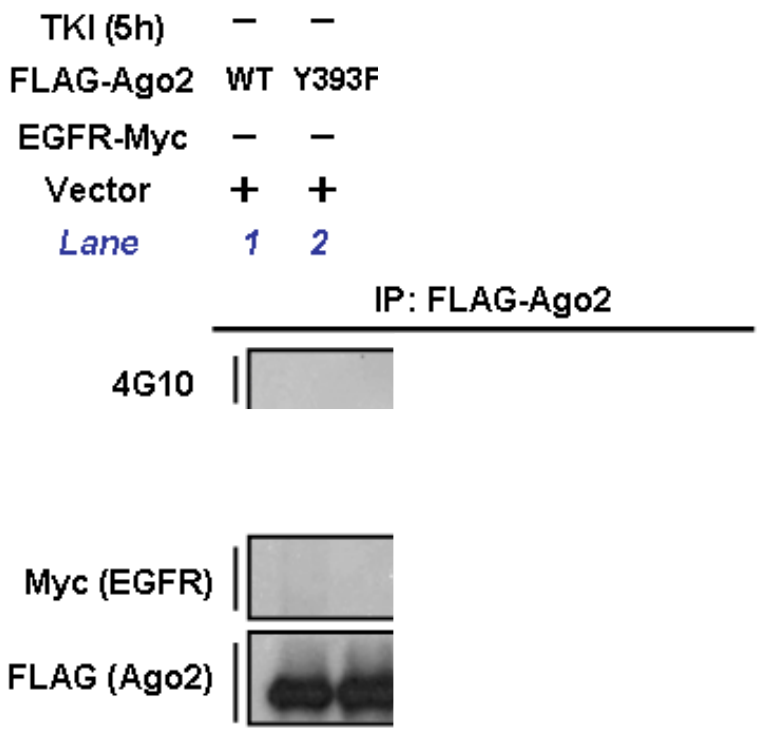
DPYVREFG	Hs_AGO2
DPYVREFG	Pt_AGO2
DPYVREFG	Bt_AGO2
DPYVREFG	Mm_AGO2
DPYVREFG	Rn_AGO2
DPYVREFG	Dr_AGO2

Identified one highly conserved residue in AGO2 (**Tyr393**) as potential site for EGFR kinase activity.

8 - EGFR kinase activity on AGO2

4G10 = Anti-phosphotyrosine antibody

Under hypoxia.



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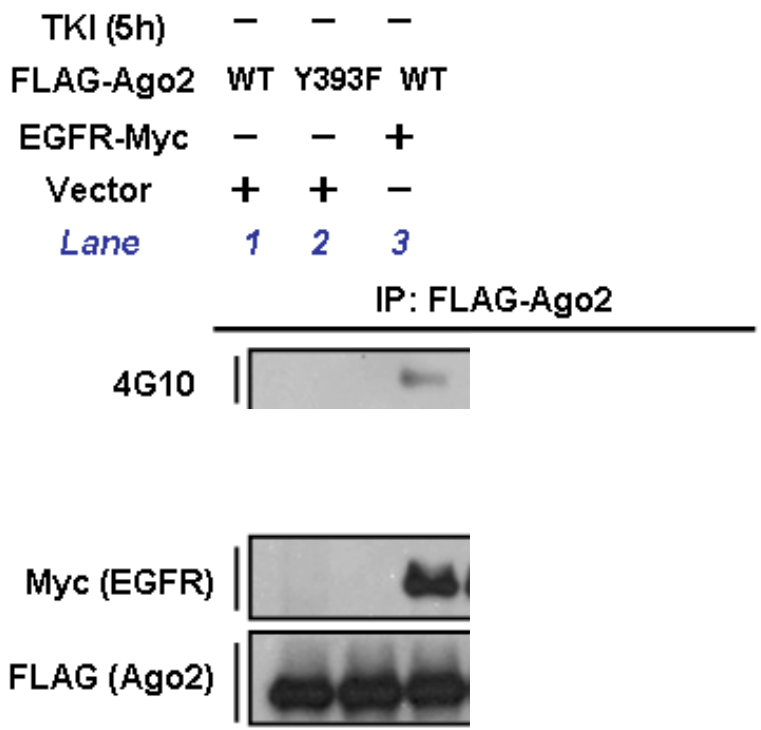

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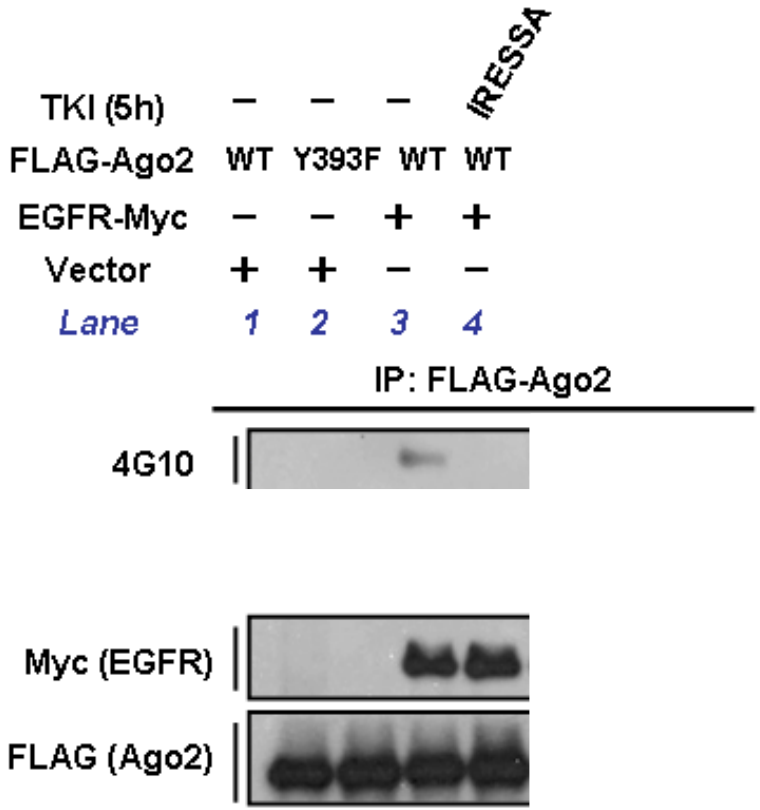
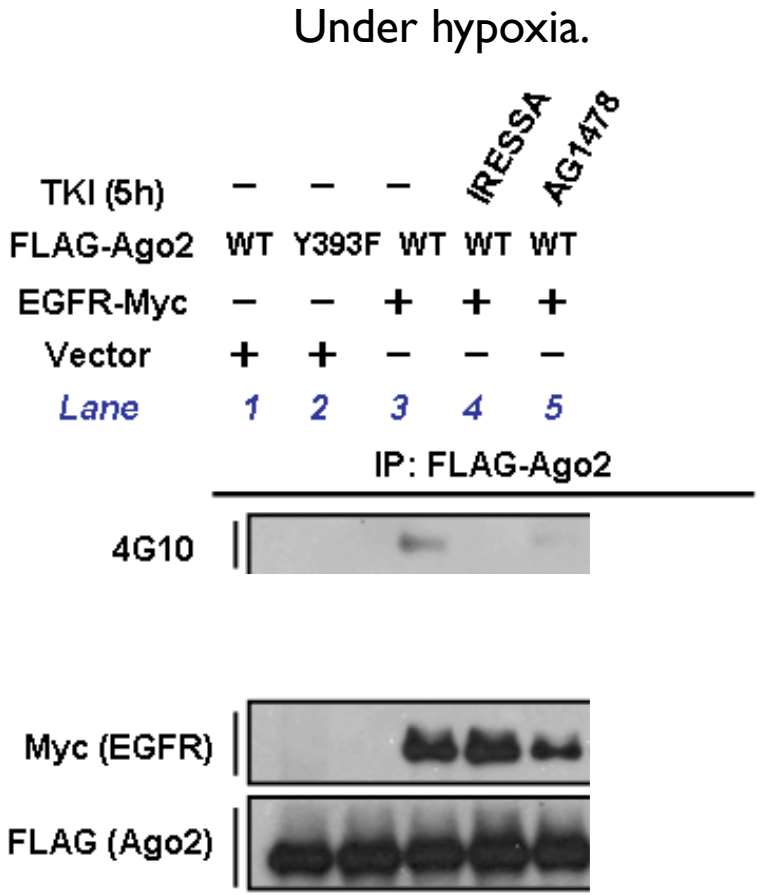
Under hypoxia.



4G10 = Anti-phosphotyrosine antibody

IRESSA is a Tyr Kinase Inhibitor

8 - EGFR kinase activity on AGO2

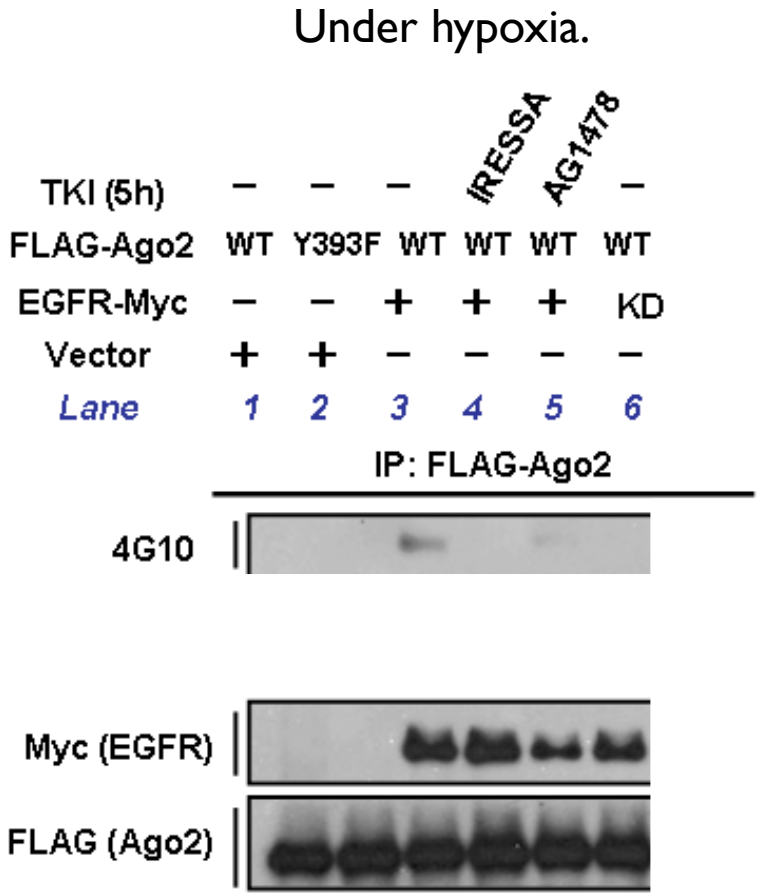


4G10 = Anti-phosphotyrosine antibody

IRESSA is a Tyr Kinase Inhibitor

AG-1478 is a selective EGFR inhibitor

8 - EGFR kinase activity on AGO2



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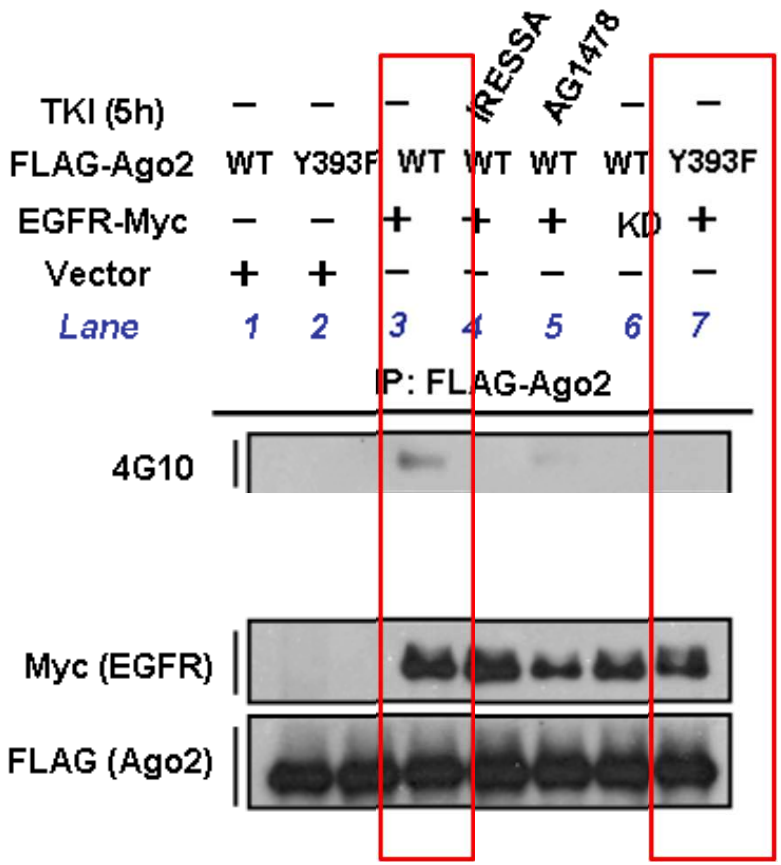
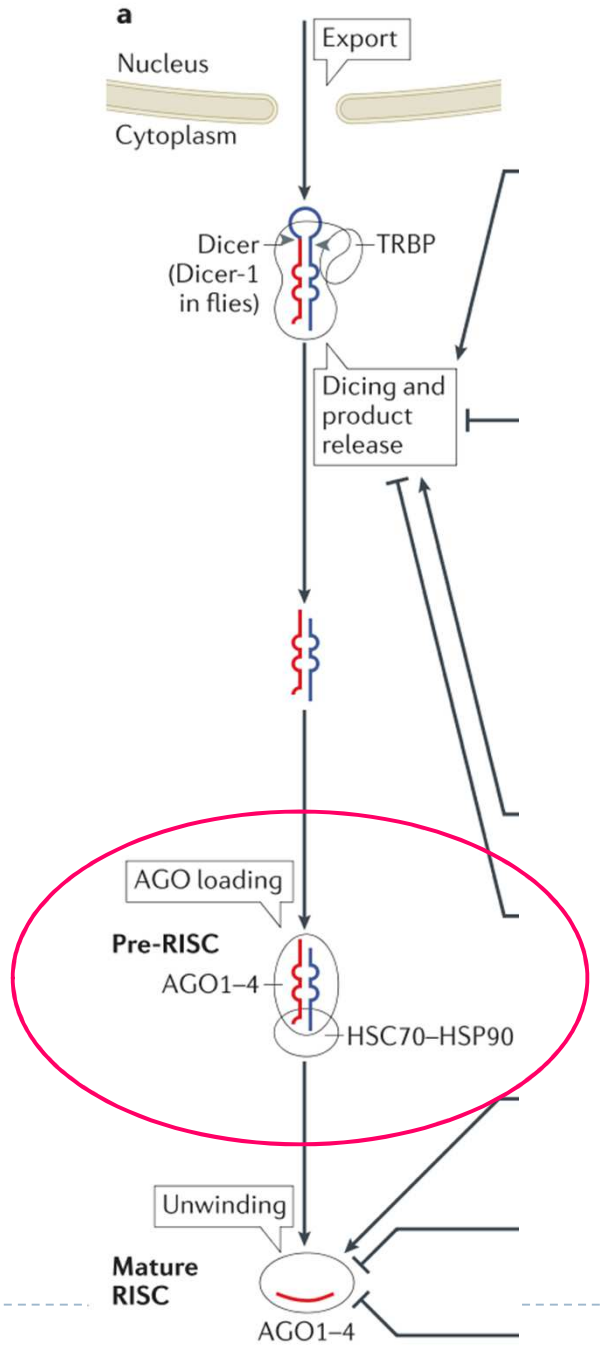


4G10 = Anti-phosphotyrosine antibody

IRESSA is a Tyr Kinase Inhibitor

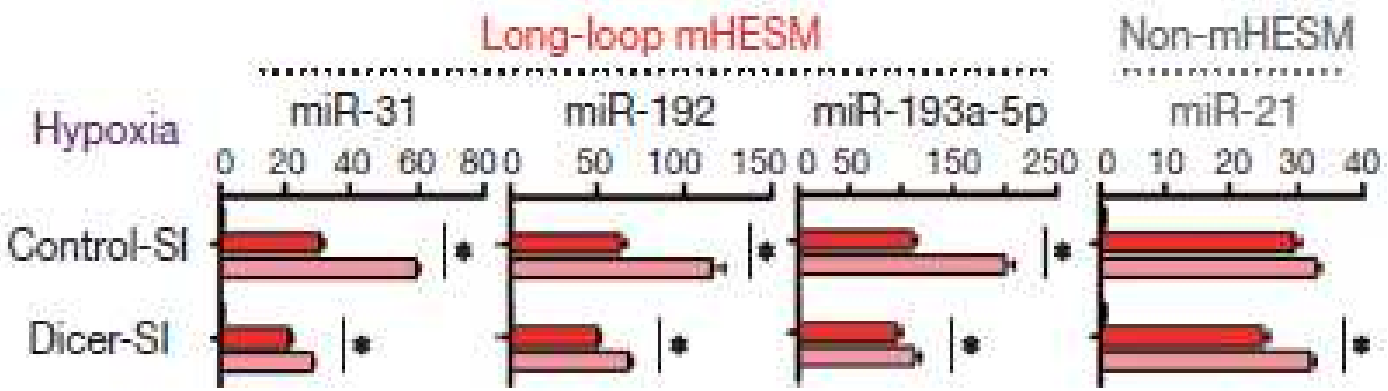
AG-1478 is a selective EGFR inhibitor

EGFR specifically phosphorylates AGO2 Tyr393.



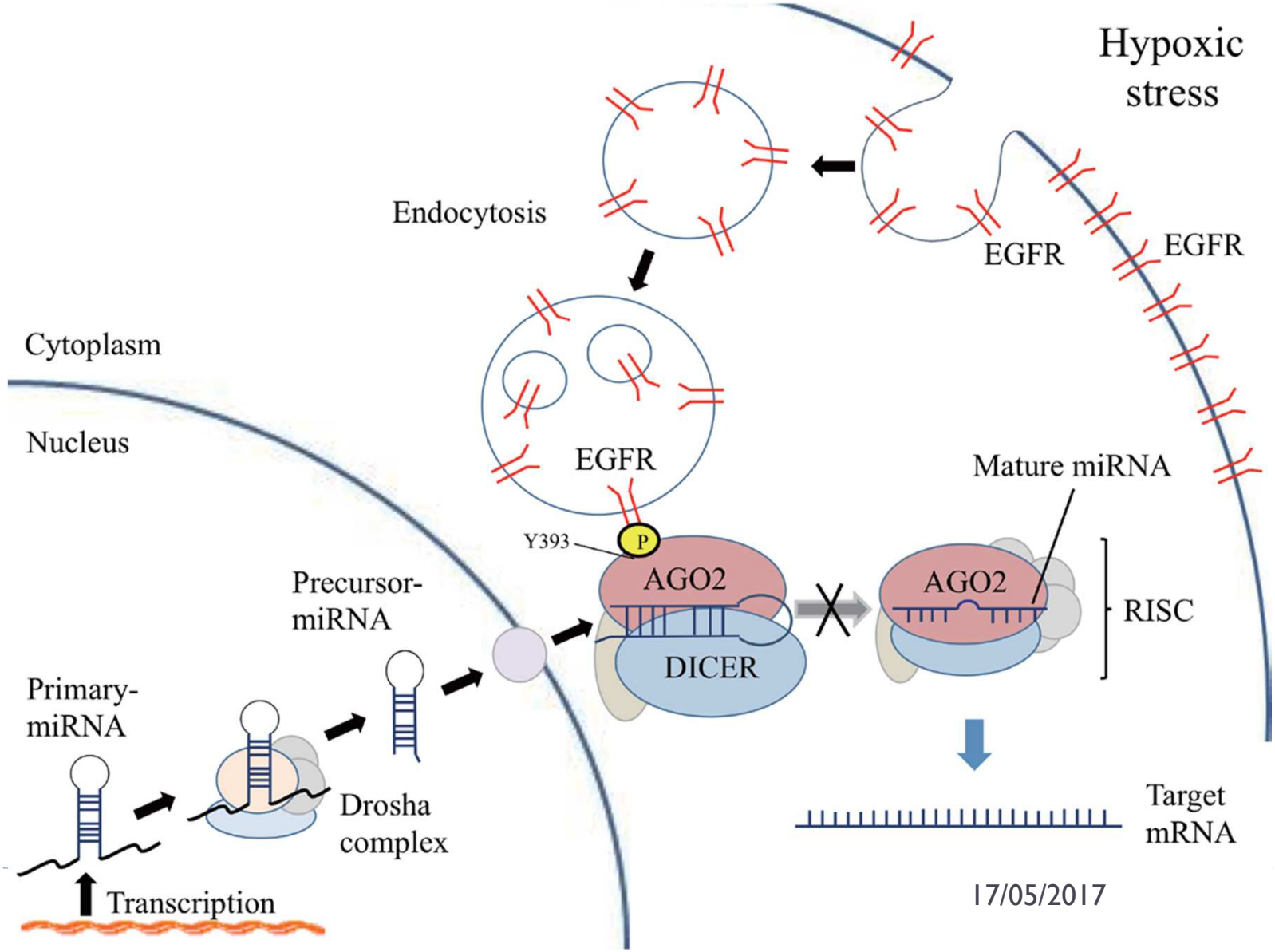
have a role in RISC assembly that resembles the function of the RLC component Dcr-2 (REFS 119,164–167). A recombinant human Dicer–TRBP complex has been shown to bind to siRNA duplexes *in vitro*[168,169]. It has also been reported that the RLC has both pre-miRNA processing activity and target cleavage activity *in vitro*[170]. These findings support the idea that miRNA duplex loading may be coupled with Dicer-dependent pre-miRNA processing in humans (known as the 'Dicer-dependent AGO loading' model). However, *Dicer1*-knockout mouse embryonic stem cells are able to undergo siRNA-directed gene silencing[98,99], which strongly indicates that Dicer is not important for small RNA loading into AGO proteins. Moreover, in flies and mammals, Dicer has been reported to be dispensable for asymmetric RISC assembly *in vitro* and also in cells[133,153,160,171,172]. Thus, the RLC may not be essential for small RNA loading on *D. melanogaster* AGO1 and human AGO proteins, although it is important for loading onto *D. melanogaster* AGO2.

10 – Dicer's silencing



mHESM maturation is dicer-dependent.

Summary


Hypoxic stress
Endocytosis
EGFR
EGFR
EGFR
Cytoplasm
Nucleus
Y393
P
AGO2
DICER
AGO2
Mature miRNA
RISC
Precursor-miRNA
Primary-miRNA
Drosha complex
Transcription
Target mRNA

Conclusions

1. Hypoxia upregulates EGFR;
2. EGFR compromises miRNA maturation;
3. EGFR interacts with the N-terminal domain of AGO2;
4. EGFR-AGO2 are co-localized in low-pH compartments;
5. EGFR specifically phosphorylates Tyr 393 of AGO2;
6. The Y393 phosphorylation reduces the interaction of AGO2 with Dicer, compromising miRNA maturation.