# identifying the phosphorylation sites on 4E-T protein

Navid Taheri

Department of Biochemistry, McGill University
Montreal, Quebec, Canada
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Abstract

4E-T, a novel binding partner of eIF4E protein, transports 4E from the cytoplasm to the nucleus. This protein is also involved in mRNA degradation. 4E-T binds to eIF4E and carries eIF4E-protein along with the mRNA to P-bodies, which is the site of mRNA degradation.

In this study, we show that 4E-T is a phospho-protein and is phosphorylated on multiple sites. We tried to identify some of the phosphorylation sites on this protein. By using overlapping PCR, a triple phospho mutant of this protein was made.

We also investigated if 4E-T is phosphorylated by the S6 kinase (S6K). Our results indicate that even though 4E-T contains the phosphorylation motif of S6 kinase, S6K is not a kinase of this protein.

Moreover, we examined if 4E-T phosphorylation on Ser213, 353 resides changes the interaction between this protein and 4E-HP. We could not find any evidence showing that the binding of these proteins together is dependent of phosphorylation of 4E-T on Ser213, 353 amino acids.

Finally, we show that the interaction between 4E-T and 4E proteins is not regulated by phosphorylation.

The Phospho-triple mutant of 4E-T protein can be used a strong tool to elucidate the function of this protein and how the interaction between this protein and its partners is regulated.

Résumé

4E-T, un nouveau partenaire de liaison à la protéine 4E, transporte 4E du cytoplasme au noyau. Il a aussi été démontré que cette protéine est impliquée dans la dégradation de l'ARNm. 4E-T lie 4E et la transporte, ainsi que l'ARNm, au P-bodies, site de la dégradation des ARNm.

Ici, nous démontrons que 4E-T est une phosphoprotéine et qu'elle est phosphorylée en plusieurs endroits. Nous avons tenté d'identifier certains des sites de phosphorylation de cette protéine. En utilisant l'overlapping PCR, un phospho-mutant triple de cette protéine a été créé.

Nous avons aussi vérifié si 4E-T est phosphorylée par la kinase S6 (S6K). Nos résultats indiquent que, même si 4E-T contient le motif de phosphorylation de la kinase S6, S6K n'est pas une kinase de cette protéine.

De plus, nous avons examiné si la phosphorylation de 4E-T change l'interaction entre cette protéine et 4E-HP. Nous n'avons trouvé aucune preuve que la liaison de ces protéines entre elles serait phospho-dépendante.

Finalement, nous démontrons que l'interaction entre 4E-T et 4E n'est pas régulée par la phosphorylation.

Le phospho-mutant triple de 4E-T peut être utilisé comme un puissant outil pour élucider la fonction de cette protéine et comment l'interaction entre cette protéine et ses partenaires est régulée.

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Introduction

In this introduction we elaborate on the proteins involved during eukaryotic translational processes. Eukaryotic mRNA is capped at the 5' end. The cap structure is composed of 7-methylguanosine ($m^7$GPPPX, where m is a methyl group and X could be any nucleotide). The protein that recognizes and binds to the cap structure is the eIF4E protein. Along with eIF4E, there is an attachment of two other proteins to mRNA: eIF4A and eIF4G. eIF4A is a helicase protein; eIF4G is a large scaffolding protein, which binds to 4E and eIF3 proteins. eIF3 also recruits the 40S ribosomal subunit to the mRNA. The 40S ribosome with its associated initiation factors scans the messenger-RNA until it encounters the first AUG codon. Once the first AUG codon (also known as initiation codon) is found, the 60S ribosome attaches the 40S subunit to form the 80S active ribosome.

The complex of three proteins together is called eIF4F (eukaryotic initiation factor 4F). These three proteins are: eIF4E, eIF4A, and eIF4G. The formation of this complex is thought to be the rate limiting step in translational initiation (77).

Initial identification of eIF4E protein was done by using the ability of this protein to bind to the cap structure. eIF4E was purified on a column containing sepharose beads coupled to $m^7$GDP (2, 3).

The region from the end of the cap structure up to the first AUG on mRNA is called the 5'-Untranslated Region (UTR). eIF4A is an RNA helicase protein and it is responsible for unwinding any secondary structure in 5'UTR of mRNA. This facilitates the mRNA scanning process for the ribosome. For unwinding mRNA, eIF4A consumes an ATP molecule. Two RNA

binding proteins, eIF4B and eIF4H stimulate the ATPase and helicase activities of eIF4A protein (5, 6).

The scaffolding protein, eIF4G, has binding sites for multiple proteins. eIF4G binds to eIF4E, eIF4A and eIF3 proteins. eIF3 has a binding site for the 40S ribosomal subunit as well. This means eIF4G protein indirectly brings the 40S to the mRNA. eIF4G also has a binding site for the PolyA Binding Protein (PABP) (4).This interaction leads to the circularization of mRNA (7, 8). Furthermore, eIF4G can recruit MNK kinase. This kinase phosphorylates 4E protein on Ser209 (9, 10). Phosphorylation of eIF4E protein enhances translation (12, 13). Lachance and colleges reported the importance of 4E phosphorylation for growth and development in Drosophila (13). In addition, Wendel group showed that in tumor development in mice, eIF4E phosphorylation is important (14). In contrast to reports showing phosphorylated eIF4E as an enhancer of translation, there is some evidence showing the opposite effect. Knauf and Ross reported the inhibitory effect of eIF4E phosphorylation on translation (15, 16).

There are different ways to phosphorylate the 4E protein. Growth factors, mitogens, hormones, and even cellular stress can all lead to the phosphorylation of this protein.

All eukaryotes have two homologous forms of 4G protein: 4GI and 4GII; which are encoded by two different genes (4). Even though both forms of 4G protein are phosphorylated on several residues, phosphorylation of 4GI has been more studied. Phosphorylation of 4GI is done by the mTORI complex. Studies have shown that Rapamycin, an inhibitor of mTOR1 complex, inhibits phosphorylation of 4GI on several residues including Ser1108 and Ser1148, and Ser1192 (43). In contrast to Rapamycin, 4GI phosphorylation is enhanced in response to cell growth stimuli like

growth factors, serum, and insulin (43, 44). The role of Phosphorylation of 4G proteins is not clear yet.

After knowing more about the translational complex proteins, the question is what proteins are located upstream of this complex; in other words, how does the cell transmit extracellular signals to the translational machinery?

The answer is in a cascade of proteins relaying signals from the cell surface to the translational complex inside the cell. Proteins involved in this cascade act in a sophisticated harmony. Activation of the receptor protein on the cell surface leads to the activation of other proteins located downstream of this pathway until the signal reaches the translational complex. Depending on the type of signal received by cells, proteins located upstream of the pathway can activate or deactivate the downstream proteins.

Below we try to further discuss the translational signaling cascade.

Insulin, growth factors, serum, and mitogens can activate the cellular surface receptor, IRS (Insulin Receptor Substrate). Subsequently PI3-kinase binds to the receptor and gets activated. Activated PI3-kinase adds a phosphate group to phosphatidyl inositol-4, 5-biphosphate (PIP2), which is located on the plasma membrane. Addition of a phosphate group to PIP2 converts it to phosphatidyl inositol-3, 4, 5-triphosphate (PIP3). This conversion leads to the recruitment of AKT and PDK proteins to the plasma membrane. AKT and PDK are serine/threonine kinase proteins. When both AKT and PDK are present on the plasma membrane, PDK phosphorylates AKT kinase on Thr308.

This leads to the activation of AKT protein. Activated AKT in turn phosphorylates and inactivates TSC2 protein (Figure 1).

TSC2 (Tuberous Sclerosis Complex) is in complex with another protein called TSC1. TSC2 is a GTPase activating protein (GAP). TSC2 is active, when it is not phosphorylated by AKT kinase. In its active form, TSC2 hydrolyzes GTP on its downstream target protein, Rheb. Therefore, Rheb-GTP is converted to Rheb-GDP. This conversion causes inactivation of Rheb protein. When AKT inactivates TSC2 protein by phosphorylation, it leads to the accumulation of Rheb-GTP inside the cell. Rheb protein in its active form, Rheb-GTP, activates its downstream target, mTOR (70).

The mammalian target of Rapamycin (mTOR), is composed of two complexes: mTOR1 and mTOR2.

The MTOR1 complex consists of four proteins: PRAS40 (Proline Rich AKT Substrate 40KDa), Raptor (Regulatory Associated Protein of TOR), mTOR, and GBL (or called mLst8) (17).

Raptor protein recruits mTOR substrates which are 4E-Bp1, S6K, and PRAS40 (18). After recruitment of 4E-Bp1and S6K to the complex via Raptor protein, mTOR phosphorylates these two substrates. GBL causes strengthening of mTOR-Raptor association (19).

PRAS40 has an inhibitory effect on the function of mTOR protein (20, 21). PRAS40, 4E-Bp1, and S6K bind to the same motif on Raptor protein (22, 23).Therefore, PRAS40 acts as a competitor of mTOR substrates. When AKT phosphorylates PRAS40 on Thr246, the inhibitory effect of PRAS40 on mTOR decreases (20, 21). Recent studies indicate that PRAS40 itself is a substrate of mTOR protein (18, 23).

There is a drug called Rapamycin which inactivates the mTOR complex. mTOR1 is sensitive to the drug while mTOR2 is not responsive to this compound.

mTOR2 complex consists of PRR5 (or called Protor), mSIN1(mammalian stress activated protein kinase (SAPK)-interacting protein), GBL, Rictor, and mTOR proteins. mTOR2 substrates are not well characterized yet. Some recent studies indicate that mTOR2 phosphorylates AKT on Ser473.

Activity of AKT is required for activation of the translational pathway. As already mentioned, AKT phosphorylates and deactivates the TSC1/TSC2 complex. Activity of this complex has an inhibitory effect on mTOR. AKT is fully active when it is phosphorylated on two residues: Thr308 and Ser473. Phosphorylation of Thr308 is done by PDK kinase, while mTOR2 is responsible for phosphorylation of AKT on Ser473. If Rictor protein is knocked down in cells, phosphorylation of AKT on Ser473 is ablated. Consequently, mTOR2 regulates AKT activity (24).

One of the targets of mTOR1 is eIF4E-binding proteins (4E-Bps). There are three homologs of 4E-Bp protein: 4E-Bp1, 4E-Bp2, and 4E-Bp3. 4E-Bp1 is the best characterized one among the three. 4E-Bp proteins bind to eIF4E and sequester eIF4E from binding to the cap structure of mRNA, consequently leading to a decrease in cap dependent translation. Binding of 4E-Bps to eIF4E protein is regulated by phosphorylation through the mTOR1 complex. When 4E-Bp1 is hyper-phosphorylated, it detaches from eIF4E. Released eIF4E increases translation of mRNAs. Hyper-phosphorylation of 4E-Bp1 occurs via mTOR1 in response to hormones, nutrients, or growth factors (26). mTOR1 phosphorylates 4E-Bp1 on two residues: Thr37 and Thr46. Phosphorylation of these two amino acids acts as a priming event for the phosphorylation of two other residues on 4E-Bp1: Thr70 and Ser65. Ultimately, 4E-Bp1 is released from eIF4E (1, 27). In contrast, when 4E-Bp1 is hypo-phosphorylated, it binds to eIF4E and consequently cap

dependent translation decreases. 4E-Bp1 and eIF4G proteins have the same binding site on eIF4E. Therefore, these two proteins compete for binding to eIF4E protein (25).

The other target of mTOR1 complex is S6 kinase (S6K). S6K has two homologues in cells, S6K1 and S6K2. Even though these two proteins are encoded by two different genes, the phosphorylation site is conserved between the two. mTOR1 complex can phosphorylate both of these two proteins (28).

There have been more studies on S6K1 than S6K2. To have a fully phosphorylated form of S6K1, two kinases should phosphorylate it: mTOR1 which phosphorylates on Thr389, and PDK1 which adds a phosphate group on Thr229 residue (29-31).

In its active form, S6K1 binds to and phosphorylates its downstream targets. rpS6 is one of the targets of S6K1 protein. Even though it is clear that rpS6 is phosphorylated by the active form of S6K1, the role of this phosphorylation is still in debate. Some early studies indicated the positive role of rpS6 phosphorylation on translation of mRNAs containing a 5' terminal oligopyrimidine tract (TOP). In contrast to these studies, no increase in translation of 5'TOP mRNA was observed in S6K1 and S6K2 double knockout cells (32-34). Since these studies show contradictory results we cannot conclude that S6K1 exerts its effect on translation via rpS6 protein.

Although the role of phosphorylation of rpS6 is not known yet, phosphorylated rpS6 is routinely used to measure S6K and mTOR activity.

The other target of S6K1 is eIF4B protein. S6K1 phosphorylates eIF4B on Ser422. Phosphorylated eIF4B interacts more efficiently with eIF3 (36, 37). This enhanced interaction between eIF4B and eIF3 results in increased mRNA translation (35). Moreover, phosphorylation

of eIF4B may enhance the helicase activity of eIF4A protein. Phosphorylation of eIF4B via S6K occurs in response to cell growth stimuli such as serum, insulin, and phorbol esters (39, 72). Moreover, when ERK1/2 in the MAPK pathway is activated, eIF4B can be phosphorylated by RSK protein (36). eIF4B phosphorylation occurs on Ser422.

PDCD4 is another target of S6K1. A tumor suppressor protein, PDCD4, binds to eIF4A and inhibits the helicase activity of this protein; thus PDCD4 has a negative effect on translation initiation. When PDCD4 is phosphorylated by S6K1, it is targeted for ubiquitination and ultimately it is degraded. Accordingly eIF4A is released and mRNA translation increases (39).

S6K1 can also phosphorylate a kinase of eEf2 protein. eEf2 is a protein involved in the translational elongation process. When eEf2 is phosphorylated by its kinase, eEF2 activity decreases. The decrease in the activity of eEF2 leads to a reduction in protein synthesis. S6K1 phosphorylates eEF2-kinase on Ser366. This phosphorylation removes the inhibitory effect of this kinase on its substrate, eEF2, leading to enhanced protein translation (40).

Finally, S6K1 protein can phosphorylate a protein called SKAR (S6K1 Aly/REF-like target). SKAR is one of the proteins involved in the regulation of cell growth. Furthermore SKAR and S6K1 together are involved in the translation of spliced mRNAs (42). SKAR is specifically phosphorylated by S6K1, but not by S6K2 (41). The phosphorylation of SKAR via S6K1 occurs on Ser383 and Ser385 residues.

A negative feedback loop exists in the translational pathway. S6K plays an important role in this loop. When S6K is activated, it phosphorylates and activates its downstream targets, as well as phosphorylating the insulin-receptor-substrate-1(IRS-1). Phosphorylation of IRS-1 by S6K leads

to a subsequent degradation of this protein. This means the activity of S6K decreases PI3K and AKT signaling and results in the inhibition of mRNA translation (51, 52).

The presence of the S6K negative feedback loop in the translational pathway is one of the obstacles in targeting cancer cells with Rapamycin. When cells are treated with Rapamycin, mTOR1 activity is inhibited. This inhibition reduces phosphorylation and activity of S6K. Consequently, phosphorylation and subsequent degradation of IRS-1 decreases, which in turn leads to elevated PI3K and AKT activity (53). Therefore, inhibition of mTOR1 and S6K leads to increased PI3K/AKT signaling, which ultimately results in the enhancement of protein synthesis. In other words, Rapamycin might actually assist the tumorigenesis process.

It should be noted that AKT is not the only protein acting on the TSC complex affecting TSC complex activity. The other protein acting on the TSC complex is a protein called AMPK (AMP activated protein kinase). AMPK is a kinase protein and it phosphorylates TSC2. Phosphorylation of TSC2 leads to activation and increase in Gap activity of TSC2 protein toward its downstream target, Rheb. Consequently, Rheb-GTP is hydrolyzed to Rheb-GDP. The inactive form of Rheb, Rheb-GDP, inhibits mTOR1, and subsequently inhibition of translation occurs (48, 73).

MPK protein is activated when the ratio of AMP to ATP inside the cell increases (47). There is also a serine/threonine kinase called LKB1 which can phosphorylate and activate AMPK (45, 46). Knockout LKB1 cells fail to inhibit mTOR1 even in the presence of AMPK activators (49). In malignant tumors of some tissues including the testes, colon, and breast, mutation in LKB1 has been reported (50).

One of the other proteins in the translational pathway is a protein called 4E-T (eIF4E Transporter). This protein binds to eIF4E and translocates it from the cytoplasm to the nucleus (67). Another study shows the role of 4E-T protein in mRNA degradation. Ferraiuolo and coworkers showed that 4E-T colocalizes with mRNA decapping factors in P-bodies, which are the site of mRNA degradation. The group also reported the presence of eIF4E, in addition to 4E-T, in P-bodies. Since eIF4E and 4E-T bind together, it is logical to think that 4E-T binds to eIF4E and brings eIF4E with attached mRNA to P-bodies, where mRNA gets degraded. Furthermore, knocking down 4E-T in cells increases mRNA stability (68).

Finally Minshal and colleges showed that in early Xenopus oocytes, CPEB protein interacts with 4E-T and eIF4Eb (an ovary specific eIF4E). Their study indicates that CPEB, pairing with 4E-T and eIF4Eb, along with its protein partners inhibits protein synthesis (69).

Another protein in the translational pathway is a protein called 4E-HP. This protein, which is a homologous protein of eIF4E (4E-HP), shares 30% identity and 60% similarity to eIF4E. Both of these two proteins have cap binding affinity and indeed they compete with each other for binding to the cap structure of mRNAs. Even though the mechanism of cap binding is the same between the two, 4EHP binds to the cap structure of mRNA with lower affinity than eIF4E (54, 55). 4E-HP does not bind to eIF4G. As a result, it prevents formation of the eIF4F complex and it acts as a translational inhibitor (56, 57).

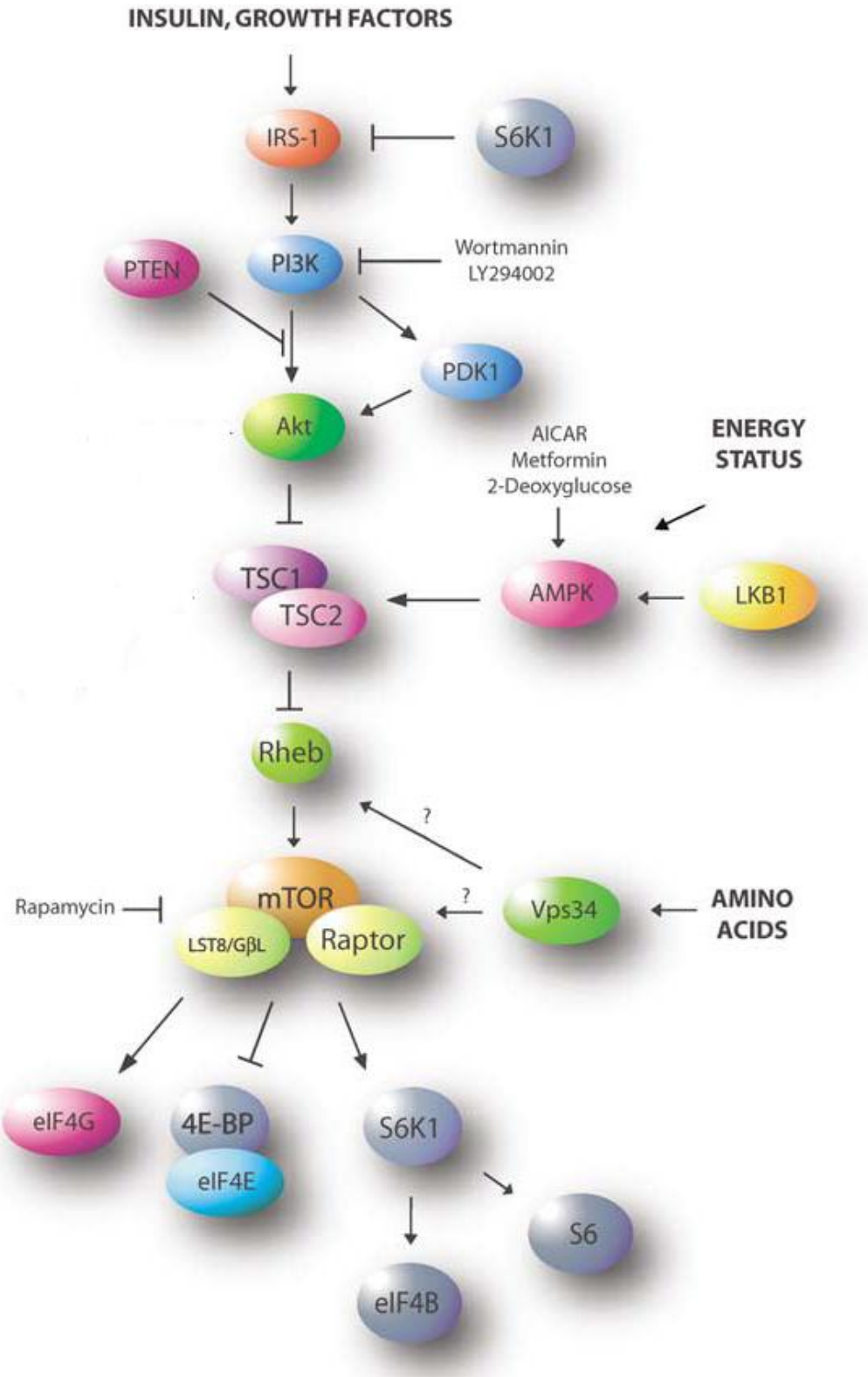
Human 4E-HP is not well characterized yet. Most of the studies on this protein have been conducted in drosophila. In this organism, 4E-HP binds to the cap structure of Caudal mRNA. In addition, it interacts with another protein called Bicoid. Bicoid binds to a region in the 3’ UTR of the Caudal mRNA. This region on mRNA is called the Bicoid Binding Region (BBR). Since 4E-

Hp binds to the 5’ region of mRNA and Bicoid binds to the 3’ UTR region upon binding of 4E-Hp to Bicoid mRNA, the messenger-RNA is circularized. This closed loop is translationally inert (58).

4E-HP and its binding partners play an important role in defining the anterior posterior axes in drosophila embryo (62). Caudal mRNAs and 4E-HP are expressed evenly throughout drosophila’s embryo. In contrast, Bicoid has a high concentration at the anterior end. Therefore the Caudal mRNA is not translated at the anterior region of embryo. On the contrary, as we move toward the posterior part of embryo, Caudal mRNA translation increases. In other words, there exists a concentration gradient of Caudal protein in embryo, with a high concentration posteriorally and low concentration anteriorally (63- 65).

4E-HP is also capable of binding to hunchback mRNA, and inhibiting the translation of this messenger-RNA. In this case 4E-HP interacts with another protein complex known as NRE complex. This protein complex consists of Nanos, Pumilio, and brain tumor proteins (59- 61).

Hunchback mRNA and 4E-Hp are distributed evenly throughout the embryo but Nanos protein is concentrated in the posterior region of the embryo. This leads to the gradient of hunchback protein with the highest concentration in the anterior region of embryo (66).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IRS-1
S6K1
PTEN
PI3K
Wortmannin
LY294002
PDK1
Akt
AICAR
Metformin
2-Deoxyglucose
ENERGY
STATUS
TSC1
TSC2
AMPK
LKB1
Rheb
?
Rapamycin
mTOR
LST8/GβL
Raptor
?
Vps34
AMINO
ACIDS
eIF4G
4E-BP
eIF4E
S6K1
S6
eIF4B

**Figure1: The initiation of translational process via mTOR signaling**

After the binding of ligands to the IRS-1 receptor, PI3k/ AKT is activated. The active form of AKT inhibits the TSC1/TSC2 complex. When this complex is deactivated, mTOR is active and it can signal the downstream targets. mTOR is a kinase which phosphorylates 4E-Bps. This phosphorylation leads to the dissociation of 4E-Bps from the eIF4E protein. Released eIF4E can bind to eIF4G and mRNA translation proceeds. mTOR also phosphorylates S6K1. Phosphorylated S6K1 enhances protein synthesis (Figure taken from reference 74, with modifications).

## Results

### 4E-T phosphorylation by serum induction

We wanted to examine if 4E-T is phosphorylated upon serum induction. If this is observed we expect to see appearance of slow migrating phospho-bands on SDS-PAGE after addition of serum to starved cells.

Hela cells were cultured with 70% confluency. After starving the cells overnight, serum enriched media was added to cells for increasing amounts of time. Cells were harvested and whole cell extracts were analyzed by western blotting.

As serum was added to starved cells, 4E-T protein shows decreased elecrophoretic mobility (Figure 1A, upper panel compare the control lane, lane1, to serum induced lanes, lanes 2-5). Furthermore, when 4E-T is subjected to SDS-PAGE, multiple bands that correspond to this protein appear (Figure 1A, upper panel lanes 1-5). eIF3b was used as a loading control (Figure 1A, lower panel lanes 1-5).

In order to ensure that the band shift observed for 4E-T protein on SDS-PAGE is not cell type specific, we also repeated the experiment using Mouse Embryonic Fibroblast (M.E.F) cells. The results are similar to the previous band shift with Hela cells. A gel band shift is observed after addition of serum to starved cells (Figure 2B, upper panel compare lane1 to lanes 2-4). The decrease in 4E-T bands after four hours of serum induction could be due to degradation of this protein (Figure 2B, upper panel lane 5). eIF3b was used as a loading control (Figure 2B, lower panel lanes 1-5).

To examine whether the slower migrating 4E-T bands are due to phosphorylation, the Hela cell extract was treated with Lambda-phosphatase, which showed a difference in migration pattern, suggesting multiple phosphorylation sites on this protein (Experimental procedure in Materials and Methods).

The experimental results are shown in Figure 1C, Lanes 1 and 2 in the upper panel are the controls of experiment. We wanted to make sure that after serum induction of starved cells we do observe the 4E-T gel band shift as we observed in the previous two experiments. The first lane contains the cell lysate from the starved cells and the second lane shows the cell lysate from the serum induced cells. Neither of these two samples was treated with the phosphatase enzyme. As in the previous experiments, after addition of serum to starved cells a gel band shift is observed (compare lanes 1 and 2). Lane 4 (upper panel) contains the sample which was treated with the phosphatase enzyme at 37**°C**. After phosphatase treatment of the sample, the slower migrating bands disappear (lane 4, upper panel). Disappearance of the upper bands in this lane indicates that 4E-T is a phospho-protein.

We also wanted to ensure that the disappearance of upper band on the gel is due to the action of the phosphatase enzyme, and not to any other possible factor. Knowing that the phosphatase enzyme cannot cleave a phosphate group from its substrate at 4**°C**, we expect to see the phospho-bands in samples treated with phosphatase enzyme but kept on ice. Lane 3 in Figure 1C (upper panel) contains this sample. The upper band does not disappear; therefore we conclude that the disappearance of the upper bands in lane 4 is due to the action of phosphatase enzyme.

eIF3b was used as a loading control (Figure 1C, lower panel lanes 1-4).

The above experiment proves that the heavy bands observed on SDS-PAGE for 4E-T are due to the attachment of a phosphate group to this protein. In addition, Immunoprecipitation of 4E-T from Hela cells, which were transfected with HA-tagged 4E-T and metabolically labeled with $^{32}P$ phosphate, showed phosphorylation of this protein compared to the HA-transfected control (Maritza Jaramillo, unpublished data).

The above data collectively shows 4E-T as a phospho-protein. Phosphate groups bind to unknown amino acid residues of this protein. We were interested to define the phosphorylated sites on this protein. The next set of experiments was performed to reach this purpose.

**Computer analysis of potential phosphorylation sites of the 4E-T protein**

The 4E-T protein was analyzed using the Scan Site program. We set the program to select the potential phosphorylation sites with high stringency. The scanner predicts multiple phosphorylation sites on the 4E-T protein. The Scan Site analysis data are shown in Appendix 1. Moreover the predicted kinase for each potential phosphorylation site is shown in these data as well.

Even though we set the Scan-Site program to select the phospho-sites with high stringency, the computer analysis data indicates multiple phosphorylation sites on this protein. These predictions were not unexpected. When we analyzed the serum induced 4E-T protein with western blotting, several bands were observed (Figure 1A, B lanes 2-5). After phosphatase treatment of serum induced samples, all the upper bands on the SDS-PAGE collapsed into a single band, indicating the observed multiple slow migrating 4E-T bands are all due to phosphorylation of this protein (Figure 1C lane 4). These data collectively indicate that 4E-T is phosphorylated on several residues.

The Scan Site analysis predicts AKT as one of the kinases of 4E-T protein. We decided to mutant some of these AKT phosphorylation sites on 4E-T protein. AKT is one of the kinases in the translational pathway (This kinase is located upstream of mTOR protein and it phosphorylates a wide variety of proteins). The fact that 4E-T is a binding partner of eIF4E protein indicates that 4E-T belongs the translational pathway as well. Therefore it is plausible that 4E-T is phosphorylated by a kinase in the same pathway.

In order to decide which phospho-mutants to make, we further tested if AKT is a potential kinase for 4E-T protein. To reach this goal, the following experiment was done.

**4E-T is a substrate of AKT kinase**

In order to determine whether 4E-T is a substrate for AKT kinase we took advantage of AKT substrate antibody which recognizes any phosphorylated serine residue in RXRXXS motif. This motif is the AKT kinase recognition motif.

Hela cells were cultured with 40% cell confluency. The cells were transfected with the wild-type HA-4E-T plasmid or an empty vector. 24 hours following transfection, cells were serum starved for 16 hours which was then followed by serum or TPA stimulation for 30 minutes. Thirty minutes as a time period was chosen because the peak phosphorylation of 4E-T protein occurs half an hour post serum induction (Figure 1A, B lane 2).

Cells were lysed and HA-4E-T was immunoprecipitated (IP) from whole cell extracts. Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with anti-HA and AKT substrate antibodies. Lysates were also analyzed with the same antibodies.

The experimental result is shown in Figure2.

Some of the proteins in cells contain RXRXXS motif with phosphorylated serine residue, thus all these proteins would be recognized by the AKT substrate antibody. This explains why we see a smear in the Input (Figure 2 upper pane l, lanes 1-4)

Pulled down HA-4E-T is recognized by AKT substrate antibody only when the cells are induced with serum or TPA (Figure 2, upper panel compare lane 6, with lanes 7, 8). We already mentioned that 4E-T is phosphorylated upon serum induction of starved cells (Figure1A, upper panel lanes 2-5 and Figure1B, upper panel lanes 2-4). Therefore AKT substrate antibody can

recognize the phosphorylated form of 4E-T. In other words the experimental results indicate that 4E-T is phosphorylated on AKT recognition site(s).

HA was used as a loading control (Figure 2 lower panel, lanes 2-4 and lanes 6-8). As expected no HA signal is detected in the negative control lanes (Figure 2 lower panel, lanes 1 and 5).

The above data suggests that 4E-T possesses AKT recognition motifs, where they are phosphorylated. For making a phospho-mutant, three AKT phosphorylation sites out of four candidates were chosen. Serine 213, 259, and 353 were selected to be mutated to Alanine.

Unlike Serine amino acid, a phosphate group cannot bind to Alanine residues. By mutating Serine sites to Alanine we can be sure that no phosphate group would be able to attach to the protein on mutated sites.

**Making 4E-T phospho-mutant**

Using the S353A 4E-T mutant as a template followed by subsequent overlapping PCR reaction, a second mutation was introduced into this plasmid. In second mutation, Serine-213 was mutated to Alanine (The single mutant plasmid was constructed by our lab member, Dr. Maritza Jaramillo, prior to my arrival in the lab).

After making the double phospho-mutant gene fragment, it had to be inserted into plasmid.

Prior to insertion of the gene fragment into plasmid, both the double mutated 4E-T gene and vector had to be digested by restriction enzymes.

The empty PCDNA vector and the 4E-T gene fragment were digested by ApaI and NheI restriction enzymes. The 4E-T gene fragment contains ApaI and NheI restriction sites at the ends of the DNA fragment. These restriction sites were designed in the primer sequences. Moreover, in the polycloning site of PCDNA vector, these two restriction sequences are included. The restriction enzyme reaction was stopped after 2 hours to prevent over digestion of the PCR fragment or the vector.

The digested PCDNA plasmid had to be phosphatase treated to reduce the probability of self ligation. The phosphatase treatment removes the phosphate group from the open ends of vector, preventing self ligation of the linear plasmid in the subsequent ligation process. For this purpose, Calf-Intestine-Alkaline-Phosphatase (CIAP) enzyme was used. After 1 hour incubation with the phosphatase, the enzyme was inactivated by incubating the reaction tube 15 minutes at 65°C water bath.

Following deactivation of the phosphatase enzyme, the phosphatase treated plasmid and digested 4E-T gene fragment were gel purified. Then, the purified vector and gene fragment were ligated using T4 DNA ligase enzyme. Following ligation, DH5α *E-coli* strain, which is ampicillin sensitive, was transformed with the ligated products. After transformation, the bacteria was spread on ampicillin agarose plate over night.

There are two possible outcomes during the ligation process. The linear vector could be self ligated. We tried to reduce the possibility of this undesired self ligation product by phosphatase treatment of the linear vector. The other possible outcome is the ligation of 4E-T gene into vector. This is the desired ligation product. Choosing a higher ratio of gene fragment to empty vector increases the possibility of latter ligation. Any of these two ligation products could be taken up by bacteria. Since PCDNA contains an ampicillin resistance gene, any *E-coli* lacking either of these two ligation products would not be able to survive on ampicillin plates.

As a result, the observed bacterial colonies on ampicillin agar plates contain either the empty vector or the vector with the inserted 4E-T fragment in it.

We observed multiple colonies on the plates. By observation alone, colonies containing the desired plasmid, consisting of PCDNA vector with the inserted gene, cannot be identified. This purpose was achieved by picking few random colonies from the plate.

Afterward, the DNA plasmid from each chosen colony was extracted.

The purified plasmids were digested with BamHI and NotI restriction enzymes. Digested plasmids were run on 1% agarose gel.

The presence of one band indicates that the plasmid only contains the empty PCDNA (Figure 3, lanes A-C and lanes E-L). Observing two bands on the gel point to the presence of the inserted gene into vector. As seen in Figure 3 lane D, there are two bands in this lane. The upper band is the linear vector and the lower band is the double mutant 4E-T gene. Wild type 4E-T plasmid was used as a positive control (Figure 3, lane M).

After sequencing the plasmid containing the inserted gene, the sequence was aligned against the wild type 4E-T gene. The sequence alignment indicated the presence of two mutations on the 4E-T gene: S353A and S213A. Part of the 4E-T gene containing the mutation, after it was aligned with the wild type 4E-T, is shown in Figure 4A.

Following the same strategy as described above, we introduced the third mutation in the double mutant plasmid. In the third mutation we mutated Serin259 to Alanine.

Sequence alignment with the wild type 4E-T showed three mutation sites on the 4E-T plasmid: S213A, S259A, S353A (Figure 4B).

**The triple mutant 4E-T protein shows disappearance of the slow migrating 4E-T bands on SDS-PAGE**

We already discussed that the slow migrating 4E-T bands on SDS-PAGE are due to phosphorylation. Since we made a triple phospho-mutant protein, we wanted to examine if the triple mutant protein shows increased electrophoretic mobility.

Hela cells were transfected with the wild type HA-4E-T plasmid or the triple mutant HA-4E-T (S213A, S259A, and S353A). 24 hours post transfection, the cells were serum starved. Starvation lasted for 16 hours. Then a serum enriched media was added to cells for 30 minutes; cells were harvested and whole cell extracts were subjected to SDS PAGE, followed by immunoblotting with HA antibody.

Both the starved and serum-induced wild type samples show the presence of the slower migrating 4E-T band (Figure 5, upper panel lanes 1and 2). The upper band is more intense in serum induced wild type sample when compared to the starved sample (Figure 5, upper panel compare lanes 1 and 2). This was expected; upon serum induction, 4E-T phosphorylation increases.

In contrast, the triple mutant protein shows no band shift on the gel. Both the starved and serum induced triple mutant samples show a single 4E-T band on SDS-PAGE (Figure 5, upper panel lanes 3 and 4). Therefore, we were able to target some of the phosphorylation sites on 4E-T protein. eIF3b was used as a loading control (Figure 5, lower panel lanes 1-4).

Although the triple mutant 4E-T protein does not show the slower migrating phospho-bands on SDS-PAGE, we cannot conclude that we hit all the phosphorylation sites on this protein. The

protein could be phosphorylated on residues other than the ones we mutated without observing a band shift on electrophoresis one dimensional gel. Thus, the samples should be run on two dimensional gel electrophoresis. This issue of separation of phospho-isomers has been elaborated further in the discussion section.

**Studying the interaction between 4E-T and 4E-HP proteins**

Studies have shown that 4E-T and 4E-HP proteins interact with each other (75).

We wanted to study if this interaction is regulated by phosphorylation. In other words, we were interested to define if phosphorylation of 4E-T has any effect on association or dissociation of 4E-T to 4E-HP.

In order to answer the above question the following experiment was designed:

Hela cells were transfected with either the wild-type, single mutant (S353A) or double mutant (S213A and S353A) HA-4E-T plasmid. After 48 hours post transfection, the cells were harvested. HA tagged 4E-T protein was immunoprecipitated by using anti-HA antibody. Then the samples were subjected to SDS-PAGE followed by immunoblotting with anti-HA or anti-4E-HP antibodies.

The presence of HA-4E-T and 4E-HP bands in the input lanes serves as a positive control indicating that 4E-T overexpression worked efficiently and that 4E-HP protein was expressed when the experiment was done (Figure 6, lanes 1-3).

Wild type HA-4E-T and 4E-HP co-immunoprecipitate (Figure 6, lane 4). This is in agreement with previous studies which indicate that these two proteins interact with each other (75).

Both single mutant (S353A) and double mutant (S213A and S353A) HA-4E-T co-precipitate with 4E-HP (Figure 6, lanes 5 and6). No significant difference in 4E-HP band intensity is observed, when 4E-Hp is pulled down along with either single or double mutant HA-4E-T compared to the positive control (Figure 6, compare lanes 5, 6 to lane 4).

Therefore, we conclude that the phosphorylation of sites we mutated does not regulate the interaction between 4E-T and 4E-HP protein.

It should be noted that 4E-HP is always observed as a doublet band on gel. Up to this point it is not clear why the antibody recognizes double bands.

**Examining if S6K is a 4E-T kinase**

4E-T is a phospho-protein. We were interested to find out what kinase phosphorylates this protein. We studied whether S6K is a 4E-T kinase.

Located downstream of mTOR, S6K is one of the kinases in the translational pathway. This kinase phosphorylates serine residues in a RXRXXS motif. This motif is the same between S6K and AKT kinase. We previously showed that 4E-T is phosphorylated on the AKT recognition motif i.e. serine amino acids in the RXRXXS motif (Figure 2, upper panel lanes 7, 8). Therefore S6K is another potential kinase for 4E-T protein. Using M.E.F S6K double knockout cells* in the lab, we tested S6K as a potential kinase for 4E-T.

Wild type or S6K double knock out M.E.F cells were cultured with 70% confluency. After overnight starvation, the cells were induced with serum enriched media. Cells were harvested and whole cell extracts were subjected to SDS-PAGE, which was then followed by immunoblotting with anti-4E-T antibody or anti S6K antibody.

As expected upon serum induction of starved cells, 4E-T phosphorylation increases (Figure 7, upper panel compare lane 1 with lane 2 or lane 3 with lane 4).

If either the S6k1 or S6k2 isoform was the kinase of 4E-T protein among multiple kinases of this protein, we expect to observe a change in the phosphorylation pattern of 4E-T protein. Here our

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*S6K has two isoforms: S6K1 and S6K2. S6K double knockout cells lack both of these two isoforms.

experimental results show that removal of S6 kinases do not have an effect on phosphorylation patterns of 4E-T protein in either the starved or serum induced cells (Figure 7, upper panel compare lanes 3, 4 with lane 2).

4E-T band intensity in S6K double knockout samples is higher compared to wild type (Figure 7, compare lanes 1, 2 with lanes 3, 4). This is due to the presence of the negative feedback loop in the translational pathway. When the translational pathway is active, S6K phosphorylates the IRS-1 receptor and signals it for degradation. In cells lacking S6K, the cell surface receptor, IRS-1, is not degraded. Therefore the PI3K/AKT pathway remains active, phosphorylating the downstream targets, including 4E-T.

eIF3b was used as a loading control (Figure 7, lower panel lanes 1-4) and S6K was used as a positive control for the presence of S6K in wild type M.E.F and absence of S6K in double knockout M.E.F (Figure 7, middle panel lanes 1-4).

As a result, 4E-T should be phosphorylated by one of the kinases in the translational pathway other than S6K. Knowing that 4E-T is phosphorylated on AKT recognition motif, the experimental results increase the likelihood of AKT being the kinase of 4E-T protein.

**The interaction between 4E and 4E-T proteins**

A previous study has shown that 4E-T interacts with 4E protein (67). Since 4E-T is a phospho-protein we were interested to examine if binding of 4E-T protein to 4E is regulated by phosphorylation of 4E-T.

Hela cells were cultured with 70% confluency. Then the cells were treated with Nocodazole for 24 hours. Cells were harvested and whole cell extracts were subjected to SDS-PAGE followed by Western blotting with an anti-4E-T antibody, or with a $^{32}$P-HMK-eIF4E probe in a Far Western assay.

4E-T shows hyperphosphorylation upon Nocodazole treatment (78). Western blotting with anti-4E-T antibody shows the appearance of slow migrating phospho-bands in samples treated with Nocodazole (Figure 8, lower panel compare lane 1 with lane 2).

4E-T from treated and untreated cells interacts equally with eIF4E, as it is shown by Far Western analysis (Figure 8, upper panel, compare the 4E-T band in lane 1 with lane 2). Therefore there is no effect of 4E-T phosphorylation on the interaction between this protein and eIF4E.

Previous experiments have shown phosphorylation of eIF4GII after treatment of cells with Nocodazole (78). In contrast to 4E-T; phosphorylation of eIF4GII protein dramatically increases its interaction with eIF4E protein as the Far Western data show it (Figure 8, upper panel, compare the 4GII band in lane 1 with lane 2).

4E-T western blot data was used as a loading control (Figure 8, lower panel).

In this study, we tried to further characterize the 4E-T protein. We showed that 4E-T is phosphorylated on multiple residues. We also identified some of the phosphorylation sites on this protein. A triple phospho-mutant of this protein was made and the mutant plasmids were used to study the interaction between 4E-T and 4E-HP. Our data indicates that the interaction between 4E-T and 4E-HP is not dependent of phosphorylation of 4E-T on Serine213, 353 residues. In addition, we realized that binding of 4ET to eIF4E is not regulated by phosphorylation either. Our experimental results reveal that when eIF4G is phosphorylated its binding to eIF4E protein dramatically increases. Finally, in an attempt to identify the 4E-T kinase, S6K was excluded as a potential kinase for 4E-T protein.

**Discussion:**

The results presented in this study demonstrate that upon serum induction 4E-T protein is phosphorylated on multiple residues. This data is in agreement with computer based predictions which predict several phosphorylation sites on 4E-T.

In order to characterize the phosphorylation role of 4E-T, the triple phospho-mutant of this protein was made. Disappearance of the slow migrating phospho-bands on SDS-PAGE indicates that the mutated Serine residues to Alanine are the phosphorylation sites on this protein. However this does not eliminate the possibility that there could be other phosphorylation sites on 4E-T. The data in this study is obtained by using one dimensional gel analysis. In order to further characterize the phosphorylation sites on 4E-T, a 2D gel should be used. Compared to one dimensional SDS gels, 2D gels have higher resolution in separating peptides. On a 2D gel, peptides are separated based on their isoelectric point in the first dimension. Then in the second dimension, the separation is done based on the molecular weight of peptides.

Alternatively, instead of gel analysis, MudPIT (Multidimensional Protein Identification Technology) method can be taken (79). In MudPIT, peptides are first separated in two dimensional liquid chromatography and then carried directly to mass spectroscopy for further analysis. In this approach, chromatography columns with independent stationary phases are coupled together such that peptides are separated based on the charge in the first dimension and based on their hydrophobicity in the second dimension. This chromatography technique improves some of the limitations associated with the two dimensional gel electrophoresis. In comparison with 2D gels, separating peptides on MudPIT columns gives higher resolution. Furthermore the elutes are directly analyzed by mass spectroscopy (79).

In an attempt to find a kinase for 4E-T protein, we tested S6K as a potential kinase. Our data excludes the possibility of S6K being a kinase for 4E-T protein. This is despite the fact that there are multiple S6K recognition sites on this protein. Since S6K and AKT recognition motifs are the same, our data increases the possibility that AKT should be a kinase for this protein. Moreover, in S6K knockout cells, we observed stronger phosphorylation of 4E-T compared to control (Figure 7, compare lanes 3, 4 with lanes 1, 2).This is due to the fact that by removing of S6K kinase, the translational pathway becomes more active. Hyperactivity of the pathway is due to the absence of the negative feedback loop in which S6K phosphorylates the cell surface receptor, IRS1, and signals it for degradation. Pathway hyperactivity suggests that 4E-T should be phosphorylated by a kinase in the translational pathway other than S6K. Pathway hyperactivity is further evidence that suggests AKT as a potential kinase of 4E-T protein; this possibility could be further examined by using AKT knockout cell lines. AKT has three isoforms: AKT1, AKT2, and AKT3. Ideally an experiment should be conducted with a triple knockout cell line.

Our data indicates that upon phosphorylation of eIF4GII, its binding to eIF4E highly increases. Therefore, eIF4GII phosphorylation might facilitate the formation of the eIF4F complex and it could enhance translation. Since the phosphorylation role of eIF4GII protein is not yet known, the findings in this study could be used to characterize the role of eIF4GII phosphorylation. In the present study, we also show that 4E-T and 4E-HP interaction is not dependent of phosphorylation of 4E-T on Serine213, 353 residues. Furthermore, phosphorylation does not regulate the interaction between a known binding partner of 4E-T: eIF4E. Since 4E-T interacts with 4E and 4EHP, and both of these two proteins are mRNA cap binding proteins, it would be of interest to investigate whether phosphorylation of 4E-T has an effect on cap dependent translation. For this purpose a bicistronic reporter construct could be used (68). This reporter

construct contains the luciferase (LUC) and chloramphenicol acetyl transferase (CAT) cistrons, and these two cistrons are separated by poliovirus IRES (Internal Ribosomal Entry Site) (80). If a cap dependent translation proceeds, the LUC cistron is being utilized; while in cap independent translation, the CAT cistron is being translated (68).

It has already been shown that 4E-T along with 4E localizes to P-bodies (68). Phosphorylation of this protein could have an effect on its localization to P-bodies. It would be interesting to test the possible P-body colocalization by using the triple phospho-mutant of 4E-T protein. Additional work is required to elucidate the functional role of phosphorylation for this protein.

## Materials and Methods:

### Antibodies

4E-T antibody was made and purified in our lab.

eIF3b antibody was purchased from Santa Cruz biotechnology.

### Agarose gel purification

All gel purification of PCR products and plasmids were done by using the QIAGEN Kit.

### Far-Western analysis

Radioactive 4E is obtained by fusing a heart muscle kinase to eIF4E protein. Upon addition of hot phosphate, the kinase phosphorylates eIF4E protein on its recognition site. Consequently, 4E protein becomes radiolabeled. This has been previously described by Pause and colleges in 1994 (81).

### Restriction enzyme digestion

The empty plasmid and the gene fragment were digested with ApaI and NheI for 2 hours at 37 ℃ water bath. The restriction enzymes were purchased from Fermentas.

### CIAP phosphatase

After digestion of the empty vector, it was treated with CIAP phosphatase (Fermentas) for 1 hour. Then the enzyme was deactivated by keeping the reaction tube for 15 minutes at 65 ℃ water bath.

### Ligation

The amplified gene fragment was ligated into vector by using T4 DNA ligase. The reaction tube was incubated for 15 minutes at room temperature. The ligation enzyme was purchased from Fermentas.

Lambda phosphatase Treatment

Starved Hela cells were serum induced for 30 minutes. Then the cells were washed three times with PBS buffer. Samples were collected in RIPA buffer. The proteins in cell lysates were quantified using the Bradford assay. Two separate samples, each containing 50µg of protein, were treated with the lambda phosphatase. One reaction tube was kept at 37 ℃ in water bath and the other one was put in ice. After one hour, the reaction was stopped by adding Sample buffer to the tubes. The proteins were resolved on 7% SDS-PAGE.

The enzyme was purchased from the New England Biolabs.

Primers sequences

4E-T forward primer: ACACGCTAGCATGGATAGGAGAAGTATGGG

4E-T reverse primer: ACACGGGCCCCATTCACACACTTAGCATATG

4E-T S213A forward: TGGTGAGCGTAGAAGAAATGATGCTTACACAG

4E-T S213A reverse: CTCTGGTTCTTCTTCTGTGTAAGCATCATTTC

4E-T S259A forward: AGGCGACGGACAGCCGCTGTGAAGG

4E-T S259A reverse: TACCTTCCTTCACAGCGGCTGTCCGTC

4E-T S353A forward: TCAGGAAGCCGATCCAGCGCTCTTGGGTC

4E-T S353A reverse: TGT GGT GTT GAC CCA AGA GCG CTG GAT CG

Buffer B for immunoprecipitations

5ml Tris-HCl; 1M; PH = 7.4

15ml KCl; 1M

0.2 ml EDTA; 0.5 molar

Phosphate buffer Solution (PBS)

10X PBS solution is prepared as follows.

NaCl 80g

KCl 2g

$Na_2HPO_4$-$7H_2O$ 26.8g (alternatively we can use 14.4g of $Na_2HPO_4$)

$KH_2PO_4$ 2.4g

Adjust the total volume to 1 liter with distilled water.

RIPA buffer

NaCl 150g

1M Tris-HCl 5mL ; PH = 7.5

1% NP-40 or Igepal

0.5% SDS

Adjust the volume to 100 ml

Furthermore, the following protease inhibitors were added to the solution.

1mM PMSF; 2mM $Na_3VO_4$; 50mM NaF; 2µg/mL Leu

Western blotting

First, the samples were run on SDS-PAGE. Then the proteins were transferred to nitrocellulose paper overnight (using 30mV voltage). Transferral can be done in 2 hours if a high voltage (80mV) is being used. Then the membrane was blotted with milk solution (10% dry-milk in PBS solution) for 1 hour. After blotting, the membrane was washed 3 times with PBS solution. Next, the nitrocellulose membrane was incubated with the desired primary antibody for 16 hours. 4E-T primary antibody needs overnight incubation. Blotting the membrane with HA primary antibody could be done in 2-4 hours, though the overnight incubation results in more intense bands. After incubating the membrane with the antibody, the membrane was washed 3 times, 5 minutes each, with PBS solution. Then the membrane was incubated with the secondary antibody for 2 hours. The membrane was washed again with PSB as before. Following the second wash, the membrane was immersed into Horse-Radish Peroxidase solution (ECL solution). A photography film was put on a membrane and the film was developed.

Overlapping PCR

For making mutant 4E-T plasmids, the Overlapping PCR technique was used. While the base of this technique is the same as the PCR reaction, three separate reactions should be conducted to design the mutation on the plasmid. We named the three separate PRC reactions PCR1, PCR2, and PCR3 (follow the diagram on page 45). In PCR1, both the Forward Primer and End Primer1

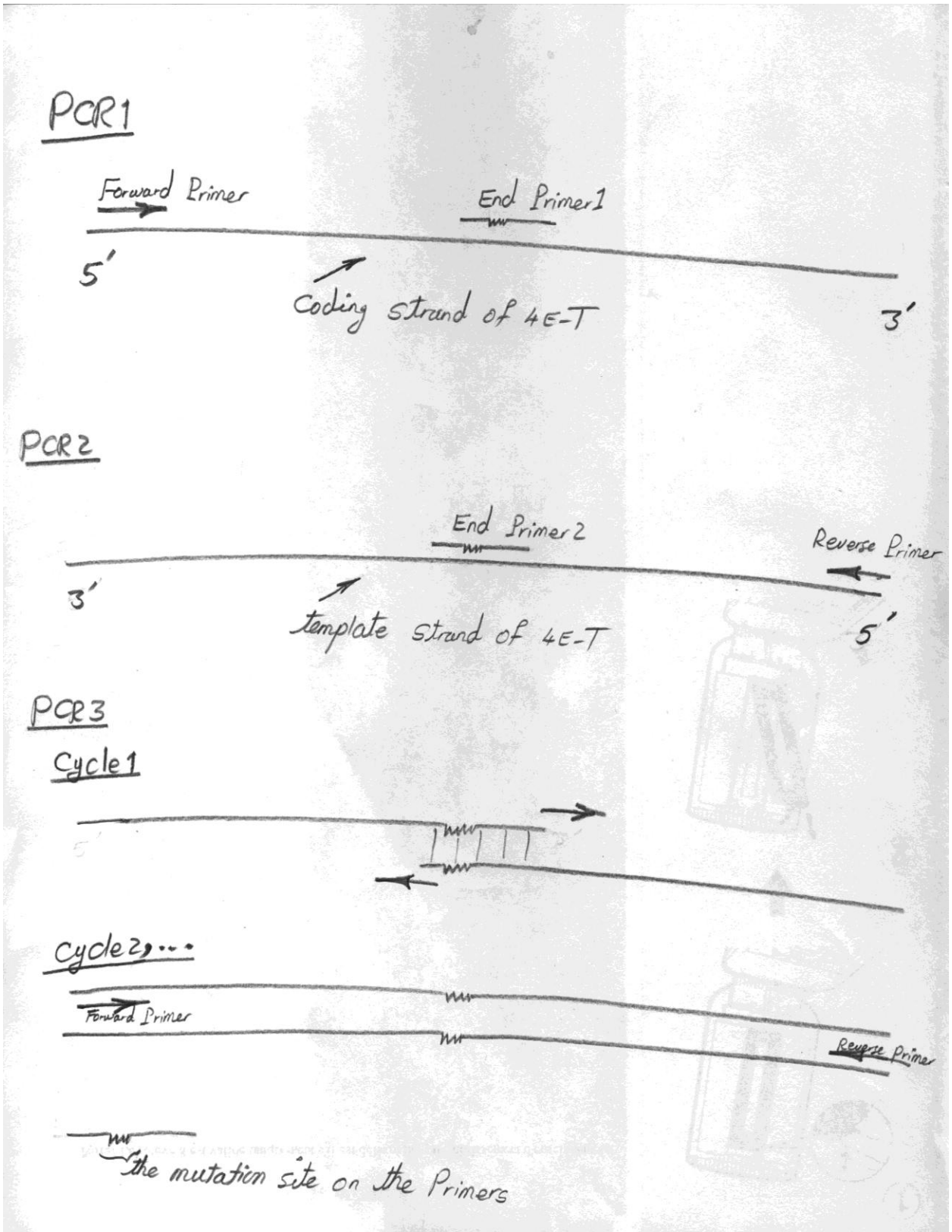
were designed complementary to the coding strand of 4E-T. The Forward Primer is complementary to the first 23 nucleotides of 4E-T gene. The End Primer1 extends the region on 4E-T gene that should be mutated. In other words, the 4E-T gene length, covered by the End Primer1, contains a triple codon which encodes one of the phospho-serine amino acids. We would like to change this serine triple codon to alanine. For this purpose, instead of designing nucleotides complementary to the serine triple codon on the End Primer 1, an alanine triple codon was inserted. In PCR2, both primers are designed complementary to the template strand. The Reverse Primer is complementary to the last 23 nucleotides of template strand of 4E-T gene and the End Primer2 is complementary to the End Primer1.

Furthermore nucleotide restriction sites, which are cleaved by ApaI or NheI restriction enzymes, was added to the Forward and Reverse Primers respectively. This design will be used for insertion of the gene fragment into the plasmid.

After designing the primers, PCR1 and PCR2 reactions were done. It should be noted that since the End Primer1 and End Primer2 are complementary, there is an overlapping part between PCR1 and PCR2 products.

In PCR3, the DNA fragments amplified in PCR1 and PCR2 reactions are used as templates, while for primers, only the Forward and Reverse Primers are being used. In the first PCR cycle, the overlapping part between PCR1 and PCR2 products holds the two DNA fragments together, allowing the DNA polymerase to use each gene fragment as a template and make a complementary strand of it. No primer is required since the overlapping part serves as a primer itself. At the end of the first cycle, a double stranded 4E-T gene, containing the desired mutation,

is made. In subsequent PCR cycles, DNA polymerase uses the Forward and Reverse primers to make a complementary DNA fragment.




Designing the overlapping PCR reaction

**Appendix1**

**Scan Site analysis**

| Phosphoserine/threonine binding group (pST_bind) | | | | |
|---|---|---|---|---|
| **14-3-3 Mode 1** | | | Gene Card **YWHAZ** | |
| Site | Score | Percentile | Sequence | SA |
| S353 | 0.2664 | 0.046 % | RSGSRSS**S**LGSTPHE | 0.603 |
| **14-3-3 Mode 1** | | | Gene Card **YWHAZ** | |
| Site | Score | Percentile | Sequence | SA |
| S342 | 0.3352 | 0.169 % | SRFSRWF**S**NPSRSGS | 0.968 |
| **Src homology 3 group (SH3)** | | | | |
| **PLCg SH3** | | | Gene Card **PLCG1** | |
| Site | Score | Percentile | Sequence | SA |
| P887 | 0.5242 | 0.198 % | ASHPLLN**P**RPGTPLH | 1.463 |
| **Basophilic serine/threonine kinase group (Baso_ST_kin)** | | | | |
| **Protein Kinase A** | | | Gene Card **PRKACG** | |
| Site | Score | Percentile | Sequence | SA |
| T257 | 0.3108 | 0.115 % | RKRTRRR**T**ASVKEGI | 1.275 |
| **PKC delta** | | | Gene Card **PRKCD** | |
| Site | Score | Percentile | Sequence | SA |
| S259 | 0.3813 | 0.099 % | RTRRRTA**S**VKEGIVE | 1.151 |
| **Akt Kinase** | | | Gene Card **AKT1** | |
| Site | Score | Percentile | Sequence | SA |
| T257 | 0.4154 | 0.059 % | RKRTRRR**T**ASVKEGI | 1.275 |
| **Akt Kinase** | | | Gene Card **AKT1** | |
| Site | Score | Percentile | Sequence | SA |
| S213 | 0.4192 | 0.064 % | GERRRND**S**YTEEEPE | 3.354 |
| **Akt Kinase** | | | Gene Card **AKT1** | |
| Site | Score | Percentile | Sequence | SA |
| S259 | 0.4405 | 0.094 % | RTRRRTA**S**VKEGIVE | 1.151 |
| **Akt Kinase** | | | Gene Card **AKT1** | |
| Site | Score | Percentile | Sequence | SA |
| S353 | 0.4449 | 0.100 % | RSGSRSS**S**LGSTPHE | 0.603 |
| **Clk2 Kinase** | | | Gene Card **CLK2** | |

| Site | Score | Percentile | Sequence | SA |
|---|---|---|---|---|
| S259 | 0.5578 | 0.179 % | RTRRRTA**S**VKEGIVE | 1.151 |

| Proline-dependent serine/threonine kinase group (Pro_ST_kin) | | | | |
|---|---|---|---|---|
| **Cdk5 Kinase** | | | Gene Card **CDK5** | |
| Site | Score | Percentile | Sequence | SA |
| S115 | 0.3718 | 0.122 % | DDLDVVF**S**PQRRSFG | 1.035 |
| **Erk1 Kinase** | | | Gene Card **MAPK3** | |
| Site | Score | Percentile | Sequence | SA |
| S693 | 0.4209 | 0.097 % | ASITSML**S**PSFTPTS | 0.450 |
| **Erk1 Kinase** | | | Gene Card **MAPK3** | |
| Site | Score | Percentile | Sequence | SA |
| S587 | 0.4480 | 0.168 % | YLRPRIP**S**PIGFTPG | 0.357 |

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Cited in: 76 and 77.

**Western blot**

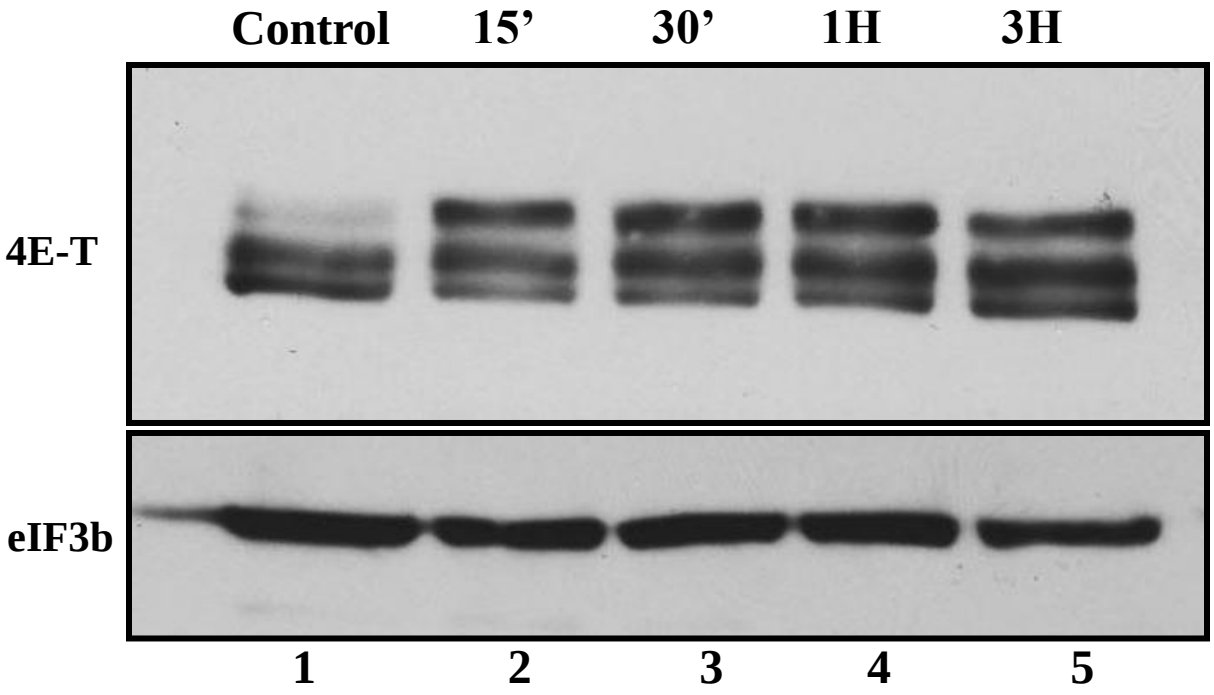



**Figure 1A: 4E-T phosphorylation. (A)** Hela cells were serum starved for 16 hours after they reached 70% confluency. Then serum enriched media was added to cells. The cells were harvested at indicated time points, and the whole cell extracts were resolved by SDS-PAGE which was followed by immunoblotting with total anti-4E-T antibody. eIF3b was used as a loading control. **(B)** The same experiment as (A) was carried out using Mouse Embryonic Fibroblast ( M.E.F) cells. **(C)** Treating 4E-T protein with the phosphatase enzyme. Starved or serum induced Hela cell lysates were treated with buffer or with phosphatase enzyme. Cell extracts were then subjected to Western blotting and analyzed by anti-4E-T antibody.

**Western blot**

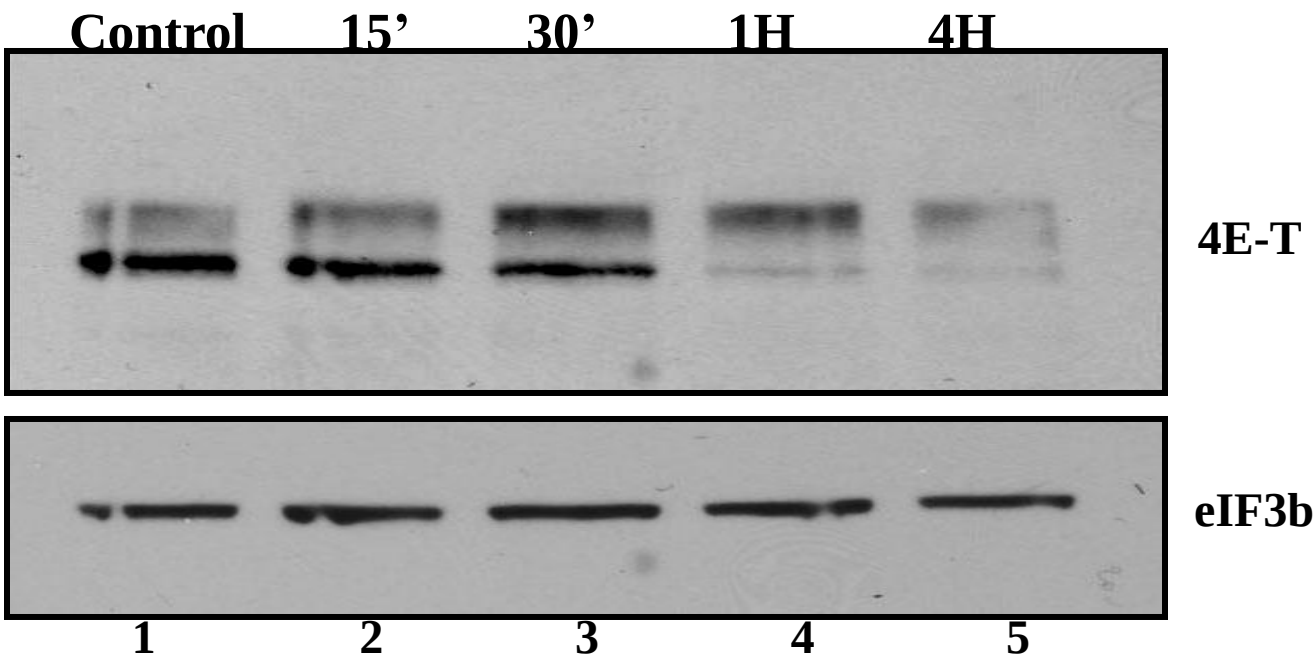



**Figure 1B**

**Western blot**

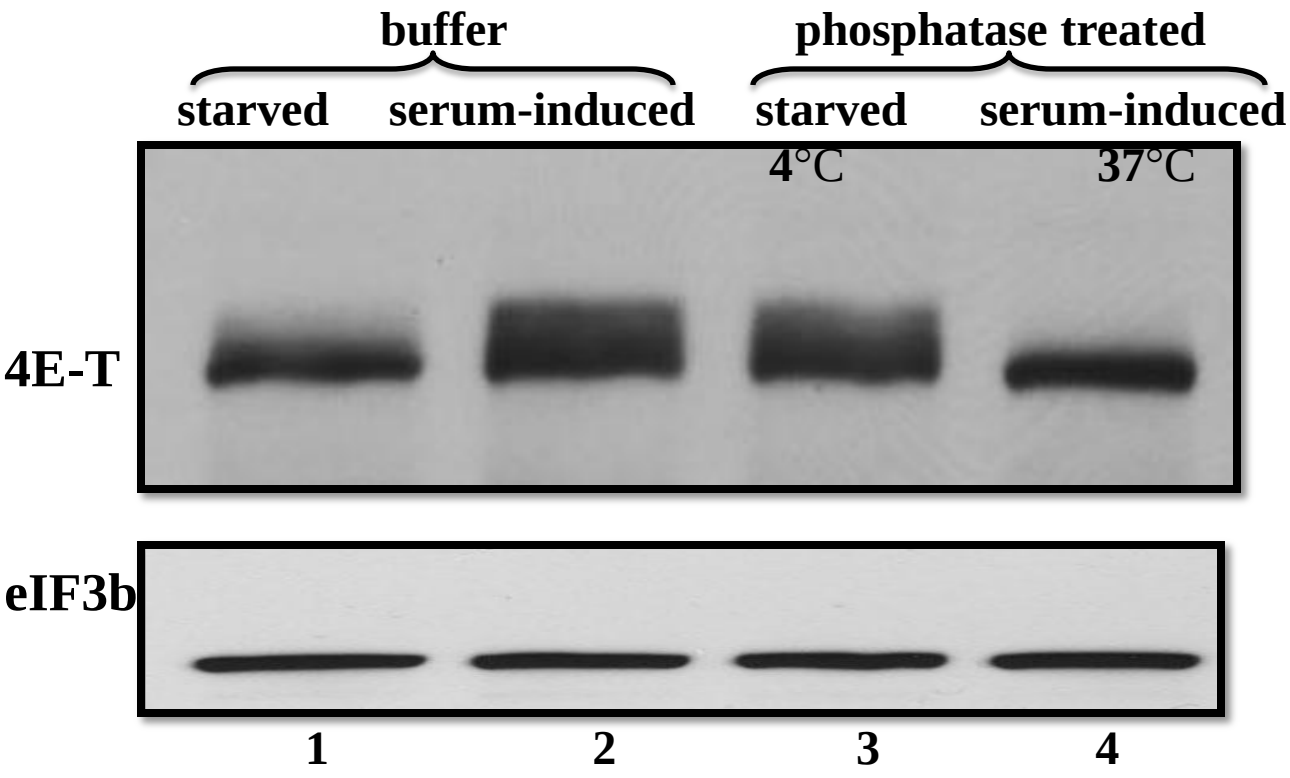



**Figure 1C**

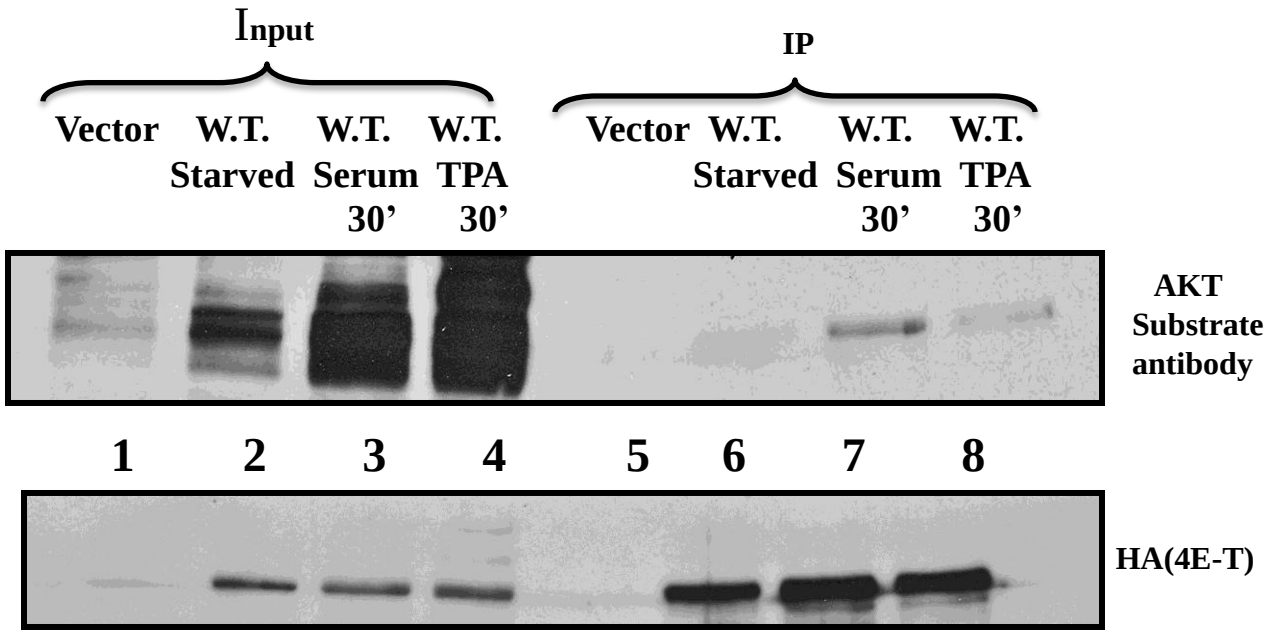



**Figure 2: 4E-T is a substrate of AKT kinase.**

Hela cells were transfected with HA-tagged wild type 4E-T plasmid. 24 hours following transfection, cells were serum starved for 16 hours. Then they were stimulated with serum or TPA for 30 minutes. Cells were lysed and HA-4E-T was immunoprecipitated (IP) from whole cell extracts. Immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-HA and AKT substrate antibodies. Lysates were also analyzed with the same antibodies.

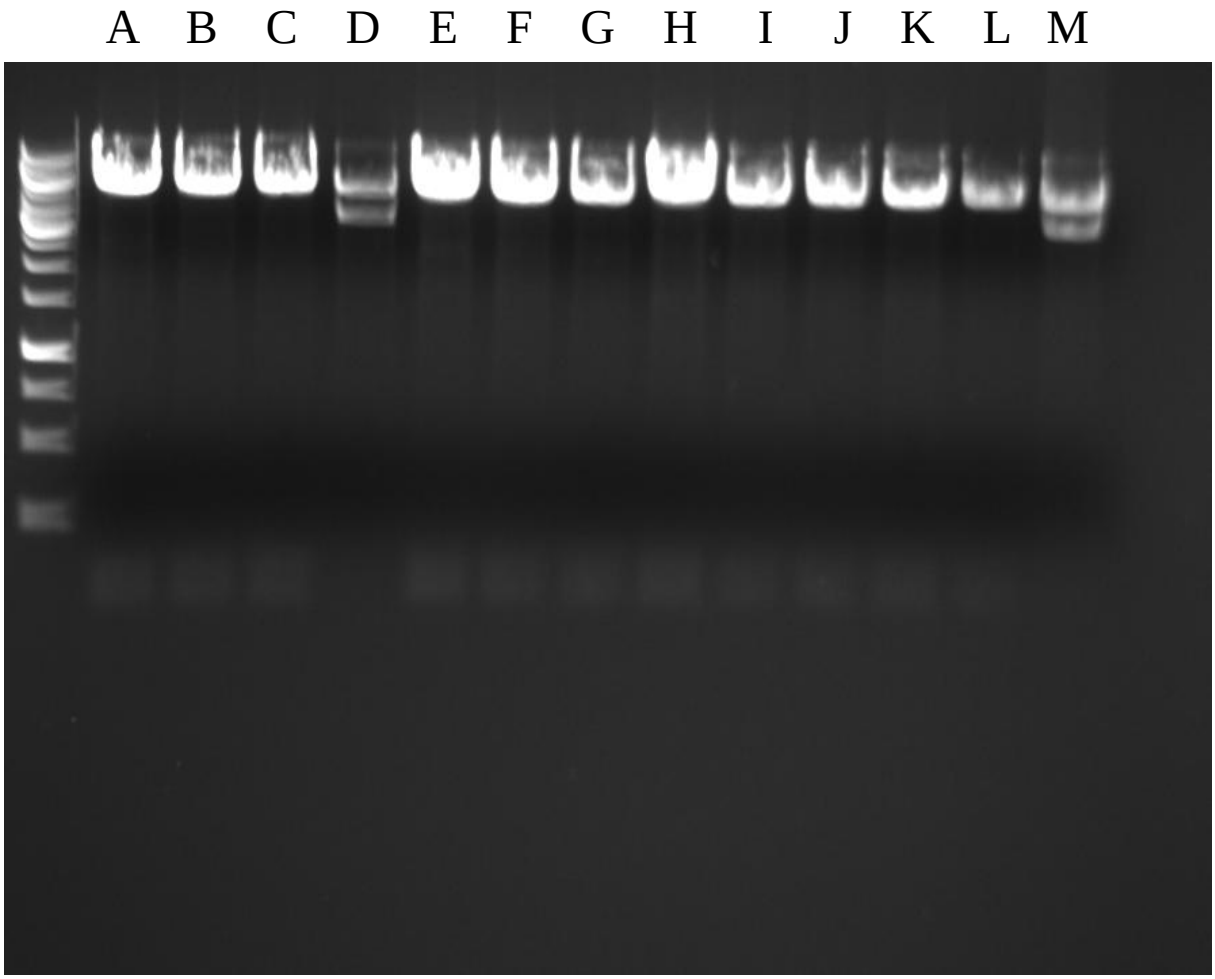



**Figure 3: Analysis of double mutant HA-4E-T (S213A and S353A) plasmid.**

5HDα bacteria were transformed with a mixture of empty PCDNA vector and double mutant 4E-T (S213A and S353A) in PCDNA vector. 24 hours after plating the bacteria on ampicillin agar plate, plasmid DNA from twelve bacterial colonies were extracted. Purified plasmids were then digested with BamHI and NheI restriction enzymes, followed by separation on agar gel and staining with ethidium bromide.

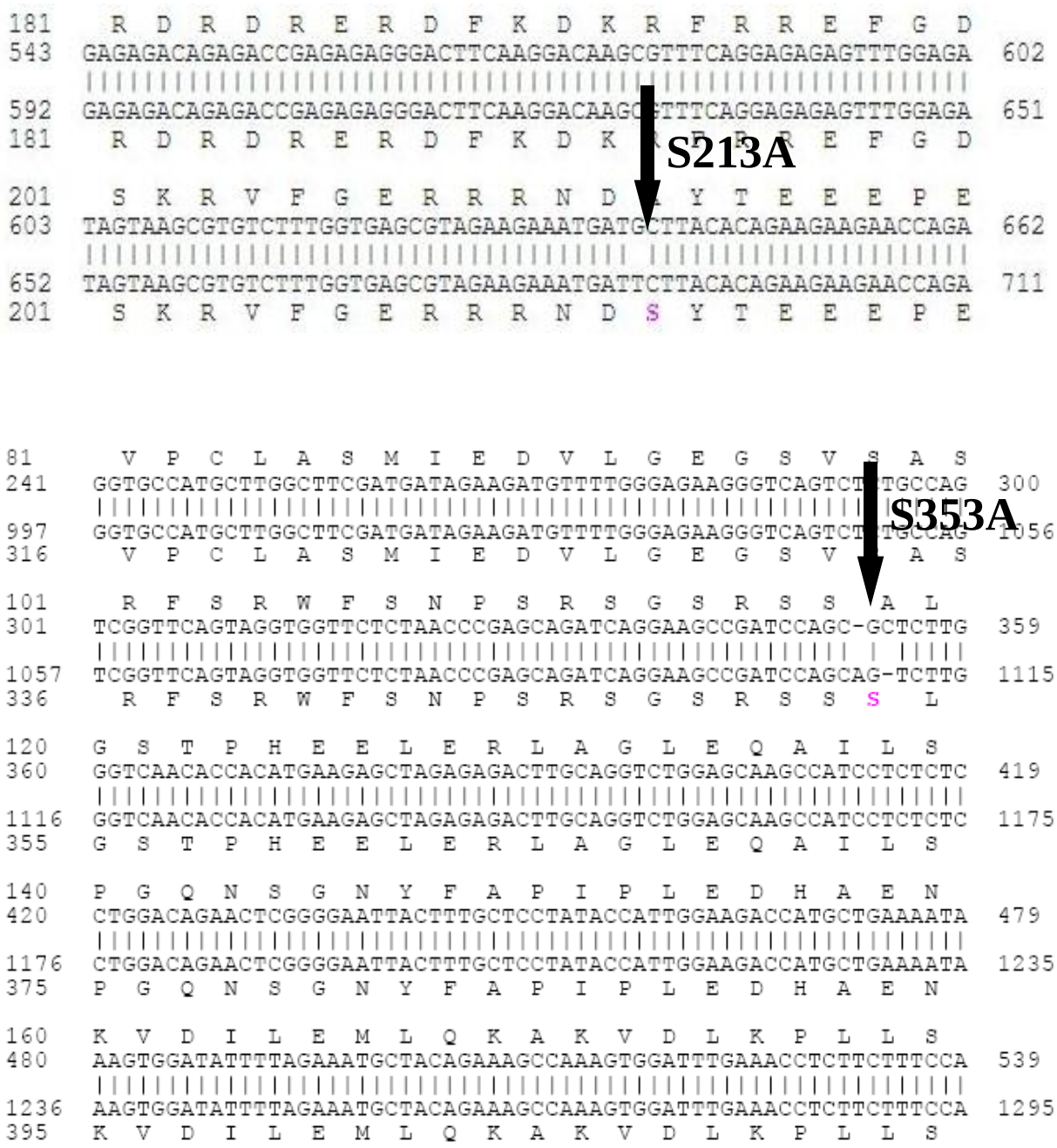



**Figure 4A: Nucleotide and amino acid alignment of wild type and mutant 4E-T**

The nucleotide sequence of mutant 4E-T was determined by direct sequencing of cloned mutant 4E-T. The sequence was then blasted against wild type 4E-T . Arrows show the two mutation sites in double-mutant 4E-T **(A)** and the three mutation sites in triple-mutant 4E-T**(B)**.

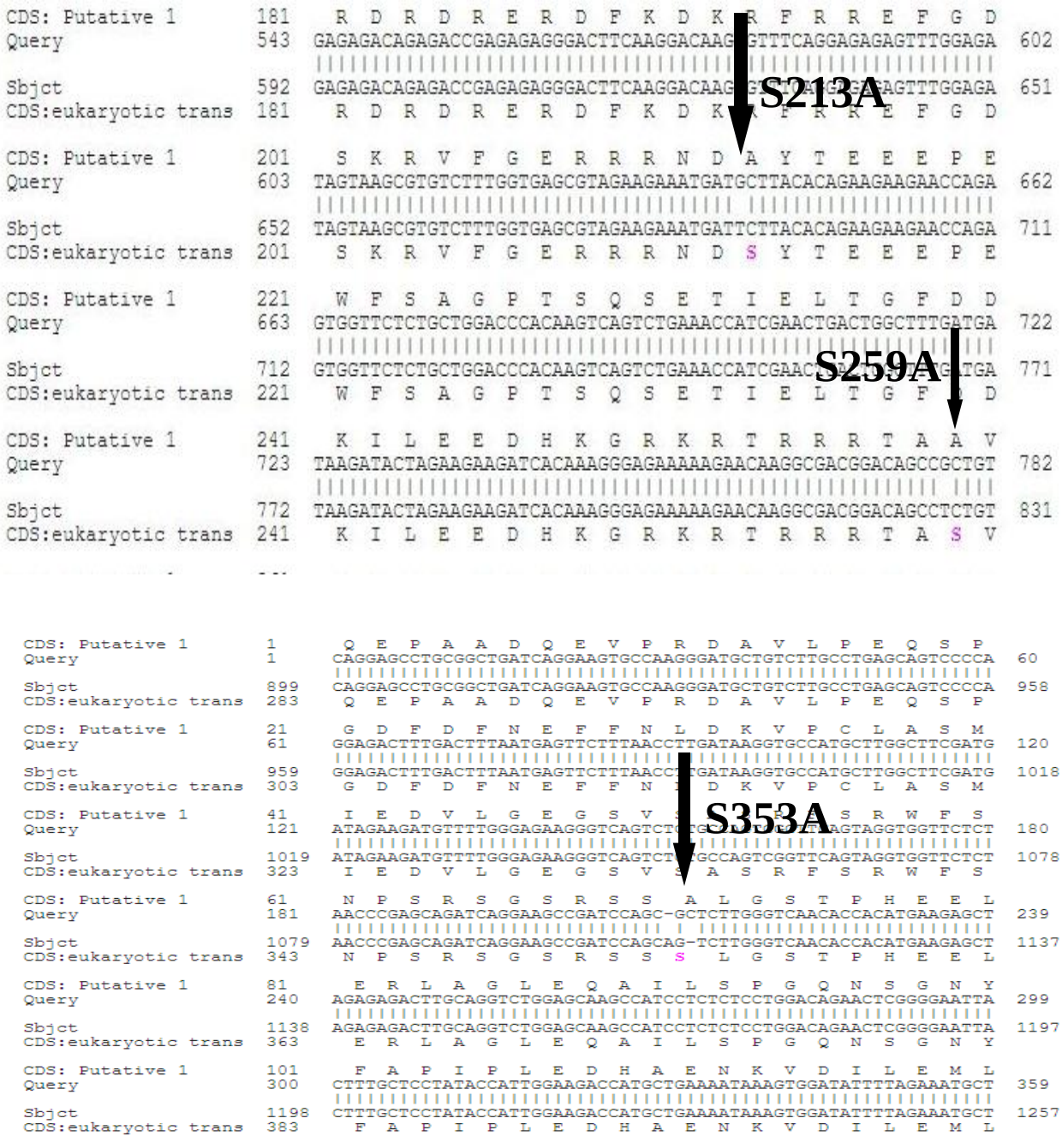



**Figure 4B**

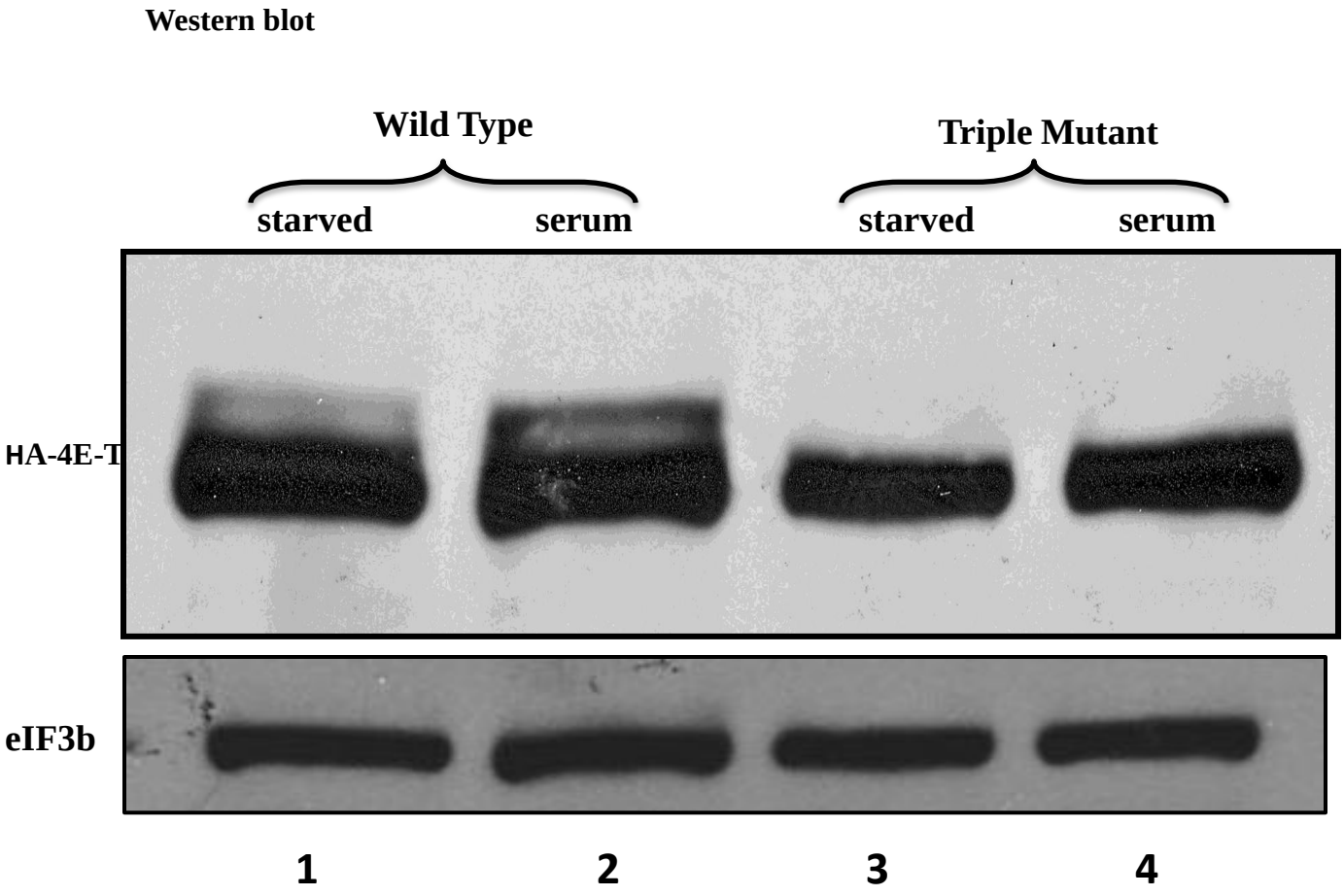
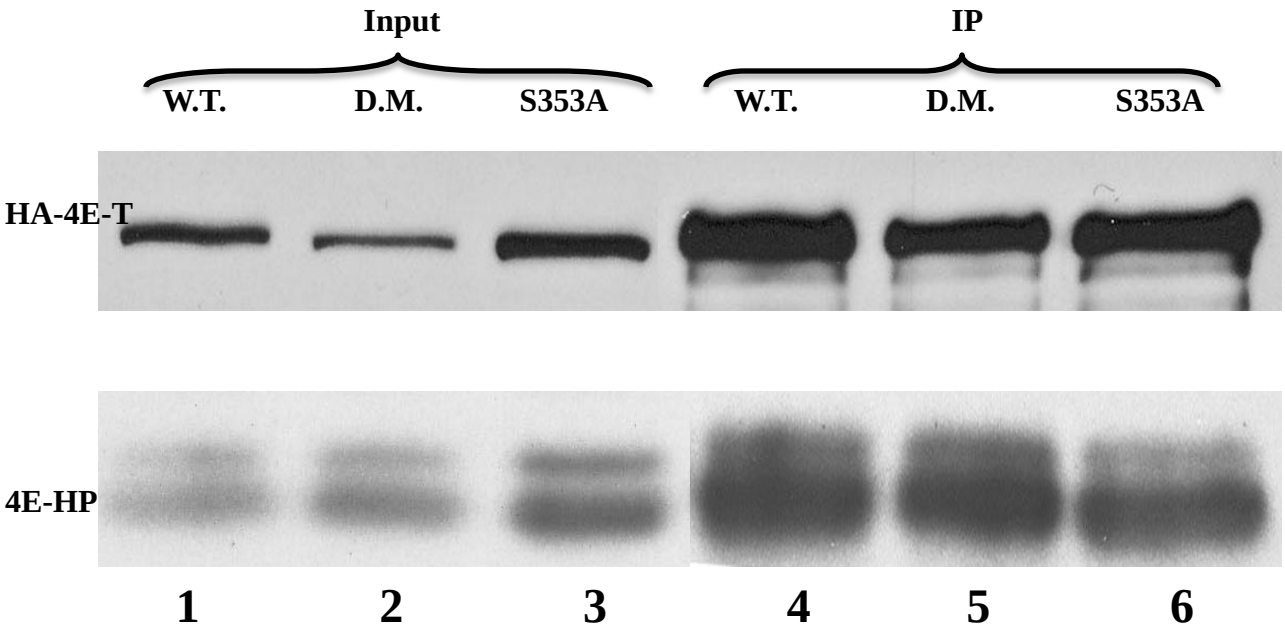



**Figure 5: Disappearance of slow migrating phospho-bands in triple mutant 4E-T .** Hela cells were transfected with HA-4E-T wild-type or HA-4E-T mutant (S213A, S259A, S353A). 24 hours after transfection, cells were starved for 16 hours, which was then followed by serum induction for 30 minutes. Cells were lysed and whole cell extracts were analysed by Western blotting with anti -A antibody.




D.M. : Double Mutant(S213A and S353A)

**Figure 6: Phosphorylation of 4E-T does not regulate its interaction with 4E-HP.**

Hela cells were transfected with wild-type HA-4E-T, HA-4E-T mutant (S353A) or HA-4E-T double mutant (S213A and A353A). 48 hours after transfection cells were harvested; HA-4E-T from cell extracts were immunoprecipitated by using anti-HA antibody. Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with anti-HA or anti 4E-HP antibodies.

**Western blot**

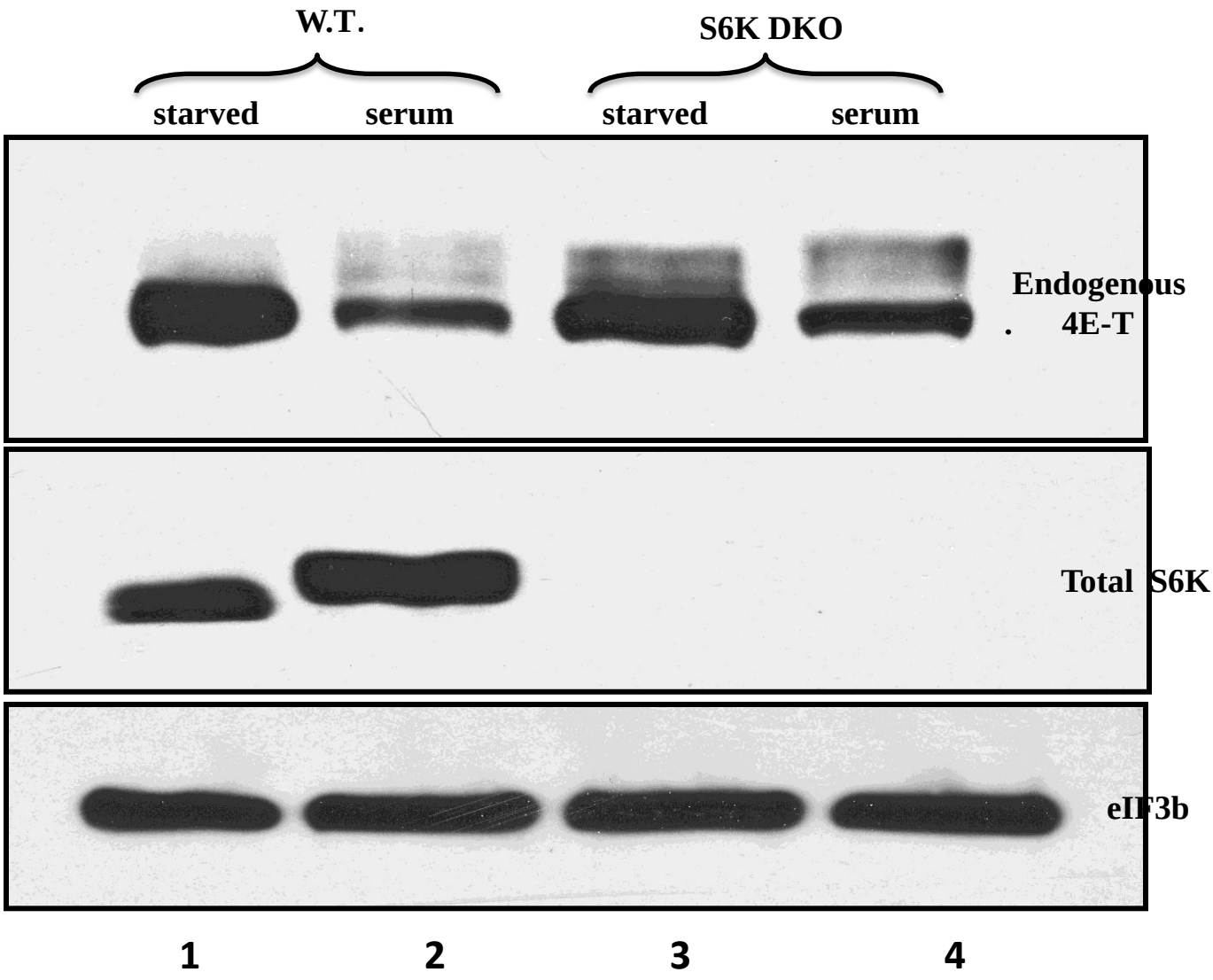



**Figure 7: S6K is not a kinase of 4E-T protein.**

Wild type or S6K Double knockout M.E.F. cells were serum starved after reaching 70% confluency. Then serum enriched media was added to cells for 30 minutes. Cells were lysed and whole cell extracts resolved by SDS-PAGE which was then followed by immunoblotting with anti-4E-T or anti-S6K antibodies.

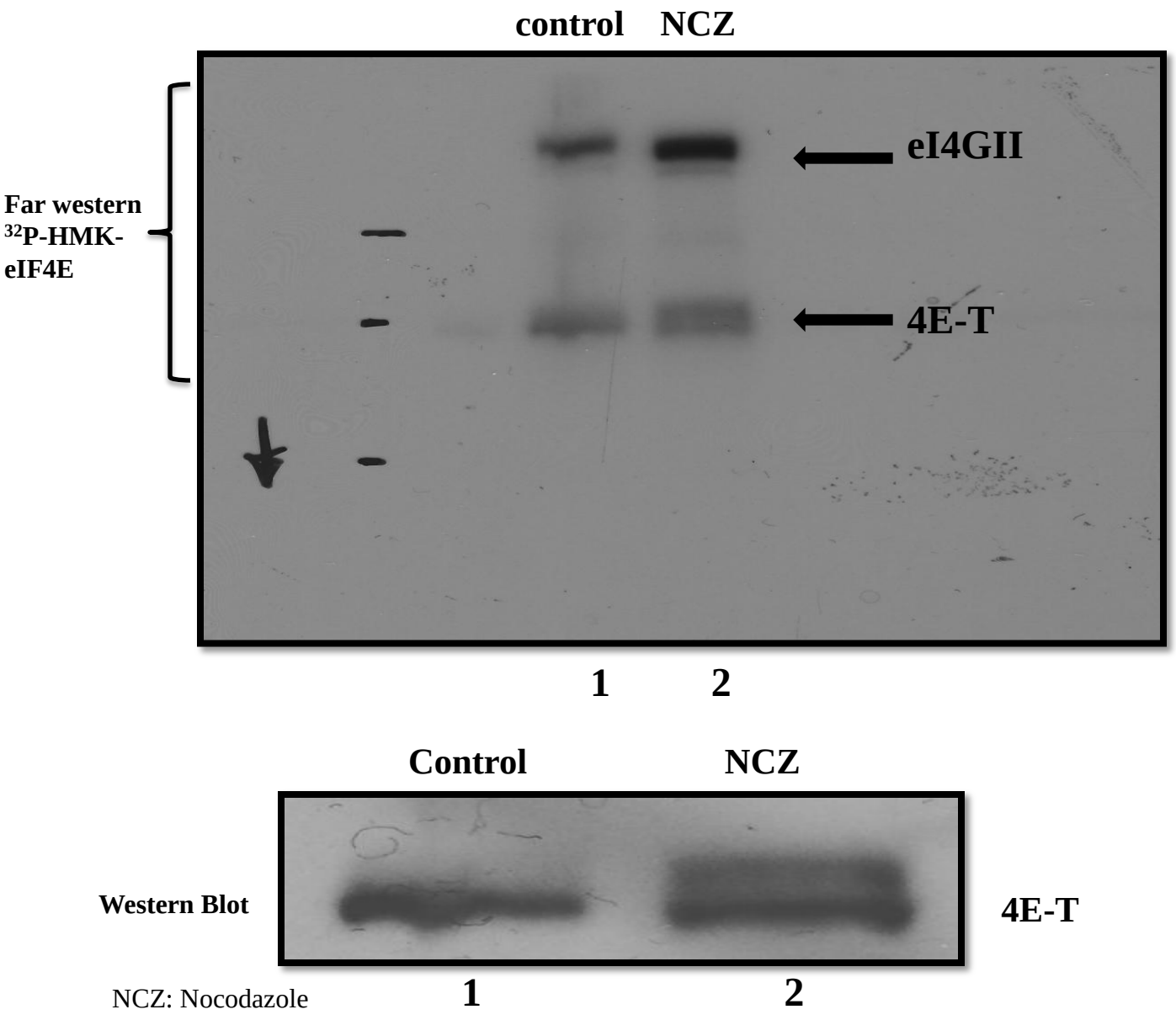



**Figure 8: While phosphorylation of 4E-T does not affect its interaction with eIF4E, eIF4GII phosphoryaltion dramatically increases its binding to eIF4E.**

Total extracts from non-treated or Nocodazole treated Hela cells were subjected to SDS-PAGE. Data was analyzed in Western blotting with anti-4E-T antibody, (lower panel) or with $^{32}$P-HMK-eIF4E probe in a Far Western assay (upper panel).