**Załącznik 3**

# Specificity of proteins from eIF4E family from different organisms towards 5' mRNA cap

AUTOPRESENTATION

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Warsaw, 2017

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## 1. Full name

Joanna Maria Żuberek

## 2. Diplomas and scientific degrees

**06.2005 - PhD in physics** (with distinction), Faculty of Physics, University of Warsaw. Title of the PhD thesis: *Molecular mechanisms of protein biosynthesis regulation – the role of phosphorylation of translation initiation factor eIF4E based on biochemical and biophysical studies*, promoter dr hab. Edward Darżynkiewicz, UW Professor

**09.1999 - MSc in physics, specialty: biophysics,** Faculty of Physics, University of Warsaw. Title of the MSc thesis: *Mechanism of action of thymidine kinase studied using emission and enzymatic kinetics methods*, dr hab. Borys Kierdaszuk

## 3. Information about current employment in scientific facilities

| | |
|---|---|
| **09.2017–09.2018** | Assistant Professor, Division of Biophysics, Institute of Experimental Physics, Faculty of Physics, University of Warsaw |
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| **10.1999–09.2004** | PhD studies at the Faculty of Physics, University of Warsaw |

*in the periods from 12.2013 to 06.2014 and from 02.2017 to 06.2017 I had my maternal leave, currently, from 06.2017 to 02.2018 I have my parental leave*

## 4. Bibliometric summary of scientific achievements

*of 13.11.2017 according to Web of Science*

Hirsch Index – 16

Total number of citation / without autocitation – 767/601

Total *Impact Factor* as per the publishing year – 164,241

Number of original papers – 44

Number of conference papers – 23

## 5. Achievements resulting from Art. 16 item 2 of the act of 14 March 2003 on scientific degrees and scientific title and about degrees and titles in arts (Dz. U. (Polish Journal of Law) No. 65, Pos. 595 as amended.):

### a) title of scientific achievement

**Specificity of proteins from eIF4E family from different organisms towards 5' mRNA cap**

### b) Authors, titles of publications, publishing year, publishing house name,

*Asterix marks correspondence authorship of habilitation applicant. Italics marks authors who performed their studied under direct supervision of the habilitation applicant.*
*(**H2.** DK –MSc student; **H5.** DK –PhD student; **H6**. AK –MSc student/PhD student; **H8**. AS –engineering and technical worker)*

**H1.** Yoffe Y, **Zuberek J.,** Lerer A., Lewdorowicz M., Stepinski J., Altmann M., Darzynkiewicz, E., Shapira, M. Binding Specificities and Potential Roles of Isoforms of Eukaryotic Initiation Factor 4E in Leishmania. 2006 ***Eukaryotic Cell*** 5: 1969-1979 IF=4,303/Cyt. 29

**H2.** **Zuberek J**., *Kubacka D*., Jablonowska A., Jemielity J., Stepinski J., Sonenberg N., Darzynkiewicz. E. Weak binding affinity of human 4EHP for mRNA cap analogs. 2007 ***RNA*** 13: 691-697 IF=5,111/Cyt. 24

**H3.** Freire E.R., Vashisht A.A., Malvezzi A.M., **Zuberek J.**, Langousis G., Saada E.A., Nascimento J de F., Stepinski J., Darzynkiewicz E., Hill K., De Melo Neto O.P., Wohlschlegel J.A., Sturm N.R., Campbell D.A. eIF4F-like complexes formed by cap-binding homolog TbEIF4E5 with TbEIF4G1 or TbEIF4G2 are implicated in post-transcriptional regulation in Trypanosoma brucei. 2014 ***RNA*** 20: 1272-1286 IF=4,622/Cyt. 12

**H4.** Freire E.R., Malvezzi A.M., Vashisht A.A., **Zuberek J.,** Saada E.A., Langousis G., Nascimento J.D.F., Moura D., Darzynkiewicz E., Hill K., de Melo Neto O.P., Wohlschlegel J.A., Sturm N.R., Campbell D.A. Trypanosoma brucei translation initiation factor homolog EIF4E6 forms a tripartite cytosolic complex with EIF4G5 and a capping enzyme homolog. 2014 ***Eukaryotic Cell*** *13*: 896-908 IF=3,179/Cyt. 10

**H5.** *Kubacka D*., Miguel R.N., Minshall N., Darzynkiewicz E. Standart N., **Zuberek J.*** Distinct Features of Cap Binding by eIF4E1b Proteins. 2015 ***Journal Molecular Biology***. 427: 387-405 IF=4,333/Cyt. 2

**H6.** *Kropiwnicka A.*, Kuchta K., Lukaszewicz M., Kowalska J., Jemielity J., Ginalski K., Darzynkiewicz E., **Zuberek J.*** Five eIF4E isoforms from Arabidopsis thaliana are characterized by distinct features of cap analogs binding. 2015 ***Biochemical Biophysical Research Communication*** 456: 47-52 IF=2,297/Cyt. 4

**H7.** **Zuberek J.***, Kuchta K., Hernandez G., Sonenberg N., Ginalski K. Diverse cap-binding properties of Drosophila eIF4E isoforms. 2016 ***Biochimica et Biophysica Acta, Proteins and Proteomics*** 1864: 1292-1303 IF=3,016/Cyt. 0

**H8.** **Zuberek J.*,** *Stelmachowska A.* Tryptophan Residues from Cap Binding Slot in eIF4E Family Members: Their Contributions to Near-UV Circular Dichroism Spectra. 2017 ***Journal Physical Chemistry and Biophysics*** *7: 250*

## c) Description of the scientific aim of the above mentioned works and the obtained results along with description of their potential application.

### 5.1 General presentation of the subject and the main aim of the work

On their 5' end, eukaryotic mRNAs have a structure composed of 7-methylguanosine connected via atypical 5'-5'-triphosphate bond to the first transcribed nucleotide ($m^7$GpppN, where N – any nucleotide), known as cap (Figure 1A) [1]. It plays a key role in all stages of mRNA metabolism in the cell, beginning from mRNA splicing [2,3], to its nuclear-cytoplasmic export [4,5], to initiation and inhibition of translation [6–8] to mRNA degradation in the cell [9,10].

In all these stages, different cap binding proteins interact with the cap structure. A common structural motif of cap binding proteins is formation of a three layer system, the so-called sandwich stacking, where cap's 7-methylguanine ring intercalates between two aromatic rings [11–14] or between an aromatic ring and another amino acid residue that stabilizes binding [15–17].

One of these proteins is eukaryotic translation initiation factor 4E – eIF4E, a small (about 25 kDa) monomeric protein of highly conserved sequence and structure (from yeast to human) with a characteristic motif with the following topology: β1β2α1β3β4α2β5β6α3β7α4β8, forming a structure called *the cupped hand* [11]. In eIF4E proteins 7-methylguanine cap moiety forms a cation–π sandwich by stacking between two tryptophan residues. In the case of the human eIF4E, these are Trp56 and Trp102. Additionally, the base ring is stabilized by three hydrogen bonds between N(1)H and 7-methylguanine amino group and carboxy group of glutamic acid (Glu103 in human) and between oxygen atom at 6th position of $m^7$Gua and amide group of tryptophan corresponding to human Trp102, as well as by van der Waals interaction of the $m^7$Gua methyl group with the third tryptophan present in the cap binding slot (Trp166 in human protein). Negatively charged phosphate groups of the cap are stabilized via hydrogen bonds with basic residues present at the entrance to the cap binding slot. In the human protein, these are Arg112, Arg157, Lys159, Lys162 and Lys206 (Figure 1B)[18,19].

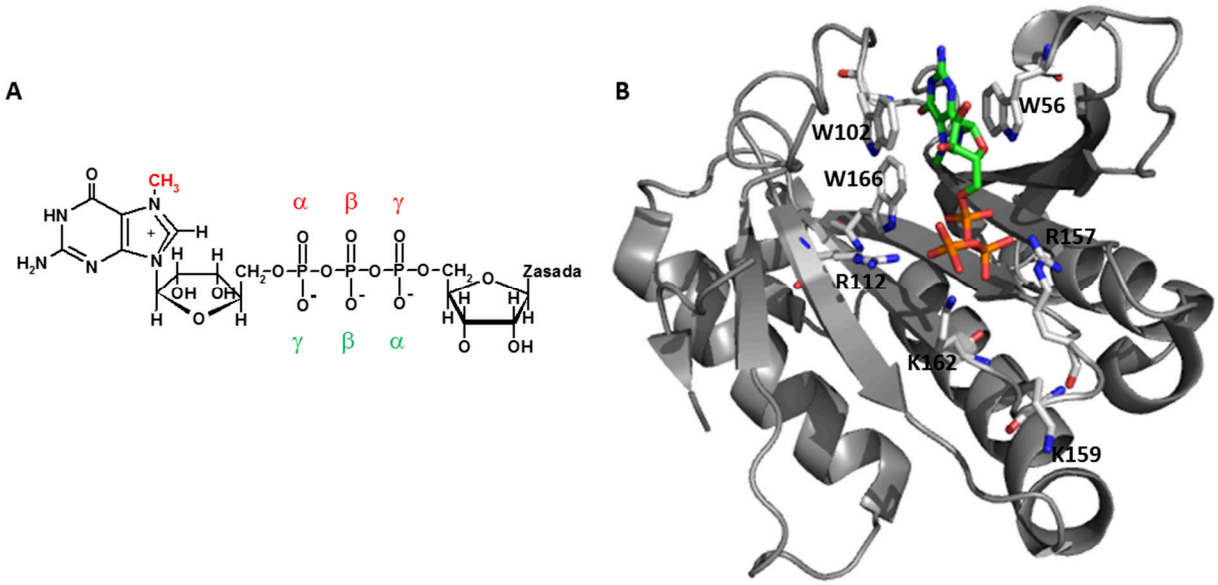


**Figure 1**. *(A) Chemical structure of analog of 5' mRNA cap. The figure shows accepted numeration of the phosphate groups for mononucleotide cap analogs (red) and for dinucleotide analogs (in green), used in the text. (B) Crystallographic structure of complex of human eIF4E with cap analog $m^7$GTP (PDB: 1IPC,* [19]*). The $m^7$GTP cap analog is shown as balls and the sticks represent amino acid residues that participate in direct cap structure*.

### eIF4E functions in the cell

It seems that all cell functions of eIF4E are connected with the presence of two binding sites at the protein [20]: one for the cap at the concave part of the protein surface in a narrow hydrophobic slot, and the second one for a broad class of 4E interacting protein, at the dorsal surface of eIF4E in a hydrophobic-acidic region which is located at the opposite face of the cap binding site [21–24].

The key cellular role of eIF4E is its participation in formation of 48S initiation during translation initiation [6,25]. Binding of eIF4E with the cap and eIF4G protein, which is a kind of "scaffold" for other complex-forming factors, is a translation initiation limiting step. Interaction of eIF4E with eIF4G is globally regulated by the so-called 4E binding proteins (4E-BPs) that compete with eIF4G for a common binding site on eIF4E, hence inhibiting the process of translation initiation. Repression of translation initiation with participation of eIF4E occurs also locally for specific mRNAs in particular stages of organism development. Here, the mechanism of translation inhibition dependent of 3'UTR mRNA sequence is mainly observed. eIF4E interacting with the cap of a given mRNA is bound by a specific protein partner that binds a specific sequence in mRNA 3'UTR directly or via other proteins which makes formation of initiation complex impossible (Figure 2) [7].

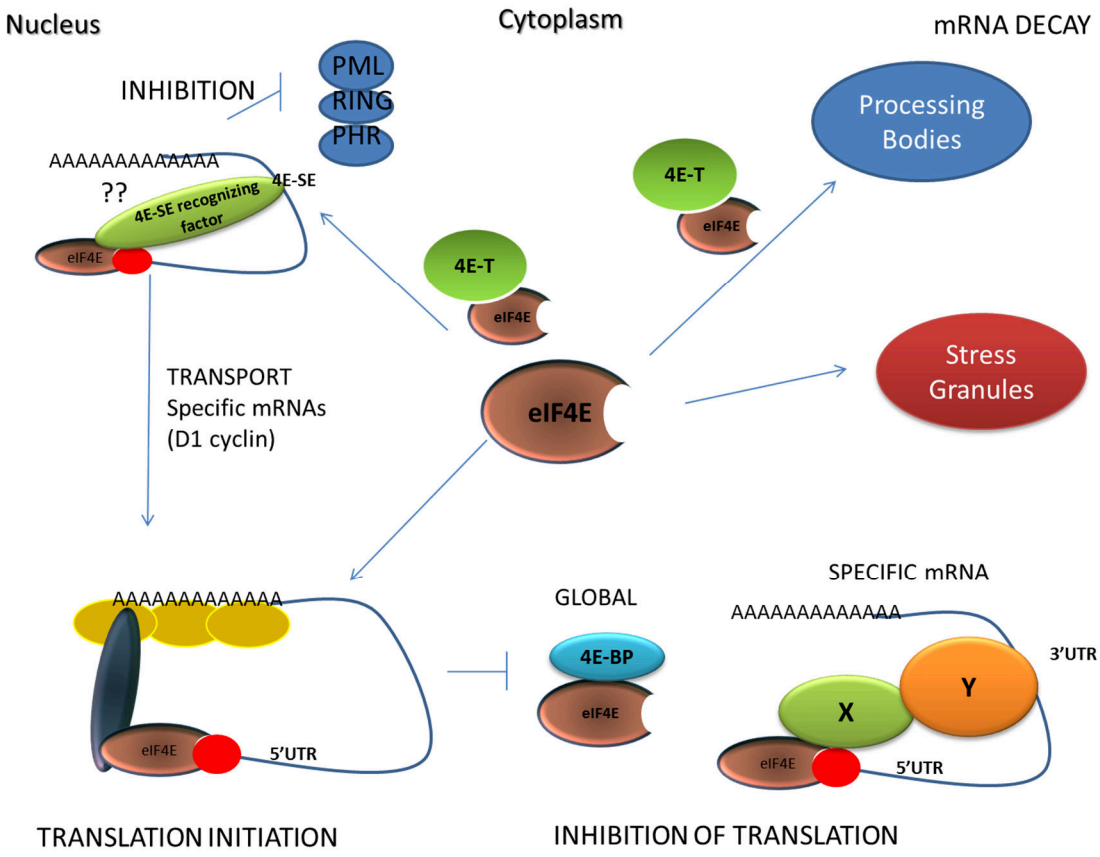


**Figure 2**. *Scheme presenting participation of eIF4E in mRNA metabolism in the cell* [6,20,22,26].

eIF4E is also engaged in nuclear-cytoplasmic export of specific mRNAs possessing the so-called eIF4E-sensitive element (4E-SE) in the 3' UTR, which facilitates recognition of mRNA by eIF4E [5,20]. eIF4E is also present in cytoplasmic granules known as *Processing Bodies* (P-Body) involved in mRNA degradation and in stress granules (SG). 4E-Transporter protein (4E-T) participates in transport of eIF4E both to the nucleus and to P-bodies [27,28] (Figure 2).

### eIF4E protein family

Initially, eIF4E was thought to be a single protein not belonging to any multiple members family, since upon protein isolation from mammalian cells using affinity chromatography only a single 25 kDa polypeptide was obtained [29]. When 15 years after discovery of eIF4E, the work by Browning et al. was published with a surprising result that in wheat there are two eIF4E isoforms (called eIF4E and eIF(iso)4E) [30], it was assumed that such anomaly is limited for angiosperms [31,32]. In the same time, a single eIF4E gene copy was identified and confirmed using Southern analysis in *Sacharomyces cerevisiae* [33]. Moreover, mammalian eIF4E was able to functionally replace yeast eIF4E in yeast strain depleted of endogenous eIF4E, which confirmed functional and structural similarity of the proteins [33]. However, 11 years later, three works were published simultaneously, showing that eIF4E is a member of a bigger protein family. The presence of three eIF4E isoforms was shown in *Caenorhabditis elegans*, the second eIF4E isoform (so-called 4EHP) was discovered in human [34], and the third eIF4E isoform - nCBP in *Arabidobsis thaliana* was indicated [35]. From that time on, the number of publications on the presence and role of eIF4E isoforms in a given organism has risen dramatically [36–40]. In 2005, there was a bioinformatic work from the laboratory of prof. Rosmery Jagus showing a

phylogenetic analysis of eIF4E protein family in eukaryotic organisms prepared based on analysis of Genebank sequences. In terms of taxonomy, members of eIF4E family can be divided into 8 subgroups, and these, apart from subgroup 8 containing atypical protozoan eIF4Es, can be grouped into three structural classes. It was shown that members of eIF4E family have a characteristic conserved central and C-terminal region of sequence – called the core, characterized by a common pattern of aromatic amino acid residues: Trp, and Phe and His in the following sequence: H(x5)**W**(x2)W(x8–12)**W**(x9)F(x5)FW(x20)F(x7)W(x10)W(x9–12)W(x34–35)W(x32–34)H. Analysis of the presence of the conserved tryptophan residues in identified eIF4E isoforms has shown that in some taxonomic subgroups there are amino acid substitution in positions corresponding to Trp43 and Trp56 of human eIF4E-1a. This feature became of the main indicators that characterize the three structural classes among proteins from the eIF4E family. In members of Class I, Trp is always present in positions corresponding to Trp43 and Trp 56 of human eIF4E-1a. This class includes all known eIF4Es functioning as translation factors (currently often referred to as eIF4E-1 or eIF4E-1a), identified in animals, plants and fungi. In many organisms, duplication of eIF4E-1 gene was observed; e.g. in mammals, there are two Class I isoforms, a fresh water fish *Danio rerio* has three isoforms, and nematode *C. elegans* has four of them. Members of Class II have Tyr, Phe or Leu in position corresponding to human Trp43 and Tyr or Phe in position corresponding to human Trp56. In most organism, there is only one isoform of eIF4E within this class (eIF4E-2), with the exception of amphibians and Osteichthyes (bony fish). Members of Class II show about 30–35% of identity with members of Class I of the given family. eIF4E (eIF4E-3) isoforms included in Class III were identified only in animals, in particular in chordates; they are also found occasionally in certain types of cnidaria, molluscs, insects or arachnids. In isoforms of this class, only substitution of Trp56 with Cys or Tyr was observed; these proteins show 25–30% of sequence identity with members of Class I within the given family.

### Potential role of eIF4E isoforms – the scientific objective of the work

Identification of individual members of eIF4E family in a given organism has posed a new, still open question: what is the potential role of eIF4E isoforms in a particular organism. Why some organisms have multiplicated eIF4E gene, and others don't? Currently, the role of eIF4E isoforms in nematode *C. elegans* [36,41–44] is known most extensively; however, a lot is also known about the role of eIF4E-2 (4EHP) and eIF4E-3 in mammals. In mammals, eIF4E-2 isoform most probably plays the role of translation inhibitor. It was observed in mice that during oocyte maturation meIF4E-2 binds the transcription factor Prep1, resulting in inhibition of translation of mRNA for homeotic gene *Hoxb4* [45], and that along with GIGYF2 protein and protein with a zinc-finger motif – ZNF598, it is a component of mRNA translation inhibition factors during embryonic development [46]. Translational regulation of homeotic genes with use of eIF4E Class II isoform was also observed in *Drosophila melanogaster*. During embryonic development, d4EHP (deIF4E-8) complexed with appropriate protein partners locally inhibits translation of mRNA *caudal* and mRNA *hunchback,* which are important for establishing the correct anterior-posterior axis polarity in the *Drosophila* embryo [47,48]. The presence of 4EHP was also observed in a complex silencing mRNA translation *via* miRNA [49]. Recent studies suggest an alternative role of eIF4E-2. They indicate that in human cells upon extreme lack of oxygen (*hypoxia*), this isoform is used in the process of alternative cap-dependent translation initiation [50–52]. Recent studies on murine eIF4E-3 isoform suggest that this isoform can act like a tissue-specific tumor suppressor [17].

Understanding the physiological role of individual members of eIF4E families is hence of great importance to understand the basic molecular processes in the cell. Explanation of the role of eIF4E proteins in maturation and differentiation of cells, embryonic development or cancerous transformation may have important implications in search for novel therapeutic strategies. However,

we cannot explain the roles of individual members of eIF4E families without identification of similarities in the molecular mechanism of recognition of 5' mRNA cap structure that influence different activity and role of individual eIF4E isoforms within the family for a given organism, as well as between individual organisms.

## 5.2 Presentation of the scientific achievement

The presented scientific achievement "*Specificity of eIF4E family proteins from different organisms towards 5' mRNA cap*" is based on comparative research on eIF4E proteins: in human as a representative of mammals, in the African clawed frog (*Xenopus laevis*) - as a representative of amphibians, in fruit fly (*Drosophila melanogaster*) – insect representative and a model organism, in thale cress (*Arabidopsis thalina*) as a representative of angiosperms (flowering plants) and eIF4E from yeast *Saccharomyces cerevisiae* as a representative of fungi and a model organism. The research was expanded with atypical eIF4E protein family from trypanosomatid parasites. The presented results are based mainly on equilibrium association constants ($K_{as}$) determined for the studied eIF4E proteins in complex with a series of cap analogs, using fluorescent titration, sequence and structure analysis supported by homology modeling for the unresolved structures, as well as on the changes in tertiary structure and changes in tryptophan residues surrounding recorded in the near-UV circular dichroism spectra.

### A. Diverse affinity for cap among "canonical" eIF4E translation factors from different organisms

In translation initiation in eukaryotic organism, there are certain differences, e.g. in the process of pre-initiation complex formation and regulation of activity of individual components of such complex, in particular of eIF4F. In plants are two separate eIF4F complexes: eIF4F composed of eIF4E and eIF4G, and eIF(iso)4F composed of eIF(iso)4E and eIF(iso)4G [30–32,53] and as research shows they are preferentially used to translation of a group of specific mRNAs. Until now, no homologs of 4E-BP proteins that regulate availability of eIF4E for formation of eIF4F complex were found in plants [53]. In this context, an important question arises: what is the difference in affinity similarity of eIF4E translation factors from different organisms and which sequential and structural components are involved. Do the changes in the molecular mechanism of cap binding by eIF4E from a given organism translate into differences in formation and interaction mechanisms between eIF4E and its individual partners during gene expression.

Association constants for canonical eIF4E factors with a series of cap analogs determined in the same conditions based on isotherms of fluorescent titration presented in publications: **H2, H5, H6, H7, H8** show that the occurring sequential and structural evolutionary changes in canonical eIF4E factors are mainly aimed at increasing the affinity of eIF4E for cap. Canonical human or murine eIF4E bind $m^7$GTP over 10-fold stronger than their counterpart from fruit fly, plants or yeast, but only 1.5 times stronger than eIF4E from amphibians (Table 1). We showed (**H6-H8**) that changes in the network of electrostatic interactions that stabilize cap's phosphate chain binding in the cap binding slot play an important role; this is especially true for a visibly weaker stabilization of γ-phosphate in the case of deIF4E-1 and for β-, γ- and δ-phosphate of cap in AteIF4E and yeIF4E. In contrast to mammalian eIF4Es, where the β-phosphate group of cap is stabilized by two hydrogen bonds with side chain of Arg157 and Lys162, for eIF4E from thale cress and from yeast the β-phosphate is stabilized by only one hydrogen bond with Arg157 in yeIF4E and Arg178 or Arg183 in AteIF4E. Presented in publication **H7** comparison of sequences and homology modeling showed that the main reason for weaker binding of γ-phosphate in eIF4E from invertebrates, plants and fungi is lack of additional α-helix present after β4-

strand. It is composed of the following sequence of amino acid resiudes: KQQRRS with large side groups present only in vertebrates. The additional α-helix moves a spatially neighboring loop β5β6 towards the cap binding slot, which allows stabilization of cap's γ- and δ-phosphate by Lys159 in vertebrates. We observed (**H5-H7** *and unpublished data for eIF4E from S. cerevisiae*) that in all studied canonical eIF4Es, the presence of the second base in the cap analog results in destabilization of binding, however, energetic cost of the presence of the second base for the yeast and plant protein is over 50% lower than in the case of vertebrate eIF4Es. In none of the isoforms, formation of stabilizing contacts for the second cap base was observed, and weaker destabilization of dinucleotide bond of cap analog as compared to its mononucleotide counterpart in yeIF4E and AteIF4E is most probably caused by lack of contact between cap γ-phosphate and side groups of positively charged amino acid residues in yeIF4E and AteIF4E present at the entrance to the cap binding slot which are not destabilized by the presence of a labile second base of the cap. Analysis of specificity of the studied canonical eIF4Es from different organisms for methylation of cap guanosine in N7 position, presented in works **H5-H8**, indicates that also in vertebrate eIF4Es there were sequential and structural changes that lead to selection of the most optimal orientation of Trp indole rings and N7-methylguanosine of the cap for the cation-π interaction.

Comparative analysis of 4E translation factor isoforms in plants, performed in **H6**, unexpectedly showed that the second factor, eIF(iso)4E, evolutionarily present as late as in the flowering plants shows very low affinity for cap analogs. AteIF(iso)4E binds $m^7$GpppG 10-fold weaker than AteIF4E, and $m^7$GTP 5-fold weaker. Analysis of the modeled structures indicates that the main cause of low affinity of eIF(iso)4E towards cap is shortening of the loop between β7-strand and β8-strand by 6 amino acid residues. Shortening of the loop makes it impossible to form α-helix that forms a closing edge of the cap binding slot in other eIF4Es.

To sum up, all identified sequential and structural differences between canonical eIF4E from the studied organisms show that upon cap binding in invertebrates, plants and insects, the structure of eIF4E remains more "open" than in eIF4Es from vertebrates. Closing of eIF4E may occur as a result of interaction with 4E binding proteins (4E-BP), and the type of the structural changes leading to the closure of cap binding slot and increase in eIF4E affinity towards the cap depends on the protein partner, and hence on the process into which eIF4E is currently involved in the cell.

### B. Diversity of cap affinity among class I eIF4E isoforms and identification of the related sequential and structural changes

In studies on the members of eIF4E families, a hypothesis set forth in 2005 by Hernandez and Vazguez-Pianola [54] is still valid; it states that only one member of eIF4E family plays the role of the main translation factor, being present in all tissues of the organism and being continuously expressed during all stages of development and life of the organism, and the other factors are engaged into specific processed or regulation of genes active e.g. at a given development stage. Hence, my next question was whether the potential physiological role of eIF4E family members within the same structural class is reflected in their different specificity towards the cap analogs. If this is the case, then what changes occur in the molecular mechanism of cap binding in those isoforms and which sequential and structural changes are involved. Is this significant for the choice of protein partners for interaction of a given eIF4E isoform?

In vertebrates, in Class I, apart from the canonical eIF4E (currently referred to as eIF4E-1a), there is eIF4E-1b isoform, the expression of which is restricted to ovaries, oocytes and early embryogenesis [55]. In mammals, its role is unknown; however, in an African clawed frog it was identified as a component of CPEB mRNP complex that inhibits translation of maternal mRNAs in oocytes [56]. In fruit fly, there are as many as six genes coding for seven Class I eIF4E isoforms,

however, apart from deIF4E-1 which is the main translation factor, only the role of deIF4E-3 is known – its presence is necessary for proper course of spermatogenesis (**D16**). In *A. thaliana*, there are four eIF4E isoforms classified into Class I, with the presence of eIF4E-1b and eIF4E-1c isoforms is related to a double duplication of *eIF4E* gene [57].

All Class I eIF4E isoforms from human, fruit fly and thale cress have amino acid residues determined to be crucial for cap binding, however their specificity towards cap varies (Table 1) as shown in studies described in **H5-H7**.

**Table 1.** *Equilibrium association constants for complexes of Class I eIF4E with cap analog $m^7GTP$ determined using fluorescence titration.*

| eIF4E Class | *H. sapiens* (**H2, H5, H7,H8**) | | *D. melanogaster* (**H7**) | | *A. thaliana* (**H6**) | |
|---|---|---|---|---|---|---|
| | eIF4E name | $K_{as}$ [$\mu M^{-1}$] for $m^7GTP$ | eIF4E name | $K_{as}$ [$\mu M^{-1}$] for $m^7GTP$ | Name eIF4E | $K_{as}$ [$\mu M^{-1}$] for $m^7GTP$ |
| **Class I** | heIF4E-1a* | 70.1 ± 1.2 | deIF4E-1* | 8.94 ± 0.27 | AteIF4E* | 5.79 ± 0.13 |
| | heIF4E-1b | 22.0 ± 1.4 | deIF4E-2 | 8.47 ± 0.28 | AteIF4E-1b | 3.12 ± 0.06 |
| | | | deIF4E-3 | 19.29 ± 0.52 | AteIF4E-1c | 5.72 ± 0.10 |
| | | | deIF4E-4 | 2.16 ± 0.07 | AteIF(iso)4E* | 1.07 ± 0.05 |
| | | | deIF4E-5 | 17.9 ± 1.4 | | |
| | | | deIF4E-7 | 5.3 ± 0.1 | | |
| **Class II** | heIF4E-2 (h4EHP) | 0.70 ± 0.04 | deIF4E-8 (d4EHP) | 2.89 ± 0.12 | nCBP | 0.68 ± 0.01 |
| **Class III** | heIF4E-3 | 5.5 ± 0.2 | not present | | not present | |

*- canonical eIF4E

Although sequences of human isoforms eIF4E-1a and eIF4E-1b are 61% identical and sequence changes are mainly in the N-terminal end of the proteins, heIF4E-1b isoform shown 2–3-fold lower affinity for mononucleotide cap analogs and about 1.5 times lower affinity for dinucleotide cap analogs as compared to heIF4E-1a (**H5**). Research performed in **H5** with use of a series of cap analogs with different substitutions at N7 position of cap guanine showed significant difference in the structure of the hydrophobic pocket that binds 7-methylguanine of the cap between the two proteins. This indicated e.g. 5-fold higher affinity of heIF4E-1b to GTP and 3-fold higher affinity to $bn^7GDP$ as compared to heIF4E-1a, which indicates a non-optimal position of aromatic rings in a three-layer system for cation-$\pi$ interaction. Gross lability of the hydrophobic pocket itself is confirmed by high affinity towards cap analogs with big substitutes at N7 position of guanine, such as e.g. benzyl group. Analogical lability of the hydrophobic cap-binding pocket was also observed for eIF4E-1b from *X. laevis* (**H5**). Homology modeling of XeIF4E-1b structure, presented in **H5** indicates that the presence of Ser and Arg in eIF4E-1b proteins in positions corresponding to Glu105 and Lys106 in heIF4E-1a may directly influence the change of Trp102 orientation, and the presence of Ala at Ser199 position may influence its orientation by change in orientation of His200. Trp56 participation in stacking interaction is probably weakened by the change in the residue surrounding, in particular due to the change in Phe28 position resulting from presence of two serines at positions 86 and 87 in eIF4E-1b, instead of methionine and proline like in heIF4E-1a. Probably, bigger lability of hydrophobic cap-binding pocket

in eIF4E-1b can be related to potentially different degree of cap methylation in maternal mRNAs in oocytes and egg [58–60].

A completely different type of changes in cap affinity was observed in closely related members of Class I eIF4Es in *A. thalina* (**H6**). AteIF4E-1b and AteIF4E-1c show 56% and 57% of sequence identity with AteIF4E sequence and the changes in the sequence are present mainly in the N-terminal part of the protein and involve amino acid residues related to interaction of eIF4E with eIF4G. Sequence identity between AteIF4E-1b and AteIF4E-1c is 95% Determined equilibrium association constants ($K_{as}$) for complexes of AteIF4E-1c with a series of cap analogs are comparable to those obtained for the canonical AteIF4E, whereas in the case of AteIF4E-1b, they are about 2-fold lower, except of the constant for $m^7$GMP, which is comparable in AteIF4E-1c and AteIF4E-1b, and at the same time 2.5-fold lower than for AteIF4E. The results suggest that the main changes between the isoforms are related to the network of interactions between the cap phosphate chain and positively charged amino acid residues in the cap binding slot. 5% difference in sequence identity between AteIF4E-1c and AteIF4E-1b is mainly due to changes in amino acid residues localized in the vicinity of the cap binding slot. Based on the comparative analysis of the modeled structures of eIF4E isoforms, we assumed that the change of hydrophobic Phe147 and Thr145 in AteIF4E-1c to methionine and serine in AteIF4E-1b may lead to weaker stabilization of Arg188 side chain (corresponding to Lys162 in heIF4E-1a), which results is a weaker binding of $\beta$- and $\gamma$-phosphate groups of cap in AteIF4E-1b. The role of AteIF4E-1c and AteIF4E-1b isoforms are yet to be determined, however these isoforms interact with plant eIF4G and are able to functionally replace yeast eIF4E in yeast cells, which indicates that they can play the role of translation factors [57].

*Drosophila* eIF4E isoform belong to Class I, studied within **H7**, show diversity in formation of molecular contacts with the cap structure. deIF4E-3 isoform has the highest affinity for cap. Sequential and structural changes that occurred for this isoform led to selection of in particular contacts stabilizing the binding of $\alpha$-phosphate group of cap, and weakened the contacts formed between positively charged side groups of amino acid residues in the cap-binding slot and $\beta$-, $\gamma$-phosphate moiety of cap. The determined equilibrium association constant for deIF4E-3 with $m^7$GMP is even 3.6 times higher than for the human eIF4E-1a. Unfortunately, sequence and structure analysis performed within this project did not provide an equivocal answer to the question which changes are responsible for such a strong stabilization of cap $\alpha$-phosphate group in deIF4E-3. Analysis performed for deIF4E-3 allowed us to identify binding of the second base via stacking interaction with His 234 ring, which is atypical for eIF4E protein family. Analogous stabilization of the second base was also found in deIF4E-5, where Phe222 can participate in the stacking interaction. Stabilization of the second cap base is hence characteristic for a different cap-binding protein – the nuclear cap binding complex (CBC) [12]. Significantly higher affinity of deIF4E-3 for cap most probably helps it win concurrence with deIF4E-1 for binding of caps in mRNAs that are translated during spermatogenesis, and due to the fact that expression of deIF4E-3 in fruit fly is limited to testes and it occurs at the early stages of spermatogenesis, it will not interfere with other processes of translation initiation, where deIF4E-1 takes part (**D16**). In deIF4E-5 isoform, we observed stronger binding of $\alpha$-phosphate moiety of cap, as compared to canonical deIF4E-1, but lack of destabilization of binding of further phosphate groups as it was for deIF4E-3. In the case of deIF4E-7 isoform, analysis of changes in free enthalpy of binding ($\Delta\Delta G^o$) of individual structural elements of cap indicates that in all stages of cap binding there were minor changes in the molecular contacts. And the performed sequence and structure analyses showed that unstructured very long 249 amino acid N-terminus present in this isoform does not influence cap binding. In the case of deIF4E-4 that has the weakest cap binding, sequence and structure changes led to destabilization of stacking interaction between indole rings of Trp and 7-methylguanine of cap, resulting from e.g. increased lability of Trp residues and non-closing of eIF4E structure upon cap binding. This confirms 2-fold increase of deIF4E-4 affinity for cap we observed, when it is complexed

with a 13 amino acid fragment of eIF4G. Binding cooperativity was not observed for the other studied eIF4E isoforms from fruit fly and human (**H7**). This suggests that for physiological role of deIf4E-4, interaction with protein partners will be important.

To sum up, the results obtained in **H5-H7** show that single changes of amino acids residues in the sequence of eIF4E isoforms around the cap binding slot have direct or indirect influence on the placement of amino acids residues that are directly involved in cap binding and result in diverse affinity of Class I members of eIF4E family towards the structure of 5' mRNA end. The observed differences in the molecular mechanism of cap binding are connected with all its elements: stabilization of 7-methylguanosine by stacking interaction with indole rings of tryptophan residues, binding of the phosphate chain or the second base of the cap. Identification of sequential and structural changes in isoforms responsible for their different affinity towards cap wouldn't be possible, though, without earlier determination of association constants for their complexes with a series of modified cap analogs and characterization of affinity differences for individual elements of the cap structure.

It should also be noted that the identified differences in the molecular mechanism of cap binding in members of eIF4E family Class I are reflected in their function within the cell, which is confirmed by those few cases of Class I eIF4E isoforms with identified function.

### C. The influence of amino acid composition in the "sandwich-stacking" in eIF4E on affinity for cap

In Class II and III eIF4E proteins, there is a difference in the layout of the aromatic rings involved in stacking interaction, where for Class II eIF4E, II there was a substitution of Trp at position 56 with an aromatic amino acid Tyr or Phe, and in Class III isoforms, at position 56, there is a non-aromatic Cys or aromatic Tyr. The first question I asked upon the start of analysis of Class II and III eIF4Es from the chosen organisms was how the change influences cap binding specificity of these eIF4E isoforms and is it reflected in analogical substitutions of Trp56 in the human eIF4E-1a – the canonical translation initiation factor.

In **H2** and **H8**, results for a series of tryptophan mutants of heIF4E-1a were presented. Expression constructs for the studied mutants were obtained using oligonucleotide-directed mutagenesis with use of PCR, and then the proteins were obtained by overexpression in *E. coli* cells and purified from the inclusion bodies. Studies on mutants interaction with cap analogs performed using fluorescence titration, presented in table 2, showed that Trp102 is the key tryptophan residue in the system of aromatic rings involved in stacking in heIF4E-1a. Its substitution by a different aromatic amino acid residue weakens protein binding to $m^7GTP$ over 10-fold. What is interesting, the research showed that the most efficient combination of aromatic rings for heIF4E-1a is Tyr56/Trp102, not the naturally occurring Trp56/Trp102.

**Table 2** *Equilibrium association constants for complexes of human eIF4E mutated within the aromatic amino acid residues involved in cap $m^7$Gua stacking, with cap analog $m^7$GTP*

| Stacking configuration | **Trp** | **Tyr** | **Phe** | **Cys** | **Ala** |
|---|---|---|---|---|---|
| | $K_{as}$ [$\mu M^{-1}$] for the complex of mutant heIF4E-1a with $m^7$GTP (Class I) | | | | |
| [X] - 56<br>***$m^7$Gua***<br>[Trp] - 102 | **70.1 ± 1.2** | 108.9 ± 7.8 | 27.4 ± 2.9 | 0.31 ± 0.03 | 0.21 ± 0.0 |
| [Trp] - 56<br>***$m^7$Gua***<br>[X] - 102 | | 7.07 ± 0.39 | 2.44 ± 0.10 | --- | 0.15 ± 0.01 |
| [X] - 56<br>***$m^7$Gua***<br>[X] - 102 | | 12.33 ± 0.35 | 1.18 ± 0.10 | --- | no binding |

In the light of the results presented in **H2** and **H8**, evolutionary substitution of Trp56 by a different aromatic amino acid in Class II and III isoforms does not directly indicate weaker cap binding by the isoforms. However, the change of Trp56 to non-aromatic amino acid residue cysteine or alanine in heIF4E-1a that disturbs the three-element stacking significantly weakens cap binding, is in agreement with the previously presented results of inability to bind $m^7$GDP-Sepharose by analogous mutants where tryptophan residues were substituted by leucines [13,61,62]. Biological studies [39] showed, however, that murine eIF4E-3, which has cysteine in the position corresponding to Trp56, is able to bind to $m^7$GTP-Sepharose.

The performed studies on interaction of human Class II eIF4E – heIF4E-2 (W->Y) and Class III – heIF4E-3 (W->C) with cap analogs, presented in **H1** and **H8**, showed that analogous substitution in the sandwich stacking set in heIF4E-1a do not directly reflect the molecular mechanism of cap binding in these isoforms. Human eIF4E-2 isoform with maintained system or aromatic rings binds $m^7$GTP 100-fold weaker than heIF4E-1a, and about 10-fold weaker than eIF4E-3 with only one Trp interacting by stacking with $m^7$Gua. For both isoforms, three characteristic elements of cap binding by eIF4Es are maintained:

(i) specificity towards guanosine methylation at N7 position,
(ii) stabilization (via electrostatic interaction) of cap binding with elongation of phosphate chain,
(iii) destabilization of cap binding by the presence of the second base.

However, analysis of changes in Gibbs free energy ($\Delta\Delta G^o$) showed a much weaker selectivity of heIF4E-2 towards N7 guanosine methylation. Analogous weak selectivity towards $m^7$G is shown by nCBP – eIF4E isoform from thale cress classified into Class II (**H6**); what is interesting, deIF4E-8 Class II isoform from fruit fly (**H7**) does not show this feature. Comparative sequential and structural analysis of the three proteins presented in **H6** and **H7** indicated that the labile loop $\beta1\beta2$, where Trp56 is located, is longer by a few amino acid residues in Class II isoforms (heIF4E-2 – 5 aa, nCBP and deIF4E-8 – 3 aa), as compared to Class I eIF4E. This can lead to configuration of tyrosine residue that is energetically unfavorable for cation-$\pi$ interaction; and even more so, since in heIF4E-2 [63] and the modeled nCBP structure (**H6**) a new short $\alpha$-helix is formed and makes the loop more rigid. Performed homology modeling did not show formation of analogous helix in deIF4E-8, though. A significant difference in molecular mechanism of cap binding in Class II eIF4Es shown by research of interaction with cap analogs of different length of the phosphate chain (**H2**, **H6** and **H7)**, is a smaller contribution

of electrostatic interactions that stabilize binding of cap phosphate groups. As structural analysis shows, this is connected with a different layout of network of amino acid residues with positively charged side group in the cap binding slot of these proteins.

To summarize, the results from **H2**, **H6**, **H7** show that in eIF4E proteins, the key aromatic amino acid residues for cap $m^7$Gua binding via stacking interaction is tryptophan corresponding to Trp102 as per numeration for the human protein, and the presence of a different amino acid residue in position equivalent to Trp56 in the human eIF4E is not the only cause of changes in affinity of Class II and III eIF4E isoforms towards the mRNA cap.

### D. Picture of changes in conformation and surrounding of eIF4E cap binding tryptophans in near ultraviolet CD spectra

For many years, circular dichroism (CD) spectroscopy has been used to study protein structures in solution and their changes upon e.g. ligand binding. The commonly used CD spectroscopy in the far UV range provides the information on the secondary structure content of the protein as a result of different processes [64]; however, a much less often used CD spectroscopy in the near UV range provides information on the tertiary structure of the protein [64,65]. Signals in CD spectra recorded in the range 250–320 nm result from electron transitions of aromatic amino acid residues, the symmetry of which was disturbed by interaction with the surrounding. The shape and intensity of CD signals is hence a characteristic and individual feature of the protein and depends among other factors on the number of aromatic amino acid residues in the protein, their spatial arrangement and distance to the neighboring amino acid residues, and in particular to other aromatic and polar amino acid residues, or e.g. their participation in formation of hydrogen bonds [65].

Evolutionary conservation of 8 tryptophan residues in a characteristic sequence mode set also by the presence of conserved phenyloalanine and histydyne in the sequence of eIF4E family from *Metazoa*, *Viridiplantae* and *Fungi* [66], direct participation of three tryptophan resiues in cap binding and their specific substitutions in Class II and III eIf4E proteins evoked a question whether the near-UV CD spectroscopy could be a useful method to tract changes in tryptophan residues surrounding in eIF4E and the changes in their interaction with cap. This resulted in work **H8**.

Research performed within **H8** showed that near-UV CD spectrum of heIF4E-1a is a typical spectrum observed for model tryptophan derivatives imitating the presence of tryptophan residue in the peptide bond and tryptophan proteins classified as Type I, where the CD bands arise from Trp $^1L_b$ electron transition. It is dominated by two vibrionic CD bands at 291 nm (0 - 0) and 284 nm (0 + 847 $cm^{-1}$) with the same positive sign Conservation of the above profile of CD spectrum was observed for all canonical eIF4Es from other organisms **H8**, as well as in human eIF4E isoforms except of heIF4E-3, where Trp band originating from type $^1L_B$ 0-0 transition is overlapped by a broad band from tyrosine and phenylalanine residues. Such a "simple" profile of a near-UV CD spectrum is very rarely in proteins with more than one tryptophan molecule and it indicates that for most tryptophan residues in eIF4E, CD bands arise from $^1L_B$ transitions of similar energy. The observed conservation of the near-UV CD spectrum profile in eIF4Es is hence another feature that highlights the structural conservation of this family of protein.

Considering the conservation of the CD spectrum profile, identification of contribution from individual Trp residues into the observed CD bands would provide a deeper insight into the molecular mechanism of structural and functional meaning of evolutionary conservation of tryptophan moieties in eIF4E and also observation of conformation changes in tryptophan residues participating in cap binding and their subtle differences between individual proteins from the eIF4E family. Identification

of signals from individual tryptophan residues may be obtained by analysis of CD spectra for mutant proteins, where individual tryptophan residues were substituted with non-aromatic amino acid residue e.g. alanine or a different aromatic amino acid residue e.g. phenylalanine which would disturb the protein spatial structure to a minor extent. In eIF4E, only Trp56 and Trp102 are located on mobile loops and the other six Trp residues are located in the regions of secondary structures, which was the reason why the obtained heIF4E-1a mutants with alanine or phenyloalanine instead of tryptopan at positions 43, 46, 73, 113, 130 and 166 destabilized the eIF4E structure and induced strong protein aggregation, thus preparation of reliable CD spectra for these proteins was impossible. CD spectra for single and double eIF4E mutants with Trp56 or/and Trp102 were substituted by alanine, tyrosine or phenyloalanine showed that for the apo protein, only Trp102 contributes to the observed CD bands. The minor input of Trp56 to eIF4E1a spectrum is most likely connected with significant rotation observed in structure of apo-heIF4E-1a.

A wide range of heIF4E-1a mutants at Trp56 or/and Trp102 that bind or don't bind mRNA cap allowed identification of changes in CD signals from tryptophan residues that occur upon cap analog binding. In wild type heIF4E-1a upon cap binding, two additional CD bands occur; one is negative in the range 295–330 nm with minimum at 297 nm and one positive in the range 255–270 nm with maximum at 262 nm. These bands are probably connected with Trp $^1L_a$ transitions. In the case of CD bands arise from Trp $^1L_b$ transitions, upon cap binding, there is an increase in intensity of 284 nm band and the intensity of 291 nm band is unchanged. Upon cap binding, the profile of heIF4E-1a CD spectrum changes from Type I to Type III.

The performed analysis of CD spectra for mutants complexed with cap analogs showed that:

(i) as a result of cap binding, local changes in eIF4E structure occur and influence conformation or surrounding of the other conserved tryptophan residues that do not participate in direct cap binding and which give negative CD signal from $^1L_b$ transitions. These changes are also connected with the change in localization of Trp56 and Trp102 or other aromatic amino acid residues substituted in these positions, resulting from cap binding, that lead to dipole-dipole coupling between the orbitals of excited states or aromatic amino acid residues at position 56 or 102 and other Trp of eIF4E.

(ii) as a result of interaction of Trp56 with the cap, additional signals appear in the CD spectrum of the complex: a positive CD signal from $^1L_b$ transitions and positive signal from $^1L_a$ transition, in the range between 295–310 nm,

(iii) as a result of Trp102 interaction with the cap, additional signals appear in the CD spectrum of the complex: a negative signal from $^1L_a$ transition in the range between 295–330 nm and a positive signal connected with $^1L_b$ (0+847cm$^{-1}$) transition, but only in the presence of an aromatic amino acid residue at position 56, which is most probably due to a dipole-dipole coupling between orbitals of excited states of Trp102 and the aromatic amino acid at position 56.

The changes observed in CD spectra of heIF4E-1a mutant spectra as a result of interaction with the cap, attributed to individual tryptophan residues, are reflected in the spectra of complexes of human eIF4E isoforms. In eIF4E-3 isoform (W56->C, in heIF4E-3 this is Cys69), we observed no signals from Trp56 or presence of aromatic amino acid in this position, only the signal connected with Trp102 (in heIF4E-3, this is Trp115) was observed. In CD spectrum of the complex of the yeast eIF4E with m$^7$GTP, we observed only signals from Trp56 (in yeIF4E, this is Trp58), and no signal from Trp102 (in yeIF4E, this is Trp104), which is connected with reverse orientation of Trp58 in yeIF4E in a three-layer system sandwich stacking, which is confirmed by NMR structure of the complex [67].

To summarize, analysis of CD signals of eIF4E in the apo form and in complexes with cap analogs, presented in **H8** shows that near-UV CD is a very useful method to analyze the changes in the system of aromatic rings that form the three-layer sandwich system of stacking that binds 7-methylguanosine of cap in eIF4E proteins; these changes result from local structural and sequential changes in these proteins or are forced by introduction of other functional groups into the N7 position of guanine in new variants of cap analog, currently widely tested as potential eIF4E inhibitors in tumor cells [68–71].

The second advantage of near-UV CD spectroscopy for eIF4E proteins which I was able to show in **H8** is the use of this method to determine the binding constants based on the recorded changes in the protein ellipticity as a function of the ligand concentration. Association constants obtained by means of analysis of titration isotherms for heIF4E-1a with four cap analogs with ellipticity changes in the range of the CD band 295–330 nm are in agreement with constants obtained in the same conditions based on fluorescence titrations.

### E. Specificity of cap binding and potential role of eIF4E proteins in Trypanosomatides

In contrast to eIF4E proteins from organisms from the kingdom of animals, green plants and fungi, some protozoans have atypical eIF4E isoforms, which are difficult to classify into one of the three defined structural classes, due to broad, random introduction of amino acid sequences in the defined structural core of eIF4E [66]. A very interesting family among parasitic protozoans are *Trypasomatidae*, which covers *Leishmania* and *Trypanosoma* geni, which upon their travel among the insect vector and mammalian host cause serious parasitic diseases in humans and domestic animals. For example, *Trypanosoma brucei* causes sleeping sickness and over 30 species from *Leishmania* genus cause a disease known as leishmaniasis, with its most severe form, the so-called visceral leishmaniasis (also known as kala-azar), which without treatment results in death in 95% of cases. This family is also interesting due to the presence of atypical molecular features related to gene expression [72]. Protein coding genes are organized into large transcription units, which form a polycistrone pre-mRNA that is further processed into monocistrone mRNA vis *trans*-splicing and polyadentylation [73]. During trans-splicing, a 39-nucleotide *Spliced Leader* (SL RNA) is attached to the 5' end of the formed RNAs; on its 5' end, this SL RNA has a unique structure of cap not present in any other eukaryotes: **$m^7Gpppm_3^{6,6,2'}Apm^{,2'}Apm^{,2'}Cpm_2^{3,2'}U$, known as cap-4** [74,75]. The aim of publication **H1** prepared in cooperation with the chemical group of Prof. Edward Darzynkiewicz and the group of Professor Michal Shapira from Ben Gurion University of the Negev and of publications **H3 and H4** prepared in cooperation with professor David Campbell from the University of California at Los Angeles, was to obtain eIF4E isoforms present in *Leishmania major and Trypanosoma brucei* and to characterize their specificity towards the cap, as well as to determine the potential biological role of these proteins. A major problem in treatment of diseases caused by these parasitic protozoans is a limited number of drugs, with the majority of them being toxic and requiring hospitalization. Hence these works and many other biological studies on these organisms is also aimed at identification of potential therapeutic targets, in this case connected with specificity of eIF4E isoforms towards the cap.

In **H1**, based on analysis of sequence homology in a genome of *L. major*, four eIF4E isoforms were identified; these are referred to as Leish4E-1, Leish4E-2, Leish4E-3 and Leish4E-4, and show a low degree of sequence homology to eIF4E from yeast or higher eukaryotes. The methods of 3D structure predicting used in this work show that despite low homology, the structures of eIF4E isoforms from *Leishmania* have a $\alpha/\beta$ fold characteristic for eIF4E proteins, however certain differences are observed for the cap binding motif itself. A characteristic system of three tryptophan residues (Trp56, Trp102 and Trp166 according to numeration for the human protein) that participate in cap binding is

conserved only in Leish4E-1 and Leish4E-2 isoforms. In Leish4E-4 isoform, there is a tyrosine in the position corresponding to Trp56 and in Leish4E-3 - a non-aromatic amino acid, methionine. From the classical set of basic amino acid present at the entrance to the cap binding slot and interacting with the cap phosphate chain, only arginine corresponding to Arg157 from the human protein is conserved in all isoforms. Until now, despite continuous efforts, no one was able to obtain crystallographic structures of any of the isoforms, which would verify out theoretical predictions based on modeling.

The performed studies on interaction of eIF4E isoforms from *Leishmania* with cap-4 and its intermediated and other cap analogs using time-synchronized fluorescence titration, optimized by me for these proteins, showed their diverse specificity towards the modified cap analogs. The studies indicated that similarly like in other eIF4E family proteins, these isoforms have a visible specificity towards methylation of the guanine ring at N7 position and a characteristic destabilization of cap binding as a result of addition of a second base to $m^7$GTP. As indicated based on analysis of changes in Gibbs free energy ($\Delta\Delta G^o$), Leish4E-1 and Leish4E-2 most probably bind the second base, just like deIF4E-3 and deIF4E-5 isoforms from fruit fly (**H7**).

A very interesting yet surprising result was an over 100-fold weaker (as compared to the murine eIF4E) affinity of eIF4E isoforms from *L. major* not only to $m^7$GTP, which was expected, but also to cap-4, present only in protozoans from genus *Kinetoplastida*. Comparative studies performed by me for the murine protein and Leish4E-1 isolated both from the soluble fraction and from inclusion bodies, not included in the discussed work, showed equivocally that the manner of the protein purification has no influence on the association constants I obtained and presented in the publication. Also the results of the experiment presented in **H1** showing that none of LeisheIF4E isoforms is able to functionally replace yeast eIF4E in yeast strain with no endogenous eIF4E confirm the structural and functional difference of these isoforms.

Among *Leishmania* eIF4E isoform, only Leish4E-2 shows a marked preference towards cap-4. Association constant for the complexes of $m^7$GTP and cap-4 with Leish4E-1 and Leish4E-4 isoforms are comparable. And Leish4E-3 is the isoform with a marked preference for $m^7$GTP binding and destabilization of binding due to the presence of the second base or methylation of bases and sugars present in cap-4 is analogous to that observed for mouse protein. The isoform with the strongest binding of modified cap analogs is Leish4E-4 with substitution of Trp56 with Tyr. Also Leish4E-3 with substitution of Trp56 to methionine binds $m^7$GTP 2-fold and 10-fold stronger than Leish4E-1 and Leish4E-2, where the system of two tryptophan residues that participate in cap N7-guanine stacking is conserved. These results, analyzed along with the previously discussed results for human eIF4E isoforms and mutants of heIF4E-1a (**H2, H8**) again show that cap 7-methylguanine stacking between two aromatic amino acid residues are not necessary or sufficient for efficient cap binding by eIF4E and that local structural changes and amino acid substitution influence the binding affinity.

Studies of affinity of Leish4E isoforms towards cap along with analysis of isoform distribution in polysome fractions and with "pull down" experiments that investigate interaction of *Leishmania* eIF4Es with human 4E-BP1 protein allowed determination of two potential candidates, Leish4E-1 and Leish4E-4 as possible translation factors in *L. major*. Further studies on Leish4E-1 and Leish4E-4, performed in cooperation with professor Michel Shapira (**D6**) showed that in promastigota from, Leish4E-4 is the translation factor.

Since professor David Campbell invited me to cooperate with him, in the case of eIF4E isoforms from *T. brucei* I characterized affinity of new eIF4E isoforms discovered by professor's group, called TbEIF4E-5 and TBEIF4E-6, which is presented in **H3 and H4**. Phylogenetic analysis and sequence homology showed a much more close homology between these proteins than with the other TbEIF4E isoforms that were previously identified and characterized [76]. In both proteins, cap binding is carried out by an atypical system of aromatic amino acid residues that participate is stacking interactions,

where also Trp102 is substituted in contrast to Trp56 as it was in the previously studied isoforms. In TbEIF4E-5 isoform, this is Trp/Tyr, and in TbEIF4E-6 – Phe/Phe. Among the remaining tryptophan residues conserved in the eIF4E sequence, Trp43 and Trp166 are conserved in both, additionally Trp73 is conserved in TbEIF4E-5. Similarly like in the previously characterized eIF4E proteins, both isoforms have a marked specificity towards methylation of the guanine ring at N7 position and a characteristic destabilization of cap binding as a result of addition of a second base to $m^7$GTP. They do not show, however, preference for cap-4 binding. Association constants determined for the complexes of these proteins with $m^7$GTP and cap-4 are very similar, although TbEIF4E-5 binds both ligands about 4-fold stronger that TbEIF4E-6. The obtained association constants are in the same range as constants obtained for Leish4E-4 and Leish4E-1, which is another confirmation of the distinctive features of eIF4Es from *Trypansomatidae*.

Moreover, biological studies presented in **H3 and H4** showed that the presence of both proteins, TbEIF4E-5 and TbEIF4E-6, is limited to the cytoplasm only and their presence influences the mobility of the cell, and in the case of TbEIF4E-6, also attachment of flagellum in a flagellar form of the protozoan. Both proteins interact with chosen homologs of TbEIF4G in larger protein complexes related to post-transcriptional regulation of gene expression. TbEIF4E-6 interacts with TbEIF4G-5 complexed with a protein known as TBG5-IP with two domains of predicted secondary structure, which is present in enzymes connected to mRNA cap biosynthesis. And TbeIF4E-5 interacts with TbEIF4G-1 and TbEIF4G-2, forming complexes analogous to those of eIF4E.

***Summary:***

The most important achievements resulting from the presented research introduced in the mentioned works (**H1-H8**) are as follows:

- Characterization of specificity of proteins from eIF4E family from seven organism for mRNA cap. Identification of sequence and structure changes that influence affinity diversity among the eIF4E family proteins for the cap within a given organism and between eIF4E proteins from a given class, and derived from different organisms. Indicating that these changes do not involve only the so-called "canonical motif" of cap binding amino acid residues. Identification of relationship between affinity of eIF4E isoforms for cap and the protein function in the organism.

- Indicating that based of the study on affinity of tryptophan eIF4E mutants, that key amino acid residue crucial for formation of an efficient stacking interaction in eIF4E, is the tryptophan corresponding to Trp102 in the human eIF4E-1a protein. And the evolutionary change of Trp56 to a different amino acid does not mean direct increase or decrease in cap binding, because effective contribution of aromatic amino acid residue in this position to stacking interaction is also influenced by local structural changes in a given form of eIF4E.

- Showing that in the case of eIF4E proteins, circular dichroism in near-UV range (near-UV CD) is a precise, fast and cheap method that allows studying local conformation changes of conserved tryptophan residues in the solution that result from sequence and structure changes present in eIF4E isoforms as well as changes resulting from formation of complexes with cap analogs.

## 6. Presentation of the other scientific and research achievements

### 6.1. Presentation of research activity before and after obtaining the PhD degree

My mains research and scientific interests before and after obtaining the PhD degree are strictly connected to the topics discussed to the presented scientific achievement and concern investigations of the murine and human canonical eIF4E factor (eIF4E-1a). The studies are conducted in two aspects: on one hand, they concern regulation of eIF4E-1a activity in the cell, by phosphorylation and interaction with 4E binding proteins, and on the other hand, the influence of chemical cap analogs with potential therapeutic application on eIF4E binding. These two seemingly separate research paths are connected by a protooncogenic nature of eIF4E and the set therapeutic targets that aim at eIF4E phosphorylation, interaction with 4E binding proteins (4E-BP) and inhibition of eIF4E activity by modified cap analogs or mRNA-based vaccines with extended lifetime in the cell and with increased translation level [77,78].

Before I obtained my PhD degree, the main subject of my research was investigation of the influence of serine 209 phosphorylation in mammalian eIF4E on the affinity of the protein towards the cap analogs. Using a modern (at that time) technique of protein ligation using inteins (*Intein-mediated Protein Ligation, IPL*) [79,80], I managed to obtain murine eIF4E with selective phosphorylation of serine 209, by ligation of eIF4E protein fragment (28–204) with a synthetic peptide that corresponded to the terminus of eIF4E(205–217) with phosphoserine (**M1**). Studies on cap analog affinity to phosphorylated eIF4E and a series of meIF4E-1a where serine 209 was replaced with amino acids with different side chains: i) positively charged – lysine, ii)negatively charged – glutamic acid and iii) neutral – alanine, performed using fluorescence titration, showed that eIF4E phosphorylation results in binding weakening by electrostatic repulsion between the negatively charged phosphate group of serine and cap phosphate chain (**M1, M3, M5**). Research with atypical cap analogs with increased number (up to six) of phosphate groups in the chain had a crucial role in explaining the mechanism of binding weakening (**M3, M5, M13**). The obtained results were in contrast with the literature hypothesis set forth based on the crystallographic structure of eIF4E-m$^7$GDP complex that eIF4E phosphorylation should increase the affinity as a result of forming a salt bridge between the phosphoserine 209 and lysine 159 that would close the cap in the slot like a clamp (the so-called *clamping model*). Presented in **M5** analysis of Gibbs free enthalpy ($\Delta\Delta G^o$) determined based on equilibrium association constants for the complexes of meIF4E mutant with alanine instead of lysine at position 159, as well as its phosphorylated counterpart with a series of cap analogs, showed that the presence of Lys159 has a positive influence on cap binding, irrespective however of the presence of phosphoserine, which equivocally excludes the possibility of salt bridge formation. Salt bridge formation is also excluded by the results of protonation state calculation for the chosen eIF4E amino acids, including lysine 159 and phosphoserine 209 performed using Monte-Carlo method and presented in **D5**.

After I obtained my PhD degree, my participation in studies on regulation of eIF4E activity concerns mainly interaction with eIF4E binding proteins (**H7**, **D6, D16, D18**). In cooperation with Greco Hernandez, PhD (*currently the National Institute of Cancer, Mexico*) in the work described in **D16**, on the role of fruit fly eIF4E-3 isoform in a cell during spermatogenesis, we showed that this isoform interacts with both eIF4G (eIF4G-1) and with its eIF4G-2 isoform, which is the key regulator of translation initiation during spermatocyte meiosis. In **H7**, we characterized interactions of deIF4E isoform with 13-amino acid peptide fragment of deIF4G in two- and three-element complexes with cap analogs. Only for deIF4E-4 isoform, we observed cooperativity in formation of deIF4G-deIF4E-4-cap complex. Also for deIF4E-4, we observed the highest binding constant for deIF4G, which may

result, as shown based on the modeled complex structure, from substitution of one of conserved eIF4E amino acid residues binding the canonical binding motif (C-motif - **Y**XXXX**LL**) of 4E-BP protein. Substitution of methionine with glutamic acid in deIF4E-4 results in ability of the isoform to form additional bonds with conserved lysines located in the C-motif of 4E-BP.

On this subject, I also cooperate with the group of Professor Ryszard Stolarski, in particular with Anna Modrak, PhD; we investigate the mechanism of interaction of human eIF4E-1a with one of 4E binding proteins – 4E-BP1. In **D18**, we showed that human 4E-BP1 markedly prefers binding with eIF4E complexed with mRNA, and on the other hand, its binding facilitates dissociation of eIF4E from the cap.

At the end of my PhD studies I started intensive cooperation with the chemical group of Professor Darzynkiewicz's Team, and this cooperation was continues after I obtained my PhD degree, in close collaboration with Jacek Jemielity, PhD, and Joanna Kowalska, PhD, on projects concerning the chemical modification of cap analogs, which will be of extreme usefulness in studies on the processes of mRNA metabolism. They can be applied in the methods of molecular biology and biotechnology or in the structural studies, or they can have significant medical meaning, especially in cancer therapy, where overexpression of eIF4E is observed[77,81,82]. My contribution to the studies covers mostly i) determination of association constants for the complexes of murine eIF4E with newly synthesized cap analogs using fluorescence titrations, ii) analysis of the influence of the introduced modifications in the cap structure on the mechanism of protein-cap interaction at the molecular level and iii) an attempt to connect the identified changes with the observed biological properties of the modified analogs.

The first group of the compounds I studied comprises dinucleotide cap analogs with various number of phosphate groups, with modifications of the ribose ring of the 7-methylguanosine at positions C2' and C3' known as Anti-Reverse Cap Analogs – ARCA (**M2, M13, D1, D28**). Introduction of methyl group in one of the mentioned positions or removal of hydroxyl ensure 100% introduction of cap analogs to the mRNA chain synthesized in the process of in vitro transcription in the correct orientation, while non-modified cap analog $m^7$GpppG is incorporated into mRNA in about 30% of cases in the reverse orientation Gpppm$^7$G-mRNA ([83,84], **M2**). Studies presented in **M2** showed that ribose modifications do not influence eIF4E affinity towards the cap analogs, whereas elongation of the phosphate chain significantly increases their affinity towards the 4E factor, which also results in a more efficient *in vitro* inhibition of translation by tetra- and pentaphosphate as compared to $m^7$GpppG. mRNA capped with ARCA analogs is translated 2-fol more effectively. However in the case of *in vitro* translation, positive influence of the cap phosphate chain elongation on the increase in efficiency was not observed, most probably due to the fact that eIF4E protein interacts here also with the further part of mRNA chain and with other translation factors, not only with the cap structure itself. Chosen ARCA compounds found their commercial applications in *in vitro* transcription systems with use of phage polymerases.

Another group of the studied compounds comprises mono- and dinucleotide cap analogs with modification in the phosphate chain and includes three subgroups of analogs. The first subgroup includes analogs where the bridging oxygen atom was replaced with -$CH_2$ group (**D9, D22, D29, D36**), or with -NH group (**D12, D33**), -$CCl_2$ or -$CF_2$ group (**D25**). The second group includes analogs, where a non-bridging oxygen atom was replaced by a sulfur (S) or selenium (Se) atom or by boranyl group ($BH_3$) (**D3, D4, D7, D8, D10, D11, D20, D22, D23, D27, D30, D34, D35, D37, D38**). The third subgroup comprises analogs with both types of modifications in one compound (**D23**). The aim of these modifications was to obtain cap analogs with increased affinity towards eIF4E, resistant against degradation by decapping enzymes, e.g. DcpS or Dcp1/Dcp2 [9,85]. Cap analogs with high eIF4E affinity and resistant to hydrolysis by pyrophosphatases (including DcpS enzyme) could possibly be good translation inhibitors in tumor cells with overexpression of eIF4E protein [81,82,86]. And cap analogs

resistant to the activity of Dcp1/Dcp2 enzyme could, after incorporation to mRNA, expand its cellular lifespan and could be used to design therapeutic mRNAs [87,88].

From among the designed and tested compounds with substitution of the bridging oxygen in the cap phosphate chain, the most promising group seem to be analogs with -$CCl_2$ modification (**D25**). As compared to analogs with methylenebisphosphonate (-$CH_2$) modification that have significantly lower affinity towards eIF4E in comparison to standard cap analogs (**D9, D22, D29, D36)**, or analogs with imidophosphate residue (-NH) that doesn't cause a marked change in eIF4E binding (**D12, D33)**, analogs with dihalogenemethylenebisphosphonate modification have increased affinity towards eIF4E. Research presented in (**D25**) suggest that high electronegativity of chloride and fluoride atoms can be one of the causes; high electronegativity results in increase of electrostatic effect of the interaction between the cap phosphate chain and positively charged residues of lysines and arginines in eIF4E. Presented in (**D25**) structures of eIF4E complexes with $m^7GppppG$ and $m_2^{7,2'O}GppCl_2ppG$ also showed a significantly different conformation of the tetraphosphate chain in $m_2^{7,2'O}GppCl_2ppG$. β-phosphate of $m_2^{7,2'O}GppCl_2ppG$ is oriented towards the exterior of the cap binding slot and forms contacts with Lys159, not with Lys162, as it is in the complex with $m^7GppppG$. We also showed that the studied -$CCl_2$ cap analogs are more efficient translation inhibitors, they undergo weak hydrolysis by human DcpS; moreover, their introduction into the mRNA transcript elongates its lifetime in the cell by increasing its resistance against hydrolysis by the enzymatic Dcp1/Dcp2 complex. Also for mRNA transcripts capped with ARCA-type – $CCl_2$ analogs, we noted a significant increase in translation efficiency, both *in vitro* in rabbit reticulocyte lysate (RRL) and *in vivo* in HeLa cells. Despite weaker binding of methylenebisphosphonate analogs to eIF4E which results most probably from increase in p$K_a$ of the phosphate group neighboring with $CH_2$ group and impossibility to form hydrogen bonds by the group (**D25**), these analogs, as well as analogs with imidophosphate modification, have found application in studies on mRNA degradation paths owing to their resistance against hydrolysis by decapping enzymes [89–92].

Research on the second subgroup of the compounds, thio-, borano- and selenium-phosphate analogs showed that these are compounds with large application potential e.g. in cancer therapy or in obtaining stable mRNAs for use in gene therapy (patents: P1, P2, 95-97). Substitution of non-bridging oxygen atom with a heteroatom present in these compounds creates a stereogenic center and the above mentioned compounds are obtained as diastereoisomers (**e.g. D3**). Affinity of these compounds towards eIF4E, regardless of the modified site, is comparable or in some analogs several fold higher than in non-modified analogs. What is interesting, for modifications at positions β and γ, significant differences were observed, even 3-fold, in affinity of diastereoisomers towards eIF4E. This is most probably due to orientation of the modified phosphate group and its ability to create hydrogen bonds with side chains of amino acids in the cap binding slot of eIF4E (**D4**, **D7**, **D8**, **D10**, **D20**). Thio- and boranophosphate analogs have a different susceptibility towards DcpS enzyme. In the case of thiophosphates, both diastereoisomers γ-S are resistant against DcpS (**D4, D7, D20**), and in the case of boranophosphates, the obtained results were surprising (**D20**). From among γ-BH3 analogs, which were expected to be resistant against DcpS hydrolysis, only $S_p$ isomer is not hydrolyzed, and high resistance (80–90%) against hydrolysis is shown by both diastereoisomers of β-$BH_3$. Non-hydrolysable boranophosphate analogs have, however, about 2–3-fold lower affinity to DcpS than γ-S analogs. Also, not all ARCA analogs modified at β position expand mRNA cellular lifetime upon being built into mRNA molecule and slowing down its degradation in the 5'-3' direction. Increase in lifetime of mRNA with attached modified cap is influenced by e.g. efficiency of incorporation of a given analog to a cap, eIF4E affinity and susceptibility to hydrolysis by Dcp1/Dcp2 enzymatic complex. Promising analogs that showed very weak (10-20%) susceptibility to hydrolysis by Dcp1/Dcp2 proved to be: $m_2^{7,2'O}Gpp_SpG$–D2, $m_2^{7,2'O}Gpp_Sp_SG$–D4, $m_2^{7,2'O}Gpp_Sp_SpG$–D1D2, $m_2^{7,2'O}Gpp_{BH3}pG$ –D2, $m^7Gpp_{BH3}pm^7G$. However, during the works on application of mRNA in gene therapy, the following analogs seem promising:

$m_2^{7,2'O}Gpp_SpG$–D1 and $m_2^{7,2'O}Gpp_{BH3}pG$ –D1 ([93], **D20**). Upon introduction to human immature dendritic cells (hiDC), mRNA terminated with these analogs show higher stability and are translated more effectively. In the case of mRNA transcripts terminated with $m_2^{7,2'O}Gpp_SpG$–D1, studies performed in the laboratory of prof. Sahin from Jan Gutenberg University showed clear stimulation of synthesis of antigen protein in immature DCs induced by these transcripts, upon injection into murine lymphatic nodes. Stimulation of protein synthesis results in increased production of antigen-specific T lymphocytes, hence activating the immune system in mice[93]. Works on anti-cancer vaccines based on these mRNAs are highly advanced [98].

A side aspect of my biophysical research on eIF4E proteins is a problem of whether the choice of expression and isolation method I used influences their activity. The first method of expression and isolation of eIF4E from *E. coli* was developed by Edery et al. in 1988 [94] and was based on purification of eIF4E from the soluble fraction, using affinity chromatography with $m^7$GDP-Sepharose resin. In the following years, the method was modified. However, due to eIF4E tendency to aggregate during expression in *E. coli* and to close the protein in cytoplasmic granules known as inclusion bodies, in 1997 Marcotrigiano et al. [11] developed a method to isolate murine eIF4E from inclusion bodies by use of a denaturing agent (guanidine hydrochloride) and subsequent folding of eIF4E to the native form by removal of denaturing agent via dialysis. The last method was developed and adapted by me to obtain (on my own) recombined eIF4E from various organisms studied in the presented works. My doubts about correct folding of eIF4E *in vitro* resulted in a side research (publication is in preparation), where I compare the properties of human eIF4E-1a and yeast eIF4E, especially in terms of their affinity towards cap. I obtained the proteins using three different overexpression procedures in *E. coli* and purified them from soluble fraction using affinity resin: $m^7$GDP-Sepharose and nickel resin (metal ion affinity chromatography), where in the second case, eIF4E proteins had polyhistidine tag on the N– or C-terminus of the protein, as well as using my standard method of eIF4E isolation from inclusion bodies. At the very beginning, analysis of mass of the obtained eIF4E by use of gel filtration showed that attachment of affinity tag on the C-terminus of the protein results in its rapid and strong aggregation, in contrast to the other proteins where only monomers were observed. Earlier biophysical (**M7**) and structural studies [19] and prediction of eIF4E isoform structures via homology modeling (**H6, H7**) showed a significant meaning of eIF4E C-terminus for interaction with 5' mRNA end and also for formation of the correct eIF4E structure. The aggregation observed upon attachment of six histidine residues at the C-terminus of eIF4E again confirms the structural and functional role of the protein C-terminus and the choice of the label and attachment site may influence the protein properties.

The performed studies on interaction of the other eIF4Es (obtained using different methods) with a series of cap analogs using fluorescence titration showed that eIF4E obtained from the soluble fraction and from inclusion bodies (with protein *in vitro* refolding) have the same specificity towards the studied cap analogs. Analysis of fluorescence investigation on the fraction of active protein shows that in the case of the commonly used purification on $m^7$GTP-Sepharose, about 30–40% of the protein is active, whereas in the protein fraction from inclusion bodies or that purified using metal ion affinity chromatography, about 70 to 90% of the protein is active. Analysis of potential changes in the secondary and tertiary structure of the protein arising possibly due to different method of obtaining and purification, performed via near and far-UV CD spectroscopy, didn't show any such changes.

To summarize, the presented results indicate that by isolation of eIF4E from inclusion bodies and its subsequent *in vitro* refolding into the native form , we obtain a fully functional eIF4E protein and that isolation method cannot be the reason for differences in the protein specificity towards the 5' mRNA cap I observed in the presented works.

## 6.2 Publications that form the remaining scientific achievements after obtaining the PhD degree

### 6.2.1. Philadelphia List publications

D1. Grudzien-Nogalska E., Stepinski J., Jemielity J., Zuberek J., Stolarski R., Rhoads R.E., Darzynkiewicz E. (2007) Synthesis of Anti-Reverse Cap Analogs (ARCAs) and their Applications in mRNA Translation and Stability. Methods Enzymology 431: 203-227, IF 1,640/Cyt. 29

D2. Lewdorowicz M., Stepinski J., Kierzek R., Jemielity J., **Zuberek J.**, Yoffe Y, Shapira M., Stolarski R., Darzynkiewicz E. Synthesis of Leishmania cap-4 intermediates, cap-2 and cap-3. (2007) Nucleosides Nucleotides Nucleic Acids 26: 1339-1348, IF 0,671/Cyt. 1

D3. Kowalska J., Lewdorowicz M., **Zuberek J.**, Bojarska E., Stepinski J., Stolarski R., Darzynkiewicz E. Jemielity J. (2007) Assignment of the absolute configuration of P-chiral 5' mRNA cap analogues containing phosphorothioate moiety. Nucleosides Nucleotides Nucleic Acids 26: 1301-1305, IF 0,671/Cyt. 2

D4. Kowalska J., Lewdorowicz M., **Zuberek J.**, Grudzien-Nogalska E., Bojarska E., Stepinski J., Rhoads R.E., Darzynkiewicz E., Davis R.E., Jemielity J. (2008) Synthesis and characterization of mRNA cap analogs containing phosphorothioate substitutions that bind tightly to eIF4E and are resistant to the decapping pyrophosphatase DcpS. RNA 14: 1119-1131, IF 5,840/Cyt. 47

D5. Szklarczyk O., **Zuberek J.**, Antosiewicz J.M. (2009) Poisson-Boltzmann model analysis of binding mRNA cap analogues to the translation initiation factor eIF4E. Biophysical Chemistry 140: 16-23, IF 2,362/Cyt. 5

D6. Yoffe Y., Léger M., Zinoviev A., **Zuberek J.**, Darzynkiewicz E., Wagner G., Shapira M. (2009) Evolutionary changes in the Leishmania eIF4F complex involve variations in the eIF4E-eIF4G interactions. Nuclei Acid Research 37: 3243-3253, IF 7.479/Cyt. 23

D7. Kowalska J., Lukaszewicz M., **Zuberek J.**, Ziemniak M., Darzynkiewicz E., Jemielity J. (2009) Phosphorothioate analogs of $m^7$GTP are enzymatically stable inhibitors of cap-dependent translation. Bioorganic & Medicinal Chemistry Letters 19: 1921-1925, IF 2,650/Cyt. 20

D8. Kowalska J., Lukaszewicz M., **Zuberek J.**, Darzynkiewicz E., Jemielity J. (2009) Phosphoroselenoate dinucleotides for modification of mRNA 5' end. Chembiochem 10: 2469-2473, IF 3,332/Cyt. 18

D9. Rydzik A., Lukaszewicz M., **Zuberek J.**, Kowalska J., Darzynkiewicz Z.M., Darzynkiewicz E., Jemielity J. (2009) Synthetic dinucleotide mRNA cap analogs with tetraphosphate 5˘,5˘ bridge containing methylenebis(phosphonate) modification. Organic & Biomolecular Chemistry 7: 4763-7476, IF 3,550/Cyt. 28

D10. Strenkowska M., Kowalska J., Lukaszewicz M., **Zuberek J.**, Su W., Rhoads R. E., Darzynkiewicz E., Jemielity J. (2010) Towards mRNA with superior translational activity: synthesis and properties of ARCA tetraphosphates with single phosphorothioate modifications. New Journal of Chemistry 34, 993 – 1007, IF 2,631/Cyt. 15

D11. Su W, Slepenkov S., Grudzien-Nogalska E., Kowalska J., Kulis M., **Zuberek J**., Lukaszewicz M., Darzynkiewicz E., Jemielity J., Rhoads R.E. (2011) Translation, stability, and resistance to decapping of mRNAs containing caps substituted in the triphosphate chain with BH3, Se, and NH. RNA 17: 978-988, IF 6,051/Cyt. 16

D12. Rydzik A. M., Kulis M., Lukaszewicz M., Kowalska J., **Zuberek J.,** Darzynkiewicz Z. M., Darzynkiewicz E., Jemielity J. (2012). Synthesis and properties of mRNA cap analogs containing imidodiphosphate moiety – fairly mimicking natural cap structure, yet resistant to enzymatic hydrolysis. Bioorganic & Medicinal Chemistry 20: 1699-1710, IF 2,903/Cyt. 23

D13. Kowalska J., Osowniak A., **Zuberek J.**, Jemielity J. (2012) Synthesis of nucleoside phosphosulfates. Bioorganic & Medicinal Chemistry Letters 22: 3661-3664, IF 2,338/Cyt. 9

D14. Szczepaniak S.A., **Zuberek J.**, Darzynkiewicz E., Kufel J., Jemielity J.(2012) Affinity resins containing enzymatically resistant mRNA cap analogs-a new tool for the analysis of cap-binding proteins. RNA 18: 1421-1432, IF 5,095/Cyt. 7

D15. Jemielity J., Lukaszewicz M., Kowalska J., Czarnecki J., **Zuberek J**., Darzynkiewicz E. (2012) Synthesis of biotin labelled cap analogue - incorporable into mRNA transcripts and promoting cap-dependent translation. Organic & Biomolecular Chemistry 10: 8570-8574, IF 3,568/Cyt. 11

D16. Hernández G., Han H., Gandin V., Fabian L., Ferreira T., **Zuberek J.**, Sonenberg N., Brill J.A., Lasko P. (2012) Eukaryotic initiation factor 4E-3 is essential for meiotic chromosome segregation, cytokinesis and male fertility in Drosophila. Development 139: 3211-3220, IF 6,3208/Cyt. 14

D17. Warminski M., Kowalska J., Buck J., **Zuberek J**., Lukaszewicz M., Nicola C., Kuhn A.N., Sahin U., Darzynkiewicz E., Jemielity J. (2013) The synthesis of isopropylidene mRNA cap analogs modified with phosphorothioate moiety and their evaluation as promoters of mRNA translation. Bioorganic & Medicinal Chemistry Letters 23: 3753-3758, IF 2,331/Cyt. 8

D18. Modrak-Wojcik A., Gorka M., Niedzwiecka K., Zdanowski K., **Zuberek J.**, Niedzwiecka A., Stolarski R. (2013) Eukaryotic translation initiation is controlled by cooperativity effects within ternary complexes of 4E-BP1, eIF4E, and the mRNA 5' cap. FEBS Letters 587: 3928-3934, IF 3,582/Cyt. 5

D19. Nowakowska M, Kowalska J, Martin F, d'Orchymont A, **Zuberek J**, Lukaszewicz M, Darzynkiewicz E, Jemielity J. (2014) Cap analogs containing 6-thioguanosine--reagents for the synthesis of mRNAs selectively photo-crosslinkable with cap-binding biomolecules. Organic & Biomolecular Chemistry 12: 4841-4847, IF 3,487/Cyt. 4

D20. Kowalska J., Wypijewska del Nogal A., Darzynkiewicz Z.M., Buck J., Nicola C., Kuhn A.N., Lukaszewicz M., **Zuberek J**., Strenkowska M., Ziemniak M., Maciejczyk M., Bojarska E., Rhoads R.E., Darzynkiewicz E., Sahin U., Jemielity J. (2014) Synthesis, properties, and biological activity of boranophosphate analogs of the mRNA cap: versatile tools for manipulation of therapeutically relevant cap-dependent processes. Nucleic Acids Research 42: 10245-10264, IF 8,808/Cyt. 14

D21. Zytek M., Kowalska J., Lukaszewicz M., Wojtczak B.A., **Zuberek J.**, Ferenc-Mrozek A., Darzynkiewicz E., Niedzwiecka A., Jemielity J. (2014) Towards novel efficient and stable nuclear import signals: synthesis and properties of trimethylguanosine cap analogs modified within the 5',5'-triphosphate bridge. Organic & Biomolecular Chemistry 12: 9184-9199, IF 3,487/Cyt. 8

D22. Ziemniak M., Kowalska J., Lukaszewicz M., **Zuberek J**., Wnek K., Darzynkiewicz E., Jemielity J. (2015) Phosphate-modified analogues of $m^7$GTP and $m^7$Gppppm$^7$G – synthesis and biochemical properties. Bioorganic & Medicinal Chemistry 23: 5369-5381, IF 2,793/Cyt. 4

D23. Strenkowska M., Grzela R., Majewski M., Wnek K., Kowalska J., Lukaszewicz M., **Zuberek J**., Darzynkiewicz E., Kuhn A.N., Sahin U., Jemielity J. (2016) Cap analogs modified with 1,2-dithiodiphosphate moiety protect mRNA from decapping and enhance its translational potential. Nucleic Acids Research 44: 9578-9590, IF 9,202/Cyt. 4

D24. Warminski M., Sikorski P.J., Warminska Z., Lukaszewicz M., Kropiwnicka A., **Zuberek J.**, Darzynkiewicz E., Kowalska J., Jemielity J. (2017) Amino-Functionalized 5' Cap Analogs as Tools for Site-Specific Sequence-Independent Labeling of mRNA. Bioconjugate Chemistry 28: 1978-1992, IF 4,818/Cyt. 0

D25. Rydzik A.M., Warminski M., Sikorski P.J., Baranowski M.R., Walczak S., Kowalska J., **Zuberek J.**, Lukaszewicz M., Nowak E., W Claridge T.D., Darzynkiewicz E., Nowotny M., Jemielity J. (2017) mRNA cap analogues substituted in the tetraphosphate chain with CX2: identification of O-to-CCl2 as the first bridging modification that confers resistance to decapping without impairing translation. Nucleic Acids Research 45: 8661-8675, IF 10,162/Cyt. 0

### 6.2.2. Articles in publishing houses beyond the Philadelphia List

D26. Stepinski J., Worch R., **Zuberek J**., Bojarska E., Jemielity J., Lewdorowicz M., Stolarski R., Haber D., Darzynkiewicz E. (2005) Synthesis and preliminary characterization of mRNA 5'cap analogues containing ribavirin. Chemistry of Nucleic Acid Components, Collection Symposium Series 7: 479-480.

D27. Kowalska J., Lewdorowicz M., **Zuberek J**., Bojarska E., Darzynkiewicz Z.M., Grudzien E., Davis R.E, Rhoads R.E., Stepinski J., Stolarski R., Darzynkiewicz E., Jemielity J. (2005) Synthesis and Properties of phosphorotioate 5' mRNA cap dinucleotides. Chemistry of Nucleic Acid Components, Collection Symposium Series. 7: 417-419.

D28. Jemielity J., Stepinski J., **Zuberek J**., Grudzien E., Lewdorowicz M., Stolarski R., Rhoads R.E., Darzynkiewicz E. (2005) Dinucleotide analogues of the mRNA cap that enhance translational efficiency. Chemistry of Nucleic Acid Components, Collection Symposium Series 7: 361-365.

D29. Kalek M., Jemielity J., Grudzien E., **Zuberek J**., Darzynkiewicz Z.M., Bojarska E., Stepinski J., Stolarski R., Davis R.E., Rhoads R.E., Darzynkiewicz E. (2005) Synthesis and biochemical properties of the novel enzymatically table mRNA cap analogues with versatile potential applications. Chemistry of Nucleic Acid Components, Collection Symposium Series 7: 355-359

D30. Kowalska J., Ziemniak M., Lukaszewicz M., **Zuberek J**., Strenkowska M., Darzynkiewicz E., Jemielity J. (2008) Phosphorothioate analogues of m7GTP: strong inhibitor of translation with increased resistance towards enzymatic degradation. Chemistry of Nucleic Acid Components, Collection Symposium Series 10: 487-490

D31. Szczepaniak S.A., Jemielity J**., Zuberek J**., Darzynkiewicz E., Kufel J. (2008) Synthesis of nonhydrolyzable cap anlog substituted Sepharose for affinity purification of decaping enzymem. Chemistry of Nucleic Acid Components, Collection Symposium Series 10: 461-464

D32. Rydzik A., **Zuberek J**., Kowalska J., Darzynkiewicz E., Jemielity J. (2008) Bisphosphonate modification In tetraphosphate 5'mRNA cap analogs- synthesis and biochemical properties. Chemistry of Nucleic Acid Components, Collection Symposium Series. 10: 444-447

D33. Kulis M., Kowalska J., **Zuberek J.**, Darzynkiewicz E., Jemielity J. (2008) Imidodiphosphate modification of dinucleotide mRNA cap analogs. Chemistry of Nucleic Acid Components, Collection Symposium Series 10: 389-392

D34. Kowalska J., Grudzien-Nogalska E., Lewdorowicz M., **Zuberek J.**, Bojarska E., Stepinski J., Rhoads R.E., Darzynkiewicz E., Davis R.E., Jemielity J. (2008) Phosphorothioate analogues of mRNA cap with superior biological properties Chemistry of Nucleic Acid Components, Collection Symposium Series 10: 362-365

D35. Kowalska J., **Zuberek J**., Darzynkiewicz Z.M., Lukaszewicz M., Darzynkiewicz E., Jemielity J. (2008) Synthesis and properties of boranophosphate mRNA cap analogues. Chemistry of Nucleic Acid Components, Collection Symposium Series 10: 383-385

D36. Rydzik A., **Zuberek J.**, Kowalska J., Lukaszewicz M., Darzynkiewicz E., Jemielity J. (2008) Synthesis and biochemical studies of tetraphosphate 5' mRNA cap analogs bearing bisphosphonate modification. Nucleic Acids Symposium Series 52: 287-288

D37. Kowalska J., **Zuberek J.**, Darzynkiewicz Z.M., Lukaszewicz M., Darzynkiewicz E., Jemielity J. (2008) The first examples of mRNA cap analogs bearing boranophosphate modification. Nucleic Acids Symposium Series 52: 289-290

D38. Kowalska J., Lukaszewicz M., **Zuberek J**., Ziemniak M., Strenkowska M., Darzynkiewicz E., Jemielity J. (2008) m7GTP alphaS is a strong and stable inhibitor of cap-dependent translation. Nucleic Acids Symposium Series 52: 291-292

D39. Szczepaniak S.A., Jemielity J., **Zuberek J.**, Kufel J., Darzynkiewicz E. (2008) Bisphosphonate mRNA cap analog attached to Sepharose for affinity chromatography of decapping enzymes. Nucleic Acids Symposium Series 52: 295-296

D40. Jemielity J., Lukaszewicz M., Kowalska J., Czarnecki J., **Zuberek J**., Darżynkiewicz E. (2011) Synthesis and properties of dinucleotide cap analog for mRNA 5' end labeling Chemistry of Nucleic Acid Components, Collection Symposium Series 12: 351-353

D41. Warminski M., Kowalska J., Nowakowska M., **Zuberek J**., Lukaszewicz M, Darzynkiewicz E., Jemielity J. (2011) Synthesis and properties of new thio-substituted mRNA cap analogs J. Nucleic Acid Components, Collection Symposium Series 12: 378-380

D42 Ziemniak M, Mugridge J., Kowalska J., Stelmachowska A., **Zuberek J.**, Edward Darzynkiewicz E., Gross J.D., Rhoads R.E. Jemielity J. (2014) Utility of chemically modified cap analogues in studying Dcp1/2 decapping complex mechanism of action. Chemistry of Nucleic Acid Components, Collection Symposium Series 14: 155–158

D43. Kowalska J., Baranowski M.R., Nowicka A., Kasprzyk R., **Zuberek J.**, Wojcik J., Jemielity J. (2014) Synthesis and properties of nucleotides containing a fluorophosphate moiety. Chemistry of Nucleic Acid Components, Collection Symposium Series 14: 159–162

D44. Majewski M., Strenkowska M., **Zuberek J.**, Kowalska J., Jemielity J. (2014) Synthesis and properties of cap-decorated gold nanoparticles. Chemistry of Nucleic Acid Components, Collection Symposium Series 14: 287–288

D45. Walczak S., Wanat P., Nowicka A., **Zuberek J**., Kowalska J., Jemielity J.(2014) Synthesis and properties of dinucleotide cap analogs containing a triazole ring within the oligophosphate bridge. Chemistry of Nucleic Acid Components, Collection Symposium Series 14: 289–290

D46. Tomasiewicz Z., Warminski M., Ubych K., Lukaszewicz M., **Zuberek J.**, Kropiwnicka A., Darzynkiewicz E., Kowalska J., Jemielity J. (2014) Properties and applications of amino- or carboxyfunctionalized mRNA 5'-cap analogues and their conjugates Chemistry of Nucleic Acid Components, Collection Symposium Series 14: 387–388

### 6.2.2. Patents

P1. Kowalska J. Jemielity J., Darżynkiewicz E., Łukaszewicz M., **Żuberek J.** "Nowe boranofosforanowe analogi dinukleozydów, ich zastosowanie, cząsteczka RNA, sposób otrzymywania RNA, oraz otrzymywania peptydów lub białka." Patent No. P 215513 granted on 31.12.2013 by Urząd Patentowy RP.

P2. Kowalska J. Jemielity J., Darżynkiewicz E., Rhoads R.E., Lukaszewicz M., **Zuberek J**. "mRNA Cap Analogs"US Patent US8519110, przyznany 27.08.2013.

## 6.3 Publications that form the remaining scientific achievements before obtaining the PhD degree

### 6.3.1. Philadelphia List publications

M1. **Zuberek J.**, Wyslouch-Cieszynska A., Niedzwiecka A., Dadlez M., Stepinski J., Augustyniak W., Gingras A-C., Zhang Z., Burley S. K., Sonenberg N., Stolarski R., Darzynkiewicz E. (2003) Phosphorylation of eIF4E attenuates its interaction with mRNA 5' cap analogs by electrostatic repulsion; Intein-Mediated Protein Ligation strategy to obtain phosphorylated protein. RNA 9: 52-61, IF 5,099/Cyt. 82

M2. Jemielity J., Fowler T., **Zuberek J**., Stepinski J., Lewdorowicz M., Niedzwiecka A., Stolarski R., Darzynkiewicz E., Rhoads RE. (2003) Novel "anti-reverse" cap analogs with superior translational properties. RNA 9: 1108-1122, IF 5,099/Cyt. 110

M3. **Zuberek J.,** Jemielity J., Niedzwiecka A., Stepinski J., Wyslouch-Cieszynska A., Stolarski R., Darzynkiewicz E. (2003) Influence of the length of the phosphate chain in mRNA 5 ' cap analogues on their interaction with eukaryotic initiation factor 4E. Nucleosides Nucleotides Nucleic Acids 22: 1707-1710, IF 0,781/Cyt. 4

M4. **Zuberek J**., Jemielity J., Stepinski J., Lewdorowicz M., Niedzwiecka A., Haber D., Stolarski R., Rhoads R.E., Darzynkiewicz E. (2003) Binding studies of eukaryotic initiation factor eIF4E with novel mRNA dinucleotide cap analogues. Nucleosides Nucleotides Nucleic Acids 22: 1703-1706, IF 0,781/Cyt. 1

M5. **Zuberek J**., Jemielity J., Jablonowska A., Stepinski J., Dadlez M., Stolarski R., Darzynkiewicz E. (2004) Influence of electric charge at residues 209 and 159 on the interaction of eIF4E with the mRNA 5' terminus. Biochemistry 43: 5370-5379, IF 3,922/Cyt. 49

M6. Lewdorowicz M., Yoffe Y., **Zuberek J.**, Jemielity J., Stepinski J., Kierzek R., Stolarski R., Shapira M., Darzynkiewicz E. (2004) Chemical synthesis and binding activity of trypanpsomatid cap-4 structure. RNA 10: 1469-1478, IF 4,430/Cyt. 25

M7. Yoffe Y., **Zuberek J.**, Lewdorowicz M., Zeira Z., Keasar C., Shaanan B., Orr-Dahan I., Jankowska-Anyszka M., Darzynkiewicz E., Shapira M. (2004) Cap binding activity of a eIF4E homologue from Leishmania. RNA 10: 1764-1775, IF 4,430/Cyt. 26

M8. Kowalska J., Lewdorowicz M., **Zuberek J**., Bojarska E., Wojcik J., Cohen L.S., Davis R.E., Stepinski J., Stolarski R., Darzynkiewicz E., Jemielity J. (2005) Synthesis and properties of mRNA cap analogs containing phosphorothioate moiety in 5',5'-triphosphate chain. Nucleosides Nucleotides Nucleic Acids 24: 595-600, IF 0,429/Cyt. 8

M9. Kalek M., Jemielity J., Grudzien E., **Zuberek J.**, Bojarska E., Cohen L.S., Stepinski J., Stolarski R., Davis R.E., Rhoads R.E., Darzynkiewicz E. (2005) Synthesis and biochemical properties of Novel mRNA 5' cap analogs resistant to enzymatic hydrolysis. Nucleosides Nucleotides Nucleic 24: 615-621, IF 0,429/Cyt. 25

M10. Stepinski J., **Zuberek J.**, Jemielity J., Kalek M., Stolarski R., Darzynkiewicz E. . (2005) Novel dinucleoside 5',5'-triphosphate cap analogues Synthesis and affinity for murine translation factor eIF4E. Nucleosides Nucleotides Nucleic Acids 24: 628-633, IF 0,429/Cyt. 7

M11. Westman B., Beeren L., Grudzien E., Stepinski J., Worch R, **Zuberek J.**, Jemielity J., Stolarski R., Darzynkiewicz E., Rhoads RE., Preiss T. (2005) The antiviral drug ribavirin does not mimic the 7-methylguanosine moiety of the mRNA cap structure in vitro. RNA, 11: 1505-1513, IF 5,842/Cyt. 29

### 6.3.2. Articles in publishing houses beyond the Philadelphia List

M12. **Zuberek J**., Stepinski J., Niedzwiecka A., Stolarski R., Salo H., Lonnberg H., Darzynkiewicz E. (2002) Synthesis of tetraribonucleotide cap analogue $m^7GpppA^{m2'}pU^{m2'}pA^{m2'}$ and its interaction with eukaryotic initiation factor eIF4E. Chemistry of nucleic Acid Components, Collection Symposium Series 5: 399-403.

M13. Jemielity J., **Zuberek J.**, Stepinski J., Lewdorowicz M., Niedzwiecka A., Haber D., Tolvert F., Stolarski R., Rhoads R.E., Darzynkiewicz E. (2002) Synthesis, physico-chemical and biochemical properties of the novel tri-, tetra-, and pentaphosphate mRNA cap analogues. Chemistry of nucleic Acid Components, Collection Symposium Series 5: 159-168.

## 7. Activity in development of new scientific and research infrastructure in the Division of Biophysics and new initiative in the teaching process

The presented scientific and research activity before and after obtaining the PhD degree would not be possible without the enormous work I have made in creating and development of ”***Gene Expression Laboratory***” initiated by Prof. Edward Darzynkiewicz in the Division of Biophysics of the Faculty of Physics UW, where I obtained the majority of the studied proteins. The current state of the laboratory allows us to obtain different biological objects (including recombined proteins, RNA and DNA molecules) for biophysical research using a broad spectrum of molecular biology methods as well as to conduct simple biochemical and biological experiments *in vitro.*

My engagement in development of "*Gene Expression Laboratory*” started just after the initiation of my PhD studies in the Faculty of Physics, UW, under the supervision of Prof. Edward Darzynkiewicz in the Division of Biophysics. I created the infrastructure, instrument and methodology basics. Thanks to kindness of Professor Michał Dadlez and Aleksandra Wysłouch-Cieszyńska PhD from the Institute of Biochemistry and Biophysics of PAN (Polish Academy of Sciences), during the first two years of my PhD studies I could work and study the basic techniques of molecular biology in their laboratories, which was a great challenge for me as a physicist. I offered my whole experience gathered in IBB and then expanded in specialistic courses both on molecular biology methods and organization and functioning of such laboratories to the Division of Biophysics, becoming a research, teaching and technical employee of the Gene Expression Laboratory.

Organization of the Laboratory, as well as my experience in molecular biology methods allowed me to expand the experimental scope of "Biochemistry Laboratory" in the Division of Biophysics, and to create my own "Molecular Genetics Laboratory" for the students of the Faculty pf Physics at the Biophysics specialization that was previously led at the Institute of Biochemistry and Biophysics of PAN. I also participated actively in creating of the biological part of curriculum for a new study mayor of 1st degree "Application of Physics in Biology and Medicine" for the specialty of "Molecular Biophysics" and "Molecular Design and Bioinformatics" at the Faculty of Physics. Within the scope of this mayor, I co-created the infrastructure of "Molecular Biology Laboratory" and I prepared my own programs, student's guides and all necessary biological materials for the lab classes: " Laboratory of General Microbiology and Bacterial Genetics ” and "Molecular Biology Laboratory”. Within the scope of ZFBM, I prepared and conducted a lecture entitled "Molecular Biology part 2" presenting the modern techniques of molecular biology.

During my work at the Faculty of Physics, I supervised three licencjat works (2009, 2012, 2013, 2015) and three master theses (2007, 2009 and 2015). The theses by Dorota Kubacka entitled "Stacking interactions in recognition of 5` mRNA cap structure by human eIF4E izoforms " was awarded with a 1st degree award named ofter Arkadiusz Piekara for master thesis in 2007 granted by the Polish Physics Society. I was also an informal supervisor of PhD theses prepared in the Faculty of Physics UW, which resulted in publications **H5 and H6**. Currently, I am a direct supervisor of a PhD student at his 3rd year of PhD studies.

In 2006 and 2007, I organized and performed a 7-day workshop entitled "The basics of microbiology and molecular biology" for the students of 2nd year of 1st degree studies with a major in Physics and in 2006, 2007, 2011 and 2012 I held holiday practices for students.

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