UNIVERSITÀ POLITECNICA DELLE MARCHE

DIPARTIMENTO SCIENZE DELLA VITA E DELL'AMBIENTE

Corso di Laurea Magistrale in Biologia molecolare e applicate

(CLASSE LM-6)

PRODUZIONE E CARATTERIZZAZIONE DELLE PROTEINE eIF5A E DHS UMANE PER L'IDENTIFCAZIONE DI NUOVI INIBITORI

PRODUCTION AND CHARACTERIZATION OF THE HUMAN PROTEINS eIF5A AND DHS FOR THE IDENTIFICATION OF NEW INHIBITORS

Laureando
Kazi Razrul Islam

Relatore
Chiar. ma Prof.ssa Anna La Teana

Correlatore
Dott.ssa Cristina Maracci

Sessione Straordinaria

Anno Accademico 2021/2022

Summary in Italian.

Il cancro è una delle malattie caratterizzate dalla più alta mortalità in tutto il mondo. L'assenza di farmaci efficaci e diagnosi precoci non riesce a prevenire la morte del paziente. Uno dei biomarcatori coinvolti nella tumorigenesi è eIF5A, un fattore della traduzione eucariotico coinvolto nella fase di allungamento della sintesi proteica. Esistono due isoforme di eIF5A: eIF5A1 ed eIF5A2, che sono espresse da geni distinti ma sono correlati tra di loro a livello patologico, cioè entrambe le forme vengono rilevate nelle cellule tumorali. È stato dimostrato che eIF5A è coinvolto nella patogenesi di diverse malattie, incluso il cancro. eIF5A1 è espresso in tutte le cellule, mentre eIF5A2 è espresso prevalentemente in tessuti tumorali. Entrambe le isoforme sono coinvolte nello sviluppo e nella progressione del cancro e sono quindi utilizzate come marcatori per la prognosi. eIF5A subisce una modifica post-traduzionale non canonica, ovvero, la lisina 50 viene convertita in Ipusina, un aminoacido che si trova esclusivamente in eIF5A. La reazione di ipusinazione coinvolge due enzimi: DHS e DOHH. Nella prima reazione, l'enzima deossi-ipusina sintetasi (DHS) catalizza il trasferimento del gruppo 4-amino butilico dalla spermidina al gruppo ε-amino del residuo di lisina in posizione 50 della proteina eIF5A per formare il residuo deossi-ipusina. La seconda reazione è eseguita da deossi-ipusina Idrolasi (DOHH) tramite idrossilazione del precursore intermedio per forma il residuo Ipusina, coì il fattore eIF5A viene

attivato. Dato che la reazione è reversibile, è sufficiente bloccare un enzima per interrompere l'intero processo biologico, perciò, abbiamo pensato di bloccare il primo enzima che è deossi-ipusina sintetasi (DHS) attraverso l'uso di inibitori. Uno dei potenti inibitori che è stato scoperto è il GC7, ma è importante continuare a cercare inibitori più potenti e specifici. Per poter testare grandi quantità di potenziali inibitori è necessario avere a disposizione un processo sperimentale rapido ed efficiente. Perciò, lo scopo di questa tesi è di produrre eIF5A e DHS ricombinanti, analizzare la loro interazione e sviluppare un protocollo sperimentale che permetta di analizzare velocemente potenziali nuovi inibitori del DHS.

INTRODUCTION

1.1 Summary of the thesis

Cancer is the leading cause of death worldwide. The absence of obvious symptoms and the absence of sensitive biomarkers in the early stages of carcinoma limits early diagnosis. One of the biomarkers involved in tumorigenesis is eIF5A. eIF5A is a eukaryotic translation factor involved in the elongation phase of protein synthesis. Two isoforms of eIF5A are known: eIF5A1 and eIF5A2, which are expressed from distinct but related genes. The two human isoforms share 84% sequence identity and 94% similarity. It has been demonstrated that eIF5A is involved on the pathogenesis of several diseases, including cancer. eIF5A1 is ubiquitously expressed, while eIF5A2 is expressed in selected tissues, such as in the brain and testis. Both isoforms are involved in cancer development and progression and are thus used as markers for prognosis despite their distinct expression patterns. The activity of eIF5A depends on the unique post-translation modification of a conserved lysine residue (Lys 50 in Human), which is obtained by conjugation of the aminobutyl moiety of spermidine to the conserved lysine residue. The post-translational synthesis of Hypusine involves two enzymatic steps: the first step is catalyzed by deoxhypusine Synthase (DHPS) and the second by deoxyhypusine Hydroxylase (DOHH). eIF5A is conserved between Eukarya, Archaea and Bacteria. In Bacteria, β-lysilynation of a conserved Lysine residue substitutes the conserved Hypusine.

The most powerful inhibitor of the DHSP enzyme is the spermidine analogue N^1 -guanyl- 1,7 -diaminoheptane (GC7), which binds in a specific long pocket at the interface between two DHS monomers. This inhibitor showed a potent antiproliferative effect on several cancer cell lines where both eIF5A isoforms are overexpressed.

1.2 Eukaryotic translation initiation factor (eIF5A)

eIF5A was originally purified from ribosomes of rabbit reticulocytes in 1976 and named IF-M2Bα; later, the name of IF-M2Bα was changed in Eukaryotic initiation factor (eIF5A) to conform to the uniform nomenclature of initiation factors (Kemper, Berry, and Merrick 1976).

The eukaryotic translation factor is a small acidic protein, highly conserved in all three kingdoms, with homologues in Archaea (aIF5A) and in Bacteria. This initiation factor was shown to be essential in Eukarya and Archaea but not in Bacteria. (EF-P)(Balibar, Iwanowicz, and Dean 2013; J. H. Park et al. 2011). The three-dimensional structure of human eIF5A shows that it is mainly composed of β-sheets, as shown in figure1.

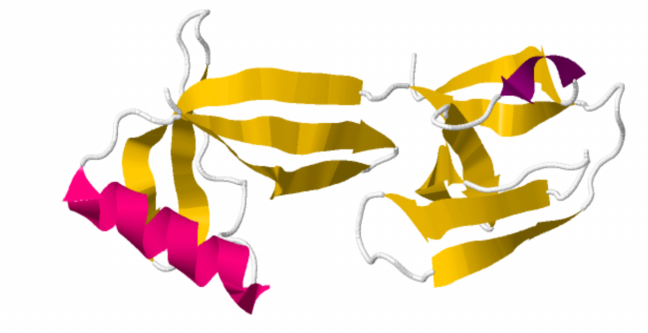

Figure 1. crystal structure of human eukaryotic translation initiation factor eIF5A (PDB ID code 3CPF)

eIF5A is composed of two different domains: the basic N-terminal domain is folded in an SH-3 like barrel and found in other proteins related to translation. The C-terminal domain consists of a five-stranded beta-barrel known to bind nucleic acids and typical of other translation machinery components, like eIF1A1, eIF2α and other ribosomal proteins. (Dias et al. 2013).

1.3 eIF5A comparison with other organisms.

eIF5A is conserved among Eukarya and Archaea. In addition, the ortholog EF-P shows a structural similarity with a/eIF5A, with some important differences. (Hanawa-Suetsugu et al. 2004) (figure 2).

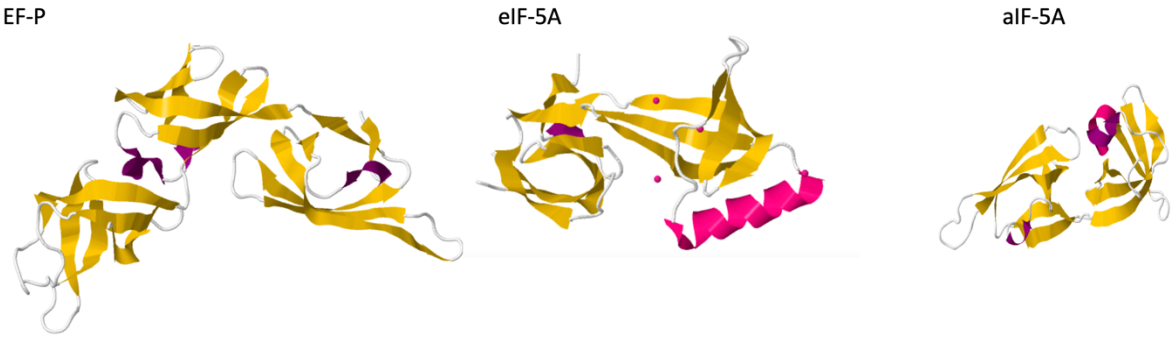

Figure 2. First Structure, is aIF5A (PDB ID code: 1IZ6) Second Structure, eIF5A (PDB ID code: 3CPF) third structure is (PDB ID code: 1UEB)

Eukaryotic and Archaeal aIF5A have a very similar conformations.(Hanawa-Suetsugu et al. 2004)

eIF5A is conserved between Eukarya, Archaea and Bacteria as demonstrated throw the multialignment shown in figure 3. In the scheme a star indicates that the amino acid is conserved while the two dots that the amino acid is similar. Therefore, those protein structure are very similar to each other (Clustal Omega).

```
------------------MISVTDLRPGTKVKMDGGLWECVEYQHQKLGRGGAKVV-AKF 41
MADDLDFETGDAGASATFPMQCSALRKNGFVVLKGRPCKIVEMSTSKTGKHGHAKVHLVG 60
-------------MGDKTKVQVSKLKPGRYIIIDDEPCRIVNITVSSPGKHGSAKARIEA 47
                  :. : *: .  : :..   . *:   .. *: *   .

KNLETGATVERTFNSGEKLEDIYVETRELQYLYPEGEEMVFMDLETYEQFAVPRSRV-VG 100
IDIFTGKKYEDICPSTHNMDVPNIKRNDFQLIGIQDGYLSLLQDSGEVREDLRLPEGDLG 120
VGIFDGKVRSIVKPTSAEVDVPIIDKKTAQVIAITPDTVQIMDMETYETFEVPIDTG-VA 106
 .:  *   .    :  :::   :. .  * :      : ::: .      :      :.

AEF---FKEGMTALGDMYEGQPIKVTPPTVVELKVVDTPPGVRGDTVSGGSKPATLETGA 157
KEIEQKYDCGEEILITVLSAM----TEEAAVAIKAMAK---------------------- 154
DEIRDQLKEGI--NVEYWETL----GRIKIMRIKGEGE---------------------- 138
 *:    . *        .           : :*

VVQVPLFVEPGEVIKVDTRTGEYVGRA  184
---------------------------  154
---------------------------  138
```

Figure 3- Multialignment between Human sapience (PDB ID: 3CPF), Pyrococcus Horikoshii (PDB ID: 1IZ6) and Thermus Thermophilus (PDB ID: 1UEB). it has been used CLUSTAL OMEGA, where there is star, it is means the amin acid conserved between three kingdoms while there two dots it is means the amino acid is similar

1.4 Structural Characterization of eIF5A

eIF5A is a protein involved in the elongation phase of protein synthesis and its function depends on the unique post-translation modification of a conserved lysine residue called Hypusine. Due to the involvement of eIF5A in cancer and other diseases, much effort is being put into the development of compounds that may inhibit this factor, therefore knowledge of the three-dimensional structure is of fundamental importance.

As mentioned above, the crystal structure of eIF5A1 has been solved: it is organized in two domains: the N-terminal domain consist of 83 amino acids (residue 1-83), while the C-terminal includes 68 amino acids (residue 84-151).

The N-terminal domain is positively charged and is arranged in six β-strands (denominated β1-β6) that fold into a partially open β-barrel. The loop connecting β1 and β2 also includes a 3_{10}-helix turn (residue 22-24).

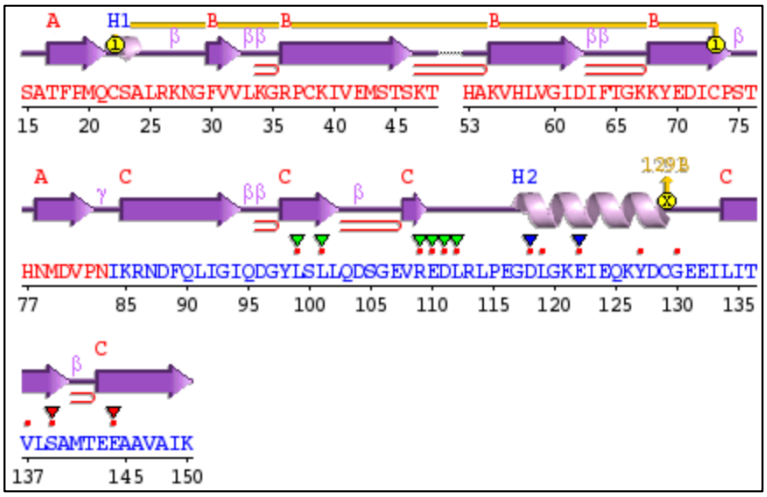

Figure 4. PDB sum topology representation of eIF5A.

Regarding the quaternary structure of eIF5A, the protein was shown to exist as a homodimer both in solution and *in vivo*. Gentz and coworkers demonstrated that formation of eIF5A dimer in S. cerevisiae requires the presence of the Hypusine residue as well as the presence of cellular mRNA molecules. (Gentz, Blatch, and Dorrington 2009). More recent experiments performed in yeast showed that eIF5A forms dimers, both *in vitro* and *in vivo*, in an RNA-dependent manner, but regardless of the presence of the Hypusine residue (Dias et al. 2013).

1.5 Post-translation modification and Hypusination

eIF5A is the only protein known to contain an Hypusine residue. Hypusination consist in the conversion of a conserved lysine residue into a nonstandard amino acid using spermidine as a donor (Shiba et al. 1971). This amino acid was discovered in bovine brain extracts. Its name derived from the two components:

hydroxyputrescine and lysine. After a few years, it was discovered that Hypusine is incorporated exclusively in the translation factor.

Spermidine is a polyamine synthesized from putrescine, which derives from the urea cycle by the catalytic decarboxylation of ornithine ((Igarashi and Kashiwagi 2018). The hypusination reaction is carried out in two independent enzymatic steps: the first step consists of the transfer of the aminobutyl residue, generated upon the cleavage of spermidine, on the specific lysine residue of eIF5A (Lys 50 in mice and human, Lys 51 in yeasts) with the formation of an imine intermediate (Dhp), as shown figure 5. The second step of hypusination is carried out by the deoxyhypusine hydrolase (DOHH) in the presence of oxygen and NAD^+ with the formation of the active hypusinated from of eIF5A (figure 5)(M. H. Park and Wolff 2018a).

The first reaction, carried out by DHS, is reversable and requires NAD^+ as a cofactor. DHS catalyzes the covalent binding of the aminobutyl moiety of spermidine to the ε-amino group Lys50, which is located on an exposed loop in the N-terminal domain of eIF5A. This reaction involves the formation of an imine intermediate, deoxyhypusine residue, and can be reconverted in spermidine in the presence of NADH (M. H. Park and Wolff 2018b).

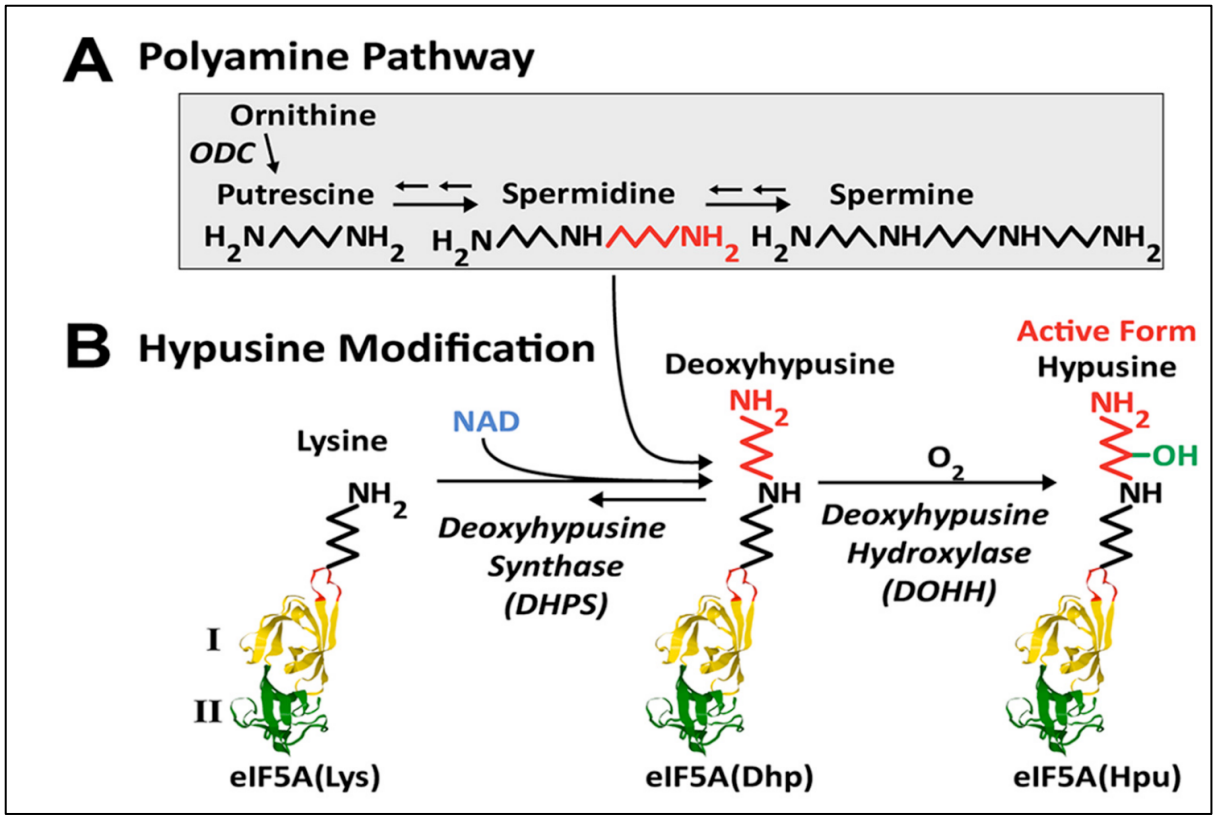

Figure 5. Schematics of the hypusination pathway. Putrescine is generated from ornithine and converted to spermidine and spermine. In the lower part: Hypusination of eIF5A by two enzymatic steps catalyzed by Deoxyhypusine Synthase and Deoxyhypusine Hydroxylase. The aminobutyl moiety of spermidine(red) is reversibly bound by DHS on Lys50 of eIF5A precursor (M. H. Park and Wolff 2018b)

1.6 Differences between Isoforms of eIF5A

Two different isoforms of eIF5A are coded by the human genome, both of which are involved in cancer development and progression and are used as markers for diagnosis and prognosis. In addition, two or more isoforms of eIF5A have been identified in Eukarya (Jenkins, Hååg, and Johansson 2001). In *S. cerevisiae* there are two eIF5A genes: TIF51A and TIF51B. The first gene is expressed under aerobic condition while the latter is expressed under anaerobic conditions ((Schnier et al. 1991) . The two proteins perform, most likely, the same function in *S. cerevisiae*, and inactivation of one or both genes is not viable, confirming

the essential nature of eIF5A. In humans, the two isoforms of eIF5A, eIF5A1 and eIF5A2, are differentially expressed. eIF5A1 is expressed in all cells at high levels, especially in proliferating cells, as shown figure 6, whereas eIF5A2 is expressed at very low levels in normal tissues with the important exception of the testis (figure 7). Both isoforms are overexpressed in many malignancies.

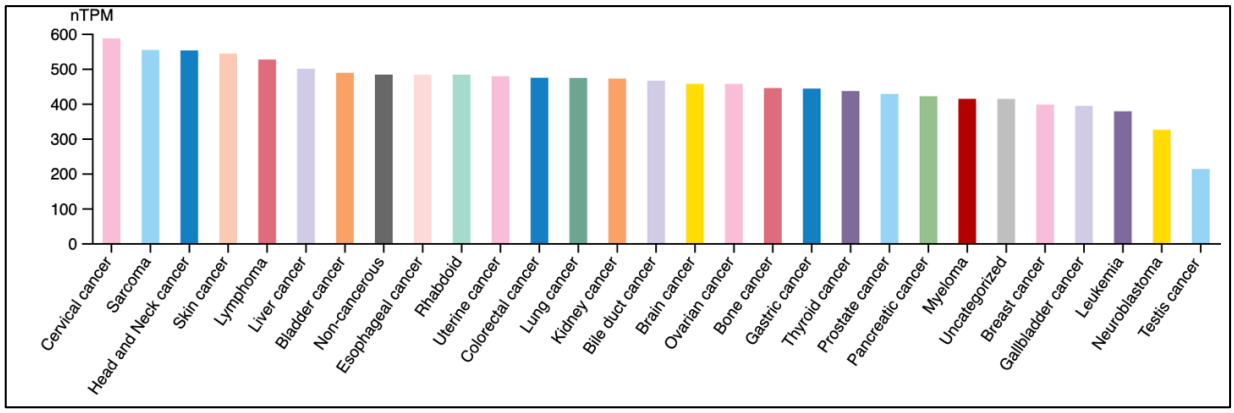

Figure 6. Level of expression of eIF5A1 isoform in different organs, (The Human protein Atlas).

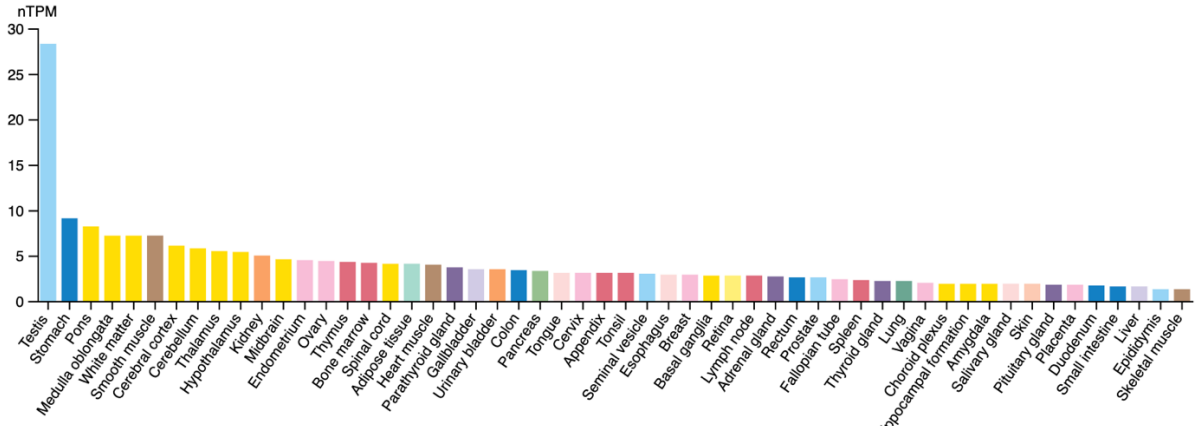

Figure 7. Level of expression of eIF5A2 isoform in different organs. The highest level of expression is observed (the Human protein Atlas).

eIF5A and *eIF5A2* genes are located on different chromosomes, as shown in figure 8; *eIF5A* is located on 17p12-p13, while *eIF5A2* is located on 3q25.q27.

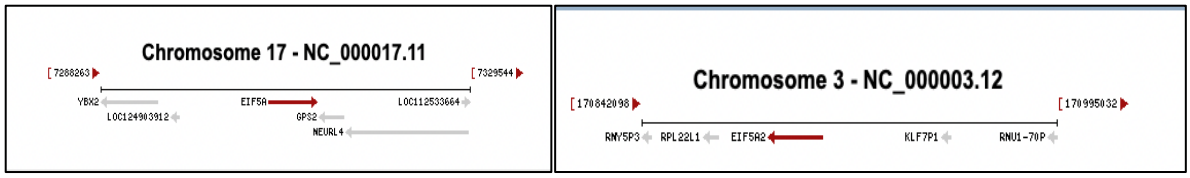

Figure 8. localization genes, it took from NCBI, first eIF5A1, it located chromosome 17. While second eIF5A2. It located chromosome 3.

1.7 Function of eIF5A in translation

Initially, eIF5A was found to stimulate the methionyl-puromycin synthesis, which suggested a role in the formation on the first peptide bond (Caraglia et al. 2013). However, the role of eIF5A as an initiation factor was questioned when it was found that depletion of eIF5A in yeast caused only a slight decrease in the global rate of protein synthesis (Kang and Hershey 1994). As a result, Pelechahano and coworkers have shown that eIF5A is a key factor required for the translation of a subset of specific mRNAs. Furthermore, in other studies, it was demonstrated that eIF5A interacts with structural components of the translation machinery and is involved in the elongation step of translation, as suggested by analyzing the profile of polysomes in yeast after gene depletion. *S. cerevisiae* mutant strains depleted of eIF5A showed a marked inefficiency in the elongation phase (Saini et al. 2009). The interaction of eIF5A with the ribosome has been demonstrated by Jao and Chen, who provided a list of eIF5A-tagged interacting partners (Jao and Chen 2006). 14 out of 19 proteins are ribosomal

proteins belonging either to the 40S or the 60S subunits. Therefore, they suggested that eIF5A binds the 80S ribosome during the elongation phase of translation and this binding requires mRNA and the conserved Hypusine modification (Jao and Chen 2006).

Later on, *in vivo* and *in vitro* assays revealed that eIF5A plays a crucial role in the translation of specific proteins containing consecutive proline residues (Gutierrez et al. 2013), much similar to the function of the paralog EF-P.

The experiment consisted of a set of dual-luciferase reporter constructs where the 5' and 3' luciferase open reading frames were separated by sequences encoding ten consecutive codons of each of the 20 amino acids. The experiment confirmed that the expression of the peptides containing a minimum of three consecutive proline residues dependended on eIF5A(Gutierrez et al. 2013)

In the absence of eIF5A, the ribosome stalls on polyproline stretches because of the cyclic side chain that makes it a poor acceptor as well as a poor donor in the peptidyl transferase reaction. (Schmidt et al. 2016)

Recent cryo-electron microscopy (Schmidt et al. 2016) and X-Ray diffraction(Melnikov et al. 2016) studies in yeast have demonstrated that eIF5A binds to 80S ribosomes between the P and the E site, thereby interacting with tRNAs, rRNA and ribosomal proteins as shown figure 9.

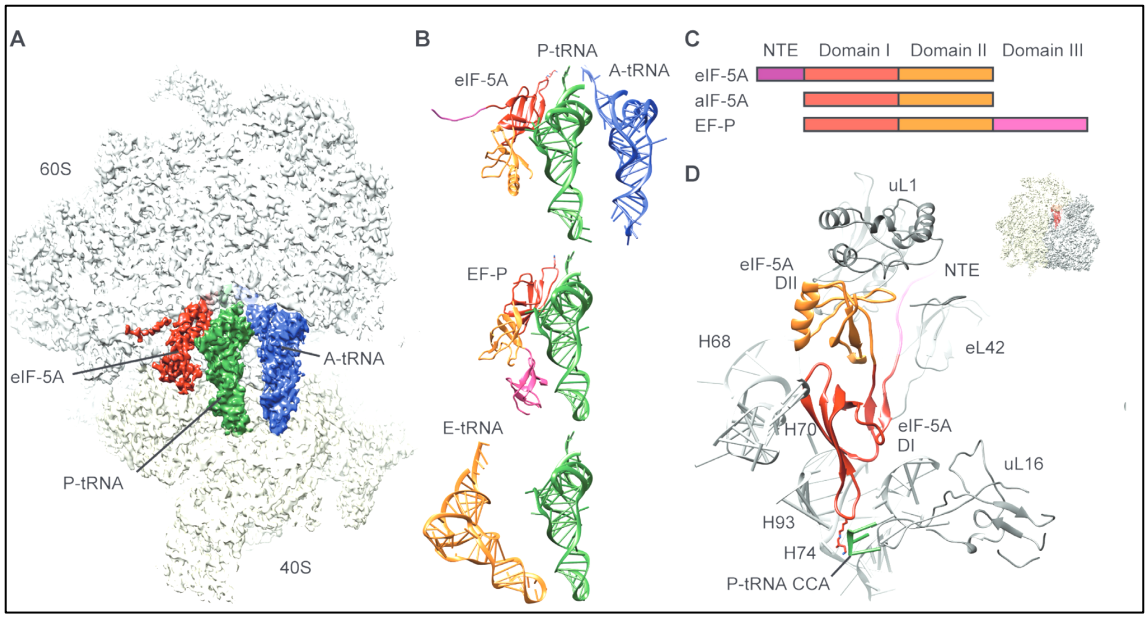

Figure 9. Cry-EM structure of eIF5A bound to the yeast ribosome. (A)Transverse section of the cryo-EM map of eIF5A-80S complex, which it is shown the eIF5A binding site. P-tRNA (green) and A-tRNA(blue). (B) comparison of ribosome binding positions of eIF5A (dark red). EF-P(magenta) and E-site tRNA(orange)(Schmidt et al. 2016)

In addition, eIF5A is involved also in the initiation and termination phase of synthesis protein. During the initiation phase, eIF5A participates in controlling the fidelity of the start codon selection(Manjunath et al. 2019). During the termination step, eIF5A stimulates the hydrolysis of peptidyl-tRNA catalyzed by the termination factors eRF1 eRF3 (Schuller et al. 2017)

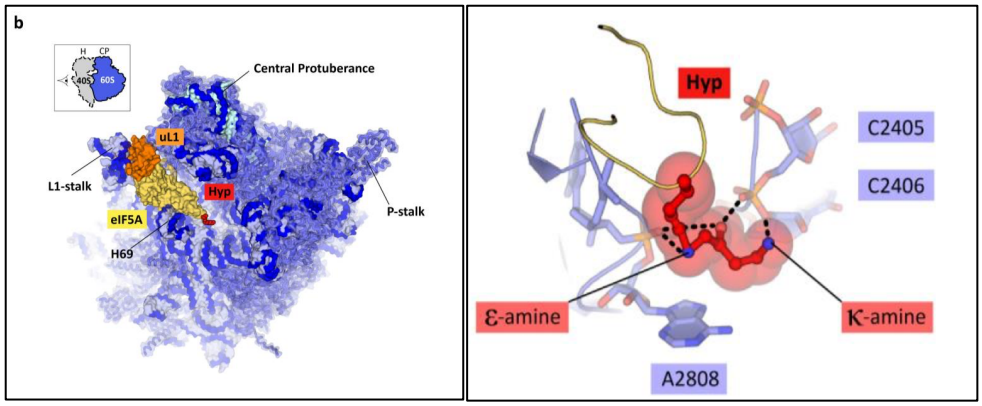

Figure 10. Electron density map reveals ribosome-bound eIF4A. (left) it is observed 80S ribosome/eIF5A structure. views of the 60S ribosomal subunit shows the eIF5A-binding sire and eIF5A.(right) the Hypusine-binding site.(Schmidt et al. 2016)

1.8 eIF5A is involved in pathological cellular processes.

eIF5A1 and eIF5A2 share 84% sequence identity and 94 % similarity (Clement et al. 2003) despite their genes being located on different chromosomes. eIF5A is up regulated in several malignancies and is found at elevated levels in peripheral blood cells in patients of chronic myeloid leukemia, revealing its role in cell proliferation. It has been demonstrated that inhibition of the hypusination process in cancer cells reduces their proliferation (Tong et al. 2009)

eIF5A is also involved in many others pathological processes, such as HIV-infection, diabetes, malaria(Bevec et al. 1996). Studies on the immunodeficiency virus (HIV) revealed that eIF5A interacts with viral mRNAs, acting like a nucleocytoplasmic transport for viral mRNAs through the RRE (Rev responsive elements) or IRES (Internal Ribosome Entry Site) and these interactions are

mediated by a stable binding of the hypusinated protein with specific mRNAs (Liu et al. 1997)

Hypusination of eIF5A is involved in the inflammatory damage of β cells in the diabetic disease; depletion of eIF5A caused by inhibition of hypusination with GC7 reduced hyperglycemia in diabetic mice (Maier et al. 2010)

1.9 The Deoxyhypusine Synthase

Deoxyhypusine synthase (DHS) is a cytosolic enzyme involved in the hypusination process. DHS is encoded by the *DHPS* gene that situated on chromosome 19 in humans (Jones et al. 1996). Deoxyhypusine synthase and eIF5A are conserved all over the eukaryotic kingdom and both essential for cell proliferation and survival (Umland et al. 2004). It has been shown that inactivation of the two genes of eIF5A or the Deoxyhypusine synthase gene causes loss of cell viability.

DHS binds specifically to eIF5A and spermidine, however it can also bind homospermidine, aminopropyl cadaverine, N8-methyl and N8-ethyl spermidines (J.-H. Park et al. 2003).

DHS catalyzes the transfer of 4-aminobutyl moiety of spermidine eIF5A Lys50 (J.-H. Park et al. 2003). DHS is a tetrameric enzyme, composed of four identical subunits of 40 kDa, as shown figure 11. DHS is an extremely conserved protein, both in function and structure; human and *S. cerevisiae* share 68% of identical

residues (Wolff et al. 2007), while the identity between the human and archaeal protein is 50 %(Bult et al. 1996).

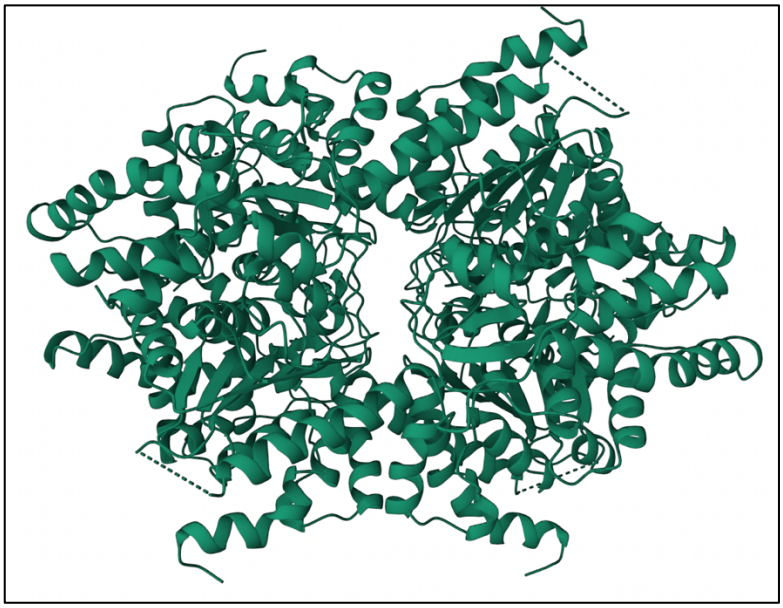

Figure 11. Human Deoxyhypusine synthase DHS (PDB code ID: 1DHS)

1.1.1 The role of DHS in the hypusination pathway

Chang and his coworkers have demonstrated that DHS catalyzes the NAD-dependent transfer of the butylamine from the spermidine to a specific lysine residue of eIF5A and also proposed that the binding of spermidine to the enzyme is recognizable only in the presence of NAD or NADH, which induces a conformational change of the enzyme as well as help the binding of the spermidine. (Lee and Park 2000). The complete maturation of Hypusine occurs in four steps, as shown figure 12. In the first step, in the presence of NAD^+, DHS

catalyzes the formation of dehydrospermidine through the oxidation of spermidine and generates NADH (Wolff, Park, and Folk 1990).

In the second step, the dehydrospermidine is cleaved to produce diaminopropane, and the residual butyloamine moiety is connected to the DHS Lys329 via an imine bond. (Joe et al. 1997). However, in absence of the eIF5A precursor, the enzyme can catalyze an abortive reaction with the production of 1,3-diaminopropane, Δ1-pyrroline, and NADH (Wolff, Park, and Folk 1990). If the specific residue Lys329 is mutated, this will lead to a totally inactive human enzyme (Joe et al. 1997; Umland et al. 2004). In the third step, the same butylamine moiety is transferred from the enzyme-imine intermediate to the ε-amino group of a specific lysine of the eIF5A (Lys 50 in human), with formation of eIF5A-imine intermediate. In the last step, DHS catalyzes the reduction of the eIF5A Lys 50 Imine-intermediate to deoxyhypusine via NAD regeneration (Wolff, Park, and Folk 1990).

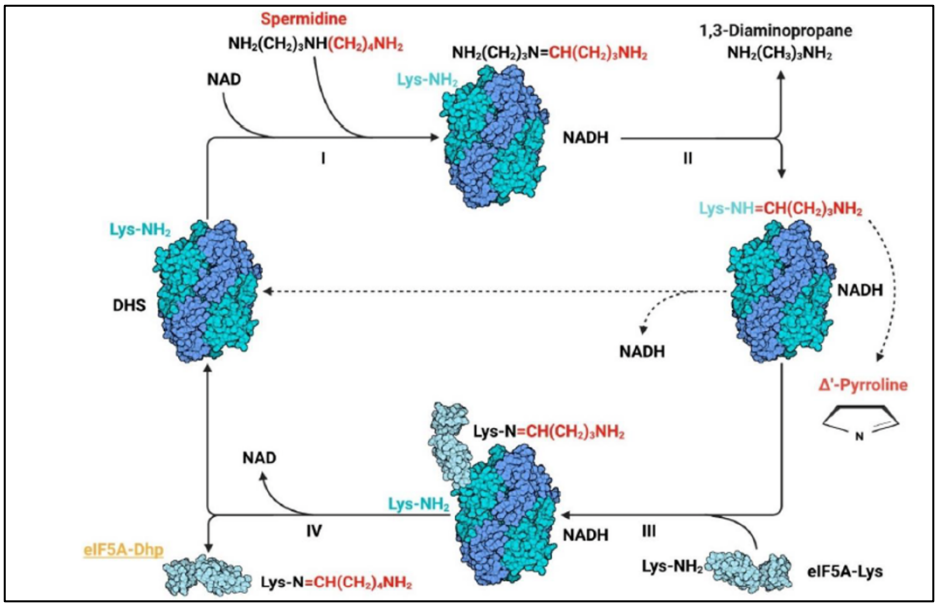

Figure 12. Schematic representation of the DHS reaction. The reaction of Hypusination constituted from four steps (D'Agostino et al. 2020)

1.1.2 Comparison of human DHS with other Kingdoms

As mentioned above, DHS is conserved among Eukarya and Archaea; the *DHSP* gene has been found in all sequenced archaeal genomes and post-translation modification is found in all archaeal organisms, albeit with some variation. Some species express the hypusinated version, whereas others only the deoxyhypusinated form and a few contain both versions. (M. H. Park and Wolff 2018b).

The archaeal DHS shares between 31 and 52 % of amino acid identity and similarity with the human version (figures 13 and 14).

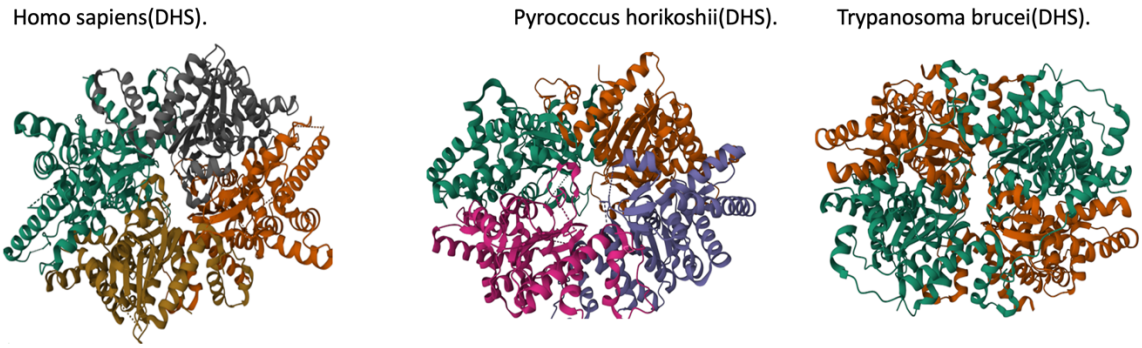

Figure 13. first structure, DHS of Homo sapience, second structure, DHS of pyrococcus horikoshii, and last structure, DHS of Trypanosoma. Brucei.

```
--------------------MSGVPFPSRVIGDLDYSNLLNIGQEEAIRCVLNAYPNIGL 40
MEGSLEREAPAGALAAVLKHSSTLPPESTQVRG------YDFNRGVNYRALLEAFGTTGF 54
----------MKAKDIVLKKSEK--IEGVEVKG------PWLDDAQSLEEVVSYYYRIGF 42
                     .     .  : .        :.     . ::. :   *:

EATNLGRARRIVQRALN-------------------DNGMDGNKVMLAYTSNLISSGLRD 81
QATNFGRAVQQVNAMIEKKLEPLSQDEDQHADLTQSRRPLTSCTIFLGYTSNLISSGIRE 114
QATHLGRAIEIWRKVE------------------EKRERGEEIRVFLGYTSNIISSGLRE 84
:**::*** .  .                          .      ::*.****:****:*:

TFACLARENRIGAVVTTAGGVEEDVIKCLGDTLVGDFALNDHALRNNGLNRVGNLLVPND 141
TIRYLVQHNMVDVLVTTAGGVEEDLIKCLAPTYLGEFSLRGKELRENGINRIGNLLVPNE 174
IIAWLVKEKKVDVIVTTAGGVEEDFIKSLKPFILGDWEVDDAELRKKGVNRIGNIFVPND 144
 :  *.:.: :..:**********.**.*    :*:: : .  **::*:**:**::***:

NYRNFEDFFVPLLRRLHEQQRDSRWTTKTTPSQIIAEIGAALESVRPNDCGSSLIYWCYR 201
NYCKFEDWLMPILDQMVMEQNT--EGVKWTPSKMIARLGKEI------NNPESVYYWAQK 226
RYIEFEKYMIPFFERVLKIEEK--LSRPLTASEFIYEMGRYMDEKLGKEKEKSVIYWAYK 202
.* :**.:::*:: ::   :.        * *::* .:*  :      :  .*: **. :

NDIPVFSPAFTDGSMGDMIYFYNYSR--KGLVVDPVPDVRRLRQLGCKSTNVGRITCIVL 259
NHIPVFSPALTDGSLGDMIFFHSY--KNPGLVLDIVEDLRLINTQAIFAK---CTGMIIL 281
NNIPIFCPAITDGSIGDMLYFFKEERRDSRLIIDIANDIVKLNNLAITAK---ETASIIL 259
*.**:*.**:****:***::*..       *::* . *:  :.  .  :.       *:*

GAGLPKHHLLRN----VQADAVVYVTTGSDADGCESSCNVMADRANGLLSPNCDVVRVHG 315
GGGVVKHHIANANLMRNGADYAVYINTAQEFDGSDSGARPDEAVSWGKIRVDAQPVKVYA 341
GGSLPKHAIINANLFRGGTDYAIYISTAVPWDGSLSGAPPREGVSWGKIKAKADYVEVWG 319
*..: ** : .       :* .:*:.*.   **. *..      : * :  ..: *.* .

DATIISPLLLLRSSDGKEKVGVREDGN- 342
DASLVFPLLVAETFAQKMDAFMHEKNED 369
DATLIFPILVWMVMKARGQGYAQ----- 342
**::: *:*:      : .   :
```

\

Figure 14. Multialignment of the DHS, Structural comparison of the eukaryotic DHS (PDB Id: code 6XXM) with P. horikoshii (PDB ID: 7CMC) and T. brucei (PDB ID: 6DFT). Amino acid sequences alignment did throw Clustal omega. where there is star, it is means the amin acid conserved between three kingdoms while there two dots it is means the amino acid is similar.

1.1.3 Structural features of Eukaryotic DHS

The first structure of DHS was resolved in 1998 (Liao et al. 1998). DHS exists as a tetramer in solution, with four identical subunits organized in closely associated pairs to forms dimers: A1A2 and B1B2, with two active sites, situated in each dimer interface.

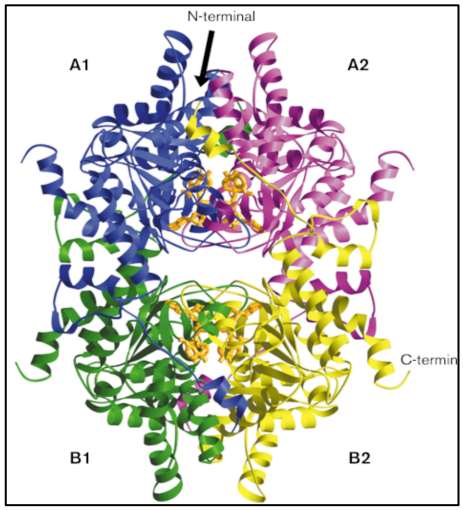

Figure 15. Structure of DHS tetra model with four bound NAD molecules, (PDB ID:1DHS) and organize in two dimers. A1(Blu) A2(magenta) and B1(green) B2(yellow). Two NAD molecules (orange stick model) are bound in each dimers interface.

Each monomer includes a central six-strand parallel β-sheet, and a small antiparallel β-sheet and 16 helices. Near the N-terminal end of each monomer there is a 35-residues insertion of the other monomer, composed of two helices, α2 and α3, with helix α3 packed parallel to the C-terminal helix α7. Among β2 and αC2 there is a group of five helices (helices α4, α6 αB1, αB2 and αC1) packed

on the edge of the parallel sheet on the opposite side from the N-terminal tail (as shown figure 16).

A three-stranded antiparallel β-sheet (composed of strands βa, βb and βc) and a short helix α5 are inserted between helix α4 and helix α6 of the cluster and are located at the C-terminal end of the parallel sheet. One side of this small subdomain constitutes part of the dimer interface that contains two active sites.

The N-terminal tail composed (9-39 residues) and has two turn α helix at its end; this tail from one monomer, for example B2, extends though the side of the small subdomain of the opposite monomer A2 to the A1A2 dimer interface, where the short helix blocks in a ball-and-chain disposal the entrance to the active site formed at A1A2 interface, causing an inactive conformation of enzyme as shown figure 14 (Liao et al. 1998).

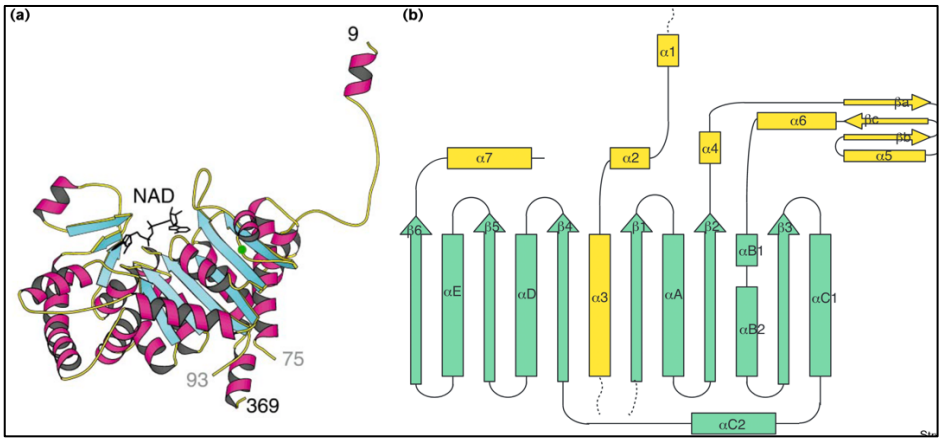

Figure 16. Ribbon representation of the monomer of DHS with bound NAD (shown as a black model) Schematic diagram of the secondary structures. DHS contains a Rossmann fold(Shown in green) with insertion (in yellow) at the N and C terminus and between the secondary β strand and the secondary α helix of the Rossmann fold (Liao et al. 1998)

1.1.4 GC7 Inhibits the Reaction of hypusination

To date, several inhibitors have been developed to block the hypusination reaction. The most powerful inhibitor is N1-guanyl 1,7-diaminoheptene (GC7), which i\s able to target the DHS in the first step of hypusination (as shown figure 17) (D'Agostino et al. 2020).

This inhibitor was discovered by Jakus and his coworkers examining several spermidine analogues as potential inhibitors of Deoxyhypusine synthase (DHS)(Jakus et al. 1993). GC7 is a spermidine analogue constituted by guanidino moiety and an amino group.

GC7 has been tested in several cancer cells, however, because of important limitations, such as its reduced bioavailability due to the presence of polyamine oxidases in the bloodstream, it cannot be used in the clinical practice. In addition, GC7 might affect the composition of the overall cellular polyamines pool.

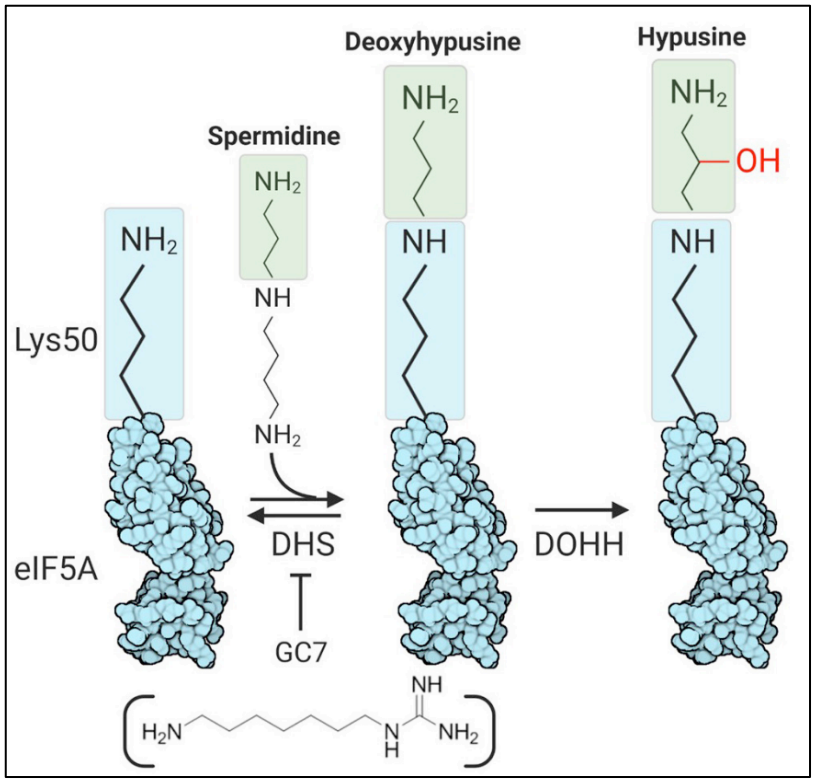

Figure 17. rection of hypusination carried out by two enzymes: DHS and DOHH, first reaction is reversible rection and second is irreversible. If it blocks DHS will be block the reaction of hypusination. Therefore, it used inhibitor GC7 to block DHS. (D'Agostino et al. 2020)

1.1.5 Aim of the thesis

The scope of this thesis is 1to produce humans, recombinant eIF5A and DHS via cloning, expression, and purification. eIF5A DHS are conserved in all eukaryotes. eIF5A is involved in variou1s pathologies, such as HIV-1 infection, malaria, and diabetes, and is overexpress1ed in several cancer types. Therefore, finding novel molecules able to inhibit the 1process of hypusination is crucial. One of the most powerful inhibitors of the hy1pusination process is the spermidine analog N1-guanil 1.7-diaminoheptane (GC7). Unfortunately, this inhibitor shows poor selectivity and restricted bioav1ailability and presents several side effects. It has

been confirmed by several studies that the molecule is extremely effective on different types of cancer cell lines. Therefore, our aim is to produce eIF5A and the first enzyme involved in the1 hypusination process, so that we can study the interaction between eIF5A and 1DHS and design specific inhibitors of the eIF5A-DHS complex formation. The structure of human eIF5A2 is unknown; this prompted us to predict its structure using Alphafold and then to compare it to the eIF5A structure, thereby highlighting the differences between the isoforms. Ultimately, this work will open new roads in the drug design for specific inhibitors of eIF5A.

2.Materials and Methods

2.1 The Principle of Ligation-Independent Cloning

Ligation independent cloning (LIC) is a technique developed in the 1990s as an alternative to restriction enzyme and ligation cloning. This method uses the

ability of the T4 DNA polymerase to catalyze a 3'→ 5' exonuclease activity in the absence of deoxy nucleotides. Ligation-independent cloning vectors contain a specific LIC sequence in the MCS, constituted by a 30-nucleotide stretch containing only three of the four bases (A, T and C) and a unique SspI restriction site in the middle, which generates blunt ends.

The vector is first linearized at the unique restriction site and then subjected to T4 DNA polymerase treatment in the presence of dGTP to generate 15-nt LIC overhangs. The insert is prepared by PCR using primers containing the complementary LIC sequence and then digested with T4 DNA polymerase in the presence of dCTP. This way, cloning requires only the annealing of the vector and insert, without the need for additional ligation steps (figure 18).(Gradia et al. 2017).

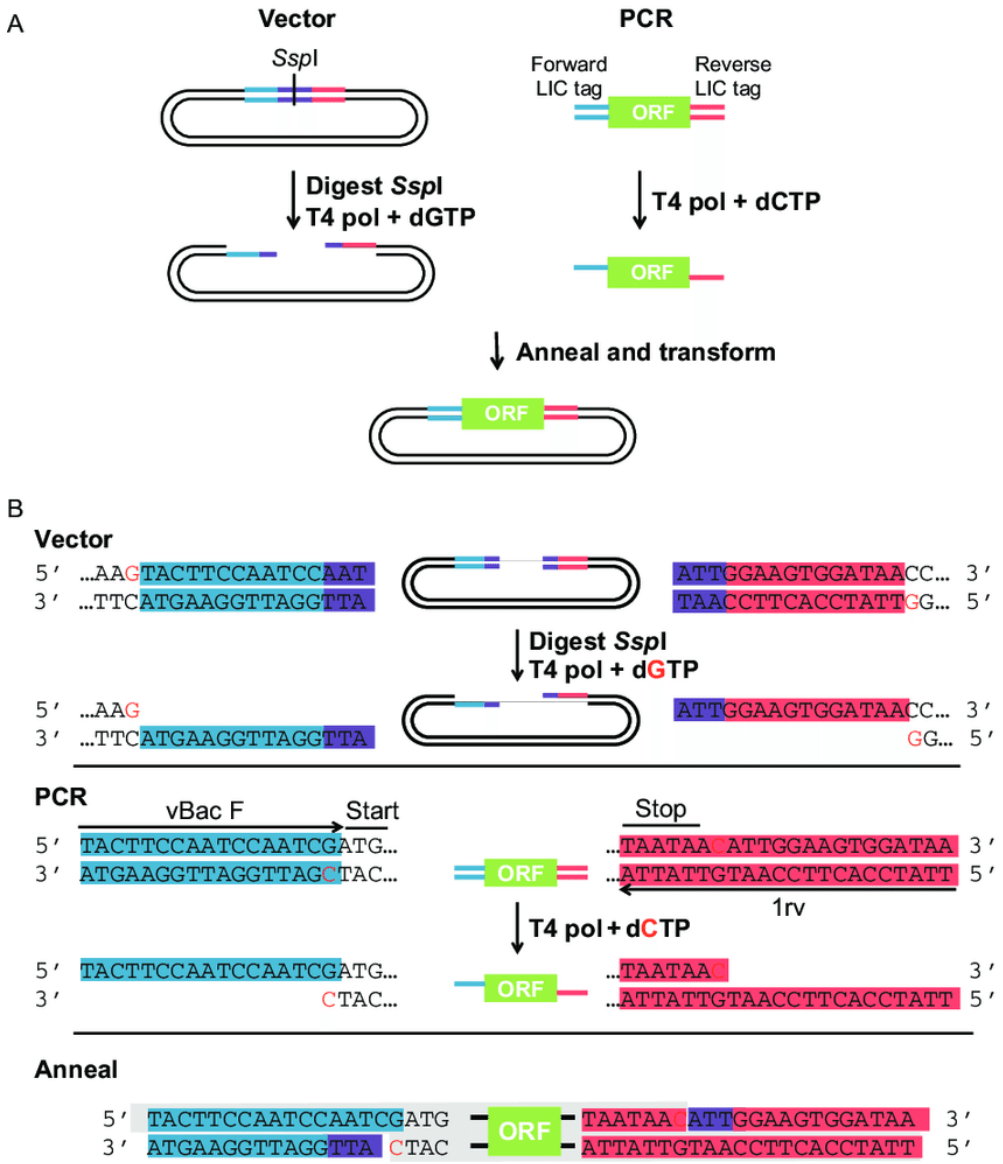

Figure 18: Schematics of the Ligation-Independent Cloning steps

2.2 the pMCSG7 Expression Vector

The pMCSG7 was first produced from Midwest Center for structural Genomics (MCSG). This ligation independent Cloning (LIC) expression vector was used for the cloning of the eIF5A and DHS genes.

The vector contains the following elements (Figure 19)

- An Ampicillin selection marker, which confers resistance to the antibiotic

- A strong T7 transcription promoter
- A lac operator that controls the expression of the target gene
- A Multiple Cloning Site (MCS) including a LIC sequence with a unique SspI restriction site
- An RBS sequence, which recruits the ribosome for translation.
- 6X His-tag, used for the purification of the protein of interest.
- A TEV cleavage sequence.
- T7 transcriptions terminator

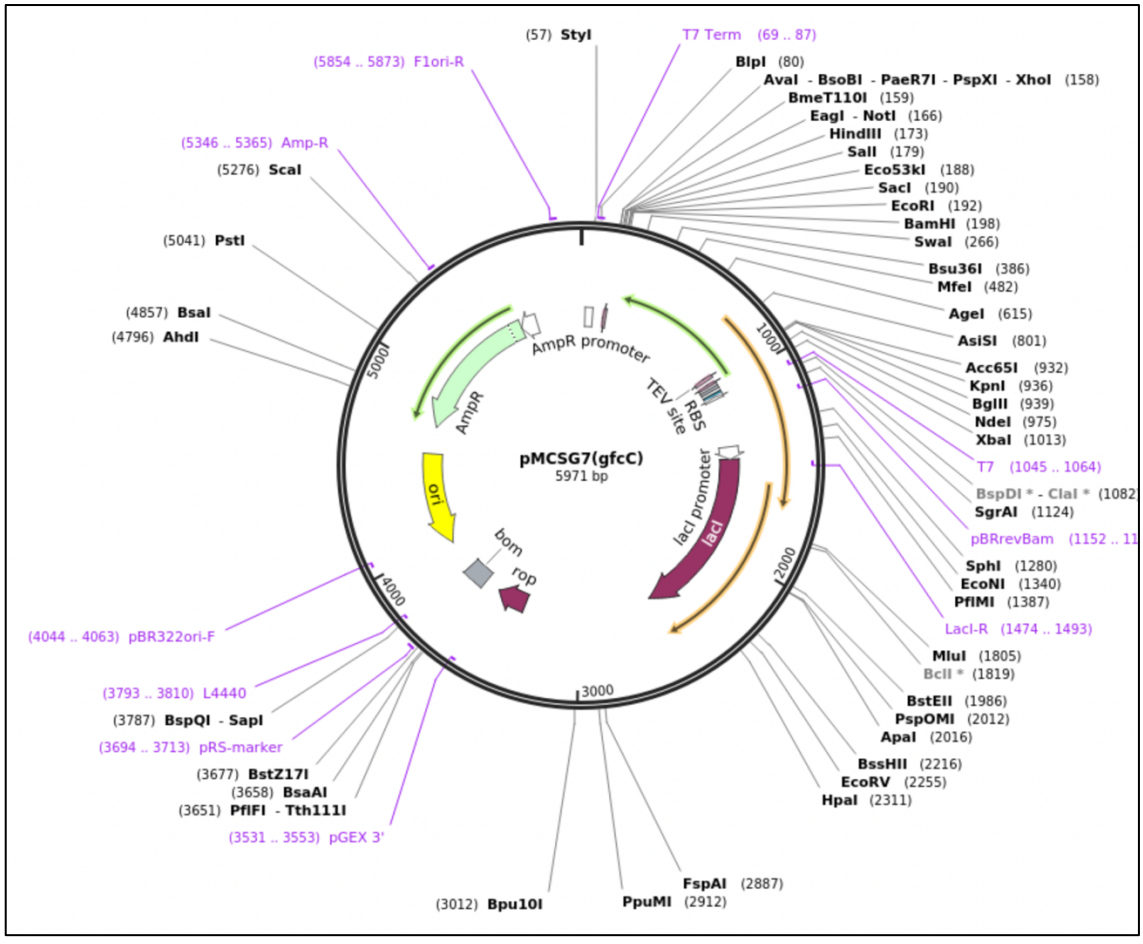

Figure 19. Vector map of the pmCSG7. Its image come from addgene.

2.3 Preparation of the vector for the ligation-independent cloning

The pMCSG7 vector was linearized with SspI, which generates blunt ends in the middle of the LIC sequence. The reaction mix was prepared with the following components:

Buffer Cutsmart 10x	4 µl
SspI 20 U/ul	1 µl
pMCSG7 414 ng/ µl	5 µl
H20 sterile	30 µl
Final volume	40 µl

Table 1. linearization Plasmid(pMCSG7) with enzyme SspI.

The reaction was incubated for 1 hour and 30 minutes at 37 °C, followed by. enzyme inactivation at 65 °C for 20 minutes. The extent of linearization was checked by electrophoresis on an agarose gel, followed by purification of the linearized vector by gel extraction.

2.4 Purification of the linearized pMCSG7 vector by agarose gel electrophoresis.

DNA molecules of differing sizes can be separated by gel electrophoresis on an agarose gel. Agarose is a water-soluble sugar at boiling temperature that becomes solid when it cools, forming a matrix through hydrogen bonds between linear chains. If a gel is subjected to the action of an electric field, molecules of DNA migrate in the gel depending on their charge, size, and structure.

The electrophoresis apparatus is connected to a power supply of electric current and the DNA molecules that are charged negatively charged due to the presence of phosphate groups, migrate from the negative pole (cathode) to the positive pole (anode) (figure 20)

These fragments separated by electrophoresis can be easily visualized by adding an intercalating substance to the gel, that emits a fluorescence when exposed to UV light.

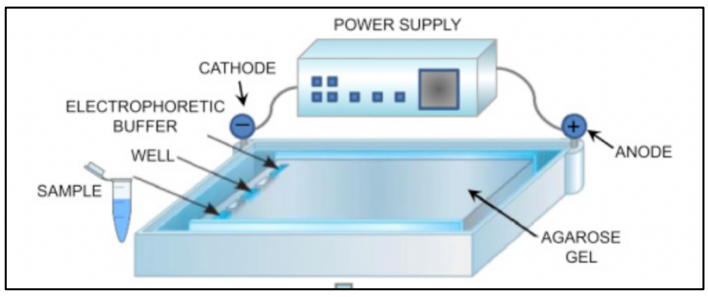

Figure 20. Agarose gel electrophoresis of DNA.

The linearized pMCSG7 was loaded on a 1% agarose gel; after the electrophoretic rues, the DNA band corresponding to the linearized vector was the excised from the gel, and the DNA was extracted using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel).

2.5 Amplification of the eIF5A open reading frame from plasmid pcDNA3.1

The pcDNA: eIF5A vector has been kindly gifted by Dr. Dario Benelli, Sapienza of University Rome. In order to clone eIF5A into a bacterial expression vector, the insert has been amplified using ligation-independent cloning (LIC) primers. The PCR reaction was set up as shown in table 1.

Component (initial concentration)	1X
pcDNA3.1 (2,45ng/µl)	2 µl
Buffer 10X	2,5 µl
$MgSO_4$(25 mM)	1,5 µl
dNTPs(25mM)	1,5 µl
Primer F-LIC (10 µM)	1 µl
Primer R-LIC (10 µM)	1 µl
KOD (1U/µ) (71086-5)	0,5 µl
H_2O	15 µl
Vf	25 µl

Table 2. protocol reaction of PCR for extraction of eIF5A with primers LIC.

The primers used for the amplification of eIF5A are the following:

Forward Primer: 5'-
TACTTCCAATCCAATGCCATGGCAGATGACTTGGACTTCG- 3'
Reverse Primer: 5' –
TTATCCACTTCCAATGTTATTATTTTGCCATGGCCTTG – 3', where the portion highlighted in red is complementary to the insertion site in the vector.

The program used for the amplification is described in Table 3.

Program	TEMPERATURE	TIME	CYCLES
Initial denaturation	98 °C	2'	1X
Denaturation	98 °C	30''	30X
Anneling	58 °	30"	
Elongation	70 °C	1'	
Final extension	70 C°	4'	1x

Table 3. PCR program for the amplification of eIF5A from pcDNA3.1(+/-)

2.6 Total RNA extraction from Hek293 cells and cDNA synthesis

To clone the *DHPS* open-reading frame, total RNA was extracted from human cells using the Genejet RNA Purification Kit (ThermoScientific). Hek293 cells have been used because they are widely used in cell biology research because of their reliable growth and low propensity for mutations.

The total RNA was extracted following the manufacturer's protocol.

Before proceeding with the cDNA synthesis, 2 µg of total RNA were subjected to DNase digestion, to avoid contamination with genomic DNA.

The reaction mix was prepared as shown in table 4.

(2 ug) total RNA	5 µl
DNase (2U)	2 µl
Buffer(10X)	2 µl
H20 sterile	11 µl
Vf	20 µl

Table 4: Protocol digestion with DNase.

The reaction was incubated for 15 minutes at room temperature and stopped by addition of 2 µl EDTA (Ethylenediaminetetraacetic).

In the next step, the first strand of the cDNA was synthesized by reverse transcription, in the presence of Oligo dT and Random 6-er primers.

The total RNA was first incubated at 65 °C for five minutes with the primers (table 5).

Total RNA DNase I	4 µl
Oligo dT	0,5 µl
Random 6-er	0,5 µl
H_2O Sterile	1 µl
Vf	6 µl

Table 5. RT-cDNA synthase RevertAid Thermo.

After the incubation, the reverse transcription reaction was set up as described in Table 5 and incubated 42 °C for 60 minutes.

5 X Buffer	**2 µl**
Ribolock	0,5 µl
MulV RT	0,5 µl
dNTPs	1 µl
Vf	10 µl

Table 6. protocol of RT-cDNA synthase.

The reaction was then stopped by incubation at 70 °C for five minutes.

2.7 Amplification of the DHPS open reading frame

2 µl of the cDNA synthesized as described in Section 2.6 was used as template for the amplification of the *DHPS* open reading frame (ORF) as follows.

H20 PCR	26, 5 µl
10 X Buffer	5 .0 µl
Mg SO4	3. 0 µl
dNTPs	7,5. 0 µl
KOD Polymerase	1 .0 µl
cDNA	2 ul µl
Primer-F (10 µM)	2, 50 µl
Primer -R (R10 µM)	2, 50 µl
VF	50 ul

Table 7. PCR reaction mix for the amplification of the DPHS *ORF*

The sequence of the LIC primers used for the amplification was:

Primer LIC -FW: 5' –

TACTTCCAATCCAATGAAGGTTCCCTGGAACGGGAG 3'

Primer LIC-Rev: 5' -

TTATCCACTTCCAATGTTATCAGTCCTCGTTCTTCTCATGC – 3,

where the portion highlighted in red is complementary to the insertion site in the vector.

The program used for the PCR is described in Table 8.

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	2’	35 cycles.
Denaturation	95 °C	30’’	
Anneling	58 °C	30’’	
Extension	70 °C	1’and 30’’	
Final Extension	70 °C	5’’	

Table 8. Program of PCR for Amplify my insert (cDNA)

The PCR product was then subjected to electrophoresis and isolated for the gel band as described below.

2.8 Purification of the eIF5A and DHS open reading frames by agarose gel electrophoresis.

The eIF5A and DHS PCR products were purified after agarose gel electrophoresis as described in Section 2.4. The DNA bands were the excised from the gel, and the PCR products were extracted using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). The concentration of the purified DNA fragments was determined by spectrophotometric measurement to be 12,6 ng/µl for the DHPS and 314,5 ng/µl for the eIF5A products.

2.9 T4 DNA Polymerase treatment of vector and inserts for ligation-independent cloning

The linearized pMCSG7 vector and the eIF5A and DHS ORFs were treated with T4 DNA polymerase to generate the single-strand LIC overhangs. The reaction mixes were prepared as indicated in table 9 and 10 for the vector and inserts, respectively.

LIC Treatment of the vector.

Mix	**Ci**	**Cf**	**Vi**	**Vf**
H_20 PCR				20 µl
T4-pol(buffer)10X	10	1	2 µl	
BSA(UI)	100	1	0,2 µl	
dGTP(mM)	100	2,5	0,5 µl	
Linearized Vector ng/ µl	9		17,05 µl	
T4 DNA polymerase U/ µl	3	0.0375	0,25 µl	

Table 9. LIC treatment Vector of DHS

LIC treatment of the insert.

MIX	Ci	Cf	Vi	Vf
H_2O PCR			13.05 µl	20
T4-pol(buffer)10X	10	1	2 µl	
BSA(UI)	100	1	0,2 µl	
dCTP (mM)	100	2,5	0,5 µl	
T4 DNA Polymerase U/ul	3	0,0375	0,25 µl	

Table 10. LIC treatment insert of DHS.

8 µl of the insert mix was combined with 2 µl each of the purified inserts.

The T4 DNA polymerase treatment was carried out for 60 minutes at room temperature, followed by inactivation at 75 °C for 20 minutes.

2.1.1 Vector and insert annealing

In the annealing step, the overhangs in the vector and insert pair to generate a circular vector with two one-strand nicks, which can be repaired by bacteria without the need for additional ligation steps.

2 µl of vector were combined with 4 µl of each insert. The reaction was incubated at 22 °C for 60 minutes and then stopped by addition of 2 µl of a 25mM EDTA solution, followed by incubation at 22 °C for 5 minutes. A negative control was set up by incubating 2 µl of vector in the absence of inserts.

2.1.2Transformation of LIC cloning reactions in GC5 E. coli competent cells

Transformation is the technique that allows to introduce exogenous DNA molecules into bacteria.

Some bacteria are naturally capable of absorbing exogenous DNA molecules, while others, such as *E. coli*, acquire this ability upon specific treatments that alter the permeability of the bacterial membrane.

The permeability of the bacterial membrane can be increased by chemical transformation, based on the treatment of the bacteria with CaCl2, or by electroporation, which consists of a short, intense electrical discharge that causes pores in the membrane to open.

The competent *E. coli* cells obtained are mixed with the plasmid of interest and must be subjected to a short heat shock for transformation to take place, after which they are allowed to grow in rich medium before being plated on solid medium.

2 µl of each LIC reaction was combined with 50 µl of *E. coli* GC5 competent cells. The cells were first incubated on ice for 30 minutes, followed by a short heat shock at 42 °C for 30 seconds.

400 µl of pre-warmed rich LB medium were added to the cells; the cells were then allowed to recover for 1h at 37 °C

200 µl of each transformation were plated on solid LB plates containing 100 µg/ml Ampicillin. The plates were then incubated over-night at 37 °C.

The following day, the colonies grown on each plate were screened by colony PCR, to verify the presence of the inserts.

2.1.3Colony PCR screening

Colony PCR is a method used for rapidly screening colonies of bacteria that have grown on selective media following a transformation step, to verify that the desired genetic construct is present, or to amply a portion of the construct.

Each colony was picked with a sterile tip and swirled in 20 µl of sterile water. The cells were lysed at 100 °C for 5 minutes followed by centrifugation at 11000 x g for 6 minutes. 2 µl of the supernatant was used as template for the PCR.

MIX	**Ci**	**Cf**	**Vi**	**Vf**
PCR-grade H2O			167,4 µl	270 µl
KOD 10X	10	1	27 µl	
$MgSO_4$(mM)	25	1,5	16,2 µl	
dNTPs(mM)	2	0,2	27 µl	
KOD Polymerase (U/ µl)	1	0,02	5,4 µl	
T7 Promoter primer (µM)	10	0,5	13,5 µl	
T7 Terminator primer (µM)	10	0,5	13.5	

Table 11. PCR Colony for DHS and eIF5A

Each PCR reaction was set up with 23 µl of Mix and 2 µl of cell lysate. Universal T7 promoter and T7 terminator primers were used for all constructs.

Program			
Initial denaturation	95 C°	2’	**35 cycles**
Denaturation	95 C°	30’’	
Annealing	58 C°	30’’	
Extension	70 C°	1’	
Finale Extension	70 C°	5’	

Table 12. Program of PCR

The results were verified by agar gel electrophoresis and the positive colonies were grown in 10 ml LB medium supplemented with Ampicillin for plasmid extraction.

2.1.4 Plasmid extraction and restriction analysis.

To extract of the pMCSG7 + eIF5A and pMCSG7+DHS plasmids, the Genelute plasmid Mini preparation kit (lot SLCE 47665) was used according to the manufacturer’s protocol. The plasmids were quantified by spectrophotometer measurement and subjected to Sanger sequencing (service provided by BMR Genomics). The sequencing confirmed the presence and correct insertion site for both pMCSG7 + eIF5A and pMCSG7 + DHS.

2.1.5 Transformation of pMCSG7+DHS and pMCSG7+eIF5A inBL21(DE3) +pLysS cells.

The *E. coli* BL21 (DE3) pLysS strain was used for the expression of eIF5A and DHS. This strain expresses the T7 polymerase gene upon induction with the non-hydrolysable allolactose analogue isopropyl-β-D-thiogalactoside (IPTG), which binds to the lacUV5 promoter. Thus, upon induction with IPTG, large amounts of the protein of interest can be produced.

50 ng of pMCSG7+eIF5A and pMCSG7+DHS were added to a 50-µl aliquot of competent cells each and incubated on ice for 30 minutes. Cells were then subjected to heat-shock transformation: the cells were first incubated for 45 seconds at 42°C using a thermostatically controlled bath and immediately placed back on ice for two minutes. Next, 1 ml of LB medium was added to the mixture and the cells were incubated for 1 hour at 37°. 200 µl of the cell suspension were plated on solid LB medium containing 100 µg/ml Ampicillin and 34 µg/ml Chloramphenicol; the plates were then incubated overnight at 37°.

2.1.6 Large scale expression of eIF5A and DHS.

A large-scale expression of a protein of interest requires the gradual propagation of a single bacterial colony in liquid medium in order to obtain a sizeable amount of cells.

A single colony was picked from each of the BL21(DE3) + pLysS plate and inoculated in a flask containing 25 ml LB supplemented with Ampicillin and Chloramphenicol (LB-Amp+Chlo). The flasks were incubated at 37 °C over-night with moderate shaking (180 rpm) in a Shel Lab incubator. The following day, the optical density (OD) of the cultures was measured at 600 nm using a spectrophotometer. For the large-scale expression, a suitable volume of saturated culture was transferred to two Erlenmeyer flasks containing 500 ml LB-Amp+Chlo each at 0.1 OD/ml. The cultures were incubated at 37°C, 180 rpm; spectrophotometer measurements were performed every hour until the cultures entered the logarithmic growth phase and reached 0.7 OD/ml.

500 µl of each culture were taken as pre-induction controls: the cells were centrifuged at 12000 x g for 3 minutes and resuspended in 120 µl/OD 2X SDS-PAGE loading buffer (0.125 M Tris-HCl pH 6.8, 25% glycerol, 4% SDS, 5% β-Mercaptoethanol, 0.001% Bromophenol Blue).

After that, the expression of eFI5A and DHS was induced by addition of 0,5 mM IPTG (Isopropyl -β-D-1-tiogalattopiranoside). Induction was allowed for two and half hours at 37 °C and 180 rpm. After that, 500 µl of each culture was collected and used as a post-induction control; the cells were centrifuged at 12000 x g for

3 minutes and resuspended in 120 µl/OD 2X SDS-PAGE loading buffer. The remaining cultures were centrifuged at 4000 rpm in a Beckman JLA8.100 rotor at 4°C and the cells pellets were stored at -80°C.

The extent of protein expression was evaluated by loading the pre- and post-induction sample on a poly-acrylamide gel.

2.1.7 SDS-PAGE Protein Electrophoresis

Electrophoresis is the most widely used technique to separate mixtures of proteins of different sizes; the separation of the various protein components is usually performed on polyacrylamide gels, with the porosity best suited to the size of the proteins to be separated.

The speed with which a protein migrates through the gel depends on the size and the charge of the protein ant on the intensity of the applied electric field. The net charge of a protein depends, in turn, on the pH of the buffer used for the electrophoretic run. Most commonly, proteins are separated under denaturing conditions in the presence of sodium dodecyl sulphate (SDS): this is referred to as the SDS-PAGE technique (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis). In the presence of SDS and other specific experimental conditions, the secondary structures of proteins are denatured, and the molecules are saturated by negative charges, allowing the migration of the proteins to the

anode with a speed essentially dependent on the size (molecular weight) of the different protein in the mixture.

The pre- and post-induction samples were loaded on a 12.5 % SDS-PAGE gel and stained with Coomassie blue to allow the visualization of the protein bands on the gel.

2.1.8 Western Blot analysis

Western Blot is a molecular biology technique used to detect the presence of a specific protein in a given sample. This technique uses three key elements to achieve its task of separating a specific protein from a complex. First the proteins are separated by size, then they are transferred to a solid support, usually a nitrocellulose membrane, and finally the protein of interest is labelled with the use of specific antibodies.

The first step, i.e., protein separation by size, was performed by SDS-PAGE electrophoresis, as described in Section 2.16.

Next, the transfer was performed using Trans-Blot® Turbo™ Transfer System (Bio-Rad) using the standard SD method (1A, 25V, 30 minutes) (Figure 21). The membrane was saturated with Tris- and Tween-buffered saline (TBST: 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) supplemented with 3% bovine serum albumin (BSA) to avoid unspecific binding of the antibodies. The membrane was then incubated with the anti-His primary antibody (Invitrogen),

diluted 1:10,000 in TBST-BSA overnight at 4°C. The following day the membrane was washed 3 times in TBST to remove the unbound antibody and then incubated with the secondary anti-mouse antibody conjugated with horseradish peroxidase (HRP), diluted 1:10000 in TBST-BSA for 1 hour at room temperature. Finally, after four washes with TBST, the membrane was developed using two substrates capable of reacting with the peroxidase bound to the secondary antibody, for which the Clarity™ Western ECL Substrate kit (Bio-Rad) was used. 500 µl of each solution were added and incubated in the dark for 5 minutes to allow the chemiluminescent reaction to take place. Results and images were then visualized using a Chemidoc (Bio-Rad).

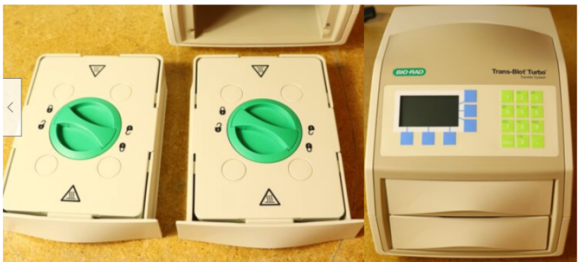

Figure 21. Trans -blot SD semi-Dry Transfer Cell, Bio-Rad.

2.1.9 Affinity purification of eIF5A and DHS

To perform in vitro binding experiments, eIF5A and DHS must first be purified from the heterogeneous mixture of *E. coli* cell lysate containing other proteins, nucleic acids, lipids, and other molecules.

Several features can be exploited to facilitate the purification of proteins of interest, such as the differences in chemical and physical properties between the protein to be purified and the others in the mixture.

eIF5A and DHS were expressed with a N-terminal tail of 10 histidine, also known as His-tag. The His-tag has a strong affinity for nitrilotriacetic acid (NTA) resins complexed with bivalent nickel ions. His-tag-containing proteins thus bind specifically to the resin. Elution of the tagged proteins under specific conditions can lead to substantial purification of the recombinant protein in a single step.

For the purification of eIF5A and DHS, the same protocol was used, albeit with different buffer compositions, as indicated in the following.

<u>Buffers for eIF5A purification</u>

1. Lysis buffer: 50 mM Tris/ HCl pH= 7, 5, 1 mg/ml Lysozyme, 1 mM PMSF, 15 mM Imidazole
2. Wash buffer: 50 mM Tris-HCl pH=7.5, 500 mM NaCl, 20 mM Imidazole
3. Elution buffer: 50 mM Tris-HCl pH=7.5, 150 mM NaCl, 250 mM Imidazole
4. Dialysis buffer: 50 mM Tris-HCl pH=7.5, 150 mM NaCl

<u>Buffers for DHS purification</u>

1. Lysis buffer: 50 mM Tris-HCl pH=7.8, 500 mM NaCl, 40 mM Imidazole, 5 mM Beta-Mercaptoethanol, Lysozyme 1 mg/ml, 1 mM PMSF
2. Wash buffer: 50 mM Tris-HCl pH=7.8, 500 mM NaCl, 40 mM Imidazole

3. Elution buffer: 50 mM Tris-HCl pH=7.8, 500 mM NaCl, 250 mM Imidazole

4. Dialysis buffer: 50 mM Tris-HCl pH=7.8, 500 mM NaCl

The frozen cell pellets were resuspended in 15 ml of Lysis buffer and kept on ice for 30 minutes. Cell membranes were ruptured by 5 1-minute cycles of sonication at 60% amplitude and 2 s pulses; cell debris and were removed by centrifugation at 12000 x g for 30 min at 4°C in a JLA8.1 rotor. After that, the supernatant was applied to 2 ml of Ni-NTA resin, pre-equilibrated in the respective Wash buffer. Binding of the tagged proteins was carried out overnight on a rotator at 4 °C. The following day the resin slurry was loaded on a plastic column containing a filter and the flow-through was collected. The resin was then washed with 10 volumes of Wash buffer. Elution was carried out by loading 15 1-ml aliquots of the respective Elution buffer on the column. Fractions were screened for the presence of eIF5A and DHS via SDS-PAGE. The fractions containing the largest amount of purified protein were collected, concentrated and buffer-exchanged into Dialysis buffer on Centricon filters (Millipore), with a cut-off of 3 kDa for eIF5A and 10 kDa for DHS. The concentration of the proteins was determined at the spectrophotometer, by measuring the absorbance at 280 nm and applying the Lambert-Beer Law. The molar extinction coefficients ε were obtained from the Expasy Protparam online tool (https://web.expasy.org/protparam/).

2.2.1 histidine tail cleavage with TEV enzyme

The TEV protease is a cysteine protease from the Tobacco Etch Virus (TEV). The optimal recognition site for this enzyme is the sequence Glu-Asn-Leu-Tyr-Phe-Gln (Gly/Ser) and cleavage occurs between residues Gly or Gly/Ser.

For the TEV digestion of DHS, 2 mg of the protein were incubated overnight at room temperature with 40 µg of TEV protease in the presence of EDTA.

DHS	1900 µl
TEV	13,5 µl
EDTA	1,9 µl

Table 13. TEV digestion of DHS.

The cleavage extent was determined the following day by loading 10 µg of protein on a 15% SDS-PAGE gel.

2.2.2 Native PAGE

Native gel electrophoresis allows for the separation of proteins under native conditions, that is, they have kept their optimal folding. This technique allows the separation of the proteins based on the molecular weight, structure, and charge of the protein, and is particularly useful to detect dimers and protein complexes. The protocol used is the same as for the standard SDS-PAGE gel electrophoresis,

except for the absence of SDS in the gel and in the running buffer. It follows tables 14 below for the experiments.

To detect the eIF5A

-DHS complex formation, reactions were set up in DHS Buffer (Tris-HCl 50 mM pH= 8.5, NaCl 200 mM) as indicated in Table 14.

Components	Ci	Cf	1	2	3	4	5	Vf
eIF5A ug/ µl	4,7	0,5	3.19 µl		3.19 µl	3,19 µl	3.19 µl	30 µl
DHS ug/ µl	0,5	0.4		24 µl		24 µl	24 µl	
NAD mM	10	1			3 µl		3 µl	
Spermidine mM	50	2			3 µl		3 µl	
Buffer			26.81 µl	6.00 µl	20.81 µl	2,81 µl	3.19 µl	

Table 14. the protocol that I used for demonstration hypusinated eIF5A migrates more slowly than non-hypusinated protein.

Samples were incubated 30 minutes at 37 °C and the Native PAGE gel 10% was prepared according to published protocols. The samples were run at 150 V for 2 hours in an ice bath in Tris/Glycine running buffer.

2.2.3 Bioinformatics Analysis

Study of eIF5A isoform 2 using NCBI

I used NCBI tools for searching Isoform 2 of eIF5A (eIF5A2). Briefly, to arrive at the expected result I retrieved the eIF5A2 amino acidic sequence in FASTA format and I use this as query sequence in different programs.

The National Centre for Biotechnology Information (NCBI) houses a series of databases relevant to biotechnology and biomedicine. It is an important resource for bioinformatic tools and services. The major NCBI database is GenBank, where all publicly available DNA sequences are stored. GenBank can be accessed in different ways: *via* the "nucleotide database" or by aligning sequences using the Basic local Alignment Search Tool (BLAST).

BLAST can be used to find region of similarity between sequences. This program compares nucleotide or protein sequences and calculates the statistically significant of matches (Figure 3).

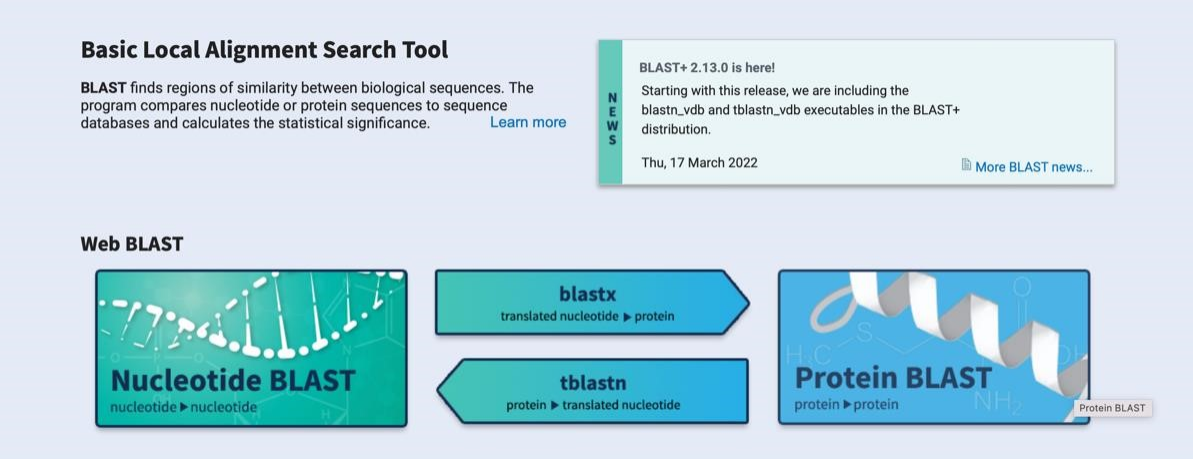

Figure 22. A look at the blast webserver.

BLAST can be found in different flavours:

BLASTn (nucleotide BLAST). It compares one or more nucleotide query sequences to a subject nucleotide sequence or a database of nucleotide sequences.

BLASTx (translated nucleotide sequence searched against protein sequences). It compares a nucleotide query sequence that is translated in six reading frames,

against a database of protein sequences that translates the query nucleotide sequence in all six reading frames.

tBLASTn (protein sequence searched against translated nucleotide sequences). It compares a protein query sequence against the six-frame translation of a database of nucleotide sequences.

BLASTp (Protein BLAST). It compares one or more protein query sequences to subject protein sequence or a database of protein query sequences for trying to identify a protein.

For the purpose of this work, I retrieved the amino acidic sequence of eIF5A2 from the paper of Tong et al. (Proteins 75(4):1040-52009) and I used BLASTp to obtain the FASTA format of this protein isoform. As reported in Figure 23, the amino acid sequence of eIF5A2 is stored in the NCBI protein database under the NCBI Identifier NP_065123.

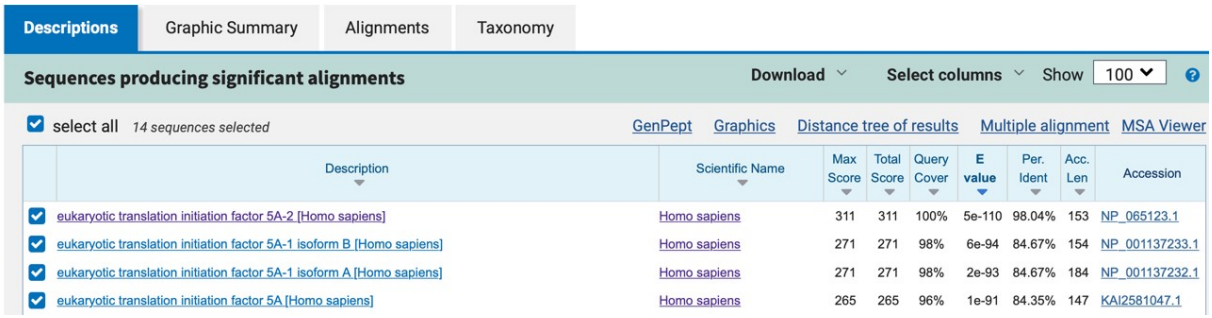

Figure 23. BLASTp result

Structural Prediction of eIF5A2 employing AlphaFold2

Since no PDB structure is available for eIF5A2 to obtain its 3D structure I employed AlphaFold2 structure prediction software. This innovative software,

which aims to solve protein folding, is based on an artificial intelligence program developed by deep Mind which performs prediction of protein structures.

For predicting eIF5A2 3D structure I followed a five-step workflow.

First Step. AlphaFold2 is provided with the amino acid sequence of eIF5A2 (Figure 5). The key assumption for protein folding is that all the information necessary to do it can be acquired from the amino acid sequence.

Second Step: The data pre-processing is carried out to generate the backbone atoms' random coordinates from the amino acid sequence.

Third Step: AlphaFold2 uses multiple sequence alignment (MSA) to look for templates in structure databases based on sequence similarity. At this point, the software constructs an initial representation of the structure starting from a pairwise alignment between the query (the provided amino acidic sequence) and the templates.

Fourth Step. The MSA and the pairwise structures alignment are passed through a deep learning model called “evoformer”, which has 48 blocks of neural networks to exchange information between the amino acid sequences and the pairwise structural characterization of the geometry. This helps to refine the model over several iterations. Then, another deep learning model called “structure model”, optimize the previously obtained structures.

Fifth Step. The result is a refined folded protein structure with the corresponding coordinates for each atom.

Results

3.1. Amplification of the insert(eIF5A) and DHS by PCR and purification from the agarose gel.

The eIF5A open reading frame is contained within the mammalian pcDNA 3.1 vector that cannot be expressed in *E. coli* (as shown figure 24). Therefore, I amplified the insert via PCR using LIC primers specific for eIF5A. The amplification product was loaded onto an agarose gel to verify the size of the amplicon.

As shown in the figure 24, the PCR reaction amplified a DNA fragment of the expected size (465 bp). Which, I checked with standard. Now, I can proceed for the purification of the insert and quantification because I am sure that is my desired insert.

Figure 24. amplification of the eIF5A ORF. Ladder: 3 µl of GeneRuler" 1 kb DNA ladder. Lanes 2 and 3 are corresponded fragments of eIF5A.

The ORF coding for DHS was obtained via RT-PCR on a total RNA sample from Hek293 cells (Section 2.2). Following the manufacturer's protocol, the total RNA

was extracted from 4 million cells; the procedure yielded 100 µl of total RNA in a concentration of 400 ng/µl, roughly 10 µg RNA per million cells.

The DHS ORF was then amplified by PCR using LIC primers. The size of the amplicon was assessed by electrophoresis on a 1% agarose gel, as shown figure 25. The gel shows a marked band in the expected size (1026 bp). The two bands that are observed and are circled with rectangle are the unbound primers.

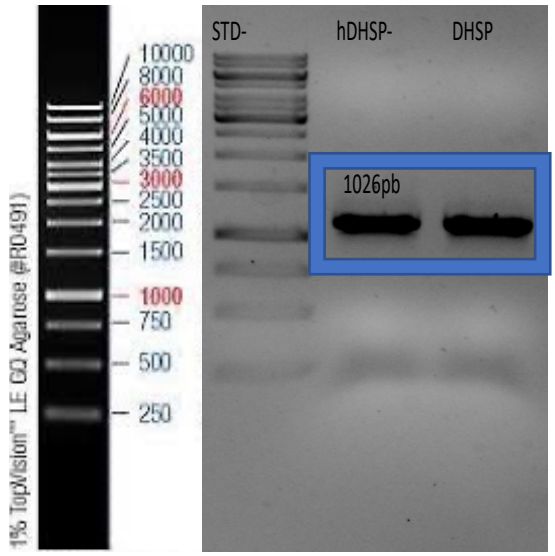

Figure 25. Amplification of DHS total RNA. Std: *3 µl of GeneRuler" 1 kb DNA ladder*

3.2 pMCSG7 Vector linearization

Vector linearization is the first important step in Ligation Independent Cloning, because it defines the site in which the insert will be incorporated. A blunt linearization also provides symmetrical DNA extremities accessible to the T4 DNA polymerase (see also Section 2.5). To obtain a suitable amount of linearized vector, 1 µg of plasmid was incubated with the SspI restriction enzyme. The extent of linearization was assessed by electrophoresis on a 0.8% agarose gel

(figure 26). The gel showed a single band in the expected size (5286 bp), indicating that the vector was completely linearized. Now, it is ready to be purified and quantified because I'm sure the plasmid has been linearized with restriction enzymes and I can proceed for ligation independent cloning.

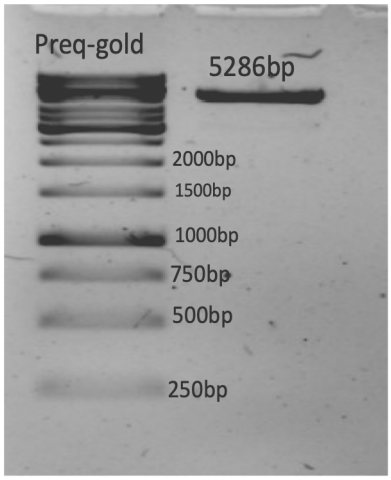

Figure 26. Vector linearization with SspI restriction enzyme on a 0.8 % agarose gel*). As shown in the figure a single band in the expected size.*

3.3 Extraction of the insert DHS and eIF5A from agarose gel.

Extraction of the DNA bands from agarose gel allows to isolate DNA fragments based on size. This is a commonly used technique for the cloning of desired inserts.

For extraction of the insert DHS and eIF5A the QIAquick Gel Cat N° 2804 kit was used. This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard purification or agarose gels (See section 2.1 for details)

After elution of the DNA fragments, the concentration was determined at the Nanodrop spectrophotometer.

The concentration of the eIF5A DNA was 314, 515 ng/µl, while the concentration of the DHS DNA was 12, 583 ng/ µl.

3.4. PCR colony screening of DHS and eIF5A transformants.

The inserts and vector for ligation independent cloning were treated with T4 DNA polymerase and annealed according to the standard procedure described in section 2.1.2 and the product of the reaction was used to transform GC-5 *E. coli* competent cells. As mentioned in Section 2.7, numerous transformants were obtained for DHS, while only two colonies were obtained for the eIF5A construct. To verify he presence of the inserts in the pMCSG7 plasmid constructs, colony PCR was performed from randomly picked colonies on the DHS plate and both colonies from the eIF5A plate. The results were analyzed by agarose gel electrophoresis as shown in figures 27 and 28.

Three out of the 10 colonies tested from the DHS plate were positive for the presence of the insert, whereas both colonies, 5A1 and 5A2, from the eIF5A plate showed an insert in the correct size. These colonies were used to start a large scale expression and purification of the proteins of interest from BL21(DE3) pLysS *E. coli* cells.

GEL A

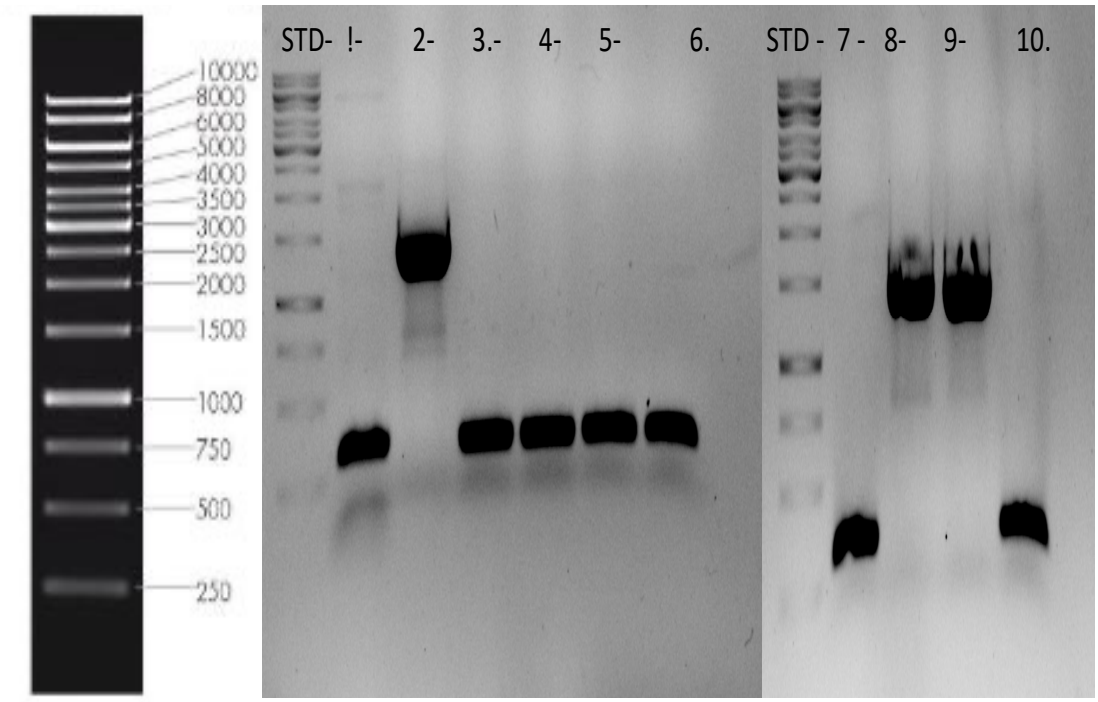

Figure 27. Colony PCR screening for the DHS gene insert. Colonies 2, 8 and 9 produced a DNA amplicon in the expected size. Std: PeqGold 1kb DNA ladder.

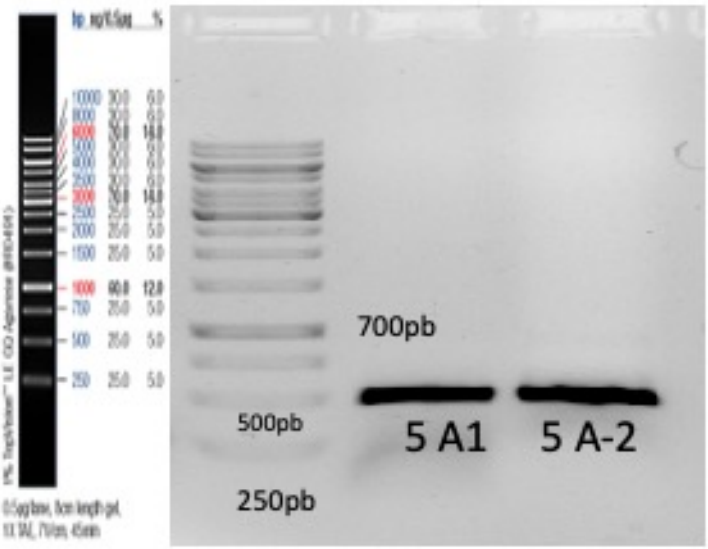

Figure 28. Colony PCR screening for the eIF5A gene insert. Both colonies produced a DNA amplicon in the expected size. Std: PeqGold 1kb DNA ladder (465 pb)

3.5 Expression of eIF5Aand DHS on large scale

In order to express the recombinant proteins, the pMCSG7 + DHS and pMCSG7 + eIF5A were first isolated from *E. coli* GC-5 cells and used to transform the *E. coli* expression strain BL21 (DE3) pLysS. For the DHS construct, the plasmids obtained from two of the positive colonies (colony 8 and 9) were used to transform BL21(DE3) pLysS cells, in order to screen for the best protein-expressing strain. The cells were grown in 1l liquid LB medium and induced with 0.5 mM IPTG as described in paragraph 2.1.1. To check whether protein overexpression occurred, pre- and post-induction samples were loaded onto a SDS-PAGE gel (Figure 29). Concerning DHS expression, a marked band corresponding to the size of the protein (41 kDa) appeared in the colony 8 post-induction sample, whereas only a faint band appeared in the colony 9 post-induction sample. After induction, we obtained 0, 98 g of cells from colony eight and 1,26 g from colony nine cells.

Conversely, despite a band corresponding to the size of His-tagged eIF5A appeared in the post-induction sample, we decided to unambiguously confirm the identity of the protein by Western Blot using an anti-Histidine tag antibody.

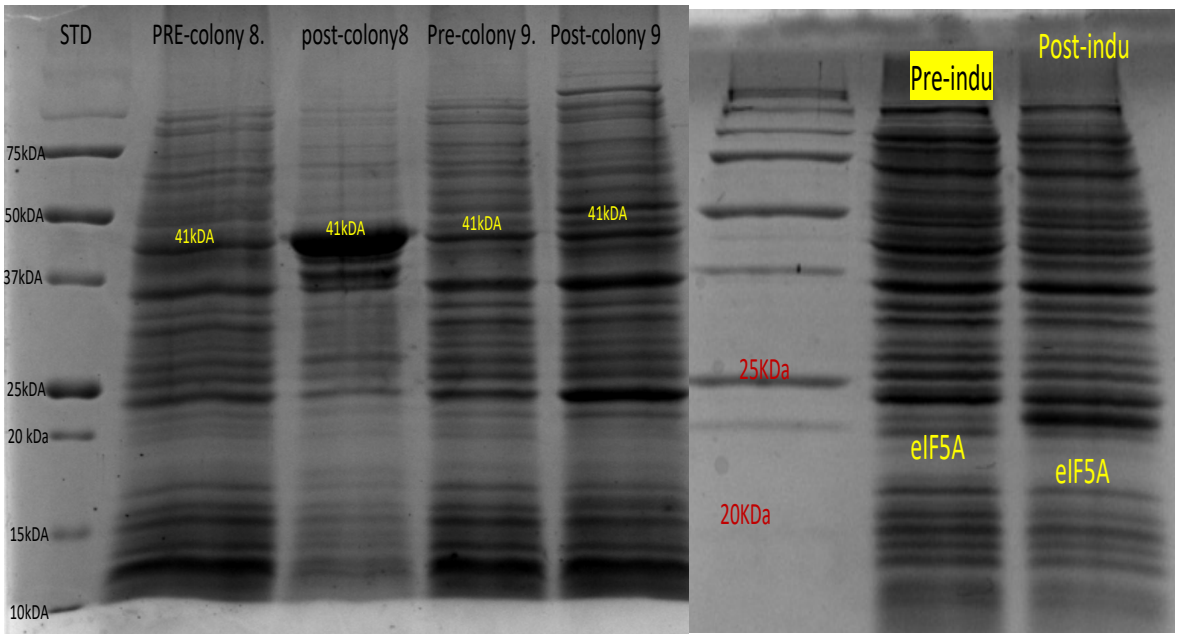

Figure 29. Analysis of protein expression levels by SDS-PAGE. The choice of acrylamide concentration depends on the size of the protein. On the left, the pre and post-induction samples from the DHS-expressing strains are shown, while on the right side the pre and post-induction samples from the eIF5A. eIF5A strain are shown..

3.6 Western Blot Using Ant-Histidine antibody for eIF5A detection.

Western Blotting is a technique used to detect a specific protein in a heterogeneous sample. This technique allows to detect with certainty the presence of the protein of interest because it uses specific antibodies (See paragraph 2.1.8) The results of the Western Blot showed that the band corresponding to the size of eIF5A bears a 6X Histidine tag; this confirms that eIF5Ais correctly expressed in the BL21 (DE3) + pLysS cells (Figure 30).

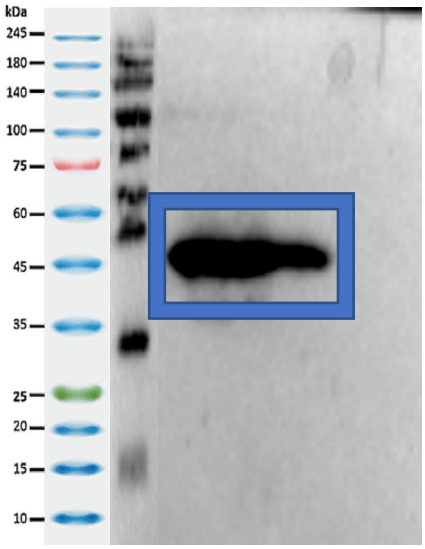

Figure 30. Western Blot analysis of a eIF5A post-induction cell sample. Lane 1: Standard (Protein Marker: PM2610). Lane :2 eIF5A N-terminal His tagged in E. coli BL21 (DE3)+ pLysS cells.. The protein of interest is expressed because it corresponds the correct size as shown with the blue rectangle.

3.7 Large-scale purification of eIF5A and DHS.

The cells obtained after induction of the expression of eIF5A and DHS were lysed in their respective Lysis Buffer, ruptured by sonication and centrifuged to obtained clear total lysate for further affinity chromatography purification of the recombinant proteins (See Section 2.1.6) for details on the purification protocol) Samples were collected throughout the purification procedure for SDS-PAGE screening (Figures 31-33) to identify the fractions containing the most proteins. eIF5A is a soluble protein and is found in all soluble fractions throughout the purification protocol. The eluted protein was pure and did not necessitate further purification steps.

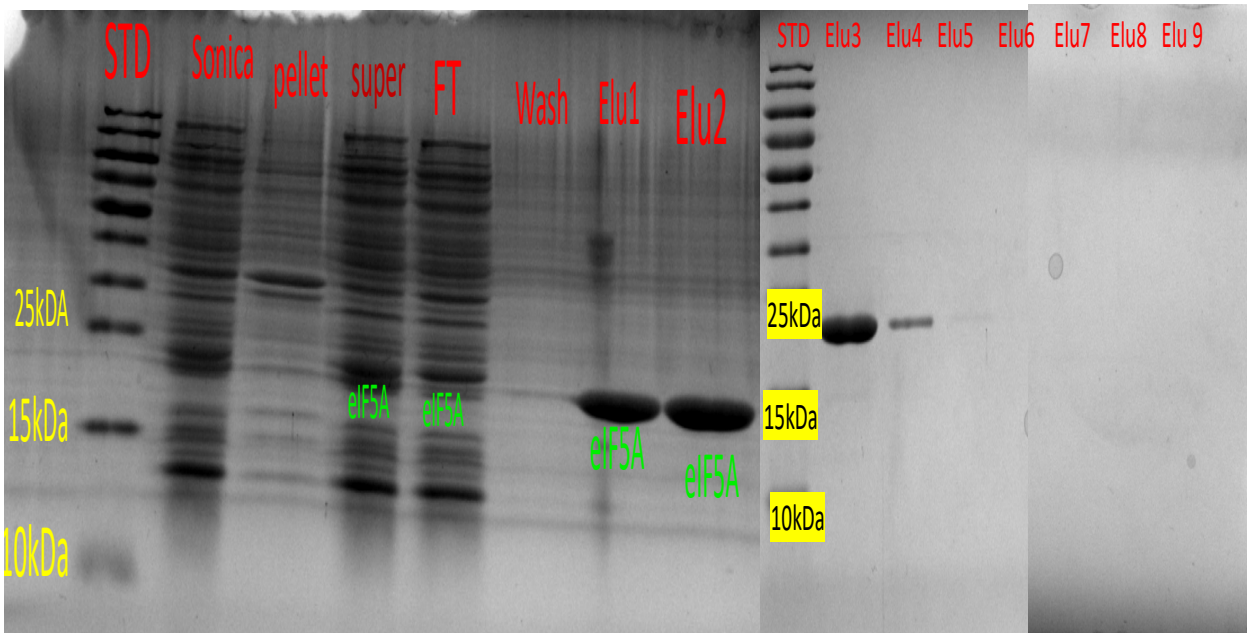

Figure 31. Purification of recombinant eIF5A. Proteins were separated by SDS-PAGE and stained with Coomassie Blue. Starting from the left: Standard – Sonication-pellet-supernatant-Flowthrough – wash- Elution fraction.

Regarding DHS, two different colonies were expressed: in the samples obtained from the expression in colony eight, most protein was found the first pellet, however, the soluble protein was still more abundant than what was observed in the samples obtained from colony nine. For this reason, we decided to purify the protein from the samples from colony eight.

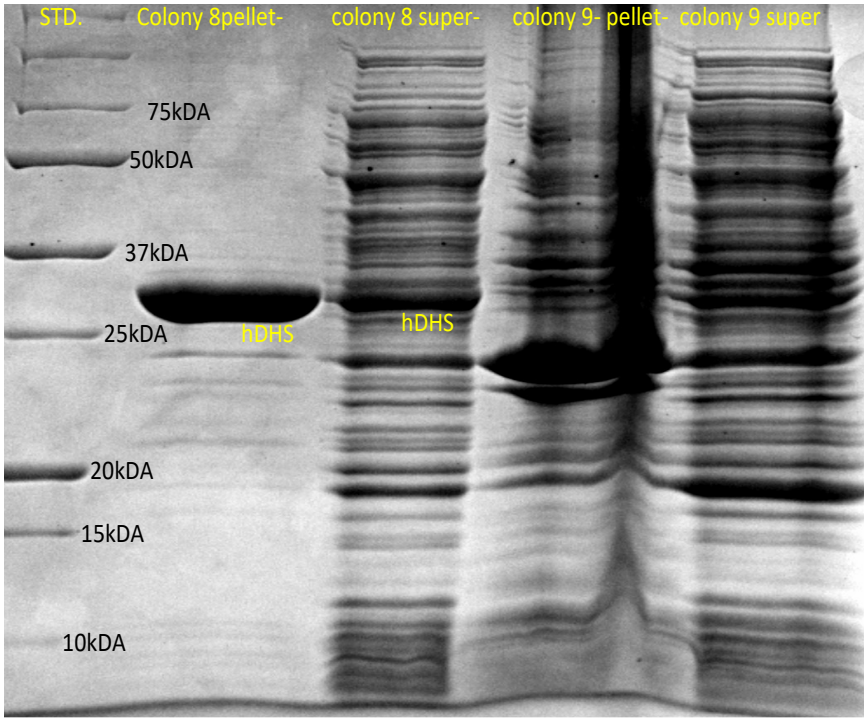

Figure 32. Crude cell lysates and first pellet from the purification of DHS from colony 8 and 9. From the left: Standard- colony 8(pellet- supernatant), colony nine (pellet- supernatant).

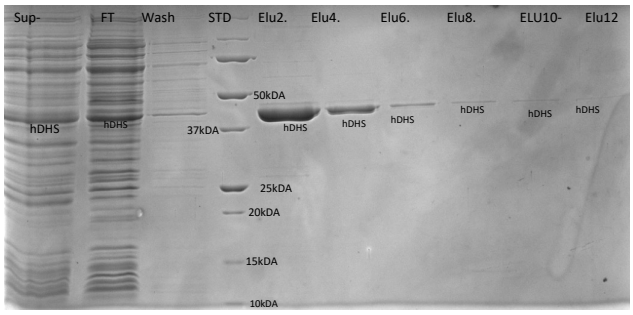

Figure 33. production and purification of DHS. Proteins were separated by SDS-PAGE gel 13 %. Starting from the left: Supernatant- Flowthrough- Wash-Standard- Fraction from 2 to 12. First observation: All most protein is present on the flowthrough; therefore, it must be eluted. Second observation: I have the protein up to 12 fractions, but its concentration decreases by increasing the elution.

The fractions containing the largest portion of the purified eIF5A, and DHS were pooled buffer-exchanged into their respective storage buffer.

Because of the tetrameric structure of DHS, we decided to cleave the N-terminal 6X Histidine tag from the recombinant protein, which might interfere with the correct folding of the enzyme.

3.8. Enzymatic cleavage of the polyhistidine-tage

The 6X Histidine tag was removed from the DHS protein using the TEV protease (see Section 2.2.1); to verify that the digestion has taken place correctly, we loaded pre- and post-cleavage samples on a SDS-PAGE. As it can be seen in Figure 34, the protein was completely cleaved and migrates as a lower band compared to the His-tagged DHS.

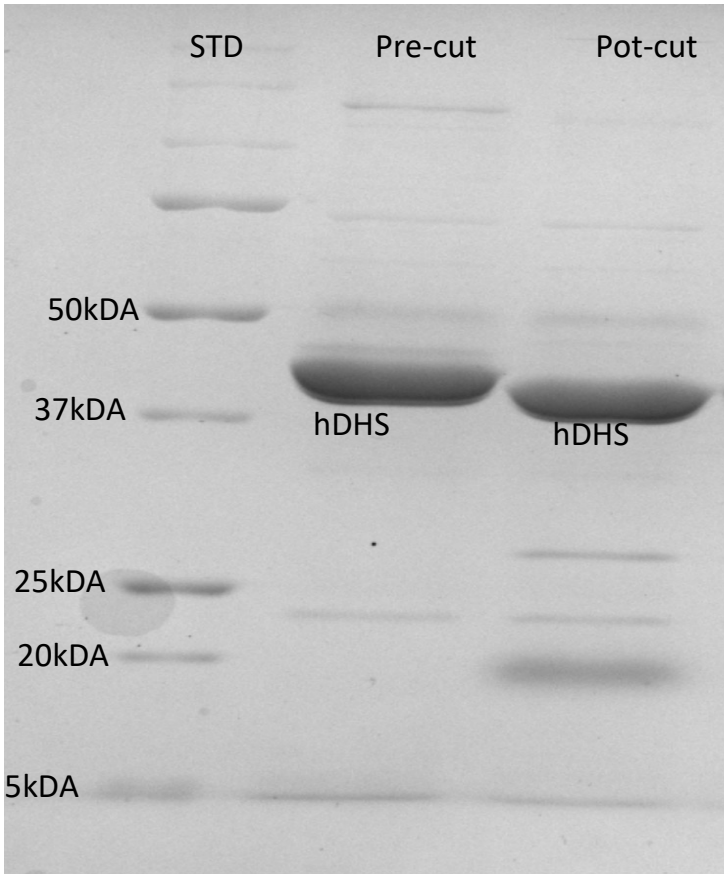

Figure 34. TEV cleavage of DHS. The difference in size between the pre- and post-cleavage samples indicates that the TEV enzyme processed the recombinant protein to completion.

3.9. Gel electrophoresis under native conditions to detect the eIF5A-DHS complex formation.

Native gel electrophoresis is one of the most powerful techniques for studying the composition and structure of native proteins, because both the conformation and the biological activity of proteins remain intact during this technique. This technique allows separation based on the molecular weight, structure, and charge of the protein. It is a technique that allows the separation of single proteins and protein complexes in a non-denaturing system on the basis of their isoelectric point. The characteristics of this electrophoretic run allow the study of the

interaction of protein complexes. In this study, the between eIF5A and DHS was tested. It has been demonstrated that deoxyhypusinated eIF5A migrates slower than non-hypusinated protein on a native gel. Furthermore, the eIF5A-DHS complex can be separated from the DHS tetramer by native PAGE. This prompted us to verify the activity of the purified recombinant DHS, by se(Lee and Park 2000) thing up an *in vitro* reaction in the presence of NAD^+ and spermidine.

The samples were then loaded on a native PAGE gel (Figure 35). The results of this experiment showed that the interaction between eIF5A and DHS occurs and depends on the presence of the NAD^+ cofactor and the donor spermidine; in the absence of either component no complex was observed. Notably, in the presence of both NAD^+ and spermidine the eIF5A band shifted upwards, indicating that deoxyhypusination occurs correctly.

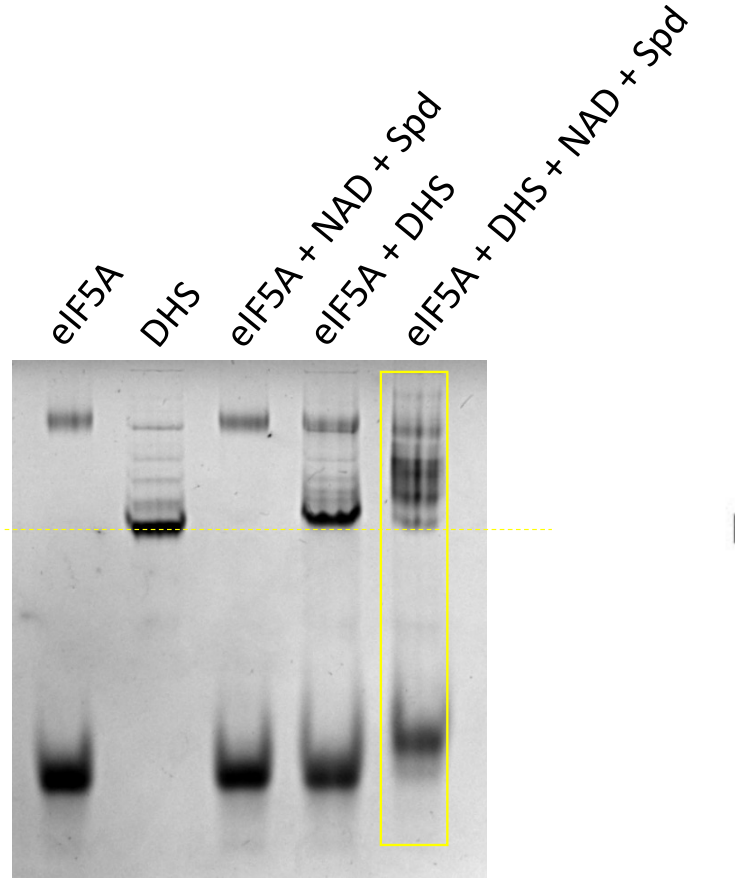

Figure 35. Native PAGE analysis of the eIF5A-DHS complex formation. The gel shows that deoxyhypusinated eIF5A migrates slower than non-hypusinated protein, as highlighted with the yellow rectangle.

Next, we wanted to determine if an antibody raised against Hypusine would recognize deoxyhypusinated eIF5A; this would allow us to screen potential novel inhibitors of the hypusination process by using very small amounts of recombinant proteins. The samples prepared for the native PAGE experiments were loaded on SDS-PAGE gels and analyzed by Western Blot using an anti-Histidine and an anti-Hypusine primary antibodies (Figure 36). The results show

that deoxyhypusinated eIF5A is efficiently recognized by the anti-Hypusine antibody. Now, we can conclude that the recombinant DHS enzyme is active and that we can use Western Blot to screen for DHS inhibitors.

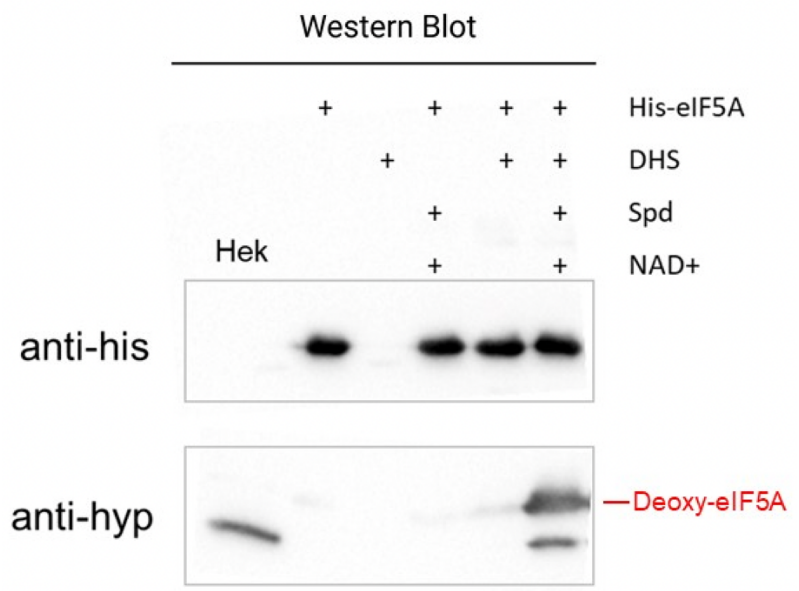

Figure 36. Testing DHS activity by Western Blot, Hek: 15 ug of Hek-293T cell extract (positive control for anti-Hypusine, negative control for anti-Histidine)

3.1.1 Structure Prediction of eIF5A2 using Alphafold.

Figure 37 shows the most important result obtained using AlphaFold2. It is evident that the central part of eIF5A2_is covered by more than 7000 amino acidic sequences with high sequence identity. As we expected, the terminal part of the query protein has less coverage with a lower level of sequence identity.

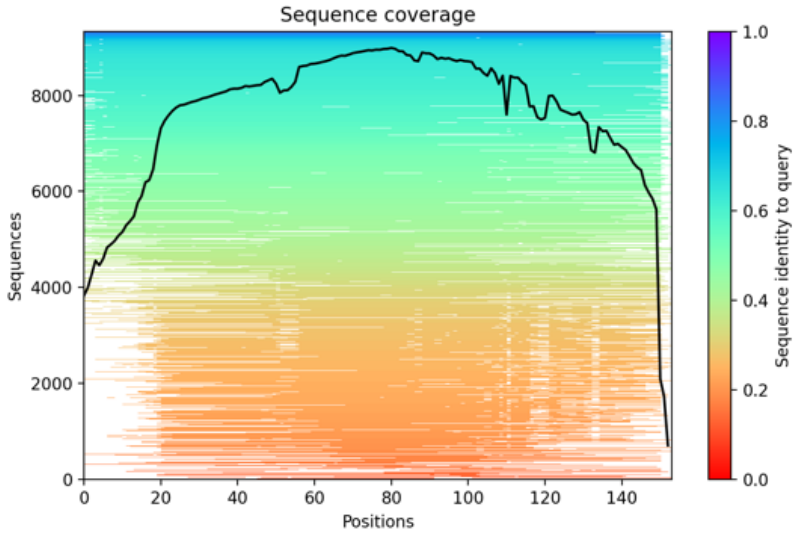

Figure 37. AlphaFold2 result for amino acid sequence coverage per position.

Overall, we have obtained a good result. For subsequent analysis, the best structure for eIF5A2 obtained through AlphaFold2 was used. The 3D ribbon structure of eIF5A2 illustrated in Figure 42 is very similar to that of eIF5A1.

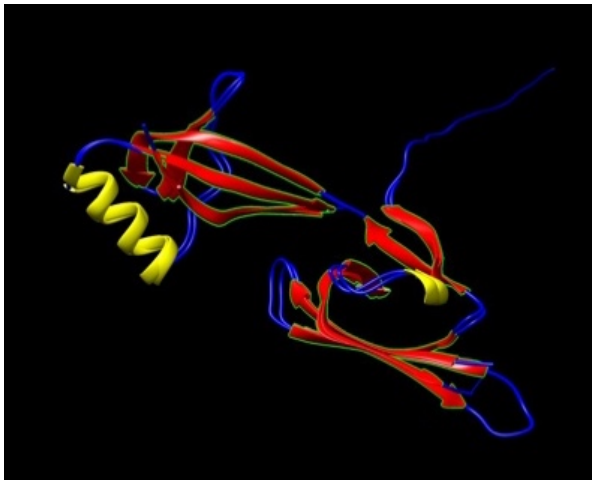

Figure 38. eIF5A2 3D structure in ribbon style. The secondary elements are depicted with different colours: β-strands are in red, α-helix are in yellow, and loop are in blue

3.1.2 Structural comparisons of eIF5A isoforms

To understand the fine molecular differences between the two different isoforms of eIF5A we performed structural comparison. We loaded on Chimera software the AlphaFold2 predicted structure of eIF5A2 and the 3CPF PDB structure of eIF5A1. Using the Chimera pre-installed Matchmaker software, we superimposed both structures using the Needleman-Wunsch global alignment algorithm. The superimposition result is shown in Figure 39. The two structures are basically identical even though the AlphaFold2 predicted structure presents a long N-terminal unstructured part. This latter features arises from the inaccuracy of AlphaFold2 to predict this segment (as previously seen in Figure 39). The root mean square deviation (RMSD) between the two structure is of 1.40 Å, demostrating the high similarity between the two eIF5A structures.

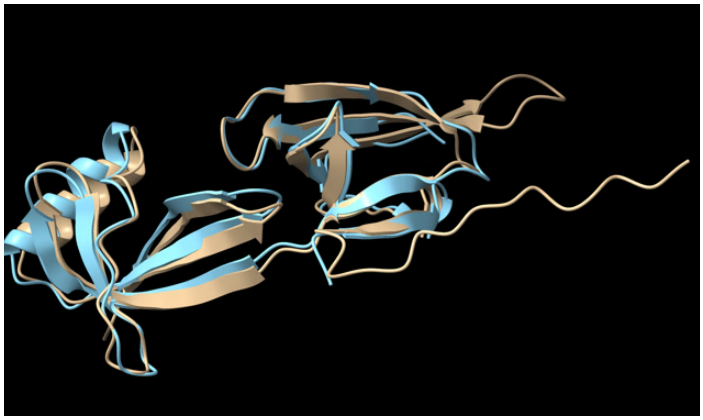

Figure 39. Ribbon representation of the superimposition between the two different isoforms of eIF5A. In the picture eIF5A1 and eIF5A$_2$ are depicted in cyan and tan, respectively.

Of course, all the structural discrepancies are derived from the differences in the amino acidic sequences of the two isoforms (Figure 40). The reported sequence identity is indeed 83.33% between the crystallized isoform 1 and the predicted isoform 2 of eIF5A.

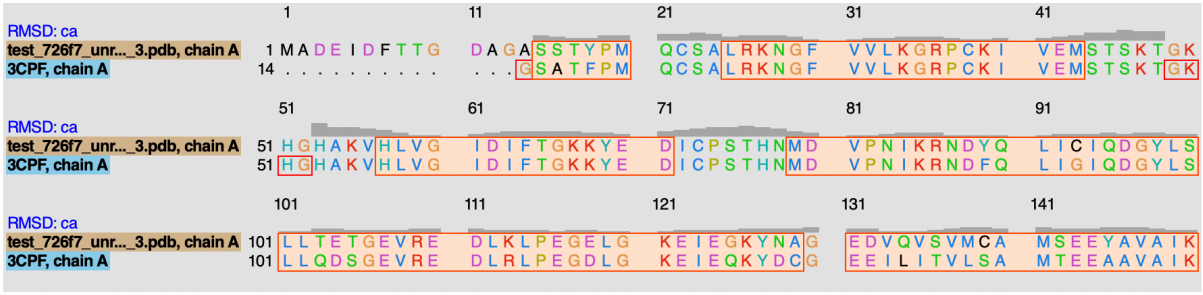

Figure 40. Global Alignment between the predicted eIF5A$_2$ and the crystallized eIF5A isoforms.

Using a computational study that included searching, use of prediction software and molecular programs, we were able to construct a structure for the second isoform of the translation factor eIF5A for which a PDB structure is missing. As expected on the basis of the high level of sequence identity, the predicted structure of eIF5A2 is very similar to eIF5A1 with the exception of its N-terminal end

4. Discussion.

The eukaryotic translation factor is a small acidic protein, highly conserved in all kingdoms of life, with homologues in Archaea (aIF5A) and in Bacteria. This initiation factor was shown to be essential in Eukarya and Archaea but not in

Bacteria. The eukaryotic proteins perform functions at different levels of translation, but the main task is the promotion of the synthesis of proteins containing consecutive proline residues. In fact, eIF5A is a protein mainly involved in the elongation phase of protein synthesis and its function depends on the unique post-translation modification of a conserved lysine residue into Hypusine. This reaction is carried out via two consecutive enzyme reactions, the first one catalyzed by DHS and the second one by DOHH. DHS is conserved between Eukarya and Archaea domains. Hypusination consists in the conversion of a conserved lysine residue into a nonstandard amino acid using spermidine as a donor. Spermidine is a polyamine synthesized from putrescine, which derives from the urea cycle by the catalytic decarboxylation of ornithine.

As many other translation factors, eIF5A is highly involved in different pathologies such as cancer, HIV-1 infection. Diabetes and malaria. Because of this, eIF5A together with the enzymes involved in the hypusination pathway are considered very interesting therapeutic targets leading researchers to search for new inhibitor molecules. As mentioned above, the polyamine GC7 is the most powerful inhibitor of DHS known to date. This spermidine analogue can block hypusination, but it is not sufficiently selective and specific to be used in clinical practice.

Based on this premise, the aim of this thesis was to produce recombinant eIF5A and DHS proteins to develop an *in vitro* screening assay for potential DHS inhibitors. These proteins have been produced using genetic engineering

techniques such as cloning, expression, and purification from BL21 (DE3) pLysS cells. The results presented demonstrate that the recombinant DHS enzyme is active and efficiently modifies the recombinant eIF5A.

In the protein data bank (PBD) only the structure of the eIF5A1 isoform is present; for this reason, and because eIF5A2 is involved in a large number of cancer types, I predicted the structure of eIF5A2 using the powerful Alphafold tool.

These results of this Thesis will be useful to everyone who wants to produce recombinant eIF5A and DHS for biochemical studies.

4.1Future perspectives

Considering that GC7 is not sufficiently selective, need higher concentration and there are side effects for patients. Starting from this consideration and driven by the idea that knowing the molecular determinants of the GC7 binding in homologous proteins can open new frontiers for design of inhibitors specific for hypusination, my colleague Beatrice Campanelli identified a promising inhibitor related to GC7, by studying the molecular dynamics of its interaction with DHS. The *in vitro* experiments shown in this thesis can be easily applied to the study of novel molecules, because they require very little amount of purified proteins.

The same protocols that were used to clone, express and purify eIF5A can be used to produce recombinant eIF5A2. Because of its role in cancer, it is crucial to determine its 3D structure. Furthermore, the structure of the eIF5A-DHS complex can be solved by X-ray crystallography, paving the way to molecular dynamics studies to better understand the interaction between DHS and its inhibitors.

Bibliography

Balibar, Carl J., Dorothy Iwanowicz, and Charles R. Dean. 2013. 'Elongation Factor P Is Dispensable in Escherichia Coli and Pseudomonas Aeruginosa'. *Current Microbiology* 67(3): 293–99.PMID: 23591475 DOI: 10.1007/s00284-013-0363-0

Bevec, Dorian et al. 1996. 'Inhibition of HIV-1 Replication in Lymphocytes by Mutants of the Rev Cofactor EIF-5A'. *Science* 271(5257): 1858–60. PMID: 8596953 DOI: 10.1126/science.271.5257.1858

Bult, Carol J. et al. 1996. 'Complete Genome Sequence of the Methanogenic Archaeon, ***Methanococcus Jannaschii*** '. *Science* 273(5278): 1058–73. PMID: 8688087DOI: 10.1126/science.273.5278.1058

Caraglia, M. et al. 2013. 'EIF5A Isoforms and Cancer: Two Brothers for Two Functions?' *Amino Acids* 44(1): 103–9. doi:10.1007/s00726-011-1182-x.

Clement, Paul M. J. et al. 2003. 'Identification and Characterization of Eukaryotic Initiation Factor 5A-2: Human EIF5A-2'. *European Journal of Biochemistry* 270(21): 4254–63. https://doi.org/10.1046/j.1432-1033.2003.03806.x

D'Agostino, Mattia et al. 2020. 'Insights Into the Binding Mechanism of GC7 to Deoxyhypusine Synthase in Sulfolobus Solfataricus: A Thermophilic Model for the Design of New Hypusination Inhibitors'. *Frontiers in Chemistry* 8: 609942. https://doi.org/10.3389/fchem.2020.609942

Dias, Camila Arnaldo Olhê, Wanius Garcia, Cleslei Fernando Zanelli, and Sandro Roberto Valentini. 2013. 'EIF5A Dimerizes Not Only in Vitro but Also in Vivo and Its Molecular Envelope Is Similar to the EF-P Monomer'. *Amino Acids* 44(2): 631–44. https://doi.org/10.1007/s00726-012-1387-7

Gentz, Petra M., Gregory L. Blatch, and Rosemary A. Dorrington. 2009. 'Dimerization of the Yeast Eukaryotic Translation Initiation Factor 5A Requires Hypusine and Is RNA Dependent: Dimerization of Yeast Eukaryotic Translation

Initiation Factor 5A'. *FEBS Journal* 276(3): 695–706. https://doi.org/10.1111/j.1742-4658.2008.06817.x

Gradia, Scott D. et al. 2017. 'MacroBac: New Technologies for Robust and Efficient Large-Scale Production of Recombinant Multiprotein Complexes'. In *Methods in Enzymology*, Elsevier, 1–26. https://linkinghub.elsevier.com/retrieve/pii/S0076687917300897 (February 8, 2023). DOI: 10.1016/bs.mie.2017.03.008

Gutierrez, Erik et al. 2013. 'EIF5A Promotes Translation of Polyproline Motifs'. *Molecular Cell* 51(1): 35–45. https://doi.org/10.1016/j.molcel.2013.04.021

Hanawa-Suetsugu, Kyoko et al. 2004. 'Crystal Structure of Elongation Factor P from *Thermus Thermophilus* HB8'. *Proceedings of the National Academy of Sciences* 101(26): 9595–9600. https://doi.org/10.1073/pnas.0308667101

Igarashi, Kazuei, and Keiko Kashiwagi. 2018. 'Effects of Polyamines on Protein Synthesis and Growth of Escherichia Coli'. *Journal of Biological Chemistry* 293(48): 18702–9. https://doi.org/10.1074/jbc.TM118.003465

Jakus, J., E.C. Wolff, M.H. Park, and J.E. Folk. 1993. 'Features of the Spermidine-Binding Site of Deoxyhypusine Synthase as Derived from Inhibition Studies. Effective Inhibition by Bis- and Mono-Guanylated Diamines and Polyamines'. *Journal of Biological Chemistry* 268(18): 13151–59. https://doi.org/10.1016/S0021-9258(19)38631-4

Jao, David Li-En, and Kuang Yu Chen. 2006. 'Tandem Affinity Purification Revealed the Hypusine-Dependent Binding of Eukaryotic Initiation Factor 5A to the Translating 80S Ribosomal Complex'. *Journal of Cellular Biochemistry* 97(3): 583–98. https://doi.org/10.1002/jcb.20658

Jenkins, Zandra A., Petra G. Hååg, and Hans E. Johansson. 2001. 'Human EIF5A2 on Chromosome 3q25–Q27 Is a Phylogenetically Conserved Vertebrate Variant of Eukaryotic Translation Initiation Factor 5A with Tissue-Specific Expression'. *Genomics* 71(1): 101–9. https://doi.org/10.1006/geno.2000.6418

Joe, Young Ae, Edith C. Wolff, Young Bok Lee, and Myung Hee Park. 1997. 'Enzyme-Substrate Intermediate at a Specific Lysine Residue Is Required for Deoxyhypusine Synthesis'. *Journal of Biological Chemistry* 272(51): 32679–85. https://doi.org/10.1074/jbc.272.51.32679

Kang, H.A., and J.W. Hershey. 1994. ‘Effect of Initiation Factor EIF-5A Depletion on Protein Synthesis and Proliferation of Saccharomyces Cerevisiae.’ *Journal of Biological Chemistry* 269(6): 3934–40. https://doi.org/10.1016/S0021-9258(17)41723-6

Kemper, W. M., K. W. Berry, and W. C. Merrick. 1976. ‘Purification and Properties of Rabbit Reticulocyte Protein Synthesis Initiation Factors M2Balpha and M2Bbeta’. *The Journal of Biological Chemistry* 251(18): 5551–57.

Lee, Chang Hoon, and Myung Hee Park. 2000. ‘Human Deoxyhypusine Synthase: Interrelationship between Binding of NAD and Substrates’. *Biochemical Journal* 352(3): 851–57. https://doi.org/10.1016/S0021-9258(17)33095-8

Liao, Der-Ing, Edith C Wolff, Myung Hee Park, and David R Davies. 1998. ‘Crystal Structure of the NAD Complex of Human Deoxyhypusine Synthase: An Enzyme with a Ball-and-Chain Mechanism for Blocking the Active Site’. *Structure* 6(1): 23–35. https://doi.org/10.1016/S0969-2126(98)00004-5

Liu, Yi Ping, Martin Nemeroff, Yong Ping Yan, and Kuang Yu Chen. 1997. ‘Interaction of Eukaryotic Initiation Factor 5A with the Human

Immunodeficiency Virus Type 1 Rev Response Element RNA and U6 SnRNA Requires Deoxyhypusine or Hypusine Modification'. *Neurosignals* 6(3): 166–74. https://doi.org/10.1159/000109123

Maier, Bernhard et al. 2010. 'The Unique Hypusine Modification of EIF5A Promotes Islet β Cell Inflammation and Dysfunction in Mice'. *Journal of Clinical Investigation* 120(6): 2156–70. https://doi.org/10.1172/JCI38924

Manjunath, Hema et al. 2019. 'Suppression of Ribosomal Pausing by EIF5A Is Necessary to Maintain the Fidelity of Start Codon Selection'. *Cell Reports* 29(10): 3134-3146.e6. https://doi.org/10.1016/j.celrep.2019.10.129

Melnikov, Sergey et al. 2016. 'Crystal Structure of Hypusine-Containing Translation Factor EIF5A Bound to a Rotated Eukaryotic Ribosome'. *Journal of Molecular Biology* 428(18): 3570–76. https://doi.org/10.1016/j.jmb.2016.05.011

Park, J. H. et al. 2011. 'Production of Active Recombinant EIF5A: Reconstitution in E.Coli of Eukaryotic Hypusine Modification of EIF5A by Its Coexpression with Modifying Enzymes'. *Protein Engineering Design and Selection* 24(3): 301–9. https://doi.org/10.1093/protein/gzq110

Park, Jong-Hwan, Edith C. Wolff, J.E. Folk, and Myung Hee Park. 2003. 'Reversal of the Deoxyhypusine Synthesis Reaction'. *Journal of Biological Chemistry* 278(35): 32683–91. 10.1074/jbc.M304247200

Park, Myung Hee, and Edith C. Wolff. 2018a. 'Hypusine, a Polyamine-Derived Amino Acid Critical for Eukaryotic Translation'. *Journal of Biological Chemistry* 293(48): 18710–18. https://doi.org/10.1074/jbc. https://doi.org/10.1074/jbc.TM118.003341

. 2018b. 'Hypusine, a Polyamine-Derived Amino Acid Critical for Eukaryotic Translation'. *Journal of Biological Chemistry* 293(48): 18710–18.

Saini, Preeti, Daniel E. Eyler, Rachel Green, and Thomas E. Dever. 2009. 'Hypusine-Containing Protein EIF5A Promotes Translation Elongation'. *Nature* 459(7243): 118–21. https://doi.org/10.1038/nature08034

Schmidt, Christian et al. 2016. 'Structure of the Hypusinylated Eukaryotic Translation Factor EIF-5A Bound to the Ribosome'. *Nucleic Acids Research* 44(4): 1944–51. https://doi.org/10.1093/nar/gkv1517

Schnier, J et al. 1991. 'Translation Initiation Factor 5A and Its Hypusine Modification Are Essential for Cell Viability in the Yeast Saccharomyces

Cerevisiae'. *Molecular and Cellular Biology* 11(6): 3105–14. https://doi.org/10.1128/mcb.11.6.3105-3114.1991

Schuller, Anthony P. et al. 2017. 'EIF5A Functions Globally in Translation Elongation and Termination'. *Molecular Cell* 66(2): 194-205.e5. https://doi.org/10.1016/j.molcel.2017.03.003

Shiba, Tetsuo et al. 1971. 'Hypusine, a New Amino Acid Occurring in Bovine Brain'. *Biochimica et Biophysica Acta (BBA) - General Subjects* 244(3): 523–31. https://doi.org/10.1016/0304-4165(71)90069-9

Tong, Yufeng et al. 2009. 'Crystal Structure of Human EIF5A1: Insight into https Functional Similarity of Human EIF5A1 and EIF5A2'. *Proteins* 75(4): 1040–45. https://doi.org/10.1002/prot.22378

Umland, Timothy C., Edith C. Wolff, Myung Hee Park, and David R. Davies. 2004. 'A New Crystal Structure of Deoxyhypusine Synthase Reveals the Configuration of the Active Enzyme and of an Enzyme·NAD·Inhibitor Ternary Complex'. *Journal of Biological Chemistry* 279(27): 28697–705. https://doi.org/10.1074/jbc.M404095200

Wolff, E. C., K. R. Kang, Y. S. Kim, and M. H. Park. 2007. 'Posttranslational Synthesis of Hypusine: Evolutionary Progression and Specificity of the Hypusine

Modification'. *Amino Acids* 33(2): 341–50. https://doi.org/10.1007/s00726-007-0525-0

Wolff, E C, M H Park, and J E Folk. 1990. 'Cleavage of Spermidine as the First Step in Deoxyhypusine Synthesis. The Role of NAD.' *Journal of Biological Chemistry* 265(9): 4793–99. https://doi.org/10.1016/S0021-9258(19)34042-6