Universidade de Aveiro Departamento de Ciências Médicas
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Estarão as proteínas associadas à tradução presentes e funcionais no espermatozoide?

Are translation-related proteins present and functionally active in sperm?

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Margarida Fardilha, Professora Auxiliar com Agregação do Departamento de Ciências Médicas e coorientação da Doutora Joana Vieira Silva, Investigadora de Pós-Doutoramento do Instituto de Biomedicina da Universidade de Aveiro (iBiMED); do Instituto de Investigação e Inovação em Saúde (I3S) da Universidade do Porto; e da Unidade para a Investigação Multidisciplinar em Biomedicina (UMIB) da Universidade do Porto.

Dedico este trabalho aos meus pais, Isabel e Victor, e à minha irmã, Mariana.

À minha avó, Augusta, que desde o primeiro momento acreditou em mim.

o júri

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palavras-chave

Tradução, espermatozoide, inibição da tradução, *surface sensing of translation*

Resumo

Os espermatozoides são células haploides altamente diferenciadas originadas a partir de sucessivas divisões mitóticas e meióticas – a espermatogénese. Durante este processo, o espermatozoide sofre alterações estruturais e funcionais a nível nuclear e citoplasmático. Devido a essas alterações, o silenciamento da expressão génica é amplamente aceite nos espermatozoides de mamíferos. Uma nova perspetiva surgiu quando a síntese de novas proteínas foi revelada em espermatozoides em condições capacitantes, gerando discussão sobre um dogma estabelecido.

Assim, este trabalho visa caracterizar e avaliar a atividade translacional em espermatozoides em condições capacitantes. Para alcançar este objetivo foram realizadas abordagens a nível bioinformático e experimental.

A análise bioinformática revelou a presença de 315 proteínas relacionadas com a tradução em espermatozoides. A análise simultânea dos processos biológicos e dos compartimentos celulares de cada proteína levou à identificação de 31 proteínas exclusivamente ligadas à tradução. Complementarmente, a análise da rede de interacção proteína-proteína revelou que estas 315 proteínas estão fortemente conectadas e interrelacionadas. Além disso, a análise de enriquecimento desta rede, para os processos biológicos e para os compartimentos celulares, evidenciou uma forte associação das proteínas com processos relacionados com a tradução e uma localização preferencial em regiões citoplasmáticas e mitocondriais.

Experimentalmente, a técnica de SUnSET estabeleceu de forma inequívoca a existência de atividade translacional através da incorporação de puromicina nas cadeias nascentes de polipéptidos. Foi observada uma diminuição da incorporação de puromicina em condições com inibidores translacionais citoplasmáticos e mitocondriais, o que nos leva a acreditar na coexistência de ambas as formas de tradução no espermatozoide.

Em suma, estes resultados evidenciam a existência de atividade translacional em espermatozoides de mamífero.

Keywords Translation, spermatozoa, inhibition of translation, surface sensing of translation

Abstract

Spermatozoa are highly differentiated haploid cells originated through a sequence of mitotic and meiotic divisions – the spermatogenesis. During this process, spermatozoa undergo major structural and functional changes at nuclear and cytoplasmic levels. Because of these changes, the silencing of gene expression is widely accepted in mammalian spermatozoa. A new perspective emerged when *de novo* protein synthesis was shown to occur in sperm cells under capacitation conditions, raising discussion on an established dogma.

The main objective of this work is to characterize and evaluate the translational activity that may occur in mammalian spermatozoa. To achieve this goal, bioinformatic and experimental approaches were performed.

Bioinformatic analysis revealed the existence of 315 translation-related proteins present in spermatozoa. Simultaneous analysis of the biological processes and cellular compartments associated with each protein led to the identification of 31 translation exclusive-proteins. Additionally, the PPI network analysis revealed that 315 overlapping proteins are strongly connected and related to each other. Also, the enrichment analysis of PPI network, for biological processes and cellular compartment, evidenced a strong association to translation-related processes and a preferential location in cytoplasmic and mitochondrial regions.

The SUnSET technique unequivocally established the existence of translational activity through the incorporation of puromycin into the nascent polypeptide chains. A decrease in puromycin incorporation was observed when using mitochondrial and cytoplasmic translational inhibitors, which leads us to believe in the coexistence of both types of translation in spermatozoa.

Together, these results evidenced the existence of translational activity in mammalian spermatozoa.

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List of Abbreviations, symbols and acronyms

^{35}S	Sulphur isotopes
aa-tRNA	Aminoacyl-tRNA
ABCE1	ATP binding cassette protein E1
A-Site	Aminoacyl site
ATP	Adenosine triphosphate
AUG	Translation initiation codon
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CH	Cycloheximide
CO_2	Carbon dioxide
D-CP	D-chloramphenicol
e.g.	*exemplī grātiā* (example given)
EC	Extracellular compartment
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
eRF	Eukaryotic release factor
E-Site	Exit site
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDR	False Discovery Rate

FSC	Forward scatter
GDP	Guanosine diphosphate
GO	Gene ontology
GTP	Guanosine-5'-triphosphate
i.e.	*id est*
iBiMED	Institute for biomedicine
IF	Insoluble fraction
IM	inner membrane
IP	Immunoprecipitation
kDa	Kilodalton
Met	Methionine
MFI	Mean fluorescence intensity
Min	Minutes
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
MRP	Mitoribosomal proteins
mt-DNA	Mitochondrial DNA
mtEF	Mitochondrial elongation factor
mtIF	Mitochondrial initiation factor
mt-mRNA	Mitochondrial mRNA
mtRF	Mitochondrial release factor
mtRRF	Mitochondrial ribosomal recycling factor
mt-rRNA	Mitochondrial rRNA

mt-tRNA	Mitochondrial tRNA
NC	Negative control
ODF	Outer dense fibres
OM	Outer membrane
OXPHOS	Oxidative phosphorylation system
PABP	Poly-A binding protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
P_i	Inorganic phosphate
PIC	43S pre-initiation complex
PPI	Protein-Protein interaction
P-Site	Peptidyl site
PTC	Peptidyl transferase centre
RNE	Redundant nuclear envelopes
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal RNA
RT	Room temperature
S	Svedberg unit
SCA	Sperm class analyser
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
SF	Soluble fraction

SPM	Sperm preparation medium
SSC	Side scatter
SUnSET	Surface sensing of translation
TBST	Tris-buffered saline with Tween 20
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane
tRNA	Transfer RNA
WR	Working reagent

1 Introduction

1.1 From spermatogenesis to capacitation: the life journey of the spermatozoon

The male reproductive system is responsible for the production of spermatozoa (spermatogenesis) and male sex hormones (steroidogenesis) and allows the delivery of male gametes into the female reproductive tract (1). Testicles, where the spermatozoa production occurs, have an oval shape and are located outside of the body in separate compartments within the scrotum (1). Testicular parenchyma is composed of highly convoluted seminiferous tubules surrounded by interstitial tissue. Histologically, seminiferous tubules are a stratified layer of developing male germ cells (spermatogonia, spermatocytes and spermatids) and sustaining Sertoli cells (figure 1.1) (2).

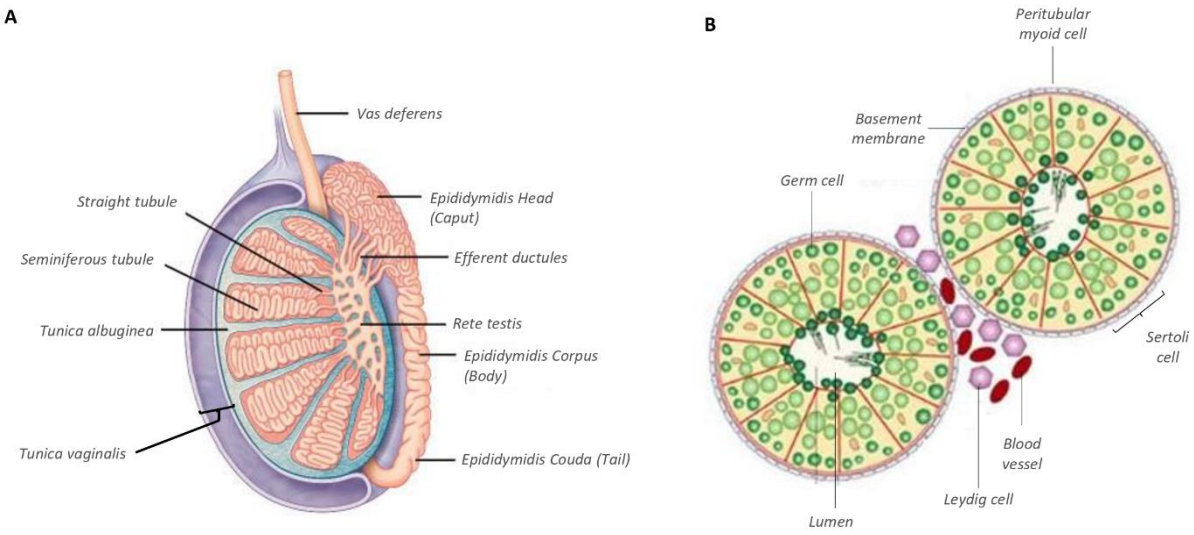

Figure 1.1 – **Testis**. (**A**) Sagittal schematic view showing the location of the seminiferous tubules, the vas deferens and epididymis. (**B**) Schematic cross-section of seminiferous tubules: germ cells at different stages of maturation (green) embedded in somatic Sertoli cells (outlined – yellow). The Leydig cells are in the interstitium of seminiferous tubules. Morphologically mature spermatozoa are represented in the lumen of the tubule (adapted from (3,4)).

1.1.1 Spermatogenesis: a spermatozoon is born

Spermatogenesis encompasses a set of morphological and functional changes that occur in spermatogonia, which will give rise to spermatids and ultimately to spermatozoa. This complex network of processes occurs within the seminiferous tubules (1). In humans, spermatogenesis lasts approximately 72 days, but some experiments showed that this process may take less time (approximately 64 days). Also, spermatogenesis has a highly variable duration amongst different

individuals (5,6). Spermatogenesis involves a set of mitotic and meiotic divisions and can be divided into four stages: (i) self-renewal and differentiation of spermatogonia, (ii) meiosis, (iii) spermiogenesis and (iv) spermiation (figure 1.2) (1). Protein translation is transversal and essential to all phases of spermatogenesis. In the first stage, spermatogonia suffer a set of mitotic divisions that originates a population of cells composed of type A long, type A dark, type A pale and type B spermatogonia. Type B spermatogonia originate the primary spermatocytes through mitotic divisions (7–10). In the meiotic phase, primary spermatocytes undergo two meiotic divisions which originate haploid spermatids as final cells. At phase (iii), these spermatids differentiate into spermatozoa through the following changes: formation and development of acrosome and flagellum; nuclear maturation and reshaping; and removal of cytoplasm (7,8,11). Hence, and after these changes, newly spermatozoa can be released to the tubule lumen during spermiation (12). The spermatogenesis process is highly regulated by complex signalling mechanisms involving the hypothalamic-pituitary-gonadal axis, leading to the continuous production of spermatozoa since puberty through the whole male reproductive life span (13).

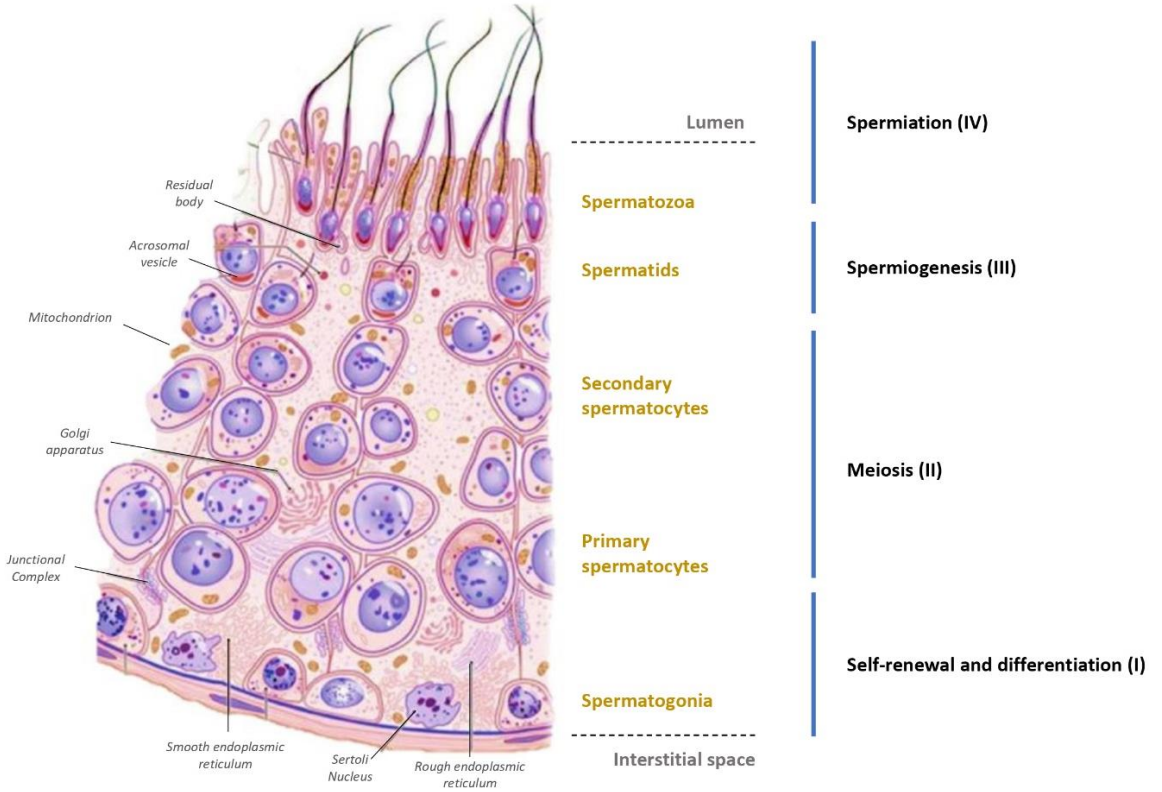

Figure 1.2 – Spermatogenesis. Stratified layer of seminiferous tubules composed by the male germ cells at different stages of differentiation and somatic Sertoli cells (overlined). The organelles of Sertoli cells are identified in the figure (adapted from (14)).

1.1.2 Maturation in the epididymis: improving sperm configurations

When spermatozoa leave the seminiferous tubules, they are already highly morphologically differentiated, although immobile and incapable of fertilizing. Thus, two additional sperm maturation steps are required: one occurring in the male reproductive tract (epididymal maturation) and the other occurring in the female reproductive tract (capacitation – section 1.1.3). The epididymis is a long tubule, highly convoluted and sensitive to androgens, and can be divided into 4 anatomical regions: initial segment (proximal to the testis), caput (connection between the initial segment and corpus), corpus (intermediate portion) and cauda (which connects to vas deferens) (1). The epididymis has an important role in spermatozoa transport, development of progressive motility, storage and protection. All these modifications are ensured by the biochemical environment that is generated within the lumen of the epididymis (15,16). During the epididymal maturation, the sperm proteome suffers several alterations (figure 1.3). Considering that spermatozoa are translationally silent (see details in section 1.2), these proteomic alterations appear to result from the exchange of proteins between the epididymis and spermatozoa. Some exchanged proteins have a significant role in sperm functions (such as motility, capacitation and the acrosome reaction) (17–23), while others tag the defective and/or dead spermatozoa during epididymal transit (24,25). The current hypothesis is that these proteins are produced by the epididymal epithelium and then acquired by the spermatozoon by (a) direct absorption of soluble proteins; (b) incorporation of exosomes (epidymosomes) synthesized by epididymal epithelium; or (c) tight junctions between sperm surface and apical surface of epithelial cells (figure 1.3) (16). However, there are still gaps in the full picture of the mechanisms behind the exchange of proteins between the epididymal fluid and the maturing spermatozoa. Additionally, part of the alterations in sperm proteome result from post-translational modifications (figure 1.3) (15,16).

1.1.3 Capacitation: the key of a successful fertilization

In the early 1950s, two researchers, Chang and Austin, independently observed that ejaculated spermatozoa are not able to penetrate the zona pellucida of female gametes. They need a time-dependent process where the sperm undergoes structural and functional modifications to fertilize the oocyte – the capacitation (26,27). It begins with the removal of the stabilizing factors, acquired from the seminal plasma, continues during transportation in the female reproductive tract and ends when the spermatozoon is able to attach to the zona pellucida, culminating in the acrosomal reaction (28). Structurally, the sperm membrane becomes more fluid, with lateral movement of cholesterol to the apical region of the sperm head and efflux of cholesterol from the plasma membrane to the extracellular space (29,30). Intracellularly, changes occurs in cAMP and calcium ions (Ca^{2+}) levels, accompanied by changes in pH, activation of various protein kinases and formation of reactive

oxygen species (29,30). These changes lead to activation of signalling pathways which are necessary for acrosome reaction. Although there is a large gap in proteomic comparisons between ejaculated spermatozoa and *in vitro* capacitated spermatozoa, evidence from the available studies shows that consistent alterations in the differential expression of certain proteins (figure 1.3) (31–33). Specifically, there is an increase in the proteins that are required for the fertilization and a decrease in the proteins involved in the stability of the spermatozoa, especially those found in the acrosome membrane (34). How these changes occur is still an issue of interest and debate. Similar to what happens during epididymal maturation, some of the changes occurring in the proteome are resultant from post-translational modifications (figure 1.3) (35–37).

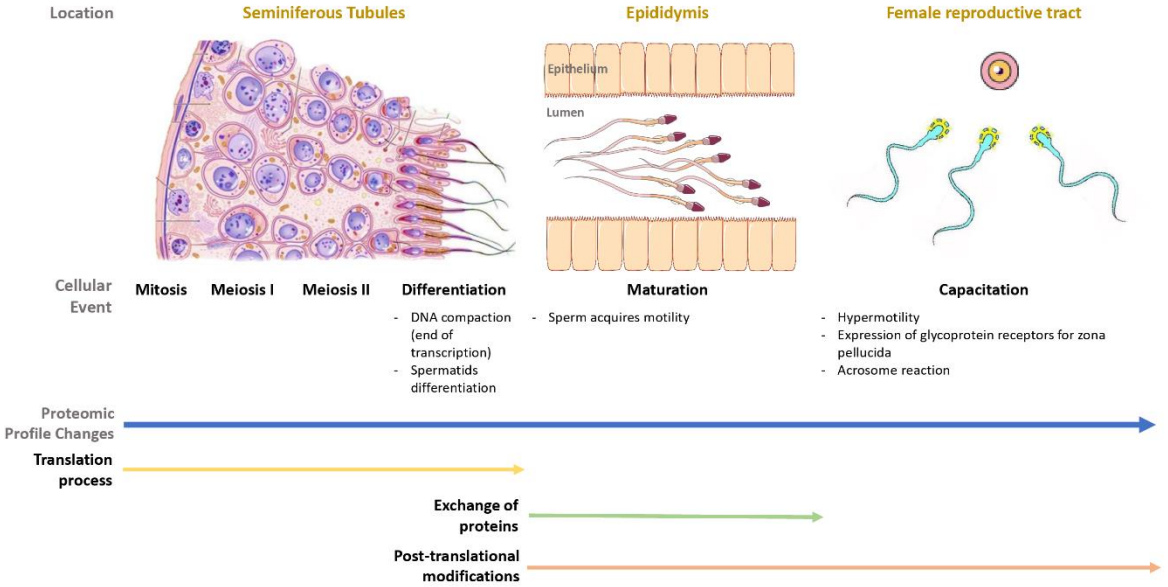

Figure 1.3 – Sperm development events and proteomic profile changes. During the spermatogenesis and sperm maturation, the proteomic content of the spermatozoon suffers major alterations. These alterations result of the protein translation (in spermatogenesis), exchange of proteins (in epididymis) and post-translational modifications (both in the epididymis and the female reproductive tract) (adapted from (14)).

1.2 The Spermatozoon: an atypical cell

Spermatozoon is a highly differentiated haploid cell formed during spermatogenesis. Regarding the structure, the spermatozoon may be divided into two major regions: the head and the tail (figure 1.4). Both regions are covered by plasma membrane.

The sperm head comprises the nucleus and the acrosome (figure 1.4). In the late stages of spermatogenesis, the somatic histones are replaced by protamines, leading to hypercondensation of the chromatin (38). This histone-to-protamine exchange is a gradual process (39). Nevertheless, the

replacement of histones by protamines is incomplete: a small fraction of histones remain bound to DNA (1% to 15%, depending on species). In human spermatozoa, there are ~10% to 15% of histones in nuclear constitution (38–40). Because of these modifications, and despite the remaining nucleosomes, it is widely accepted that spermatozoa are transcriptionally silent. Furthermore, also the majority of the nuclear envelope and of nuclear pore complexes are removed, except for the redundant nuclear envelopes (RNEs) at the base of the nucleus in some species (41). The nucleus is protected by a rigid shell strengthened by disulphide (S-S) bonds between the structural proteins combined with other proteins – the perinuclear theca (42). Half to two-thirds of the sperm's head is occupied by the acrosome, a structure derived from the Golgi complex (43). This structure is composed by a double membrane (internal and external) that surrounds a dense matrix containing a variety of proteases necessary for the digestion of fertilization site in the zona pellucida. The acrosome equatorial region is a folded-over complex of perinuclear theca, inner and outer membranes, and displays receptors involved in the fusion between sperm and oocyte (44).

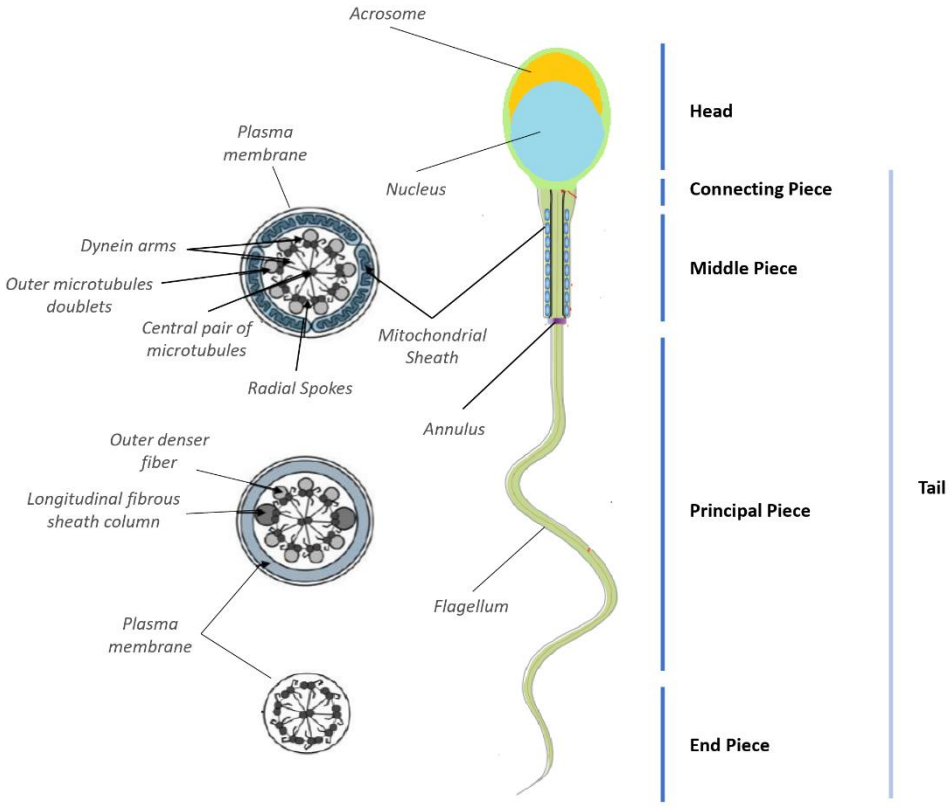

Figure 1.4 – Spermatozoon structure and flagellum ultrastructure. The spermatozoon can be divided into two parts: the head (nucleus and acrosome) and the tail (connecting, middle, principal and end pieces). The details of the ultrastructure of the flagellum are detailed in the text of section 1.2 (adapted from (45)).

The flagellum is the longest part of the spermatozoon, is essential for mobility and can be divided into the connecting piece, mid-piece, principal piece and end piece (figure 1.4) (46,47). The axoneme extends over the entire length of the spermatozoon. It consists of a ring of 9 pairs of microtubules around a central pair (48). The peripheral pairs are accompanied by nine outer dense fibres that provide flexible support during the movement. These microtubules are partially fusion by nexins. The peripheral microtubules also have small arms (internal and external) constituted by dynein's and that are responsible for the propellant movements. In addition, from the peripheral pairs, there are nine radial peduncles that project to the central pair in a helical form (47). In the middle piece, the axoneme is covered by a ring of nine outer dense fibres (ODFs). Both structures are surrounded by a mitochondrial sheath that is unique to this region (more details about sperm mitochondria in section 1.4.5). The *annulus* signals the end of the mid-piece and the beginning of the principal piece (46). From this point onwards, the outer fibres are replaced by two longitudinal columns of fibrous coating. In the final part of the principal piece, these columns disappear leaving only the axoneme covered by the membrane (figure 1.4) (46).

Another relevant structural characteristic in the spermatozoon is it reduced cytoplasm. Parallel to nuclear remodelling, there are also major changes in sperm cytoplasm that lead to removal of cytoplasm along with other cellular organelles (including cytoplasmic ribosomes). Due to such event, the cytoplasmic protein translation machinery is removed (49). Additionally, some evidence suggests that full-length 28S and 18S rRNA, both components of the 80S eukaryotic ribosome, were absented in mature spermatozoa (50). These two types of rRNA are necessary for any translational active cell. Like in somatic cells, 28S and 18S rRNA are the most abundant transcripts in spermatozoa, yet they are fragmented in these haploid cells. The degradation of these transcripts appear to ensure the cessation of translation (50).

Thus, it is widely accepted that spermatozoa are translationally silent. The cytoplasmic changes described above seem to contribute to this silencing. However, as explained in sections 1.1.1-3, spermatozoa are derived from translationally active cells and significant changes in the sperm proteome occur during the maturation and capacitation processes. Could translation process be excluded *a priori* in this type of cells? Could this process exist in a residual way in spermatozoa? And if it exists, is it necessary for normal cell physiology? In the next sections, the essential aspects of protein translation will be described, as well as the evidence described in the literature that supports the existence of translation in spermatozoa.

1.3 Molecular basis of cytoplasmic protein translation

Protein biosynthesis is a core process in cell biology where mRNA is translated into functional peptides/proteins which will play different roles in the physiology and structure of the cell. This process requires extensive biological machinery such as ribosomes, translation factors, tRNA and enzymes. Protein synthesis is highly regulated and changes in this process may cause various diseases (51,52). In this section, the key molecular aspects of the translation process will be elucidated.

1.3.1 Ribosome: the machine responsible for the process

The ribosome is one of the most complex and bigger molecular machines known. The different components of the ribosome are measure in Svedberg (S) units which corresponds to the sedimentation coefficient when subjected to ultracentrifugation. The mammalian ribosome – 80S ribosome – is composed of 80 different proteins and four chains of rRNA (28S, 5S, 5.8S and 18S) (53). Those proteins and rRNA assembly into two unequal subunits: the large subunit (60S) and the small subunit (40S) (figure 1.5) (53,54). The assembly of ribosomal proteins occurs simultaneously with maturation and folding of the pre-rRNA into mature rRNA (55). During the maturation process, the rRNA undergoes a set of modifications that contribute to ribosome stabilization. They are also present in the functional sites of the ribosomes, facilitating the recognition of conserved mRNA and tRNA sites (56). The small subunit contains 33 different proteins and 18S rRNA and works as a decoder centre allowing the interaction between mRNA and tRNA and consequently the formation of proteins from mRNA (55). In the interface of the small subunit are located the mRNA-binding sites and the three tRNA-binding sites (A, P and E) (figure 1.5). Also, the decoding centre is located on the surface of this interface where codon (of mRNA) and anticodon (of tRNA) are paired to ensure translation fidelity (54). On the other hand, the large subunit is responsible for catalysing the peptide bond formation and is composed of 47 different proteins and 5S, 5.8S and 28S rRNA and catalyse the peptide bond formation (55). In the interface of 60S subunit, there are also the tRNA-binding sites (A, P and E) and the peptidyltransferase centre (PTC) which is responsible for the formation of the peptide bond between two amino acids (figure 1.5) (54). Since the peptidyl transfer is dependent on the recognition of a codon, the translation process requires communication and coordination between the two subunits (51).

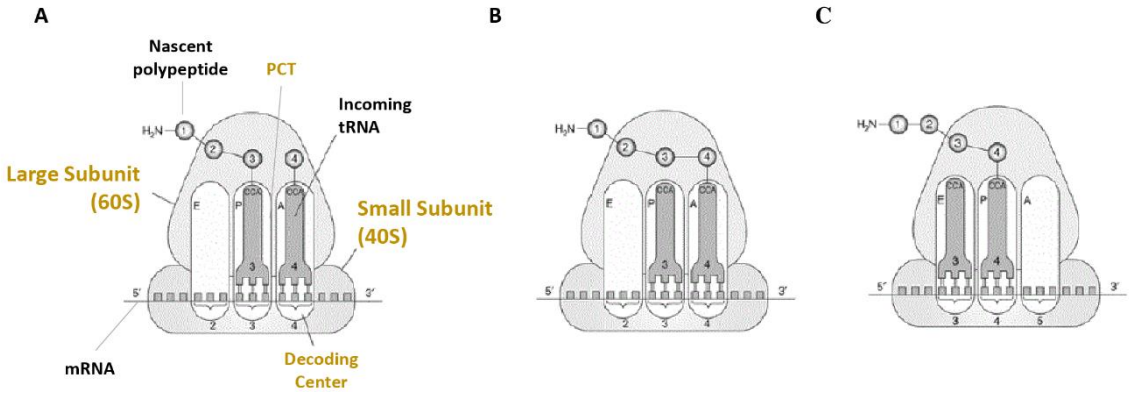

Figure 1.5 – The 80S mammalian ribosome. Schematic representation of eukaryotic ribosome during the translation elongation. The tRNA can bind in three sites of the ribosome: A (aminoacyl), P (peptidyl) or E (exit) site. (A) The codon/anti-codon pair is established at the A site by the decoding centre of the 40S subunit. (B) The PTC promotes the formation of a peptide bond between the amino acids in the A and P sites and leaves a deacylated tRNA at the P site. (C) The translocation of mRNA leads to the occupation of the P site by the peptidyl-tRNA and a deacylated tRNA at the E site, leaving the A site free for next cycle of elongation (adapted from (51)).

1.3.2 Translation Initiation: the predominant level of regulation

The initiation phase is the rate-limiting step of translation and is largely regulated. Thus, the initial steps of eukaryotic translation of mRNA require many eukaryotic initiation factors (eIF) (table 1.1) (57). The translation initiation may be divided into three contiguous stages: (I) formation of the 43S pre-initiation complex, (II) mRNA recruitment and (III) scanning and ribosome assembly (figure 1.6).

Table 1.1 – Eukaryotic initiation factors of cytoplasmic translation in mammalian cells.

Factor	Function	References
eIF1	Formation of the 43S pre-initiation complex (binds to P-site of small subunit); selection of the start site; control of GTPase activating protein activity of eIF5	(58–61)
eIF1A	Formation of the 43S pre-initiation complex (binds to A-site of small subunit); selection of the start site	(60)
eIF2	Formation of the ternary complex; "bridge" between the 40S subunit and Met-tRNA$_i$; GTPase activity	(62)
eIF3	Scaffold protein; selection of start site; prevent the premature assembly of the ribosome subunits	(63)
eIF4A	Unwinding of the secondary mRNA; loading of the mRNA into the 43S PIC	(64,65)
eIF4B	Unwinding of the secondary mRNA; loading of the mRNA into the 43S PIC	(64,65)
eIF4E	Cap binding protein; formation of complex eIF4F	(66)
eIF4F	Complex formed by the eIF4E and the eIF4G	

eIF4G	Scaffold protein; formation of the eIF4F complex; loading of mRNA into the 43S PIC	(67,68)
eIF5	GTPase activating protein; recycling of the eIF2·GDP complex	(60,67,69)
eIF5B	GTPase activity; promotes the ribosome assembly	(70)
eIF6	Binds to 60S subunits and prevents premature ribosome assembly	(71,72)
PABP	Interaction with the poly-A tail on mRNA and eIF4G	(73)

In the early steps of the initiation phase, the 80S ribosome dissociates into its 60S and 40S subunits. The 60S subunit binds to the eIF6 (71,72). At the same time, the eIF2 associates with the GTP and the Met-tRNA$_I$ to form the ternary complex (62). This tRNA has structural features distinct from the Met-tRNA used in the elongation, which prevents its binding to elongation factors (74). Then, the 40S subunit binds to eIF1, eIF1A, eIF3, eIF5 and the ternary complex to form 43S pre-initiation complex (PIC). During the start site recognition, the eIF5 promotes the cleavage of GTP in the ternary complex and release of eIF2·GDP from the 43S pre-initiation complex (PIC) (figure 1.6) (60,67,69).

After the formation of the 43S PIC, mRNA is recruited through interaction with eIF4 factors and poly-A binding proteins (PABP). The mRNA cap structure is recognized by eIF4E and this factor to eIF4G to form the eIF4F complex (66). Besides the interaction with the eIF4E, the eIF4G anchoring other proteins (such as eIF4A, eIF4B, PABP and eIF3) that contribute to the preparation and loading of the mRNA into the 43S PIC (67,68). The eIF4A and the eIF4B interfere with the secondary structure of the 5' terminal of the mRNA, preparing it for binding to the 43S PIC (64,65). The eIF4G also promotes the "close loop" circular conformation of mRNA through its interactions with eIF4E and PABPs. This conformation seems to be helpful in the last steps of translation (73). Thereby, the messenger ribonucleoprotein (mRNP) is ready to be loaded onto PIC through the interaction between eIF3 and eIF4G (figure 1.6) (75).

When the mRNA is loaded into the 43S PIC, the scanning starts in the 5' to 3' direction until the initiation codon (AUG) is identified (76). The scanning conformation of 43S PIC is facilitated by eIF1 and eIF2 interactions (58). The eIF5 interacts with the eIF2 which stimulate the eIF2 GTPase activity. Also, the eIF5 prevents the premature release of P_i from the 43S PIC (61,77). When the 43S PIC finds the AUG codon in the context of the P site, the codon/anti-codon pair is formed which promotes the complete accommodation of the Met-tRNA$_i$ into the P site. This event allows the interaction of the eIF5 with the eIF1A and the release of P_i which leads to the scanning arrest and conversion to the "close" conformation of 43S PIC (78,79). The "close" 43S PIC releases the eIF5·eIF2·GDP complex which cleans the surface of the 40S subunit. The eIF5B·GTP facilitates the junction between the 40S and 60S subunits by interaction with eIF1A. The hydrolysis of GTP causes the conformational rearrangement of the ribosome (70). The complex eIF5B·GDP has low affinity

and is dissociated from the assembled ribosome (figure 1.6) (80). The ribosome is at this step ready for the next stage of translation: the elongation.

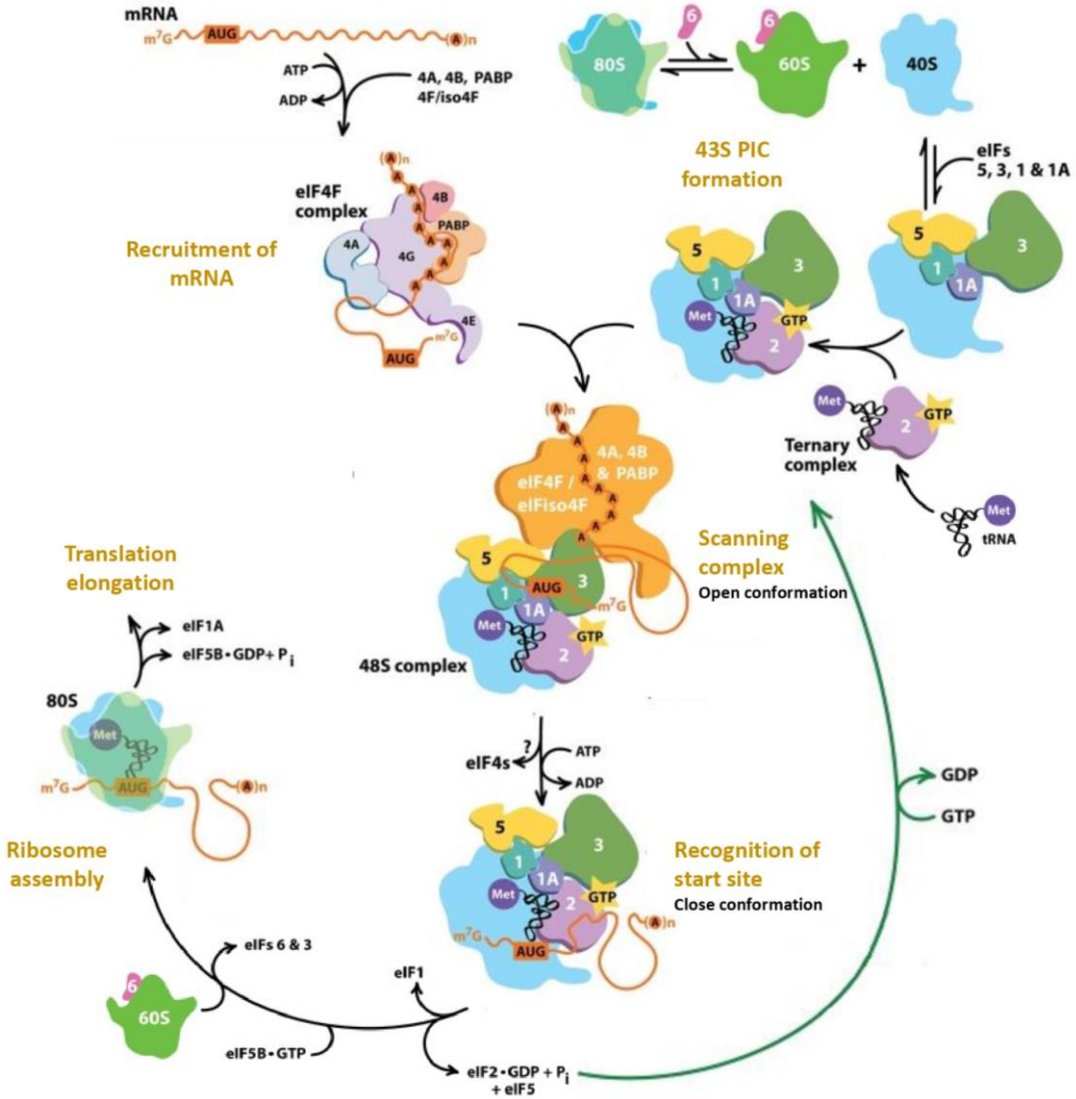

Figure 1.6 – Translation Initiation. Overview of the steps of cytoplasmic translation initiation on mammalian cells (adapted from (81)).

1.3.3 Translation elongation: a peptide arises

When subunits assembly is completed, the Met-tRNA$_i$ is placed at the P site of the ribosome and a second codon is present in A site awaiting for interaction with its respective anti-codon of the aminoacyl-tRNA ((aa)-tRNA) (figure 1.7). An important step of elongation is the binding of the (aa)-tRNA and the eukaryotic elongation factor (eEF) 1A in a GTP-dependent manner (82). The eEF1A·GTP directs the delivery of (aa)-tRNA to the A site when the corresponding codon is present. At the A site, the eEF1A promotes GTP hydrolysis, which allows the accommodation of the (aa)-

tRNA (83). The eEF1A·GDP is then released and recycled by eEF1B (82). The interaction between codon/anti-codon pair stimulates the peptide bond formation by PTC between the Met-$tRNA_i$ and the (aa)-tRNA. This quick reaction leaves a deacylated tRNA at the P site (82). Subsequently, translocation of the mRNA occurs leading to the occupation of the P site by the peptidyl-tRNA and the passage of the deacylated tRNA to the E site (that then is ejected from the ribosome), leaving the A site free for the next elongation cycle (84). The binding of eEF2·GTP to the ribosome promotes GTP hydrolysis and the release of P_i which induces passage from the locked to the unlocked conformation of the ribosome and allows the movement of mRNA and tRNAs. The P_i release is also important for eEF2 dissociation (figure 1.7) (82). Although they do not directly contribute to the elongation step, aminoacyl-tRNA synthetases play an important role in the backstage, by promoting the formation of aminoacyl-tRNA through the coupling of amino acids and tRNAs in an ATP-dependent reaction (figure 1.7) (85). The elongation phase occurs until a stop-codon is found.

Table 1.2 – Eukaryotic elongation and termination factors of cytoplasmatic translation in mammalian cells.

Factor	Function	References
Elongation Phase		
eEF1A	Binds to aminoacyl (aa)-tRNA and GTP	(82)
eEF1B	Recycling of the eEF1A·GDP complex	(82)
eEF2	Translocation process	(82)
Termination Phase		
eRF1	High-fidelity recognition of stop codon; peptide release	(86,87)
eRF3	GTPase activity; peptide release	(88)
ABCE1	ATPase activity; peptide release; ribosome recycling	(89–91)

1.3.4 Translation termination and ribosome recycling: the final acts

Upon recognition of a stop-codon (UAA, UGA, UAG) at the A site the elongation phase ends and the termination begins (figure 1.7) (92). This phase of translation is mediated by two eukaryotic release factors (eRF) (table 2) and culminates in the release of the polypeptide (88). The eRF1 is responsible for stop-codon recognition and its middle domain is analogue to tRNA, entering in the PTC and promoting cleavage of the polypeptide (86,87,93,94). The eRF3 promotes the polypeptide hydrolytic release and increases the termination efficiency (88). Prior to the stop codon recognition, a cytosolic complex composed of these factors is formed - eRF1·eRF3·GTP (the eRF1 slows down the GTP hydrolysis). When a stop codon is recognized at the A site, the complex is recruited, GTP hydrolysis occurs and the binding of eEF1A·(aa)-tRNA is prevented (figure 1.7) (92).

In the current view, translation termination is followed by ribosome recycling, a fundamental step to maintaining the ribosome homeostasis. During termination, the ATP binding cassette protein E1 (ABCE1) binds to eRF1 and stimulates the hydrolytic cleavage of the nascent chain. After that it uses the energy of ATP hydrolysis to dissociate the 40S and 60S subunits (89–91). The corporation between ABCE1 and eRF1 is important for both reactions.

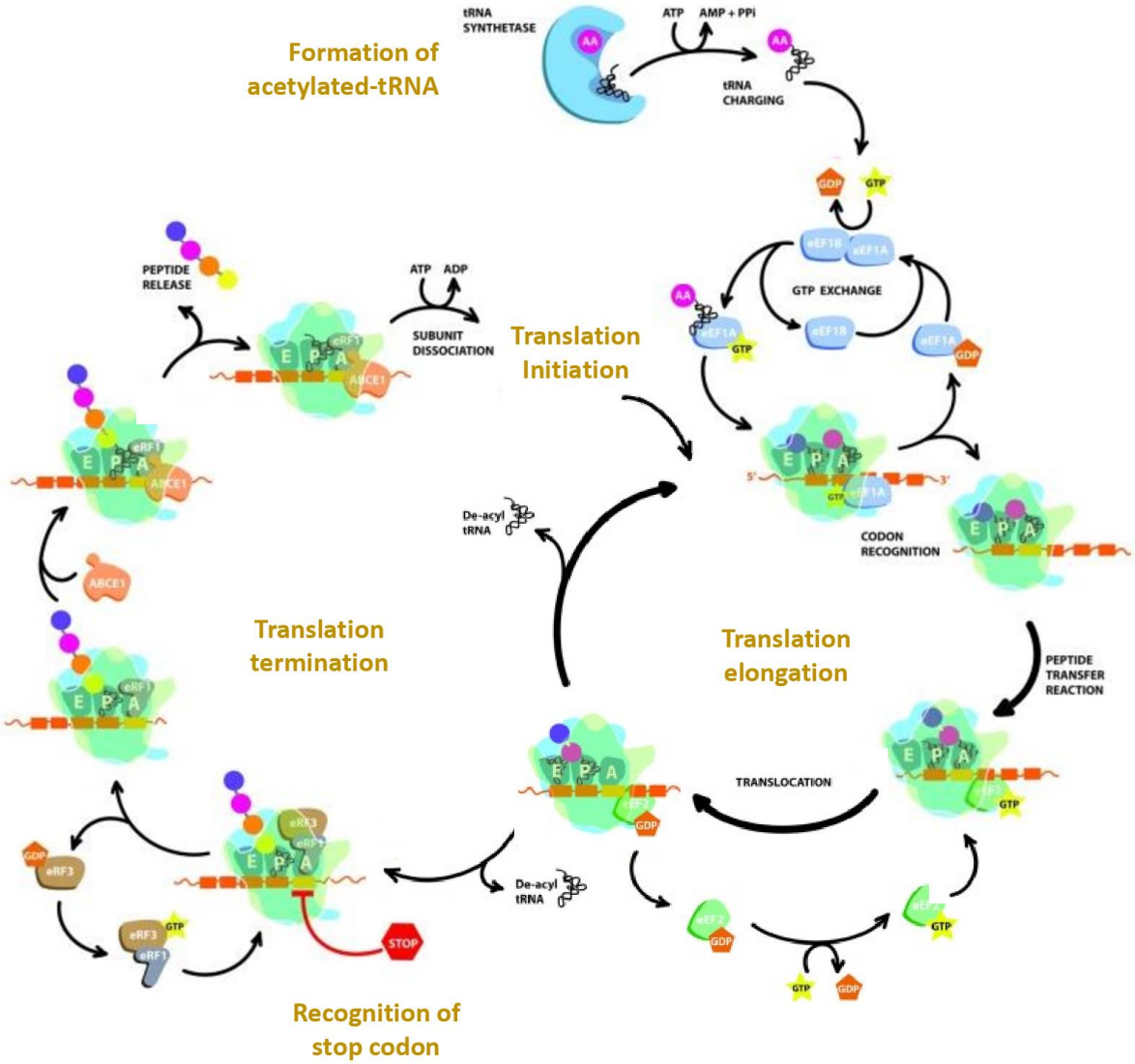

Figure 1.7 – Translation Elongation and Termination. Overview of the steps of cytoplasmic translation elongation and termination on mammalian cells (adapted from (81)).

1.4 Mitochondria: more than a power source

Mitochondria are distinct organelles, responsible for the major energy production in aerobic conditions. Since they have their own genome and protein translation machinery, they are also one

of the places where protein synthesis is possible. In this section, the major aspects related to mitochondrial translation will be detailed.

1.4.1 Mitochondria of mammalian cells: a brief overview

Mitochondria are unique cellular organelles involved in multiple cellular functions with variable morphology. Structurally, mitochondria are composed of two membranes, the outer (OM) and the inner membrane (IM), which define two compartments: the intermembrane space (between the membranes) and the mitochondrial matrix (delimited by IM) (figure 1.8). These two compartments are interconnected by transmembrane proteins allowing the exchange of molecules between compartments. The IM and the mitochondrial matrix are the most specialized structures where many enzymes, mostly organized in multi-complexes, exist (95). The IM is the site of ATP production via oxidative phosphorylation (96). Mitochondria contain their own circular genome (mtDNA), a double-strand of circular DNA located in the mitochondrial matrix, that encodes 2 mt-rRNAs, 22 mt-tRNAs and 13 polypeptides of the enzymatic complexes in the respiratory chain (97,98). However, the vast majority of mitochondrial proteins (~ 1500 proteins) are encoded by the nuclear genome (99).

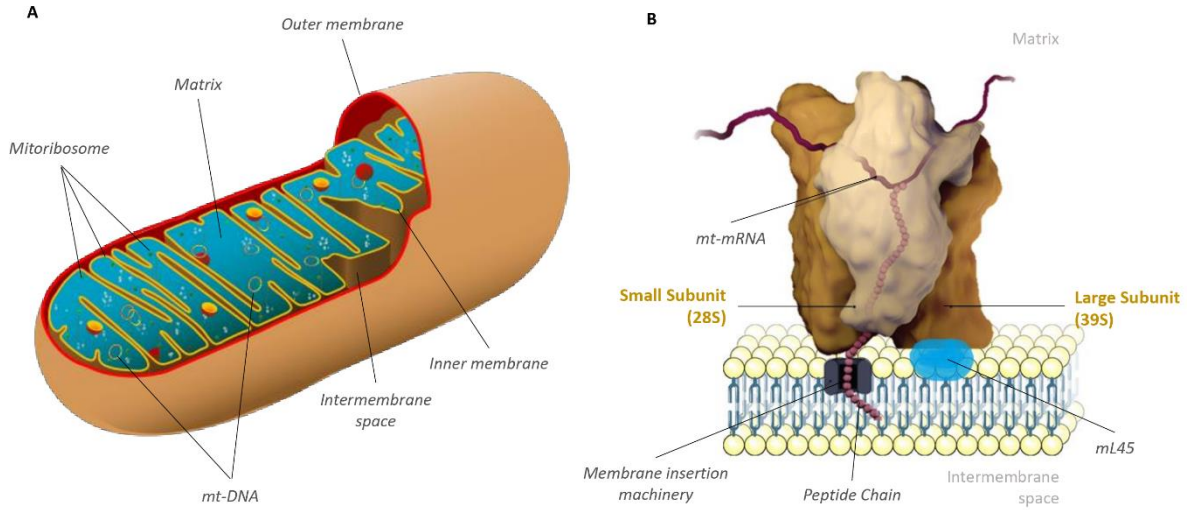

Figure 1.8 – The mitochondrion and the mitoribosome. (**A**) Schematic representation of the mammalian mitochondrion and (**B**) the association between mitoribosome and the inner membrane of the mitochondrion (adapted from (100)).

1.4.2 Mitoribosome: a special ribosome

The mitochondrial polypeptides are synthesized within the mitochondrion through organelle specific machinery, the mitochondrial ribosome or mitoribosome. Mitoribosomes have a lower sedimentation

coefficient (~55S) and are composed by a 28S small subunit and a 39S large subunit (figure 1.8). The small subunit is formed by 12S rRNA and by 30 mitoribosomal proteins (MRPs) while the large subunit is composed of 16S rRNA, a structural Val-tRNA (in human cells) and by 50 MRPs (101–104). Among these 80 MRPs, 36 are exclusive of mammalian mitoribosomes (101,104,105). The arrangement of specific proteins on the outer surface of the subunits suggests that the functional nuclei (mRNA recognition site in the small subunit and PTC in the large subunit) were preserved throughout evolution (101,103,104). Another important difference is the bridges between subunits: these bridges are ensured by certain proteins, contrary to what happens in cytoplasmic and bacterial ribosomes (RNA-RNA intersubunit bridges). Additionally, the lower number of intersubunit bridges allows greater flexibility in the subunits conformation (106). The mitoribosomes are present in the mitochondrial matrix in association with the IM, which facilitates the insertion of the recently synthesized protein into the membrane (figure 1.8) (101). This association is promoted through the mL45 protein that functions as an anchor of the large subunit. Furthermore, the polypeptide exit tunnel region is adapted to the transit of hydrophobic peptide (101,103,104).

1.4.3 Mitochondrial protein translation

The mitochondrial translation is a key process in mammalian mitochondria to produce the components of oxidative phosphorylation complexes. After the transcription of polypeptides gene sequences, the mitochondrial mRNA (mt-mRNA) are matured by addition of a poly(A) tail (~50 nucleotides) (107). The mature mt-mRNA has some particular features: (a) does not possess a leader sequence neither a cap structure on the 5' end, (b) the translation start codon is the second codon in 5' end (108) and (c) the mitochondrial gene code is different from the universal (for example, AGG and AGA – arginine codons – are termination codons instead of UAA and UAG) (109). The mitochondrial translation process can also be divided in initiation, elongation, termination and ribosome recycling (figure 1.9) (110).

The first step in the initiation of mitochondrial translation is the dissociation of 55S mitoribosome by the mitochondrial initiation factor (mtIF) 3 and the formation of the 28S·mIF3 complex. This ensures that premature association of subunits does not occur. The next step is the recruitment of mt-mRNA by the small subunit (figure 1.9) (103,104). As start codons are recognized the triples AUG, AUA and AUU. Simultaneously, the complex mtIF2·GTP recruits the formylated Met-tRNA (f-Met-tRNA). The mtIF2·GTP complex ensures that f-Met-tRNA is delivered in the P site. If a positive interaction between codon and anticodon is formed, interaction with large subunit is allowed. The association of the subunits leads to GTP hydrolysis which releases the mtIF2 and mtIF3 (figure 1.9) (111).

Once the 55S mitoribosome is assembled, the elongation phase can start. At this point, a ternary complex is formed by mitochondrial elongation factor (mtEF) Tu, GTP and (aa)-tRNA. The ternary complex enters the A site of the mitoribosome and a codon-anticodon pair is formed which triggers the GTP hydrolysis and the release of the mtEF-Tu·GDP (110). The mtEF-T**s** promotes the exchange of GDP by GTP, which allows the regeneration of the mtEF-TU·GTP complex. After the release of the mtEF-Tu factor, mitoribosome catalyses the formation of the peptide bond, leaving a deacylated tRNA at the P site and a peptidyl-tRNA at the A site (110,112). The translocation of the mitoribosome is catalysed by mtEF-G1, allowing the passage of tRNA deacylated to the E site (103,104) and the peptidyl-tRNA for the P site (110,112).

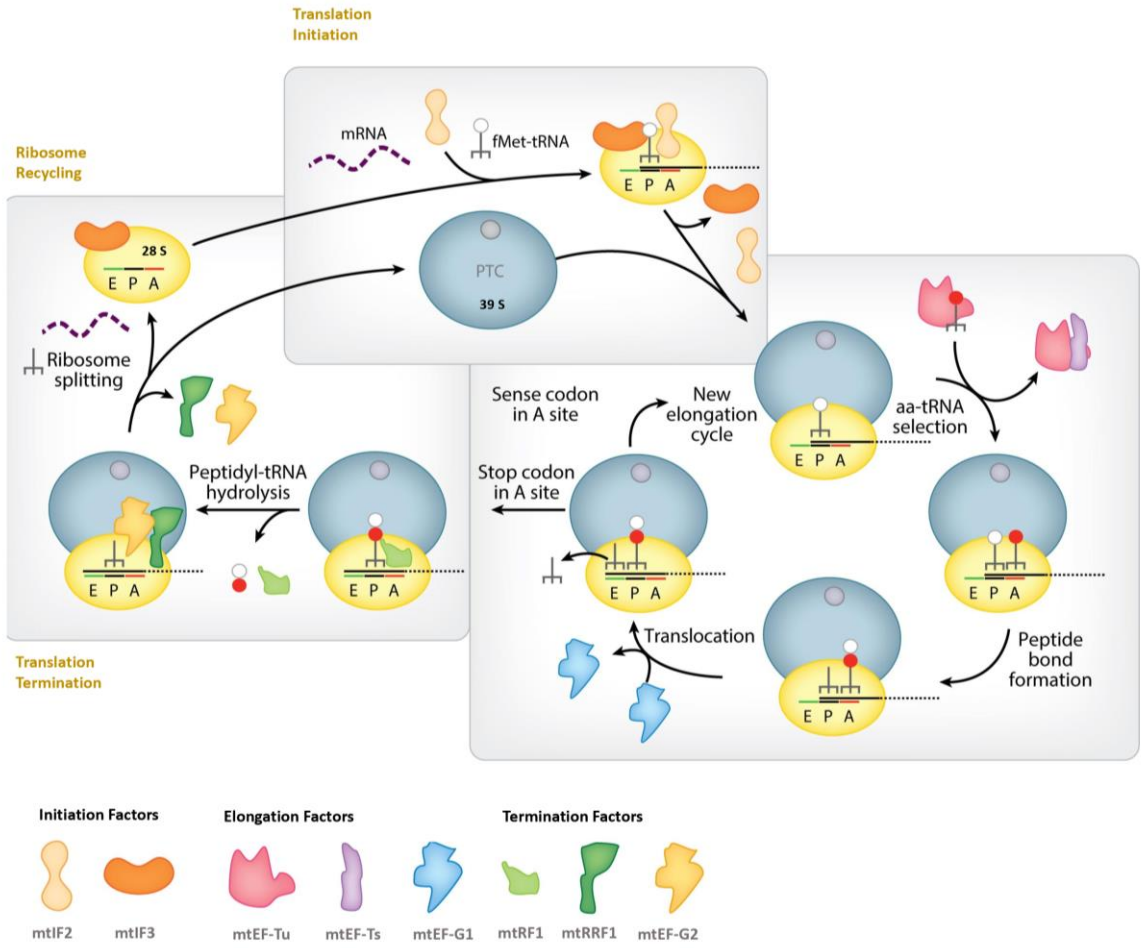

Figure 1.9 – Mitochondrial Translation. Overview of the translation process that occurs in mammalian cells mitochondria (adapted from (110)).

The mitochondrial translation stops when a stop codon (UAA and UAG) is recognized at the A site. Contrarily to what happens in cytoplasmic translation, the recognition of the stop codon is done by a specific protein - mtRF1a. When this codon is recognized, the structure of mtRF1a changes, allowing it to enter in the PTC. This change promotes the hydrolysis between the tRNA and the final

amino acid and the presence of GTP allows the release of the polypeptide from the 39S subunit (110,112). After the polypeptide release, two recycle factors - mtRRF1 and mtEF-G2 - bind to the A site promoting the dissociation of 55S mitoribosome and the release of mt-mRNA and deacylated tRNA. Finally, these two factors are released, and a new mitochondrial translation cycle can be started (112–114).

1.4.4 The relationship between cytoplasmic ribosomes and mitochondria: a history of efficiency

Despite mitochondria ability to performing protein synthesis, most of the mitochondrial proteins (including mitoribosome proteins and mitochondrial translation factors) are encoded by nuclear DNA and synthesized by cytosolic ribosomes (115). Mitochondrial proteins are synthesized as precursors with specific targeting signals that determine its destination. Protein import and sorting are performed by highly specialized translocase machines (like TOM and TIM) which recognize the targeting signals and allow the passage of the stated precursors across the mitochondrial membranes (reviewed in (116)). When the protein reaches the correct compartment, it is cleaved and folded, becoming functional. Over the last years, several studies have reported that some cytoplasmic ribosomes are near to the outer membrane and hence they can perform translation on-site (reviewed in (117)). Protein synthesis near to the action sites have been described in different cellular localization and is advantageous since it is possible to reduce the energy costs of translocation and allows on-site expression regulation (118). Additionally, some cellular compartments are more resistant to stress, which may allow translation under adverse conditions (119). As reviewed by Zhang and Xu, the on-site translation may be advantageous in mitochondrial homeostasis, since it allows localized protein synthesis required for damaged mitochondrial components, which "saves" mitochondria from complete degradation (120). Either way, between mitochondria and the cytoplasmic ribosome there is a sophisticated mechanism of translational control that ensures that cytoplasmic and mitochondrial translation are synchronized (121).

1.4.5 Sperm Mitochondria

During the cytoplasm elimination that occurs in spermatogenesis, most mitochondria are also eliminated (122). While a somatic cell may have between 383 to 882 mitocondria, mature mammalian spermatozoa only have 50 to 75 remaining mitochondria, reorganized into tubular structures that surround the anterior portion of the ODFs in the region of the middle piece (figure 1.4) (122). These remaining mitochondria present a more condensed morphology, compared to somatic forms, with a more compacted matrix that allows greater energy efficiency (123–125). Another feature of the sperm mitochondria is the presence of the mitochondrial capsule. This

keratinous structure is formed by disulphide bridges (S-S) between proline-rich and cysteine-rich regions of selonoproteins residues. Also, this mitochondrial capsule may grant protection to sperm mitochondria (126,127). As reviewed by Amaral and colleagues, the integrity of mitochondria and mt-DNA is crucial for spermatozoa functionality and genetic and proteomic alterations in mitochondria have been associated with infertility conditions (130). Interestingly, the paternal mt-DNA is not passed to offspring during embryogenesis since the paternal mitochondria are degraded (129). Initially, and due to the compartmentalization of the mitochondria, it was thought that the ATP generated in these organelles was not the primary energy source of the spermatozoa. Studies carried out in recent years have shown that both glycolysis (in the principal piece of sperm tail) and oxidative phosphorylation (in mitochondrial sheet) are used as complementary energy sources in response to the energy demand that is generated by the movement of spermatozoa (130,131). In addition to this role in metabolism, and despite the changes it undergoes during spermatogenesis, does the sperm mitochondria retain the protein translation process?

1.5 Spermatozoa: cells with silenced protein translation (?)

Over recent years, some studies on mature spermatozoa have shown evidence that has led to questioning the dogma of translational silencing in spermatozoa. These studies focused mainly on the transcripts present in mature spermatozoa (including mRNAs and rRNAs). However, the most relevant evidence supporting the revision of this dogma was presented by Gur and Breitbart (132). Here, these major findings will be reviewed.

1.5.1 The presence of mRNAs in mature spermatozoa: raw material for translation (?)

Considering the nuclear modifications of spermatids (see section 1.2), it is widely accepted that transcription is terminated gradually and there are no transcription processes in mature spermatozoa. Due to transcriptional silencing, several mRNAs are transcribed in the early stages of spermatogenesis, stored and maintained under translational repression. In the late stages of spermatogenesis, those mRNAs are translated and resulting proteins perform their activity (133,134). However, more than 4,000 different types of mRNA were found in mature human spermatozoa (135,136). The role of these mRNA remains controversial. There are evidences that mRNAs are delivered into oocytes and are necessary to early stages of embryogenesis (137–139). On the other hand, it seems that some of the mRNAs are fundamental for the normal motility and capacitation of the spermatozoa (reviewed in (140)). Could these mRNAs undergo translation in mature spermatozoa?

1.5.2 Intact cytoplasmic rRNA: ribosomes are present (?)

As described above, the ribosome is a molecular machine composed by RNP and rRNA. The rRNA is crucial for the correct function of this molecular machine. The existence of intact mature forms of rRNA allows to infer the existence of ribosomal subunits, which may or may not be functional. In mature spermatozoa, intact forms of 12S and 16S rRNA were detected, both components of mitoribosomes (136,141,142). Thus, the mitoribosomes subunits appear to be present in mature sperm. On other hand, the presence of intact forms of 18S and 28S, both components of 80S ribosomes, is still a matter of debate. Initial evidence suggests that these two transcripts are the most abundant in spermatozoa, however they would be fragmented (50). New evidence showed that fragmentation could have an experimental and non-physiological origin, that is, methods of RNA extraction may have led to the fragmentation of cytoplasmic rRNAs (143,144). In addition to the presence of intact 18S and 28S, these recent studies also identified small subunits of cytoplasmic ribosomes in mature sperm (143,144). Can both mitochondrial and cytoplasmic ribosomes be present and functional in mature spermatozoa?

1.5.3 Translation in spermatozoa: experimental evidence

A new perspective emerged when Gur and Breitbart proved *de novo* protein synthesis in mammalian spermatozoa (132). They incubated sperm with labelled amino acids ([^{35}S]-Methionine and [^{35}S]-Cysteine), under capacitation conditions to check their eventual incorporation into the newly synthesized polypeptides. Then, they used several mitochondrial inhibitors of translation (e.g. chloramphenicol (CP)), which resulted in total blockage of labelled amino acid incorporation. However, when they used cycloheximide (CH), a cytoplasmic 80S ribosome inhibitor, the incorporation of labelled amino acid was not affected. Thus, this study suggested that protein translation in sperm involves ribosomes inside or near mitochondria (possibly the mitoribosomes) but not the cytoplasmic, being a capacitation-dependent process. Additionally, and to identify the cellular site of translation within the sperm cells, Gur and Breitbart used BODIPY-lysine-tRNA^{Lys} that labels newly made proteins. A high level of fluorescence was detected in the middle piece (where the mitochondria are exclusively located) and a low level was detected in the principal piece of the tail. Furthermore, the labelling was inhibited by CP but not by CH, reinforcing their previous findings (132,145).

Additionally, Rajamanickam and colleagues showed the increase of sperm Na/K-ATPase isoform (ATP1A4) content in the sperm plasma membrane (raft and non-raft fractions) through mitochondrial translation (146). ATP1A4 is a testis-specific isoform and is responsible for the maintenance of Na^+ and K^+ balance between the intra- and extracellular environment (147). A specific ligand of this

enzyme is ouabain, a protein secreted by adrenal gland, that is also present in the vaginal fluid (148). Given the high affinity of ATP1A4 for ouabain, Rajamanickam and colleagues hypothesized that this enzyme has a role in sperm physiology (146). They showed that the increase in ATP1A4 content was accompanied by an increase in enzymatic activity, which excluded the possibility of relocation of the enzyme during the capacitation process (146). Moreover, and using the same tRNA label technology of Gur and Breitbart, Rajamanickam and colleagues proved *de novo* synthesis of ATP1A4. Similarly to Gur and Breitbart's experiment, the ATP1A4 translation was decreased when CP was used (146).

Both studies showed that translation occurred upon capacitation. Therefore, Gur and Breitbart proposed two possible hypotheses for the occurrence of this process: (i) replacement of degraded proteins and/or (ii) production of specific elements required for capacitation. Also, these authors proposed that sperm translation occurs in CP-sensitive ribosomes that probably are inside or near the mitochondria (figure 1.10) (145). Further studies are needed to support these hypotheses.

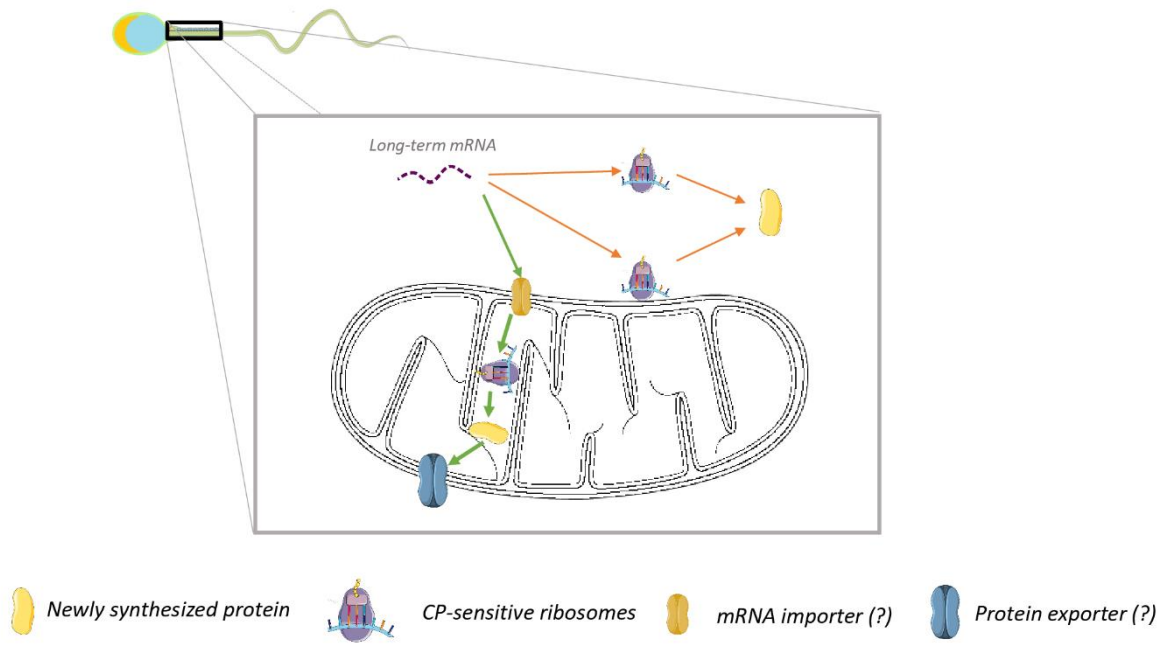

Figure 1.10 – Protein translation in spermatozoa. Proposed model by Gur and Breitbart for protein translation in CP-sensitive ribosomes in spermatozoa present inside or outside of the mitochondrion (145).

1.6 Aims

The working **hypothesis** of this project is that spermatozoa are translationally capable, being a crucial cellular process for gamete competence and fertility. Thus, the **main objective** of the present work is the characterization and evaluation of protein synthesis in mammalian spermatozoa. To achieve that, three specific aims were established:

I. Identification of translation-related proteins present in spermatozoa through *in silico* analysis;

II. Monitoring and assessment of the functionality of the protein synthesis machinery in bovine spermatozoa under capacitation conditions using Surface Sensing of Translation (SUnSET);

III. Characterization of the newly synthesized proteins by mass spectrometry analysis.

2 Bioinformatic Approach

2.1 Methods

2.1.1 Data collection

2.1.1.1 Collection of translation-related proteins

To identify the proteins associated with the translation process, a search was carried out on a Gene Ontology database, AmiGO2 (http://amigo.geneontology.org/amigo – version 2.5.12 – amigo2b). This database gathers a controlled vocabulary of terms covering biological concepts, and a great number of genes or products whose attributes have been annotated as gene ontology (GO) terms. The expression "*protein translation*" was searched in AmiGO2 query resulting in 88 annotations. After a brief analysis of "Inferred Tree View", the GO:0006412 term was selected, since it was the most general term related to protein translation. Next, the protein list was filtered to *Homo sapiens* and to *protein* and the output was revised to exclude duplicates, using Excel for that purpose. To solve redundancy, all proteins were annotated through the UniProtKB/Swiss-Prot accession number by using Retrieve/ID mapping tool of the UniProt database**.** Within this tool, the protein list was uploaded and "UniProtKB AC/ID" and "UniProtKB" options were selected. After that, only reviewed proteins were maintained. Considering the focus on cytoplasmic and mitochondrial translation, the GO:0002181 and GO:0032543 terms were also selected since they present the proteins involved in these two specific forms of translation. The procedure described above was applied to these two sets of proteins. All data were downloaded on Oct 10th, 2018.

2.1.1.2 Collection of sperm proteins

An exhaustive literature search was conducted using the PubMed database to identify human sperm proteomic studies published in English language. A list of all the sperm proteins identified in the proteomic studies available online until April 30th, 2018 was compiled. All studies included were performed using ejaculated human spermatozoa samples (human epididymal spermatozoa and animal models were not included). Only proteomic studies in which a false discovery rate (FDR) < 5% of protein identification was set and proteins identified with at least two peptides were included (33,149,158–167,150,168–177,151,178–187,152,188,153–157). Duplicates were removed and protein isoforms were separated in an independent list. As described before, to bypass redundancy,

the proteins list was annotated using the UniProtKB/Swiss-Prot accession number by using Retrieve/ID mapping tool. Only reviewed proteins were selected and used in the subsequent analyses.

2.1.2 Compilation of the translation-related proteins with expression in spermatozoa

To obtain the list of translation-related proteins present in spermatozoa, a Venn diagram analysis was performed using the Jvenn Tool (http://jvenn.toulouse.inra.fr/). The resulting list was evaluated to find potential protein isoforms of translation proteins. Since isoforms have a specific designation (UniProtKB followed by a hyphen and isoform number), analysis by Venn diagram is not possible. So, Excel was used to perform this analysis. Also, it was relevant to understand if the cytoplasmic and mitochondrial translation processes have common proteins and if these proteins are present in sperm cells. To achieve this purpose, a second Venn diagram analysis was performed using the Jvenn Tool.

2.1.3 Characterization of translation-related proteins with expression in spermatozoa

To identify the role of each translation-related protein present in spermatozoa, the UniProt database was used to gather information regarding biological processes (data was downloaded on Oct 11th, 2018). Since each protein may have been annotated for different processes, including processes not related to translation, a set of GO terms related to the different steps of the translation was selected. This selection was based on children terms of GO:0006412 and QuickGO definitions (www.ebi.ac.uk/QuickGO/) for GO terms that had the potential to be associated with the translation process. Subsequent analysis was performed using Excel resources.

The cellular compartment of each protein was also annotated using the GO vocabulary stored in the UniProt database (data was downloaded on Oct 17th, 2018). The initial analysis was performed in Excel. As a supplementary analysis, and since each protein may be annotated for more than one compartment, a Venn diagram analysis was performed considering different cellular compartments.

After this characterization, it was verified whether there were proteins exclusively linked to the translation process (*i.e.* proteins that were annotated only for biological processes related to translation and located in the proper cellular compartment for this process to occur). Initially, the 315 proteins were filtered based on their biological process, according to the GO terms previously defined. After this filtration, each protein was individually analysed and all the proteins presenting

annotations to other biological processes not related to translation were excluded. In a second step, the annotations for the subcellular localization of the previously identified proteins were considered. Each protein was analysed individually and only those that had annotations to the cytoplasm and/or mitochondria were included. In this second filtering, the GO terms defined for each cell compartment were considered. This entire filtering process was performed with Excel resources and the QuickGO definitions were used in the decision process for some GO terms.

2.1.4 Development of translation-related proteins PPI network and network analysis

To predict protein interactions (both physical and functional), a **PPI** network was constructed for translation-related proteins identified in sperm. The interactions were identify using the STRING database (https://string-db.org/ - version 10.5). The UniProtKB list containing translation-related proteins was uploaded to "Multiple Protein" tab and all associations between input list and proteins found by STRING were manually checked. Only interactions from "experiments" with an interaction score equal to or greater than 0.7 were considered. The data was downloaded on Jan 5th, 2019. Additionally, the physical associations and direct interactions between the translation-related proteins were also collected from HIPPIE database (http://cbdm-01.zdv.uni-mainz.de/~mschaefer/hippie/network.php). Only interactions between translation-related proteins with a high confidence level (0.72) were considered. The data was downloaded on Jan 9th, 2019. These two tables were imported to Cytoscape (version 3.7.0), originating two independent networks. Cytoscape was also used to import a network for translation-related proteins from IMEx database. The generated network was filtered based on taxonomic names ("*human*") and confidence score intact miscore (> 0.70). After this filtration, only the nodes corresponding to the translation-related proteins were selected and from this selection a subnetwork was created (used in subsequent analysis). Duplicate edges and self-loops of the three previous networks were removed. The three networks were merged ("union" option) in Cytoscape, based on the gene name of each protein. Duplicate edges, self-loops and nodes of the merged network were removed. The topological properties of the merged network (average neighborhood, clustering coefficient, shortest path length and others) were analysed through Network Analyzer tool (189).

Through the ClueGo plug-in (version 2.5.3) and CluePedia, an enrichment analysis was performed in order to identify the specific biological processes and cellular compartment associated with the merged network. It was considered a GO level range between 8 and 12 and only pathways with a p-value <0.01 were considered. For the remaining statistical options, the default parameters were

adopted. For both biological processes and cellular compartments, the data available on Jan 23th, 2019 were considered.

2.2 Results

2.2.1 Translation-related proteins

With the aim to collect all proteins known to date to be involved in the translation process, a search was conducted in AmiGO2, using the accession GO:0006412 ("Translation"), since it entails any cellular metabolic process in which a protein is formed from a sequence of mature mRNA or circRNA, according to the QuickGO definition. The entry GO:0006412 retrieved 1625 annotations, restricted to *Homo sapiens* and *proteins*, however, after excluding all duplicates, the dataset included only 637 different proteins. These 637 proteins were annotated in UniProt database, using Retrieve/ID mapping tool. Only 631 were mapped successfully and, from those, only 623 presented a reviewed status (supplementary table S1). From now on, all references to translation proteins are relative to these 623 proteins.

The translation process can occur in the cytoplasm or in the mitochondrion, each process having a specific set of proteins associated. To determine if these specific proteins were present in sperm cells, an additional search was conducted in AmiGO2 using the accessions GO:0002181 "Cytoplasmic Translation" and GO:0032543 "Mitochondrial translation". Both accessions are considered the most suitable ancestor term to these specific translation processes. The entry GO:0002181 retrieved 139 annotations, restricted to *Homo sapiens* and *proteins*. After excluding duplicates, 96 were successfully mapped on UniProt. Only 93 proteins have a reviewed status and from now on these proteins will be referred as cytoplasmic translation proteins (supplementary table S2). The entry GO:0032543 retrieved 261 annotations. After removing duplicates, 135 different proteins were listed and all of them were mapped successfully with Retrieved/ID mapping tool. All these 135 proteins presented a reviewed status and hereafter will be referred as mitochondrial translation proteins (supplementary table S3) (Table 2.1).

2.2.2 Sperm proteins

A literature search was conducted using the PubMed database to identify human sperm proteomic studies. By merging data from the 42 available papers, a list of 7622 different proteins present in human spermatozoa was created (33,149,158–167,150,168–177,151,178–187,152,188,153–157). From those, 491 proteins were isoforms (supplementary table S4). The remaining 7131 proteins were analysed with Retrieved/ID mapping tool and 7129 proteins were successfully mapped.

Only 6475 proteins presented a reviewed status and henceforth will be referred as sperm proteome (supplementary table S5) (Table 2.1).

Table 2.1 – Summary of the proteins annotated to translation processes and identified in sperm cells. Contains the number of proteins after the removal of duplicates, the total of proteins mapped in the UniProt database and the total of proteins with a reviewed status, that were used in subsequent analysis. (*used for subsequent analysis)

Proteome	Reference	Total Proteins	Total Proteins w/out duplicates	Total Proteins Mapped	Total Proteins reviewed*
Translation	AmiGO2	1625	637	631	623
Cytoplasmic Translation	AmiGO2	139	97	96	93
Mitochondrial Translation	AmiGO2	261	135	135	135
Spermatozoa	(33,149,158–167,150,168–177,151,178–187,152,188,153–157)	7645	7131	7129	6475

2.2.3 Translation-related proteins expressed in spermatozoa

The cross comparison of the sperm proteome with translation-related proteins resulted in a list of 315 different proteins (figure 2.1 A). The sperm proteome was also crossed with mitochondrial translation-related proteins and cytoplasmic translation-related proteins (figure 2.1 B). As shown in figure 2.1 B, there are 38 mitochondrial translation-related proteins and 63 cytoplasmic translation-related proteins in sperm cells. Moreover, this analysis revealed that mitochondrial translation and cytoplasmic translation processes have independent protein machinery. Together, these results indicate that theoretically there is translation machinery present in sperm cells for both mitochondrial and cytoplasmic translation processes.

The 315 translation-related proteins were also crossed with the list of protein isoforms present in sperm. The results show that 21 proteins have one isoform expressed in sperm cells. The list of proteins and respective isoform are described in table 2.2.

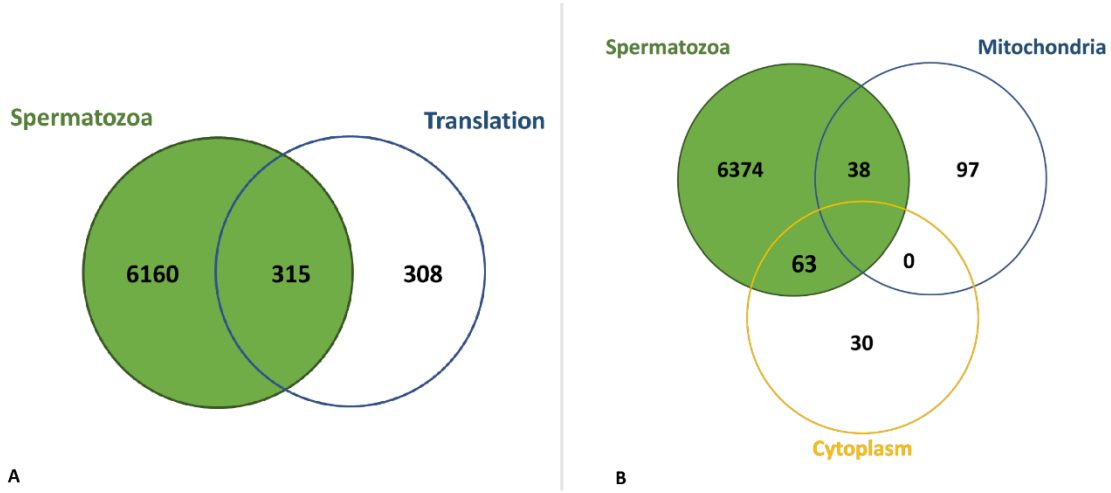

Figure 2.1 – Cross comparation of sperm proteome and translation-related proteins. (**A**) Venn's diagram illustrating common proteins between sperm proteome and translation proteins. Translation: Translation proteins; Sperm: Sperm Proteome. (**B**) Venn's diagram illustrating common proteins between sperm proteome, mitochondrial translation proteins and cytoplasmic translation proteins Cytoplasm: Cytoplasmic Translation proteins; Mitochondria: Mitochondrial Translation Proteins; Spermatozoa: Sperm Proteome

Table 2.2 – Translation proteins and respective isoforms found in spermatozoa.

UniProtKB	Protein Name	Isoform	Isoform Name
O43390	Heterogeneous nuclear ribonucleoprotein R	O43390-2	71 kDa protein
O60506	Heterogeneous nuclear ribonucleoprotein Q	O60506-1	Isoform 1 of Heterogeneous nuclear ribonucleoprotein Q
P05067	Amyloid-beta A4 protein	P05067-1	Isoform APP770 of Amyloid beta A4 protein (Fragment)
P06748	Nucleophosmin	P06748-1	Isoform 1 of Nucleophosmin
P11940	Polyadenylate-binding protein 1	P11940-1	Isoform 1 of Polyadenylate-binding protein 1
P23381	Tryptophan-tRNA ligase, cytoplasmic	P23381-1	Isoform 1 of Tryptophanyl-tRNA synthetase, cytoplasmic
P29692	Elongation factor 1-delta	P29692-2	Isoform 2 of Elongation factor 1-delta
P30050	60S ribosomal protein L12	P30050-1	Isoform 1 of 60S ribosomal protein L12
P34896	Serine hydroxymethyltransferase, cytosolic	P34896-1	Isoform 1 of Serine hydroxymethyltransferase, cytosolic
P40763	Signal transducer and activator of transcription 3	P40763-1	Isoform 1 of Signal transducer and activator of transcription 3
P43897	Elongation factor Ts, mitochondrial	P43897-1	Isoform 1 of Elongation factor Ts, mitochondrial

P54136	Arginine-tRNA ligase, cytoplasmic	P54136-1	Isoform Complexed of Arginyl-tRNA synthetase, cytoplasmic
P55884	Eukaryotic translation initiation factor 3 subunit B	P55884-2	Isoform 2 of Eukaryotic translation initiation factor 3 subunit B
P62847	40S ribosomal protein S24	P62847-1	Ribosomal protein S24 isoform d
P62913	60S ribosomal protein L11	P62913-1	Isoform 1 of 60S ribosomal protein L11
P63241	Eukaryotic translation initiation factor 5A-1	P63241-2	Isoform 2 of Eukaryotic translation initiation factor 5A-1
Q07666	KH domain-containing, RNA-binding, signal transduction-associated protein 1	Q07666-1	Isoform 1 of KH domain-containing, RNA-binding, signal transduction-associated protein 1
Q14103	Heterogeneous nuclear ribonucleoprotein D0	Q14103-1	Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0
Q14240	Eukaryotic initiation factor 4A-II	Q14240-2	Isoform 2 of Eukaryotic initiation factor 4A-II
Q15046	Lysine-tRNA ligase	Q15046-2	Isoform Mitochondrial of Lysyl-tRNA synthetase
Q96J94	Piwi-like protein 1	Q96J94-1	Isoform 1 of Piwi-like protein 1

2.2.4 Characterization of the translation-related proteins present in spermatozoa

To understand the role of 315 proteins identified as being related to the translation process, a GO analysis was performed. Many of these 315 proteins have also been associated with biological processes that do not contribute to the protein translation process at all. So, the biological process analysis was restricted to processes related to translation. Since each protein may be annotated with global or specific terms, three groups were defined: global terms (common to cytoplasmic and mitochondrial processes), cytoplasmic or mitochondrial specific terms (figure 2.2).

Looking for global terms, 33% of translation proteins identified in spermatozoa have a role in translation initiation being this the most represented biological process. Another highly represented biological process is tRNA aminoacylation (9,84%), which corresponds to the process where mature tRNA molecules suffer an acylation and get ready for translation. There are also proteins with a role in translation elongation and termination phases. The percentage of proteins with a positive role in regulation is higher (~12,06%) when compared to those with a negative role (~9,84%). Comparatively, the percentage of proteins annotated for cytoplasmic translation (9,52 %) is bigger than the percentage of proteins annotated for mitochondrial translation (2,54%). Also, most of the proteins with specific annotation for cytoplasmic processes have a role in the initiation phases (GO:0001732, GO:0001731, GO:1902416 and GO:0002188).

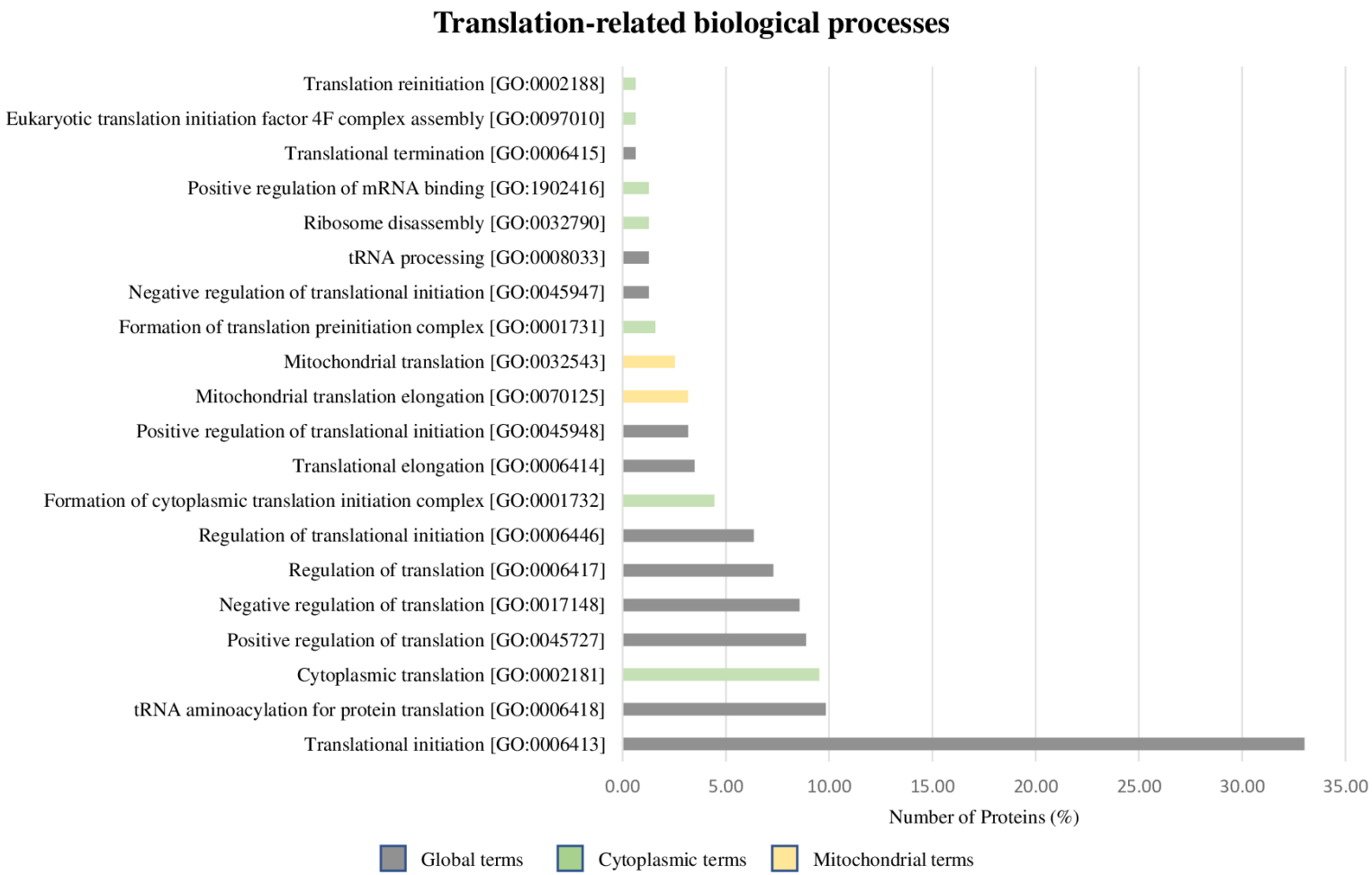

Figure 2.2 – Biological process analysis. Most represented biological processes (top 20) associated with translation-related proteins present in spermatozoa. The green bars represent cytoplasmic specific terms, yellow bars represent cytoplasmic specific terms and grey bars represent global terms. Source: UniProt database.

Another important aspect to be considered is the cellular localization of these proteins (figure 2.3). Approximately 45,71% of the translation proteins present in sperm cells have an annotation for cytoplasm (GO:0005737) and, comparatively, there are only 16,11% of the proteins with an annotation to the mitochondria (GO:0005739). This result was expected given the previously obtained results (figure 2.1 and 2.2). Nevertheless, it was also verified that 7,94% of the proteins have annotations for the mitochondrial matrix (GO:0005759) and 7,62% for mitochondrial inner membrane (GO:0005743). Through the analysis of figure 2.3, it is possible to infer that the annotations for the cytoplasmic ribosome (GO:0005840) and its subunits (GO:0022625 and GO:0022627) are among the most common. Another common location is the nucleus (GO:0005634) for which 42,86% of the proteins were annotated.

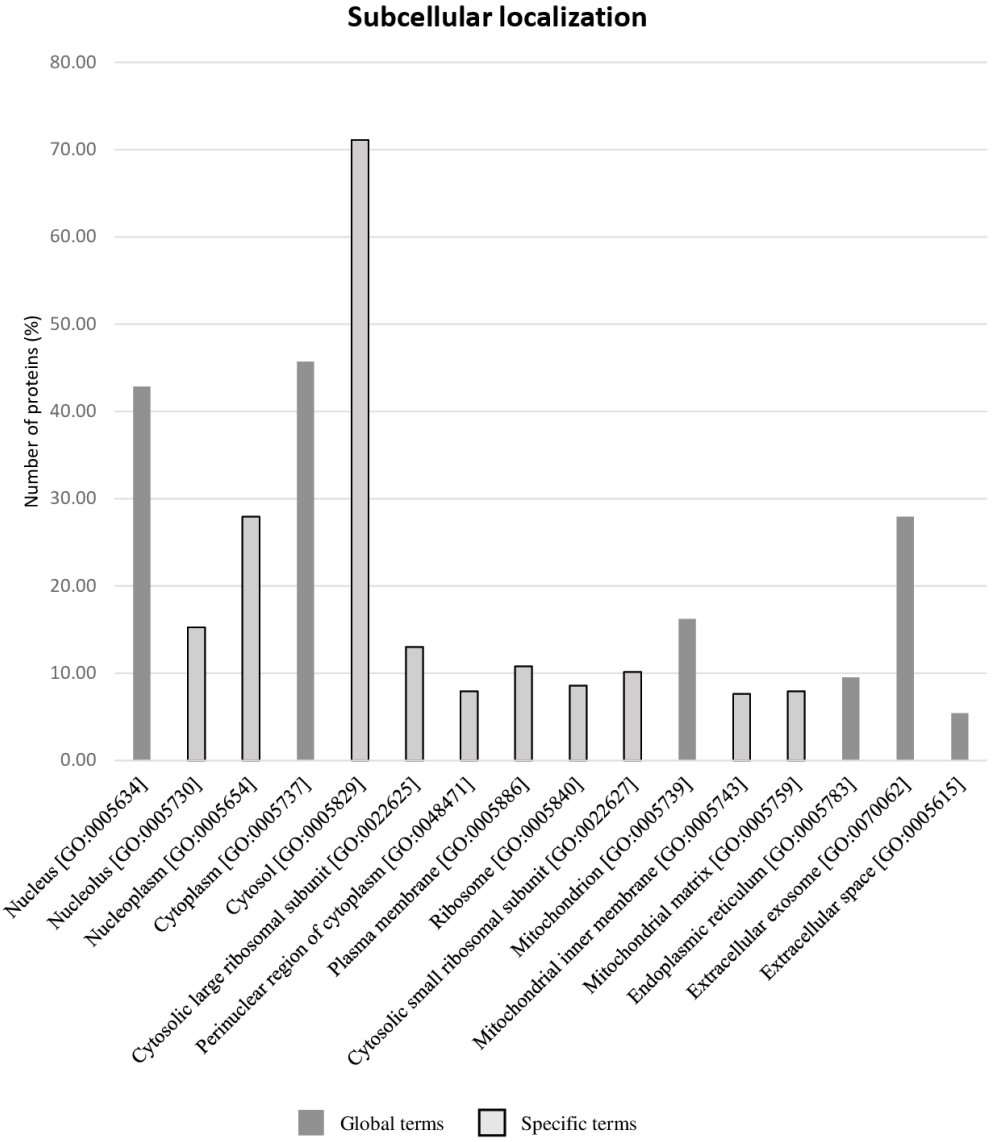

Figure 2.3 – **Cellular compartment analysis.** The most common (top 15) subcellular localizations associated with translation-related proteins present in spermatozoa. The dark grey represents global GO terms and the light grey represents the more specific GO terms. Source: UniProt Database.

Curiously, the most common subcellular locations include the nucleoplasm (GO:0005737), the endoplasmic reticulum (GO:0005787) and annotations for extracellular components (GO:0070062 and GO:0005615). However, the first two organelles are absent in sperm cells and these cells do not have secretory functions. To understand if the proteins were annotated for these three subcellular locations, a Venn diagram analysis was performed. As major compartments were considered the following: nucleus, cytoplasm, mitochondria, endoplasmic reticulum and extracellular compartment. As shown in figure 2.4, most of the proteins have annotations for more than one compartment and only 89 proteins have a unique location for one of the considered compartments. Only two proteins (UniProtKB: P62341 and Q8WU17) have exclusive annotations for endoplasmic reticulum, so it is plausible to hypothesize that these proteins occupy a different subcellular location in spermatozoa. One protein was identified only in the extracellular compartment (UniProtKB: Q9Y450). However, it has been found that this protein also has annotations for components of the intracellular space (GO:0030014, GO:0005622 and GO:0016020).

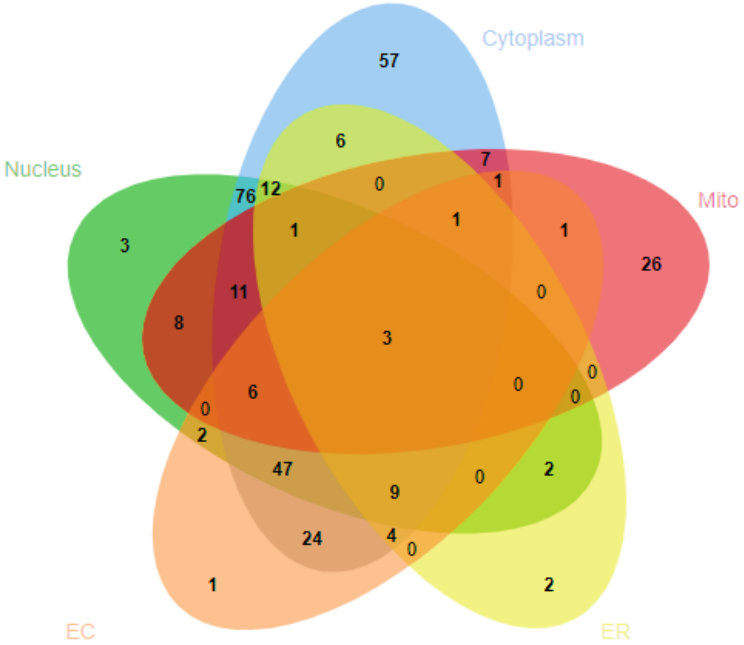

Figure 2.4 – **Common proteins to cellular compartments.** Venn diagram illustrating common translation-related proteins present in sperm between the five major compartments taken in consideration: nucleus, cytoplasm, mitochondria (Mito), endoplasmic reticulum (ER) and extracellular compartment (EC).

Finally, the 315 proteins were filtered to identify proteins exclusively linked to translation. First, only proteins that have exclusive annotations for translation-related processes were selected. A total of 46 proteins that fulfilled this requirement were identified. These 46 proteins were further filtered according to their cellular location. As translation only occurs in two cellular compartments, cytoplasm and mitochondria, proteins were selected if annotated only for these two compartments. From this selection, 31 proteins were identified (table 2.3). This analysis revealed that in sperm cells there is a total of 31 proteins linked exclusively to the translation process and that are simultaneously in the correct location for its occurrence.

Table 2.3 – Proteins exclusively linked to translation found in spermatozoa.

UniProtKB	Gene name	Protein Name	Biological Process	Celular Compartment
A2RTX5	TARSL2	Threonine-tRNA ligase 2, cytoplasmic	threonyl-tRNA aminoacylation [GO:0006435]	cytoplasm [GO:0005737]
B5ME19	EIF3CL	Eukaryotic translation initiation factor 3 subunit C-like protein	formation of cytoplasmic translation initiation complex [GO:0001732] translational initiation [GO:0006413]	eukaryotic 43S preinitiation complex [GO:0016282] eukaryotic 48S preinitiation complex [GO:0033290] eukaryotic translation initiation factor 3 complex [GO:0005852]
O43432	EIF4G3	Eukaryotic translation initiation factor 3 subunit C-like protein	regulation of translational initiation [GO:0006446]	cytosol [GO:0005829] eukaryotic translation initiation factor 4F complex [GO:0016281]
O60841	EIF5B	Eukaryotic translation initiation factor 5B	regulation of translational initiation [GO:0006446]	cytoplasm [GO:0005737] cytosol [GO:0005829]
O75822	EIF3J	Eukaryotic translation initiation factor 3 subunit J	formation of cytoplasmic translation initiation complex [GO:0001732] translational initiation [GO:0006413]	cytosol [GO:0005829] eukaryotic 43S preinitiation complex [GO:0016282] eukaryotic 48S preinitiation complex [GO:0033290] eukaryotic translation initiation factor 3 complex [GO:0005852]

O95363	FARS2	Phenylalanine-tRNA ligase, mitochondrial	phenylalanyl-tRNA aminoacylation [GO:0006432] tRNA aminoacylation for protein translation [GO:0006418] tRNA processing [GO:0008033]	mitochondrial matrix [GO:0005759] mitochondrion [GO:0005739]
P23588	EIF4B	Eukaryotic translation initiation factor 4B	eukaryotic translation initiation factor 4F complex assembly [GO:0097010] formation of translation preinitiation complex [GO:0001731] regulation of translational initiation [GO:0006446] translational initiation [GO:0006413]	cytosol [GO:0005829] eukaryotic translation initiation factor 4F complex [GO:0016281] polysome [GO:0005844]
P26640	VARS	Valine-tRNA ligase	tRNA aminoacylation for protein translation [GO:0006418] valyl-tRNA aminoacylation [GO:0006438]	cytosol [GO:0005829]
P47813	EIF1AX	Eukaryotic translation initiation factor 1A, X-chromosomal	translational initiation [GO:0006413]	cytosol [GO:0005829]
P49589	CARS	Cysteine-tRNA ligase, cytoplasmic	cysteinyl-tRNA aminoacylation [GO:0006423] tRNA aminoacylation for protein translation [GO:0006418]	cytoplasm [GO:0005737] cytosol [GO:0005829]
P82650	MRPS22	28S ribosomal protein S22, mitochondrial	mitochondrial translational elongation [GO:0070125] mitochondrial translational termination [GO:0070126]	mitochondrial inner membrane [GO:0005743] mitochondrial ribosome [GO:0005761] mitochondrial small ribosomal subunit [GO:0005763] mitochondrion [GO:0005739]

P82663	MRPS25	28S ribosomal protein S25, mitochondrial	mitochondrial translational elongation [GO:0070125] mitochondrial translational termination [GO:0070126]	mitochondrial inner membrane [GO:0005743] mitochondrion [GO:0005739] ribosome [GO:0005840]
P82909	MRPS36	28S ribosomal protein S36, mitochondrial	mitochondrial translational elongation [GO:0070125] mitochondrial translational termination [GO:0070126] translation [GO:0006412]	mitochondrial inner membrane [GO:0005743] mitochondrial oxoglutarate dehydrogenase complex [GO:0009353] mitochondrial small ribosomal subunit [GO:0005763] mitochondrion [GO:0005739]
P82921	MRPS21	28S ribosomal protein S21, mitochondrial	mitochondrial translation [GO:0032543] mitochondrial translational elongation [GO:0070125] mitochondrial translational termination [GO:0070126] translation [GO:0006412]	mitochondrial inner membrane [GO:0005743] mitochondrial small ribosomal subunit [GO:0005763]
P82930	MRPS34	28S ribosomal protein S34, mitochondrial	mitochondrial translation [GO:0032543] mitochondrial translational elongation [GO:0070125] mitochondrial translational termination [GO:0070126]	mitochondrial inner membrane [GO:0005743] mitochondrial small ribosomal subunit [GO:0005763] mitochondrion [GO:0005739]
Q15031	LARS2	Probable leucine-tRNA ligase, mitochondrial	leucyl-tRNA aminoacylation [GO:0006429] mitochondrial translation [GO:0032543] tRNA aminoacylation for protein translation [GO:0006418]	mitochondrial matrix [GO:0005759] mitochondrion [GO:0005739]

Q5T160	RARS2	Probable arginine-tRNA ligase, mitochondrial	arginyl-tRNA aminoacylation [GO:0006420] mitochondrial translation [GO:0032543] tRNA aminoacylation for protein translation [GO:0006418]	mitochondrial matrix [GO:0005759] mitochondrion [GO:0005739]
Q7L2H7	EIF3M	Eukaryotic translation initiation factor 3 subunit M	cytoplasmic translational initiation [GO:0002183] formation of cytoplasmic translation initiation complex [GO:0001732] translational initiation [GO:0006413]	cytosol [GO:0005829] eukaryotic 43S preinitiation complex [GO:0016282] eukaryotic 48S preinitiation complex [GO:0033290] eukaryotic translation initiation factor 3 complex [GO:0005852] eukaryotic translation initiation factor 3 complex, eIF3m [GO:0071541]
Q7L3T8	PARS2	Probable proline-tRNA ligase, mitochondrial	prolyl-tRNA aminoacylation [GO:0006433]	mitochondrial matrix [GO:0005759]
Q969S9	GFM2	Ribosome-releasing factor 2, mitochondrial	mitochondrial translation [GO:0032543] mitochondrial translational termination [GO:0070126] ribosome disassembly [GO:0032790] translational elongation [GO:0006414]	mitochondrial matrix [GO:0005759]
Q96DV4	MRPL38	39S ribosomal protein L38, mitochondrial	mitochondrial translational elongation [GO:0070125] mitochondrial translational termination [GO:0070126]	mitochondrial inner membrane [GO:0005743] mitochondrial large ribosomal subunit [GO:0005762] mitochondrion [GO:0005739]

Q96GW9	MARS2	Methionine-tRNA ligase, mitochondrial	methionyl-tRNA aminoacylation [GO:0006431] tRNA aminoacylation for protein translation [GO:0006418]	mitochondrial matrix [GO:0005759]
Q99613	EIF3C	Eukaryotic translation initiation factor 3 subunit C	formation of cytoplasmic translation initiation complex [GO:0001732] positive regulation of mRNA binding [GO:1902416] positive regulation of translation [GO:0045727] translational initiation [GO:0006413]	cytosol [GO:0005829] eukaryotic 43S preinitiation complex [GO:0016282] eukaryotic 48S preinitiation complex [GO:0033290] eukaryotic translation initiation factor 3 complex [GO:0005852]
Q9BQ48	MRPL34	39S ribosomal protein L34, mitochondrial	mitochondrial translational elongation [GO:0070125] mitochondrial translational termination [GO:0070126] translation [GO:0006412]	mitochondrial inner membrane [GO:0005743] mitochondrial large ribosomal subunit [GO:0005762] mitochondrial ribosome [GO:0005761] mitochondrion [GO:0005739]
Q9BW92	TARS2	Threonine-tRNA ligase, mitochondrial	mitochondrial threonyl-tRNA aminoacylation [GO:0070159]	mitochondrial matrix [GO:0005759] mitochondrion [GO:0005739]
Q9BYJ9	YTHDF1	YTH domain-containing family protein 1	positive regulation of translation [GO:0045727] positive regulation of translational initiation [GO:0045948]	cytoplasm [GO:0005737]
Q9BZE1	MRPL37	39S ribosomal protein L37, mitochondrial	mitochondrial translational elongation [GO:0070125] mitochondrial translational termination [GO:0070126]	mitochondrial inner membrane [GO:0005743] mitochondrial large ribosomal subunit [GO:0005762]

			translation [GO:0006412]	mitochondrial ribosome [GO:0005761] mitochondrion [GO:0005739]
Q9NSE4	IARS2	Isoleucine-tRNA ligase, mitochondrial	isoleucyl-tRNA aminoacylation [GO:0006428] tRNA aminoacylation for protein translation [GO:0006418]	cytosol [GO:0005829] mitochondrial matrix [GO:0005759] mitochondrion [GO:0005739]
Q9NWS8	RMND1	Required for meiotic nuclear division protein 1 homolog	positive regulation of mitochondrial translation [GO:0070131] translation [GO:0006412]	mitochondrion [GO:0005739]
Q9NYK5	MRPL39	39S ribosomal protein L39, mitochondrial	mitochondrial translational elongation [GO:0070125] mitochondrial translational termination [GO:0070126]	mitochondrial inner membrane [GO:0005743] mitochondrial large ribosomal subunit [GO:0005762] mitochondrial ribosome [GO:0005761] mitochondrion [GO:0005739]
Q9UGC7	MTRF1L	Peptide chain release factor 1-like, mitochondrial	mitochondrial translational termination [GO:0070126]	mitochondrial matrix [GO:0005759] mitochondrion [GO:0005739]

2.2.5 PPI network analysis

To build the PPI network, the interactions of translation-related proteins identified in spermatozoa were retrieved from three databases: STRING, HIPPIE and IMEx. Table 2.4 summarizes the number of proteins that were retrieved in each database and the number of interactions between them (according to the selected parameters).

Table 2.4 – Number of proteins retrieved in each database and the number of interactions between them.

Number	STRING	HIPPIE	IMEx	Merged
Proteins	204	238	313	313
Interactions	2926	1051	619	3310

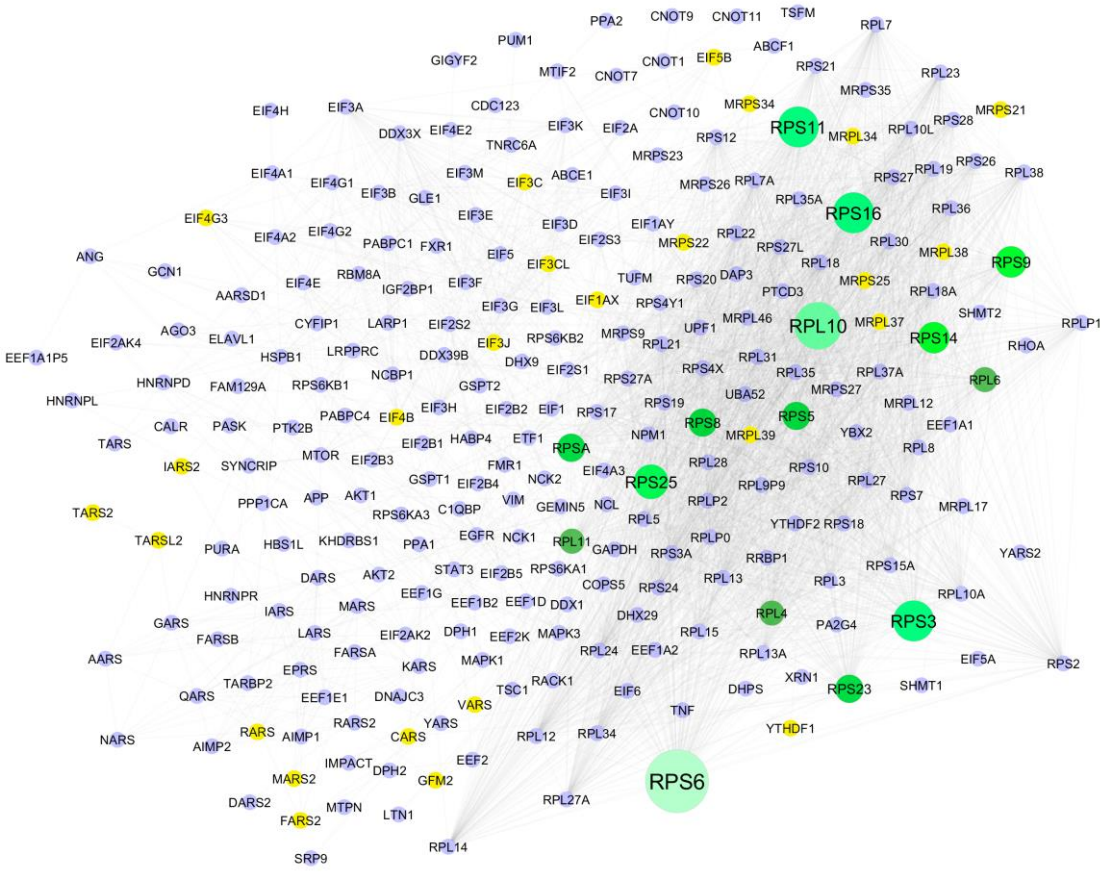

Figure 2.5 – PPI network of translation-related proteins present in spermatozoa. This network results from the merge of the three distinct networks created from the accessed databases (HIPPIE, STRING and IMEx). Each node represents a different protein. The blue nodes represent proteins with a degree less than 75. The green nodes correspond to proteins with a degree equal or greater than 75 (dark green and smaller sizes to degrees closer to 75). The yellow nodes represent the proteins linked exclusivity to translation

present in spermatozoa. Only the connected nodes are represented (N=265). Proteins are represented by the gene name.

The three networks were merged to give rise to a single network (figure 2.5). The total number of proteins in the merged network is N=313 of which 48 correspond to isolated nodes. The total number of interactions between the proteins is L=3,310. The average number of neighbors of a node in the network – averaged connectivity – is <q>= 21.15 with the power law connectivity distribution, $P(q) \sim q^{-\gamma}$, where is the degree exponent. The characteristic path length, which represents the average shortest path length between two nodes, is l=2.758 and the network diameter is 8. The network diameter corresponds to the longest distance between two nodes. So, the merged network has a spare structure with hubs (highlighted with green colours in figure 2.5). The clustering coefficient, which characterizes the degree to which neighbouring nodes in a network tend to cluster together, is C=0.402. If all of them are tightly connected to each other, then they form a clique and the clustering coefficient is 1. On the other hand, for a spare random uncorrelated network of finite size N, this coefficient is close to zero (C=<q>/N ~0.02). It should be noted that the value C=0.402 is about twenty times bigger than the clustering coefficient expected to a random network.

To determine the most relevant biological processes and cellular compartments associated with the merged network, a ClueGO plus CluePedia enrichment analysis was performed. In GO tree terms, the upper terms (GO level range: 1-4) represent more global processes with many associated genes and the lower terms (GO level range: 7-15) correspond to more detailed processes with a few associated genes (190,191). Therefore, the GO level range was defined between 8 and 12 aiming to identify more detailed terms. It is important to note that within the enrichment networks the terms are connected based on kappa score. This measure compares the number of genes shared between two terms and allows the definition of functional groups. Terms of the same group have the same colour and the most significant term defines the group name (191,192). The biological processes associated with the merged network can be observed in figure 2.6. A total of 104 different biological processes were identified. The most significant biological processes annotations among the proteins in the merged network were translation initiation (44,73%; p=2,31E-193), regulation of translation (48,56%; p=3,34E-168) and nuclear-transcribed mRNA catabolic process, nonsense-mediated decay (25,56%; p=2,78E-99). The enrichment analysis also revealed that the 12 most significant biological processes associated with the merged network are translation-related processes. The cytoplasmic translation is the seventh biological process with the most significant association (p=2,94E-90), presenting 69 associated

proteins. On the other hand, the mitochondrial translation appears in the sixteenth position (p=1,87E-36) counting only with 28 associated proteins. In enrichment analysis for cellular compartment, 21 different terms were identified (figure 2.7). The most significative terms are ribosomal subunit (30,99%; p=1,00E-114), cytosolic ribosome (26,52%; p=2,23E-111) and cytosolic part (27,48%; p=1,23E-80). Noteworthy, the cytoplasmic regions are preferably associated with the merged network.

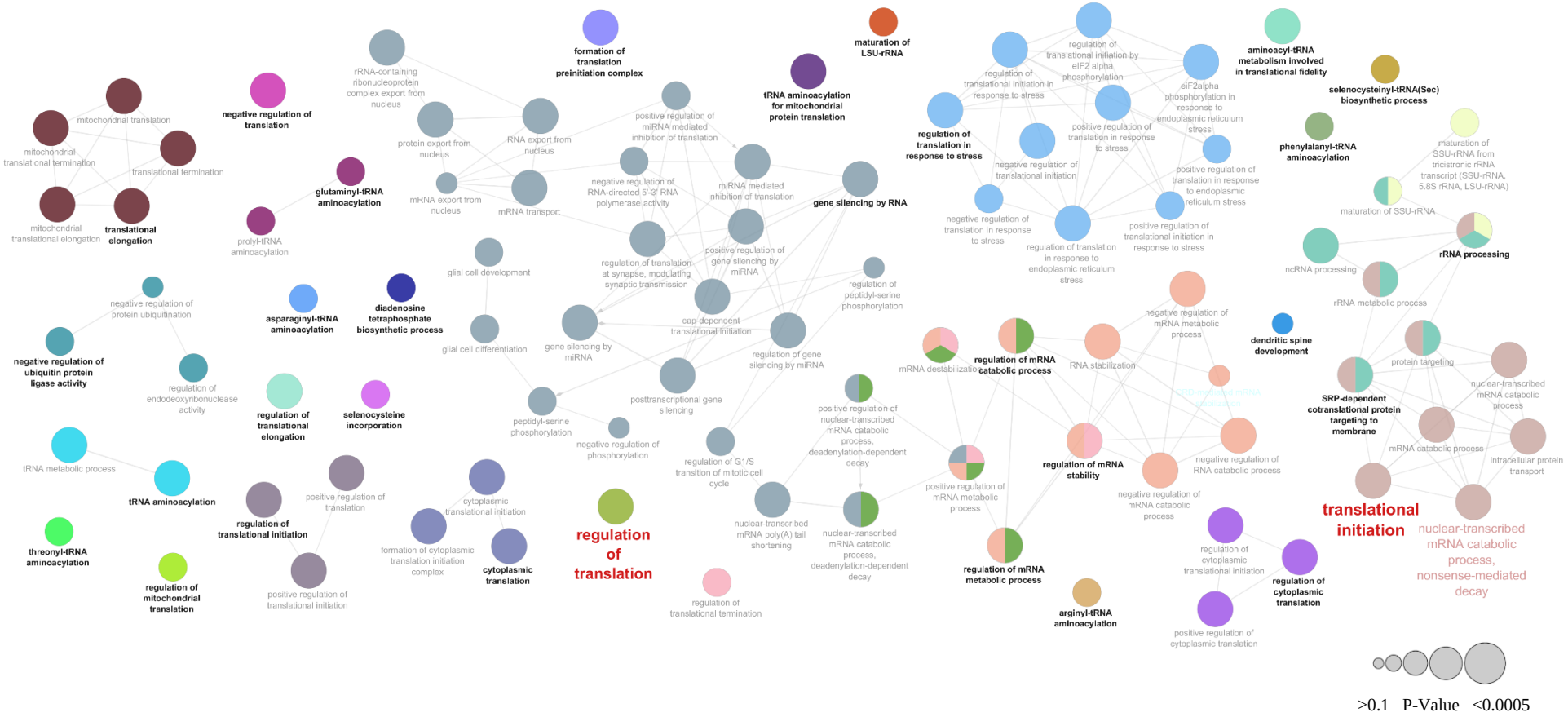

Figure 2.6 – **Biological processes enrichment analysis**. Network view for biological processes corresponding to the translation-related proteins include in the merged network, after applying the defined parameters on ClueGO. Terms are functionally grouped based on Kappa Score and are illustrated with different colours. The most significant term defines the name of the group (label black and bold). The red labels correspond to the three most significant biological processes.

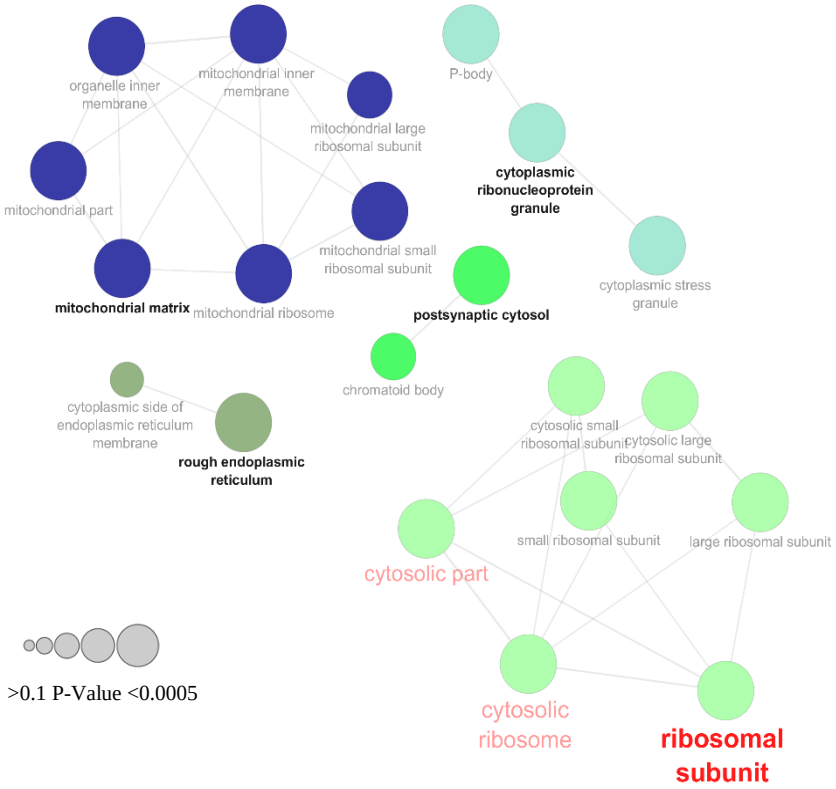

Figure 2.7 – Cellular compartment enrichment analysis. Network view for the cellular compartment corresponding to the translation-related proteins include in the merged network, after applying the defined parameters on ClueGO. Terms are functionally grouped based on Kappa Score and are illustrated with different colours. The most significant terms define the name of the group (label black and bold). The red labels correspond to the three most significant terms of cellular compartments.

3 Experimental Approach

3.1 Methods

The experimental procedures were performed at the Signal Transduction Laboratory, Institute for Biomedicine (iBiMED), University of Aveiro (Aveiro, Portugal). The details of the solutions used throughout the experimental steps are stated in the supplementary table 6.

3.1.1 Cell culture

PNT-2 cells were obtained from (217). The cells were maintained in RPMI 1640 (GIBCO BRL Life Technologies, Rockville, MD) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO BRL Life Technologies, Rockville, MD) and antibiotics (1% penicillin/streptomycin mixture). Cells were grown on 100 mm plates at 37ºC in a 5% CO_2 humidified incubator.

3.1.2 Sperm samples preparation

Fresh samples of bovine semen were obtained from LusoGenes, Lda (Aveiro, Portugal). The spermatozoa were isolated from the seminal plasma and washed three times by centrifugation (600g for 10 min at room temperature (RT)) using 1x Phosphate Buffered Saline (PBS) at 37ºC. Pellet was re-suspended in Sperm Preparation Medium (SPM) (Origio, Denmark) to a final desired concentration and incubated at 37ºC with 5% CO_2 for 3 hours. All microscopy analyses were performed using a Zeiss Primo Star microscope (Carl Zeiss AG, Germany). The concentration of sperm cells after the washing procedures was assessed using the Sperm Class Analyzer CASA System (Microptic S L, Barcelona, Spain) with SCA® v6.2.0.16 software. The samples (2 µL) were loaded into individual chambers of Leja Standard Count 8 chamber slide [20 µm depth] (Leja Products B. V., The Netherlands) which were preheated at 37ºC. Samples from different bovine individuals were used.

3.1.3 Cell viability assay

PNT-2 cells and bovine spermatozoa viabilities were measured using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation (Promega, Madison, USA) according to manufacturer's guidelines. The absorbance was measured at 490 nm using an Infinite® 200 PRO spectrophotometer

(Tecan, Switzerland). The reduction of tetrazolium compounds has been previously been used as a reliable and rigorous assessment of spermatozoa viability (193).

3.1.4 Sperm motility assay

The sperm motility parameters were assessed using the Sperm Class Analyzer CASA System (Microptic S L, Barcelona, Spain) with SCA® v5.4 software. Samples and controls (2 μL) were loaded into individual chambers of Leja Standard Count 8 chamber slide [20 μm depth] (Leja Products B. V., The Netherlands) which were preheated at 37ºC. This temperature was maintained while at least 500 sperm cells per measurement were evaluated.

3.1.5 Surface Sensing of Translation (SUnSET)

The SUnSET method allows to evaluate the translation activity in cells through the incorporation of tRNA analogue puromycin in the nascent peptide chains (194). Both types of cells, PNT-2 and bovine spermatozoa, were incubated with puromycin or with puromycin and a translation inhibitor. Cycloheximide was used as a cytoplasmic translation inhibitor (195) and D-chloramphenicol was used as a mitochondrial translation inhibitor (196). Each condition was performed in triplicate in independent assays.

3.1.5.1 PNT-2 cells assay

PNT-2 cells were used as a positive control, since the translation process is highly described in this type of cells and puromycin incorporation would occur in them. For each condition, 1 x 10^6 cells were incubated for 15 min with 1 μg/mL of puromycin at 37ºC in 5% CO_2 humidified atmosphere. Cells treated with translation inhibitors were previously incubated for 5 min with 5 μg/mL of cycloheximide or 0,1 mg/mL of D-chloramphenicol at 37ºC in 5% CO_2 humidified atmosphere. After this 5-minute incubation, puromycin was added. The medium was removed, and cells were washed with 2 mL of 1x PBS. Trypsin (Trypsin-EDTA (0.05%), phenol red, Gibco™) (500 μL) was added and incubated for 3 min at 37ºC in 5% CO_2 humidified atmosphere. After inhibition of trypsin, 100 μL of cell suspension was collected to a microtube for cell viability assay (see section 3.1.3). The remaining cells were transferred to a different tube and centrifuged at 1000 rpm for 3 min. The supernatant was removed after centrifugation and the cells were subsequently used for flow cytometry analysis (further details in section 3.1.5.3).

3.1.5.2 Bovine spermatozoa assay

Bovine spermatozoa (40 x 10^6) were capacitated for 3 hours in SPM and then incubated for 15 min with puromycin 7,5 μg/mL at 37ºC in 5% CO_2 humidified atmosphere. Spermatozoa treated with translation inhibitors were previously incubated for 5 min with 5 μg/mL of cycloheximide or 0,1 mg/mL of D-chloramphenicol at 37ºC in 5% CO_2 humidified atmosphere. After this 5-minute incubation, puromycin was added. For cell viability assay (see section 3.1.3) and motility assay 110 μL of cell suspension was collected to a microtube. The remaining sperm cells were centrifuged 5 min at 500g and the supernatant was discarded. The spermatozoa were washed with by resuspension in 1x PBS, centrifuged (500g, 5 min) and supernatant discarded, and ultimately diluted in 1x PBS for subsequent flow cytometry analysis (further details in section 3.1.5.3).

3.1.5.3 Flow cytometry analysis

Cell suspension was centrifuged at 300g for 6 min and the supernatant was discarded. Then, cells were fixed in 400 μL of 4% paraformaldehyde (PFA). The pellet was resuspended and incubated for 15 min at RT. Cells were harvested by centrifugation at 4ºC (300g, 6 min). The supernatant was discarded, and the cells were washed three times with 400 μL of cold 1x PBS (in each wash cell suspension was centrifuged at 300g for 6 min and supernatant discarded). PNT-2 cells were resuspended in 100 μL of fluorescence activated cell sorting (FACS) solution with 0,1% saponin. Bovine spermatozoa were resuspended in 150 μL of FACS solution with 0,1% saponin. The cell suspension was transferred to a 96 round bottom well plates (Thermo Fisher Scientific) (50 μL per well). The plate was centrifuged at 4ºC (400g, 3 min), the supernatant was discarded and to the plate were added 50 μL of anti-puromycin Alexa488 conjugate (clone 12D10, Millipore, USA) diluted (1:100) in FACS solution. The unstained control was incubated only with 50 μL of FACS solution. In the third well of bovine spermatozoa was added 50 μL of anti-IgG2a Alexa488 (clone MOPC-173, Thermo Fisher Scientific) (anti-isotype control) diluted (1:40) in FACS solution. Cells were resuspended and incubated 30 min on ice in the dark. Then 100 μL of FACS solution was added per well, resuspended, centrifuged at 4ºC (300g, 3 min) and the supernatant discarded. Cells were washed twice with 150 μL of cold FACS solution and once with 150 μL of cold 1x PBS. Finally, the pellet was resuspended in 200 μL of cold 1x PBS, filtered and the FACS analysis was carried out. Events were collected on a BD Accuri™ C6 Cytometer and the data was acquired and analysed using BD Accuri™ C6 software (BD Biosciences).

3.1.6 Western Blot analysis

3.1.6.1 PNT-2 cells lysates

PNT-2 cells (0,5 x 10^6 cells) were incubated with 1 μg/mL of puromycin for 15 min at 37ºC in a 5% CO_2 humidified atmosphere. PNT-2 cells were also incubated with CH (5 μg/mL) or with D-CP (0,1 mg/mL) for 5 min followed by incubation with puromycin (1 μg/mL) for 15 min at 37ºC in a 5% CO_2 humidified atmosphere. Negative controls were performed in the absence of puromycin, CH or D-CP. The medium was removed, and cells were washed with 2mL of 1x PBS. Trypsin (500 μL) was added and incubated for 3 min at 37ºC in 5% CO_2 humidified atmosphere. Cells were collected to a tube and centrifuged at 1000 rpm for 3 min. The supernatant was removed after centrifugation and cells were subsequently lysed with 50 μL of 1% Sodium Dodecyl Sulfate (SDS) on ice for 15 min. The PNT-2 cells were centrifuged at 4ºC (16000g for 5 min) and the supernatant was kept.

3.1.6.2 Bovine spermatozoa lysates

Bovine spermatozoa (30 x 10^6 cells) were incubated with different concentrations of puromycin (5 μg/mL; 7,5 μg/mL or 10 μg/mL) for 15 min at 37ºC in a 5% CO_2 humidified atmosphere. Bovine spermatozoa were also incubated with CH (5 μg/mL) or with D-CP (0,1 mg/mL) for 5 min at 37ºC in a 5% CO_2 humidified atmosphere. After these treatments, puromycin (7,5 μg/mL) was incubated for 15 min at 37ºC in a 5% CO_2 humidified atmosphere. Negative controls were performed in the absence of puromycin, CH or D-CP. The sperm cells were centrifuged for 5 min at 500g and the supernatant was discarded. Cells were washed twice by resuspension in 1x PBS (centrifuged for 5 min, 500g) and subsequently lysed with 50 μL of 1% SDS on ice for 15 min. The sperm cells were ultimately centrifuged at 4ºC (16000g for 4 min) and the supernatant was kept.

3.1.6.3 Bicinchoninic acid (BCA) assay

Lysates were mass normalized using the bicinchoninic acid (BCA) assay (Fisher Scientific, Loures, Portugal). Samples were prepared by adding 5 μL of each sample plus 20 μL of 1% SDS in a 96-well plate. Standard protein concentrations were prepared as described in Supplementary Table 7. Samples were prepared in duplicate. The Working Reagent (WR) was prepared by mixing BCA reagent A with BCA reagent B in the proportion of 50:1. Then, 200 μl of WR was added to each well (standards and samples) and the plate was incubated at 37ºC for 30 min. Once the 96-well cooled to RT, the absorbance was spectrophotometrically measured at 562 nm using an Infinite® 200 PRO

(Tecan, Switzerland). A standard curve was obtained by plotting BSA standard absorbance *vs* BSA concentration and used to determine the total protein concentration of each sample.

3.1.6.4 Western blotting

The cells lysates were resolved by a 10% SDS-polyacrylamide gel electrophoresis (PAGE) run. Proteins were electrotransferred onto nitrocellulose membranes. The gel was run at 200 V and electrotransferred at 200 mA for 2 hours. For loading control, the membrane was incubated with Ponceau S solution (Sigma Aldrich, 0.1% [w/v] in 5% acetic acid) for 15 min at RT with slow agitation. The membranes were washed with distilled water until the protein bands were well defined and analysed using densitometer GS-800. To remove the staining, the membrane was washed with 1x Tris-buffered saline containing 0,1% of Tween 20 (TBST). Pixel intensity was quantified using Image Studio Lite software (version 5.2.5). The intensity from an empty lane within the membrane was subtracted from all signals and all data were normalized to the Ponceau control. Then, non-specific protein-binding sites on membrane were blocked with 5% non-fat dry milk solution in 1x TBST at RT for 1 hour. The membranes were incubated with primary antibody for 1 hour at RT (mouse anti-puromycin Alexa488 conjugated, diluted 1:25000 in 5% non-fat dry milk solution in 1x TBST). After the incubation, membranes were washed two times for 10 min with 1x TBST. Then, the membranes were incubated with secondary antibody for 1 hour at RT (IRDye anti-mouse, diluted 1:10000 in 5% non-fat dry milk solution in 1x TBST). After the incubation, membranes were washed two times for 10 min with 1x TBST and immunodetected using Odyssey Infrared Imaging System (LI-COR® Biosciences, US).

3.1.7 Immunoprecipitation of puromycin-peptides

3.1.7.1 Cells extracts for IP

PNT-2 cells (1,0 x 10^6 cells) were incubated with 1 µg/mL of puromycin for 15 min at 37ºC in a 5% CO_2 humidified atmosphere. The medium was removed, and cells were washed with 2mL of 1x PBS. Trypsin (500 µL) was added and incubated for 3 min at 37ºC in 5% CO_2 humidified atmosphere. Cells were transferred to a tube and centrifuged at 1000 rpm for 3 min. Bovine spermatozoa (30 x 10^6 cells) were incubated with 7,5 µg/mL of puromycin for 15 min at 37ºC in a 5% CO_2 humidified atmosphere. The spermatozoa were centrifuged for 5 min at 500g and the supernatant was discarded. Cells were washed twice by resuspension in 1x PBS, centrifuged at 5 min, 500g and supernatant discarded. For both type of cells, negative controls were performed in the absence of puromycin.

For immunoprecipitation assay, both PNT-2 cells and spermatozoa were lysed in 1x RIPA buffer (500 µL) (0.5 M Tris-HCL, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) (Millipore Iberica S.A.U, Madrid, Spain) supplemented with protease (10 mM benzamidine, 1.5 µM aprotinin, 5 µM pepstain A, 2 µM leupeptin, 1 mM PMSF) and phosphatase inhibitors (1 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 50 mM beta-glycerophosphate, 1 mM sodium orthovanadate) for 30 min on ice and sonicated three times for 5 sec on ice. Then, the samples were centrifuged at 16000g for 15 min at 4ºC. A major part of the of the supernatant (approx. 90%) was used for the subsequent steps (soluble fraction). The remaining 10% of the soluble fraction was saved and the pellet was re-suspended in 50 µL of 1% SDS (insoluble fraction).

3.1.7.2 Indirect immunoprecipitation

RIPA supernatant lysates were pre-cleared using 10 µL of Dynabeads® Protein G (life Technologies AS., Madrid, Spain) per condition, during 30 min with rotation at 4º C. The pre-cleared extracts were collected for new microtubes and an indirect immunoprecipitation approach was performed using 1 µg of anti-Puromycin (clone 12D10, Millipore) or 1 µg of anti-IgG2a Alexa488 (clone MOPC-173, Thermo Fisher Scientific) (only for sperm extracts) incubated overnight with rotation at 4ºC. After the incubation, 50 µL of beads were added per condition and incubated 1 hour with rotation at 4ºC. Then, the supernatant was removed to a new microtube and stored (unbound IP fraction) and the beads were washed two times with 500 µL 1x PBS during 10 min with rotation at 4ºC. Then, the beads were re-suspended in 1x loading buffer (30 µL) and boiled for 5 min. The eluted fraction was then collected (IP fraction).

3.1.7.3 Western blot analysis of the IP samples

As a technical control of the IP protocol, the 10% of soluble fractions and insoluble fractions; unbound IP fractions; and 15% of IP fractions were resolved by a 10% SDS-polyacrylamide gel electrophoresis (PAGE) run. Proteins were electrotransferred onto nitrocellulose membranes. The western blotting analysis was performed as described in section 3.1.6.3. The IP fractions were resolved by a 10% SDS-polyacrylamide gel electrophoresis (PAGE) run. For loading control, the gel was incubated with Coomassie Brilliant Blue solution overnight at RT with slow agitation. The gel was washed with 20% methanol solution until the protein bands were well defined for further analysis using densitometer GS-800.

3.1.8 Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistic Software 25. First, the Shapiro-Wilk test and the Levene's test were performed to validate the assumptions required by the parametric tests. Since these were not validated, a nonparametric method was used: (i) Kruskal-Wallis H Test, for comparisons between more than two independent conditions; (ii) Mann-Whitney U Test for comparisons between two independent conditions. The significance level was set at 0.05.

3.2 Results

3.2.1 Strategy for assessment of translation in bovine spermatozoa

To assess the translational activity in bovine spermatozoa, the surface sensing of translation (SUnSET), a nonradioactive fluorescence-activated cell sorting-based assay, was used. The SUnSET allows the direct monitorization and quantification of protein synthesis in individual cells from a heterogeneous cell population (194). Contrary to traditional radioactive methods, the SUnSET uses puromycin, an aminonucleoside antibiotic produced by *Streptomyces alboniger*, that is a structural analogous of amino acylated-tRNA (aa-tRNA). During the elongation phase of translation, the puromycin molecule enters in the A-site of the ribosome, is incorporated into the nascent polypeptide through a peptide bond and prevents the elongation, inducing the premature chain release (fig. 3.1) (197,198). When used in minimal concentrations (< 10 µg/mL), puromycin incorporation in newly proteins chains may be used as a direct measure of the rate of mRNA translation *in vitro* (199–201).

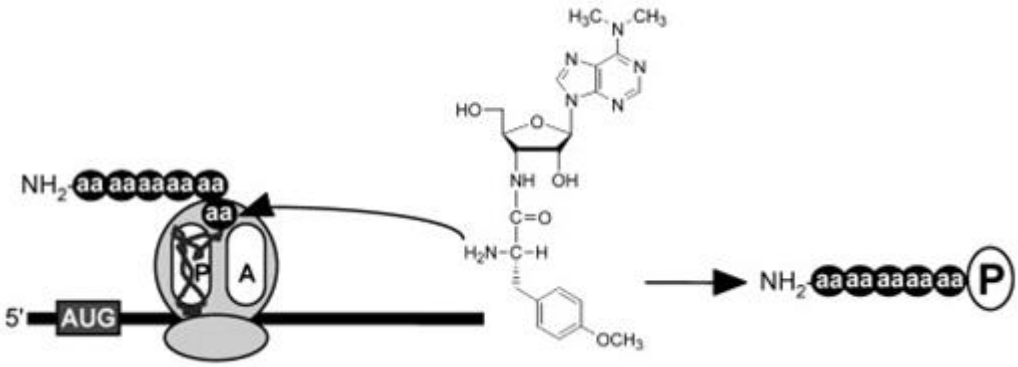

Figure 3.1 – Puromycin action. Schematic representation of puromycin incorporation during the elongation phase of translation and subsequent chain release (adapted from (197)).

3.2.2 Impact of experimental treatments in PNT-2 cells and bovine spermatozoa viability

Since translation is a highly energetic depend-process (see sections 1.3.2-4 and 1.4.2), it is important to ensure that the cell is metabolic functional after experimental treatments. To achieve this purpose, a metabolic assay was performed using Cell Titer 96®. In this assay, the tetrazolium compound is reduced into a coloured formazan (absorbance at 490 nm) by succinate dehydrogenase system in active mitochondria (193,202). The PNT-2 cells and capacitated bovine spermatozoa (capacitation for 3 hours) were exposed to puromycin, puromycin with cycloheximide (CH) or puromycin with D-

Chloramphenicol (D-CP). As a negative control, cells without any treatment were used. In both PNT-2 cells and bovine spermatozoa no significant differences were observed between the negative control and each experimental condition. (figure 3.2 A and B). Moreover, among the experimental conditions no significant differences were found (figure 3.2). These results suggest that the experimental treatments used do not interfere with cell viability.

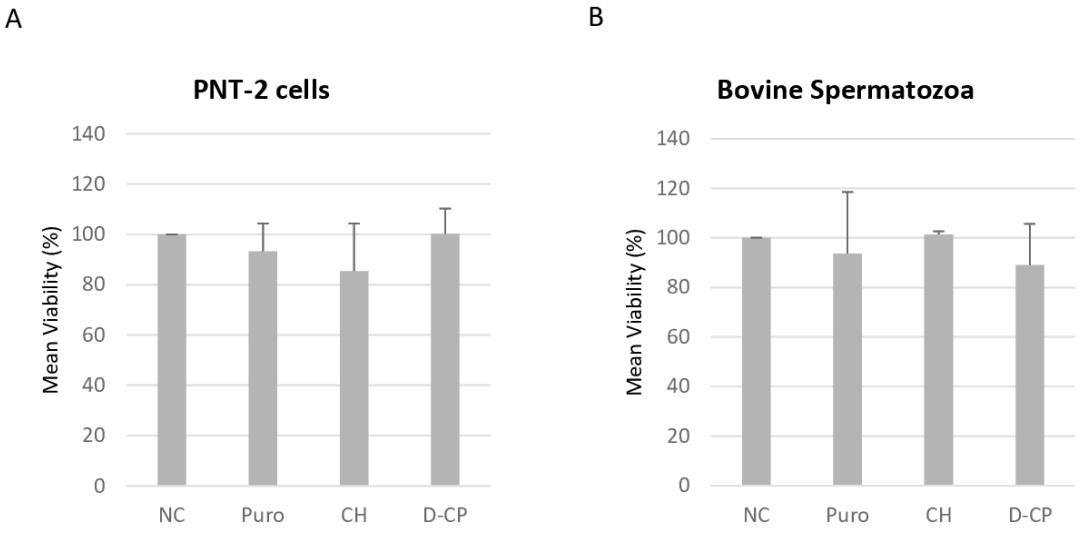

Figure 3.2 – **Impact of the experimental conditions on cellular viability in (A) PNT-2 cells and (B) capacitated bovine spermatozoa**. Viability was assessed by metabolic assay (Cell Titer 96®). Experiments were performed in duplicate. Graph bars represent the mean values and error bars correspond to the standard deviation (SD). Results are represented as fold change over control. NC, Negative Control; Puro, Puromycin; CH, Cycloheximide; D-CP, D-chloramphenicol.

3.2.3 Impact of experimental conditions in bovine spermatozoa motility

Capacitated bovine spermatozoa were treated with puromycin or with puromycin and a translation inhibitor (CH or D-CP). Motility parameters were assessed after 15 min (puromycin) or after 20 min (CH and D-CP). For reference, a negative control was performed in the absence of experimental treatments. The fast progressive (figure 3.3 A), medium progressive (figure 3.3. B) and non-progressive motility (figure 3.3 C), as well the immobility (figure 3.3 D) were assessed in each sample. There were no significant differences between the experimental conditions for any motility parameter, suggesting that there was no influence of treatment on spermatozoa motility. Although not statistically significant, in conditions treated with CH and D-CP is possible to observe a decrease in fast progressive motility (9,77 + 1,1% and 10,54 + 3,78%, respectively) compared to the negative control (15,85 + 0,1%). In contrast, the results show an increase in non-progressive motility (33,82 + 2,80% and 34,48 + 2,13%, respectively) in the CH and D-CP conditions compared to the negative

control (28,51 + 1,37%). In D-CP condition is also observed an increase of 2,89% in immotile spermatozoa.

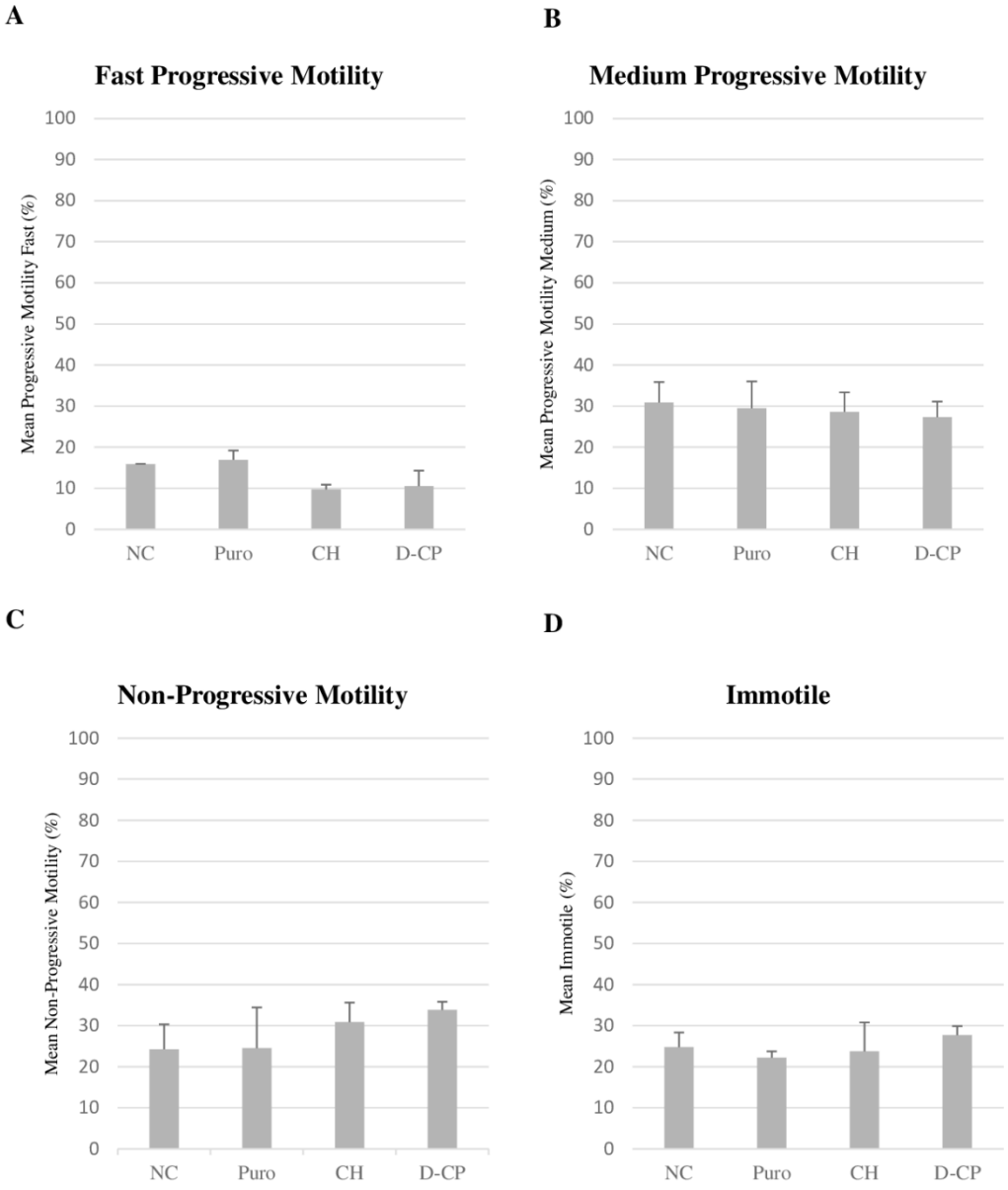

Figure 3.3 – Impact of the experimental conditions in motility parameters of capacitated bovine spermatozoa: (**A**) fast progressive motility; (**B**) medium progressive motility; (**C**) non-progressive motility and (**D**) immotile spermatozoa. Bovine spermatozoa were capacitated for 3 hours and subsequently treated with puromycin (7,5 μg/mL) for 15 min. Also, bovine spermatozoa were treated with CH – a cytoplasmic translation inhibitor - (5 μg/mL) and D-chloramphenicol – a mitochondrial translation inhibitor – (0,1 mg/mL). Experiments were performed in triplicate. Graph bars represent the mean values and error bars correspond to

the standard deviation (SD). NC, Negative Control; Puro, Puromycin; CH, Cycloheximide; D-CP, D-chloramphenicol.

3.2.4 Assessment of translation activity by SUnSET

Through flow cytometry it is possible to characterize and define different cell subpopulations in a heterogeneous sample. The proper assessment of a specific cell type requires the initial establishment of a gate. For that purpose, two non-fluorescent parameters are considered: forward scatter (FSC) and side scatter (SSC). The FSC is correlated with the cell size and SSC is proportional to the granularity/density of the cell (203). The suitable gate for bovine spermatozoa was previously established through work done at Signal Transduction Laboratory. The gate was established where the cell population was dense (supplementary figure S1). It should be noted that within this gate it is possible to distinguish two subpopulations of spermatozoa since a semen sample contains a morphologically heterogeneous population of spermatozoa (relative to size and state of maturation) (204).

3.2.4.1 Assessment in a positive-control cell type

To ensure that the technique selected for translation evaluation is adequate, PNT-2 cells were used as positive-control since these cells present active translation. As unstained control of the experiment, PNT-2 cells incubated with puromycin (1 µg/mL) were used. As experimental conditions, PNT-2 were exposed to puromycin or puromycin with a translation inhibitor. In the overlay histogram, the experimental conditions revealed a shift to the right in FL1-A axis compared to the unstained control (figure 3.4 A). The FL1-A axis corresponds to the mean fluorescence intensity (MFI) emitted by the anti-puromycin Alexa 488 antibody. Comparatively to the unstained control, puromycin MFI had a significant increase in the puromycin condition (figure 3.4 C). Comparatively to puromycin (1 µg/mL) condition, the shift of D-CP (0,1 mg/mL) and CH (5 µg/mL) conditions were smaller (figure 3.4 A). Despite the shift reduction, both inhibitors showed a significant increase in the puromycin MFI in comparison to the unstained control (figure 3.4 C). In the conditions with inhibitors, a non-significant reduction of puromycin MFI was also observed. The puromycin MFI reduction was particularly considerable in CH condition (~73%) (figure 3.4 C).

3.2.4.2 Assessment in capacitated bovine spermatozoa

Bovine spermatozoa were capacitated (for 3 hours) and then exposed to the same experimental conditions as PNT-2 cells. Only the puromycin (7,5 µg/mL) concentration was bigger due to

optimization work at Signal Transduction Lab. Additional to the unstained control, an isotype control was also performed. In the overlay histogram, the experimental conditions revealed a shift to the right in FL1-A axis in comparison to the unstained and isotype controls (figure 3.4. B). The unstained and isotype controls presented similar shifts (figure 3.4. B) and non-significative differences were found in puromycin MFI between the two controls (figure 3.4. D). On the other hand, a significant increase of puromycin MFI was observed in experimental conditions compared to the unstained and isotype controls (figure 3.4. D). Among the experimental conditions, non-significant differences were observed (figure 3.4 D). However, in D-CP and CH conditions a non-significant decrease in puromycin MFI was observed (figure 3.4 D). These results suggest that spermatozoa have translational activity and both cytoplasmic and mitochondrial translation appear to be present in this type of cell.

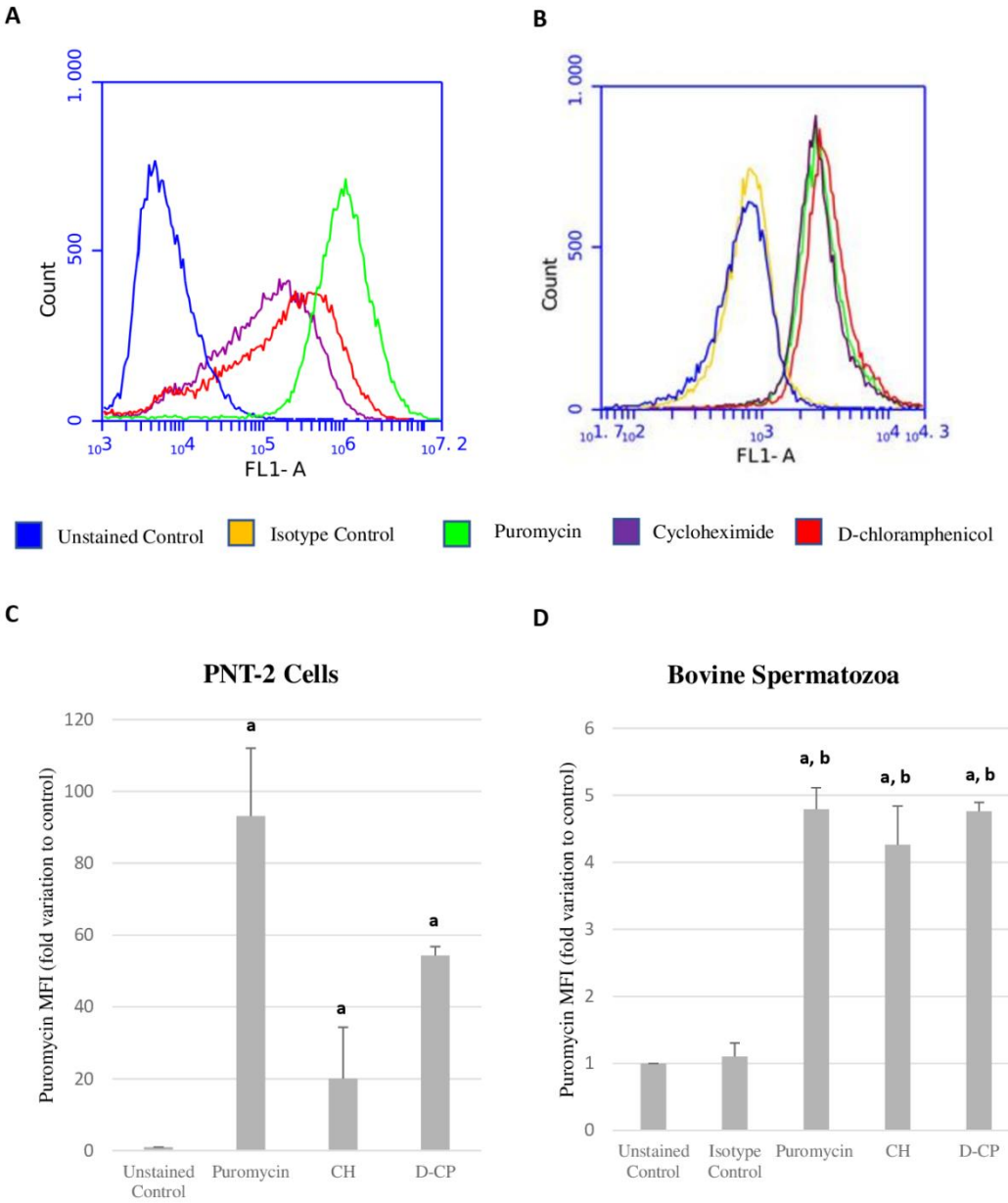

Figure 3.4 – **Assessment of protein translation**. Flow cytometry plots (A and B) and histograms (C and D) of average of mean fluorescence intensity (MFI) of puromycin in PNT-2 (positive control) and in bovine spermatozoa. Translation evaluation was done using the SuNSET method, where puromycin is incorporated into the nascent polypeptide chain. PNT-2 cells were treated with puromycin (1 µg/mL) for 15 min. Spermatozoa were capacitated for 3 hours and subsequently treated with puromycin (7,5 µg/mL) for 15 min. Both cell types were treated with cycloheximide – a cytoplasmic translation inhibitor - (5 µg/mL) and D-chloramphenicol – a mitochondrial translation inhibitor – (0,1 mg/mL). In conditions with inhibitors, they were incubated for 5 min, then puromycin was added. The samples were left unstained (negative control) or stained for anti-puromycin and analysed by flow cytometry. Experiments were performed in triplicate. The plots show representative MFI measurements for (A) PNT-2 cells and (B) bovine spermatozoa, and (C and D) the histograms represent the average of MFI for each type of cells. MFI was normalised to the unstained

control of each condition. Graph bars (C and D) represent the mean values and error bars correspond to the standard deviation (SD). Statistically significant findings compared with the unstained control are indicated with a (a) and compared with the isotype control are indicated with a (b). p<0,05. CH, Cycloheximide; D-CP, D-chloramphenicol.

3.2.5 Assessment of translation activity by western blot analysis

In order to identify different puromycin-peptides, PNT-2 cells and capacitated bovine spermatozoa were exposed to conditions with puromycin. Additionally, and to understand the impact of translation inhibitors in the expression of these peptides, PNT-2 cells and capacitated spermatozoa bovine were also incubated with CH or D-CP followed by incubation with puromycin. For both types of cells, negative controls were performed in the absence of puromycin and translation inhibitors. The cells lysates were run in a 10% SDS-PAGE gel. All protein bands detected in the immunoblotting (figure 3.5 A, supplementary figure S2 A) were quantified and normalized with their respective loading control (Ponceau S staining) (figure 3.5 B, supplementary figure S2 B).

In PNT-2 cells lysates, and comparatively to the negative control, there is puromycin incorporation in all conditions (supplementary figure S2 B). There is a significant increase of puromycin incorporation in puromycin condition. Also, these results show a decrease in puromycin incorporation in inhibitors conditions. The decrease is more pronounced when cytoplasmic inhibitor was used (supplementary figure S2 B).

In spermatozoa lysates, a significant puromycin-incorporation in all conditions was observed in comparison to the negative control (figure 3.5 B), except for cycloheximide condition. In the presence of inhibitors, and in comparison, with puromycin 7,5µg/mL, a decrease in puromycin incorporation was observed. The puromycin-incorporation is lower in the cycloheximide condition (figure 3.5 B).

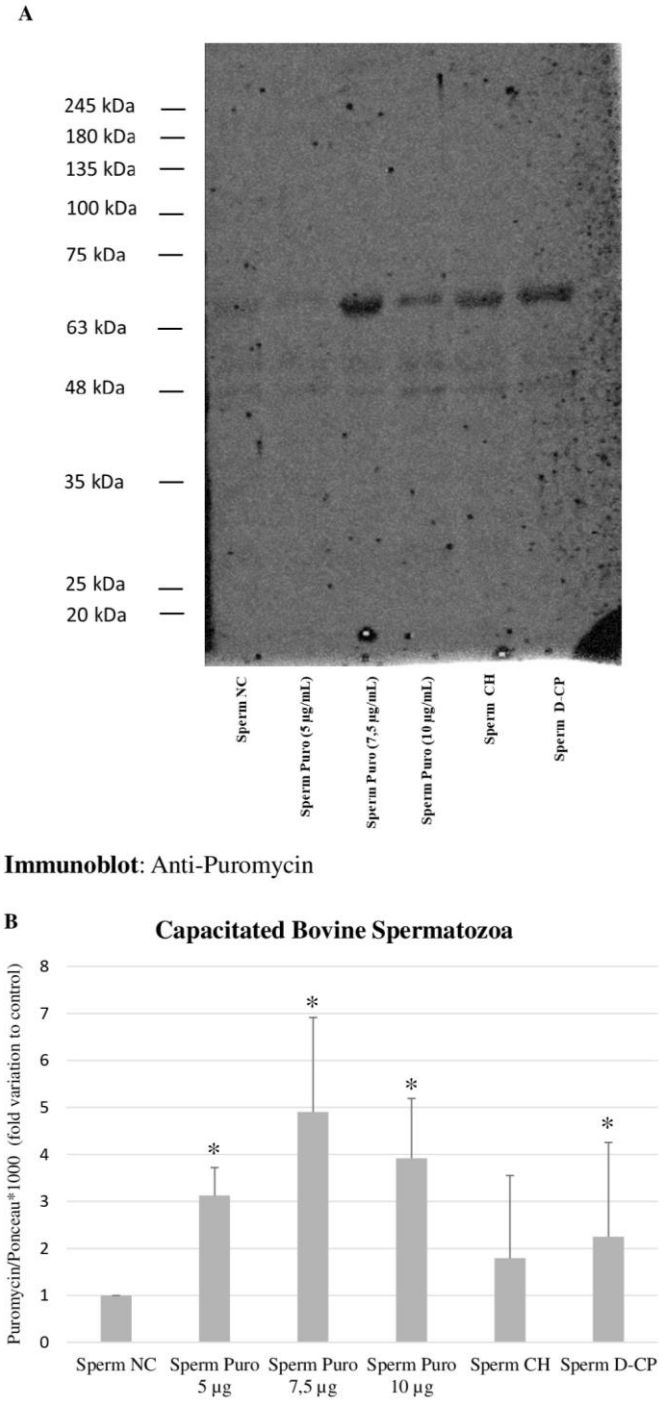

Figure 3.5 – Immunoblot results of puromycin-peptides using anti-puromycin mouse antibody. (A) Bovine spermatozoa soluble lysates corresponding to 20 µg of protein were loaded, followed by immunoblot with anti-puromycin mouse antibody. **(B)** Pixel intensity was quantified using Image Studio Lite software and Ponceau S staining was used as a loading control. Experiments were performed in triplicate. Graph bars represent the mean values and error bars correspond to the standard deviation (SD). Statistically significant findings compared with the negative control are indicated with a (*). *p<0,05. Molecular weight markers are

indicated to the left (Protein Marker – NZYColour Protein Marker II). NC, negative control; Puro, puromycin; CH, cycloheximide; D-CP, D-Chloramphenicol.

3.2.6 Characterization of the newly synthesized puromycin-peptides

To determine which proteins are *de novo* synthesized in bovine spermatozoa, an indirect immunoprecipitation (IP) was performed. When puromycin is incorporated in the nascent peptide chains, the elongation phase stops and then the puromycin-peptide is released. Thus, the anti-puromycin antibody can be used to immunoprecipitate these peptides for further analysis in mass spectrometry.

PNT-2 cells and capacitated bovine spermatozoa were incubated with puromycin. Negative controls were performed in the absence of puromycin. Both cell types were lysed in RIPA lysis buffer originating a soluble fraction (SF). The SF was used in subsequent IP steps. The insoluble fraction (IF) was solubilized by the addition of 1% SDS. As a technical control, 10% of SF and IF of each sample were resolved by electrophoresis and transferred onto nitrocellulose membranes. Then, membranes were stained with Ponceau S (for loading control) and incubated with the anti-puromycin antibody (Figure 3.6.).

The Ponceau analysis of SFs reveals that there are proteins in both PNT-2 samples, but for spermatozoa, the staining reveals only one protein band in all sperm samples (figure 3.6 A). This protein band has a weight between 68 kDa and 75 kDa – probably corresponding to albumin (~ 69 kDa), an abundant protein in both semen and SPM. Also, this analysis reveals that solubilization of sperm proteins failed and therefore the IP protocol for this cell was conditioned. In PNT-2 samples, puromycin-peptides were detected in both lanes (figure 3.6 B). The incorporation of puromycin-peptides was greater in puromycin condition (figure 3.6 C).

In IFs, the Ponceau analysis revealed the presence of proteins in all samples of PNT-2 cells and spermatozoa (figure 3.6 D). The anti-puromycin staining exposed puromycin-peptide in all samples except in spermatozoa negative control (figure 3.6 E). This analysis also appears to support the translational activity of spermatozoa by the incorporation of puromycin.

Despite the results obtained and described previously, the IP protocol was conducted to the end. The IP fractions obtained in each condition were run in 10% SDS-PAGE followed by staining of the gel with Coomassie Brilliant Blue (supplementary figure S3). In spermatozoa samples, only the heavy and light chains of the IgG2aκ were detected. In PNT-2 cells samples, the IgG chains were also detected. In addition, the staining reveals two proteins bands (one at 245 kDa and another at ~ 48

kDa) in both samples of PNT-2 and a third band (between 63 kDa and 75 kDa) in PNT-2 cells incubated with puromycin.

The IP protocol needs to be optimized to ensure the solubilization of sperm proteins.

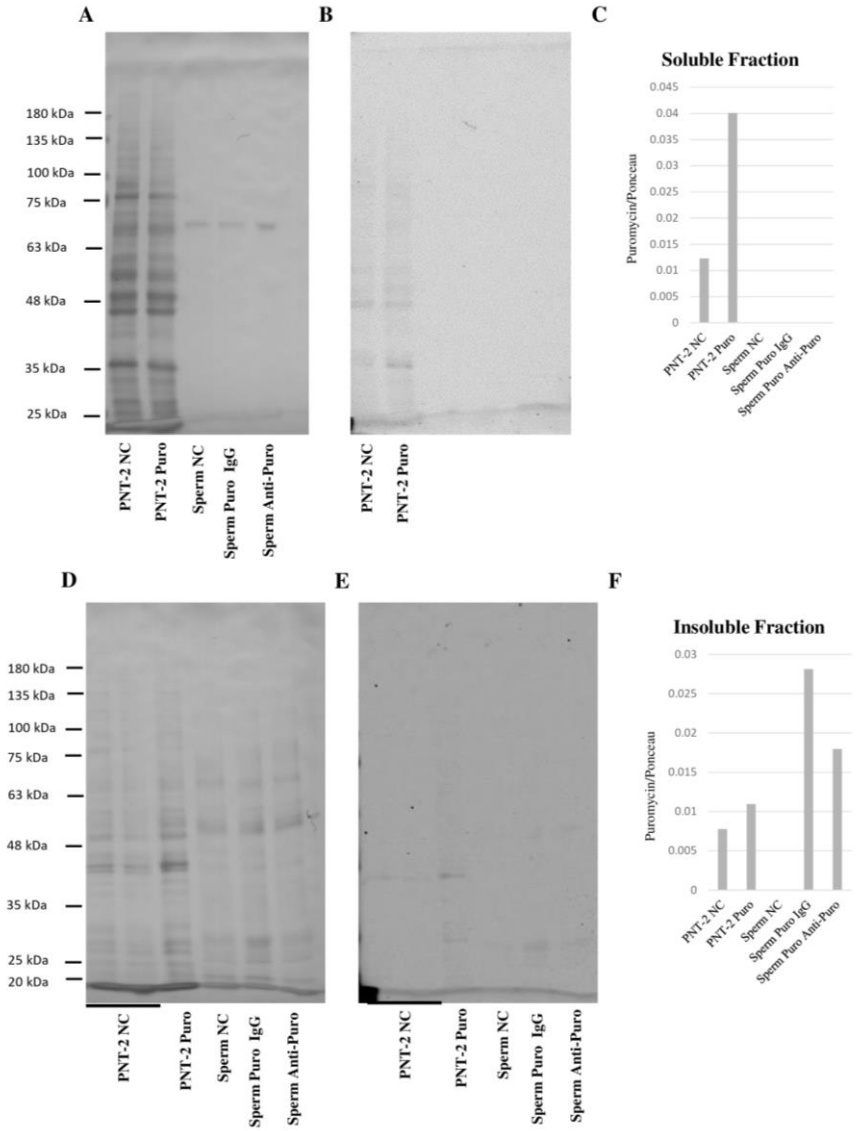

Figure 3.6 – Immunoblot of soluble fractions (SF) and insoluble fractions (IF) of PNT-2 cells lysates and capacitated bovine spermatozoa. Both fractions were obtained from 1 x 10^6 PNT-2 cells or from 30 x 10^6 bovine spermatozoa. Images **A-C** correspond to SF and images **D-F** correspond to IF. The samples of each fraction were run in a 10% SDS-PAGE and proteins transferred onto a nitrocellulose membrane followed by Ponceau S staining (loading control) (A and D) and immunoblot with mouse anti-puromycin (B and E). Pixel intensity was quantified using Image Studio Lite software and normalized to Ponceau S staining (C and F).

Molecular weight markers are indicated to the left (Protein Marker: NZYColour Protein Marker II). NC, negative control; Puro, puromycin.

4 General Discussion

The current dogma assumes that spermatozoon is a cell with silenced gene expression. This highly differentiated haploid cell presents unique characteristics that over the years led the scientific community to believe that two basal processes of somatic cells - transcription and translation - would be absent. However, a new perspective emerges when Gur and Breitbart prove *de novo* protein synthesis (132). This perspective was also confirmed by experiments of Rajamanickam and colleagues (146). Moreover, and with the advance in RNA quantification technologies, some transcriptomic evidences appear to support the existence of protein translation in spermatozoa (see section 1.5.1-2). Thus, **does protein translation occurs in spermatozoa**? In the present work, an *in silico* analysis and an experimental approach were conducted to answer this question.

4.1 The presence of protein-related proteins in spermatozoa

The first step of the bioinformatic analysis was the collection of translation-related proteins present in spermatozoa. The Venn diagram analysis revealed that 315 of the translation-related proteins found in somatic cells were identified in human spermatozoa (figure 2.1 A). The translation process in mammalian cells can occur in two different compartments: cytoplasm and mitochondria. These two compartments have independent protein machinery and, for both processes, proteins were identified in the spermatozoa (figure 2.1 B) (82,205,206). Interestingly, there are more cytoplasmic translation-related proteins (63 proteins) in spermatozoa in comparison to mitochondrial ones (38 proteins). Also, the cytoplasmic translation-related proteins set appears to be more complete (63 proteins out of 93 are present in spermatozoa) which may be indicative that the cytoplasm translation may be more likely to occur in spermatozoa.

In the analysis of the biological processes, the role of these 315 proteins in the translation was evaluated. In a global perspective, the analysis revealed the existence of annotated proteins for all phases of translation as well as proteins with regulatory functions (including positive and negative regulation) (figure 2.2). There are several proteins with annotations for translation initiation. As described before in section 1.3.2, for cytoplasmic translation initiation a considerable number of factors are recruited. In addition, each of these factors present a set of regulatory proteins that promote or inhibit their role in the translation pathway (81,207). On the other hand, the mitochondrial translation initiation is simpler than the cytoplasmic form (see section 1.4.3). Yet, this phase also has a tight regulation and therefore also requires more protein factors than the other phases (110).

Also, from a global perspective, the tRNA aminoacylation was the second most common annotation. Through this reaction, the (aa)-tRNA are formed. The aminoacyl-tRNA synthetases are responsible for the bond formation between the 20 standard amino acids and their tRNA-related molecules (208). In mammalian cells, there are two families of aminoacyl-tRNA synthetases (class I and class II) which correspond to 38 different proteins (209). Of the 315 translation-related proteins found in spermatozoa, 31 of them correspond to aminoacyl-tRNA synthetases (figure 2.2). This result is strongly indicative of the existence of molecular machinery for tRNA formation, a key molecule for protein synthesis.

By analysing the more specific terms, there are more annotated proteins for the cytoplasmic translation (9,52%) compared to mitochondrial translation (2,54%). Also, the most frequent specific terms associated with cytoplasmic translation are related to the initiation phase. This evidence is in line with what was previously discussed. These results support the hypothesis of cytoplasmic translation in spermatozoa.

In a complementary way, the analysis of the cellular compartment allows to determine in which subcellular region of spermatozoa the identified translation-related proteins may perform their functions. This analysis was performed in two steps: (a) evaluation of the annotations associated with each protein; (b) a Venn diagram analysis to see if proteins annotated for non-sperm organelles could be in other locations. In a global perspective and considering the more generic GO terms, there are more proteins annotated to the cytoplasm (45,71%) than for the mitochondria (16,91%) (figure 2.3). In the cytoplasm, protein synthesis occurs at sites where there are ribosomes. Ribosomes may exist as free particles in the cytoplasm or associated agglomerates - polyribosomes. They may also be in association with the membranes of the rough endoplasmic reticulum (RER) and the nuclear outer membrane (210). Therefore, it is not surprising that cytosol annotation is the most common (71,11%) (figure 2.3). Additionally, there are proteins annotated for cytosolic ribosome (and subunits) which was expected given the existence of ribosomal proteins in the 315 translation-related proteins (figure 2.3). Considering specific GO terms for mitochondria subcellular locations, the mitochondrial matrix and the mitochondrial IM were identified. Both locations are associated with protein synthesis in mitochondria (205). Other common GO terms identified among our dataset were the nucleus, the nucleoplasm and the nucleolus. How do proteins related to a cytoplasmic or mitochondrial process have an annotation to the nucleus? The most likely answer is that a considerable percentage of these proteins have other functions than their role in translation. This may justify their presence in the nucleus of somatic cells. In spermatozoa, they may perform the same alternative roles in the nucleus during the sperm life journey or may be stored for delivery during fertilization events (211). Through the Venn diagram analysis (figure 2.4) was observed that most of the proteins are common to the

cytoplasm or to the mitochondria. For proteins with annotations for nucleoplasm and extracellular space was verified that these proteins have annotations to other subcellular locations and therefore must be located there. For the proteins annotated to the ER, the same was verified except for two proteins (UniProtKB: P62341 and Q8WU17) that have an exclusive location for this organelle in somatic cells (212,213). Both proteins are involved in specific-organelle functions and their functions are not described in spermatozoa. The Venn diagram analysis also revealed that cytoplasm (57 proteins) has more exclusive proteins than mitochondria (26 proteins) So, hypothetically, if this set of 315 translation-related proteins exhibited unique translation functions, 310 proteins would be in the right places to perform their translation functions (excluding the three proteins exclusively located in the nucleus and the two exclusively located in the ER). The Venn diagram analysis also supports the hypothesis of cytoplasmic translation.

To identify translation-exclusive proteins present in spermatozoa, only proteins exclusively annotated to translation processes and located in the cytoplasm or mitochondria were selected. A total of 31 proteins were identified as translation exclusive proteins (table 2.3). Among those 31 proteins, 17 proteins act in mitochondrial translation. Most of the 31 proteins have a role as translation factors (EIF3CL, EIF5B, EIF3J, EIF4B, EIF1AX, EIF3M, GFM2, EIF3C, MTRF1L), ribosomal proteins (MRPS22, MRPS25, MRPS36, MRPS21, MRPS34, MRPL38, MARS2, MRPL34, MRPL37, MRPL39) or aminoacyl-tRNA synthetases (TARSL2, FARS2, VARS, CARS, LARS2, RARS2, PARS2, TARS2, IARS2). Although these proteins are not enough for the translation to occur, they are the evidence that there is residual translation machinery in the spermatozoa.

In the last part of the bioinformatic approach, the interactions between the 315 translation-related proteins identified in sperm were characterized. This methodology allowed the identification of a functional relationship between these proteins. The interactions were collected in three distinct databases and after that merged through the Cytoscape resources. In each database, only interactions with a high confidence level and from experimental approaches were included to ensure that information obtained from the merged network was as close as possible to the cellular reality. A total of 313 proteins and 3310 interactions between them were kept in the merged network. The clustering coefficient of the network (equal to 0.402) reveals a strong relationship between the analysed proteins (214,215). In the network, the hubs correspond to cytoplasmic ribosomal proteins (RPS6, RPL10, RPS9, RPL6, RPS14, RPS8, RPSA, RPS25, RPL11, RPL4, PRS3 and RPS23) (figure 2.5). In the translation process, the ribosomal proteins are an interface for the binding of translation factors, regulatory proteins, mRNA and tRNA (216). It was expected that within this set of proteins, ribosomal proteins would be strong candidates for those with a high node degree.

To identify the biological processes and cellular compartments associated with the merged network a ClueGo plus CluePedia enrichment analysis was performed. There are 104 biological processes associated with the merged network, being the translation initiation the most relevant one (p-value=3,34E-168) with 48,56% of the proteins (figure 2.6). This result is consistent with those obtained previously in the biological process analysis. Additionally, there is a significant association with translation-related processes. Again, the hypothesis of cytoplasmic translation is supported by the enrichment analysis. For cellular compartment, the ribosomal terms (ribosomal subunit, cytosolic ribosome, cytosolic part, small ribosomal subunit, cytosolic small ribosomal subunit, cytosolic large ribosomal subunit, large ribosomal subunit) were the most significant among the 21 identified terms (figure 2.7). Compared with previously performed cell compartment analysis, enrichment analysis provided more specific terms for subcellular locations. Yet, as previously observed (figure 2.3), the cytosolic components of the ribosome are among the most common sites for the localization of the 315 proteins analysed. The enrichment analysis of cellular compartment also supports the preferential localization of the 315 proteins in cytoplasm.

All bioinformatic analysis was performed based on data obtained for somatic cells. Thus, this becomes one of the major limitations of the bioinformatic study, due to the lack of information regarding the biological processes and cellular compartments of proteins identified in spermatozoa. Hypothetically, if all 315 translation-related proteins that were identified in the spermatozoa had only translation functions, the data obtained in this analysis would point to a greater possibility of cytoplasmic translation occurrence rather than mitochondrial translation. These data are contradictory to the data obtained experimentally, which showed that the mitochondrial translation would be the primordial form in spermatozoa (132,146).

4.2 Experimental evidence of translation in capacitated bovine spermatozoa

The work performed by Gur and Breitbart proved that protein translation occurs in spermatozoa under capacitation conditions (132). Through the labelling of sperm's nascent polypeptides with radiolabel amino acids, they showed that the translation process was completely inhibited by D-CP and in parallel was not affect by CH (132). Moreover, through the incorporation of BODIPY-lysine-$tRNA^{Lys}$, they observed that translation apparently occurs in the sperm midpiece (132). The major conclusion of this work is that sperm protein translation is capacitation-dependent and sensitive to mitochondrial translation inhibitors.

To evaluate the translation activity in capacitated bovine spermatozoa, a non-radioactive method, called surface sensing of translation (SUnSET), was used (194). This approach is based on the

puromycin characteristics that allow its incorporation in nascent polypeptide chains. In this way, the translation elongation stops and a puromycin-peptide is released from the ribosome (194,197,198).

As positive control, PNT-2 cells were used since they show a rapid growth rate and consequently high translation rates of mRNA (217). The results prove the effectivity of the SUnSET technique in the monitorization of mRNA translation rates, since it is possible to observe a clear shift of the anti-puromycin staining in cells with puromycin-peptides (figure 3.4 A and C). In spermatozoa, the experimental approach using the SUnSET technique also allowed the monitorization of puromycin incorporation in the newly synthesized peptides, which supports the hypothesis of protein translation in capacitated bovine spermatozoa (figure 3.4 B and D). Noteworthy, as expected, the protein translation levels in spermatozoa are considerably smaller than in PNT-2 cells (figure 3.4 A and C). This evidence is not surprising since during the last stages of spermatogenesis the spermatozoon loses a large part of cytoplasm (including protein translation machinery) (7,8,11,49) and mitochondria (which also has protein synthesis machinery) (122).

To understand which ribosome-type (cytoplasmic or mitochondrial) are involved in spermatozoa protein translation, two translation inhibitors were used (CH and D-CP). In PNT-2 cells, both inhibitors decrease the incorporation of puromycin, where the decrease in CH condition is more pronounced, as expected (figure 3.4 A and C). In the spermatozoa, a non-significant decrease in puromycin incorporation was observed in the presence of both inhibitors (figure 3.4 B and D). Contrary to expected, the decrease in puromycin incorporation is greater in CH condition. This evidence contrasts with results obtained by Gur and Breitbart that suggested major involvement of mitochondrial ribosomes in spermatozoa translation (132).

In a complementary way, and to corroborate these results, capacitated bovine spermatozoa were incubated with different concentrations of puromycin or with puromycin and D-CP or CH. The sperm cells lysates were resolved by electrophoresis and transferred into a nitrocellulose membrane followed by incubation with anti-puromycin antibody. Using the 7,5 µg/mL puromycin as reference, there is a decrease in puromycin incorporation when the translation inhibitors are present (figure 3.5 B), being more pronounced in cytoplasmic translation inhibition. This result does not corroborates the observations of Gur and Breitbart, where the D-CP has a most significant impact in spermatozoa translation (132).

Comparative to the experiment of Gur and Breitbart, a shorter incubation time (5 min) was used when the D-CP was applied. Most of the proteins take about 20 seconds to some minutes to be synthesized (218). Thus, translation is a fast process and therefore translation inhibition can be observed in short incubation times. Although D-CP plays a role in inhibiting mitoribosome through

the PTC, this antibiotic also causes mitochondrial stress which leads to the decrease of the ATP synthesis (219–223). In this way, if the ATP synthesis is decreased, the translation processes (both cytoplasmic and mitochondrial) would be affected given their high energy demand. Also, Gur and Breitbart showed in their work that the spermatozoa translation is dependent on ATP mitochondrial synthesised (132). When they used a mitochondrial uncoupler (carbonylcyanide ptrifluoromethoxyphenyl hydrazone – FCCP) the incorporation of labelled amino acid was completely inhibited (132). For a better understanding of the impact of D-CP on ATP synthesis, it would be necessary to complement this work with an ATP quantification assay.

The impact of the experimental conditions was assessed through the metabolic assay of the Cell Titer 96® and no significant differences were observed. In fact, puromycin (199–201), CH (195,224,225) and D-CP (219,226,227) were used at the range of described concentrations that do not affect cell viability and proliferation.

The effect of the experimental treatments was also evaluated in sperm motility parameters. No significant differences were observed. Under CH and D-CP conditions a decrease, although not significant, was observed in fast and medium progressive motility (figure 3.3 A and B). For these two conditions, there was also an increase in the percentage of spermatozoa with non-progressive mobility (figure 3.3 C). These results may be indicative that the newly synthesised proteins have a role in sperm motility. Further analysis will be necessary.

After establishing that the translation occurs in spermatozoa, it is important to understand what the roles of newly synthesized proteins are. To achieve this purpose, an indirect IP approach was performed. This protocol failed because cell lysis and subsequent solubilization of sperm proteins did not occur as expected (figure 3.6 A and D). All sperm proteins remained in the insoluble fractions of sperm extracts (figure 3.6 D). Since the entire IP procedure is performed on the soluble fraction, it was not possible to isolate the puromycin-peptides for further analysis. Additional work is required to optimize this procedure. Perhaps the optimization may be accomplished by using a stronger lysis buffer than RIPA. Note that in the immunoblot performed on the insoluble fraction samples, puromycin-peptides were detected, contributing to the support of the translation hypothesis in spermatozoa.

In a brief view, the experimental results obtained allow to clearly infer that the translation is likely to occur in capacitated spermatozoa. These results also showed that puromycin incorporation is decreased when CH is used, which is suggestive of the existence of cytoplasmic translation in spermatozoa. This evidence contrasts with the published evidence (132,146). On the other hand, the

results also show the decreased incorporation of puromycin when the mitochondrial translation is blocked, which is in line with the first evidence of translation in spermatozoa (132,146).

4.3 Integration of *in silico* and *in vitro* results: a hypothetic model of translation in spermatozoa

Mitochondrial and cytoplasmic translation have distinct protein machinery and different levels of complexity. One of the major differences of mitochondrial translation is the reduced number of translation factors that are required and the special proprieties of the mitoribosome (reviewed in (228)). Also, the mitochondria have a specific gene code (109) and do not require the existing of the cap structure neither leader sequence on the 5' end of the mRNA (108). Most of the mitochondrial proteome is nuclear-encoded (mt-DNA only encoded 13 polypeptides) (97,98). The complexes I, III, IV and V subunits of the oxidative phosphorylation system (OXPHOS) are encoded in nuclear and mitochondrial DNA. Thus, there needs to be a tight synchronization between cytoplasmic and mitochondrial translation for perfect assembly of the subunits of the OXPHOS. This will prevent the accumulation of unproductive and disassembled subunits in the IM of the mitochondria (229–231). In human mitochondria, the synthesis of COX1 (mitochondrial-encoded) is stopped in the absence of the COX4 (nuclear-encoded) (232).

Based on these evidences and in the bioinformatic and experimental results obtained, we suggest that the translation of long-term mRNA into spermatozoa may occur in cytoplasmic ribosomes. If among the long-term mRNAs there are transcripts for mitochondrial proteins (particularly for OXPHOS complexes), it is also likely that mitochondrial translation will occur in mitoribosomes. Hypothetically, and as already described (117), cytoplasmic ribosomes may be associated with the outer membrane of the mitochondria, which would justify two things: (a) its permanence after the removal of the majority of the cytoplasm; (b) the location of the translation in the midpiece of spermatozoa demonstrated by the incorporation of BODIPY-lysine-$tRNA^{Lys}$ (132). The location close to the mitochondria favoured the energy supply for the cytoplasmic translation (figure 4.1).

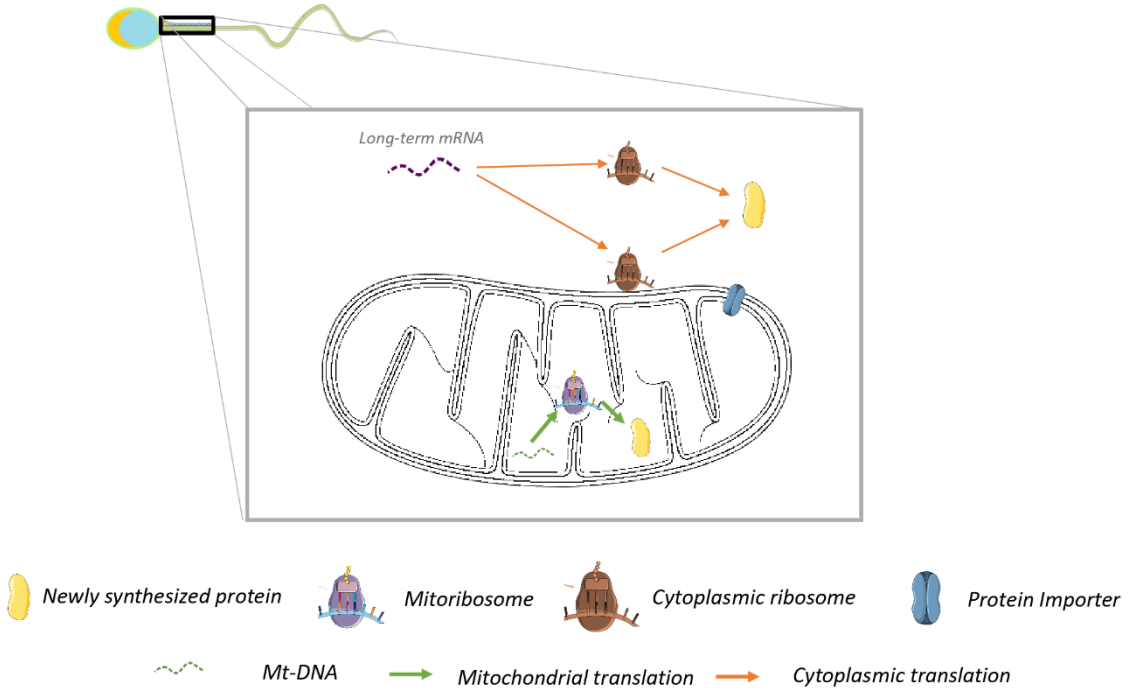

Figure 4.1 - Hypothetic model of translation in spermatozoa. Long-term mRNAs are translated by cytoplasmic ribosomes (coupled or near to mitochondria) and mt-mRNA are translated by mitoribosomes. Proteins are translocated to their active sites.

5 Conclusion and Future Perspectives

5.1 Conclusions

The main objective of this thesis was the characterization and evaluation of the protein synthesis in mammalian spermatozoa using both bioinformatic and experimental approaches.

To achieve this goal, the presence of translation-related proteins in spermatozoa was initially evaluated. For this, the spermatozoon proteome was compared with the translation proteome, which resulted in the identification of 315 overlapping proteins. From those, 63 correspond to cytoplasmic translation-related proteins and 38 are mitochondrial-translation related proteins. Additionally, in terms of cellular compartment distribution, most of the 315 proteins have annotation for cytoplasm related terms. After analysing the biological processes and cell compartments to which each protein was associated, 31 proteins exclusively associated with translation were identified in human spermatozoa. These proteins only perform translation-related functions and are located either in the cytoplasm or in the mitochondria. Analysis of the PPI network revealed that these 315 proteins are highly related and interconnect with each other. The enrichment analysis also reveals a preference for cytoplasmic translation and localization. Together this data supports the hypothesis that cytoplasmic translation may occur in mammalian spermatozoa.

Through the SUnSET technique was evidenced unequivocally the existence of translational activity in capacitated bovine spermatozoa. The use of mitochondrial and cytoplasmic inhibitors revealed a decrease in puromycin incorporation. The decrease was bigger when CH was used. Immunoblot results also confirmed the existence of translational activity. However, these results apparently revealed a more marked decrease in puromycin incorporation during D-CP treatment. Perhaps both translation processes coexist in the spermatozoon (as proposed in section 4.3 and figure 4.1).

To determine the functions of newly synthesized proteins, an indirect IP approach was used to isolate puromycin-peptides for analysis by mass spectrometry. However, this protocol, for methodological reasons, did not go as expected and the characterization of the proteins was not possible.

As a concluding remark, protein translation occurs in capacitated bovine spermatozoa and appears to be performed by both mitochondrial ribosomes and cytoplasmic ribosomes. These evidences open the door to a new perspective on the physiology of spermatozoa.

5.2 Future perspectives

Future work should be developed to validate the obtained bioinformatic results. Particularly, it would be important to prove experimentally the presence of the translation machinery.

Given the characteristics of the cytoplasmic membrane and the sperm mitochondria, it would also be interesting to isolate the sperm mitochondria and characterize their proteome to understand if the translation machinery is still present.

The SUnSET technique should also be performed in other mammalian spermatozoa (such as equine and human samples). Also, the results obtained demand for the use of the other elongation inhibitors, namely emetine, in place of cycloheximide. Emetine is a competitive inhibitor of the puromycin reaction to validate the cytoplasmic translation process. It would also be important to test other inhibitors of the mitral-like translation, as well as inhibitors of OXPHOS.

Finally, and to understand whether the translation is necessary or exists only in a residual form, future work should be done to characterize puromycin-peptides by mass spectrometry.

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Supplementary Tables

Supplementary table S1*

Table S1 – Reviewed translation-related proteins

Supplementary table S2*

Table S2 – Reviewed cytoplasmic translation-related proteins

Supplementary table S3*

Table S3 – Reviewed mitochondrial translation-related proteins

Supplementary table S4*

Table S4 - List of proteins Isoforms present in sperm

Supplementary table S5*

Table S5 – Reviewed proteins present in human spermatozoa

*Available at: https://drive.google.com/file/d/1lws41wF43vTVuY_Y5irQDSvvSNtOkKNQ/view?usp=sharing

Supplementary table S6

Table S6 – Solutions used in experiments.

General Use	
1x Phosphate-Buffered Saline (PBS)	For 1 L, add 100 mL of 10x PBS (stock) (Fisher Bioreagents) to 900 mL of deionized water.
Cell Culture	
Culture Medium	For 500 mL of RPMI 1640, add 50 mL of Fetal Bovine Serum (FBS) and 5 mL of penicillin/streptomycin mixture.
Translation Inhibitors	
D-Chloramphenicol 100 µg/mL	For 1 mL, dissolve 1 mg of d-chloramphenicol in 1 mL ethanol, add 9 mL MiliQ water.
Cycloheximide 1 mg/mL	For 1 mL, dissolve 1 mg of cycloheximide in 1 mL deionized water.

Emetine 20 mg/mL	For 1 mL, dissolve 20 mg of emetine in 1 mL deionized water.	
Surface Sensing of Translation (SUnSET)		
Puromycin 100 µg/mL	For 10 mL, dissolve 1 mg of puromycin in 10 mL deionized water.	
Flow Cytometry		
4% Paraformaldehyde (PFA)	For 50 mL, dissolve 5.40 mL of 37% PFA (stock) in 44.6 mL 1x PBS.	
Fluorescence activated cell sorting (FACS) solution including 0,1% saponin	For 10 mL dissolve 0,025 g of BSA in 5 mL of PBS and add 2 mL saponin and 0,1 mL sodium azide and make up to 10 mL with 1x PBS.	
Anti-puromycin Alexa488 diluted in 1:100 FACS solution	For 100 µL, add 1 µL Anti-Puromycin Alexa488 to 100 µL FACS solution.	
Anti-IgG2a Alexa488	For 100 µL, add 1 µL Anti-Puromycin Alexa488 to 4.5 µL FACS solution.	
Western Blot		
Running gel 10% (2 gels, 1.5 mm thickness)	ddH2O Tris 1.5 M pH8.8 Acrylamide 40% Bisacrylamide 2% SDS 10% APS 10% TEMED	7.720 mL 5.000 mL 4.920 mL 1.960 mL 0.200 mL 0.100 mL 0.020 mL
Stacking gel 4% (2 gels, 1.5 mm thickness)	ddH2O Tris 0.5 M pH6.8 Acrylamide 40% Bisacrylamide 2% SDS 10% APS 10% TEMED	4.736 mL 2.000 mL 0.784 mL 0.320 mL 0.080 mL 0.040 mL 0.008 mL
Tris-HCl 1.5 M pH 8.8 buffer	For 1 L, dissolve 181.5 g Tris in 800 mL deionized water. Adjust pH at 8.8 with HCl and make up to 1 L with deionized water.	
Tris-HCl 0.5 M pH 6.8 buffer	For 1 L, dissolve 60 g Tris in 800 mL deionized water. Adjust pH at 6.8 with HCl and make up to 1 L with deionized water.	
10% APS (ammonium persulfate)	For 10 mL of deionized water add 1 g of APS.	
10% SDS (sodium dodecilsulfate)	For 500 mL of deionized water dissolve 50 g of SDS.	
4x Loading gel buffer	For 10 mL, add 44 mL glycerol, 250 µL Tris-HCl 0.5 M pH 6.8 buffer, 0.8 g SDS, 0.2 mL β-mercaptoethanol and 3.3 mL deionized water. Add bromophenol blue (a small amount). Keep it at **RT** for short periods or at 4ºC for longer periods.	
Tris-Gly 10x (stock)	For 1 L, dissolve 30.30 g Tris (250 mM) and 144.10 g Gly (1.92 M) in 1 L of deionized water.	
Running buffer 1x	For 1 L, add 800 mL deionized water, 100 mL Tris-Gly 10x and 10 mL 10% SDS. Make up to 1 L with deionized water.	
Transfer buffer 1x	For 1 L, add 100 mL Tris-Gly 10x to 700 mL of deionized water and 200 mL methanol.	

10x Tris buffered saline (TBS) (stock)	For 0.5 L, dissolve 6.055g Tris in deionized water and adjust pH at 8.0. Add 43.8325 g NaCl and make up to 500 mL with deionized water.
1x TBST (TBS + Tween 20)	For 1 L, add 100 mL TBS 10x and 500 µL Tween-20 to 900 mL of deionized water.
Ponceau S	For 0.1 L, dissolve 0.1 g Ponceau S (0.1%) in 5 mL acetic acid (5%) and make up to 100 mL with deionized water.
Immunoprecipitation	
Colloidal Coomassie Solution	For 1 L, dissolve 1.2 g of Coomassie G-250 (0.12%) in 200 mL methanol and 800 mL deionized water.
Fixation solution	For 1 L, add 500 mL of deionized water to 100 mL acetic acid and 400 mL methanol.
Distaining solution	For 1 L, add 800 mL of deionized water to 400 mL methanol.

Supplementary table S7

Table S7 – Standards for BCA assay

Tube Name	Volume of BSA	From Tube	Volume Lysis Buffer (RIPA 1x)	Final [BSA]
A	30 µL	BSA stock	0 µL	2000 µg/mL
B	37.5 µL	BSA stock	12.5 µL	1500 µg/mL
C	32.5 µL	BSA stock	32.5 µL	1000 µg/mL
D	17.5 µL	B	17.5 µL	750 µg/mL
E	32.5 µL	C	32.5 µL	500 µg/mL
F	32.5 µL	E	32.5 µL	250 µg/mL
G	32.5 µL	F	32.5 µL	125 µg/mL
H	10 µL	G	40 µL	25 µg/mL
I	0 µL	-	40µL	Blank

Supplementary Figures

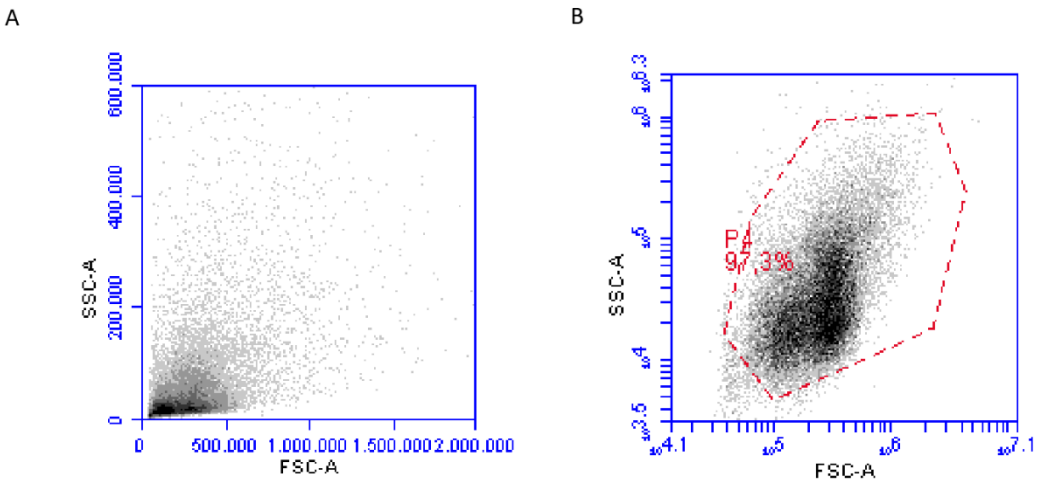

Figure S1 – Bovine spermatozoa density plot and gate establishment. (**A**) Bovine spermatozoa density plots (FCS vs SSC in linear scale); (**B**) Bovine spermatozoa gate establishment (FSC and SSC logarithmic scale). FSC, forward scatter; SSC, side scatter.

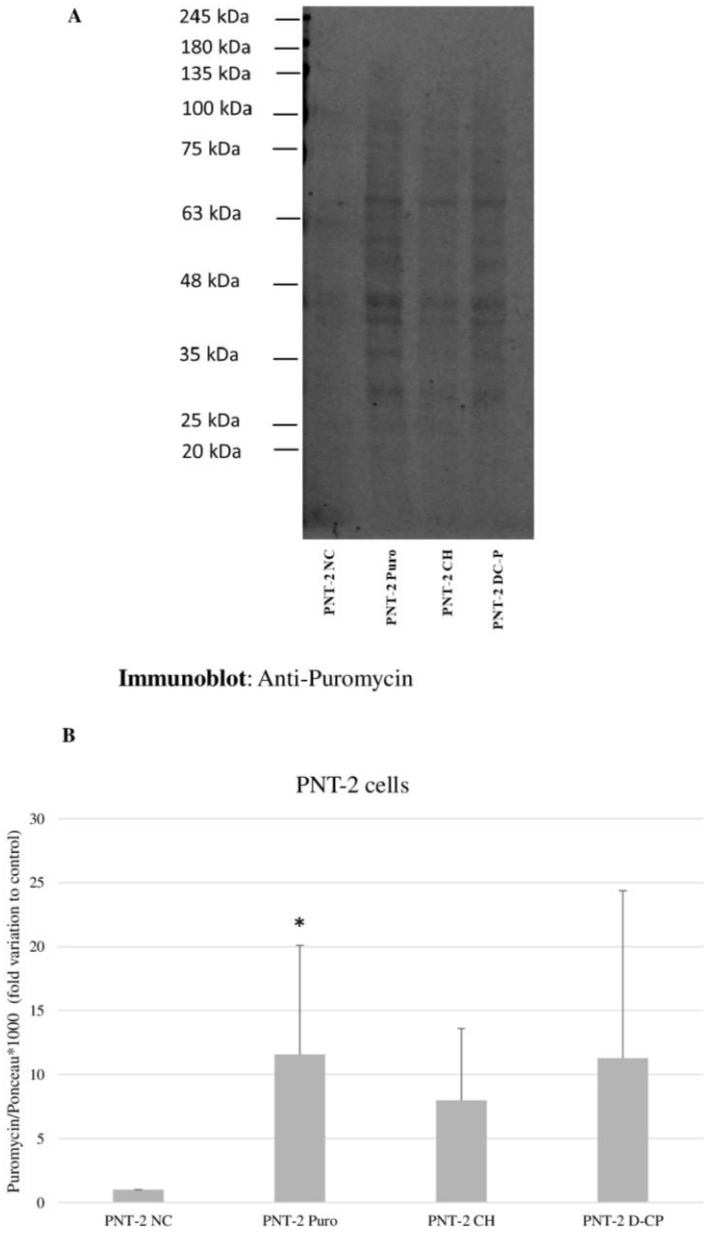

Figure S2 – Immunoblot results of puromycin-peptides using anti-puromycin mouse antibody. (A) PNT-2 soluble lysates corresponding to 20 µg of protein were loaded, followed by immunoblot with anti-puromycin mouse antibody. **(B)** Pixel intensity was quantified using Image Studio Lite software and Ponceau S staining was used as a loading control. Experiments were performed in triplicate. Graph bars represent the mean values and error bars correspond to the standard deviation (SD). Statistically significant findings compared with the negative control are indicated with a (*). *p<0,05. Molecular weight markers are indicated to the left (Protein Marker – NZYColour Protein Marker II). NC, negative control; Puro, puromycin; CH, cycloheximide; D-CP, D-Chloramphenicol.

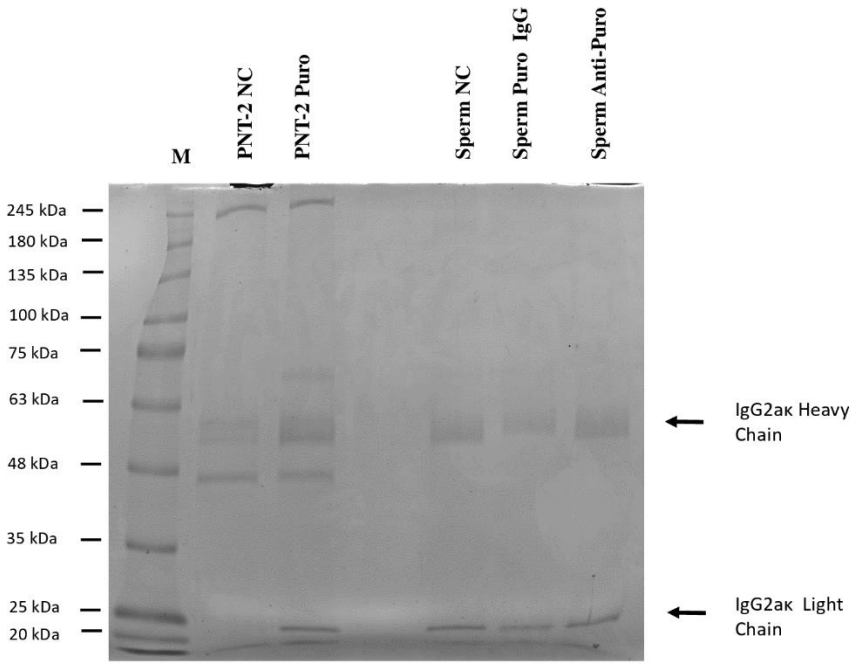

Figure S3 – Coomassie Brilliant Blue staining used as a loading control for 10%-SDS PAGE of IP fractions samples. Of each IP fraction sample, 20 µL was run in the SDS PAGE followed by Coomassie staining. In PNT-2 cells and spermatozoa samples heavy and light chains of the IgG2aκ where identified. Marker (M) – NZYColour Protein Marker II

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Are translation-related proteins present in sperm?

Pedro O. Corda, BSc [1], Joana Vieira Silva, PhD [1,2,3], Margarida Fardilha, PhD [1]

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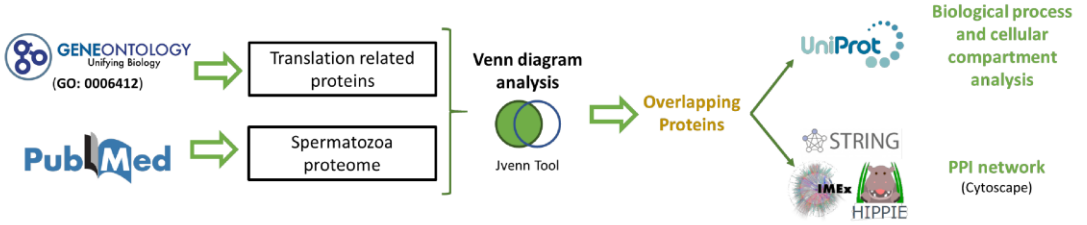
Background

Spermatozoa are highly differentiated haploid cells [1]. It is widely accepted that during cytoplasm removal in the final stage of spermatogenesis, the elimination of translational machinery may occur, possible leading to the lack of translation in spermatozoa [2]. Recently, a new perspective emerged when protein translation was described during capacitation [3]. These interesting results suggest that mitochondrial ribosomes might be involved in translation in spermatozoa [3]. Despite the loss of most cytoplasm, **is it possible to have residual translation machinery in spermatozoa?**

Bioinformatic Approach



Results

A total of 6475 reviewed proteins were identified in human sperm and 623 reviewed proteins were identified as being related to translation. Of the 623 translation-related proteins, **315** are present in the sperm (Fig. 1). Of these 315 common proteins, 71,1% have annotation for the cytosol (Fig. 2). Since each protein displays annotations for more than one biological process (including non-translational processes) and for different subcellular compartments, the 315 proteins were filtered to determine those that were exclusively linked to the translation. A total of **31 proteins** were identified as being exclusively linked to translation, located either in the mitochondria or in the cytoplasm (both sites where the translation occurs in somatic cells) (Fig. 3). After collecting the interactions between the 315 proteins, an unique network was created. The **merged network** has a spare structure with hubs and a clustering coefficient of 0.402 (Fig. 4).

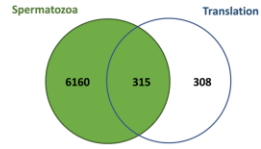


Fig. 1 – Venn's diagram illustrating common proteins between spermatozoa proteome and translation proteins.

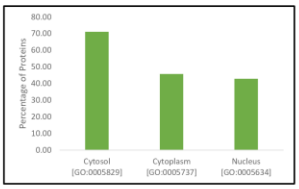


Fig. 2 - The three most common subcellular localizations associated with translation proteins present in sperm.

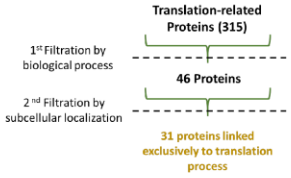


Fig.3 - Translation-exclusive proteins found in spermatozoa after biological and subcellular compartment filtration.

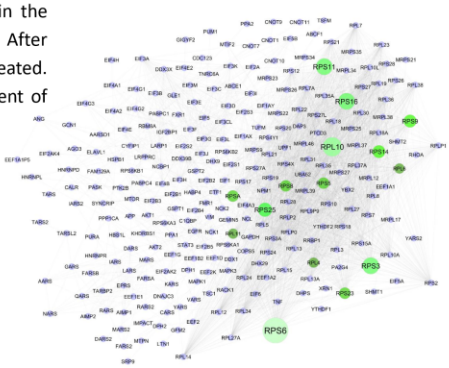


Fig. 4– PPI network of translation-related proteins present in spermatozoa. Each node represents a different protein. The green nodes correspond to hubs (proteins with a degree ≥ 75). Only the connected nodes are represented (N=265). Proteins are represented with gene name.

Conclusions

- There are translation-related proteins present in spermatozoa;
- A considerable percentage of the proteins show annotation for the cytosol, one of the main protein translation sites;
- In spermatozoa, there are at least 31 proteins linked exclusively to the translation and located in the correct place for this process to occur;
- Overlapping proteins are strongly connected and related to each other.

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Acknowledgments: This work was supported by Institute for Biomedicine – iBiMED (UID/BIM/04501/2013 and POCI-0145-FEDER-007628) and the project pAGE funded by the Operational Programme Competitiveness and Internationalization Operational Program - COMPETE 2020(CENTRO-01-0145-FEDER-000003). This study was also supported by FEDER Funds through the Competitiveness and Internationalization - COMPETE 2020 and by National Funds through FCT –Foundation for Science and Technology under the project PTDR/BBBBQB/3804/2014.

FCT COMPETE 2020 PORTUGAL 2020

Figure S4 – Are translation-related proteins present in spermatozoa? – in X Jornadas Nacionais de Ciências Biomédicas, 13-16th March 2019, Aveiro, Portugal.

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Are translation-related proteins present and functionally active in sperm?

Pedro O. Corda, **BSc** [1], Joana Vieira Silva, PhD [1,2,3], Margarida Fardilha, PhD [1]

[1] Department of Medical Sciences, Institute of Biomedicine – iBiMED, University of Aveiro, Aveiro, Portugal; [2] Reproductive Genetics & Embryo-fetal Development Group, Institute for Innovation and Health Research (I3S), University of Porto, Porto, Portugal; [3] Unit for Multidisciplinary Research in Biomedicine (UMIB), Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal

Background

Spermatozoa are highly differentiated haploid cells originated through a sequence of mitotic and meiotic divisions – the spermatogenesis [1]. Due the cytoplasmic changes that occurs during the last steps of this process is widely accepted that **translational activity is silenced in spermatozoa** [2]. A new perspective emerged when *de novo* protein synthesis was shown to occurs in sperm cells under capacitation conditions, opening the discussion to established dogma [3]. Thus, **the main goal of the present work is the characterization and evaluation of protein synthesis in mammalian spermatozoa.**

Bioinformatic Approach

The sperm proteome (6475 reviewed proteins) was collected and cross-linked with the translation-related proteins (623 reviewed proteins) which led to the identification of **315 overlapping proteins** (Fig. 1). Among the overlapping proteins, **31** were identified as **proteins exclusively linked to translation** based on filtration for biological processes (translation-related processes) and cellular compartments (cytoplasm or mitochondria) (Fig. 2).

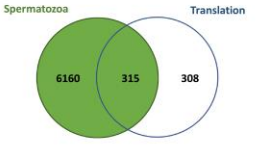

Fig. 1 – Venn's diagram illustrating common proteins between spermatozoa proteome and translation proteins.

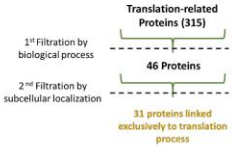

Fig. 2 - Translation-exclusive proteins found in spermatozoa after biological and subcellular compartment filtration.

A **protein-protein interaction network of the 315 translation-related proteins present in spermatozoa was constructed** (Fig. 3). The **enrichment analysis** revealed that the 12 most significant biological processes are translation-related, being the translation initiation (p=2,31E-193) the most significative. Also, the analysis revealed that cytoplasmic translation (p=2,94E-90) is more strongly associated with the network than mitochondrial translation (p=1,87E-36) (Fig. 3). For cellular compartment, there are strong associations with cytoplasmic locations, being the ribosomal subunit (p=1,00E-114) the most significative (Fig. 4).

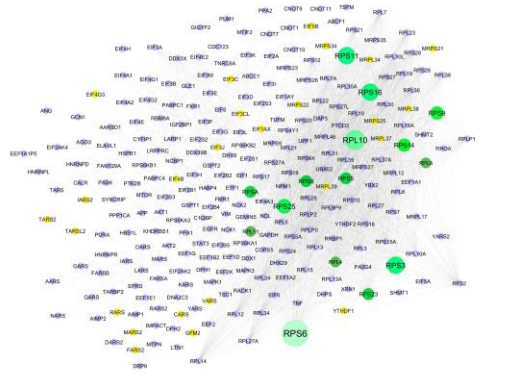

Fig. 3– PPI network of translation-related proteins present in spermatozoa. Each node represents a different protein. The green nodes correspond to hubs (proteins with a degree ≥ 75). The yellow nodes represent the proteins linked exclusivity to translation. The merged network has a clustering coefficient of 0.402. Only the connected nodes are represented (N=265). Proteins are represented with gene name.

References

1. Turek PJ. Male Reproductive Physiology.
2. Breucker H, Schäfer E, Holstein AF. Morphogenesis and fate of the residual body in human spermiogenesis.
3. Gur Y, Breitbart H. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes.
4. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis.

Acknowledgments

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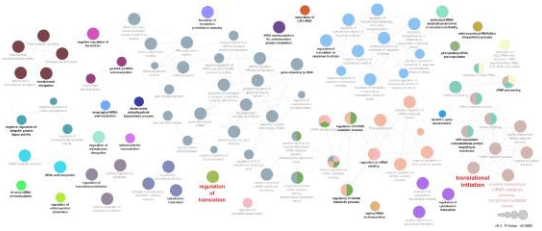

Fig. 4 - Network view for biological processes corresponding to the translation-related PPI network, after applying the defined parameters on ClueGO. Terms are functionally grouped based on Kappa Score and are exhibit with different colours. The most significant term defines the name of the group (label black and bold). The red labels correspond to the three most significant biological processes.

Experimental Evidences

The translational activity was assessed using the **SUnSET technique** [4] where the puromycin (an analogue of aminoacylate tRNA) is incorporated in the nascent polypeptide chains being used as a direct measure of the rate of mRNA translation through immunolabeling. There is a significant puromycin incorporation in sperm (Fig. 5). There is a decrease in the incorporation of puromycin in both inhibitors conditions, being more prominent in cycloheximide condition (Fig. 5B).

To assess the impact of translation inhibition on the levels of signaling proteins essential for sperm function (e.g. GSK3 and PPP1CC2), bovine spermatozoa were incubated under capacitation conditions with or without translation inhibitors (Fig. 6). There is a decrease in **GSK-3** and **PPP1CC2** quantity in conditions with translation inhibitors after 4 hours of incubation.

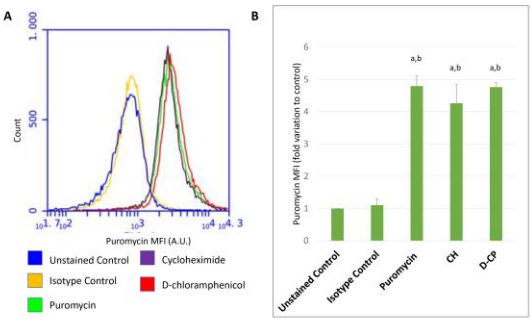

Fig. 5 – (**A**) Flow cytometry plot and (**B**) histogram of average of puromycin mean fluorescence intensity (MFI) in bovine spermatozoa. Spermatozoa were capacitated for 3 hours and subsequently treated with puromycin (7,5 µg/mL) for 15 minutes. Sperm cells were treated with cycloheximide – a cytoplasmic translation inhibitor - (5 µg/mL) and D-chloramphenicol – a mitochondrial translation inhibitor (0,1 mg/mL). In conditions with inhibitors, they were incubated for 5 minutes, then puromycin was added. The samples were left unstained (unstained control), stained with anti-IgG2a (isotype control) or with anti-puromycin and analysed by flow cytometry. Experiments were performed in triplicate. Graph bars represent the mean values and error bars correspond to the standard deviation. Statistically significant findings compared with the unstained control are indicated with a (a) and compared with the isotype control are indicated with a (b). p<0,05. CH, Cycloheximide; D-CP, D-chloramphenicol.

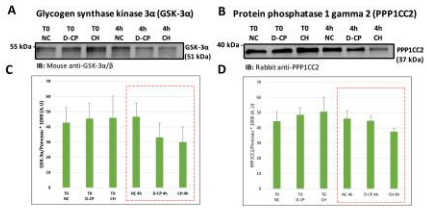

Fig 6 – (A,B) Immunoblots with anti-GSK-3α/β and anti-PPP1CC2. **(C,D)** Pixel intensity quantification. Bovine spermatozoa were incubated with or without translation inhibitors (CH or D-CP) for 4 hours under capacitation conditions. Experiments were performed in triplicate. Graph bars represent the mean values and error bars correspond to the standard deviation. NC, negative control; D-CP, D-chloramphenicol; CH, Cycloheximide; IB, immunoblot.

Conclusions

- Translation-related proteins are present in sperm;
- There are at least 31 proteins in sperm linked exclusively to the translation and located in the correct place for this process to occur;
- Sperm translation-related proteins are strongly connected and related to each other;
- The enrichment analysis revealed a preference for cytoplasmic translation;
- SUnSET results evidenced unequivocally the existence of *de novo* protein synthesis in capacitated bovine spermatozoa;
- Signalling proteins GSK-3α and PPP1CC2 might be translated during sperm capacitation.

Figure S5 – Are translation-related proteins present and functionally active in spermatozoa? – in V Simpósio de Pós-Graduação do iBiMED, 27th June 2019, Aveiro, Portugal.