MASTER IN BIOMEDECINE

Deciphering the mitochondrial co-translational import mechanism in mammals and identification of the main actors

Absin, Sébastien

Award date:
2022

Awarding institution:
University of Namur

Faculté de Médecine

Deciphering the mitochondrial co-translational import mechanism in mammals and identification of the main actors.

Mémoire présenté pour l'obtention

du grade académique de master en sciences biomédicales

Sébastien ABSIN

Janvier 2022

Université de Namur
FACULTE DE MEDECINE
Département des sciences biomédicales
Rue de Bruxelles 61 - 5000 NAMUR
Téléphone: + 32(0)81.72.43.22
E-mail: manon.chatillon@unamur.be - http://www.unamur.be/

Deciphering the mitochondrial co-translational import mechanism in mammals and identification of the main actors.

ABSIN Sébastien

Abstract

Mitochondrial post-translational import is considered as the canonical way for mitochondrial protein import, but new evidences suggest an alternative import mechanism for mitochondrial proteins. Indeed, the existence of a co-translational protein import process (translation-coupled translocation of mitochondrial proteins) has been more recently described in yeast and in higher eukaryotes.

In addition to the role played by Tom20 in the mitochondrial co-translational import process in yeast, RNA-binding proteins (RBPs) seem also involved but are still poorly characterized in mammals. To investigate the question, an unbiased proximity labeling technique has been developed in the laboratory to identify the Tom20 proxisome and screen for trans-acting proteins involved in mitochondrial co-translational import. In order to validate the candidates identified in the BioID screening, specific co-translational import reporters have been constructed and functionally characterized. The CLUH protein was selected as a hypothesis-driven candidate and SND1 was selected as a candidate from the results obtained after the BioID experiment screening the Tom20 proxisome. Both candidates were selected due to their RBP function in mammalian cells. Indeed, linked to mitochondrial physiology, Clueless, the *Drosophila* homolog of CLUH, was found to bind both ribosomes and mitochondria. For SND1, its enrichment at the human mitochondria surface was verified by Western Blotting and ultra-resolution confocal microscopy observations. Based on these features, we evaluated the role of CLUH and SND1 in the co-translational import mechanism in human cells. However, we show here that individual CLUH and SND1 depletion in the HCT116 human cell line did not disrupt the mitochondrial co-translational import process as could be assessed with our specific reporters. This suggests at least no decisive contribution of both the CLUH and SND1 proteins in the human mitochondrial co-translational import mechanism.

Mémoire de master en sciences biomédicales
Janvier 2022
Thesis Supervisor: Sébastien Meurant and Patricia Renard

Acknowledgements.

- To Sébastien Meurant, tutor.
- To Patricia Renard, promotor.
- To the URBC staff members and PhD students of the NARILIS laboratories.

Table of content.

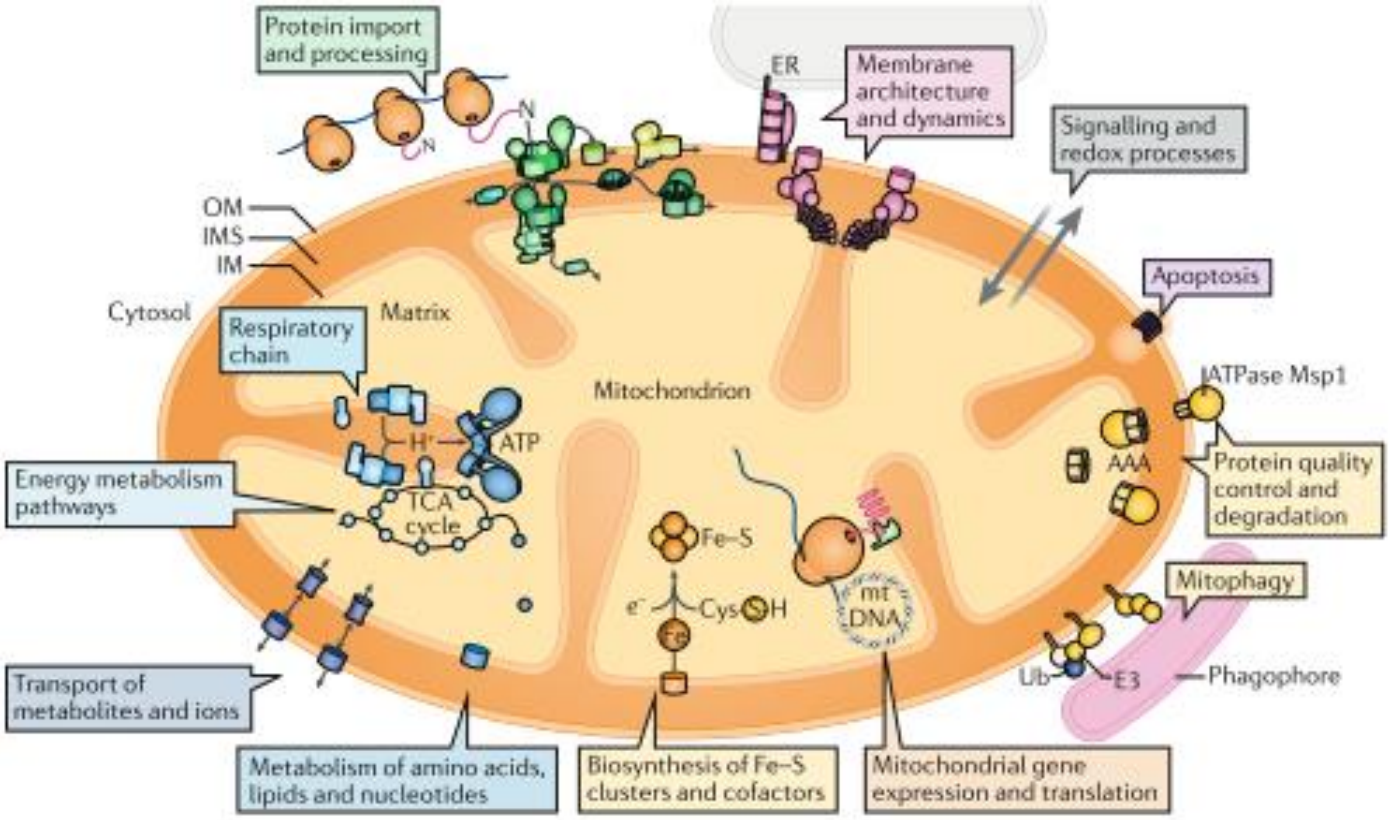


Figure 1. Overview of mitochondrial functions (Pfanner et al., 2019)**.**

Mitochondria have four different compartments with the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM) and the matrix. The mitochondrial proteins are distributed in the different compartments and ensure the multiple functions of the organelle, such as ATP generation, ROS signaling and apoptosis. Additional mitochondria activities participate in cellular dynamics with mitochondrial membrane architecture modulation, including permeabilization of mitochondrial membrane in programmed death mechanisms, signalization platform and oxidoreduction activities, protein quality control and degradation inducing mitophagy and deficient organelle elimination. In the matrix, nuclear-encoded metabolism enzyme handle tricarboxylic acid cycle, amino acid metabolism and urea cycle. There is also mitochondrial DNA maintenance and expression machineries packed in the matrix (Rich & Maréchal, 2010).

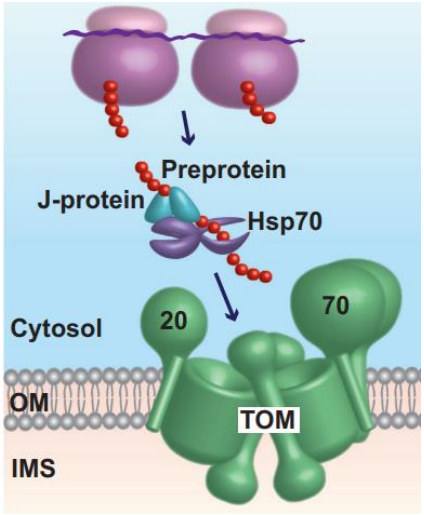


Figure 2. Post-translational preprotein delivery machinery to yeast and human mitochondria (Becker et al., 2019).

Cytosolic free ribosomes actively translating mRNA encoding for mitochondrial proteins are shown on this figure to complete mitochondrial preprotein synthesis prior to their recognition by the chaperone (Hsp70/Hsp90)/cochaperone (for example, represented J-protein) system. This complex allows preprotein targeting to the Translocon of the Outer mitochondrial Membrane (TOM) complex and interaction of the chaperone with the TOM70 receptor.

I. Introduction

1. Mitochondrial evolution in eukaryotes

Mitochondrion is an eukaryotic organelle with endosymbiotic origins. From its acquisition through the engulfment of the proteobacterium form of the mitochondrion into a protoeukaryote cell, the ancestor bacteria profoundly evolved while eukaryotic cellular complexity required mitochondrial activities specialization (Archibald, 2015). As an essential component of eukaryotic cells, the mitochondria ensure a multitude of functions such as energy production, thanks to ATP synthesis, cell signaling and even cell death control with the regulation of apoptotic mechanisms (Wiedemann & Pfanner, 2017) (fig. 1).

Therefore, mitochondrion becoming a specialized organelle of eukaryotic cells, its co-evolution led to the loss of some prokaryotic features (Archibald, 2015). Indeed, mitochondrial immersion into the eukaryotic intracellular networking with functionally different compartments imposed adaptative mitochondrial refinement such as mitochondrial genome simplification and spacial relocalization of the vast majority of prokaryotic genes from mitochondrion to the cell nucleus (Koch et al., 2021; Marc et al., 2002). Consequently, as reviewed by Wiedmann *et al.*, the human mitochondrial genome nowadays maintains only 13 protein-coding genes, encoding mitochondrial inner membrane subunits of the oxidative phosphorylation complex, in contrast to up to 1,500 human nuclear genes encoding all the other mitochondrial proteins (Calvo et al., 2016; Hansen & Herrmann, 2019; Pfanner et al., 2019; Wiedemann & Pfanner, 2017). Therefore, due to this dual genetic origin of mitochondrial proteins, a proper mitochondrial protein targeting and import system is of critical importance to ensure the different functions of mitochondria.

Mitochondrial basic protein import mechanism consists in the post-translational import of cytosolic precursor proteins targeted to mitochondria. Classically, mRNAs encoding mitochondrial proteins transit from the nucleus to the cytoplasm and are translated into preproteins by cytosolic free ribosomes (Hansen & Herrmann, 2019; Vardi-Oknin & Arava, 2019; Yogev et al., 2007) (fig. 2). Some cytosolic factors, such has chaperones or cochaperones, can recognize those signals and associate with the preproteins in order to guide them to the organelle surface receptors (Wiedemann & Pfanner, 2017). Among chaperone proteins, the most abundant cytosolic heat shock proteins 70 (Hsp70) are 70 kDa molecular weight proteins, constituted of an amino- terminal ATPase domain and a carboxyl-terminal domain which binds to hydrophobic polypeptides and protein domains in exchange of ATP consumption. By binding to the hydrophobic region of the precursors, the chaperones prevent misfolding and aggregation of mitochondrial protein precursors (Becker et al., 2019).

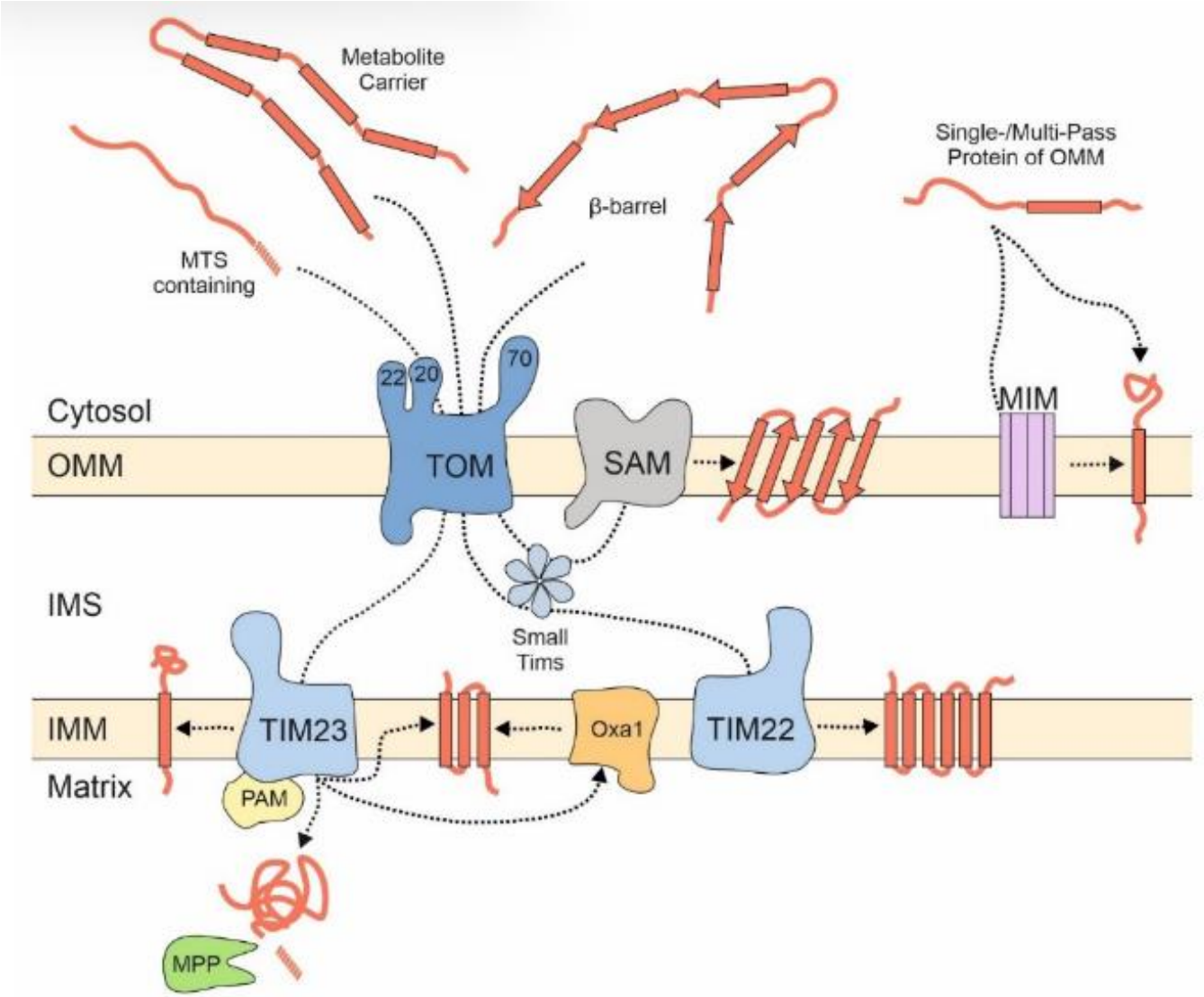


Figure 3. Summary of the different mitochondrial proteins import pathways (Koch et al., 2021)**.**

When preproteins arrive at the mitochondrial outer membrane, while proteins containing α-helical membrane anchors are being inserted in the outer membrane through the mitochondrial import complex (MIM) pathway, the vast majority of the preproteins are assimilated into the mitochondria thanks to the TOM translocase. The TOM complex ensures the translocation of preproteins into the mitochondrial intermembrane space. Then, depending on the presence or not of a cleavable presequence in their structure, two distinct preproteins groups are then redirected to their final submitochondrial localization. The presequence pathways targets matrixial import through the TIM23 translocase, thanks to the driving force of the inner membrane potential and the ATP-consuming translocase-associated motor (PAM) activity. Maturation of preprotein includes the presequence cleavage by the matrixial MPP. For non-cleavable precursors proteins, either the mitochondrial intermembrane space import and assembly (MIA) system (not shown: see Becker et al., 2019) or the small TIM proteins (chaperones) can take respectively in charge cysteine-rich precursors destined to the intermembrane space proteins and hydrophobic proteins destined to the outer membrane or inner membrane of the mitochondria. Small TIM relays to the β-barrel pathway to help outer membrane insertion of substrate proteins of sorting and assembly machinery (SAM complex) and to the TIM22 complex for inner membrane insertion of non-cleaveable precursors. This non-exhaustive list might later extends following further discoveries of new import pathways. The inner membrane Oxa1 homolog proteins have been described to play an important role in inner membrane protein insertion in both yeast and human cells, while Oxa1 deleterious mutation leads to complete mitochondrial respiratory chain deficiency (Sylvestre et al., 2003).

The distribution of preproteins preclude the existence of a multitude of sorting pathways in the mitochondria which further reroute their distribution into the different mitochondrial subcompartments (i.e., the outer membrane, the interspace membrane, the inner membrane and the matrix) (Wiedemann & Pfanner, 2017). Among them, the import of matrixial mitochondrial proteins, known as the presequence pathway, is the main import pathway used by 60% of total mitochondrial proteins (Fig. 3) (Pfanner et al., 2019). In this particular case, the N-terminal part of the preprotein contains an 8 to 80 amino acids long helicoidal amphipathic structure, called the "mitochondrial targeting signal" (MTS), allowing recognition and translocation through the uptake channels of the outer membrane. This phenomenon basically relies on the involvement of key translocases known as the Translocon of the Outer mitochondrial Membrane (TOM) complex – a protein assembly of 3 receptors subunits (Tom20, Tom22 and Tom70) and 2 to 3 β-barrel embedded pores (Tom40) (Becker et al., 2019) – and the Translocon of the Inner mitochondrial Membrane 23 (TIM23) complex– a protein assembly of a permeability modulation receptor (Tim50), TOM complex coordination subunit (Tim21) and an import motor assembly moiety taking in charge unfolded protein (Hansen & Herrmann, 2019; Neupert & Herrmann, 2007) (Wiedemann & Pfanner, 2017). At the recognition site of the TOM complex, the Tom20 receptor recognizes the MTS of the preprotein. Then, the preprotein is redirected to the Tom40 translocation channel which further releases it into the intermembrane space of the mitochondria (Hansen & Herrmann, 2019; Neupert & Herrmann, 2007; Verner, 1993). Import completion arises from a final translocation step through the mitochondrial inner membrane requiring the mitochondrial presequence translocase (TIM23) and a membrane potential as the driving force, to sort the preprotein out of the intermembrane space toward the matrix (Neupert & Herrmann, 2007). The presequence translocase-associated motor (PAM) ensures the translocation to the matrix by the ATP-dependent mtHsp70 chaperone and several other co-chaperones. The mitochondrial processing peptidase (MPP) finally cleaves off the presequence signal of the preprotein and releases it in the matrix under its mature form (Pfanner et al., 2019). Moreover, alternative import pathways requiring preprotein substrate recognition by the Tom70 receptor involve numerous chaperone proteins containing inner targeting signals which interact with tetratricopeptide repeat domains in the receptor. Docking of chaperone proteins to Tom70 implies an additional ATP consumption to release the preprotein and allow its final translocation as described above (Becker et al., 2019).

2. Translating ribosomal complexes targeted to eukaryotic OMM and ER surface

Beside the largely described mitochondrial post-translational import pathways, growing evidence has demonstrated the existence in yeast of an alternative co-translational import mechanism of mitochondrial proteins into the organelle (Becker et al., 2019). The co-translational import system conjugates the localized translation of mitochondrial protein-encoding mRNAs in close vicinity to the outer mitochondrial membrane (OMM) with the simultaneous translocation of the nascent-chain peptide into the organelle lumen (Eliyahu et al., 2010).

First, pioneering observations made by Kellems *et al.* and then by Verner *et al.*, between 1973 and 1993, have unveiled the existence of mRNA segregation to close vicinity to the mitochondria in yeast. Observations of growing yeast mitochondria micrographs by transmission electronic microscopy also revealed the presence of ribosomes bound to the mitochondrial outer membrane (Verner, 1993). Mitochondrial fractionations of yeast treated with a translation inhibitor (cycloheximide) highlighted enhanced association of actively translating ribosomes with mitochondria (R. E. Kellems & Butow, 1972). Moreover, following cycloheximide preincubation, the ribosomal resistance to dissociation was analyzed by sequential KCl and puromycin treatment of the cells. Results indicated that a partial release of engaged ribosomal complexes in presence of KCl needed cotreatment with puromycin to completely take them off from the yeast mitochondrial surface. This suggests the presence of a limited number of contact sites maintaining ribosomal association at the mitochondrial membrane (R. E. Kellems & Butow, 1972). This was the first evidence demonstrating translation activity of 80S cytosolic ribosomes at the surface of yeast mitochondria (Fujiki & Verner, 1993; Rod E. Kellems et al., 1974).

More recently, proximity-specific ribosome profiling experiments were performed in yeast and human HEK-293T cells (Vardi-Oknin & Arava, 2019; Williams et al., 2014). The cells were genetically engineered to express a recombinant *Escherichia coli* biotin ligase enzyme called BirA fused to different endogenous mitochondrial outer membrane carrier proteins (yeast Om45 and human Tom20). Ribosomal proteins of the large ribosomal subunit (yeast Rpl16 and human Rpl10A) were in parallel fused with a biotin acceptor peptide called AviTag (Williams et al., 2014). Practically, because *in vivo* biotinylation can only occur upon enzyme-substrate close interactions (Branon et al., 2018), isolation of tagged ribosomes after a short pulse of biotinylation followed by a purification step with streptavidin allowed researchers to confirm ribosomal proximity to both the yeast and the human mitochondria. The pulled-down material was further analyzed to identify the associated mRNAs, showing that hundreds of proteins destined to mitochondria were actively translated nearby outer mitochondrial membrane in yeast and human HEK-293T cells (Lesnik et al., 2015; Vardi-Oknin & Arava, 2019) In addition, numerous nuclear-encoded transcripts encoding mitochondrial protein were enriched in polysomes bound to mitochondria purified from HeLa mitochondrial fractions (Sylvestre et al., 2003).

Then, experiments focused on the transcriptome in human HEK293T cells establishing spatial repartition of transcripts. Screening of mRNA localized on the OMM was performed after endogenous expression of a fusion protein composed of Tom20 and “APEX2” derived from soybean ascorbate peroxidase, in living-cells (Fazal et al., 2019; Lam et al., 2014). Indeed, upon rapid treatment of cells with hydrogen peroxide in presence of biotin-phenol, the enzyme catalyzed the generation of an oxidized radical form of its substrate which is instantly tagging any surrounding protein and mRNA with biotin (Fazal et al., 2019; Lam et al., 2014). Then, streptavidin beads were used for enrichment of the mRNA prior to their sequencing (Fazal et al., 2019). As a result, asymmetric distributions of mRNA were detected at the reticulum endoplasmic surface, the mitochondrial outer membrane, the nucleus and other subcellular localizations and linked with the localization of proteins they encode (Fazal et al., 2019).

Taking the example of the endoplasmic reticulum (ER) as an eukaryotic organelle specialized in cellular protein trafficking, it acts as an exchange platform rerouting protein toward multiple cellular destinations. The ER co-translational import machinery has been extensively described (Aviram & Schuldiner, 2017). Considered as a referent model in cells, it could be extrapolated to other subcellular localizations enriched in translating mRNAs and actively importing newly synthetized proteins.

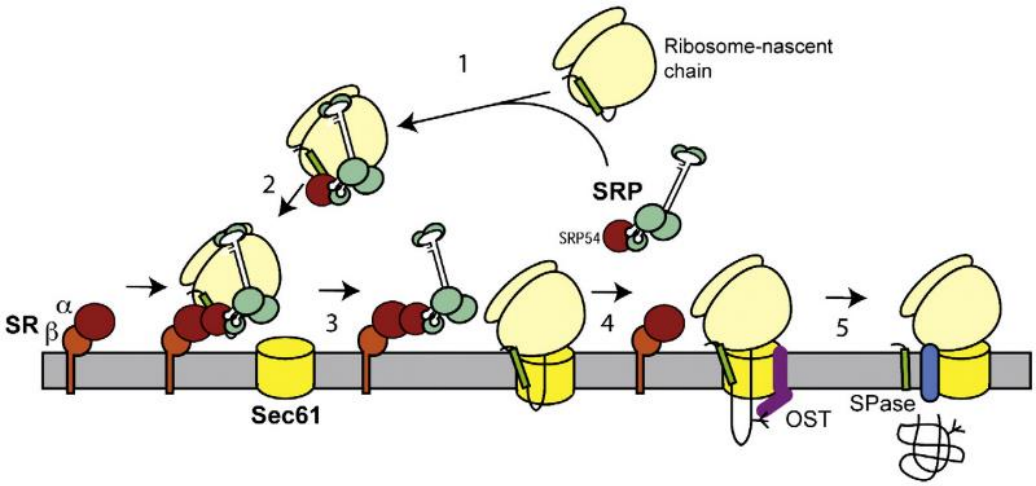


Fig. 4. The mammalian co-translational protein targeting cycle to the endoplasmic reticulum surface (Nyathi et al., 2013).

The SRP cycle consists in first the nascent polypeptide chain (signal sequence) recognition, binding and inhibition of elongation at the ribosomal surface (Halic & Beckmann, 2005). Second, prior SRP and SR binding with GTP allows efficient ribosomal complex targeting to the ER surface (Halic & Beckmann, 2005), so that the SRP bound to the ribosomal complex associates with the SR receptor (composed of α and β subunits) on the ER membrane. Third, GTP hydrolysis results in elongating preprotein transfer to the Sec61 translocon channel and SRP release to the cytosol. Fourth, protein synthesis resumes simultaneously with preprotein translocation through Sec61 pore. Fourth, protein synthesis recapitulates simultaneously to its insertion into the translocon pore. Fifth, signal peptidase (SPase) and oligosaccharyl transferase (OST) are recruited to the translocon to respectively cut the signal peptide and add N-glycan to the elongating protein.

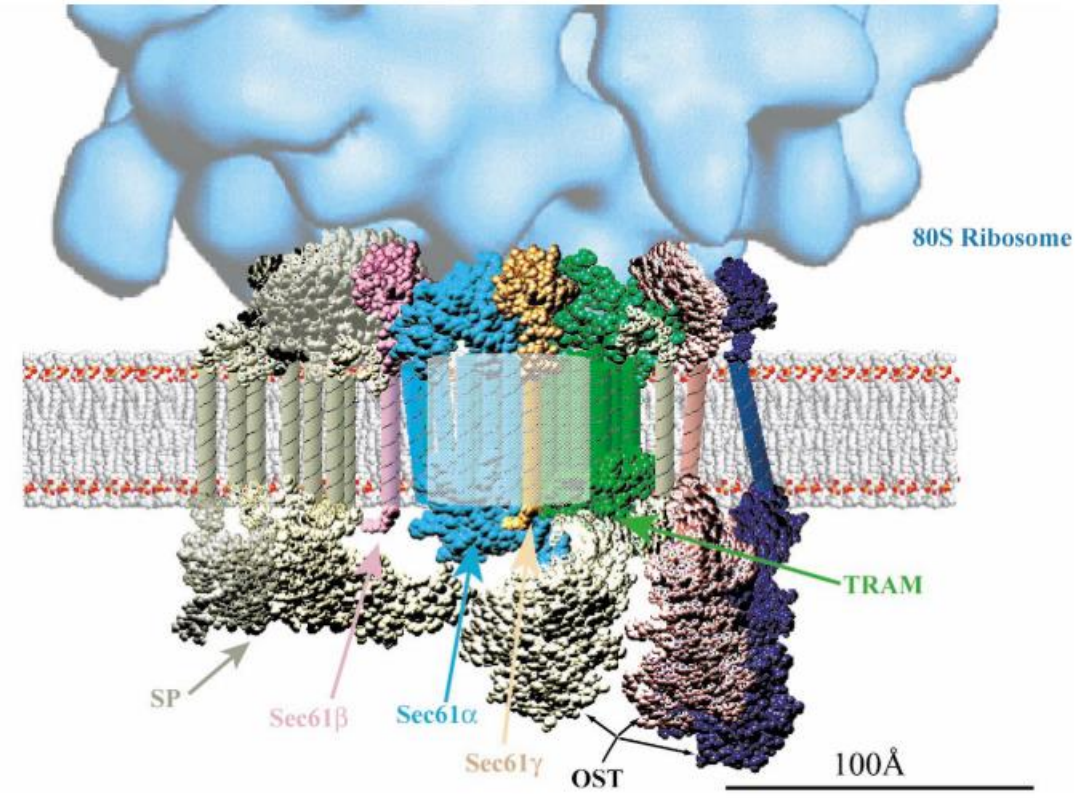


Figure 5. Ribosome docking on Sec61 complex at the surface of endoplasmic reticulum (Johnson & Van Waes, 1999).

This structural overview of the translocon protein assembly in the ER membrane shows the mammalian translocation-associated membrane protein (TRAM) reported to help integration and translocation of most protein substrates of the translocation pore, the translocon subunits (Sec61α, Sec61β and Sex61γ), annex proteins signal peptidase (SP) and oligosaccharyltransferase (OST) playing a role in post-translocation maturation of imported proteins (Johnson & Van Waes, 1999).

In the ER model, the signal peptide emerging from ribosome has to be recognized and to be combined to cytosolic factors which importantly hide highly hydrophobic nascent polypeptide chain to the cytosol and guide ribosomal complex to the ER (Kogan & Gvozdev, 2014; Lesnik et al., 2015). First, the classical guiding element to the ER is the signal recognition particle (SRP). It will combine to peptide showing endoplasmic reticulum targeting signal. In addition, a second partner called the nascent polypeptide chain complex (NAC) is a cochaperone of the ribosomal complex composed of cytosolic α and β homolog proteins which regulate ribosomal translation efficiency in higher eukaryotes by preventing protein misfolding and aggregation (Avendaño-Monsalve et al., 2020; Kogan & Gvozdev, 2014; Wang et al., 2010). Simultaneously binding to a distinct part of the elongating polypeptide chain, NAC formation at the surface of the ribosomal complex is mediated by specific interactions between the N-terminus part of β-NAC (Ott et al., 2015) with ribosomal proteins in direct surrounding of ribosomal exit tunnel and subsequent heterodimerization with α-NAC. Consequently, α-NAC enters into competitive association with the elongating polypeptide chain to protect it from cytosolic ubiquitin ligase (Kogan & Gvozdev, 2014). So, formation of this triple complex "NAC-nascent polypeptide-SRP" is favorably targeting ribosomal complex to ER.

ER cotranslational protein import mechanism implies SRP recognition of the signal peptide and targeting of the ribosomal complex to an ER anchored receptor (Fig. 4-5). In other words, the SRP recognizes early stages of ribosomal translation and eventually binds to hydrophobic signal sequences of emergent nascent polypeptide chains exiting the ribosome (Aviram & Schuldiner, 2017; Gamerdinger et al., 2015). Thanks to this association, the translating ribosomes are targeted to the endoplasmic reticulum membrane and further bind to the ER-localized SRP receptor (SR). This receptor is composed of the soluble SRα subunit and the SRβ membrane-embedded subunit. Then, following GTP hydrolysis, SRP dissociates from the receptor and targets other ribosomal substrates to the ER. Finally, the canonical Sec61 translocon – a heterotrimeric complex composed of Sec61α, Sec61β and Sec61γ in mammalian cells (Johnson & Van Waes, 1999) – is activated upon ribosomal docking to the endoplasmic reticulum surface, further causing conformational changes of the import pore. The hydrophilic environment of the import gate is favorable for the nascent-chain peptide co-translational import and reduces the tendencies for hydrophobic protein residues aggregation (Aviram & Schuldiner, 2017). In addition, docking of the ribosomes to the targeted membrane eventually spares the energy consumption required when cytosolic factors, such as chaperone and co-chaperone systems, carry precursor proteins in classical post-translational uptake mechanisms (Lesnik et al., 2015).

Interestingly, when the SRP factor is specifically depleted in yeast, a class of mRNA destined to endoplasmic reticulum is mistargeted to mitochondrial surface (Ingolia et al., 2019). This suggests a two-sided hypothesis: first, as specific depletion of one member of an interconnected functional mechanism disrupts it as a whole, we can then identify a particularly critical actor which cannot be rescued by the existence of redundant partners of the same mechanism in cells. Second, as this depletion leads to mistargeting to another organelle, then we can suppose the existence of a similar existing factor exhibiting the same roles and properties at the other organelle surface. It could be possible that the mistargeting of the ER proteins to mitochondria is part of cross-recognition of ER substrates by the co-translational machinery of the mitochondria.

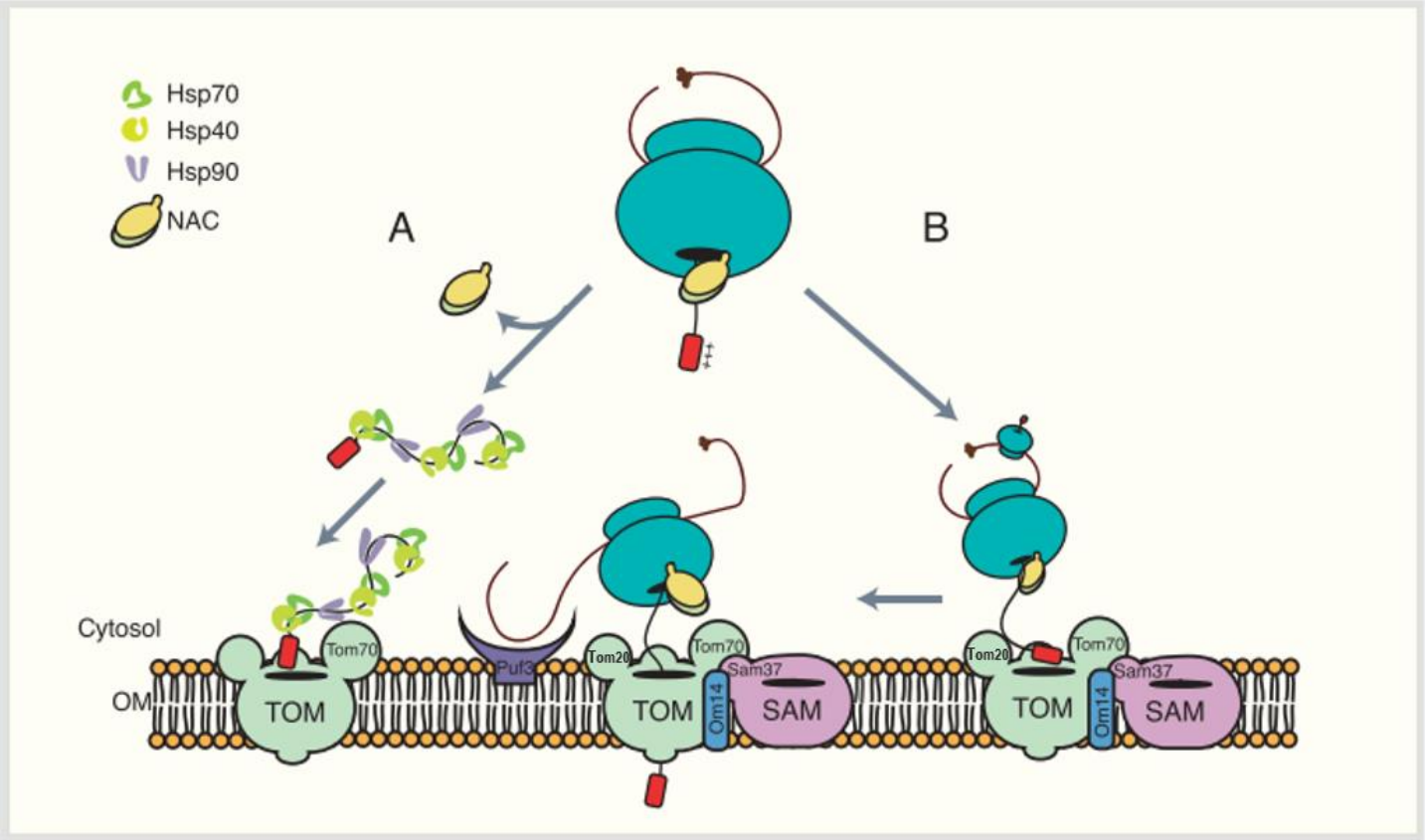


Figure 6. Summary scheme representing yeast post-translational transport of mitochondrial preproteins to the TOM complex and yeast co-translational receptors targeting actively translating ribosomes to mitochondria (Avendaño-Monsalve et al., 2020)**.**

(A) In the left side of the scheme, preproteins are being completed in the cytosol by free ribosomes recognized by the chaperon system composed of Hsp90 and Hsp70/Hsp40-based ribosome-associated complex (Ott et al., 2015) and transported to the OMM TOM complex. (B) On the right side of the scheme, the ribosome actively translating mitochondrial protein is labeled with the NAC complex when highly hydrophobic residues of the nascent polypeptide chain are displayed to the cytosol. Then, Tom20p subunit of the TOM complex and Om14 are respectively interacting with the MTS amphipathic signal contained in the N-terminal part of the precursor protein and with the αβ-NAC of the tagged ribosome as specific OMM receptor of this complex. The presence of Tom20p has been shown to increase mRNA asymmetric segregation on the yeast mitochondrial outer membrane (Eliyahu et al., 2010). Altogether, those two receptors gather mRNA and ribosomes to close vicinity of the mitochondria. In addition, Sam37 is a mitochondrial membrane protein which constitutes an additional cytosolic domain at the yeast mitochondrial surface strengthening αβ-NAC association to mitochondria (Wenz et al., 2015). This receptor is promoting transient association of the TOM complex and the SAM complex during β-barrel protein insertion. Finally, Puf3 anchored trans-acting factor increases ribosomal adhesion to the mitochondrial surface by binding to the 3' untranslated part of the mRNA encoding for mitochondrial preprotein. Local translation of the mitochondrial protein resumes, and translocation occurs simultaneous at the mitochondrial surface.

So, since SRP-dependent translocation in the endoplasmic reticulum requires the binding of cytosolic factors guiding the ribosome to the co-translational receptor, we can hypothesize that similar cytosolic factors and/or mitochondrial receptors exist and contribute to the mitochondrial co-translational import machinery.

Later on, experiments on growing yeasts allowed to identify mitochondrial receptor of ribosomes actively translating mitochondrial preproteins. When cells were treated with a polypeptide chain elongation inhibitor called puromycin (Enam et al., 2020) causing mRNA expulsion from ribosomal complexes (Fazal et al., 2019), the complete mitochondrial dissociation of the engaged ribosomal complexes was reported (Verner, 1993). If we refer to the existence of cytosolic factors binding to substrate proteins of the ER co-translational import mechanism, the disruption of mitochondria-ribosome association could be explained by lack of binding of cytosolic factors such as the nascent polypeptide chain complex (NAC). Initially supposed to target associated ribosomes to the yeast mitochondria outer membrane, NAC formation could be dependent on the presence of emerging polypeptide chains explaining why puromycin would repress the accumulation of ribosomes at the surface of mitochondria. At the opposite, this is consistent with cycloheximide treatment of growing yeasts, causing stabilization of actively translating ribosomes and exposition of the nascent-chain peptide in the cytosol, which can be recognized by cytosolic factors combining to ribosomes and targeting them to the yeast mitochondria outer membrane. This hypothesis was verified when more recent publications identified the yeast mitochondrial outer membrane protein Om14 as the receptor interacting with the NAC complex of the ribosomes. This receptor plays an important role in ribosome docking at the surface of mitochondria and promotes co-translational import of mitochondrial proteins (Fig. 6) (Lesnik et al., 2014).

Furthermore, in yeast, it has been shown that the Sam37 subunit of the SAM complex recruit ribosomes. Sam37 makes a link between both the TOM and the SAM machineries localized on the mitochondrial outer membrane in yeast (Wenz et al., 2015). This interaction between Sam37 and the ribosome allows to bring actively translating ribosomal complex to close vicinity of the TOM complex (Becker et al., 2019). This was confirmed with the implication of the Tom20p subunit of the yeast mitochondria outer membrane in the reinforcement of ribosomal adhesion to the yeast mitochondria (Eliyahu et al., 2010). Subcellular fractionations of Tom20p-depleted yeast strain revealed a loss of mRNA segregation to mitochondria (Eliyahu et al., 2010). For this reason, in this context, the MTS part of the preprotein is expected to play a role through its recognition by the TOM complex and more precisely by TOM20 (Fig. 6).

Another factor which might account for co-translational import mechanism is the presence of cis-acting elements in mRNA sequences, which are necessary addressing signals for the targeting of the mRNA toward the mitochondrial outer membrane. These signals add a layer of regulation for mRNA stability and segregation in close vicinity of the human mitochondria.

In the paper of Fazal *et al*. enriched nuclear-encoded mitochondrial transcripts were purified at the surface of human HEK293T mitochondria following *in vivo* proximity labeling and RT-qPCR technics. This paper showed that 2 different categories of mitochondrial transcripts are localized on the mitochondrial surface. After cell treatment with puromycin, all translation in cells was interrupted and transcripts were released from ribosomal complexes. Subsequently, a first group of enriched mRNAs displayed a self-capability to independently segregate to the mitochondrial surface: mRNA-dependent category (puromycin-insensitive). Then, a second condition where cells were treated with cycloheximide (CHX) allowed to identify another group of mRNAs that segregated to the OMM when cytosolic ribosomes were actively translating them: transcript of actively translating mRNA category (cycloheximide-insensitive) (Fazal et al., 2019). Interestingly, when cells were treated with uncoupling agent, mRNA enrichment at the OMM resulted in a similar transcript profiling to the one obtained after puromycin treatment. The two transcript profiles generated between cell treated with puromycin and cells treated with CHX are distinct: mRNAs (in the puromycin condition) that spontaneously segregate to the OMM encode mitochondrial ribosome components and respiratory chain complexes, while mRNA trapped in stabilized ribosomal complex encode for other mitochondrial components, most of those presenting MTS. Moreover, mRNA-dependent of ribosomal complexes weren't retrieved at the mitochondrial outer membrane after puromycin treatment (Fazal et al., 2019).

Concerning the mRNA-dependent category with ribosome targeting to mitochondria, it has been shown that the 3' untranslated regions of at least 100 mRNAs encoding mitochondrial proteins is required for the mRNAs to be efficiently segregated. This information is even essential to target ribosomal complexes to the yeast mitochondria (Sylvestre et al., 2003). Thus, intrinsic information at the 3' untranslated region of mRNA influences segregation of the transcript in cells. Such information confers to the mRNA a structural potency allowing the recognition and binding of partner proteins such as RNA binding proteins involved in mRNA transport (Berkovits & Mayr, 2015). Depending on the length of this non-coding sequence, different splicing forms direct the mRNA at different cell locations, indicating the existence of cis acting-dependent mRNA segregation mechanisms in cells (Berkovits & Mayr, 2015). According to prediction score analyses, 3' untranslated regions with G/U rich sequences and AAUAAA sequences in their tails are predicted to be localized at the mitochondrial surface (Fazal et al., 2019). In the same manner, 5' UTR of mRNA may also be involved in mRNA targeting, but to a lesser extent (Fazal et al., 2019). Therefore, these non-coding regions of the mRNA strengthen transcript targeting to the mitochondria thanks to the presence of partner proteins called hereby "trans-acting factors".

Another layer of complexity added to the co-translational mechanism includes thus the presence of trans-acting factors, such as proteins able to recognize several patterns of ribonucleotide sequences and regulate transcripts fate at the OMM. Those factors can play different roles either directly on the mRNA encoding for mitochondrial proteins, or on other proteins participating in localized translation of mRNA.

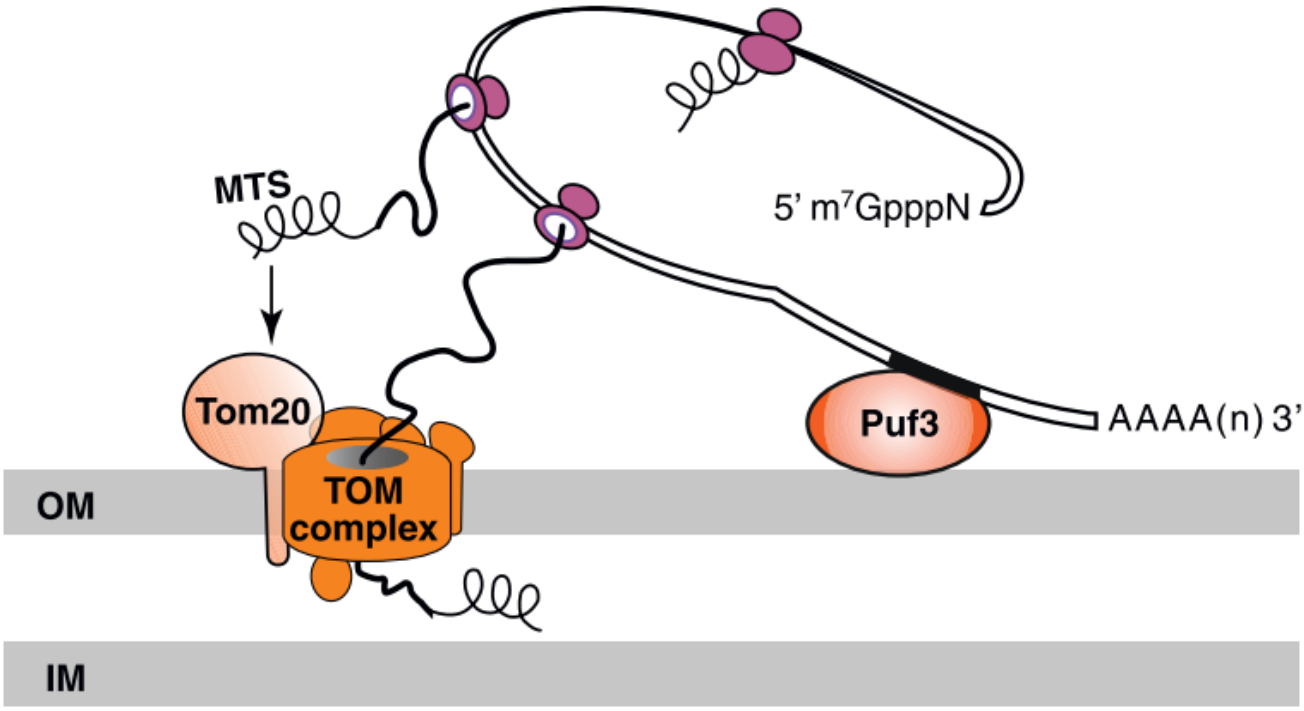


Figure 7. Dynamic action of PUF family proteins in localized translation and co-translational import into yeast mitochondria (Quenault et al., 2011)**.**

The yeast Puf3 protein interacting with 3'UTR mRNA sequences, in coordination with the tethering action of the Tom20p protein, interacting with the MTS emerging from actively translating ribosomes, favor localized translation of mRNA encoding mitochondrial proteins at the surface of mitochondria. In addition, Puf3p acting as a scaffolding protein can specifically recruit effector partner proteins in the proximity of bound mRNA. Related recruited effectors activities can either stabilize mRNA, allowing longer half-life and increased protein synthesis, or reduce mRNA half-life and modify mitochondrial metabolism and response to cellular needs.

A well described trans-acting factor in yeast is the Pumilio family of RNA binding proteins and more precisely Puf3. This RBP is localized on the surface of mitochondria where it has been shown to ensure the asymmetric distribution of mRNA encoding for mitochondrial proteins from nuclear origin (M. Garcia, X. Darzacq, T. Delaveau & R. H. Singer, 2007). This RNA binding feature of the protein is associated with the presence of Pumilio domain in its structure, allowing interaction with specific sequences in the 3'UTR of mRNA. Puf3 regulates transcripts translation rate and yeast mitochondrial biogenesis since large majority of Puf3-interacting mRNAs encode mitochondrial proteins (M. Garcia, X. Darzacq, T. Delaveau & R. H. Singer, 2007; Quenault et al., 2011). In addition, Puf3 is able to control mRNA half-life and stability at the yeast mitochondrial surface by recruiting exonucleases which shorten mRNA 3'poly-A tails (M. Garcia, X. Darzacq, T. Delaveau & R. H. Singer, 2007; Quenault et al., 2011). The yeast Puf5p was also described to recruit diverse protein partners such as translation repressors, decapping enzymes, explaining its role on several regulation levels around mRNA in yeast (Quenault et al., 2011). More interestingly, this RNA-interacting properties of Puf family proteins is conserved from yeast to higher eukaryotes, including human (Quenault et al., 2011).

Subsequent observations unveiled the essential combination of the MTS cis-acting element interacting with Tom20p receptor of the yeast mitochondrial outer membrane, and the 3' untranslated regions in mRNAs acting as additional cis-acting elements binding to Puf3p trans-acting factor, to promote adhesion of active ribosomal particles to the mitochondrial surface (Fig. 6-7). If the expression of those 2 trans-acting elements is suppressed, it induces yeast growth defect under conditions which require optimal mitochondrial functions (Eliyahu et al., 2010) and it can be even lethal when yeasts are grown on non-fermentable carbon source (Lesnik et al., 2015).

As trans-acting factors promote the co-translational import of mitochondrial proteins in yeast, the next step is to investigate the existence of similar elements in other eukaryotic models.

In *Drosophila*, two protein complexes were found to have a role in the localized translation of mitochondrial proteins at the mitochondrial surface: a complex composed of the mitochondrial outer membrane MDI (Mitochondrial DNA Insufficient) protein and the translational stimulator Larp (La-related RNA binding protein fly homolog) which form the MDI-Larp complex (Zhang et al., 2016); and a complex composed of the kinase PTEN- induced putative kinase (PINK1) and Parkin protein (E3 ubiquitin ligase) which form the PINK1-Parkin complex (Zhang et al., 2016). MDI is a conserved A kinase anchoring protein (AKAP) able to tether partner proteins among which Larp, but also signaling proteins. Interestingly, the MDI human homologue protein called AKAP1 is also localized on the human mitochondria surface. The protein complex MDI-Larp was described to localize cytosolic ribosomes at the close vicinity of mitochondria and to allow a local translation of nuclear mRNAs encoding for mitochondrial proteins by targeting specific pattern in the 5' untranslated regions of mRNAs (Zhang et al., 2016). Moreover, when MDI fly mutants contained deleterious mutations in mitochondrial DNA, it appeared that unhealthy mitochondria accumulated. The authors proposed that MDI-Larp complex might play a role in mitochondrial clearance of defective organelles, as a process of quality control of mitochondria population. As a result, damaged mitochondria are removed in a process called mitophagy (Zhang et al., 2016). Mitophagy is a mechanism targeting, among other, impaired mitochondria following inner membrane potential drop. The involvement of the PINK1-Parkin couple has been extensively described (Eiyama & Okamoto, 2015). PINK1

expression is maintained at a minimal level in cells containing healthy mitochondria, being translocated into mitochondrial matrix where its mature form is cleaved by the rhomboid-like serine protease PARL nearby the inner membrane of the mitochondria. However, upon mitochondrial uncoupling, PINK1 can homodimerize and associate to the TOM complex. Then PINK1, activated by autophosphorylation, promotes Parkin targeting to the mitochondria and phosphorylates ubiquitin S65 which will allow formation of the ubiquitin-thioester required for the E3 ubiquitin ligase activity of Parkin. Ubiquitination of OMM proteins is the starting point of mitophagy (Eiyama & Okamoto, 2015).

In addition, the *Drosophila* Clueless protein was demonstrated to bind ribosomes at the surface of mitochondria (Sen & Cox, 2016). Furthermore, its mammalian homolog protein called CLUH (clustered mitochondria homolog) was described as an RNA binding protein which specifically binds nuclear transcripts encoding mitochondrial proteins (Gao et al., 2014). Finally, coexpression and gene ontology analyses in human and mouse showed that CLUH expression is associated with the expression of genes related to mitochondrial functions, translation and ribosome biogenesis, concordantly with a potential role in hypothetical localized translation on the mitochondrial surface in human cells (Gao et al., 2014). For these three reasons, CLUH was considered in this work as a potential candidate playing a role in the mitochondrial post-translational import in mammals.

3. The mitochondrial co-translational import mechanism as an alternative and adapted protein uptake solution in human

At this point, all previously discussed elements highlighted the existence of localized translation at the surface of mitochondria, in yeast and higher eukaryotes. But still, lack of evidence showing coupled translation and translocation at the mitochondrial surface cannot prove implication in a co-translation mechanism.

Chasing of preproteins into mitochondria was analyzed following cycloheximide addition to detect involvement of translation in the translocation of the preprotein into yeast mitochondria. They discovered that the yeast cytochrome oxidase subunit IV (COXIV) protein couldn't be imported into the mitochondria if translation was inhibited. Similarly, uncoupling the mitochondrial inner membrane potential, leading to import deficiency, and subsequent restauration of the potential led to the accumulation of the precursor into cytosolic pool. This suggests the existence of strictly co-translationally imported proteins into the mitochondria (Fujiki & Verner, 1993). Studies and observations performed in yeast have shown 3 mitochondrial preproteins (i.e. fumarase, superoxide dismutase 2 and adenylate kinase) which cannot enter mitochondria after cytosolic translation completion (Angermayr et al., 2001; Luk et al., 2005; Yogev et al., 2007). This underlines the fact that alternative mechanisms take place to compensate efficiency limitations of the mitochondrial post-translational import mechanism. Indeed, for the example of fumarase, post-translational import incompetency was demonstrated in yeast treated with uncoupling agent wherein accumulating preproteins in the cytosol couldn't be chased into mitochondria after mitochondrial inner membrane potential reestablishment (Yogev et al., 2007). Further experiments dealing with mitochondrial preprotein post-translational import limitations led researchers to determine fumarase post-translational import deficiency root cause. While isolated MTS element of the preprotein was characterized to be an efficient leader information to import protein into the yeast mitochondria in a post-translational manner, folded structure of the whole fumarase obtained after cytosolic protein synthesis may further induce congestion of the translocase systems (Yogev et al., 2007). This highlights the fact that the structural conformation of the preprotein may be a limiting factor in the post-translational import mechanism. Furthermore, the combined suppression of the activity of cytosolic chaperone-cochaperone system (e.i. Hsp70/Ssa1) and blocking of the Tom20 outer membrane receptor (with addition of antisera) showed that the import rate of fumarase into yeast mitochondria only slightly reduced. This allowed to provide additional evidence of mitochondrial alternative import mechanism whereas authors suggested involvement of coupled translation and translocation of the yeast fumarase into the mitochondria (Yogev et al., 2007).

In order to show the existing co-translational protein import, elegant experiments use reporter constructs consisting in fusion proteins constituted of the MTS of mitochondrial preproteins fused to the amino acid sequence of the dihydrofolate reductase (DHFR) enzyme. The DHFR moiety of the reporter constructs is classically used for its ability to be stabilized by folate substrate or analogous compounds, trapping the enzyme in the cytosol when translation completion occurs prior to its import into mitochondria (Fujiki & Verner, 1993). Such reporter constructs were shown to be imported into the mitochondria in a co-translational manner in presence of methotrexate (a folate analog) in HeLa cells when they were constituted of the presequences of the ornithine transcarbamylase (preOTC), the arginase II (preARG2) or the aldehyde deshydrogenase 2 (preALDH2) (+ the 10 first corresponding amino acid of the mature form of the protein) fused to the DHFR enzyme. Results after fluorescence microscopy observation were positive, demonstrating that related proteins could be imported into mitochondrial via the co-translational import mechanism as an alternative route instead of the post-translational machinery (Mukhopadhyay et al., 2004).

Even if the existence of the co-translational mechanism in human mitochondria has been observed, nowadays protein complexes actively regulating translation on the surface of mammalian mitochondria are only poorly known. This project aims at identifying those mRNA-interacting proteins. Such potential trans-acting factor of the co-translational import mechanism in human mitochondria will be hereby named as “candidate”.

In practice, exploratory validation of trans-acting candidates is performed in HCT116 cells using the specific co-translational import reporters, in knock-down conditions of the candidates, and by immunofluorescence confocal microscopy observation. If the candidate has a role in the co-translational machinery, knock-down effects will impair mitochondrial import of the reporter construct known to be co-translationally imported into the mitochondria (table 1). Although the possibility that the reporter could be optionally imported into the human mitochondria in a post-translational manner cannot be discarded, the addition of folate analog to cell culture, causing post-translational machinery impairment, allow to identify clearly co-translational import candidate.

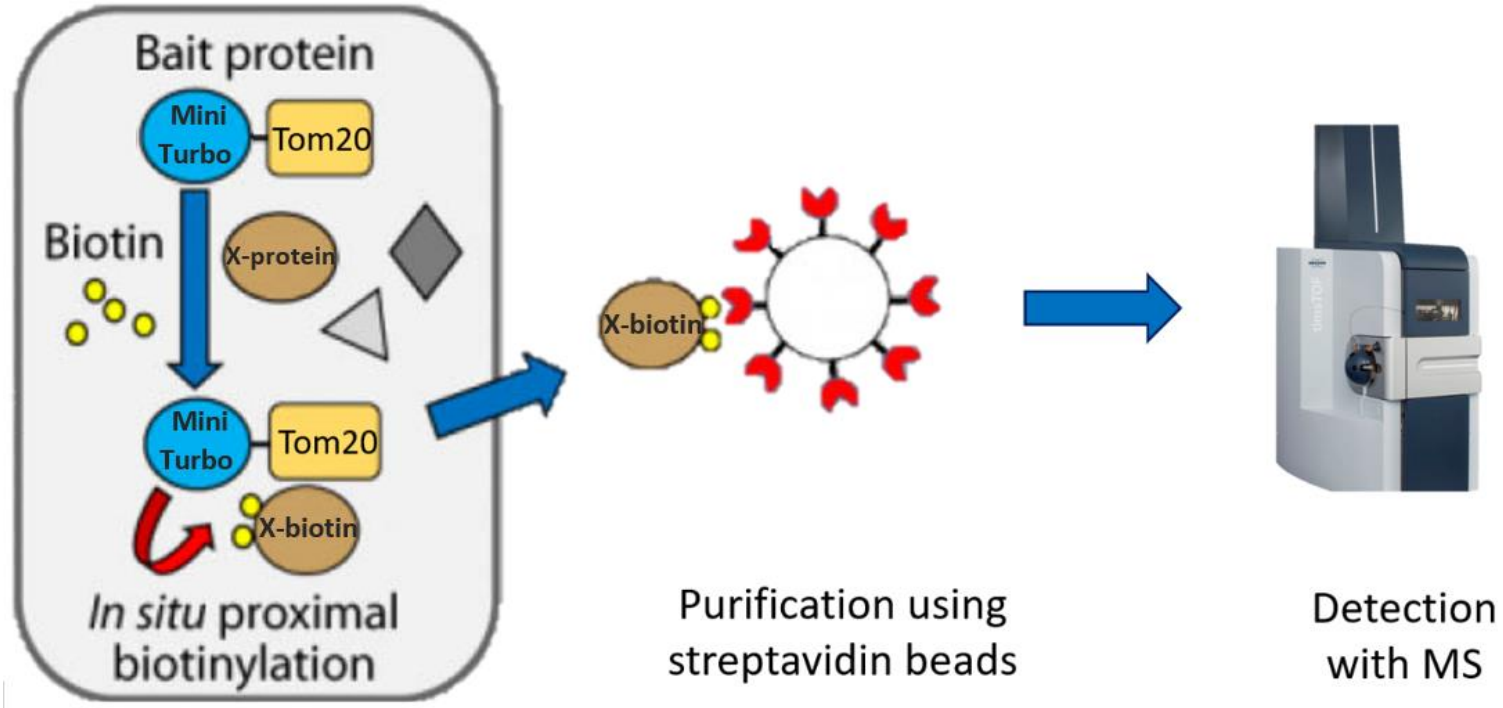


Figure 8. *In vivo* BioID experimental model to screen human Tom20 proxisome. [1]

HCT116 cells were genetically modified to endogenously express fused Tom20-MiniTurbo protein at the surface of human mitochondria. Upon short pulse of *in situ* proximal biotinylation mediated by the MiniTurbo biotin ligase enzyme, proteins in direct surrounding become tagged with a covalent biotin. This biotin is used for a subsequent streptavidin-bead purification step to obtain Tom20 protein proxisome in the eluate. Finally, an unbiased detection method implies mass spectrometry.

[1]Figure mounting based on online web ressources (last visited in January 2021): https://www.medicalexpo.de/prod/bruker-daltonics-inc/product-75820-853493.html, https://www.tebu-bio.com/blog/2015/06/17/bioid-proximity-dependent

4. Experimental approach: Human Tom20 proxisome screening

Human proteome screening in direct proximity of the Tom20 subunit at the OMM has been analyzed by a technique which is known as proximity labeling experiment. And since Tom20 plays an important role in mRNA targeting to mitochondria, it was decided to useTom20 as the bait protein for the BioID assay (Fig. 8).

The BioID enzyme is a mutant of the *Escherichia coli* biotin ligase (called BirA*) which biotinylates any nucleophile amino acid residues in close contact with it (few nanometers) (Branon et al., 2018; Roux et al., 2012). This biotinylation activity, introduced *in vivo*, fits with proteome screening by biotinylation in localized areas around carrier endogenous protein in mammalian cells since biotin grafting occurs in cells in a non-toxic manner. Then, the BioID technique evolved towards more rapid mutants of the original BioID enzyme called TurboID and miniTurboID. Those new generation enzymes have a reduced biotinylation time required to obtain sufficient protein material to screen in an unbiased manner with the mass spectrometer, and miniTurboID is even more precise in its biotinylation behaviour because it is not creating a lot of background signals by biotinylating substrate proteins before introduction of exogenous biotin to cell medium. For this reason, and because the miniTurboID is a smaller enzyme of 28 kDa, containing a N-terminal deletion and 13 mutations in comparison to the BirA* enzyme (Branon et al., 2018), it was this biotin ligase that was selected for Tom20 proxisome screening.

A BioID-competent HCT116 knocked-in cell line was thus constructed using the CRISPR-Cas9 technology, as described in Vandemortele *et al.* (Vandemoortele et al., 2017). The HCT116 cells were modified to express a chimeric protein composed of the miniTurboID enzyme fused to the carboxy-terminal end of the Tom20 endogenous gene (Branon et al., 2018). Upon biotin addition, the endogenously expressed modified biotin ligase will biotinylate all the proteins, on their lysine residues, found in close vicinity (10 nm range) of TOM20. In parallel, a control HCT116 cell line was similarly constructed with the miniTurboID enzyme fused to the mitochondrial outer membrane carnitine palmitoyltransferase 1A (CPT1A) coding sequence, to discriminate background noise from TOM20 proxisome. CPT1A has been selected as a mitochondrial outer membrane protein which is not related to the TOM complex. Following streptavidin pull-down, the enriched proteins of the two BioID conditions were identified and quantified by mass spectrometry.

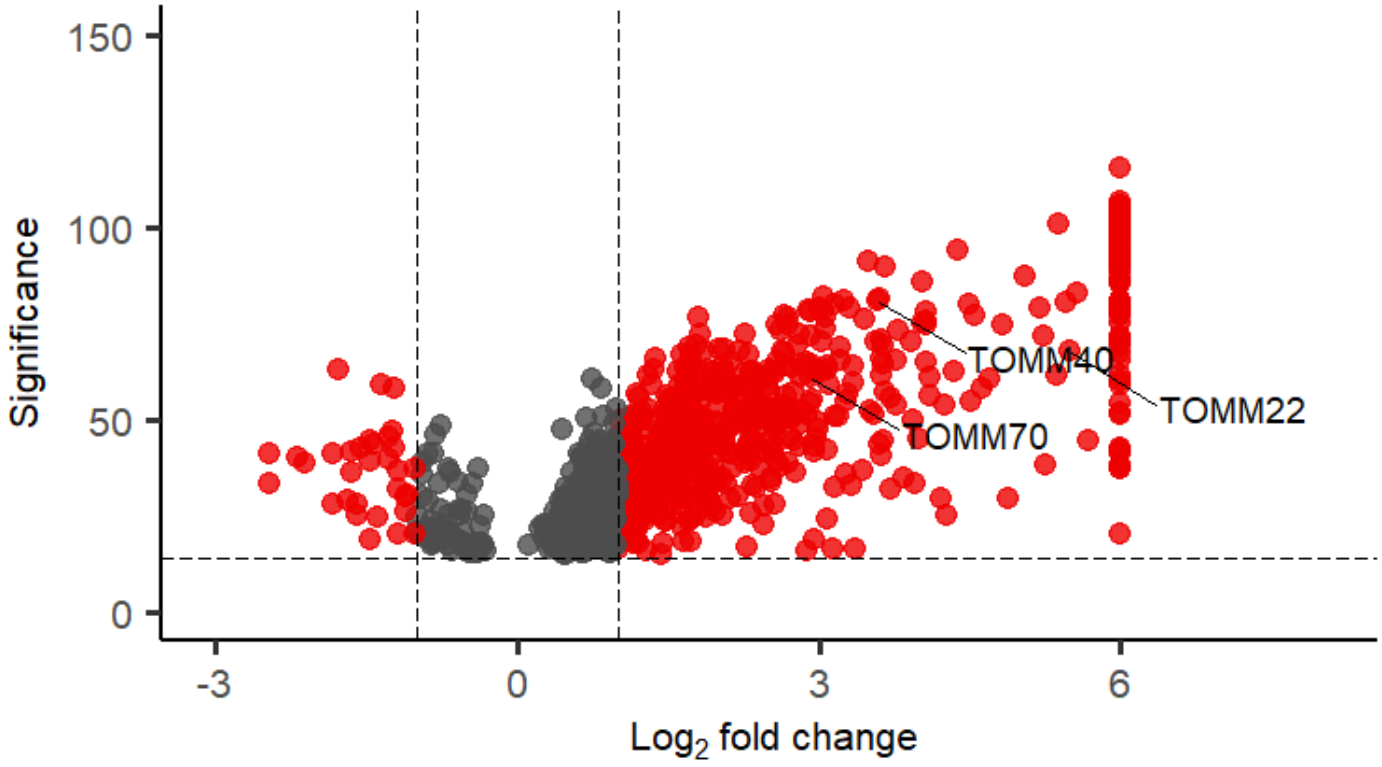


Figure 9. Result of the BioID experiment in HCT116 cells comparing the Tom20 proxisome to the CPT1A control proxisome.

A total amount of 1,021 proteins was identified in the BioID experiment. TOMM40, TOMM22 and TOMM70 are shown on the volcano plot to indicate an enrichment of the TOM proteins. The Y-axis shows the significance value derived from mathematical conversion of the p-value ($-\log_{10}P$). The X-axis shows the protein enrichment in Tom20 proxisome versus CPT1A proxisome following a $\log_2$ transformation of the data. Significance thresholds are drawn at ± 1 $\log_2$-fold change between the 2 conditions. The proteins aligned on a X-value corresponding to 6 show unique appearance in Tom20 condition, and no detection in CPT1A condition.

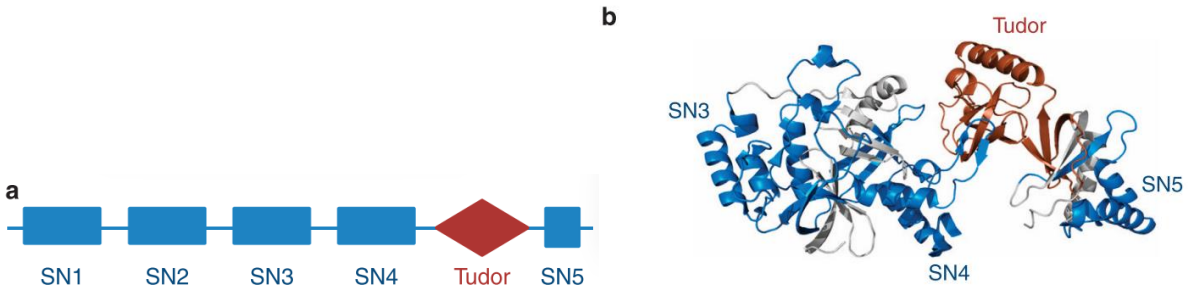


Figure 10. Schematic representation of protein domains and tertiary structure of SND1 (Gutierrez-Beltran et al., 2016).

(A) SND1 has two functional poles with, on its amino-terminal side, the presence of four repeated staphylococcal nuclease-like domains and one additional repeated domain fused to a Tudor domain on its carboxyl-terminal side (Caudy et al., 2003). (B) Crystallographic protein structure of SND1.

5. Candidate protein selection

Thanks to this proxisome profiling, the selection of candidates was performed among the proteins enriched in the Tom20 condition (Fig. 9). Taking into account that Tom20 is also involved in mitochondrial preprotein import in a post-translational manner, it is necessary to keep in mind that all proteins in the proximity of Tom20 are not mitochondrial surface trans-acting factors involved in the co-translational import mechanism. In order to discriminate interesting candidates implicated in the co-translational machinery from unrelated ones, a careful investigation of each protein associated function based on the literature and databases had to be done. The most interesting functions are, among others, protein binding to mRNA and protein involved in localized translation. But the literature analysis can also lead to collect additional information such as cellular localization(s) of those candidates, their interactome or additional role(s) and function(s) in other cell mechanisms. An example of interesting candidate would be a protein triggering translational activities and enriched in close vicinity of the mitochondrial outer membrane translocase. We will then analyse its possible involvement in the mitochondrial co-translational import process using the specific co-translational reporters.

A candidate selected from the BioID results is SND1 (Staphylococcal Nuclease Tudor containing Domain protein 1), that is 4,16-fold enriched in the TOM20 condition (p-value = 3,55E-63). By reviewing the literature, the mammalian SND1 (commonly known as "p100") is described as a 103 kDa protein evolutionary conserved between different species such as plants and *Drosophila* cells (Caudy et al., 2003).

This protein has a broad range of functions which can regulate all stages of gene expression, from transcription to post-transcriptional regulation (Gutierrez-Beltran et al., 2016).

Moreover, SND1 is associated with the ribosome and plays a role in the SRP-independent co-translational targeting of ER protein to the ER. Indeed, alternative pathways, such as the SND pathway, were identified be part of the ribosomal complex and to play a role in endoplasmic reticulum targeting of substrate proteins (Aviram & Schuldiner, 2017). So, although the mechanism hasn't been completely elucidated in yeast, we could hypothesize that the SND1 Tudor domain plays a role in scaffolding multiprotein complexes leading to ribosome retargeting to endoplasmic reticulum to allow co-translational import of the proteins retargeting to endoplasmic reticulum to allow co-translational import of the proteins (Aviram & Schuldiner, 2017; Gutierrez-Beltran et al., 2016).

SND1 is principally found in the cytosol, but also in the nucleus, where it can perform RNA interference (RNAi) activities by association with RISC complex member proteins such as the Argonaute family protein AGO2 (3,59-fold enrichment in the Tom20 condition (p-value = 1,35E-47)) and the Fragile X mental retardation autosomal homolog variant p2K (FXR1, 2,64-fold enrichment in the Tom20 condition (p-value = 2,88E-43)) (Caudy et al., 2003), the two components of the RISC complex that were also identified at the surface of human mitochondria.

The ribonucleoprotein complex RISC has endonucleolytic activities in cells (Kobayashi & Tomari, 2016). *In vivo,* presence of small interfering RNA (siRNA) will individually or in association with the RISC complex lead to mRNA site-specific targeted decay because of the activity of dicer (Caudy et al., 2003). In contrast, micrococcal nucleases domains contained in the SND1 protein do not have specific substrate affinities, but it can cleave any RNA sequences. So, among its functions, SND1 participates in local mRNA clearance agent in the complexes where it is contained.

On the other hand, SND1 homolog proteins in Arabidopsis plant (TNS1 and TNS2) have been shown to stabilize some cytosolic mRNAs in cells (Gutiérrez-Beltran et al., 2015).

Based on the fact that hundreds of transcripts encoding mitochondrial proteins are translated in close vicinity of the mitochondrial surface (Lesnik et al., 2015; Williams et al., 2014), we can hypothesize that SND1 might contribute to regulate the stability of mRNAs encoding for mitochondrial proteins. AGO2 and FXR1 proteins might also be interesting candidates to characterize in our study for their putative role in the human mitochondria co-translational import mechanism, but this won't be performed in this work.

II. Specific aims

1- During this master thesis, the first specific aim focusses on the testing of the newly generated DHFR reporter constructs in wild-type HCT116 cells. Experiment outcome consists in subcellular characterization of mitochondrial import profile of generated reporter constructs as main basis to support results. In other words, if DHFR reporter constructs are imported into the human mitochondria, this testing determines which mitochondrial machinery (co-translational and/or post-translational) participates in their import. Then, reporter imported into the mitochondria in a co-translational manner will be selected for the next experiments.

2- Next, testing of the co-translationally imported DHFR reporters is performed in knocked-down HCT116 cells for the trans-acting candidate protein of interest. Outcome of this experiment allows to validate putative implication of the candidate protein in the co-translational import machinery.

Based on a hypothesis-driven question by reviewing the scientifical literature, we first hypothesized that CLUH is an interesting trans-acting candidature protein that might contribute to the mitochondrial co-translational import. Therefore, the second specific aim realized in this work will assess the CLUH putative implication in the co-translational import mechanism in CLUH knocked-down HCT116 cells.

3- BioID experiments results obtained by upstream workscreened Tom20 protein proxisome. Because the Tom20 subunit is known to favorize transcripts encoding for mitochondrial protein targeting at the yeast mitochondrial surface, we want to identify important trans-acting partners proteins of the co-translational machinery at the human mitochondrial surface. To do so, a protein candidate called SND1 has been selected based on its specific enrichment in the Tom20 proxisome and on literature reviewing. Prerequisite experiments have been done in order to confirm SND1 enrichment in close vicinity to mitochondria in HCT116 cells such as western blot analyses on mitochondrial fractions and ultra-resolution confocal microscopy observations. Subsequently, third specific aim of this master work consists in the testing of SND1 putative role in the mitochondrial co-translational import process in SND1 knocked-down human HCT116 cells.

III. Material and methods

Cell culture

The HeLa and HCT116 cell lines are maintained in Dulbecco's Modified Eagle Medium (DMEM) with a high glucose concentration of 4,5 g/L, 3.97 mM of L-Glutamine, 1 mM of sodium pyruvate (#41966-029, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO_2. Expanding cells were maintained under 80% confluency and passed by using trypsin-EDTA (Thermo Fisher Scientific).

Transfection of reporter constructs

HCT116 and HeLa cells were seeded on coverslips in 24-well plates at 45,000 cells per square centimeter. After 24 hours, cells were transfected with 150 ng of reporter plasmid constructs (Genscript): DHFR_ATG (the DHFR coding sequence initiated by a start codon), preCOX4I1-DHFR, preARG2-DHFR, preALDH2-DHFR, preOTC-DHFR, 5'UTR-preTOP1MT-DHFR-3'UTR, 5'UTR-preETFDH-3'UTR and 5'UTR-prePHB-3'UTR using the 30-minutes preincubated (at room temperature) XTremeGENE HP Transfection Reagent (#06366236001, Roche) in Opti-MEM I serum-free medium (Gibco) (ratio: 1 mg of DNA per 2 mL of transfecting agent). After 4 hours, the medium was replaced by DMEM 10% FBS, 1% penicillin-streptomycin medium, containing or not 100 µM trimethoprim (#T7883-5G, Sigma). After 24 hours of plasmid expression, cells were prepared for immunofluorescence analysis and confocal microscopy observation.

siRNA transfection

HCT116 cells were seeded on coverslips in 6-well plates at 300,000 cells per well for western-blot analysis of siRNA silencing efficiency, or they were seeded on coverslips in 2-well plates at 30,000 cells per square centimeter for co-translational reporter assay. After 24 hours, the medium was renewed with new one DMEM 10% FBS, 1% penicillin-streptomycin, then cells were exposed to the 20-minutes preincubated (at room temperature) DharmaFECT transfection agent (#T-2001-03, Dharmacon) in Opti-MEM I serum-free medium containing 12.5 pmol of ON-TARGETplus siRNAs (Dharmacon) (**Table 1**) per well in a dropwise manner and incubated for 24 hours. Cells in 24-well plates were then further prepared for a second transfection step with co-translational reporters while cells in 6-well plate are harvested for Western-Blot analysis.

Product code	Target sequence	Reference
CLUH siRNA 1	5'CGUACAACGCGGUGGACGU3'	#J-010152-18-0002, Dharmacon
CLUH siRNA 2	5'UAUUCAAGGUGCACAGCGA3'	#J-010152-19-0002, Dharmacon
CLUH siRNA 3	5'CCAUUGGAGACACGUGAAU3'	#J-010152-20-0002, Dharmacon
CLUH siRNA 4	5'CAAGGAAAGCUCCGAGUAC3'	#J-010152-21-0002, Dharmacon
SND1 siRNA 9	5'GGAAGUCUGUUUCACGAUA3'	#J-010657-09, Dharmacon
SND1 siRNA 10	5'UGAUGGAGAACAUGCGCAA3'	#J-010657-10, Dharmacon
SND1 siRNA 11	5'CGAGAGUUCCUUCGAAAGA3'	#J-010657-11, Dharmacon
SND1 siRNA 12	5'UCAUGGUGGACGUGCGCAA3'	#J-010657-12, Dharmacon

Table 1. siRNA sequences.

Transfection of co-translational reporter constructs in HCT116 cells knocked down for SND1

24-hours after siRNA transfection, the medium is refreshed for another 24h. Thus 48 hours post-transfection, cells were transfected with 75 ng of reporter plasmid constructs: preCOX4I1-DHFR, preARG2-DHFR, preALDH2-DHFR, preOTC-DHFR, 5'UTR-preTOP1MT-DHFR-3'UTR, 5'UTR-preETFDH-3'UTR and 5'UTR-prePHB-3'UTR, as described above. After 4 hours, the medium is replaced by DMEM 10% FBS, containing or not 100 μM TMP, for another 24 hours.

Immunofluorescence analysis and confocal microscopy observation

Cells were washed 3 times with Phosphate Buffer Saline (PBS, pH 7.4, 37°C), fixed with 4% paraformaldehyde pre-warmed at 37°C during a 10-minute incubation at room temperature and washed again 3 times with PBS. Fixed cells were then permeabilized for 5 minutes with PBS-Triton (1%) (Triton X-100, Carl Roth). To limit aspecific signal, 2 washes of 10 minutes with PBS-BSA (Bovine Serum Albumin) 2% were performed. Primary antibody solutions for immunostaining were prepared in PBS-BSA (2%) (cfr. **table 2**) and incubated with fixed cell at 4°C overnight.

The next day, the coverslips were washed twice for 10 minutes with PBS-BSA (2%). Cells were then incubated with secondary antibody in PBS-BSA (2%) supplemented with of 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) (#1023627001, Sigma-Aldrich) intercalating agent (cfr. **table 3**) for 1 hour at room temperature, in the dark. Coverslips were then washed twice with PBS-BSA (2%) for 10 minutes and then left in a final PBS volume, avoiding dessication. Mounting of the slides was performed with warmed Mowiol (56°C) (#4-88, Sigma-Aldrich). Confocal microscopy analyses were performed using Leica Confocal Microscope (Leica microsystem) or Zeiss LSM900 confocal microscope.

Immunofluorescence primary antibodies				
Target	Manufacturer	Reference	Nature	Dilution
TOMM20 (KIAA0016)	Abcam plc	#EPR15581-54	Rabbit monoclonal IgG	1/100
Myc-Tag	Cell Signaling Technology Europe, B.V.	#04/2019	Mouse monoclonal IgG	1/200
CLUH (eIF3X)	Bio-Techne Corporation (Novus)	#NB100-93306	Rabbit monoclonal IgG	1/100
SND1	Abcam plc	#GR3265402-1	Rabbit monoclonal IgG	1/100
mtHSP70	Enzo	#ALX-804-077	Mouse monoclonal IgG	1/100

Immunofluorescence secondary antibodies				
Target	Manufacturer	Reference	Nature	Dilution
Rabbit (Alexa Fluor 488 nm)	Thermo Fisher Scientific	#A-11008	Goat polyclonal	1/1.000
Mouse (Alexa Fluor 488 nm)	Thermo Fisher Scientific	#A-11001	Goat polyclonal	1/1.000
Rabbit (Alexa Fluor 568 nm)	Thermo Fisher Scientific	#A-11011	Goat polyclonal	1/1.000
Mouse (Alexa Fluor 568 nm)	Thermo Fisher Scientific	#A-11031	Goat polyclonal	1/1.000

Table 2. Antibodies used for immunofluorescence analyses.

Western-Blot analyses

Cells were harvested by trypsinization, centrifuged for 5 minutes at 300 × g. The supernatant was discarded, and cell pellets were then stored at -80°C. For cell lysis, pellets were resuspended in 100 µL RIPA lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140 mM NaCl) complemented with complete Protease Inhibitor Cocktail (#04693116001, Roche, Basel Switzerland) and homemade Phosphatase Inhibitor Cocktail (25 mM Na_3VO_4 (S-6508, Sigma-Aldrich), 250 mM 4-nitrophenylphosphate (N-3254, SigmaAldrich), 250 mM di-Sodium β-glycerophosphate pentahydrate (#27874295, VWR) and 125 mM NaF (#6449/106441, Merck Millipore)) and maintained on ice. A thermomixer mixing was performed at maximum speed at 4°C for 10 minutes. Cell lysates were then centrifuged at 16.000×g and 4°C for 10 minutes, to collect supernatants. Sample protein content was determined with the Pierce detection assay (Pierce 660 nm Protein Assay Reagent (#22660, Thermo Fisher Scientific), Ionic Detergent Compatibility Reagent (IDCR) (#22663, Thermo Fisher Scientific)) according to the manufacturer recommendations.

A polyacrylamide gel composed of a running gel (10% bis-acrylamide) and a stacking gel was prepared. 20 µg of proteins were loaded in Western Blot loading buffer (0.5 M Tris at pH 6,8 (#A411.1, Carl Roth), 0.4% SDS (#A3942, Carl Roth)). The Color Protein Standard Broad Range (10-250 kDa) (#P7719S, New England BioLabs Inc.) was used as protein ladder. Protein electrophoresis was performed at 150-200 V (400 mA and 100 W). Proteins were transferred on a PolyVinyliDene Fluoride (PVDF) membrane (Immobilon-P, Merck Millipore) using the Trans-Blot Turbo Transfer System (25V, 1,3A, 10 minutes) (Bio-Rad) or liquid transfer at 100V, at 4°C for 2h. The PVDF membrane was then incubated for 1 hour with the blocking solution Intercept Blocking Buffer (TBS) (#927-60001, Li-cor Biosciences) at room temperature. The primary antibody solutions were prepared in the Intercept Blocking Buffer (TBS) with 0.1% Tween-20 (Bio-Rad) (cfr. **table 3**) **)** and incubated with the membrane, O/N on a rocker at 4°C. The secondary antibody solution was prepared in the Intercept Blocking Buffer (TBS) with 0.1% Tween-20 (Bio-Rad) as following: anti-rabbit goat polyclonal antibody (IR Dye 800CW) (1/10.000) (#926-32211, Li-cor Biosciences) and anti-mouse goat polyclonal antibody (IR Dye 680RD) (1/10.000) (#926-68070, Li-cor Biosciences). Membrane fluorescence was detected using the Odyssey Li-cor Scanner (Li-cor Biosciences) and band intensities were measured by the Odyssey Application Software (version 3.0, Li-cor Biosciences). Protein quantifications are calculated as relative protein abundance after normalization of the fluorescence intensity of each band of protein of interest over its corresponding loading control fluorescence intensity band.

HCT116 cell fractionation

HCT116 cells grew until confluency in T75 flasks. Immediately put on ice, the flask was rinsed with cold PBS (at 4°C) once, then twice with isotonic 0.25 M cold sucrose (#9286.1, Carl Roth) solution (at 4°C). Washing buffer was meticulously vacuumed 3 times before addition of sucrose 0.25 M in the corner of the flasks. Kept on ice, flasks were scraped and cells were collected into a Dounce homogenizer (equipped with a B piston). 45 Dounce strokes were performed and 10% of total homogenate volume was saved and put on ice. Cell homogenates were then centrifuged at 1.000×g for 10 min at 4°C to pellet nuclei. The supernatant was collected in calibrated tube for ultracentrifugation. Nuclear pellets were lysed in RIPA buffer supplemented with PIC and PIB 25× (see above) and supernuclease, for Western Blot analyses. Depending on the total sucrose volume added to the cells, cells were centrifuged at 8000 RPM for a specific duration to obtain mitochondrial fractions. Cytosolic fractions were saved and sampled for 1% of its total volume and conserved at -20°C. Mitochondrial fractions were lysed

in RIPA buffer supplemented with PIC and PIB 25×, and supernuclease and prepared for Western Blot analysis as described in the "Western Blot" section.

Western-Blot preparation of all samples necessitated addition of 7,5 µL of loading buffer, except for secondary supernatant fraction of variable total volume. All samples were loaded in a 10-wells gel composed of a running gel of 12% bis-acrylamide and a staking gel, like described above. Electrophoresis, band transfer on PVDF membrane and Li-cor spectrophotometric measurements of the bands were performed as described above. Immunoblotting (cfr. **Table 3**) preparation included the use of α-tubulin, SND1, TOMM20 and lamin A/C as primary antibodies and the use of secondary antibodies, diluted in Intercept Blocking solution supplemented with 0.1% Tween.

Immunoblotting primary antibodies				
Target	Manufacturer	Reference	Nature	Dilution
α-tubulin (TUBA4A(7277))	Merck Life Science BV (Sigma)	#T5168	Mouse monoclonal IgG1	1/10.000 1/20.000
SND1	Abcam plc	#GR3265402-1	Rabbit monoclonal IgG	1/1.000
TOMM20 (KIAA0016)	Abcam plc	#EPR15581-54	Rabbit monoclonal IgG	1/1.000
Lamin A/C	BD Biosciences	#612162	Mouse monoclonal IgG	1/1.000
Phospho-HSP90α	Cell Signal	#3488	Rabbit monoclonal IgG	1/1.000

Immunoblotting secondary antibodies				
Target	Manufacturer	Reference	Nature	Dilution
Mouse (IR Dye 800CW)	Li-cor Biosciences	#926-32210	Goat polyclonal	1/10.000
Rabbit (IR Dye 800CW)	Li-cor Biosciences	#926-32211	Goat polyclonal	1/10.000
Mouse (IR Dye 680RD)	Li-cor Biosciences	#926-68070	Goat polyclonal	1/10.000
Rabbit (IR Dye 680RD)	Li-cor Biosciences	#926-68071	Goat polyclonal	1/10.000

Table 3. Antibodies used for immunoblotting in Western-blot analyses.

IV. Results

1. The presequence of the human ornithine transcarbamylase (OTC) protein triggers mitochondrial co-translational import in human cells.

In order to study the co-translational import process in human cells, specific reporters were previously constructed and had to be characterized for their co-translational import feature. Therefore, the reporters were transfected in HCT116 cells in presence of an optimal concentration of trimethoprim which blocks their post-translational import into the mitochondria. As a reminder, the DHFR reporter construct is stabilized under its folded conformation upon trimethoprim treatment of the cells, causing its mitochondrial post-translational import incompetency (Baker, 1981; Fujiki & Verner, 1993). We selected both the genetic origin of the DHFR and the folate analogue compound for an improved and more specific folding efficiency with the *Escherichia coli* DHFR and the folate analog trimethoprim (TMP). Indeed, structural analysis of the bacterial enzyme revealed a much potent inhibitory concentration (IC_{50}) of the trimethoprim targeting the *E. coli* DHFR ($IC_{50} = 5 \times 10^{-9}$ M) compared to the vertebrate DHFR ($IC_{50} = 3 \times 10^{-4}$ M) (Baker, 1981). Then, optimal concentration of trimethoprim has been chosen by taking into account the balanced efficiency of the compound to block post-translationally imported constructs and the toxicity. Optimal trimethoprim concentration was determined to be 100 µM with a 24h period of incubation.

On the other hand, targeting signals to mitochondria in the precursor protein sequence are multiple. Based on the assumption that the MTS information drives the import pathway to mitochondria, a first series of DHFR-reporter constructs combined with presequences of preproteins which were observed to be imported into mitochondria in a co-translational manner were constructed. The selected presequences of the proteins expected to be imported in a co-translational manner are: presequence of the arginase II (preARG2), presequence of the aldehyde deshydrogenase II (preALDH2), presequence of the ornithine transcarbamylase (preOTC) (Mukhopadhyay et al., 2004). In addition, the presequence of the human homolog protein of the yeast cytochrome c oxidase, subunit IV of respiratory chain (preCOX4I1) was selected for its particularity to trigger both the post-translational import mechanism and the co-translational import mechanism in yeast mitochondria (**Table 4**) (Fujiki & Verner, 1993).

Studied presequence (MTS)	Expected results
preCOX4I1	Post/co-translationally imported.
preARG2	Co-translationally imported.
preALDH	Co-translationally imported.
preOTC	Co-translationally imported.

Table 4. Expected MTS-related triggered mitochondrial uptake mechanism(s) in human HCT116 cells (Fujiki & Verner, 1993; M. Garcia, X. Darzacq, T. Delaveau & R. H. Singer, 2007; Mukhopadhyay et al., 2004; Saint-Georges et al., 2008)**.**

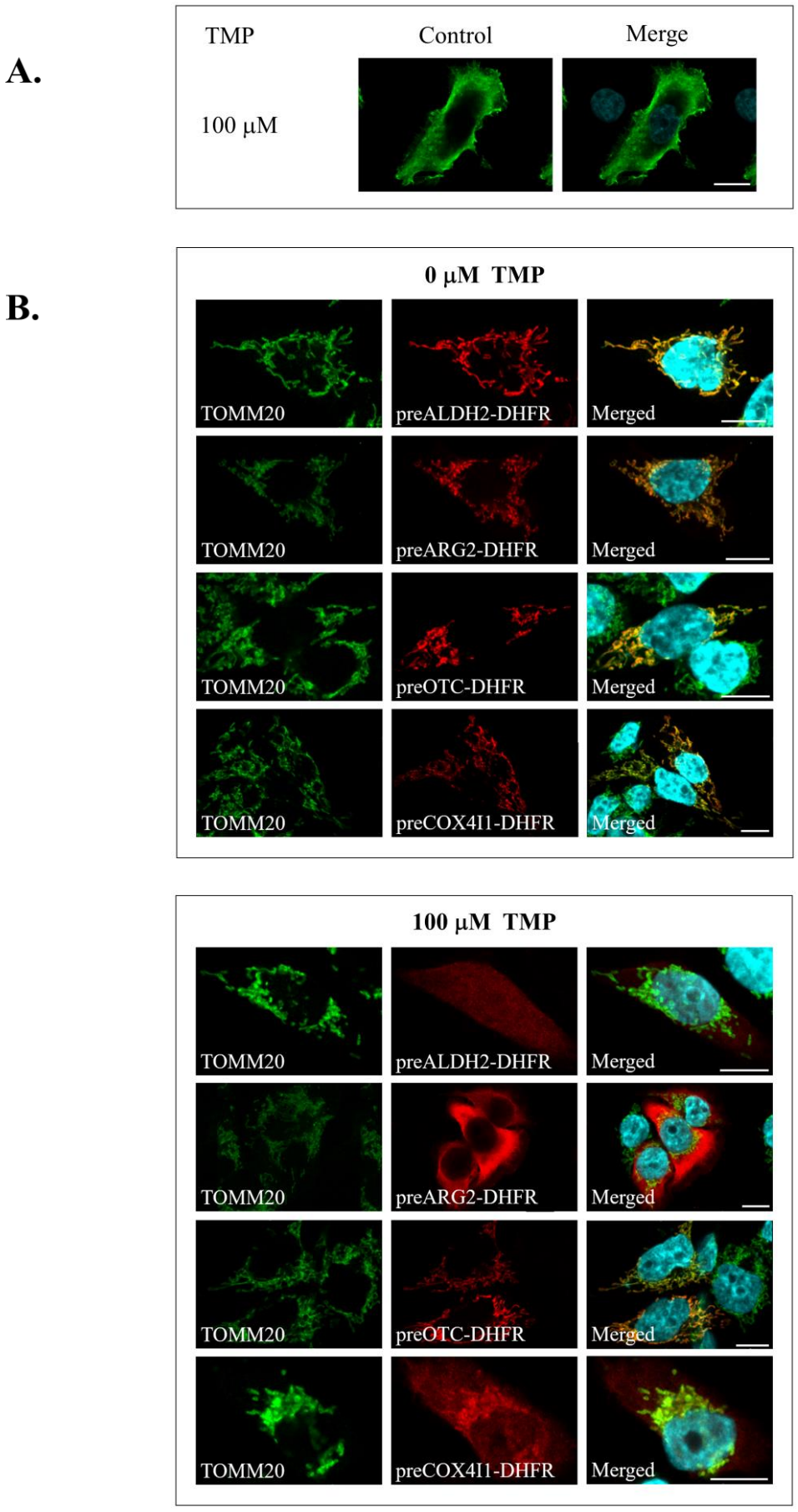


Figure 1. Subcellular localization of the MTS-containing DHFR reporter constructs. (A) HCT116 cells have been seeded on coverslips, transfected the morrow with the DHFR_ATG reporter construct, incubated for 24 hours and fixed before confocal microscopy observation. Cells were labelled using Myc-tag antibody to reveal the construct. The bar is showing 10 μm. n=2. (B) HCT116 cells were seeded on coverslips and transfected the morrow with preALDH2-DHFR, preCOX4I1-DHFR, preARG2-DHFR or preOTC-DHFR. 4h after, cells were incubated with or without 100 μM trimethoprim (TMP) for 24h and were then fixed and immunostained before confocal microscopy observation. The cells were stained with Tom20 (green) and Myc-tag (red) antibodies and nuclei were stained using DAPI. Bars are showing 10 μm, n=2.

Subsequent confocal microscopy observations of HCT116 were performed and the subcellular localization of the construct was assessed using Myc-tag antibody.

To verify correct protein sorting to mitochondria strictly based on the presence of specific cis-acting elements of interest, a negative control construct only constituted of the coding sequence of the yeast DHFR enzyme, devoid of any mitochondrial targeting signals, was expressed in cells (**fig. 1.A**). As expected, we observed a diffuse fluorescent pattern corresponding to cytosolic location, as the DHFR peptide sequence is not recognized by the protein uptake mechanisms at the human mitochondrial surface.

First, the mitochondrial targeting behavior of the different constructs has been evaluated. As previously described in yeast, the MTS of preproteins exposed to the cytosol has been shown to favor ribosomal complex targeting to the co-translational receptor apparatus on the mitochondria (Lesnik et al., 2014). Testing of the MTS-containing series of DHFR reporter constructs in HCT116 cells resulted in exclusive mitochondrial subcellular localization of all the reporter constructs after a 24-hours incubation time (**fig. 1.B**). This confirms that the MTS alone of those preproteins is sufficient to trigger the mitochondrial localization of the mature protein in human cells.

Second, post-translational import blockade was induced upon addition of 100 µM of trimethoprim to the cell culture medium. Surprisingly, we observed mitochondrial import disruption of reporter constructs containing the following preprotein amino-terminal presequences: aldehyde dehydrogenase II (preALDH2) and arginase II (preARG2) (**fig. 1.B**). This indicates that those two presequences couldn't be recognized by mitochondrial co-translational import machineries in human HCT116 cells. Only the DHFR reporter construct containing the amino-terminal presequence of the ornithine transcarbamylase (OTC) was strictly maintained in the mitochondria. Interestingly, DHFR reporter construct containing the amino-terminal presequence of the human cytochrome oxidase subunit IV (COX4I1) displayed a dual cytosolic and mitochondrial localization in cells (**fig. 1.B**).

However, not only the MTS part of precursor proteins has been shown to play a role in the co-translational import into mitochondria, but *cis*-acting information contained in the 5' untranslated and 3' untranslated regions of the mRNA has also been associated to target mRNA to the mitochondria (Berkovits & Mayr, 2015; Sylvestre et al., 2003). Based on the paper of Fazal *et al.*, additional reporter constructs have been designed. Therefore, new reporters containing the untranslated regions of the transcripts of interest were constructed and had to be characterized for their co-translational import features into human mitochondria. Two transcripts of the mRNA-dependent category (puromycin-insensitive) and one transcript of the actively translating mRNA category (cycloheximide-insensitive) have been chosen (Fazal et al., 2019). *Top1mt* is a transcript encoding human mitochondrial topoisomerase I and found to be translated in close vicinity to the human mitochondria, since cycloheximide treatment of HEK-293T cells, stabilizing ribosomal association with mitochondria, led to the enrichment of this mRNAs at the surface of mitochondria (Fazal et al., 2019). On the contrary, *Phb2* and *Etfdh* transcripts, encoding respectively human prohibitin 2 and electron-transfer flavoprotein dehydrogenase, were enriched following puromycin or CCCP treatment but negatively enriched upon CHX treatment. This suggests that the mitochondrial localization of the transcript is dependent on the transcript itself and independent of translating ribosomes (Fazal et al., 2019).

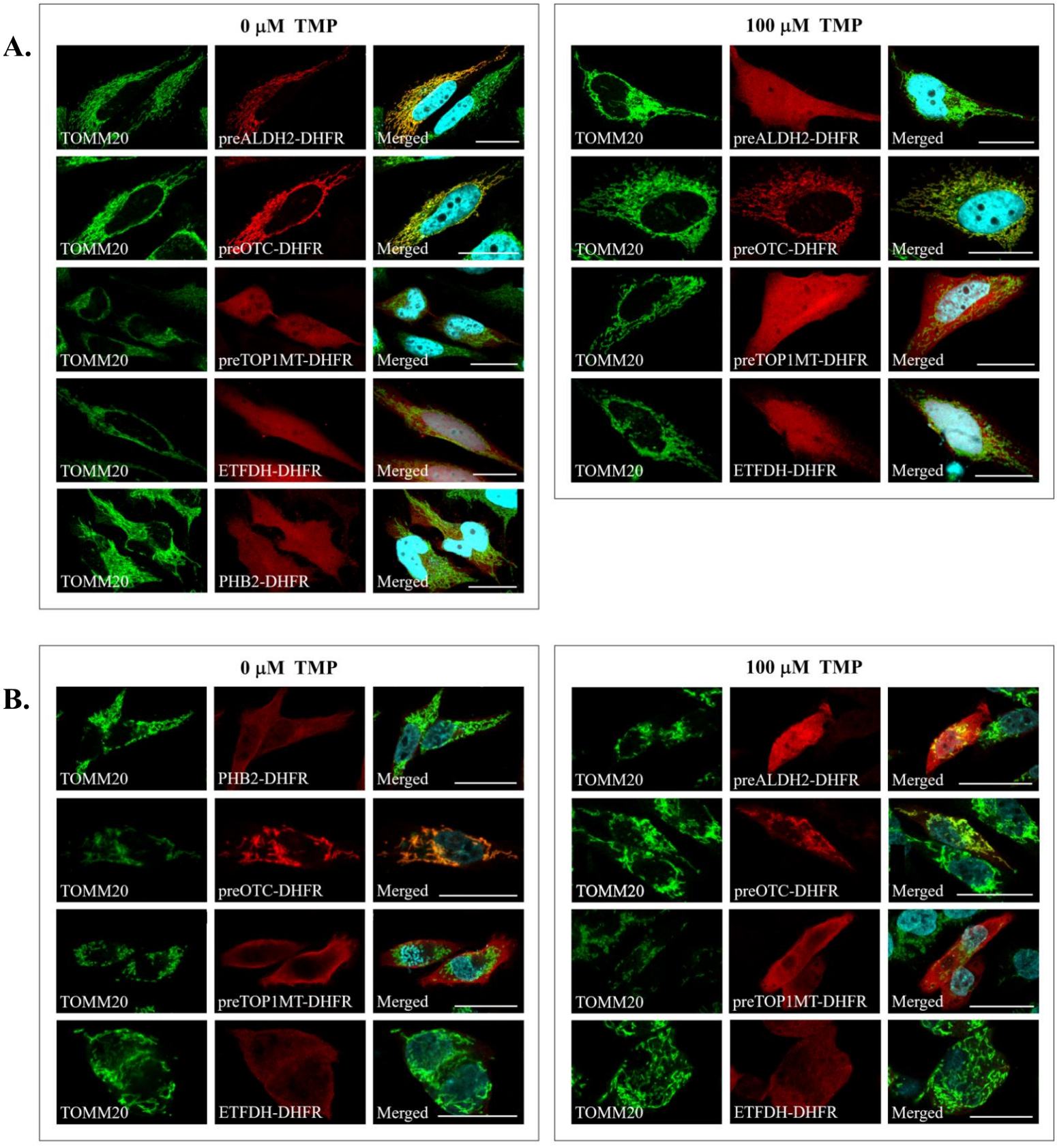


Figure 2. Subcellular localization of the MTS-containing and 3'UTR-DHFR-5'UTR reporter constructs. HeLa (A) or **HCT116** (B) cells were seeded on coverslips and transfected the morrow with preALDH2-DHFR, preOTC-DHFR, preTOP1MT-DHFR, ETFDH-DHFR or PHB2-DHFR. 4h after, cells were incubated with or without 100 μM trimethoprim for 24h and were then fixed and immunostained before confocal microscopy observation. The cells were stained with Tom20 and Myc-tag antibody and nuclei were stained using DAPI. Bars are showing 25 μm, n=1 (A) and n=1 (B).

For the DHFR reporter design, it has been decided to fuse the 5'UTR of *Top1mt*, *Phb2* and *Etfdh* (+ the MTS and the 10 first coding amino acids of the protein for the TOP1MT-DHFR reporter), to the DHFR coding sequence and finally followed by the 3'UTR of the corresponding transcripts to make the complete constructs, as schematized in the **Table 5**.

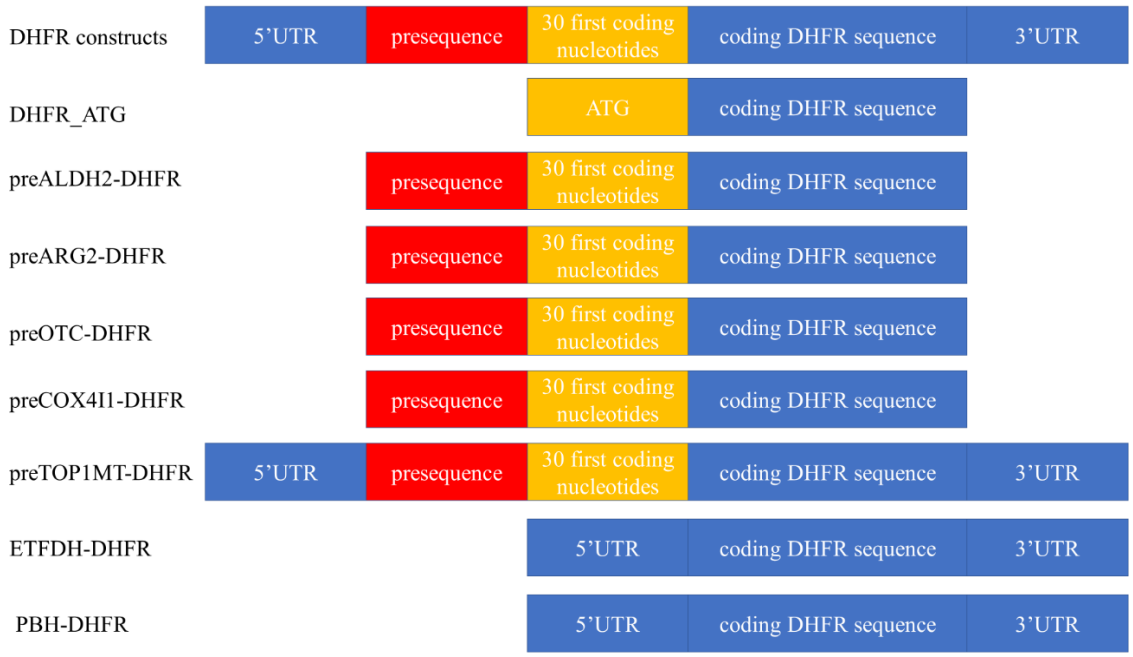


Table 5. Schematic representations of DHFR constructs.

This second series of DHFR reporter constructs was tested in HCT116 cell line (**fig. 2.B**) and in HeLa cells (**fig. 2.A**). HeLa cells were used to obtain micrographs because of biological issues in HCT116 cells, showing abnormal morphology and slowed proliferation. The results obtained from HCT116 cells and HeLa cells, showed that preALDH2-DHFR and preOTC-DHFR reporter constructs followed respectively and as expected post-translational and co-translational import into human mitochondria. However, none of the newly generated UTR-based reporter constructs allowed mitochondrial import, not even in a post-translational manner (it is to say in the absence of trimethoprim).

Consequently, only the preOTC-DHFR reporter construct has been shown to be imported into the human mitochondria in a strictly co-translational manner. This reporter will be used to detect loss of mitochondrial subcellular localization when cells are treated with trimethoprim.

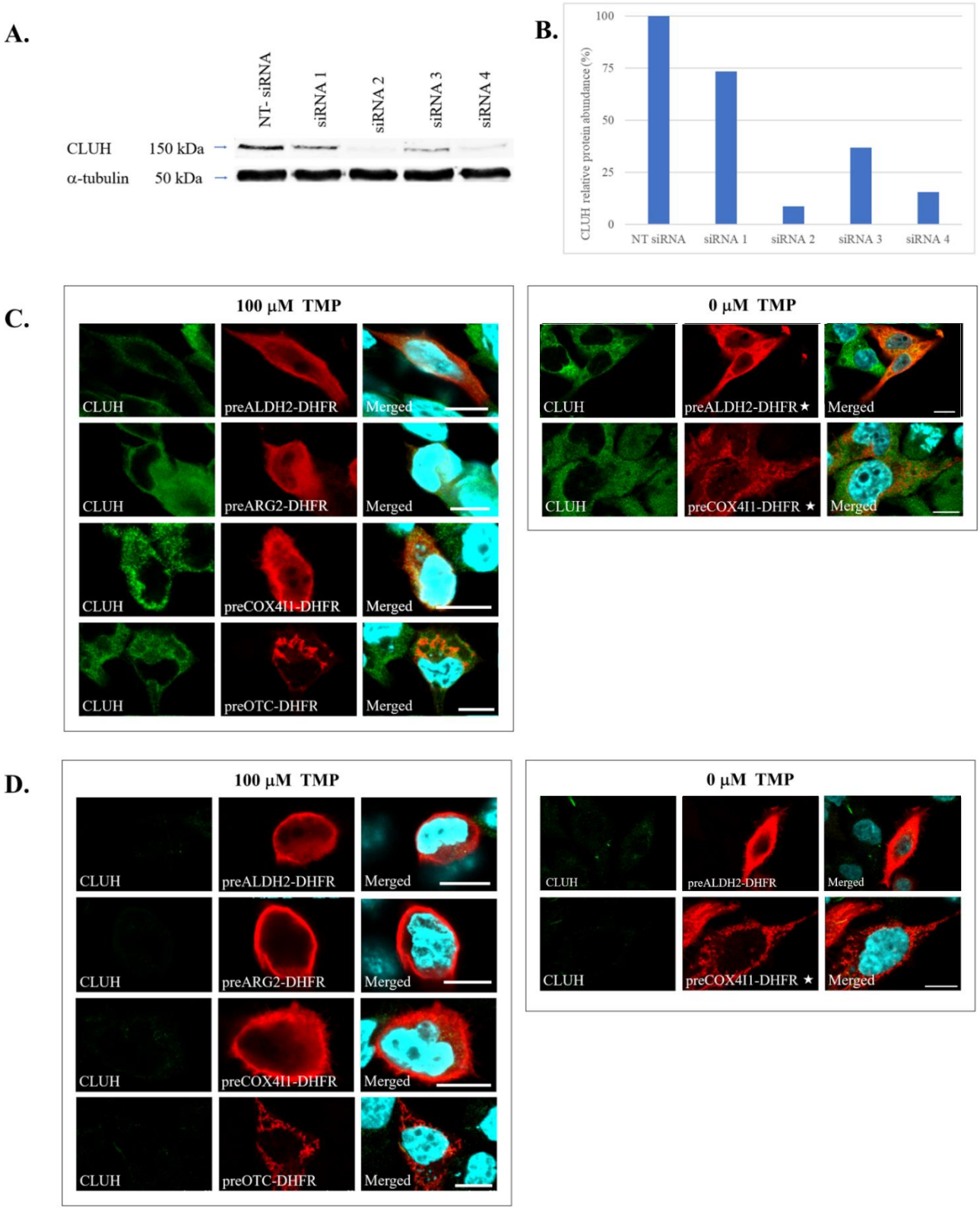


Figure 3. Effect of CLUH silencing on subcellular localization of the presequence-DHFR reporter constructs. (A-B) HCT116 cells have been seeded in a 6-well plate, transfected for 24 hours with non-targeting siRNA (NT siRNA) or siRNA targeting CLUH (siRNA 1- 4). Proteins were extracted and 20 µg of total protein per condition were resolved by SDS-PAGE. (A) Protein abundance was revealed for CLUH and a-tubulin by Western Blotting analysis. (B) CLUH and a-tubulin respective fluorescence intensities were measured by the Odyssey Application Software. Then, CLUH relative protein abundance was obtained after normalization with corresponding a-tubulin protein content and expressed as percentage to CLUH relative abundance in cells NT-siRNA treated cells. n=1. (C-E) Cells were seeded on coverslips, transfected with NT-siRNA (C), siRNA 2 (D) and incubated for 24 hours. Then, cells were transfected the morrow with post-translational (preALDH2-DHFR and preARG2-DHFR) or co-translational DHFR reporter constructs (preCOX4I1-DHFR and preOTC-DHFR). 4 hours later, cell culture medium was replaced with fresh medium containing 100 µM of trimethoprim, then cells were incubated for 24-hours (C-D) (n=2). In parallel, post-translationally imported DHFR-reporter constructs (preALDH2-DHFR) and both post-/co-translationally imported DHFR reporter construct (preCOX4I1-DHFR) were transfected in cells and incubated without trimethoprim for 24-hours (C★-D★) (n=1). Cell were fixated, stained with antibodies against CLUH (green), Myc-tag (red) and nuclei were revelated in contact with the DAPI intercaling agent before confocal microscopy observation. Bars are showing 10 µm.

2. CLUH is not involved in human mitochondria co-translational import mechanism.

Next, investigations have been performed in knocked-down HCT116 cells for the candidates. The observation of the DHFR co-translationally imported constructs into the mitochondria is the determinant factor to interpret putative role of the candidate in the mitochondrial co-translational import machinery. Subcellular localization after candidate depletion allows to deduce its implication into mitochondrial import machineries as shown in **table 6**. In this experiment, the co-translational import involvement of the candidate protein is verified if the preOTC-DHFR reporter is accumulating in the cytosol in presence of trimethoprim, and if post-translationally imported reporters are localized in the mitochondria when there is no trimethoprim. This dual observation including the preOTC-DHFR and the preALDH2-DHFR reporters is required in cells since KD effect of the candidate in cells may cause a global impairment of mitochondrial protein import activities (called primary import deficiency). An example of primary import deficiency described in yeast is the disruption of the mitochondrial inner membrane potential which causes accumulation of the preCOX4I1 precursor protein in the cytosol (Fujiki & Verner, 1993). Thus, using both the co-translationally imported preOTC-DHFR and the post-translationally imported preALDH2-DHFR reporters is a control of primary import deficiency. In addition, because the preOTC-DHFR reporter could be optionally imported in the mitochondria in a co-translational way, this reporter is tested both in presence (blocking post-translational import machinery) and in absence of trimethoprim.

Reporter / Putative role of the invalidated candidate	PreOTC-DHFR (co-translational machinery) with TMP	PreALDH2-DHFR (post-translational) without TMP
Involvement in the co-translational import	Cytosol	Mitochondria
No involvement in the co-translational import	Mitochondria	
Involvement in the post-translational import	Mitochondria	Cytosol
Primary import deficiency	Cytosol	

Table 6. Summary of possible subcellular localizations of the reporter DHFR constructs following candidate protein suppression.

The first candidate for co-translational import was CLUH. As a reminder, CLUH is a hypothesis-driven candidate as this protein has been described in mammalian cells to be a cytosolic factor which binds mitochondrial transcripts and promotes mitochondria biogenesis (Gao et al., 2014). If CLUH plays a role in the co-translational import mechanism, its silencing should affect the preOTC-DHFR reporter construct localization. This experiment is shown in **Figure 3**.

The siRNA 2 is the most efficient siRNA to knock down the CLUH protein expression in cells (**fig. 3.A – 3.B**). It reduces CLUH expression to 9% relative protein abundance in comparison to cells treated with non-targeting siRNA (**fig. 3.B**). Results for cells transfected with siRNA 2 are shown here in **Figure 3.D**.

As a control, cells transfected with non-targeting siRNA and incubated in 100 µM of trimethoprim, express the CLUH protein and display the expected subcellular localization for the different reporter constructs, as observed in **Figure 1**. The post-translationally imported reporters localize in mitochondria without trimethoprim (**fig. 3.C.★, right panel**) while they accumulate in the cytosol in the presence of trimethoprim (**fig. 3.C., left panel**). The co-translationally imported reporter showed a mitochondrial localization in presence of trimethoprim (**fig. 3.C.**).

HCT116 cells were efficiently knocked down for the CLUH protein as observed on the isolated CLUH fluorescence channel (fig. 3.D). After 24 -hours of the preOTC-DHFR reporter plasmid expression in CLUH- silenced cells and 100 µM trimethoprim condition, the mitochondrial import of the reporter is maintained in cells. This suggests that the CLUH protein depletion has no impact on the co-translational import of the reporter construct into mitochondria (Figure 3.D.). Similarly, post-translational reporter constructs preALDH2-DHFR and preARG2-DHFR were localized in the cytosol upon TMP treatment whereas preCOX4I1-DHFR still showed a dual cytosolic and mitochondrial localization (**fig. 3.D.**).

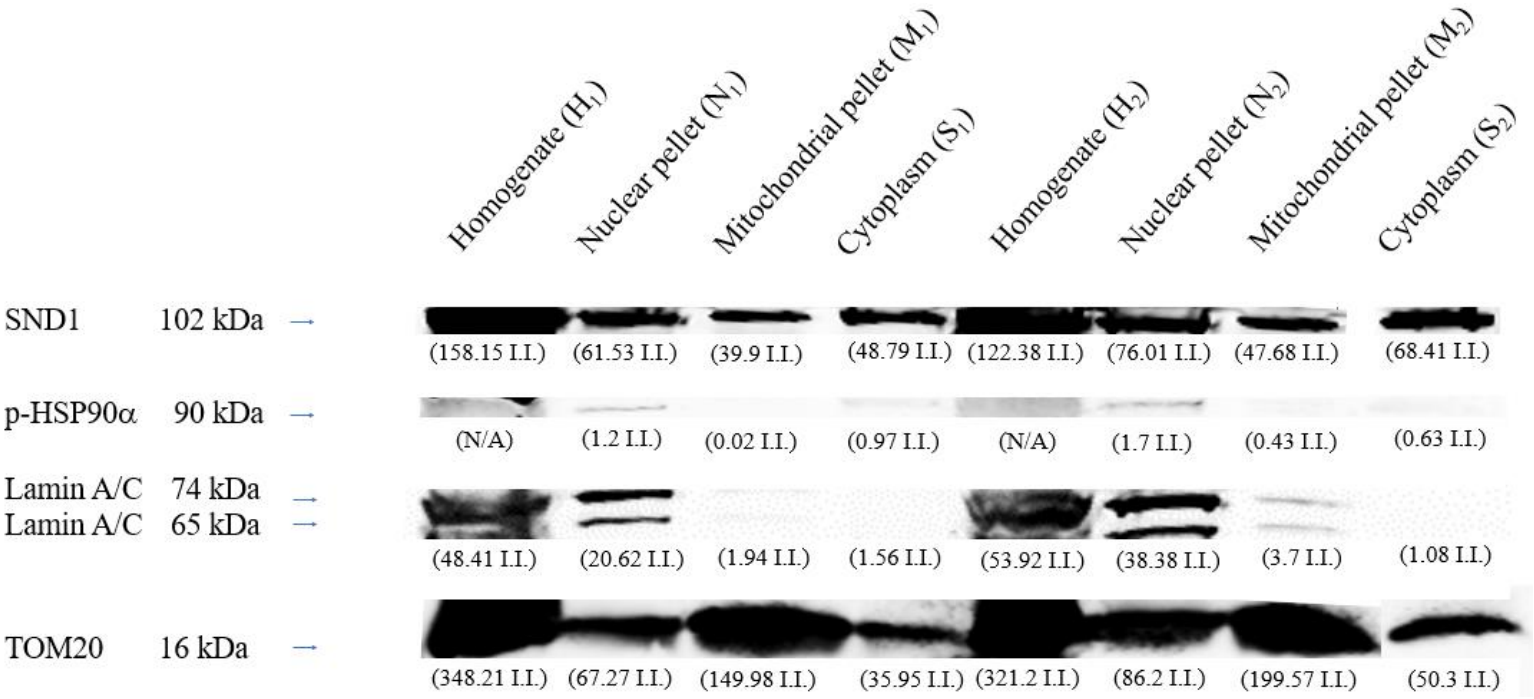
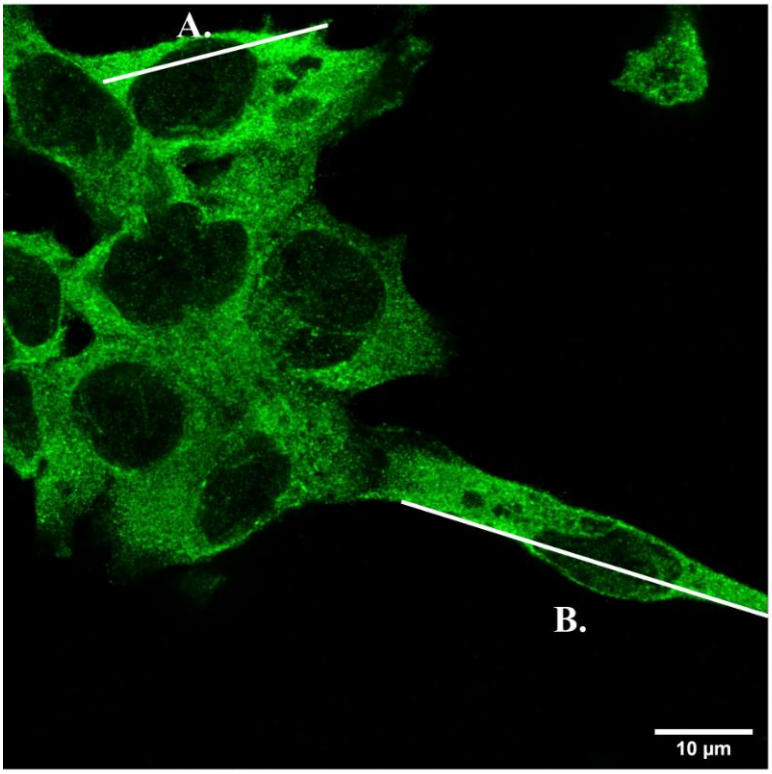
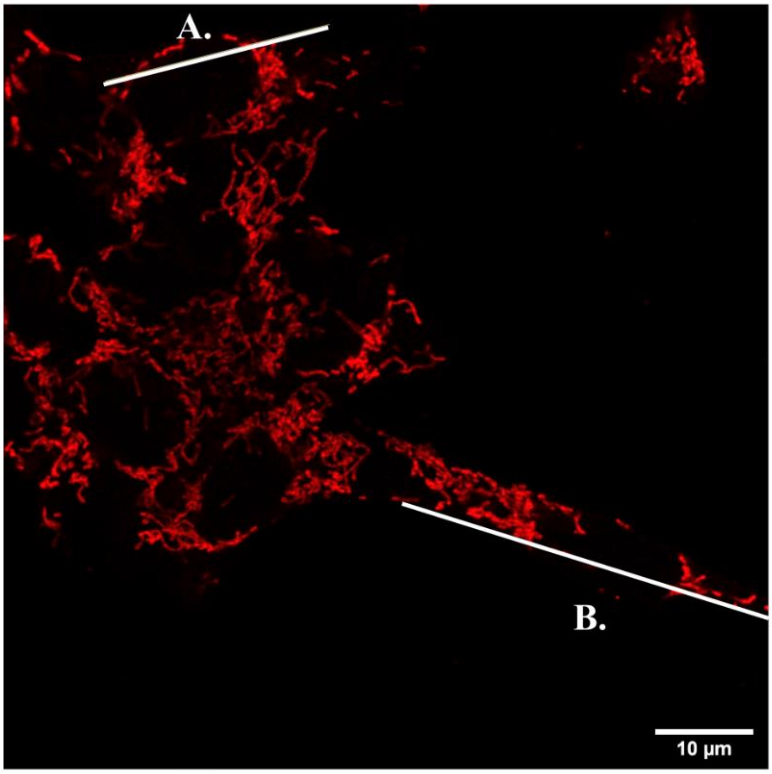
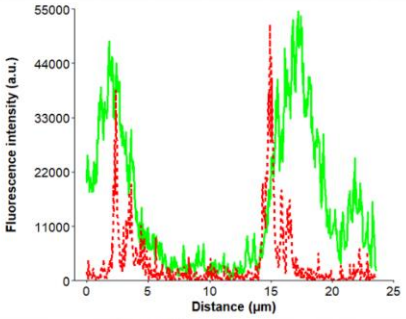


Figure 4. SND1 subcellular localization in HCT116. (A) Two flasks of HCT116 cells were grown until confluency (hereby mentioned as duplicate "1" and duplicate "2"), then cell homogenates (H) were obtained from Dounce homogenization on ice. Nuclear, mitochondrial and cytosolic fractions were obtained by differential centrifugation and analysed. Western blotting (10% of H, N and M fractions total volumes and 1% of S fraction total volume, were loaded). Band intensity values are indicated in intensity imput units (I.I. unit) (K-count function, Li-cor). n=2.





A†.



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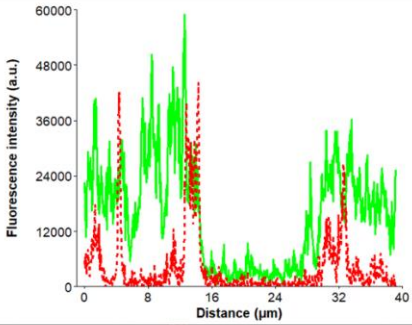

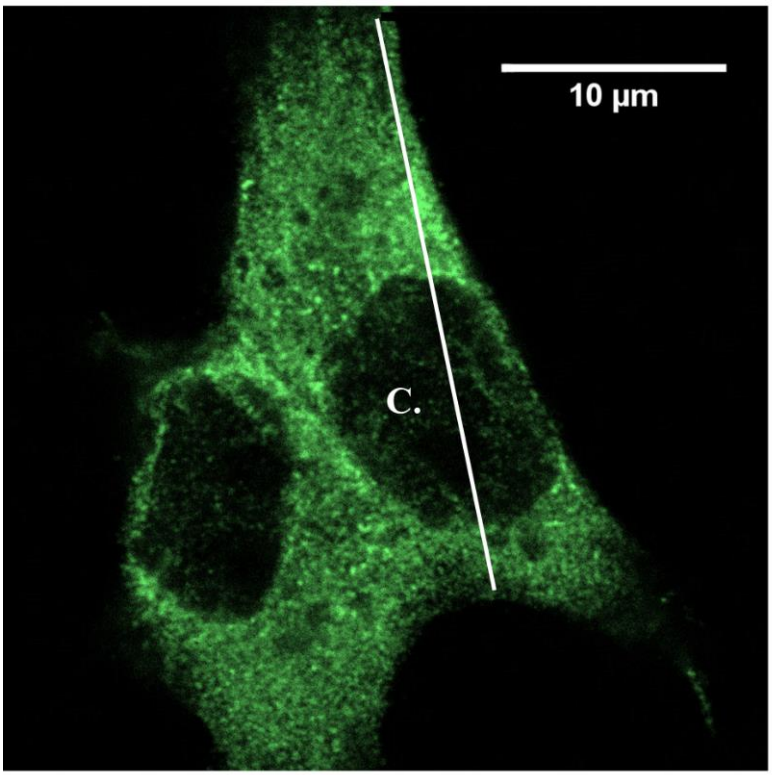

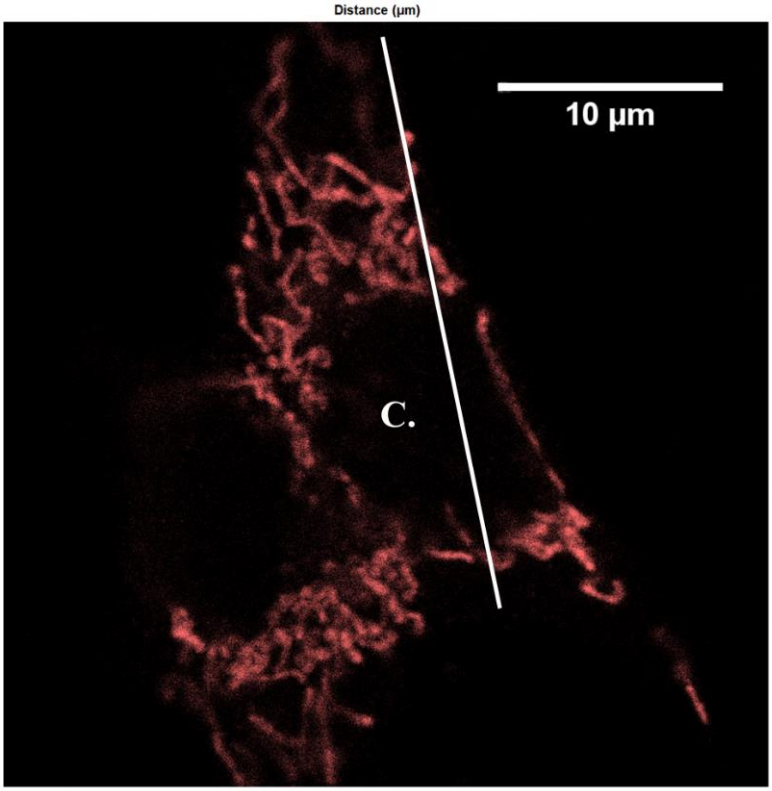

C†.

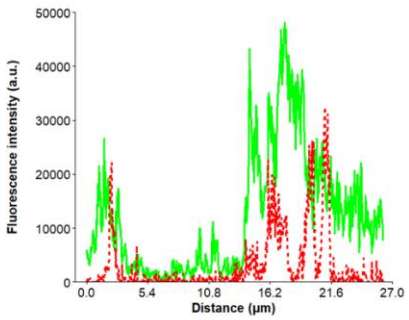

Figure 5. Subcellular localization of SND1 and mtHSP70 in HCT116. (A-C) HCT116 cells have been seeded on coverslips and fixed 24h after, before confocal microscopy observation. Cells were stained with SND1 (green) and mtHSP70 (red) antibodies. The bar is showing 10 µm. n=1. (A†-C†) Transept analyses on a distance from approximately 25 to 40 µm on HCT116 micrographs have been performed with ImageJ image analysis and R software graphical representation of the data. The green curves represent SND1 fluorescent intensity, and the red dotted curves represent mtHSP70 fluorescent intensity on the micrographies along the drawn transepts.

3. SND1 is not involved in human mitochondria co-translational import mechanism.

As a reminder, the SND1 protein has been described in mammalian cells to be a RBP which has also the ability to bind to other proteins, join the RISC complex responsible for selective mRNA decay and participate in virtually all steps of mRNA translation and protein expression in cells (Aviram & Schuldiner, 2017; Caudy et al., 2003; Gutierrez-Beltran et al., 2016). Besides, the analysis of the BioID results enabled to identify and select SND1 protein as a potential trans-acting candidate (4,16-fold change between CPT1A and Tom-20 proxisome enrichment, p-value = 3,55E-63). In order to explore its potential implication in the mitochondrial co-translational mechanism in human cells, different experiments have been performed on HCT116 cells.

First, cell fractionation has been realized in duplicate to isolate human mitochondria (M) from nuclear (N) and cytosolic fractions (S). Samples processing, migration and transfer on a PVDF membrane during the Western-Blotting step allowed to screen a panel of control proteins on the diverse fractions as shown on **Figure 4**.

Figure 4 shows limited contamination between fractions obtained by differential centrifugation. Lamin A/C, the nuclear control, is mostly enriched in the nuclear fractions as expected, while low signal was retrieved in the mitochondrial and cytoplasmic fractions. p-HSP90, the cytosolic control, is mostly enriched in the nuclear and cytosolic fraction, but there is only a low signal retrieved in the mitochondrial fraction. Next, the TOM20 marker has been immunodetected to confirm enrichment of mitochondria in the M fractions. And finally, SND1 appeared importantly enriched in the cytosolic fraction as expected, in the nuclear fraction, but also a lot in the mitochondrial fraction. This is concordant with SND1 enrichment in the BioID results obtained from the TOM20 proxisome analysis.

We then decided to investigate more precisely the subcellular localization of SND1 in HCT116 cells using super-resolution confocal microscopy. As shown on **Figure 5.B**, 3 transept analyses showing graphically the two fluorescence intensities from respectively SND1 (in green) and the matrix mitochondrial heat shock protein 70 (mtHSP70, in red) were generated. Since this graph superposes both fluorescence intensities along a virtual line drawn all over one cell surface on the micrograph, it allows to better appreciate the spatial repartition of SND1 around the mitochondrial networks in HCT116 cells.

Figure 5.A shows that SND1 distribution in the cell appears to be more localized in the cytosol than in the nucleus, respectively localized from 0 to 5 µm, then from 15 µm to 25 µm, and from 5 to 15 µm along the transept. But interestingly, it appears that the SND1 protein is localized just next to the mitochondrial network because both peak maxima are close to each other around 15 µm on the transept. The peak maxima retrieved on the **fig. 5.B** are suggesting the same proximity of the SND1 to the mitochondrial surface. However, the additional image analysis on **fig. 5.C** shows that SND1 might not interact with mitochondria surface but accumulates in close vicinity to it.

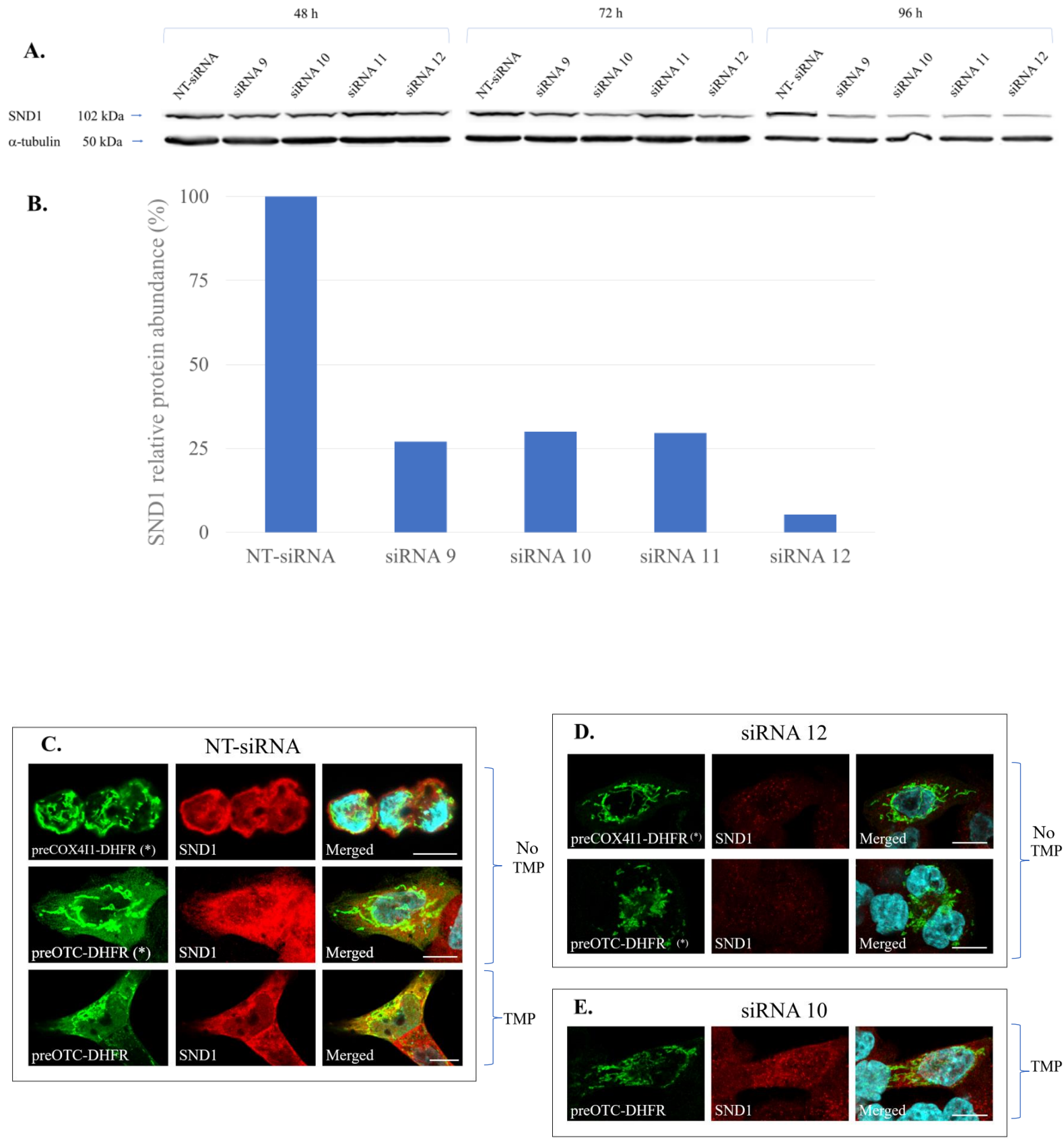

Figure 6. Effect of SND1 silencing on DHFR reporter constructs subcellular localization. HCT116 cells were seeded on coverslips and transfected for 96 hours with non-targeting siRNA (NT siRNA) or siRNA targeting SND1 (siRNA 9- 12). Total protein extraction and dosage were realized to resolve 10 µg of total proteins by SDS-PAGE. (A) Protein abundance assessment was revealed for SND1 and α-tubulin by Western Blotting analysis. (B) SND1 and a-tubulin respective fluorescence intensities were measured by the Odyssey Application Software. Then, SND1 relative protein abundance in treated cells is expressed after normalization with corresponding α-tubulin protein content. Percentage scale shows residual SND1 abundance in cells in comparison with corresponding protein abundance in NT-siRNA treated cells. n=1. (C-E) Cells were seeded on coverslips, transfected with NT-siRNA (C), siRNA 12 (D), siRNA 10 (E) and incubated for 24 hours. Then, cells were transfected the morrow with presequence-DHFR reporter constructs (preCOX4I1-DHFR and preOTC-DHFR) in HCT116 cells. 4 hours later, cell culture medium was replaced by fresh medium containing 100 µM of trimethoprim (C.-E.) or without trimethoprim (C.*-E.*). Then cells were incubated for 24-hours. Cell were fixated, stained with antibodies against SND1, Myc-Tag contained in DHFR reporter construct and nuclei were revelated in contact with the DAPI intercaling agent before confocal microscopy observation. Bars are showing 10 µm. n=1.

If SND1 plays a role in the co-translational import mechanism, its silencing should affect the preOTC-DHFR and preCOX4I1-DHFR reporter construct localization. This experiment is shown on **Figure 6**.

As shown on **Figure 6**, the siRNA 10 and 12 show the best knock-down activities with a depletion of more than 69% and 94% respectively of SND1 protein 96h post-transfection (**fig. 6.B**). Both siRNAs were thus selected for KD of SND1 in the co-translational reporter assay.

As a control, cells transfected with non-targeting siRNA express the SND1 protein and display the expected subcellular localization for the preCOX4I1-DHFR and preOTC-DHFR reporters (**fig. 2.C-2.E**).

Micrographs showing preOTC-DHFR reporter indicates no impairment of its co-translational import into the mitochondria after 24-hours plasmid expression in SND-silenced cells, as shown on **fig. 2.D** and **2.E**. The mitochondrial import of this co-translationally imported reporter was maintained in the presence of trimethoprim (**fig. 2.E**). This suggests that the SND1 protein depletion has no impact on the co-translational import of the reporter construct into mitochondria (**fig. 2.C-2.E**). However, the KD of the protein was not complete. Intense dot representing SND1 abundant areas in cells might thus still ensure its function at the mitochondrial surface.

V. Discussion

Current understanding of the co-translational import machinery in human mitochondria is still elusive but knowing the existence of a similar mechanism at the ER surface in mammalian cells can be a good basis on which comparative hypotheses can be generated.

First of all, the surface of the mitochondria displays cytosolic receptors members of the TOM assembly. Among them, the Tom20p OMM receptor participates in mitochondrial transcript asymmetric distribution and ribosomal targeting to OMM in yeast (Eliyahu et al., 2010). Trans-acting factors surrounding or/and interacting with Tom20 have been shown to be involved in mRNA translation regulation at the OMM (M. Garcia, X. Darzacq, T. Delaveau & R. H. Singer, 2007) and favorize tethering of the ribosomal complex to the OMM (Quenault et al., 2011). And because the mitochondrial co-translational import of a subset of proteins have been shown during the years in the scientifical literature, we wanted to detect other critical trans-acting partners involved in the mechanism among Tom20 proxisome proteins. Candidate proteins to study for their implication in the co-translational import mechanism in cells were CLUH and SND1. To do so, reporters were constructed by upstream work of Sébastien Meurant and in this work they were characterized in terms of post- or co-translational features.

The first series of designed reporters were generated based on the work of Mukhopadhyay *et al.*. The results show that those reporters are efficiently imported into the mitochondria, either in a post-translational or/and in a co-translational manner. So, the MTS of the preALDH2, the preARG2, the preCOX4I1 and the preOTC were recognized by the mitochondrial surface. But, unexpectedly, the presequence of the ARG2 and the presequence of the ALDH2 did not target the reporter constructs to the mitochondria in a co-translational way. Indeed, the stabilizing effect of trimethoprim on the DHFR folding in these reporter constructs caused mitochondrial import incompetency, revealing a post-translational importation mechanism driven by these presequences, as shown on **Figure 1.B**. On the contrary, the presequence of the OTC guided the reporter construct to the mitochondria in a co-translational way. This discrepancy may be explained by a possible misinterpretation of small band size differences observed by Western Blotting analysis in the original publication (Mukhopadhyay et al., 2004). Indeed, these authors based their conclusions on the co-translational character of preARG2 and preALDH2 on the fact that the presequence guiding the precursor protein to the matrix was cleaved off by the MPP. But, because such a little decrease of the molecular weight from the precursor protein form and the mature protein form represents only 2.000 Da, it may be difficult to be observed by Western-Blot analysis (Mukhopadhyay et al., 2004).

Then, because the paper of Fazal *et al.* brought interesting new information about mitochondrial transcripts from nuclear origin localized at the mitochondrial surface (Fazal et al., 2019), it was decided to choose some of them to construct new reporters (**table 6**). But the results showed that the generated reporters weren't imported into the human mitochondria. The results show that the preTOP1MT-DHFR construct allowing the synthesis of a presequence at the amino-terminal part of the preprotein is not recognized by the mitochondrial surface (**fig. 2.B**). And, even if the 10 first amino acids were included in this DHFR construct, it was still insufficient to trigger its import into the mitochondria. So, it means that other parts of the mature TOP1MT protein might be required to trigger import of the construct into the mitochondria (Hansen & Herrmann, 2019). This is an interesting preliminary result showing that the presence of an MTS in this preprotein cannot trigger its import into the mitochondria in a post-translational manner. In the same manner, ETFDH-DHFR and PBH-DHFR reporters couldn't enter the mitochondria

(**fig. 2.B**). Experiments have been reproduced in another human cell line in order to observe if import insufficiency could be cell line related. But the results showed that the reporter constructs couldn't be neither imported into the mitochondria in HeLa cells (**fig. 2.A**). Because those constructs only contained the 5'UTR and the 3'UTR of their corresponding preprotein fused to the DHFR, it means that coding sequences might be also required for their import into the mitochondria.

Concerning the second aim of this work, CLUH depletion in HCT116 didn't lead to loss of mitochondrial subcellular localization of the preOTC-DHFR reporter. The role of CLUH isn't clearly known, but it was recently described to play a central role in mitochondrial turnover and biogenesis by recruiting diverse RNA-binding proteins (RBP) and additionally sense stress signals in cells (Pla-Martín et al., 2020). But, even if the CLUH protein has been shown in higher eukaryotes to be an RNA binding protein of mRNAs encoding mitochondrial proteins (Gao et al., 2014), depletion of the CLUH protein in the HCT116 cell line didn't lead to co-translational importation arrest of the preOTC-DHFR reporter as shown in **Figure 3.D**.

Concerning the third aim of this work, SND1 depletion in HCT116 cells didn't lead to loss of mitochondrial subcellular localization of the preOTC-DHFR reporter. As shown on **Figure 6.D-6.E**, even 96-hours post-transfection of the siRNAs in cells, we can observe that a punctuated presence of SND1 remains in cells. On merged channels, during fluorescence confocal microscopy observations, it seems that SND1 may be persistent in the really neighborhood of the mitochondrial network and, thus, may supposedly pursue its functions at the mitochondrial surface. This may be explained by its association to the RISC complex which maintain its presence in cells when it is recruited. Therefore, we cannot exclude the possibility that this observation is due to experimental difficulties to knock-down SND1 expression in cells. Alternatively, another interpretation would be that SND1 have no critical implication in the co-translational import machinery of the preOTC-DHFR reporter.

Perspectives

The second series of DHFR reporters has been tested in HCT116 and in HeLa cells. The results showed no difference between the 2 cell lines. But since the paper on which mRNA asymmetric distribution was performed in HEK293T cells, maybe we should test again the reporters in the HEK293T cells in order to confirm that the mitochondrial import of those preproteins is not dependent of the cell line (Fazal et al., 2019).

As shown on the **Figure 6.A** and **6.B**, the SND1 presence in cells seems to last for a long period of time (even for more than 96 hours) post-transfection with the most efficient siRNA against SND1 (siRNA 12). As it is expected that its persistence in cells is related to its recruitment in multiprotein complexes (such as the RISC complex) where SND1 can exercises its function, it could be interesting to use ultra-resolution confocal microscopy to make more precise observations of its persisting subcellular localization 96 hours post-transfection with the siRNA 12. Maybe we could possibly see a persistent presence of the SDN1 protein in close vicinity of the mitochondrial surface in cells.

The BioID experiment results allowed to obtain a huge quantity of data. But only 1 candidate was tested in this work. Here are some other candidates which could be interesting to investigate too.

Initiation of translation in ribosomal complexes requires the combination of substrate mRNAs and cytosolic factors. Among those factors, the eIF4G is a scaffolding protein recruiting activator of the translation partners eIF4A responsible for helicase activity and the mRNA cap-binding protein, eIF4E (Ingolia et al., 2019). Many eukaryotic translation initiation factors have been enriched in the Tom20 proxisome. Among them, the most enriched are the eukaryotic translation initiation factor 4E transporter (EIF4ENIF1, 15.3-fold change enrichment, p-value = 1,10E-50) and the eukaryotic translation initiation factor 4 gamma 2 (EIF4G2, 5.0-fold enrichment, p-value = 1,91E-60).

Extensive interactions have been reported between eIF4E and oligo-uridine sequences called “4E-SE” (4E Sensitive Element) (Bong et al., 2021) in the 3’ untranslated region (UTR) of mRNAs. 4E-SE are involved in the active export of several mRNAs out of the nucleus (Ingolia et al., 2019). Therefore, eIF4E act both in mRNA transport into the cytosol as well as in translation initiation. So, we can hypothesize that eIF4E can bind to mitochondrial transcripts from the nucleus and stay attached to it during its transport in the cytosol until it arrives to the mitochondrial membrane. In addition, in *Drosophila*, the Puf homolog protein called Pum has been shown to bind mRNA and to recruit translational inhibitor d4EHP which enters into competitive interaction with eiF4E to bind mRNA cap. In *Xenopus*, it has been shown that Pum2 directly binds to mRNA cap, further preventing eIF4E binding, and inhibits ribosomal translation (Quenault et al., 2011). So, if we make a parallel to the Puf3p protein binding mitochondrial transcripts in yeast, it is possible that the involvement of translation factors in cells allows another level of mitochondrial transcript regulation. We can hypothesize that translation factors may favorize localized translation at the mitochondrial surface when other trans-acting factors aren’t interacting with actively translated mitochondrial transcripts.

So, if any protein interactant of the translation initiation factors is retrieved in the BioID experiment results, we can test that candidate protein. Of course, because knocking-down eIF4E in cell could impair global protein translation and thus cause major defects in cells, translation initiation factors cannot be candidates by themselves.

Another interesting candidate called metaxin-3 (MTX-3) showed up in the TOM20 proxisome with an enrichment value equivalent to 9,29-fold (p-value = 2,69E-69). By reviewing the literature, among the metaxin family members, human MTX-1 has been described to be localized on the mitochondrial outer membrane because the protein structure contains a signal anchor domain at the carboxyl-terminal end (Abdul et al., 2000). Observations attributed to MTX-1 a role in the mitochondrial protein import, since at least one mitochondrial protein called preadrenodoxin cannot be imported following MTX-1 inhibition in mammalian cells (Abdul et al., 2000). But it appeared that metaxin overexpression has an inhibitory effect on protein import into mitochondria. Indeed, researchers observed an increased presence of the precursor form of OTC in the cytosol when MTX-1 was overexpressed in cells (Abdul et al., 2000). MTX-3 has been identified as a vertebrate paralog of MTX-1 recruited in protein assemblies at the origin of mitochondrial cristae formation, showing involvement in the mitochondria (Huynen et al., 2016). Another important element is underlined in the study of Abdul *et al.* which pointed out the fact that precurssor form of OTC accumulated in the cytosol when Tom20 as well as when metaxin were overexpressed at the mitochondrial surface of mammalian monkey kidney-derived (COS-7) cells (Abdul et al., 2000). We could hypothesize that overexpression of cytosolic domains exposed at the mitochondrial surface could impede ribosomal complex association at the mitochondrial outer membrane. Maybe co-translational apparatus engaging actively translating ribosomal complexes is impaired at the surface of the mitochondria when the proteome equilibrium at the surface of the mitochondria is unbalanced.

These elements suggest a potential involvement of the MTX-3 in the mitochondrial co-translational import mechanism.

Besides, we can consider new experiments to try to counter possible limitation of single candidate KD at a time in cells. Indeed, it is difficult to make multiple KD in cells because this experiment might possibly have deleterious effects on cell viability. In addition, because all KD testing in HCT116 cells might fail for the reason that there could be many other trans-acting proteins which perform the same function simultaneously at the mitochondrial surface, another idea could be to explore another step of the mitochondrial co-translational import machinery circle.

This work could be extended into 4 other directions at least with…

1- We could try to detect a mammalian homolog protein which would have a high protein sequence similarity to the yeast Om14. This could allow to find a human mitochondria anchored receptor exposed to the cytosol which would interact with αβ-NAC tagged ribosomal complexes.
2- To make a new BioID experiment in which HCT116 cells would be modified to express a fused MiniTurbo enzyme to one of the SAM complex receptors. This could allow to obtain a receptor of the SAM complex proxisome screening. This is because the yeast Sam37 is known to bind to Tom22 and allow association of the TOM complex and the SAM complex to perform β-barrel protein insertion in the OMM (Wenz et al., 2015), that we could expect the same association of the two machineries in human cells for a similar function. Thus, we should obtain a very similar proxisome profile than the Tom20 proxisome because the 2 complex assemblies can localize close to each other. This can help to eliminate the majority of imported protein into the mitochondria. And since Sam37 is not expected to be involved in the mitochondrial co-translational import mechanism, we could eliminate protein retrieved in both the Tom20 proxisome and the receptor of the SAM complex proxisome from the candidate list. Indeed, there could have more chances that protein highly enriched in direct surrounding of the SAM complex are not involved in the mitochondrial co-translational import machinery.
3- Third, as well as the trans-acting factors could be present at all the steps of the mitochondrial co-translational import machinery circle, maybe it could be interesting to screen the proxisome of a ribosomal protein surrounding the exit tunnel. Indeed, following the assumption that ribosomes are targeted to an enough close vicinity to the Tom20 on the mitochondria, we could maybe retrieve interesting trans-acting candidates favorizing the mitochondrial co-translational machinery in both the Tom20 proxisome results and this new investigated ribosomal protein proxisome. We can hypotheze that a cytosolic protein recognizing the nascent polypeptide chain on ribosomal complexes actively translating mitochondrial protein (such as for example the α-NAC and β-NAC cochaperones) can interact with partner protein/receptor localized on the OMM. In the same way that Rpl131 is expected to interact with β-NAC while Rpl117 would interact with α-NAC (Kogan & Gvozdev, 2014), those two proteins could be fused to the MiniTurbo enzyme to make a new proxisome profiling.
4- A final suggestion would be to do the mRNA screening in the Tom20 proxisome. As showed in the paper of Fazal *et al.*, they screened mRNA by using an alternative enzyme to the biotin ligase used in the BioID experiment called APEX2. This proximity labeling of the transcripts worked in HEK293T cells, so this shall work also in HCT116 cells (Fazal et al., 2019).

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Annexe 4

	Ex	***TB***	***B***	***S***	***I***
	≥ 17	16-15	14-13	12-10	<10
Critères pour l'évaluation par les membres du jury					
Critères pour l'évaluation du mémoire écrit					
Intégration du sujet dans une problématique générale					
Démarche scientifique					
Pertinence de l'intro par rapport aux résultats/ au sujet					
Clarté					
Structure (regroupement des idées similaires, introduction et raisonnement clairs)					
Cohérence (bonne connaissance du sujet)					
Concision (pas de répétitions, raisonnement clairement exposé)					
Précision (les généralités sont évitées, les exemples sont précis)					
Justification et présentation des expériences					
Analyse des données					
Interprétation des résultats vis-à-vis de la littérature scientifique récente/Discussion					
Conclusions et mise en avant de perspectives					
Critères pour l'évaluation de la présentation ET de la défense					
PRESENTATION					
Clarté du support et du discours					
Structure (objectifs, introduction, résultats, conclusions, …)					
DEFENSE					
Réponses aux questions : compréhension des techniques utilisées					
Réponses aux questions : compréhension des expériences et des résultats					
Réponses aux questions : pertinence et précision du vocabulaire scientifique					
Réponses aux questions : culture générale et connaissance de la littérature					
Profondeur de l'analyse du sujet					
Critères pour l'évaluation par le promoteur					
Participation active et dynamisme au cours de l'année					
Etendue et pertinence de la recherche bibliographique					
Prise d'initiative et autonomie dans les expériences					
Résultats fiables					
Sens critique/Analyse des résultats					
Compréhension du sujet/profondeur d'interprétation					
Autonomie pour l'écriture du mémoire/degré d'intervention du promoteur					
Autonomie pour la préparation de la présentation orale/degré d'intervention du promoteur					