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Regulation of the expression of heteromeric receptor subunits by modulation of mRNA stability – a case study of TRC40 receptor

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Abstract

In recent years, the mechanisms of post-transcriptional regulation of gene expression have gained increased attention, including their importance in oligomeric protein assembly. The ER based proteins CAML and WRB have been described to form a heteromeric protein complex that functions as a receptor for the TRC40 ATPase, mediating the post-translational insertion of tail-anchored proteins into the ER membrane. A recent study showed that, even if the two proteins are not in a stoichiometric equilibrium - CAML is in ~5-fold excess over WRB -, the transient siRNA mediated depletion of each one of the two subunits, in HeLa cells, leads to the depletion of the second one. Interestingly, the silencing of CAML leads not only to the depletion of WRB protein but also to the depletion and strong destabilization of WRB mRNA, revealing a novel mechanism for the regulation of the expression of different subunits of a protein complex. In this thesis, I have followed up on this work, trying to unveil the mechanisms involved in the co-regulation of the expression and assembly of the TRC40 receptor complex. My results demonstrate that the interplay observed at the transcript level is not reciprocal, i.e. CAML mRNA is not affected by WRB siRNA mediated depletion, and that the CAML-sensitivity of the WRB transcript is attributable to a relatively small portion (370 nt) of its 3' untranslated sequence (UTR), as shown by the results obtained with the dual-luciferase reporter system. Moreover, co-IP experiments show that CAML is indeed in excess over WRB and that a WRB-free population of CAML exists. It remains however unclear, what mechanism may explain the depletion of CAML upon WRB silencing. Finally, I propose a model according to which, on the one hand, CAML protein either directly or indirectly interacts with the 3'UTR of WRB, promoting is stability, which is lost when CAML is silenced. On the other hand, CAML may be inserted in the ER membrane in an unusual way, via the TRC40 pathway, meaning that it would require a functional TRC40 receptor complex, which is lost when WRB is silenced. The resulting non-inserted CAML molecules would then be degraded by the ubiquitin-proteasome system. Future work is required to further understand the precise mechanisms involved in the co-regulation of the expression of the two subunits of the TRC40 receptor. This work could pave the way for the discovery of new post-transcriptional co-regulatory mechanisms for the expression and assembly of oligomeric protein subunits, which could very well be relevant to many different protein complexes.

Riassunto

Durante gli ultimi anni, un'attenzione crescente è stata rivolta ai meccanismi della regolazione dell'espressione genica post-trascrizionale, meccanismi che rivestono particolare importanza nell'assemblaggio di proteine oligomeriche. Le proteine del reticolo endoplasmatico, CAML e WRB, formano un complesso eteromerico che funziona come recettore dell'ATPasi TRC40, mediando così l'inserimento nella membrana dell'ER delle proteine "tail-anchored" (cioè con àncora transmembrana C-terminale). Uno studio recente ha dimostrato che anche se queste due proteine non sono in equilibrio stechiometrico - poiché CAML è in eccesso molare di 5 volte rispetto a WRB -, il silenziamento transiente tramite siRNA di ciascuna delle subunità, in cellule HeLa, causa la deplezione della seconda. Un'osservazione molto interessante di questo studio è stata che il silenziamento di CAML non solo porta alla deplezione della proteina WRB ma anche alla destabilizzazione del corrispondente mRNA, rivelando così un nuovo meccanismo per la regolazione dell'espressione delle subunità di un complesso proteico. In questa tesi sono partito da questa osservazione con lo scopo di svelare i meccanismi coinvolti nella co-regolazione dell'espressione e assemblaggio del complesso recettoriale per TRC40. I risultati ottenuti dimostrano che l'effetto del silenziamento di ciascuna delle subunità sull'mRNA dell'altra non è reciproco, poiché i livelli dell'mRNA di CAML non sono modificati in seguito a deplezione di WRB. Ho dimostrato inoltre che la sensibilità del trascritto WRB alla mancanza di CAML è attribuibile a una porzione ristretta (370 nt) della regione 3' non tradotta (3'UTR), come dimostrato da esperimenti usando il sistema reporter della luciferasi. Inoltre, esperimenti di co-IP hanno confermato l'eccesso molare di CAML rispetto a WRB e dimostrato che nel reticolo esiste una popolazione di molecole CAML non associate a WRB, lasciando quindi aperto il meccanismo tramite cui WRB, in proporzioni sub-stechiometriche, garantisce la stabilità di CAML. Propongo un modello secondo il quale, da una parte, la proteina CAML interagisce, direttamente o indirettamente, con il 3'UTR del trascritto WRB stabilizzandolo; dall'altra CAML è inserito in membrana tramite il recettore TRC40, che viene a mancare quando WRB è silenziato. Le molecole di CAML non inserite verrebbero poi eliminate dal sistema ubiquitina-proteasoma. Ulteriori studi sono richiesti per la comprensione precisa dei meccanismi coinvolti nella co-regolazione dell'espressione delle due subunità del recettore TRC40. Tali studi potrebbero aprire vie per la scoperta di nuovi meccanismi

post-trascrizionali regolatori dell'espressione e assemblaggio di oligomeri che potrebbero essere di rilevanza per altri complessi proteici.

List of abbreviations

ARE – AU-rich element

ARE-BP – AU-rich element binding protein

CAML – Calcium modulating ligand

CDH5 – Congenital heart disease 5 protein

cDNA – Complementary DNA

Co-IP – Co-immunoprecipitation

C_t – Threshold cycle

Cyt b5 – Cytochrome b5

DB – Denaturation buffer

DBC – Deoxy big chaps

DMEM – Dulbecco's modified eagle medium

dNTP – deoxynucleotide triphosphate

DRB – 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

DS – Down syndrome

EGFR – Epidermal growth factor receptor

eIF – Eukaryotic initiation factor

EJC – Exon junction complex

ER – Endoplasmic reticulum

ERAD – ER-associated degradation

FBS – Fetal bovine serum

FRET – Fluorescence resonance energy transfer

GET – Golgi to ER traffic

hERG – Human ether-à-go-go-related gene

Hsa1 – Distal region of the long arm of chromosome 21

IP – Immunoprecipitation

IRES – Internal ribosomal entry site

KH – K-homology

MOM – Mitocondrial outer membrane

mRNA – Messenger RNA

mRNP – messenger ribonucleoprotein particle

NMD – Nonsense-mediated mRNA decay

ORF – Open reading frame

PABP – Poly(A) binding protein

PAGE – Polyacrylamide gel electrophoresis

P-Body – Processing body

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PDI – Protein disulphide isomerase

PIC – Protease inhibitor cocktail

PNS – Post nuclear supernatant

Pre-mRNA – Precursor mRNA

P/S – Penicillin/streptomycin antibiotic mix

PTP1B – Protein tyrosine phosphatase 1B

qPCR – quantitative PCR

RACE – Rapid amplification of cDNA ends

RBP – RNA-binding protein

RISC – microRNA-induced silencing complex

RRM – RNA-recognition motifs

RT-qPCR – Reverse transcription quantitative PCR

SDS – Sodium dodecyl sulfate

Sgt2 – Small Glu-rich tetratricopeptide repeat-containing 2

siRNA – small interfering RNA

SND – SRP-independent pathway

SRP – Signal recognition particle

TA protein – Tail-anchored protein

TAE – Tris-acetate buffer

TCR – T-cell receptor

TMD – Transmembrane domain

TRC40 – 40 kDa subunit of the transmembrane domain recognition complex

uORF – upstream ORF

UPS – Ubiquitin proteasome system

UTR – Untranslated region

WRB – Tryptophan rich protein

1
INTRODUCTION

1.1 Protein complexes

Proteins are one of the building blocks of life. The genetic information contained in the DNA is transcribed into RNA that is then translated into protein in a flow of information that Crick defined as the Central Dogma of Biology.

During translation, the ribosome reads the information contained in the messenger RNA (mRNA), encrypted in the four-letter genetic code, and translates each triplet - codon - into the corresponding amino acid, catalysing the formation of a chain of chemically bound amino acid residues that precisely represents the sequence carried by the mRNA.

The resulting polypeptide chain represents the primary structure of a protein. The intrinsic properties of the amino acids that compose a polypeptide chain confer to them well defined physico-chemical characteristics that play a major role in the final protein structure. The process of folding the polypeptide chain into the mature protein begins with the local interaction of the different amino acids in the sequence that, by forming hydrogen bonds between them define the secondary structure of the protein. The most common secondary structures formed are α-helixes and β-sheets. Various combinations of secondary structures can be present in a polypeptide chain, forming protein domains.

The formation of the tertiary structure of a protein, the overall three-dimensional form of the protein, is mostly driven by the forces exerted by the contact of the different amino acids with the surrounding medium. The different amino acids present different levels of hydrophobicity under physiological conditions, some amino acids are very hydrophobic while others are hydrophilic. Thus, the polypeptide chain tends to turn and twist to accommodate hydrophobic amino acids in the interior of the protein, while the hydrophilic amino acids remain exposed in the exterior, shielding the interior from the aqueous medium. This way, the protein will find itself in its lowest-energy state and therefore in its most stable state. To provide strength to this conformation, and higher stability, often disulphide bridges between cysteine residues, additional hydrogen bonds or other interaction are created in this step.

In many cases, proteins are composed of multiple polypeptide chains, referred to as protein subunits. These subunits can be identical (homomers) or different (heteromers). In these cases, two or more polypeptides form a larger aggregate protein

complex by interacting with each other and often rearranging themselves to obtain a higher stability.

Throughout the maturation process the proteins are subjected to various forms of modification, called post-translational modifications, that serve to either increase or decrease their stability.

The folding and maturation of a protein or a protein complex into its final, native state, where it is in its most stable natural conformation is thus regulated at multiple levels, always driven by a common denominator, stability. When incompletely folded proteins find each other, it is highly probable that their hydrophobic regions will interact, creating aggregates of unfolded proteins. The tight regulation of folding and assembly is extremely important, since the presence of aggregated unfolded proteins can be detrimental for cellular homeostasis. Thus, when a protein for some reason loses its functional conformation, it is soon targeted for degradation, since the accumulation of unstable proteins could ultimately lead to cell death (Alberts et al. 2014; Nelson et al. 2012).

1.1.1 Pharmacological receptors

A receptor is a macromolecule, often a protein or a complex of proteins, that interacts with chemical substances such as hormones, neurotransmitters, intracellular messengers or drugs, transmitting chemical signals inside and between cells. This signal transmission triggers a response in the form of a change in cellular function.

Most drugs act by targeting cellular receptors, changing their function or activity dynamics, either as agonists or antagonists. These receptors can be divided in two main families: membrane receptors and intracellular receptors.

Some examples of intracellular receptors are the receptors for steroids, thyroid hormone, vitamin A and D, oestrogen and retinoic acid. These are generally formed by monomeric proteins composed of three main domains: (i) a C-terminal ligand binding site; (ii) a central DNA binding domain, specific for a particular DNA sequence; and (iii) a N-terminal regulatory domain responsible for gene transactivation.

The wider family of membrane receptors, on the other hand, can be further divided into three major subgroups: ligand-gated ion channels, G protein-coupled

receptors and enzyme-linked receptors. The latter classes are most commonly monomeric proteins, while the first are oligomeric proteins, which I describe here.

Ligand-gated ion channels are membrane-embedded oligomeric protein complexes that upon ligand binding, undergo a transitory conformational change that opens a channel through the membrane, through which ions flow, causing a variation in the membrane electric potential. These protein complexes, that can be either homo or heteromeric, are composed of three, four or five subunits. Depending on the ions that permeate these receptors, they can induce a ligand mediated depolarization or hyperpolarization of the membrane, which is then important for several cellular functions and responses. Examples of this class of receptors are nicotinic, glutamate, serotonin, nucleotide, GABA, and glycine receptors. The intrinsic multimeric nature of this type of receptors, makes the assembly process of the complex a key step towards their proper function. Thus, one important aspect of the biology, of oligomeric ligand-gated ion channels, is the regulation of the assembly of the receptor complex: how the expression of each subunit is controlled and how the different subunits interact with each other to create a final, functional complex (Paoletti et al. 2004; Golan et al. 2006).

1.2 Regulation of protein complex folding and assembly

1.2.1 Mechanisms of assembly

The intracellular environment is a crowed place or, to be more precise, presents a strong exclusion volume effect. This effect is defined by the fact that each individual molecule occupies a certain space that, obviously, cannot be occupied by another molecule, thus that volume is unavailable for other molecules and is then said to be excluded from the total volume. Typically, macromolecules occupy approximately 20-30 % of the intracellular milieu that is further occupied by diverse small molecules and solvents. This crowding effect is tightly related to the size of the molecule in question, so it is easy to imagine that entities such as proteins will be strongly affected by it (Ellis 2001).

Crowding is thus a very important factor when talking about the regulation of protein folding, assembly and function. This has been extensively studied regarding the problem of protein folding. If earlier studies showed that small globular proteins could be denatured and re-folded back to the native state in vitro, in the absence of any other adjuvant (Anfinsen et al. 1961), later studies started to demonstrate that this may not be the case for more complex polypeptides. In fact, it has been shown that, some proteins assemble in vitro very inefficiently, but that the addition of crowding agents greatly increases the ability of the polypeptide to fold into the native conformation in-vitro by favouring the most compact state (Galán et al. 2001). However, the effects of crowding are not always positive. It has been demonstrated that the proper refolding of reduced lysozyme was abolished due to aggregation when in the presence of crowding agents. Yet, that aggregation was avoided when protein disulphide isomerase (PDI) was added to the reaction, this due both to PDI's activity as disulphide bond catalyst and as molecular chaperone. Furthermore, it was shown that under crowding conditions, PDI was a much more effective molecular chaperone than that under dilute conditions (Van Den Berg et al. 1999).

These are but examples of a large body of work, developed over the years, that showed that the essential information for the folding of the polypeptide chain into the native, most stable conformation, is embedded in the polypeptide sequence itself and that in some cases proteins can spontaneously fold by themselves in vitro. However, the highly

crowded intracellular environment in which polypeptides are synthesised often poses a barrier for the efficient folding of the proteins. In fact, crowding should favour aggregation by increasing the thermodynamic activity of partly folded polypeptides. This effect is on the one hand, greater for smaller polypeptides, since large ones have a reduced rate of diffusion and consequently of encounter with other macromolecules, and on the other hand, enhances the aggregation of slow-folding chains, as fast-folding chains can internalize their hydrophobic surfaces before these can bind to those in other chains (Ellis 2001). Thus, the ability to fold, imprinted in the polypeptide sequence is not the sole parameter determining proper folding. The rate at which the folding happens is also important and determines the probability that a given polypeptide will interact with another one, before being completely folded, controlling in this way the formation of non-specific aggregates. So, to overcome these issues, evolution found its solution in the use of molecular chaperones that aid the polypeptide in its folding process, while preventing non-intended interactions with other proteins that could lead to aggregation. The different levels of folding assistance start already co-translationally, while the ribosome is synthesising the polypeptide; indeed, there is even evidence that folding begins inside the ribosomal exit tunnel (Nilsson et al. 2015).

The intricate processes that govern polypeptide folding are very well studied and progress is being made towards the prediction of the final native conformation of a protein, with a fairly good level of accuracy, by analysing the polypeptide sequence. Even more complex is the process of assembly of protein complexes composed of multiple subunits. It is well accepted though, that some of the forces that drive protein folding may also be important for protein complex assembly. This assembly is likely driven by the need of a subunit to find the right binding partner in order to shift towards a lower energetic state, this, while avoiding casual transient interactions that may lead to protein aggregation, and all this in the previously described crowded intracellular environment. Thus, if protein folding is very likely to be a process for which regulation starts immediately after translation, to reduce the risk of misfolding and aggregation, and to lower the overall energy needed for the process, the same is probably true for the assembly of multimeric protein complexes (Natan et al. 2017).

Indeed, considering the expected low diffusion rate of large complex subunits in crowded conditions, it is natural to predict that assembly could, at least in certain cases,

happen co-translationally (Siwiak and Zielenkiewicz 2015). The co-translational assembly of homomeric complexes can be idealized in manner more straight forward than that of heteromers. Homomers, as detailed above, are composed of a single subunit type, thus they can be formed by polypeptides being translated in the same polyribosome (Fig. 1.1-A). In this way, the nascent peptides of two monomers could begin interacting and assembling before either one of the subunits is fully translated. Another view is that a fully translated subunit can interact with the nascent chain of a second subunit initiating the co-translational assembly (Natan et al. 2017; Wells et al. 2015). The first proposed case of co-translational homomer assembly was that of β-Galactosidase in the 60's (Kiho and Rich 1964).

The assembly of heteromers, can be more complex. In prokaryotes, the hypothesis of co-translational assembly of heteromers can be interpreted in the same way as described above for homomers, since it is widely accepted that the operon architecture of prokaryotic genes generally puts together in a polycistronic messenger RNA proteins that physically interact (Dandekarab et al. 1998; Shieh et al. 2015). Thus, each subunit is translated in the same operon in a sequential manner and the co-translational assembly is facilitated by the proximity between the interacting subunits (Fig. 1.1-C) (Li et al. 2014).

Co-translational assembly of heteromeric complexes in eukaryotes, however, presents an additional challenge. In eukaryotes, each gene is translated from a monocystronic mRNA, thus, co-translational assembly can happen in two major modes (Fig. 1.1-B): (i) a fully translated subunit may interact with the nascent chain of the second subunit, initiating the assembly while it is being translated, or (ii) two different polyribosomes, translating in proximity, allow for the interaction of the nascent chains of two subunits of a protein complex. In these cases, an extra level of regulation is present, the regulation of the spatio-temporal localization of mRNA, which is very important to allow for the interaction by proximity of the two newly formed polypeptides. In agreement, Duncan and Mata (2011) have shown that 40 % of a selected number of proteins, that did not have any canonical RNA binding domains, interacted with the mRNAs of binding partners in a translation dependent manner. Moreover, Morisaki and colleagues (Morisaki et al. 2016), using recently developed microscopy techniques that allow the visualization at single molecule resolution of real-time, live translation, observed the existence of a population of polysomes that were translating two different

transcripts in a complex that could be tracked over-time. Recently, a work by Liu and colleagues (Liu et al. 2016), demonstrated that the mRNA of the two subunits of an heteromeric K^+ channel (hERG) are bound by a RNA binding protein that determines the proximity of the two transcripts, allowing the assembly of the complex co-translationally. The importance of the regulation of co-translational complex assembly for the stability and overall functionality of the resulting protein complex is thus becoming evident.

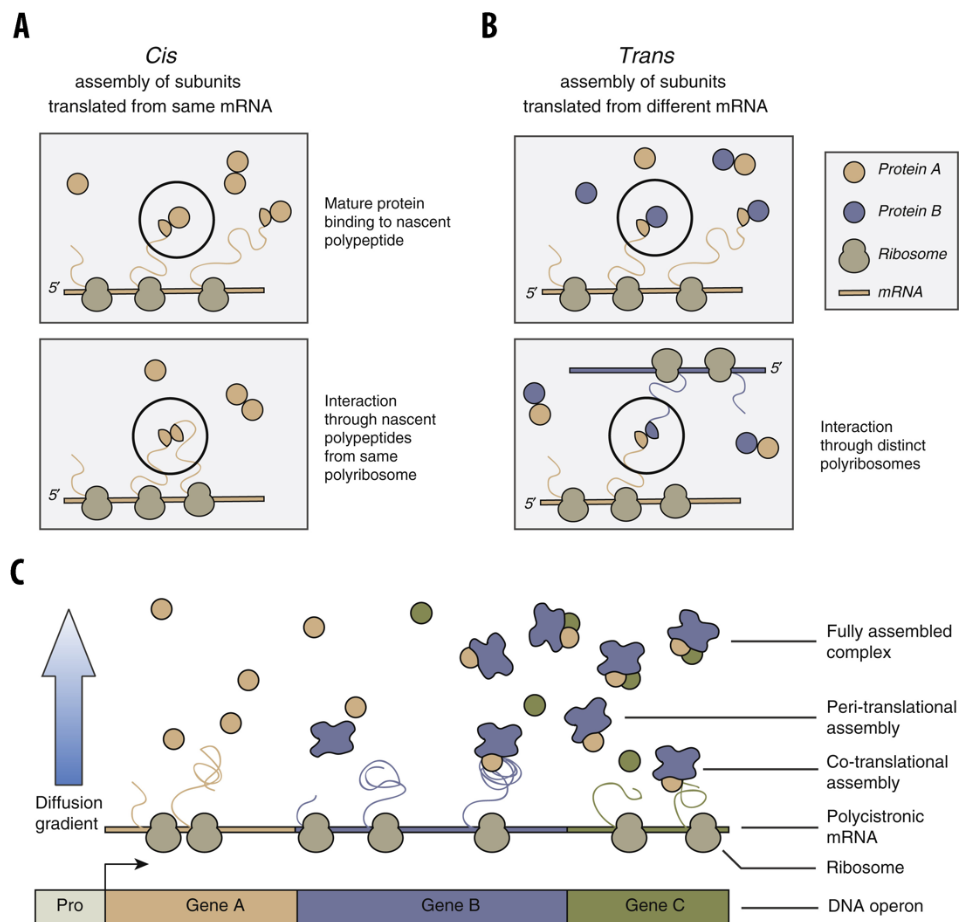

Fig. 1.1 - Models for co-translational assembly of protein complexes. (A) Assembly of homomeric complexes. (B) Assembly of heteromeric complexes. (C) Assembly of prokaryote protein complexes by polycistronic mRNA. (Adapted from Natan et al. 2017)

1.2.2 Regulation of the production of receptor subunits in the correct stoichiometric ratios

An important problem in the production of oligomeric complexes is to avoid the generation of an excess of one type of subunit over its partners. Indeed, an unassembled subunit, by exposing hydrophobic patches destined for interaction with partner subunits, could aggregate, with deleterious effects for the cell. As already mentioned, in prokaryotes this problem is solved by including the cistrons for oligomer subunits in the same operon., under the control of the same promoter. This arrangement leads to production of the subunits in stoichiometric ratio. Prokaryotes appear to exert a tight transcriptional control that guarantees the same levels of expression of genes encoding complex subunits even when these are not contained in the same operon, as demonstrated by a recent study of the group of Jonathan Weissman (Li et al. 2014).

In eukaryotes, the transcriptional regulation of genes encoding subunits of the same complex has been less investigated. However, eukaryotic cells have a safeguard against unaccompanied complex subunits: these are recognised by the ubiquitin proteasome system (UPS), and degraded. Indeed, recently a mechanism responsible for the recognition of unassembled proteins was identified (Yanagitani et al. 2017). The instability of "lonely" subunits has become particularly visible since the introduction of silencing technology. There are countless examples in which knockdown of one oligomer subunit causes depletion of the other subunits of the complex (Peth et al. 2007; Colombo et al. 2016; Rivera-Monroy et al. 2016; Shrimal et al. 2017; to cite just a few). For receptors destined to the cell surface, this quality control system operates at the endoplasmic reticulum (ER), where newly synthesised subunits are born. Incompletely assembled oligomers and single subunits are not allowed to be exported to the Golgi apparatus and are instead retro-translocated to the cytoplasm, ubiquitinilated and degraded by the proteasome in a process known as ER-associated degradation (ERAD) (Bernasconi and Molinari 2011).

In summary, to ensure efficient oligomer assembly, the eukaryotic cell controls gene transcription, maximizes assembly efficiency and disposes of any uncoupled subunits. There are, however, a number of steps in this general picture that remain to be

elucidated, as well as different behaviours and different modes of degradation of different oligomers.

In my work, I have taken the case of a heteromeric receptor composed of two subunits, the TRC40 (40kDa subunit of the transmembrane domain recognition complex) receptor, for which previous work from my laboratory indicated a novel and intriguing mechanism of subunit co-regulation. My work could pave the way for the characterisation of a new mechanism regulating oligomeric complex expression and assembly that could operate also in pharmacologically relevant protein complexes.

1.3 TRC40 pathway for Tail-anchored protein insertion

1.3.1 Tail-anchored (TA) membrane proteins

It is estimated that about 30% of the genes of most genomes code for membrane proteins (Krogh et al. 2001), which, depending on number of transmembrane domains (TMD) and topological orientation can present a wide structural diversity. Most membrane proteins are assembled in the ER. In this cellular compartment, they are inserted into the membrane, fold and assemble into protein complexes. Apart from some exceptions, membrane proteins are inserted in the membrane co-translationally, via the signal recognition particle (SRP) pathway. During translation, when the first hydrophobic element, either a cleavable N-terminal sequence or a TMD, gets out of the ribosomal tunnel, it is recognized by the SRP that binds to the domain, causing a pause in translation. SRP then brings the entire translating unit to the ER by interacting with the SRP receptor. When in the ER, the SRP hands down the translating ribosome to the Sec61 translocon, where the hydrophobic domain is introduced and translation is resumed. The translocon then mediates the lateral insertion of the TMDs in the membrane and translocation of exoplasmic domain(s) across the ER membrane (Shao and Hegde 2011) (see Fig 1.2).

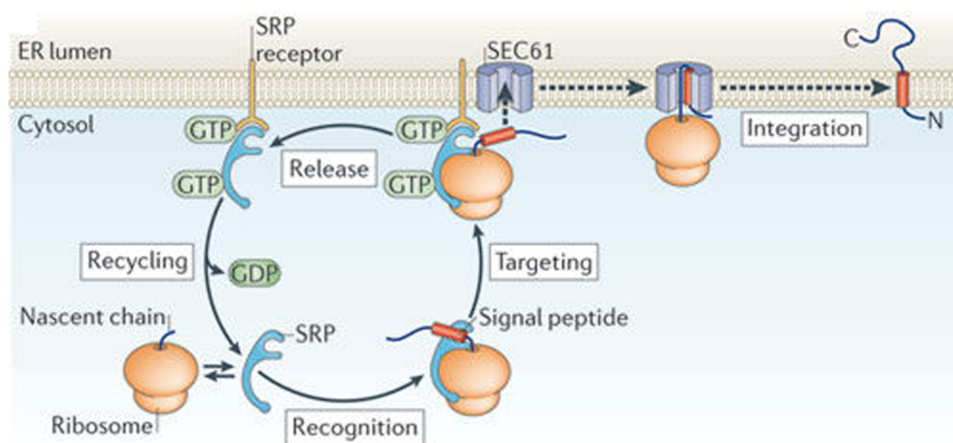

Fig. 1.2 - Representation of the SRP pathway. Adapted from Hegde and Keenan 2011

One well studied exception of this mechanism is the case of TA proteins. TA proteins are predicted to be distributed in all domains of life and they represent ~3-5% of all membrane proteins (Beilharz et al. 2003; Kalbfleisch et al. 2007; Borgese and Righi 2010). This class of protein is characterized by having a single TMD no more than ~30 residues from the C-terminus. This topology, combined with the lack of an N-terminal signal sequence, excludes TA proteins from the SRP pathway (Kutay, Hartmann, and Rapoport 1993; Borgese, Colombo, and Pedrazzini 2003). This is because, the TMD is so close to the C-terminus that it emerges from the ribosomal tunnel only upon termination and release of the translated polypeptide, thus the newly synthesized TA proteins must be inserted in the ER membrane post-translationally. Several TA proteins have been found to be players in several basic cellular processes, of which some examples are listed in Table 1.1.

Table 1.1 - Examples of TA protein targeting and function. (Adapted from Borgese and Fasana 2011)

Target membrane	TA protein	Function
ER	Sec61β	Protein translocation
	Ramp4	Protein translocation
	Synaptobrevin-2	SNARE required for synaptic vesicle exocytosis
	Sed5	Golgi SNARE
	Cyt b5	Lipid metabolism in the ER
MOM	Small TOM proteins	Protein translocation
	OMb5	Enzymatic
	VAMP-1B	SNARE
	Bcl-XL, Bak	Regulation of apoptosis
Peroxisomes	Pex26	Peroxisome biogenesis
Dual targeting: ER and MOM	Bcl-2	Regulation of apoptosis
Dual targeting: MOM and peroxisomes	Fis1	Mitochondrial and peroxisomal fission

The mechanisms of post-translational translocation of TA proteins have been the focus of a great deal of work in the past decade. Depending on the physical properties of the TMD and on the target membrane organelle, TA proteins have been shown to be targeted and to insert into membranes through diverse mechanisms.

TA proteins with low hydrophobic TMDs, like cytochrome b5 (cyt b5) and protein tyrosine phosphatase 1B (PTP1B), are able to insert into biological membranes

and even into pure phospholipid bilayers spontaneously, without the need for any additional machinery (Brambillasca et al. 2005, 2006; Colombo et al. 2009; Fueller et al. 2015). The mechanism of the *in-vivo* targeting of these proteins is somewhat obscure, although it seems that lipid composition plays an important role (Brambillasca et al. 2005). In addition, the group of Stephen High (Abell et al. 2007; Rabu et al. 2008) showed that, the chaperoning complex Hsp40/Hsc70 is able to mediate the ATP-dependent insertion of at least a subset of TA proteins containing moderately hydrophobic TMDs, like that of cyt b5, into the ER membrane. The precise mechanism by which this mediation takes place is still not completely understood since a membrane receptor for Hsp40/Hsc70 is still to be found. The current view is that this chaperone complex keeps TA proteins in an integration-competent form, preventing aggregation until the proteins reach the target membranes.

A second pathway that has been reported for a subset of TA proteins with TMDs with a higher degree of hydrophobicity involves the participation of SRP in an unusual post-translational mode. It is thought that ribosome-associated SRP captures the substrate's TMD immediately upon release from the ribosome (Abell et al. 2004).

Most importantly, a 40 kDa AAA ATPase, called TMD recognition complex (TRC)40, (previously annotated Asna1) was shown to bind to TA proteins' TMD and to be responsible for their targeting to the ER membrane (Stefanovic and Hegde 2007; Favaloro et al. 2008). These were the first of a series of studies that characterized the TRC40 pathway in mammals and the homologous GET system in yeast. This pathway is widely accepted to be the most important mechanism for the post-translational insertion of TA proteins in the ER membrane and will be further discussed in the following sections.

Recently, a set of yeast proteins, called SND (SRP-independent pathway) protein, were shown to be able to mediate the ER targeting and translocation of ER substrates (Aviram et al. 2016). The authors demonstrate that the SND pathway works in parallel with both the SRP and GET pathways, contributing to the ER targeting of both secretory and TA proteins.

An issue that remains to be completely understood is the targeting and insertion of TA proteins in other membrane organelles. For mitochondria, the most accepted hypothesis is that TA proteins are spontaneously inserted in the mitochondrial outer

membrane (MOM) (Kemper et al. 2008). It is however, unclear what is the mechanism by which those proteins are selectively targeted to the mitochondria. Recent results indicate that TA proteins with moderately hydrophobic TMDs may, by default, be spontaneously inserted in the MOM and may need some energy-dependent mechanism, other than those already discussed to be targeted to the ER instead (Costa et al. 2017 - *manuscript submitted for publication*).

The peroxisome is another organelle that appears to have its own TA protein insertion machinery that may act in parallel with the traditional ER-trafficking route. In this organelle, PEX19 and PEX3 have been shown to be responsible for the insertion of several TA proteins as well as of other membrane proteins (Halbach et al. 2006; Mayerhofer 2016).

TA proteins are, as already mentioned, quite abundant in the eukaryotic proteome. They are well distributed between all membrane systems of eukaryotic cells and there are examples of TA proteins having a role in almost every aspect of cell biology (see Table 1.1). Several TA proteins are linked to diverse pathologies, some examples of which are listed in Table 1.2. Thus, a detailed understanding of their biogenesis could provide insight into unknown roles played by TA proteins in other diseases, caused not only by mutations in the TA proteins themselves, but also by dysregulations of the cellular machinery involved in their targeting and insertion into membranes. Such knowledge could lead to the discovery of novel therapeutical targets.

Table 1.2 - Examples of pathologies associated with TA proteins. (Source: Kalbfleisch, 2007; The UniProt Consortium 2017)

TA protein	Gene	Pathology associated
Aldehyde dehydrogenase 3A2 isoform 2	ALDH3A2	Sjogren-Larsson syndrome
Apoptosis regulator Bcl2	BCL2	Chronic lymphatic leukaemia
Cardiac phospholamban	PLN	Hypertrophic cardiomyopathy; Dilated cardiomyopathy
Cytochrome b5	CYB5A	Methemoglobinemia
Dysferin	DYSF	Limb-girdle muscular dystrophy 2B; Miyoshi muscular dystrophy;
Emerin	EMD	Dreifuss-Emery muscular dystrophy
FXYD domain-containing ion transport regulator 2 isoform 1	FXYD2	Renal hypomagnesemia-2
Golgi SNAP receptor complex member 2	GOSR2	Epilepsy, progressive myoclonic 6
Golgin subfamily A member 5	GOLGA5	Papillary thyroid carcinomas
Hereditary hemochromatosis protein	HFE	Hemochromatosis 1; Microvascular complications of diabetes 7
Junctophilin-2	JPH2	Cardiomyopathy, familial hypertrophic 17
Junctophilin-3	JPH3	Huntington disease-like 2
Mitochondrial fission factor	MFF	Encephalopathy due to defective mitochondrial and peroxisomal fission 2
Monoamine oxidase A	MAOA	Brunner syndrome
Myotonin-protein kinase	DMPK	Dystrophia myotonica 1
Otoferlin	OTOF	Deafness, autosomal recessive, 9; Auditory neuropathy, autosomal recessive, 1
Potassium voltage-gated channel subfamily E member 3	KCNE3	Brugada syndrome
Regulator of G-protein signaling 9-binding protein	RGS9BP	Prolonged electroretinal response suppression
Selectin L	SELL	Immunoglobulin A nephropathy
Syntaxin-1B	STX1B	Generalized epilepsy with febrile seizures plus 9
Sodium/potassium-transporting ATPase subunit gamma	FXYD2	Hypomagnesemia 2
Syntaxin 16 isoform a	STX16	Pseudohypoparathyroidism type Ib
Syntaxin 1A	STX1A	Williams-Beuren syndrome
Tectonic-3	TCTN3	Orofaciodigital syndrome 4; Joubert syndrome 18
Threonylcarbamoyladenosine tRNA methylthiotransferase	CDKAL1	Diabetes mellitus, non-insulin-dependent
Thymopoietin isoform beta/gamma	TMPO	Dilated cardiomyopathy
VAMP-associated protein B/C	VAPB	Amyotrophic lateral scelerosis 8
Vesicle-associated membrane protein 1	VAMP1	Spastic ataxia 1, autosomal dominant

1.3.2 The GET system in yeast

As stated before, Stefanovic and Hegde found TRC40 to bind and effectively delivery TA proteins to the ER membrane in an energy dependent way (Stefanovic and Hegde 2007), an observation that was reported independently by Favaloro et al. (2008).

At this point, no other components of the TRC40 pathway were known, but its yeast homolog, Get3 had just been discovered, in a comprehensive genetic interaction study, to be part of a complex involved in Golgi to ER traffic (GET) together with Get1 and Get2 (Schuldiner et al. 2005). Thus, it was not long until all three components of the yeast GET complex were shown to participate in the post-translational insertion of TA proteins. Get3, being the homolog for TRC40, is the chaperone with ATPase activity that binds to the TA protein TMD after translation and delivers it to the ER membrane, where Get1 and Get2 are the two subunits of a membrane receptor that binds Get3 and mediates the insertion of the TA proteins (Schuldiner et al. 2008). These discoveries led to the conclusion that the initial findings on the GET complex - its involvement in Golgi to ER trafficking and all otherwise unconnected phenotypes - were secondary effects caused by an impairment in TA protein insertion. Recent work, however, showed that things are not that simple and that in fact, some components of the GET system may have additional functions (Powis et al. 2013; Voth et al. 2014).

Subsequently, Get4 and Get 5 (Jonikas et al. 2009) and Sgt2 (small Glu-rich tetratricopeptide repeat-containing 2) (Costanzo et al. 2010) were discovered as three additional members of the GET pathway. With the major players defined, the mechanism by which TA proteins are inserted in the ER membrane in yeast was elucidated using a combination of structural and functional studies.

As schematized in Fig. 1.3, after translation, TA proteins are captured at the exit of the ribosomal tunnel by Sgt2 that binds directly to the TMD of TA proteins, acting as a chaperone that prevents TA protein aggregation or mistargeting, before handing them down to Get 3. For this hand-off to occur, Sgt2 binds the ubiquitin-like domain of Get5, to interact with the Get4-Get5 sub-complex, that serves as a bridge between Sgt2 and Get3 (the latter binds to Get4) (Wang et al. 2010). Crucially, Get4 selectively binds the ATP-bound (and thus TA TMD receptive) conformation of Get3 (Chartron et al. 2010), allowing the transfer of the TA protein from Sgt2 to Get3.

Get3 is a homodimeric ATPase that, when bound to ATP is in a closed conformation that allows for the binding of TA TMDs and when ATP is hydrolysed transits to an open conformation that releases the TA proteins. Upon transfer of TA protein substrate from Sgt2 to Get3, Get3 carries the TA proteins in the cytosol, shielding and maintaining the solubility of the hydrophobic TMD, inside a hydrophobic groove that spans both Get3 monomers, arriving at the membrane in a closed conformation (Mateja et al. 2009, 2015).

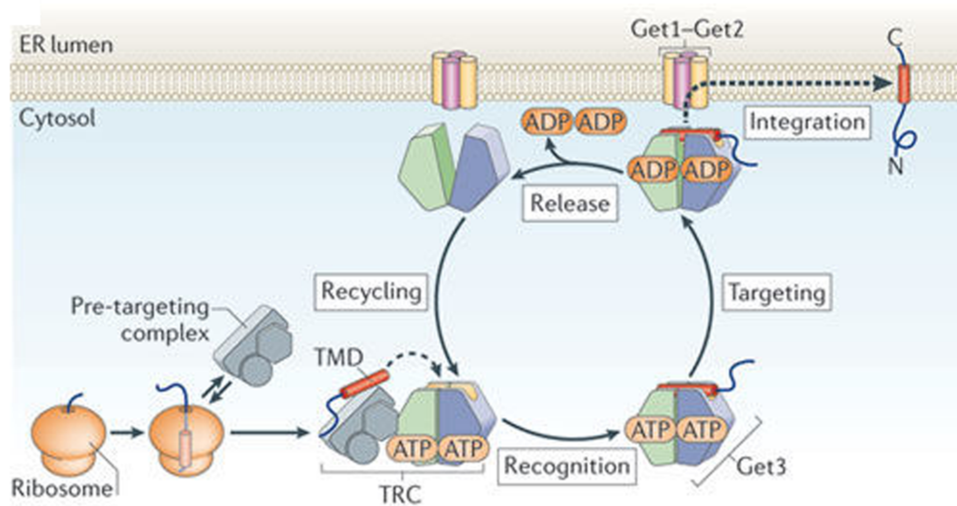

Fig. 1.3 - Representation of the GET system in yeast. Adapted from Hegde and Keenan 2011

Get3 then delivers the TA protein to its ER membrane receptor, formed by Get1 and Get2. The two monomers are bound by their transmembrane domains in a heterodimeric or tetrameric complex (Zalisko et al. 2017) and interact with Get3 via the cytosolic domains in a stepwise manner (Mateja et al. 2009, 2015; Mariappan et al. 2011; Wang et al. 2011; Stefer et al. 2011; Rome et al. 2014). First, the flexible cytosolic domain of Get2 captures the Get3/TA complex, by binding to one of the subunits of Get3, bringing it close to Get1; in this process, the ATP bound to Get3 is hydrolysed shifting the conformation into a partially destabilized closed conformation. Get1 then binds to Get3, creating a transient complex in which Get3 is bound simultaneously by Get2 on one side and Get1 on the opposite side (Zalisko et al. 2017). This transient interaction drives the Get3/TA complex from the previous partially destabilized closed conformation into an open conformation that disrupts the hydrophobic TA protein binding site, releasing the protein. After release, the TMDs of Get1 and Get2 capture the TMD of the TA proteins and may subsequently facilitate the entry of the tail-anchor into the membrane,

acting as an insertase (Wang et al. 2014). Get3 is again bound by ATP, which interrupts its interaction with Get1 and is recycled back to the cytosol where again is available for interaction with the Get4/Get5 pre-targeting complex.

1.3.3 The TRC40 pathway

The advances in the understanding the molecular mechanisms of post-translational TA protein insertion in yeast allowed for a parallel definition, mostly through homology, of the players and mechanisms that compose the TRC40 pathway in mammals.

In mammals, the pre-targeting complex is composed of the Sgt2 homolog, SGTA, a complex homologous to Get4/Get5 (TRC35/UBL4A in mammals), but that has a third component, Bag6 that seems to act as scaffold for the interaction between TRC35 and UBL4A; indeed, unlike the yeast homologs, the latter two proteins are not able to directly interact (Chartron et al. 2012). This pre-targeting complex, is then responsible for the capture of TA proteins from the ribosome and for its delivery to the TRC40 homodimer. Moreover, the Bag6 complex has been seen to be involved in protein quality control, binding a wide range of hydrophobic substrates and mediating the degradation of mistargeted proteins (Hessa et al. 2011).

As for the membrane components of the TRC40 pathway, first the tryptophan-rich basic protein (WRB) was found to be the mammalian homologue of Get1 and to be part of the membrane receptor for TRC40 (Vilardi et al. 2011). Based on bioinformatic analysis, however, no mammalian homologue was found for Get2. Nonetheless, a physical interaction assay, using TRC40 as bait, allowed the identification of CAML (calcium modulating cyclophilin ligand) as both a TRC40 and WRB interactor (Yamamoto and Sakisaka 2012). The authors showed that CAML binds TRC40 through a long cytosolic N-terminal domain (much as the interaction between Get2 and Get3) and interacts with WRB through the TMDs (again, like the yeast Get1/Get2 complex). Subsequent studies, demonstrated that the CAML/WRB complex was able to restore TA protein insertion competence both in ΔGet1/Get2 yeast cells (Vilardi et al. 2014) and in reconstituted proteoliposomes (Colombo et al. 2016), confirming the functional homology between CAML and Get2. The exact mechanisms by which the WRB/CAML protein interacts with

TRC40 and mediates TA protein insertion and how similar these processes are to what is observed in yeast is still poorly understood.

A recent study that tried to further characterize the WRB/CAML complex and its regulation (Colombo et al. 2016) will be discussed in more depth in a following section.

1.3.3.1 CAML

Before being found to be an essential component of the TRC40 pathway (Yamamoto and Sakisaka 2012), CAML had been the focus of much attention, being found to interact with numerous proteins. CAML is a ubiquitously expressed, ER-resident membrane protein with three putative transmembrane domains located in its C-terminal region and a long cytosolic N-terminal arm.

First discovered in 1994 by Richard Bram (Bram and Crabtree 1994) in a screen for cyclophilin B-binding proteins, CAML was described as being a participant in the calcium-signal transduction pathway, acting downstream of the T-cell receptor (TCR) and upstream of calcineurin by causing an influx of calcium. This action was suggested to be modulated by CAML's TMDs (Holloway and Bram 1996).

CAML was also found to interact with a member of the tumour necrosis factor receptor superfamily, inducing activation of the calcium-dependent transcription factor NF-AT in Jurkat T cells (von Bulow and Bram 1997).

It was later suggested that CAML associates directly with the kinase domain of the EGFR (epidermal growth factor receptor), indicating a potential role of CAML in receptor recycling during long-term proliferative responses to EGF (Tran et al. 2003).

Also, $GABA_A$ receptors were seen to interact with CAML, more precisely, the γ_2 subunit of the $GABA_A$ receptors. This interaction was proposed to be important for the functional expression and endocytic recycling of $GABA_A$ receptors (Yuan et al. 2008).

These are only some examples of the interactions and cellular process in which CAML has been found to take part, implying the importance of CAML for cellular homeostasis. It is a highly-conserved protein among vertebrates and is ubiquitously expressed. Moreover, CAML total knockout is lethal for mice embryos while mouse embryonic stem cells from CAML-knockout embryos grow without problems (Tran et al.

2003). To be noted, no homologues have been identified in non-vertebrate metazoans or in plants.

Presently, it is still uncertain if the many processes in which a role for CAML has been implied is but a side effect of CAML being a component of the TRC40 pathway or if, instead, CAML has other functions besides being a part of the TRC40 receptor. A recent work points to the latter as being the most plausible answer, revealing an essential role for CAML in supporting survival and mitotic progression in Myc-driven lymphomas, independent of TA protein insertion function (Shing et al. 2017). It remains to be understood if CAML in complex with WRB or WRB-free CAML may be responsible for these various functions reported for CAML.

1.3.3.2 WRB

WRB, also known as CHD5 (congenital heart disease 5 protein), is expressed from a gene located on chromosome 21 in a region thought to be responsible for CHD in Down syndrome (DS) (Egeo et al. 1998). WRB is an ER membrane protein, ubiquitously expressed, that has three transmembrane domains and a coiled-coil, cytoplasmic domain between the first and second TMDs. Recently it was shown that DS fetal fibroblasts presented an increased expression of WRB protein (Colombo et al. 2016), however the link between the higher expression of WRB and CHD occurrence in DS patients remains unclear.

Differently from CAML, WRB is conserved throughout the eukaryotic domain of life. Different animal models showed that a reduced expression or a complete knock down of WRB led to defects in cardiac development and morphology (Murata et al. 2009; Sojka et al. 2014). Other works, have implied WRB to be essential for the formation of synaptic structures in photoreceptors and inner ear cells (Daniele et al. 2016; Lin et al. 2016).

It is still uncertain if the role WRB in these pathologies and cellular processes is related to its essential role in the TRC40 pathway or not.

1.3.4 Regulation of CAML/WRB

Recently, Colombo and colleagues set out to fill the gap of knowledge that remained, concerning the function and reciprocal interactions of CAML and WRB as the two subunits of the TRC40 receptor, compared to the extensive understanding of the yeast GET system (Colombo et al. 2016).

Using a series of functional, in-vitro assays, they were able to demonstrate that both WRB and CAML are essential for TRC40-dependent TA protein insertion in microsomal extracts and that they were sufficient to confer insertion competence to liposomes. Furthermore, it was demonstrated that CAML and WRB indeed physically interact and that immune-depletion of CAML from microsomal extracts, also depletes WRB and renders the microsomal extract incompetent for insertion.

Then, using a strategy based on the application of recombinant proteins for the creation of standard curves, the authors quantified the absolute amount of CAML and WRB in rat liver microsomes and in a human neuroblastoma cell line, finding CAML to be in large molar excess over WRB (4-fold in microsomes and 7-fold in neuroblastoma cells). They also saw that, in-vitro, the excess of CAML does not interfere with TA protein insertion.

Since the WRB gene is present in the distal region of the long arm of chromosome 21 (Hsa21), which is a region considered to play a critical role in the DS phenotype, the authors used a similar strategy to quantify the absolute amounts of CAML and WRB in DS and control fetal fibroblasts. They found an increase in WRB, both at the protein and the transcript levels, in line with a previously reported 1.5-fold upregulation of Hsa21 transcripts (Prandini et al. 2007). Additionally, they again saw that an imbalance in WRB/CAML protein levels did not impair TA protein insertion.

In yeast, it had been seen that the deletion of each one of the subunits resulted in at least a partial loss of the second subunit (Mariappan et al. 2011) in agreement with the concept of quality control (see Section 1.2.2). Thus, to understand if a similar effect was observed in mammalian cells, the authors silenced each one of the subunits in HeLa cells, using RNA interference. They saw that indeed, also in mammalian cells, the depletion of one subunit led to a parallel depletion of the second one (Fig. 1.4-A; B). Subsequently, to investigate at which step the expression of each subunit was influenced by the other, they

analysed the relative transcript levels after silencing, surprisingly finding that upon CAML silencing, the levels of WRB transcript were greatly reduced, while the silencing of WRB did not seem to have such a strong effect on the levels of CAML transcript (Fig. 1.4-C; D). Furthermore, the authors showed that the reduction in WRB transcript following CAML silencing was caused by its destabilization (Fig. 1.4-E). The strong effect of WRB silencing on CAML protein and the very mild effect on transcript levels were instead in line with results obtained from a conditional knockout mouse for WRB, which has reduced levels of CAML protein but not alterations in CAML transcript (Rivera-Monroy et al. 2016).

The destabilization effect of CAML silencing on WRB transcript and the causes for the reduced protein levels upon partner silencing are still to be better elucidated. The investigation of these regulatory mechanism will be the main focus of the present work. The last chapter of this introduction is dedicated to mechanisms of post-transcriptional regulation, in order to provide a background for my studies on the regulation of the WRB transcript.

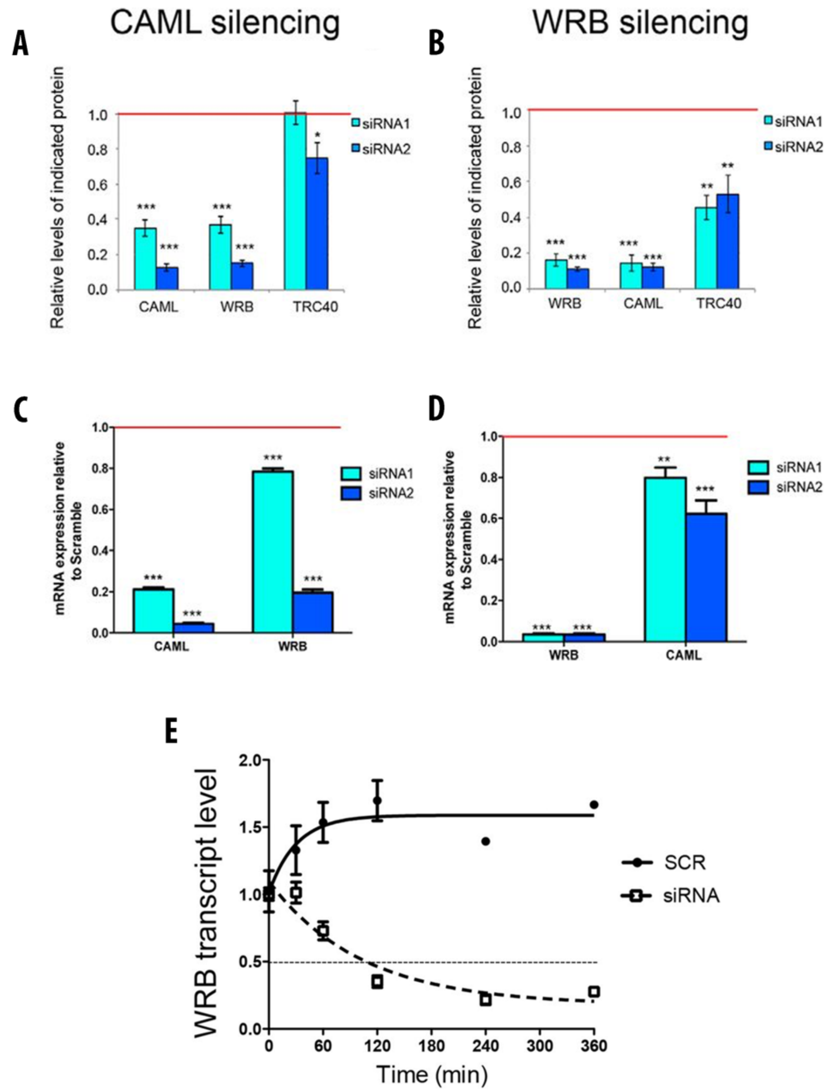

Fig. 1.1 - Effect of the silencing of the TRC40 receptor subunits on each other. The silencing of CAML leads to the reduction of WRB protein (A) and transcript (B) levels, by destabilizing its transcript (E). The silencing or WRB leads to the depletion of CAML protein (B) and a small reduction of its mRNA levels (D). Adapted from (Colombo et al. 2016).

1.4 Post-transcriptional regulation

From the moment it is transcribed, RNA passes through several steps of maturation and regulation until and while it is finally translated into protein. These steps include RNA splicing, transport, stability, localization and translation, which ultimately determine the amount and subcellular localization of the translated protein, thus impacting the cellular response to stimuli and stress. The multitude of regulatory mechanisms of mRNA fate can be appreciated in Fig. 1.5.

RNA regulation factors can be divided in two major categories, RNA-binding proteins (RBP) and non-coding RNA. Right from when transcription initiates, these factors dynamically bind the RNA forming the messenger ribonucleoprotein particle (mRNP). Most RNA regulation factors, are targeted to structures or sequences that are mRNA specific; these recognition elements are commonly present in the untranslated regions (UTR) of mRNA, at the 5' and 3' ends of the transcript. It is the unique combination of factors accompanying each mRNA, as well as their relative position along the transcript, that dictates the fate of every mRNA (Moore 2005).

Eukaryotic RNA, immediately after transcription is referred to as precursor-mRNA (pre-mRNA), which then needs to be processed to become functional mRNA. The first post-transcriptional modification to which pre-mRNAs are subjected is 5'capping. This consists of the addition to the 5' end of the pre-mRNA of a 7-methylguanosine through a 5'-5'-triphosphate linkage. The 5'cap is important for several aspects of mRNA fate. It serves to protect mRNAs from exonuclease 5'-to-3' degradation, both in the nucleus and the cytoplasm (Stevens and Poole 1995; Jinek et al. 2011). It is also necessary for efficient translation, serving as a binding site for various eukaryotic initiation factors (eIFs) and promoting the assembly of the pre-initiation complex (Jackson et al. 2010).

Another step in mRNA maturation is intron removal and exon splicing. Splicing is performed by the spliceosome and can result in alternative splicing, giving rise to different mature mRNAs. The exon-exon junctions resulting from splicing are bound by the exon junction complex (EJC), a protein complex that can either positively influence translation when it is bound in the open reading frame (ORF) or target the bound mRNA for rapid destruction via nonsense-mediated mRNA decay (NMD) when bound to the mRNA 3'UTR (Tange et al. 2004).

The last mRNA modification during maturation is polyadenylation. In this process, the pre-mRNA is first cleaved between the highly conserved AAUAAA sequence and a degenerate U/GU rich sequence, after which the poly(A) polymerase adds a 3' poly(A) tail of ~200 nucleotides (Minvielle-Sebastia and Keller 1999). Polyadenylation can take place in different sites of the mRNA, creating multiple mRNA isoforms by alternative polyadenylation. The poly(A) tail provides the mRNA with a binding site for poly(A) binding proteins (PABP), a class of regulatory proteins that influence mRNA export, stability and decay, and translation (Mangus et al. 2003).

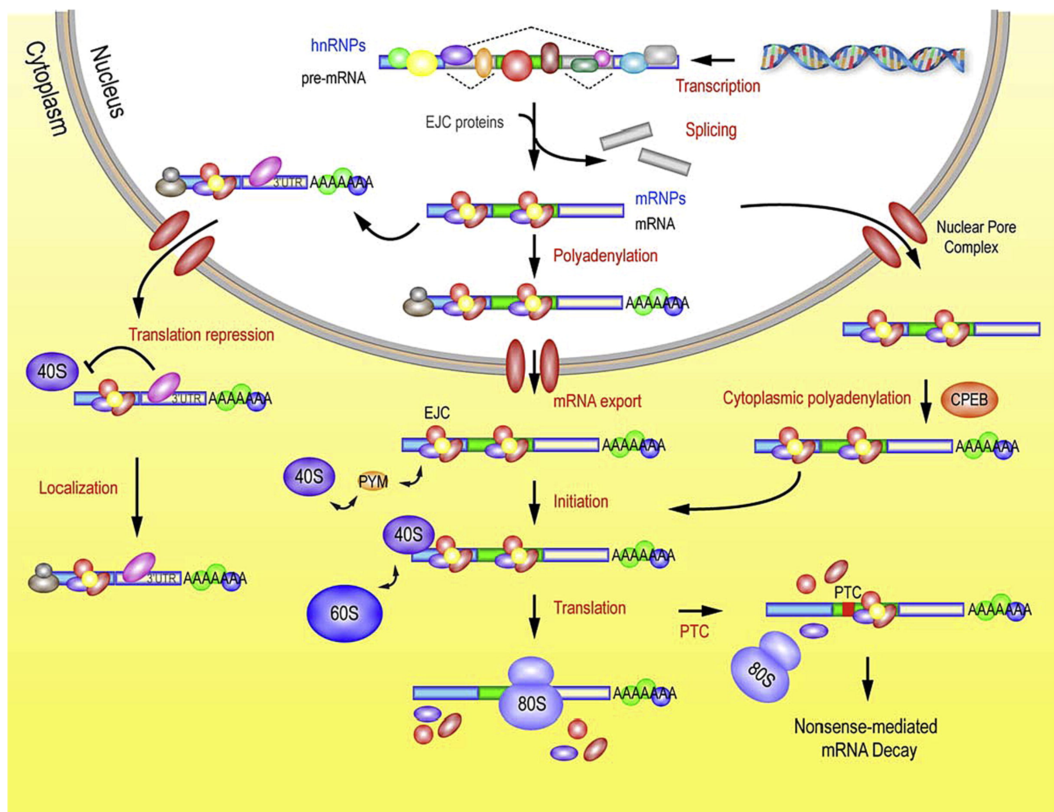

Fig. 1.2 - Representation of the various layers of post-transcriptional regulation that dictate the fate of mRNAs (Glisovic et al. 2008).

During and after mRNA maturation, several regulatory factors bind to mRNA, promoting its transport across the nuclear pore complex and into the cytoplasm and then promoting interaction with the translation machinery. As it is transiting the pore or soon after reaching the cytosol, a change in mRNP composition takes place, with some nuclear export proteins detaching from the mRNA and a concomitant engagement of the translation machinery. Some mRNAs have even been seen to associate with multiple ribosomes while still transiting the nuclear pore (Moore 2005).

Interestingly, a model has been proposed - thoroughly reviewed by Keene (2007) – that eukaryotes may present an operon-like regulation mechanism, where multiple mRNAs coding either for interacting or co-localized proteins can be bound together by a common RBP that is responsible for the physical proximity of the various transcripts, allowing a co-regulation of those mRNAs. This could for example be favourable for the assembly of multi-subunit protein complexes, recreating the simpler situation observed in prokaryotes, where these complexes are expressed in polycistronic mRNAs (see Section 1.2.1 and Fig. 1.1).

Translation of mRNA is controlled by a number of factors, including a network of simultaneous interactions between the 5'cap and eIFs present in the 5' UTR and the PABPs in the poly(A) tail of the 3'UTR. Two of the most well studied examples of modulation of translational efficiency are the internal ribosomal entry sites (IRES) and upstream ORFs (uORFs). IRES are mRNA regulatory motifs that facilitate a cap-independent mechanism of translation initiation, in which the ribosome binds to an internal site close to the translation initiation site (Filbin et al. 2009). IRES promote the recruitment of ribosomes and continued translation of essential proteins under conditions when cap-dependent translation is inhibited by stress, cell-cycle stage or apoptosis.

An uORFs is a 5'UTR regulatory mechanism. It occurs when an AUG is present in the 5'UTR, upstream to the main start codon, with an in-frame stop codon. In mRNAs that contain uORFs, the translational machinery may translate the uORF, altering the efficiency of translation of the main ORF, potentially causing great reductions in protein expression levels (Meijer and Thomas 2002). It has also been proposed that uORFs may relate to NMD, implying that the mechanism of uORF reduction of translation of the main ORF is more complex than previously thought (Mendell et al. 2004).

If the 5'UTR mostly carries motifs important for the regulation of translation, the 3'UTR can be seen as a regulatory hub, responsible for regulation of numerous processes including transcript cleavage, stability, degradation, polyadenylation, translation and mRNA localisation (Barrett et al. 2012). The 3'UTRs have the sequence targets for miRNAs, small non-coding RNAs that interact with their targets to regulate the expression. They also carry binding motifs for several RBPs, of which one of the most well-studied examples are the AU-rich elements (ARE). AREs are described as

stabilisation elements ranging from 50-150 bp and generally containing multiple copies of the pentanucleotide AUUUA (Chen and Shyu 1995). Different RBPs, or a RBP subjected to different stimuli, can bind AREs and result in different outcomes for the bound mRNA (Barrett et al. 2012).

Another important step in the life cycle of mRNA is degradation. This is true both for the degradation of aberrant mRNAs and for degradation during natural mRNA turnover. As commented in a previous section (see Section 1.2.2) regarding proteins, also for mRNA, the accumulation of defective mRNAs can have deleterious impacts on the cell by resulting in the translation of inappropriately terminated proteins. Moreover, when a protein is no longer needed, the transcript present in the cell must be degraded to stop expression. The degradation mechanisms are known to be intimately connected with translation and are regulation by a complex network of RBPs, including those that are responsible for the translation initiation itself. During natural transcript turnover, the mRNA is deadenylated and then can either be degraded 3' to 5' by the exosome or, more commonly, undergo decapping followed by 5' to 3' exonuclease degradation. Different quality control pathways exist for the degradation of aberrant mRNAs, these pathways are usually induced after the first round of translation: transcripts containing a premature stop signal undergo NMD which leads to deadenylation-independent decapping and, in metazoans, endonuclease cleavage; transcripts that lack a termination codon altogether are degraded via non-stop decay resulting in 3' to 5' exonucleolytic decay; and mRNAs that present strong stalling in translation elongation can be cleaved by endonuclease activity (reviewed in Wilusz et al. 2001; Shoemaker and Green 2012).

In eukaryotic cells, when translation is finished, polysomes are disassembled and non-translating mRNPs are targeted to specialized, non-membrane cellular compartments for storage or degradation. These compartments are composed of non-translating mRNPs and can either have several mRNA decay factors or translation initiation factors. Due to their composition, there has been much discussion as to the classification and function of these compartments, but the current status is that three kinds of compartments can exist, based on protein and mRNA composition, and genesis: processing bodies (P-bodies), GW-bodies and stress granules (Anderson and Kedersha 2006; Decker and Parker 2012; Patel et al. 2016). P-bodies in yeast were initially also called GW-bodies in higher eukaryotes due to the presence of Gw/GW182, a protein that is not

conserved in yeast. For long, these were thought to be the same homologous entity, however, recent evidence proposes a composition and function-based divergence, suggesting that both compartments can exist in higher eukaryotes. Both have been seen to be composed of translation repressor and several mRNA degradation factors such as exonucleases, decapping and deadenylation factors and modulators, among others. Supporters of the diverging model point out the presence of Gw/GW182 as a determinant factor for the GW-body as an entity on its own, because of its ability to recruit Ago-1 - a member of the microRNA-induced silencing complex (RISC) - and consequently the recruitment of RISC-targeted RNAs, implying an additional function of GW-bodies in miRNA-mediated silencing (Patel et al. 2016). Moreover, while P-bodies are thought to never associate with membranes, GW-bodies have been reported to interact with endo-lysosomal compartments and even to be secreted in exosome-like vesicles (Gibbings et al. 2009). Stress granules, on the other hand, are formed under stress conditions or when translation is artificially blocked. When cells are subjected to stress, the translation, especially of housekeeping genes, is shut down, while genes important for the stress response and for the adaptation of the cell to the new environment are upregulated; these include chaperones, and enzymes responsible for damage repair. In this process, stalled transcripts aggregate forming complexes containing several translation initiation factors, the small ribosomal subunit and several other RBPs. Transcripts can shuttle between P-bodies and stress granules, maybe undergoing degradation in the former, and being stored for future use in the latter; indeed, when the stress disappears or the cells adapt to the stress, these transcripts can return to the cytoplasm, resuming translation (Anderson and Kedersha 2006; Protter and Parker 2016).

It must be noted that the relationship between mRNA degradation pathways and P-body formation has been recently cast into doubt (Hubstenberger et al. 2017), and further work should address the real contribution of P-bodies for mRNA degradation and storage.

Here are described only some of the numerous layers of post-transcriptional regulation of mRNAs, to show the diversity of mechanisms by which different transcripts can be independently or co-regulated. The fundamental objective of these incredibly complex regulatory networks is efficient and localized protein translation and mRNA turnover.

2
AIM OF THE THESIS

Protein complexes represent a significant portion of the cellular targets of drugs. To be able to discover and design new and improved compounds for pharmacological therapies, one must better understand the very essence of the targeted receptors, including the molecular and cellular mechanisms that govern not only their response to the substance being used but also their biogenesis, assembly, subcellular localization and stability.

In the last decades, a great deal has been learned about proteins and protein structures. The application of multiple biophysical techniques, including X-ray crystallography, NMR and cryo-electron microscopy (2017 Nobel prize for chemistry), has allowed the determination of an ever-growing number of protein structures, including those of oligomeric protein complexes. This has led also to the determination of the interactions between subunits and the structural changes they undergo when forming a complex. Nevertheless, the regulatory mechanisms that govern the assembly of a multi-subunit protein complex, including spatio-temporal relations with the process of their translation on ribosomes, remain rather poorly understood.

In this work, as example of an oligomeric protein complex, we studied the regulation of the synthesis and assembly of the TRC40 receptor. The TRC40 receptor, a heterodimer composed of the WRB and CAML subunits, is responsible for the post-translational insertion of tail-anchored proteins into the ER membrane. It was recently found that this heteromeric receptor presents an inter-subunit transcript-protein cross-talk that could be important for the expression and assembly of the final complex. Thus, we intend to study this receptor expression, with the aim of understanding how the regulation of the expression of each subunit could be modulated by the second one. Our findings could be important in describing a new system for the co-regulation of the expression of the subunits of a protein complex.

3.
MATERIALS AND METHODS

3.1 Cell culture and manipulation

3.1.1 Cells

HeLa cells were the first immortalised human cells, derived from a human epidermoid carcinoma of the cervix in 1951 (Scherer et al. 1953). Since then, the use of this epithelial cell line as cell model became widely spread, due to their resistance and to the easiness in their handling. In this work, these cells were the model chosen to study the regulation of the expression of the WRB/CAML complex; they were used for steady-state analysis of transcripts and proteins, as well as for gene overexpression and silencing experiments.

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10 % (v/v) of heat-inactivated Fetal Bovine Serum (FBS, Carlo Erba), 1% (v/v) penicillin/streptomycin antibiotic mix (P/S, Invitrogen) and 1% (v/v) L-glutamine (Invitrogen). Cells were kept at 37 °C and 5 % CO_2 in a humidified incubator and routinely subcultured when optimal confluence was reached.

3.1.2 Transcription inhibition

For studies on transcript stability, transcription was inhibited using 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, Sigma). DRB inhibits transcription by inhibiting transcription elongation by RNA polymerase II (Sehgal et al. 1976; Dubois et al. 1994). Transcript stability was studied by inhibiting transcription for different time periods and analysing the mRNA levels at each time point.

For this, cells were seeded at ~35% confluency in 28 cm^2 culture dishes, one culture dish for each of the seven time points (t = 0; 0.5; 1; 2; 4; 6 and 8 hours). The next day, the cells from t = 0 were washed twice with Phosphate-buffered Saline (PBS – 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4) for removal of serum proteins and then harvested for RNA extraction by scraping in PBS. The cells were then transferred into a tube and pelleted by centrifugation at 850 g for 10 minutes at 4 °C. The supernatant was discarded and the pellet was frozen in liquid nitrogen and kept at -80 °C until use. Meanwhile, the medium of the cells of each of the other time points, was collected together and DRB was added (60 μM). The medium containing DRB was then divided by

the various culture dishes and the cells were incubated for the indicated times. At the end of incubation, cells were harvested and frozen as described above.

For the study of CAML transcript stability upon WRB silencing, three 3.8 cm^2 wells with HeLa cells were silenced for each DRB time point, and DRB treatment was initiated ~68h after WRB silencing. After incubation, cells from the three wells were harvested together in the same tube, as above.

3.1.3 Transfection

Different transfection methods are available for the insertion of external nucleic acids into cells. In this work chemical transfection was used, taking advantage of two different transfection reagents, for the introduction of either plasmid DNA for transient gene overexpression or small interfering RNA (siRNA) for gene silencing.

3.1.3.1 Gene silencing

Silencing of WRB or CAML was achieved by RNA interference. In this technique, the mRNA of a gene is degraded or its translation is blocked, in each case preventing the expression of the gene. In this work, RNA interference was performed using siRNAs, which are small double stranded RNAs, typically ranging from 20-25 nucleotides that are designed to specifically target the gene intended to be silenced. When introduced in the cell, one of the single stranded RNA fragments is loaded into the RISC complex activating it. The siRNA is then used by RISC to scan the mRNAs in the cell until the target is found and the Argonaute protein, one of the RISC components proceeds with the target mRNA degradation. The siRNAs (Invitrogen) used in this work are listed in Table 3.1. In each silencing experiment, a parallel sample is treated with a scramble siRNA (siSCR), that does not target any known human gene, as control.

The siRNAs were delivered to the cell using the Lipofectamine™ RNAiMAX transfection reagent (Invitrogen).

For silencing experiments, HeLa cells were seeded 24 h prior to the silencing at ~8% confluency in an antibiotic-free DMEM in 12-well plates, the day after, before silencing, fresh medium was added to the cells.

For CAML silencing, 10 pmol of siCAML or of siSCR was diluted in a tube with opti-MEM I Reduced Serum Media (opti-MEM, Gibco) in a final volume of 100 μL and 1 μL of Lipofectamine RNAiMAX was diluted with opti-MEM in a second tube in a final volume of 100 μL. The two dilutions were gently mixed and spun before adding the siRNA dilution to the Lipofectamine tube. After this, the reaction was mixed gently, spun and incubated for 20 minutes at room temperature to allow for complex formation. After incubation, complexes were gently added to the cells in a drop-wise manner. Cells were incubated with the complexes at 37 °C and 5 % CO_2. After 6h of incubation the medium was substituted with antibiotic supplemented DMEM and the cells were allowed to grow for 48h before being harvested for western-blot analysis or subjected to additional treatment.

Cells intended for SDS-PAGE and western blot analysis were washed twice with PBS for removal of serum proteins and harvested by direct lysis with hot Lysis Buffer (1% [w/v] SDS; 10 mM Tris-HCl pH 8.6; 1 mM PMSF; protease inhibitor cocktail [PIC]) and proteins were further denatured with the addition of 0.5 volumes of Denaturation Buffer (DB) 3X (3 % β-Mercaptoethanol; 25.5 % glycerol; 6% SDS; 60 mM Tris-HCl pH 6.8; 0.03 % Bromophenol blue). Samples were then boiled for 2 minutes at 100 °C and either used immediately or stored at -20 °C until use.

For WRB silencing, the same procedure was followed with a few changes. 10 pmol of siWRB or siSCR were mixed with 2 μL of Lipofectamine RNAiMAX and, after addition of complexes, cells were grown for 68-72h before being harvested for western-blot analysis as described above or being subjected to additional treatment.

The efficiency of silencing was analysed as a function of protein or transcript level in silenced cells relative to cells treated with siSCR.

Table 3.1 - Oligonucleotides used in this study. See text for details.

Oligo	Sequence (5'-3')
Primer 1	ttaagcttggcgcttggcttcaccg
Primer 2	cttaatgtttttggcatcttcc
Primer 3	gctctagagctgaacaggaggatggata
Primer 4	gctctagaagtgagaaaattcagctttactggg
Primer 5	gtctagagcactgttttgttttttaaga
Primer 6	gctctagatggaatttttagtagtgg
Primer 7	gcgagcacagaattaatacgact
Primer 8	cagccgcgaggctaaaaaac
Primer 9	cgcggatccaattaatacgactcactatagg
Oligo 1	agcttgggtggggcgcttggcttcaccgcgcaggcgcggtcgccgctgttgttgtggtccccat ggagctgccgtagcggacccagcacagccaggagcgtccgggccca
Oligo 2	agcttgggcccggacgctcctggctgtgctgggtccgctacggcagctccatggggaccacaacaa cagcggcgaccgcgcctgcgcggtgaagccaagcgccccaccca
Oligo 3	agcttgggtggggcgcttggcttcaccgcgcaggcgcggtcgccgctgttgttgtggtccccGtCgag ctgccgtagcggacccagcacagccaggagcgtccgggccca
Oligo 4	agcttgggcccggacgctcctggctgtgctgggtccgctacggcagctcGaCggggaccacaaca acagcggcgaccgcgcctgcgcggtgaagccaagcgccccaccca
siCAML	gcgcggaagaagaaagucatt
siWRB	ggguaguaagugucgcuuutt

3.1.3.2 Transient gene overexpression

Transient gene overexpression was carried out with the use of the jetPEI transfection reagent (Polyplus transfection). jetPEI is a polyethylenimine polymer able to create very stable, positively charged, complexes with DNA.

Transient transfections where used to study the expression of WRB-HA and FLAGCAML in CAML and WRB silenced cells, respectively.

Transfection of WRB-HA or FLAGCAML, was carried out after silencing of CAML (48h) or WRB (72h), respectively in 12-well plates. Before transfection, fresh medium was added to the cells. To control for transfection efficiency, pEGFPN1 was co-transfected with WRB-HA and FLAGCAML.

For the preparation of complexes, 1 µg of either pRK5rs-WRB-HA or pCLX31.1-FLAGhCAML and 0.5 µg of pEGFPN1 were diluted in a tube containing 150 mM NaCl in a final volume of 50 µL and 4.5 µL of jetPEI were diluted in a second tube in 150 mM NaCl in a final volume of 50 µL. The two dilutions were vortexed and spun before adding the jetPEI dilution to the tube containing the DNA. The mixture was then vortexed, spun

and incubated for 20 minutes at room temperature, to allow for the formation of complexes. After incubation, the complexes were gently added to cells in a drop-wise manner. Cells were allowed to grow in the presence of the complexes for 24h, before being harvested for western-blot analysis as described (see Section 3.1.3.1).

Luciferase constructs carrying WRB UTRs were transfected in either non-silenced or CAML silenced cells to investigate the effect of CAML on luciferase expression by each of WRB UTRs. Each of the pTKpGL4.11 constructs were co-transfected with the phRG-B plasmid, which encodes the *Renilla* luciferase and was used for transfection efficiency normalization.

In these experiments, 100 ng of phRG-B was co-transfected with a 5-fold molar excess of pTKpGL4.11 based plasmids, using 2.55 μL of jetPEI for complex formation. For preparation of complexes, the same procedure was used as above. Luciferase activity was measured 24h after transfection.

Cells intended for analysis with the Dual Luciferase Assay were washed twice with PBS before being detached by incubation with PBS with 1 mM EDTA at 37 °C for 5 minutes. Cells were then harvested and transferred into a tube and pelleted by centrifugation at 850 g for 10 minutes at room temperature. Residual EDTA was eliminated from the pellet by addition of PBS followed by another centrifugation. Cells were then lysed in 1X Passive Lysis Buffer (PLB) as part of the Dual Luciferase Reporter Assay System (Promega) manufacturer's protocol.

3.2 Molecular biology techniques

3.2.1 Construct preparation

The basic molecular biology techniques applied in this work for the preparation of constructs were performed according to the Current Protocols of Molecular Biology (Ausubel et al. 2001), for the exception of when commercially available kits were used following manufacturer's protocols.

3.2.1.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a technique that allows for the very specific amplification of a fragment of DNA. It is based on the process of DNA replication, which consists in repeated cycles of denaturation and replication of a DNA template, in which a Polymerase produces a complementary copy of a single stranded DNA sequence, if guided by the presence of a primer that first anneals to the template sequence.

In this study, mainly two different commercially available DNA polymerases were used. The GoTaq® DNA Polymerase (Promega) was used for standard control PCRs and the Expand™ High Fidelity PCR System (Roche) that exhibits proofreading activity was used for the amplification of sequences intended for subsequent cloning into expression vectors. Both enzymes were used following the manufacturer's protocols, calibrating the annealing temperature according to the primer pair being used. The various primers used for the preparation of the constructs used in this work are described in (Table 3.1). PCRs were performed in a PTC-100 thermal cycler (Bio-Rad).

3.2.1.2 Restriction Enzyme Digestion

For this study, restriction enzymes where used both in the cloning of determined DNA sequences into expression vectors and to control that the cloning was successful.

Generally, in 20-50 μL reaction volumes, 0.2-2 μg of DNA was mixed, never exceeding 0.1 μg/μL of DNA concentration, with water, 1X of the recommend buffer for the enzyme being used and a 5-fold excess of enzyme over DNA quantity (U/μg). Reactions were carried for 2 h at 37 °C. When digesting plasmids for subsequent insertion

of DNA fragments, at the end of the incubation, 1U of Thermosensitive Alkaline Phosphatase (TSAP) (Promega) was added per μg of DNA for 30 minutes at 37 ºC for the removal of phosphate groups from 5' ends. The phosphatase was then inactivated by incubating the sample at 74 ºC for 15 minutes.

After digestion, a fraction of the sample was controlled by electrophoresis on an agarose gel.

3.2.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis is the most utilized technique to separate, identify and purify DNA fragments based on their length. To visualize the fragments, usually an intercalating fluorescence probe, e.g. Ethidium Bromide (EtBr), is used.

For this work, 0.8-2 % agarose gels were prepared in Tris-acetate buffer (TAE – 40 mM Tris acetate; 2 mM EDTA) with 0.5 μg/mL of EtBr. Fluorescence from EtBr was detected using a UV transilluminator (UVITEC) and images of the gels were acquired using a UVIsafe HD5 imaging system (UVITEC).

3.2.1.4 Purification of DNA fragments

DNA fragments, intended for subsequent insertion into expression vectors, resulting either from PCR amplification or from digestion from other plasmids, were purified, prior to the ligation step, for removal of enzymes, buffers and other unwanted DNA fragments. Purification was carried by binding of DNA fragments to silica membranes followed by removal of contaminants and subsequent elution of purified DNA from the membranes. In this work, the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel) was used, following the manufacturer's protocol, to purify DNA fragments either directly from PCR samples or after separation by agarose gel electrophoresis.

3.2.1.5 Ligation of DNA fragments to plasmid vectors

When cloning a DNA fragment into a plasmid vector, the fragment is ligated to the plasmid using a DNA ligase, which is an enzyme that can catalyse the formation of a

phosphodiester bond between two DNA strands. This ligation happens between two fragments that have either sticky, complementary ends or blunt ends.

In this work, the T4 DNA Ligase (Invitrogen) was used. Briefly, in a 20 μL reaction volume, 30 ng of linearized vector were mixed with a 10-fold molar excess of the fragment to be inserted, 2 μL of 10X Ligase buffer and 1 U of T4 DNA ligase. The reaction was then incubated in a water bath at 16 °C overnight. The resulting ligation product was then transformed into competent *E. coli*.

3.2.1.6 TA-cloning

The DNA polymerases used in this work produce an amplicon that presents single 3' adenine overhangs. To allow for direct cloning of these DNA fragments, the TOPO® TA cloning® Kit (Invitrogen) was used. This kit uses a strategy based on the utilization of a topoisomerase that catalyses the ligation between the PCR product adenine overhangs and the previously linearized pCRII-TOPO thymidine overhangs. The newly formed plasmids can then be transformed into competent bacteria

The resulting plasmids were either used to sequence the PCR product or to facilitate restriction digestion of restriction sites flanking the amplicon for subsequent sub-cloning into an expression vector.

3.2.1.7 Preparation of competent bacteria

In order to allow the transformation of *E. coli* with plasmid DNA, these must be rendered competent. This can be achieved by treating the bacteria with a divalent cation - usually Ca^{2+} - solution. The divalent cation, neutralizes the negative charges of the DNA and helps permeabilize the bacterial wall, allowing the DNA to enter the bacteria. In this work, the DH5α strain of *E. coli* was used. To turn it competent, a bacterial colony grown in an antibiotic free lysogeny broth (LB) agar plate (1.5 % [w/v] Agar; 1 % [w/v] Tryptone; 0.5 % [w/v] Yeast extract; 1 % [w/v] NaCl; pH 7) overnight was inoculated into 100 mL of Super Optimal Broth (SOB) medium (2 % [w/v] tryptone; 0.5 % [w/v] Yeast extract; 0.05 % [w/v] NaCl; 2.5 mM KCl; pH 7.2) with 20 mM $MgSO_4$ and bacteria was grown at 37 °C under strong agitation until mid-log phase (OD_{600} ~ 0.1). After this, the suspension was incubated on ice for 10 minutes before being centrifuged at 3220 g for 10 minutes at

4 °C. Then, the pellet was carefully resuspended in 40 mL of ice-cold Frozen Storage Buffer (FSB - 45 mM $MnCl_2$; 10 mM CaCl2; 100 mM KCl; 3 mM Hexaamminecobalt (III) chloride; 10 mM Potassium acetate, pH 7.5; 10 % [w/v] glycerol; pH 6.4) and incubated on ice for 10 minutes before being centrifuged again at 3220 g for 10 minutes at 4°C. The pellet was resuspended in 8 mL FSB, and 140 µL of DMSO were added for cryo-preservation. The suspension was incubated on ice for 15 minutes and then another 140 µL of DMSO were added. After this, aliquots of competent bacteria were fast-frozen in liquid nitrogen. The competent bacteria were kept at -80 °C until needed for transformation.

3.2.1.8 Transformation of plasmid DNA into competent bacteria

For the transformation of bacteria with plasmid DNA, cells are subjected to a heat-shock treatment, that helps the DNA enter the bacteria. Plasmid DNAs confer to the bacteria a resistance for a determined antibiotic, which is then added to the growth media, allowing for selective growth of only transformed bacteria.

Briefly, 1 ng of plasmid DNA, 10 µL of ligation product or 6 µL of TA-cloning product, were mixed with 100 µL of Competent DH5α *E. coli* and kept on ice for 30 minutes. The mixture was then placed in a water bath at 42 °C for 90 seconds and then placed back to ice for 1 minute. At this point, 900 µL of LB medium was added to the transformed bacteria and the suspension was kept for 1 hour at 37 °C under agitation. After this, 100 µL of the suspension was spread onto a selective LB plate containing an appropriate antibiotic, when plasmid was being transformed. When a ligation or TA-cloning product were being transformed, the bacterial suspension was centrifuged for 5 minutes at 1000g, then resuspended in a small volume of supernatant, so that the complete transformation sample could be spread onto the selective LB plate.

Plasmid DNA extracted from selected bacterial colonies grown after transformation, was analysed by electrophoresis, restriction digestion or PCR for assessment of overall quality of the plasmid and of insertion and orientation of inserted fragment in the case of ligation or TA-cloning products transformation.

3.2.1.9 Plasmid DNA extraction from bacteria

After transformation, the transformed DH5α *E. coli* were grown in a selective medium to amplify the DNA plasmid of interest either in a small of medium scale. The plasmid was then extracted and purified.

For small scale plasmid extraction, 2 mL of LB with an appropriate selective antibiotic (100 μg/mL) was inoculated with a bacterial colony grown overnight at 37 ºC on a selective LB agar plate and bacteria was grown overnight at 37 ºC under strong agitation. The following morning, 1.4 mL of the bacterial suspension was centrifuged for 5 minutes at 16560 g at 4 ºC. The remaining bacteria were stored at -80 ºC in a 40 % glycerol suspension. The pellet was then resuspended in 100 μL of resuspension buffer (50 mM Tris-HCL, pH 8.0; 10 mM EDTA; 100 μg/mL RNase A) and vortexed for complete resuspension. Then, bacteria were lysed by the addition 150 μL of alkaline lysis buffer (200 mM NaOH; 1 % SDS) and the contents were gently mixed by inverting the tube. After this, the lysate was neutralized by the addition of 150 μL of neutralization buffer (3 M potassium acetate, pH 5.5) and incubated 5 minutes on ice. The lysate was then cleared from genomic DNA by centrifugation at 16560 g for 5 minutes at 4 ºC. The cleared lysate was then transferred to a new tube and plasmid DNA was precipitated by mixing with 800 μL 100 % (v/v) EtOH and centrifugation at 16560 g for 20 minutes at 4 ºC. The DNA pellet was then washed with 500 μL of 70 % (v/v) EtOH followed by centrifugation at 16560 g for 10 minutes at 4 ºC. The pellet was then allowed to dry and resuspended in 30 μl Tris-EDTA buffer (TE - 10 mM Tris-HCl, pH 8.0; 1mM EDTA). The plasmid DNA extracted from small scale preparations was mostly used for analysis of ligation products by restriction digestion and in some cases for DNA sequencing.

Medium scale plasmid extraction was used when preparing plasmids for subsequent sub-cloning or for end-point applications, such as plasmid transfection. Medium scale preparations were performed on 100 mL bacterial suspensions grown overnight in the presence of an appropriate selective antibiotic, using the QIAGEN® Plasmid Midi Kit (Qiagen), following the manufacturer’s protocol.

3.2.1.10 Constructs

The constructs and primers used in this work are illustrated in Fig. 3.1 and Table 3.1, respectively.

pRK5rs-WRB-HA was a kind gift from Fabio Vilardi (University of Göttingen, Göttingen, Germany), and its preparation had been previously described elsewhere (Vilardi et al. 2011); it consists of the WRB coding sequence fused to a C-terminal HA tag. The pCLX31.1-FLAGhCAML, containing the CAML coding sequence fused to a N-terminal FLAG tag (Holloway and Bram 1996) had been provided by Richard Bram (Mayo Clinic, Rochester, USA).

pEGFN1 (Clontech) was available in the laboratory and codes for the enhanced Green Fluorescent Protein (eGFP).

The phRG-B and pTKpGL4.11 were kind gifts from Roberta Benfante (IN-CNR, Milan, Italy). phRG-B is a promoterless mammalian vector that encodes a humanized *Renilla* luciferase. pTKpGL4.11 encodes the firefly luciferase under the Thymidine Kinase (TK) promoter.

The constructs used to study the effect of WRB UTRs in expression were built on the pTKpGL4.11 backbone. The sequence of the WRB transcript deposited on NCBI database (NM-004627) was used as reference for the preparation of a synthetic oligonucleotide cassette containing the WRB 5'UTR sequence flanked by cohesive ends complementary to the HindIII restriction site (oligo 1 and oligo 2 in Table 3.1). Due to the presence of an ATG sequence in WRB 5'UTR, a second oligonucleotide cassette (oligo3 and oligo 4 in Table 3.1) was prepared, in which that ATG was changed into GTC; this cassette was called WRB 5'mutUTR. The pTKpGL4.11-WRB5'UTR and pTKpGL4.11-WRB5'mutUTR were prepared by inserting each one of the synthetic oligonucleotide cassettes into the HindIII restriction site of pTKpGL4.11, present upstream to the luciferase coding sequence. The insertion and correct orientation of the cassette in the plasmid was controlled by PCR, using primers 1 and 2; the amplification of a DNA fragment reported on the correct orientation.

The same WRB transcript sequence was used to design a primer pair, primers 3 and 4, that carried a 5'-end XbaI restriction site, to amplify WRB 3' UTR from cDNA retrotranscribed from HeLa cell RNA. The amplified WRB 3' UTR was first sub-cloned in a TA-cloning vector and sequenced. After confirmation that it was identical to the

deposited sequence, the WRB 3' UTR was excised from the TA-cloning vector using the XbaI flanking sites and then cloned into the pTKpGL4.11, pTKpGL4.11-WRB5'UTR and pTKpGL4.11-WRB5'mutUTR XbaI site, situated downstream to the luciferase coding sequence; the resulting constructs were called pTKpGL4.11-WRB3'UTR, pTKpGL4.11-WRB5'+3'UTR and pTKpGL4.11-WRB5'mut+3'UTR. The insertion and correct orientation of the WRB 3' UTR fragment was controlled by restriction digestion with SpeI: the presence of two closely migrating bands of ~3000bp was indicative of the correct orientation.

The "AREsless" construct, pTKpGL4.11-WRB3'UTRΔARE, which has an internal portion of WRB 3' UTR without any of the three putative AREs, was prepared by amplifying the 3'RACE product B2 from the pCRII-TOPO vector using primers 5 and 6, both primers designed with 5' end XbaI restriction sites. Primer 5 was designed to be complementary to the antisense strand in a region downstream of a potential ARE site located in the 5' region of WRB 3' UTR. The amplification product was subcloned into the TA-cloning vector to be excised by XbaI restriction digestion. The excised fragment was then cloned into the XbaI site of pTKpGL4.11.

3.2.2 RT-qPCR

Real-time or quantitative PCR (qPCR) is a PCR (see Section 3.2.1.1) based method that allows for the quantification of the initial template present in the reaction. In traditional PCR, the amplification product is analysed and quantified after the last cycle, without obtaining information on the initial template quantity. In qPCR, on the other hand, the amplification product is analysed at the end of each cycle. This is achieved by using fluorescent probes that give fluorescent signals proportional to the number of amplification molecules generated.

When combined with reverse-transcription (RT-qPCR), this technique allows to measure also the RNA levels in a sample, by first retrotranscribing the RNA into complementary DNA (cDNA) and then amplifying the resulting cDNA. By using probes that are designed to be highly specific for a gene transcript of interest, one can measure the mRNA levels of that gene in a biological sample. These probes have two different fluorescent molecules, one at each end of the probe. When in close proximity, one of the

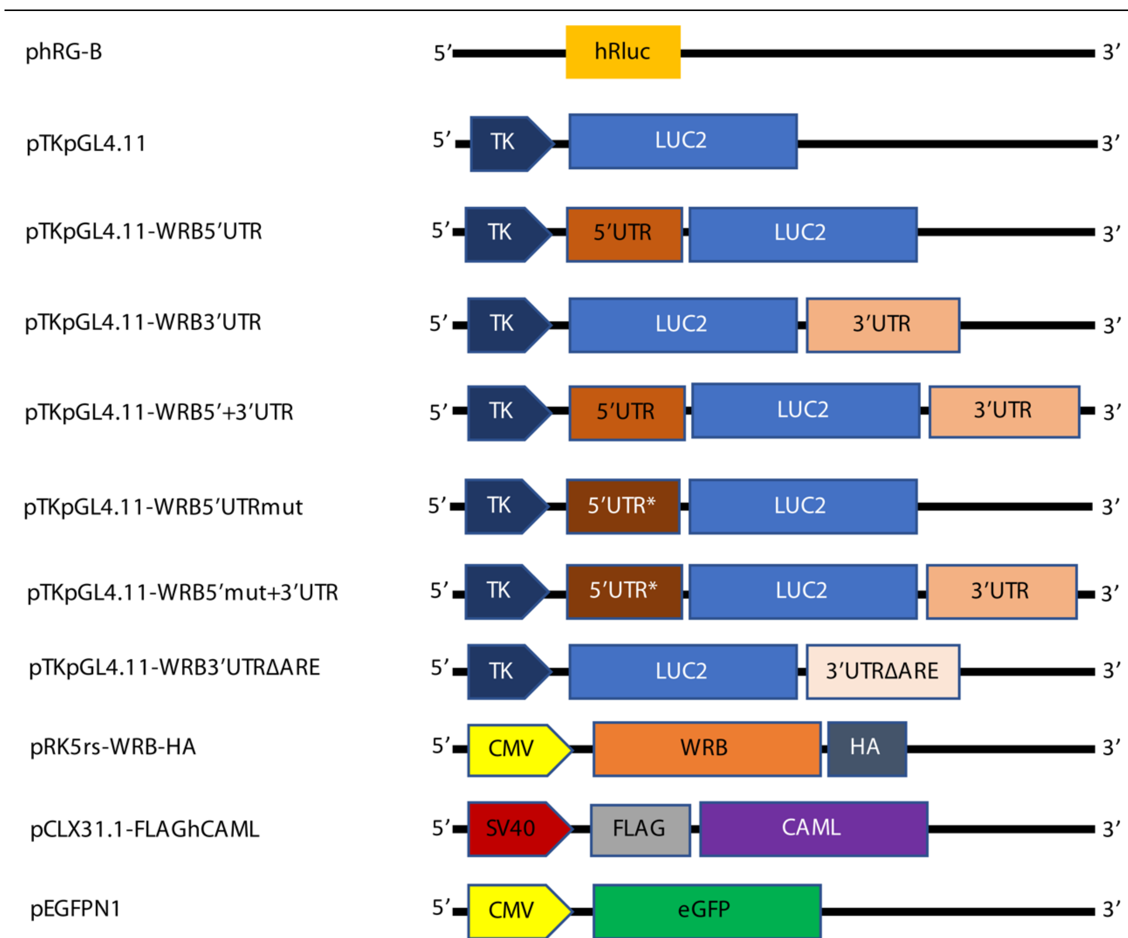

Fig. 3.3 - Representation of the constructs used in this study. See Section 3.2.1.10 for details.

two molecules, the quencher, strongly supresses the fluorescence emission of the reporter dye in a physical phenomenon called fluorescence resonance energy transfer (FRET). When in solution or bound to the target, the two molecules are near and FRET occurs, quenching the fluorescence of the reporter dye. However, during PCR using a primer pair specific for the target, both primers and probe anneal to the target and then, when the target sequence is amplified, the taq polymerase 5’ nuclease activity cleaves the probe separating the two molecules. When the two molecules are separated, FRET can no longer occur and the reporter dye emits a fluorescence signal. This approach is more specific because even if either the primers used or the probe present an off-target annealing, those off-targets will not be represented in the result, since for a sequence to be measured, a combination of both the primer and the probe annealing are needed.

In each case, after a certain number of amplification cycles, the fluorescence signal will increase sufficiently to be distinguishable from the background signal; this cycle is defined as the threshold cycle (C_t). The C_t is inversely proportional to the initial amount

of target, so it can be used to perform an absolute quantification of the number of template molecules present in the initial sample, by comparison with a standard curve composed of know amounts of target copies, or to perform a relative quantification between the template levels of two different samples.

The relative quantification between two samples is generally performed recurring to the $\Delta\Delta C_t$ method. In this method, the C_t for the gene of interest is adjusted in relation to the C_t of a normalizer gene (usually a housekeeping gene) in each sample. Then, one of the samples is used as calibrator (e.g., the t0 of a treatment or the scramble control in silencing) and the difference is usually represented by a fold difference ($2^{-\Delta\Delta Ct}$).

In this work, RT-qPCR was used to measure the relative levels of CAML and WRB transcripts using GAPDH as normalizer between samples, and t=0 or scramble samples as calibrators in transcript stability or effect of silence on transcript level experiments, respectively.

3.2.2.1 RNA extraction

RNA intended for reverse-transcription was extracted and purified from HeLa cells (prepared as described in Section 3.2.2) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Qiashreder spin columns (Qiagen) and RNase-free DNase set (Qiagen) were used for lysate homogenization and complete removal of genomic DNA, respectively. The procedure yielded generally ~30 µg of RNA that was stored at -80 ºC until use.

3.2.2.2 Reverse-transcription

For the quantification of RNA using qPCR, an essential step is the reverse-transcription of RNA into cDNA using a reverse transcriptase and a reverse transcription primer. Depending on the application, this can be achieved using different approaches, mainly by changing the kind of primer used. One can retrotranscribe total RNA using random hexamer primers, poly(A) RNA by using oligo(dT) primers, and specific RNAs using gene specific primers. Strategies for the reverse transcription of small non-coding RNAs such as miRNAs are also available.

In this work, total RNA extracted from HeLa cells was retrotranscribed using Random Hexamers (Euroclone) and SuperScript IV Reverse Transcriptase (Invitrogen). Reactions were carried out in a PTC-100 thermal cycler (Bio-Rad). For each sample, 1.5 μg of RNA was retrotranscribed following the manufacturer's protocol. The newly synthesised cDNA was either used immediately for qPCR or stored at -20 °C for at most a few days.

3.2.2.3 qPCR

Real-time PCR was performed in a QuantStudio 5 System thermocycler (Applied Biosystems) using TaqMan Gene Expression Assays (Applied Biosystems) with FAM-MGB dyes containing a probe and a primer pair, specifically targeted to CAML, WRB or GAPDH (Assay IDs: Hs00266143_m1; Hs00190294_m1 and Hs99999905_m1, respectively). The TaqMan Gene Expression Master Mix (Applied Biosystems) was used to provide the necessary reagents including the taq polymerase.

For each sample, 1 μL of cDNA was mixed with 1 μL of TaqMan Gene Expression Assay and 10 μL of TaqMan Gene Expression Master Mix in a 96-well plate in a final volume of 20 μL. Each amplification reaction was performed in triplicate and a non-template control, in which cDNA was replaced by water was done for each probe.

The amplification protocol was as follows: first a Hold Stage where samples were incubated at 50 °C for 2 minutes, followed by incubation at 95 °C for 10 minutes. Then, the PCR stage consisted of 40 cycles of an initial denaturation step of 15 seconds at 95 °C followed by a second elongation step of 1 minute at 60 °C.

C_t values were obtained using QuantStudio Design & Analysis Software (Life Technologies).

3.2.3 Rapid Amplification of cDNA Ends

The amplification of the 3' UTR of a transcript, of which the 3' end is not known or which, could present multiple polyadenylation variants, is not possible by standard PCR amplification, since in these cases it is not possible to design a specific primer for the 3' end. Amplification can be achieved using the Rapid Amplification of cDNA Ends

(RACE) method. 3' RACE takes advantage of the poly(A) tail present in mature mRNAs. In this procedure, mRNAs are reverse transcribed using an oligo-dT adapter primer that contains a poly(T) sequence followed by an adapter sequence. In this way, all polyadenylated mRNA is converted into cDNA. Then, this cDNA is used as template to amplify the target by PCR. In the first PCR, the sense primer is a gene specific primer while the antisense primer is designed to anneal with the sequence of the oligo-dT adapter. This PCR should yield the amplification of the target sequence. Often, however, to obtain more material or greater specificity, a second, nested PCR is needed. The amplification product can then be cloned as desired.

In this work, the FirstChoice RLM-RACE kit (Ambion) was used. Briefly, 1 μg of RNA extracted from HeLa cells was reversed transcribed using SuperScript IV Reverse Transcriptase and 3'RACE adapter for priming. The product of this reaction was then used as template for a first PCR using GoTaq® DNA Polymerase and primers 3 and 7 (see Table 3.1) for priming. The product of this reaction was then used as template for a second PCR using primers 8 and 9 (see Table 3.1) for priming.

The amplification products yielded in this procedure were then separated by agarose gel electrophoresis and purified, before being cloned into the pCRII-TOPO TA-cloning vector and sequenced.

3.2.4 Dual-luciferase reporter assay

Genetic reporter systems are tools commonly used to study gene expression. Their applications range from the study of receptor activity, transcription factors, intracellular signaling, protein folding, to mRNA regulation.

The Dual-Luciferase Reporter Assay System (Promega) applied in this work uses the firefly and the *Renilla* luciferases, measuring the activities of both luciferases sequentially in the same sample.

In this work cells, co-transfected, harvested and lysed as described in Section 3.1.3.2 were analyzed in 96-well white bottom plates, with the Dual-Luciferase Reporter Assay System following the manufacturer's protocol in a GloMax Discover (Promega) luminometer.

3.3 Biochemical methods

3.3.1 Immunoprecipitation and Co-immunoprecipitation

Co-immunoprecipitation (co-IP) is the most straightforward method to study protein-protein interaction. This method is based on the immunoprecipitation (IP) procedure. In a basic IP, an antibody, specific for the protein of interest, is used to bind to that protein in a solution that can have multiple proteins, for example a cell lysate. The antibodies are coupled to solid substrates, usually agarose or magnetic beads, either before or after incubation with the sample. The solid substrate is then used to collect the antibodies, which in turn collect the target proteins, allowing to wash-off the rest of the sample where all proteins that did not bind to the antibody remained. The protein can then either be eluted from the antibody and analysed or used in other applications. In an ideal, completely efficient IP reaction, all the target protein and that protein alone would be collected from the initial sample, however the efficiency of precipitation is influenced by various factors such as antibody binding affinity, wash stringency, among others.

In a co-IP reaction, the same procedure is used, but if the target protein, also referred to as bait, interacts with a second protein, that can be called prey, and if the interaction is maintained during the reaction, the prey will be "co-precipitated" together with the bait. However, co-precipitating the prey is not always easy due to the nature of the protein-protein interactions that can be weak or have low-affinity. So, the conditions in which the reaction is performed are crucial for a successful co-IP, particularly concerning the buffers chosen to lyse the cells and wash the beads. This is especially true when handling membrane proteins, due to their tendency to aggregate in solution. In these cases, a mild detergent must be used to keep the proteins soluble, but it cannot be strong enough to disrupt the protein-protein interaction.

In this work, the WRB/CAML complex was co-immunoprecipitated using either one of the two subunits as bait. The buffers used for the reaction contained Deoxy Big Chaps (DBC), since it had been demonstrated that both CAML and WRB are soluble and functional in the presence of this detergent (Colombo et al. 2016).

Hela cells, grown to confluency in a 28 cm^2 culture dish were washed twice with PBS before being harvested by scrapping in PBS. Cells were then pelleted by

centrifugation for 10 minutes at 800 g at 4 °C. The pellet was resuspended in hypotonic buffer (1 mM Tris-HCl pH7.5; 1 mM EDTA; 1 mM KCl; 1 mM NaCl) in the presence of protease inhibitors and incubated 10 minutes on ice to promote swelling of the cells by osmotic pressure. Swollen cells were homogenized by passing 50 times through a syringe needle (26Gx1/2”). Homogenized samples were then solubilized in a DBC containing buffer (0.6 % [w/v] DBC; 50 mM Hepes-K^+ pH7.4; 250 mM sorbitol; 70 mM potassium acetate; 5 mM EDTA; 2.5 mM magnesium acetate; protease inhibitor cocktail) and the nuclei were pelleted by centrifugation at 800 g for 10 minutes at 4 °C. The recovered supernatant, referred to as “post-nuclear supernatant” (PNS), was incubated for 1 hour at 30 °C

Equal aliquots of PNS were then used for co-IP reactions with either anti-CAML or anti-WRB antibodies, while one equal aliquot (Input) was immediately mixed with 0.5 volumes of DB 3X and boiled for 2 minutes at 100 °C. Each co-IP reaction was composed of PNS, a 1:1 protein G agarose slurry and 5 µg/mL of the antibody for the bait protein (either WRB or CAML antibody). A control co-IP reaction was performed in parallel, where the specific antibody was substituted with 5µg/mL of non-immune rabbit IgG (Sigma). The reactions were incubated overnight at 4 °C with mild agitation.

The next morning, the agarose beads were pelleted by centrifugation at 800 g for 5 minutes at 4 °C. The supernatant (Unbound) was recovered and immediately mixed with 0.5 volumes of DB 3X and boiled for 2 minutes at 100 °C.

The beads were washed, first with the same DBC containing buffer used to solubilize the samples and then with double-distilled H_2O. After washing, DB was added to the beads and the sample (Bound) was then boiled for 2 minutes at 100 °C.

Samples were analysed by western-blot. For this, the entire Bound samples and half of both the Input and Unbound samples (50%) were loaded on a 12.5% polyacrylamide gel. Only half of the Input and Unbound samples were analysed in order to obtain western blot bands within the range expected for those in the Bound sample to allow for a better comparison, considering that the immunoprecipitation process is frequently poorly efficient.

The bands corresponding to each protein were quantified, the values for those corresponding to the Input and Unbound samples were multiplied by two, and the IP efficiency was determined by two different methods.

Method 1:

$$IP_{Efficiency}(\%) = \frac{Signal_{Bound\ in\ IPx} - Signal_{Bound\ in\ IgG}}{(Signal_{Input})} \times 100$$

Method 2:

$$IP_{Efficiency}(\%) = \frac{(Signal_{Input} - Signal_{Unbound\ in\ IPx}) - (Signal_{Input} - Signal_{Unbound\ in\ IgG})}{Signal_{Input}} \times 100$$

Method 1 considers the signal of the protein bound to the beads, from which is subtracted the signal obtained in the IgG sample to correct for unspecific binding. However, this value may be underestimated due to protein loss during washes. Furthermore, the lanes containing the bound fraction have a much higher background, which makes the quantification more difficult.

Method 2 on the other hand considers the signal of the protein present in the Unbound sample, and subtracting it from the signal in the input, indirectly estimates the amount of protein that was bound by the antibody; the same calculation applied to the IgG sample is subtracted from this first value to correct for unspecific binding. This method, even if less direct, could be more precise since it does not suffer from the variability of the washing procedure.

To compare the co-IP efficiency obtained with the two baits (CAML and WRB), the co-IP efficiencies were further normalized to the IP efficiency of the protein used as bait.

3.3.2 SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE), is a technique widely used for the separation of proteins. In the absence of sodium dodecyl sulfate (SDS) proteins are separated according to their electrophoretic mobility which is related to the proteins'

higher order structural organization, the presence of post-translational modifications, overall surface charge and molecular weight. Although the analysis of these factors may be important for some applications, often one is interested in separating the proteins based solely on their molecular weight. For this, one can use SDS-PAGE.

In this work, protein from cell lysates prepared as described in Section 3.2.3.1 or from co-IP reactions prepared as described in Section 3.4.1 were separated in polyacrylamide gels (Stacking gel: 4 % [w/v] Acrylamide; 0.125 M Tris-HCl pH6.8; 0.1 % [w/v] SDS; 0.13 % [v/v] Temed; 0.065 % [w/v] APS; Resolving gel: 12.5% [w/v] Acrylamide; 0.75 M Tris-HCl pH 8.6; 0.1 % [w/v] SDS; 0.06 % [v/v] Temed; 0.03 % [w/v] APS) emerged in a glycine buffer (200 mM glycine; 25 mM Tris) with 0.1% SDS under constant voltage (120V) before being used for western blot.

3.3.3 Western-blot

After being separated by SDS-PAGE, proteins can be transferred onto an immobilizing membrane.

In this work, proteins separated by SDS-PAGE were transferred into nitrocellulose membranes in the presence of a tris-glycine buffer solution (200 mM glycine; 25 mM tris; 20 % [v/v] methanol) by the application of a constant electric current for a determined amount of time, either 1 A for 3 hours or 0.16 A overnight.

The membranes were then blocked with blocking buffer (20 mM tris; 150 mM NaCl; 5 % [w/v] skim milk; 0.1 % [v/v] tween-20) and incubated with the antibodies listed in Table 3.2 using the described dilutions, followed by incubation with the respective secondary antibodies conjugated with fluorescent fluorophores. The membranes were then scanned with the Odyssey CLx Infrared Imaging System (LI-COR) and the fluorescence signal from the bands was quantified using Image Studio software (LI-COR).

Table 3.2 - Antibodies used in this work for westernblot.

Type	Antibody	Description
I°	α-TUB	Monoclonal antibody raised in mouse against α-tubulin; clone B-5-1-2 (Sigma)
I°	α-WRB	Affinity purified, MBP depleted, polyclonal antibody raised in rabbit against WRBcc (Synaptic Systems)
I°	α-CAML	Polyclonal antibody raised in rabbit against CAML (gift from Richard Bram)
I°	α-GFP	Polyclonal antibody raised in rabbit against GFP (MBL)
II°	α-mouse IR680	Secondary antibody raised in goat against mouse IgG; conjugated with IRDye 680 (Li-COR Biosciences)
II°	α-rabbit IR800	Secondary antibody raised in goat against rabbit IgG; conjugated with IRDye 800 (Li-COR Biosciences)

4
RESULTS

4.1 The stability of the mRNA of the two subunits

Previous work has shown a strong destabilization of WRB mRNA upon CAML silencing (Colombo et al. 2016). This led to the formulation of the hypothesis that the transcripts of WRB and CAML could physically interact in such a way that the elimination of CAML mRNA by RNA interference could promote the concomitant degradation of WRB. The veracity of this hypothesis implied that, also in normal conditions, without undergoing silencing treatments, the two transcripts would show parallel degradation rates.

To test this hypothesis, we treated cells with the transcription inhibitor DRB, and determined the time course of WRB and CAML diminution by qPCR. As shown in Fig. 4.1, the two transcripts have very different decay rates: CAML mRNA decayed with a half-life of 4h, while WRB mRNA was quite stable, with an estimated half-life of ~20h (beyond the timescale of the experiment).

This result indicates that WRB mRNA stability does not depend on the presence of CAML transcript, and suggests instead that CAML protein, which is depleted in siRNA experiments (Colombo et al. 2016), is required for the stabilization effect.

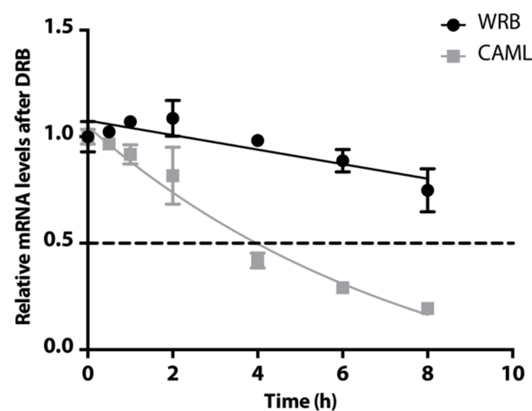

Fig. 4.4 – WRB transcript is more stable than CAML mRNA. HeLa cells were treated with DRB for the indicated times. The RNA extracted from cell lysates of each time point was subjected to retrotranscription and qPCR probing for the transcripts of WRB, CAML and GAPDH. Fold difference, determined using time 0 as experimental calibrator is shown in the graph as mean ± S.D. of triplicate measures of two independent experiments.

4.2 The effect of CAML silencing on the expression of a WRB transcript that lacks the endogenous UTRs

As already elucidated in the introduction, the UTRs are strongly involved in the post-transcriptional mechanisms that regulate, among other things, mRNA translation and stability. To investigate if the UTRs of the WRB mRNA could play a part in the destabilization of WRB transcript caused by CAML silencing, a WRB tagged construct (WRB-HA), lacking the endogenous UTRs was expressed in CAML silenced cells (siCAML).

Analysis of the protein levels, in comparison to a control (cells treated with scramble siRNA - siSCR), showed that, while the endogenous WRB is depleted upon CAML silencing (as seen before), the same is not true for WRB-HA (Fig. 4.2). The expression of WRB-HA was not affected by the silencing of CAML. This result suggests that the WRB mRNA UTRs could indeed be playing a role in the observed effect of CAML silencing.

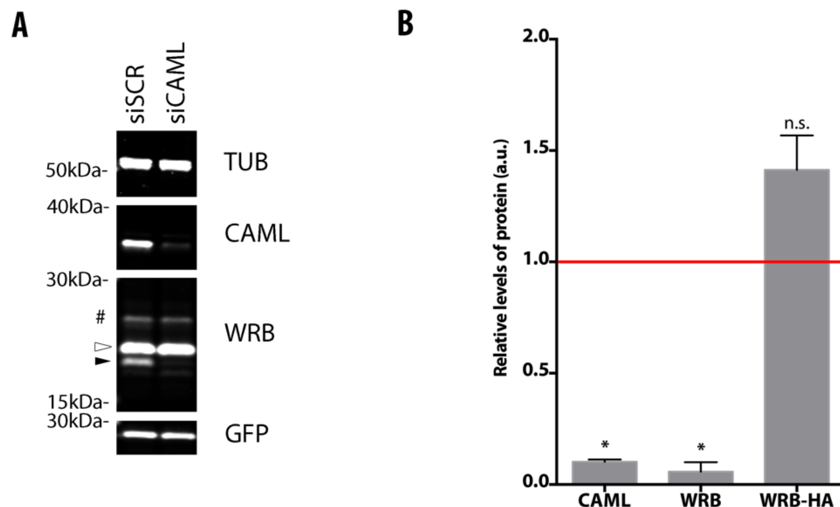

Fig. 4.2 – CAML silencing reduces the levels of endogenous WRB but not those of WRB-HA expressed from a transcript lacking the endogenous UTRs. HeLa cells were treated with either a scramble (siSCR) or a CAML targeted siRNA (siCAML) for 48h and then transfected with the pRK5rs-WRB-HA plasmid that codes for WRB fused with a HA tag, and lacks WRB endogenous UTRs, together with a GFP-encoding plasmid, as reporter for transfection efficiency. (**A**) 24h after transfection cells were lysed and equal aliquots of lysate were analysed on SDS-PAGE followed by western blotting with the indicated antibodies. The HA tagged form of WRB runs slower on SDS-PAGE and is indicated with a white arrowhead while the endogenous form of WRB, which runs faster, is indicated with a black arrowhead. The hashtag (#) indicates an unidentified band produced by the expression of the pRK5rs-WRB-HA plasmid. (**B**) The intensities of the bands corresponding to each protein were quantified and Tubulin and GFP were used as normalizers for endogenous and transfected proteins, respectively. The fold-change with respect to control cells is shown in the graph as mean ± S.D. of three independent experiments each analysed in duplicate; * indicates $p<0.05$ for differences between silenced and control cells for each protein analysed by multiple t-test, with Holm-Sidak method for multiple comparison correction, after logarithmic transformation of the normalized band intensities.

4.3 The WRB 3' UTR

The GenBank annotation for WRB (Gene ID: 7485), predicts six different mRNA isoforms resulting from alternative splicing or promoters; isoform 1 is the one that codes for full length WRB. The isoforms vary at the 5' end, with two of the isoforms potentially coding for truncated forms of WRB, that lack the N-terminal, membrane-associating portion. The relevance of these isoforms is yet to be investigated.

As stated in the introduction, the 3'UTR of a mRNA is known to be a regulatory hub, where most binding sites for RBPs that regulate mRNA stability are present. Considering this, the sequence of the 3'UTR of WRB mRNA was analyzed in greater detail using 3' RACE to look for potential variations to the annotated sequence. This technique uses a oligodT containing backward primer, and a gene specific forward primer, so as to obtain amplicons of all polyadenylated 3'UTRs of the investigated transcript (see Methods).

Surprisingly, this analysis revealed a heterogeneous population of polyadenylated 3'UTRs (Fig. 4.3-B). The main 3' RACE product populations (bands a, b and c) were cloned into a pCRII-TOPO cloning vector and some of the resulting colonies were sequenced. Sequence alignment of the 3'RACE products with the annotated WRB transcript isoform 1 (and with all other isoforms – not shown) revealed that all sequenced transcripts aligned almost perfectly at the 5' end of the annotated 3' UTR, but some were shorter in length (Fig. 4.3-C; D).

It must be noted that some transcripts originating from molecular cloning of different 3'RACE product populations are identical. This is probably due to inefficient electrophoretic separation that resulted in cross-contamination of the different populations.

Furthermore, a bioinformatic analysis of the full length WRB 3'UTR revealed the presence of three putative ARE motifs. These motifs are known to be important for the regulation of the stability of mRNA. The position of the three AREs is represented in pink on the cartoon and in the sequence (Fig. 4.3-D).

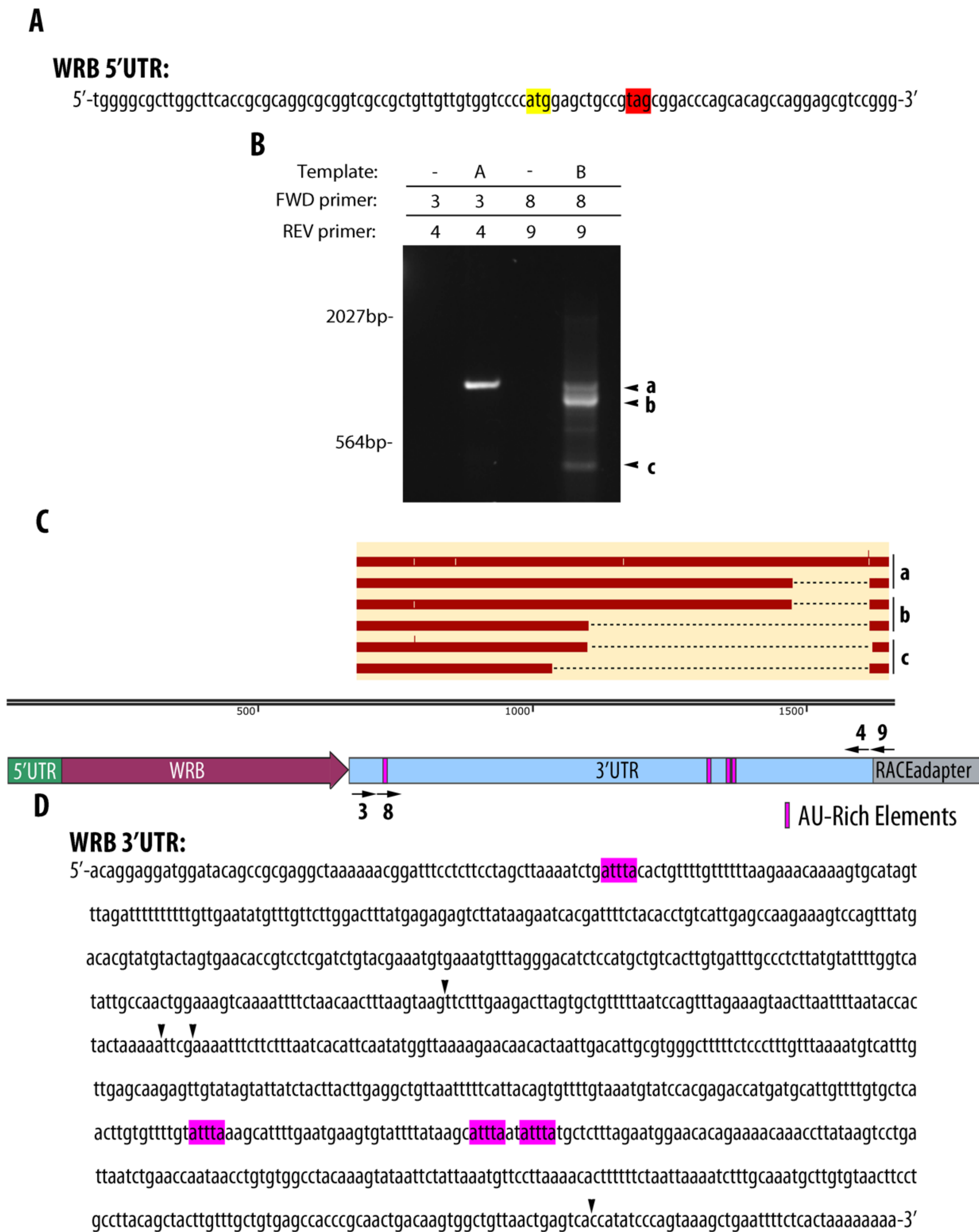

Fig. 4.3 – WRB transcript has an upstream ATG and is present in multiple isoforms that vary in 3'UTR length. (**A**) The deposited sequence for WRB 5'UTR (NM_004627.5); the presence of an upstream ATG is highlighted in yellow and in red is highlighted the in frame STOP codon. The main ORF ATG is represented in capital letters. (**B**) RNA extracted from HeLa cell lysates was retrotranscribed using either random primers or a 3'RACE adaptor to generate templates A and B, respectively. The two templates were then submitted to PCR amplification with specific primer pairs: primers 3, 4, 8 and 9, indicated in panel B. The different amplicons (a, b and c) obtained from Template B were isolated and cloned into a pCRII-TOPO cloning vector. Two constructs resulting from the cloning of each amplicon were sequenced. (**C**) Sequences from each of the produced constructs were aligned with the deposited sequence for WRB transcript, to which was added the 3'RACE adaptor sequence (schematized at the bottom of the panel). The matching regions of the sequence are shown in full red while the missing portions are represented by dotted lines. In the scheme are also represented (in pink) the four AUUUA pentamers within the 3'UTR, which are hallmarks of AREs. (**D**) The deposited sequence of WRB 3'UTR. Arrowheads indicate the positions of premature termination, with resulting truncated UTRs, identified by 3'RACE. The ARE pentamers represented in (**C**) are highlighted in pink.

4.4 The role of the UTRs in the regulation of WRB mRNA expression and on its CAML-sensitive stability

To investigate the importance of the WRB UTRs on its expression and on the CAML-sensitive stability, a dual-luciferase reporter system was used, with luciferase constructs carrying both WRB 5' and 3' UTRs.

Regarding the 5'UTR, bioinformatic analysis revealed that WRB has an upstream ATG of unknown consequence (Fig. 4.3-A). To analyse the importance of this upstream ATG, a set of luciferase constructs with this ATG mutated were designed. As described in the introduction, upstream initiation codons followed by an in frame stop codon may decrease the efficiency of translation of the main ORF. The upstream ATG of WRB transcript partially conforms to the Kozak consensus sequence, and could thus be used for translation. This would result in interruption of the scanning process, and consequent failure of the small ribosomal subunit-containing initiation complex to reach the subsequent AUG at the start of the main ORF of the transcript.

The 3'UTR sequence used was that annotated for isoform 1 of the WRB transcript, corresponding to our longest 3'RACE product.

First, the effect of each UTR, alone or combined, on the expression of luciferase was studied (Fig 4.4-A). Constructs carrying the 5'UTR were significantly less expressed (40% reduction) than the control. However, when the upstream ATG was mutated, the expression was restored to control levels. The 3'UTR, either alone or in combination with the 5'UTR did not have any effect on luciferase.

Next, the sensitivity of the different UTRs to CAML silencing was tested by expressing the various luciferase constructs in CAML silenced cells. To begin, it must be stated that treatment with different siRNAs influenced the expression of the luciferase backbone, reducing its expression by ~0.5 fold (see figure legend). The causes for this effect are not clear. Because of this, the analysis was performed on differences in fold-variation of luciferase expression with respect to the backbone control, for CAML silenced or control cells.

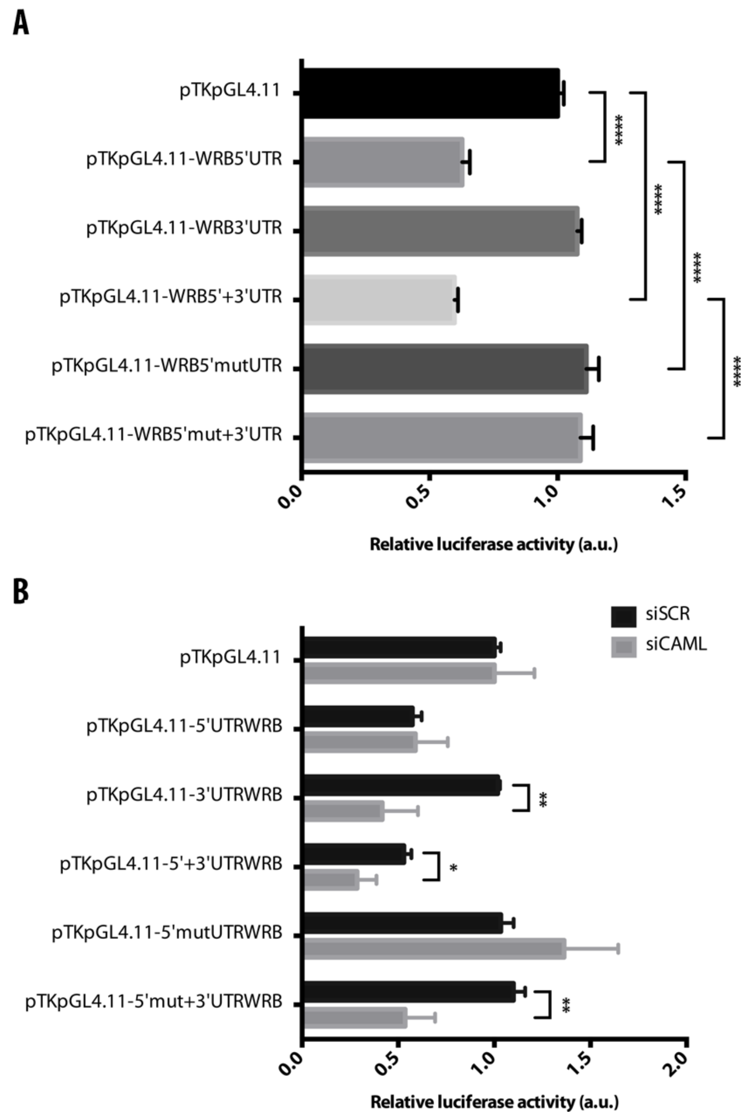

Fig. 4.5 - The UTRs regulate the expression of WRB and the 3'UTR is responsible for the CAML-sensitive stability. (**A**) Luciferase constructs driven by the TK promoter and fused with WRB's UTRs were transiently transfected in HeLa cells together with a Renilla control plasmid. **** indicates statistical significant differences between the indicated groups, analysed by unmatched analysis of variance and Tukey post-hoc test, $F (5,12) = 149.9$, $p<0.0001$. (**B**) The same Luciferase constructs were transiently transfected together with a Renilla control plasmid in HeLa cells previously treated with either a scramble (siSCR) or a CAML-targeted (siCAML) siRNA for 48h. siRNA treatment (independently of the siRNA target) had an off-target effect on the luciferase backbone, causing a 59% reduction in its activity with respect to scramble. Considering this, the analysis of the data was performed as fold-variation in Luciferase activity of each construct with respect to the backbone for CAML silenced or control samples. * and ** indicates statistically significant differences, in the fold-variation, with respect to the backbone, between the indicated groups, analysed by unpaired Student's t-test ($p<0.05$ and $p<0.01$ respectively). For both panels, the Luciferase activity, determined 24h after transfection, was measured as a function of luciferase luminescence signal normalized to Renilla luminescence signal. Values are expressed as mean ± S.D. of fold-variation of three independent experiments, each performed in duplicate.

As shown in Fig. 4.4-B, the expression of 5'UTR constructs did not change significantly in response to CAML silencing, irrespectively of the presence or not of the upstream ATG. By contrast, constructs carrying the WRB 3'UTR were significantly less expressed with respect to the backbone in CAML silenced cells (~0.5 fold). This reduced expression was observed in all the constructs containing the 3'UTR, regardless of whether it was alone or in combination with the wt or mutated 5'UTR.

As indicated above, the WRB 3'UTR, here shown to be responsible for the sensitivity of WRB to the silencing of CAML, has three putative ARE motifs. ARE motifs have been proposed to be very important for the regulation of mRNA stability. Thus, considering the presence of these putative AREs (Fig 4.3-C; D), a new luciferase construct containing a portion of the 3'UTR that excluded the AREs (Fig. 4.5-A) was designed. As shown in Fig. 4.5-B, expression of this construct was equally sensitive to CAML silencing as the one containing the full-length 3'UTR. Thus, the CAML-sensitive stability of WRB mRNA is not mediated by the ARE sites.

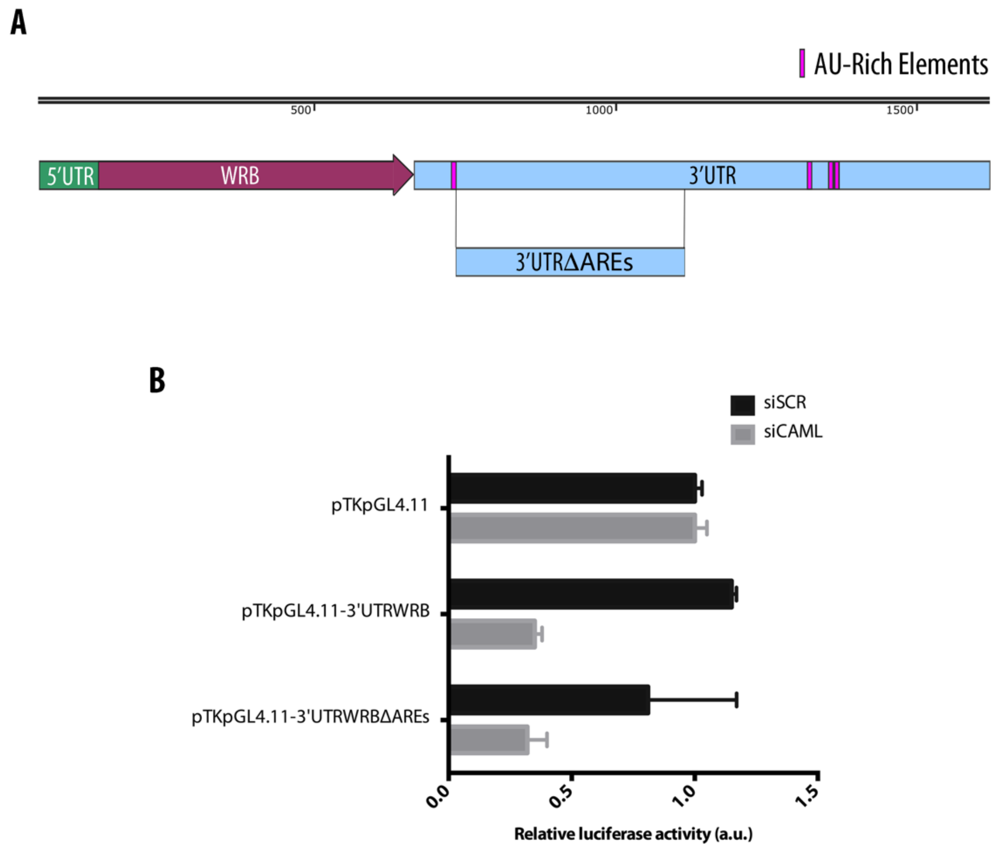

Fig. 4.6 – The stabilization of the WRB mRNA by CAML is not ARE-mediated. (**A**) Schematic representation of the AREless region of the WRB transcript cloned into pTKpGL4.11 (see Section 3.2.1.10 for details). (**B**) Luciferase constructs, with either full length or an AREless portion of WRB 3'UTR, were transiently transfected together with a Renilla control plasmid in HeLa cells previously treated with either a scramble (siSCR) or a CAML-targeted siRNA (siCAML) for 48h. Luciferase activity, determined 24h after transfection, was measured as a function of Luciferase to Renilla luminescence signals. Values are expressed as mean ± S.D. of fold-variation in Luciferase activity with respect to the Luciferase backbone of one experiment performed in duplicate.

4.5 Characterization of the WRB-CAML interaction

In yeast, Get1 and 2 form a complex, the two subunits are expressed in approximate 1:1 ratio, and deletion of one subunit causes depletion of the other one (Mariappan et al. 2011). This result is consistent with the idea that the two subunits form a 1:1 complex, and that each subunit is required for its partner's stability (see Introduction). In mammals, however, the situation appears to be more complex. On the one hand, WRB and CAML form a complex (Yamamoto and Sakisaka 2012; Colombo et al. 2016), and the overexpressed proteins are unstable in yeast unless expressed together (Vilardi et al. 2014), suggesting that the relationship between CAML and WRB is similar to the one between Get1 and 2. On the other hand, however, a study from my laboratory (Colombo et al. 2016), based on quantitative immunoblotting reported that in mammalian cells CAML is present in ~5-fold excess over WRB. This finding fits well with the multiple reported functions of CAML (Shing et al. 2017), but is difficult to reconcile with the effect of WRB depletion on CAML expression levels.

To reconcile these previous contradictory findings, I considered the possibility that, in mammals, WRB and CAML might form a super-complex, in which CAML would be in molar excess over WRB. The previous study (Colombo et al. 2016) had demonstrated that WRB is quantitatively associated with CAML, but the reverse, whether all CAML is associated with WRB, had not been tested.

To investigate the stoichiometry of the complex, I carried out co-immunoprecipitation experiments with anti-WRB and anti-CAML antibodies. If all the excess CAML were in complex with WRB, one would expect it to co-precipitate with WRB with the same efficiency as the reverse precipitation (WRB with CAML).

Cell lysates were prepared with a mild detergent (DBC) that preserves the WRB-CAML complex (Colombo et al. 2016). As shown in Fig. 4.6, using WRB as bait, also a fraction of CAML was co-precipitated (Fig. 4.6-A; B; D). In parallel, an antibody raised against CAML could immunoprecipitate native CAML itself while pulling down together a considerable fraction of WRB.

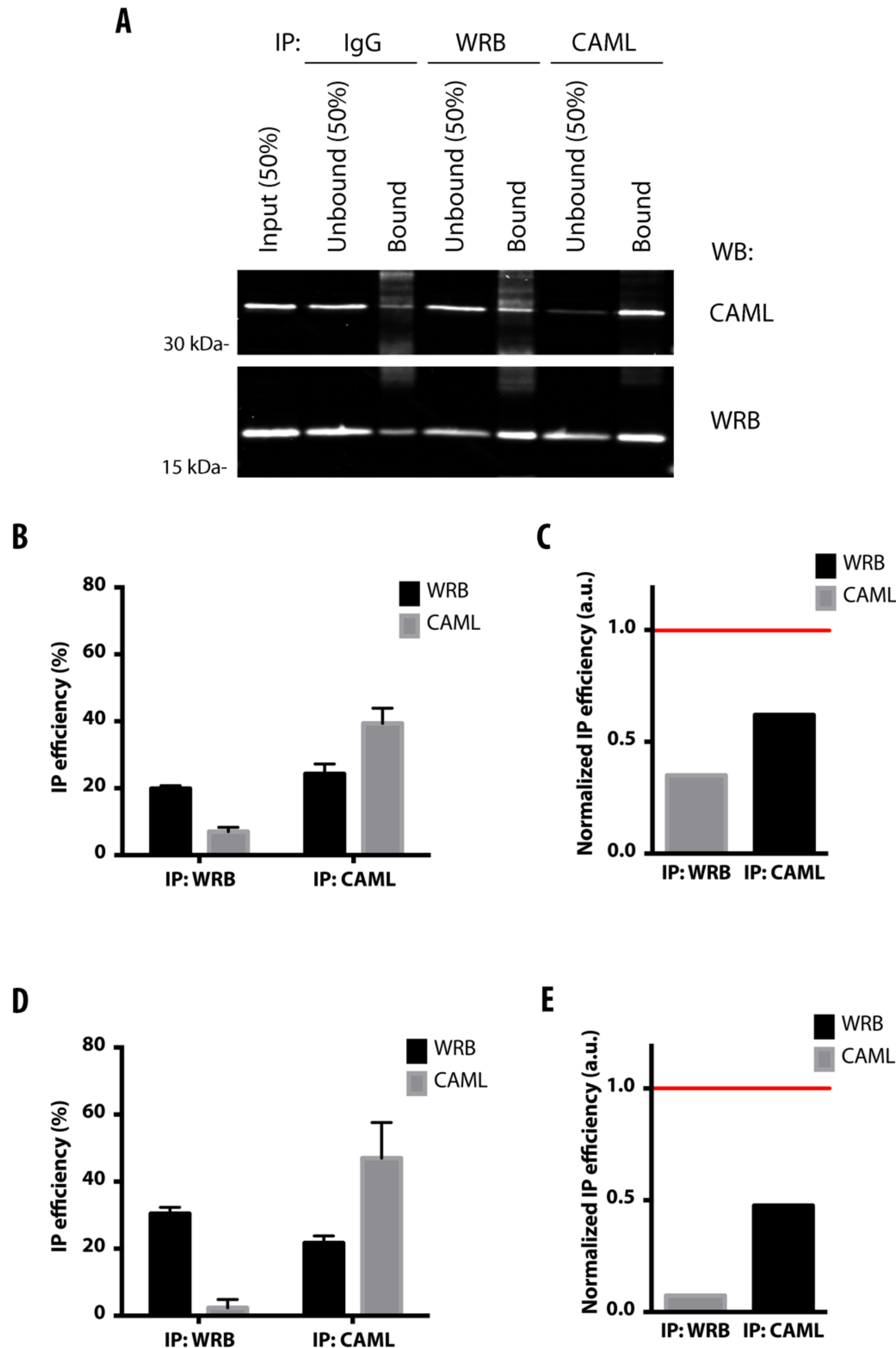

Fig. 4.7 – Co-immunoprecipitation of the WRB/CAML complex reveals the presence of a WRB-free population of CAML. (**A**) HeLa cells were mechanically lysed in a DBC containing buffer. The post-nuclear supernatant (PNS) obtained was used for co-immunoprecipitation with either anti-WRB or anti-CAML antibodies, rabbit IgG was used as control. Input, bound and unbound fractions were then analyzed on SDS-PAGE followed by western blotting with the indicated antibodies. (**B**) The bands corresponding to each protein present in the Bound fraction were quantified and the percentage of IP efficiency, quantified using "Method 1" (see Section 3.3.1). (**D**) The bands corresponding to each protein present in the Unbound fraction were quantified and the percentage of IP efficiency, quantified using "Method 2" (see Section 3.3.1). Percentage of IP efficiency is shown in the graph as mean ± half-range of two independent experiments. (**C and E**) The means of the percentage of immunoprecipitated protein obtained from each method were normalized to the percentage of precipitated bait.

The interpretation of the results was not straightforward, because, while the anti-CAML antibody was fairly efficient in immunoprecipitating its cognate antigen, the anti-WRB antibody was much less proficient in immunoprecipitation (Fig. 4.6-A; B).

As stated in the Methods section, two different procedures were used to quantify the IP and co-IP efficiency. The first one (Fig. 4.6-B) uses the amount of protein found in the "Bound" fraction, while the second one (Fig. 4.6-D) uses the amount of protein found in the "Unbound" fraction. Although some differences in the efficiency of the various IPs were observed for the different methods, the overall result was rather similar. To better appreciate the result and considering the variability in antibody efficiency, the co-IP efficiency of each prey was normalized to the IP efficiency of the respective bait (Fig. 4.6-C; E). In the hypothesis that all WRB interacts with all CAML, the relative efficiency of co-IP should be similar, independently on the bait being used. However, that was not observed. The relative amount of CAML that was pulled down with WRB was lower than the relative amount of WRB that was pulled down with CAML. This result confirms the molar excess of CAML over WRB reported by Colombo et al. (2016) and furthermore indicates the existence of a population of CAML that is not bound to WRB.

4.6 The effect of WRB silencing on CAML transcript stability

Although the silencing of CAML was seen to provoke a great destabilization of the WRB transcript, the reverse situation showed that CAML transcript levels were only slightly reduced upon WRB silencing (Colombo et al. 2016). To further investigate the effect of WRB silencing on CAML at both protein and transcript levels, I investigated whether the stability of the CAML transcript is affected by WRB depletion, as occurs in the reverse case. The results are shown in Fig. 4.7.

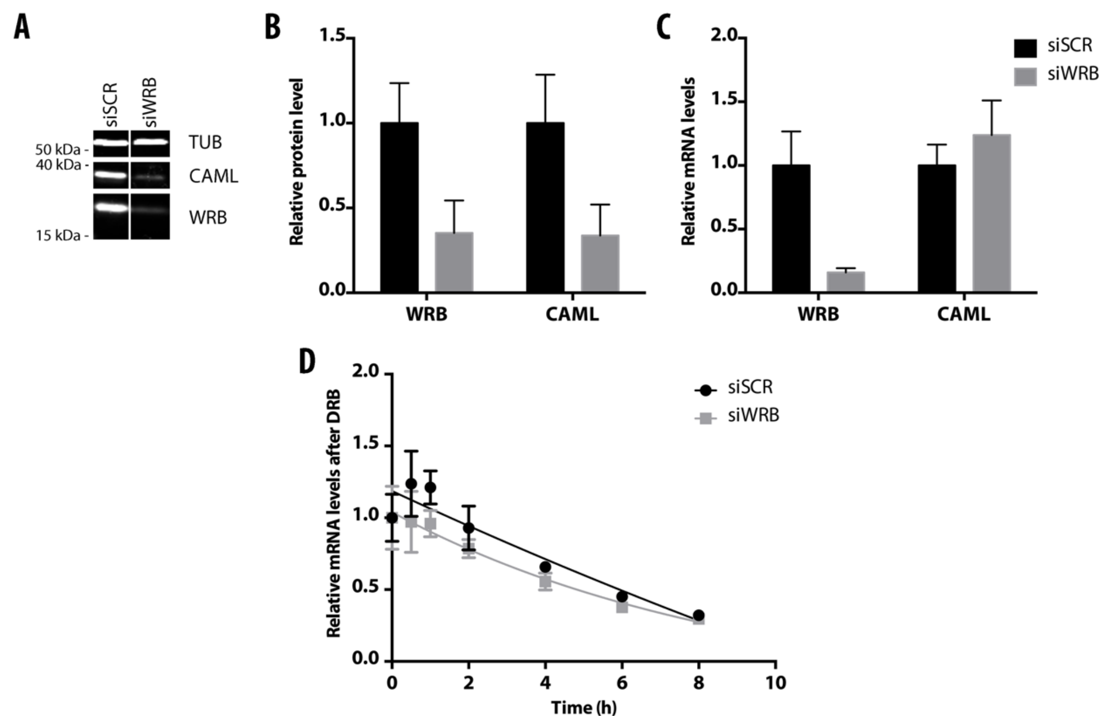

Fig. 4.8 – The silencing of WRB depletes CAML protein but does not affect either the amount or the stability of the transcript. HeLa cells were treated with either a control scramble (siSCR) or a WRB targeted siRNA (siWRB) for 68h. (**A**) Cells were lysed and equal aliquots of lysate were analysed by SDS-PAGE followed by western blotting with the indicated antibodies. (**B**) The band intensity of each protein was quantified and Tubulin was used as endogenous normalizer. The fold-variation of WRB silenced cells with respect to control is shown in the graph as mean ± S.D of two independent experiments analysed in duplicate. (**C**) RNA extracted from WRB silenced and control cells was subjected to retrotranscription and qPCR probing for WRB, CAML and GAPDH mRNA. Fold difference was determined with siSCR as experimental calibrator for each probe. The result is shown in the graph as mean ± S.D of triplicate measures of two independent experiments. (**D**) After silencing, cells were treated with DRB (60μM) for the indicated times. The RNA extracted from cell lysates of each time point was subjected to retrotranscription and qPCR probing for the transcripts of CAML and GAPDH. Fold-variation, determined using time 0 (cells not exposed to DRB) as experimental calibrator, is shown in the graph as mean ± S.D. of triplicate measures of two independent experiments.

Confirming the results of Colombo et al. (2016), the silencing of WRB, greatly reduced the levels of CAML protein (Fig. 4.7-A; B) while not having any effect of the transcript levels (Fig. 4.7-C). To understand if the silencing of WRB had any effect on the stability of CAML mRNA, the levels of CAML transcript were analysed over time after inhibition of transcription as described for the experiments of Fig. 4.1 (Fig. 4.7-D). The results show that there is no significant effect of WRB silencing on the overall stability of CAML transcript. In control cells (siSCR), the half-life of CAML transcript was ~6h, close to that found in control cells (Fig. 4.1). This value remained unchanged in cells silenced for WRB. This result indicates that the depletion of CAML protein upon WRB silencing is not due to the destabilization of its transcript, as is to be the case for the reverse situation.

The reason for the depletion of CAML protein upon WRB silencing remained elusive. It was hypothesised that it could be either due to a loss of CAML protein stability or to a potential interaction of WRB with some translational regulation factor present in CAML UTRs. To investigate this, a CAML tagged construct (FLAGCAML), that lacked the endogenous CAML UTRs, was expressed in WRB silenced cells. The results show that, although not statistically significant, there was a 40% reduction in the expression of FLAGCAML when WRB was silenced (Fig. 4.8), suggesting that WRB depletion interferes with CAML protein stability.

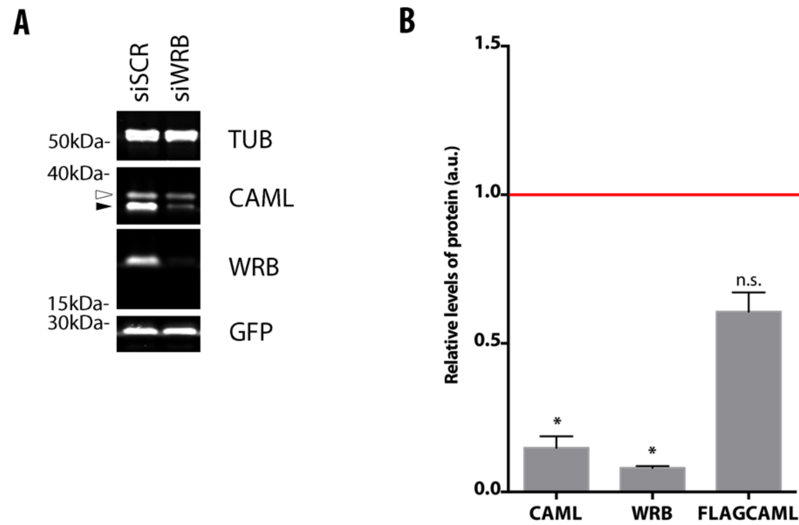

Fig. 4.9 – The effect of WRB silencing on CAML is independent of CALM's UTRs. HeLa cells were treated with either a control scramble (siSCR) or a WRB targeted siRNA (siWRB) for 72h, after which they were transfected with the pCLX31.1-FLAGhCAML plasmid, which codes for a FLAG tagged form of CAML and lacks the endogenous UTRs, together with a plasmid encoding GFP, as reporter for transfection efficiency. (**A**) After 24h of transfection cells were lysed and equal aliquots of lysate were analysed on SDS-PAGE followed by western blotting with the indicated antibodies. FLAG tagged CAML which migrates more slowly on SDS-PAGE is indicated with a white arrowhead while endogenous CAML is indicated with a black arrowhead. (**B**) The intensity of the blotted bands was quantified; Tubulin and GFP were used for normalization of endogenous and transfected proteins, respectively. The fold-change with respect to siSCR treated cells is shown in the graph as mean ± S.D. of three independent experiments, each analysed in duplicate; * indicates $p<0.05$ for differences between silenced and control cells for each indicated protein analysed by multiple t-test, with Holm-Sidak method for multiple comparison correction, after logarithmic transformation of normalized band intensities. FLAGCAML levels are reduced by WRB silencing, although the difference did not reach statistical significance.

5
DISCUSSION

In this thesis, I have investigated novel mechanisms by which two subunits of a protein complex, the TRC40 receptor, influence each other's expression in mammalian cells. My results reveal previously undescribed modalities of subunit co-regulation, which may likely be relevant also for other complexes, including oligomeric receptors and ion channels. I discuss in turn WRB regulation by CAML, and, *viceversa*, CAML regulation by WRB.

5.1 How CAML depletion affects WRB levels

5.1.1 WRB transcript stabilization is mediated by CAML protein and not CAML transcript

My research had as starting point the unexpected observation by Colombo et al. (2016) that CAML depletion causes instability of the WRB transcript. Since the siRNA technique causes the degradation of the target mRNA with consequent depletion of the protein product, my first question was whether the stabilization of the WRB transcript by CAML was due to the CAML transcript or to the CAML protein. Indeed, a recent study demonstrated that the mRNAs encoding two subunits of a heteromeric K^+-channel expressed in cardiomyocytes are physically associated, and each one influences the stability of the other one (Liu et al. 2016). We reasoned, that if this were the case also for the WRB-CAML pair, the two transcripts should show parallel degradation kinetics, revealed after a block of transcription by DRB. We found instead that the two transcripts had distinctly different stabilities, such that the one encoding WRB were hardly affected by an ~80% reduction in CAML transcript at 8 h of treatment with DRB. This result indicates that the destabilizing effect of CAML silencing on WRB transcript is mediated by CAML protein.

5.1.2 WRB transcript stabilization by CAML is mediated by the 3' UTR.

As described in the Introduction, a frequently observed phenomenon in the biogenesis of protein oligomers is the association of one subunit with the nascent polypeptide chain of other subunits of the complex (Duncan and Mata 2011). This interaction allows for efficient assembly of the complex, but could also affect the stability of the captured mRNA. On the other hand, it is well known that the UTRs, especially the 3'UTR, play a key role in determining mRNA stability. To investigate whether CAML interacts with WRB nascent chains or whether its effect is mediated by interactions of the UTRs, I expressed a tagged WRB construct that is translated from a transcript lacking the UTRs of the endogenous WRB transcript. I found that the levels of transfected tagged WRB were not affected by CAML silencing, strongly suggesting that the WRB UTRs underlie instability of the transcript in the absence of CAML. This conclusion was confirmed by my experiments with luciferase constructs carried out in CAML-depleted or control cells. Indeed, the 3'UTR reduced luciferase expression when CAML was depleted, while it had no effect in control cells. Furthermore, the 3' UTR was not only necessary, but also sufficient for this effect. Indeed, while the 5' UTR also reduced luciferase expression, this effect, which was due to the upstream AUG, was insensitive to the presence or absence of CAML.

5.1.3 AU- rich elements (AREs).

The above results confirmed that the coding sequence of the WRB transcript is not involved in the CAML effect and demonstrated that the 3'UTR is the region of the mRNA that underlies the instability of the transcript in the absence of CAML. My next step was to identify the region within the 3'UTR responsible for the instability and for the CAML-dependent stabilization. Upon analysis of the 3'UTR, I noticed three possible Class I ARE motifs; these are defined as containing 1-3 interspersed copies of the pentamer AUUUA surrounded by U-rich regions (Wu and Brewer 2012). Analysis of the WRB mRNA 3'UTR using the AREsite2 software, identified three potential AREs, two between position 655 and 703, at 247 nucleotides from the polyA, and one upstream sequence at position 63-68. I considered these three sequences as candidate mediators of the observed phenomenon. Here I give a brief summary of the features of these key

regulatory elements.

As briefly mentioned in the introduction (Section 1.4), ARE motifs are 3'UTR elements that are the target of several RBPs, composing a complex regulatory network that affects mRNA localisation, translation and stability (review: Wu and Brewer 2012). The complexity of the ARE-mediated regulation is demonstrated by several reports indicating that multiple proteins can bind to these motifs, competing between each other to regulate stability and/or translation and also the interaction of miRNAs with the target mRNAs.

RBPs bind to ARE motifs through diverse domains including RNA-recognition motifs (RRM), CCCH tandem zinc fingers and K-homology domains (KH). Moreover, a single protein can have multiple motifs, which provide them with the ability to simultaneously bind to multiple targets or multiple sites in the same target. To date, over twenty ARE-binding proteins (ARE-BP) have been identified, some of them with potentially redundant functions and other with contradictory reports on how they affect the target mRNA (Shen and Malter 2015). Some of the best characterized ARE-BPs are AUF1, TTP, TIA-1, TIAR, KSRP and HuR. These ARE-BPs are often catalogued based on their most common effect on target mRNA: TTP, KSRP and AUF1 (in an isoform dependent way) are usually reported as promoting mRNA degradation; HuR and AUF1 (again, depending on the isoform) are often referred to as mRNA stabilizers; TIA-1 and TIAR are said to be translational repressors (Khabar 2017). However, this classification is not always straightforward, and the same ARE-BP can - depending on target mRNA, type of stimulus, or environmental cues - present opposite behaviours regarding target modulation. These contrasting functions for singular ARE-BPs may be due to the fact that ARE-BPs may present a high level of crosstalk with another class of mRNA regulatory factors, miRNAs (Ho and Marsden 2014; Plass et al. 2017). This crosstalk is thought to be responsible for the complex, often contradictory, regulation of target mRNA fate by AREs. For instance, it has been suggested that a single ARE-BP can either compete with miRNAs for binding (Simone and Keene 2013) or cooperate with miRNAs to stabilize or degrade a target mRNA (Sharma et al. 2013).

Interestingly, this ARE-BP-mediated modulation of the destiny of target mRNAs is regulated also by ARE-BP post-translational modifications. The ability of these proteins to interact with target mRNAs and with each other has been seen to be strongly affected

by ARE-BP phosphorylation by MAPK and PI3K/AKT signalling cascades, allowing the ARE-BP to quickly modulate the expression of several early response genes, such as cytokines, lymphokines and proto-oncogenes (Winzen et al. 2004; Palanisamy et al. 2012; Shen and Malter 2015).

5.1.4 The WRB transcript AREs are not involved in mediating CAML-sensitive instability.

Given the widespread effect of AREs on mRNA metabolism, I investigated their potential role in mediating the effect of CAML on WRB transcript stability. I therefore produced a luciferase construct, in which the 3'UTR was truncated at position 438, with the consequent deletion of the downstream AREs; in addition, the first 68 nucleotides of the 3'UTR were deleted, with removal of the upstream ARE. Unexpectedly, I found that the resulting "mini"-3' UTR was as effective as the full-length sequence in reducing luciferase expression in the absence, but not in the presence, of CAML. Although this result did not meet my expectations, it restricts the CAML-sensitive regulatory portion of the 3'UTR to 370 nucleotides (position 69 - 439 of the 3'UTR). My future work will be directed at further restricting the active portion to identify the responsible sequence.

5.1.5 What is the mechanism through which CAML stabilizes the WRB transcript?

As illustrated in Fig. 5.1, left panel, I favour a model, in which CAML would directly or indirectly associate with the 3' UTR of the WRB transcript. This association would have three beneficial effects: (i) it would keep the WRB transcript close to the ER membrane; this would allow the hydrophobic stretches at the N-terminus (the future TMDs) to have easier access to the Sec61 translocon for subsequent insertion into the ER bilayer. In the classical model of membrane protein insertion into the ER, it is SRP, *via* the SRP receptor, that brings the ribosome-mRNA-nascent polypeptide complex to the ER membrane for subsequent delivery to Sec61 (Blobel and Dobberstein 1975; Blobel 2000). However, SRP recognition may not always be 100% efficient, and the occurrence of translation of a membrane protein in the cytosol could be very harmful to the cell. Thus, it is not surprising that ribosome-independent anchorage to the ER of mRNAs

coding for membrane proteins has been observed (Cui et al. 2015; Chen et al. 2011). The possibility of direct delivery of nascent chains to the translocon, bypassing the SRP step, has also been reported (Potter and Nicchitta 2002); (ii) it would guarantee translation of WRB to occur in proximity to its CAML partner, thus facilitating assembly (see Introduction); (iii) by restricting WRB mRNA to the ER compartment, it would protect it from access to the RNA degradation machinery of the cell, resulting in the observed stabilization.

CAML does not have any known RNA binding motifs, thus it is difficult to envisage, although it is fully possible, that it directly binds the 3' UTR of the WRB transcript. However, its interaction with WRB mRNA's 3'UTR could be mediated by another protein, or even by a miRNA (see Fig. 5.1). Indeed, the utilization of online software miRWalk2.0 to identify miRNA recognition sequences in WRB mRNA's 3' UTR identified one sequence as potential target for miRNA 24-1.

Admittedly, at the present stage of my research, I cannot exclude that CAML's effect on WRB mRNA's stability is indirect, mediated by a cascade of events related to the many reported functions of CAML (Shing et al. 2017). CAML could alter the expression levels of a stabilizing or destabilizing factor (protein or miRNA). My current research is directed at demonstrating (or excluding) the existence of a complex between CAML and WRB mRNA.

5.1.6 WRB mRNA's CAML-sensitive stability: a failsafe mechanism to avoid useless translation of a "lonely" subunit.

As described in the Introduction, quality control systems in the cell ensure that oligomer subunits, if produced in excess over their partners, are rapidly degraded, and this phenomenon is generally thought to underlie the reduction of the levels of all complex subunits, when one of them is depleted by RNA interference. My results show that, in addition to this quality control mechanism, regulation of the transcript levels may also be important. It should be noted that, while my results indicate that a free pool of CAML exists in the ER, the reverse is not true: indeed, previous results from my lab (Colombo et al. 2016) demonstrated that WRB is quantitatively associated with CAML.

Whatever the mechanism by which CAML stabilizes the WRB transcript, the final result will be that WRB is not produced in the absence of CAML. Such a regulation,

operating at the level of the transcript, is a cost-effective way of limiting the presence of a "lonely" subunit. If no CAML is around, why should the cell spend the energy to produce WRB and then degrade it? To be noted, also degradation by the ubiquitin-proteasome system is an energy-consuming process. Of course, in real life, CAML levels may vary, but are not expected to fall to nearly zero, as in siRNA experiments. Nevertheless, the physiological implications of my results are clear: WRB production is expected to be regulated by the availability of CAML, and could thus be modulated by variations in the levels of its partner.

5.2 How WRB depletion affects CAML levels

My investigation of the effects of WRB silencing on CAML transcript levels yielded results quite different from those obtained for the reverse situation. Indeed, in agreement with a recent study on conditional knockout mice (Rivera-Monroy et al. 2016), CAML transcript levels and its stability were unaffected by WRB depletion. It must be stated that the results on the transcript levels were slightly different from those obtained by Colombo et al. (2016), who did observe a 20-30% reduction in CAML transcript following WRB silencing. While at present I do not know the reason for this discrepancy, in both my hands and in the studies of Colombo et al. and of Rivera-Monroy et al, CAML protein levels did not go in parallel with those of CAML transcript: in my hands, CAML transcript was unaltered, and CAML protein was depleted to the same extent as WRB; in the study of Colombo et al., a ~25% reduction in CAML transcript levels was accompanied by depletion of 90% of CAML. These results indicate that, in the absence of WRB, CAML protein is not expressed in cells, and strongly suggests that CAML stability requires the presence of WRB protein. This conclusion was strengthened by the observation that expression of a tagged form of CAML, expressed from a cDNA lacking the UTRs, was reduced upon WRB silencing, differently from the result obtained in the specular experiment (tagged WRB expression from a cDNA lacking the UTRs was not affected by CAML silencing).

The dependency of CAML on WRB protein could be easily understood if CAML were quantitatively included in a WRB-containing complex. That this is not the case was already suggested by the work of Colombo et al. (2016), and directly shown here

by co-immunoprecipitation experiments. When normalized to the efficiency of immunoprecipitation of the antibodies, 50-70% of WRB co-precipitated with CAML, while only 10-30% of CAML co-precipitated with WRB from the same lysate. Thus, it appears that at least 50% of the CAML molecules exist free of WRB.

So, how can the destabilization of CAML in the absence of WRB be explained, given the large pool of free CAML molecules that exist without being associated with WRB? Clearly the effect of WRB on CAML cannot be mediated by a stoichiometric mechanism, and must be catalytic. I suggest that this catalytic mechanism could consist WRB assisting CAML insertion into the ER membrane. CAML's TMDs are close to its C-terminus; they might, therefore, be inefficiently recognized by SRP. Indeed, in yeast, it has been shown that recognition by SRP is optimal if the position of the hydrophobic sequence in the nascent chain is not too far removed from the N-terminus (Aviram et al. 2016). Although CAML is not a TA protein, the vicinity of its TMDs to the C-terminus might permit it to interact with TRC40; in this case, the TRC40 receptor (WRB/CAML) would be involved in its insertion into the ER membrane. In other words, CAML would use itself to integrate into the bilayer. This situation would not be unusual: for instance, also the α subunit of Sec61 requires a pre-existing Sec61 translocon to integrate into the ER membrane (Knight and High 1998). In the absence of integration into the ER membrane, CAML would be recognized by the UPS and degraded. Our model for the role of WRB in CAML biogenesis is depicted in Fig. 5.1 (right).

5.3 Conclusions

The research for this thesis has uncovered novel, and unexpected modes of regulation of the expression of oligomer subunit. Indeed, I show that, beyond the important concept of quality control, meaning with this the degradation of unassembled oligomer subunits, mRNA stability of one subunit can be affected by depletion of another one, and that protein stability may be affected also in cases in which a portion of one subunit is not incorporated in the oligomer. These novel observations may be relevant for other protein complexes, and open a large number of questions, which I summarized in the preceding sections of this discussion, and which I hope to address in my future research.

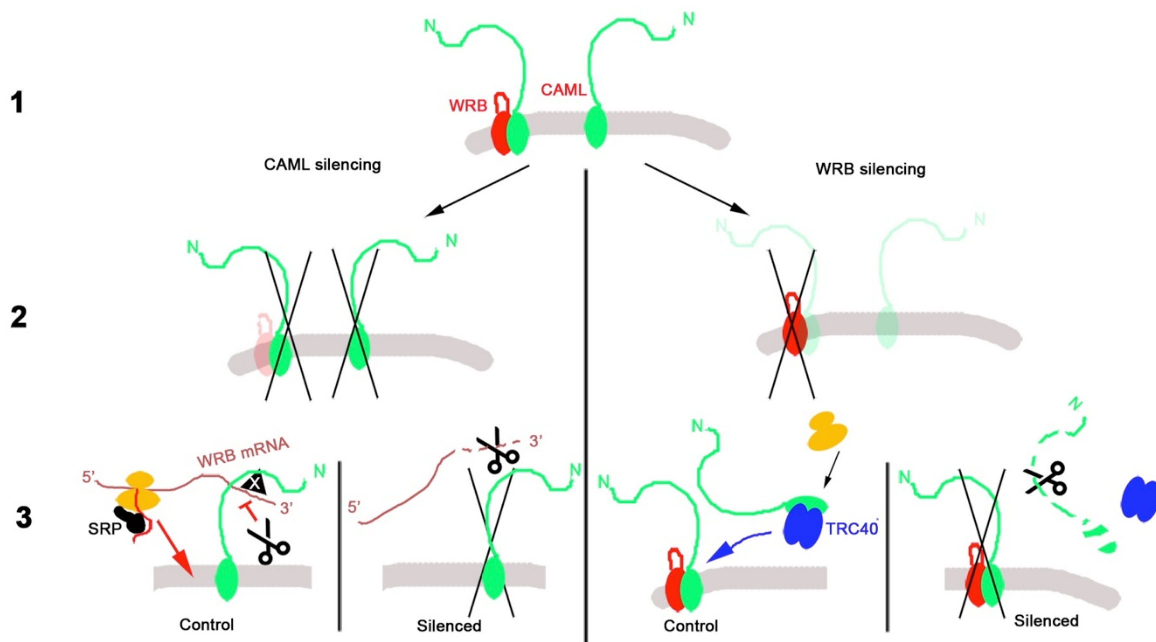

Fig. 5.1 – Models for the mechanism of reciprocal regulation of the expression of TRC40 receptor subunits. Row 1: under normal conditions WRB (red) is quantitatively associated with CAML (green), but, in addition, a pool of CAML subunits not associated with WRB is present. Row 2: silencing of CAML (left) or of WRB (right), indicated by the crosses, results in depletion of the companion subunit (represented as ghosts in light colour). Row 3: left: under normal conditions, WRB mRNA is kept close to the ER membrane thanks to the interaction of its 3' UTR with CAML. The possibility of an indirect interaction with CAML is indicated by the black triangle (X). WRB nascent polypeptide is generally recognized by SRP (black), but could also be directly delivered to the ER membrane, thanks to the CAML-mRNA association. The compartmentalization of the WRB transcript protects it from access to nucleases (indicated by the scissors). When CAML is absent (black cross) the association of the WRB transcript with the ER membrane is in part lost (mediated now only by the SRP-SRP receptor interaction). The free WRB transcript is now accessible to the RNA degradation machinery. Right: under control conditions, the C-terminal TMD of CAML is recognized by TRC40, and delivered to the ER membrane via interaction with the TRC40 receptor, composed of WRB and CAML itself. When WRB is absent, TRC40 cannot deliver its cargo to the ER, and CAML, released into the cytosol, is degraded. See text for further explanation.

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