Hubrecht Institute
Developmental Biology and Stem Cell Research

Translation Specificity and Ribosomal Heterogeneity

Dylan Mooijman[1]

Control of gene expression is essential for cellular processes to proceed correctly as disturbances in both RNA and protein levels have shown to be causative for a multitude of diseases and developmental defects (Orkin et al 1982, Kishino et al 1997). For instance the process of development is highly regulated and is especially sensitive to disturbances in gene expression. Many developmental defects are the direct result of the misregulation of one or more gene products during a specific time point during development (Chisaka and Capecchi 1991). Most of the attention on gene regulation is focused on transcription, in part because a lot is known about transcription by the use of expression-array and RNA sequencing technology. Recently, methods have become available that suggest that ribosomes are more than constitutive translation machines. This, in combination with the fact that regulation of the translation from RNA to protein has shown to be of vital importance during development (Ganapathi and Shimamra 2008) shows that a focus on translational events can be of great interest. This master thesis will focus on the methods to assess translation, the mechanisms of translational regulation in different organisms during development and the selectivity of the process and I will end with my view on current interesting perspectives in the field of translational regulation and ribosome specificity.

Pre-translation

Gene expression is a multistep process starting at the DNA level with transcription of genes to their corresponding mRNA molecules as the first step in the gene expression process with transcription being regulated by the epigenetic status of the chromatin, amount of transcription machinery on the gene and modifications of the transcription machinery. Transcription of DNA to RNA first yields primary mRNA molecules containing both exons and introns that are copies of the DNA template and require the removal of the intronic sequences to create a functional mRNA. Splicing of the primary transcript can generate different mRNA molecules and lead to functionally different proteins (Kalsotra and Cooper 2011). The spliced mRNA molecules are modified with a stretch of Adenosine nucleotides (poly A tail) on the 3' end and a 7-methylguanylate cap (m7G) on the 5' end. Both modifications prevent exonucleases from digesting mRNA transcripts (Decker 1994), but they also cooperate in a synergistic manner to increase translation to proteins (Gallie et al, 1991).

Ribosome and translation

Translation of mRNA to a sequence of amino acids to ultimately form a protein starts with the assembly of the small subunit of the ribosome. The ribosome is a multi-subunit protein/RNA complex responsible for translating the nucleotide RNA code to an amino acid (AA) code. Each 3 nucleotides on the mRNA corresponds to a AA, converting the triplet DNA code to a singlet AA code.

The ribosome consists of 2 subunits, a small 40S (the S represents Svedberg units, a measure for size) subunit comprised of 33 proteins and 1 rRNA molecule, and a large 60S subunit comprised of 46 proteins and 3 rRNA molecules. The ribosomal RNA (rRNA) is responsible for stability and the catalytic activity of the complex, while ribosomal proteins are thought to facilitate optimal rRNA processing and folding (Stillman 2001). The process of translation can be divided into four steps, the initiation step, elongation, termination and ribosome recycling. The start or initiation of translation is a highly regulated multistep process with various ribosomal and non-ribosomal factors taking part (Sonenberg and Hinnebusch 2009). The majority of translation initiation events are dependent on the 7mG 5'cap, although exceptions will also be discussed. Eukaryotic translation initiation factors (eIFs) play a pivotal role in the initiation of cap dependent translation by recognizing and binding the m7G cap structure at the 5'end or the poly A sequence at the 3'end of the mRNA. The bound eIF proteins recruit the small 40S ribosomal subunit to facilitate the start of the translation process. The small ribosomal subunit now consists of multiple eIFs (eIF1,1A,2,3 and 5) and the initiator tRNA, creating a larger 43S subunit.

[1] Hubrecht Institute-KNAW and University Medical Center UTRecht, Uppsalalaan 8, 3584 CT UTRecht, The Netherlands.

This complex moves along the mRNA scanning for the translation initiation codon, signified by the AUG triplet. Recognition of the codon triggers the termination of scanning and hydrolysis of the eIF2-GTP complex, the subsequent release permits joining of the big 60S subunit and forms the elongation competent 80S ribosome complex (Pestova et al, 2007).
The ribosome contains transfer RNAs (tRNAs) linked to different AAs. Each tRNA contains a complementary nucleotide triplet (anticodon) to a triplet on the mRNA (codon). Each triplet encodes for an AA, with the tRNAs as intermediaries between RNA and protein code. The 80S ribosome contains 3 binding sites, an animoacetyl-tRNA entry site (A), a peptidyl-tRNA site (P) and a free tRNA site (E) (Figure 1). Translation of the mRNA code to a chain of AAs and protein molecules works like a conveyor belt, with each new AA-tRNAs entering in the A site and binding to their complementary codon. After binding to the codon they move to the P site where the AA is linked to the growing peptide chain by a peptidyltransferase. The then remaining free tRNA exits the ribosome via the E site, after which the cycle can start again, generating a string of AAs that after folding correctly generates a functional protein.

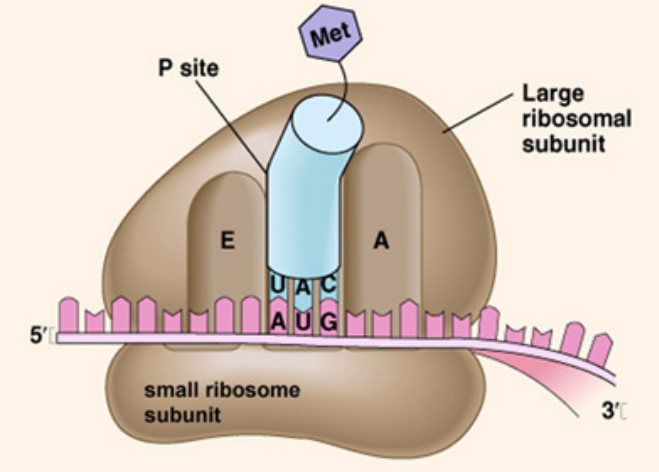

Figure 1, 80S ribosome with the initiator codon binding on the mRNA.
The initiator aminoacetyl transfer RNA (blue cylinder linked to the methionine AA) is bound to the AUG initiator codon via basepairing with the UAC anti-codon. Binding of the initiator tRNA will start elongation and allows new AA-tRNAs to enter via the A site, bind in the P site and after linking their AA to the growing peptide chain, exiting via the E site. Adapted from 'Molecular Biology of the Cell', 4th edition.

Alternative mode of initiation
Some mRNAs contain specialized internal ribosomal entry site (IRES) sequences in their 5'UTR that allows for m7G cap independent initiation (Jang et al., 1988; Pelletier and Sonenberg, 1988). An IRES element is a 450 nucleotide highly structured element first discovered in picorna viruses (Sasaki and Nakashima, 1999). The IRES is able to directly recruit eIFs and the 40S ribosomal subunit without the need for a m7G cap or scanning for the initiator codon. The ability to bypass the need for cap dependent translation is used during stress conditions, where cap dependent translation might be less efficient. This might explain that a substantial portion of IRES containing mRNAs encode for proteins that play a role in reacting to stress response and mediating apoptosis (Bushell et al, 2006; Komar and Hatzoglou, 2005).

Techniques to measure gene expression
The ability to measure gene expression has been a powerful tool to understand gene function in both a qualitative and quantitative aspect. However, most gene expression methods focus solely on transcription levels as a proxy to estimate protein abundance, creating a bias since mRNA translation is extensively regulated (Gebauer and Hentze 2004). Furthermore, inferring protein sequence from mRNA sequence is problematic because of confounding factors such as internal ribosomal entry sites, non-sense read-through and non-AUG initiation.
Methods that focus on the protein level, like quantitative proteomic measurements have arisen to address these issues, however they are limited in identifying de novo protein sequences and low abundant proteins. Analysing the translation step between mRNA and protein can provide a useful estimate of protein synthesis and can reveal additional translational regulation (Arava et al, 2003).
For translational analysis, the most obvious factor to measure is the amount of ribosomes bound to mRNAs. A method to determine the relative quantity of ribosomes associated with a mRNA involves the increased sedimentation rate of ribosome bound mRNA, as the mass of the ribosomes is the dominant factor in the sedimentation process. Therefore, sedimentation of a mRNA-ribosome complex in a sucrose gradient will be directly related to the number of ribosomes present on the mRNA. When complexes are sedimented by centrifugation through a sucrose gradient solution, complexes with a similar density as the sucrose solution will not continue to sediment (Figure 2). This allows for the separation and isolation of mRNA-ribosomal complexes with distinct density differences. The isolated mRNA-ribosome complexes can then be interrogated using a RNA detection technique to identify mRNA association with a ribosomes related to the sedimentation position.

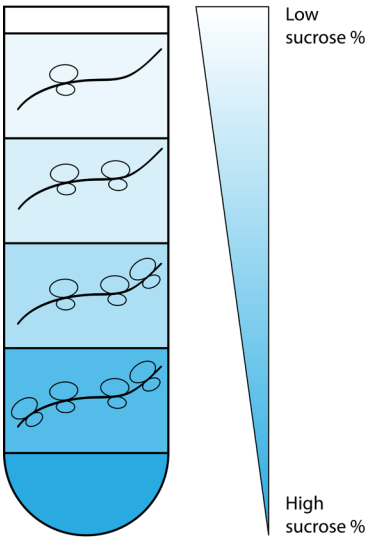

Figure 2, sucrose gradient centrifugation to isolate mRNA-ribosome complexes.
The sedimentation position of the ribosome bound mRNA is dependent on the amount of bound ribosomes (spheres) and the local sucrose concentration indicated in blue..

The existence of polysomes were detected using this method by Jonathan Warner, Paul Knopf, and Alex Rich in 1963. Polysomes or polyribosomes are clusters of ribosomes present on a mRNA (Warner et al, 1963) and are a mark of high translational activity shown by increases in polysome counts and translation upon changing conditions (Ashe et al, 2000; Dickson and Brown, 1998; Proweller and Butler, 1996; Tzamarias et al, 1989). Translational analysis by assessing polysome abundance is commonly referred to as polysome profiling.

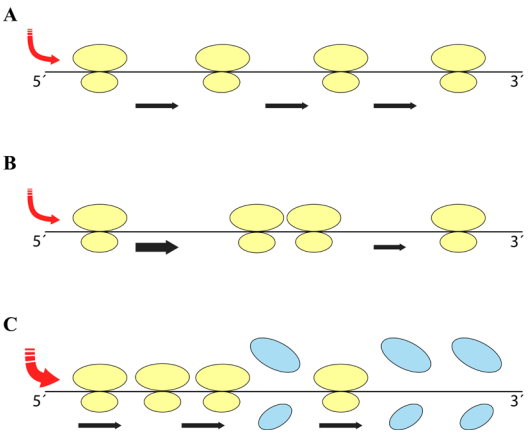

Figure 3,Ribosomal occupancy differences
Different ribosome occupancy (yellow) dependent on initiation (red arrow), pause sites and disassociation (blue).

However, this technique does not generate positional information of ribosome binding. For example, a mRNA with a slow elongation rate and a mRNA with a fast elongation rate but with a strong pausing site can both have the same overall number of ribosomes(Figure 3, A and B). The same is true for a mRNA with a fast initiation but with frequent ribosome dissociations having a comparable ribosome number as a slow initiating mRNA (Figure 3, A and C). Although the number of ribosomes on the mRNA is similar, the position for each scenario is different. A strong pause site would result in increased ribosome densities upstream of the pause site or frequent ribosome dissociation will result in decreasing ribosome numbers towards the 3´ end (Figure 3, B and C).

Being able to tell where ribosomes bind can generate information about the individual stages of translation and multiple techniques were developed for this goal. For instance, ribosome accumulation at either the initiation or termination site can be detected by using a toeprinting method . This involves annealing a radioactive primer against the mRNA and generating a cDNA product that stops extending at the bound ribosome, with the size of the cDNA product indicating the ribosome position (Hartz et al, 1988; Sachs et al, 2002). A similar method incorporating a micrococcal nuclease is also able to detect stalling sites along the mRNA (Wolin and Walter, 1988; Wolin and Walter; 1989), however these methods are low throughput and require a priori knowledge, limiting the ability to analyze multiple mRNAs.

Ribosome density mapping (RDM) is a technique that uses site specific cleavage of polysome mRNA and subsequent sucrose gradient sedimentation to identify the ribosome bound region of the mRNA (Arava et al, 2005). Site specific cleavage is mediated by annealing an RNA oligo antisense to a specific region of the mRNA creating a local double stranded RNA molecule that can be cleaved by the nuclease RNAse H. The cut polysome mRNA can then be analyzed after sucrose gradient sedimentation to determine which part of the mRNA has bound ribosomes, reflected by a higher position in the sucrose gradient. RDM allows for detection of ribosomes at different parts of the mRNA, identifying stalled ribosomes either during initiation, elongation or the termination phase, but it is still limited by the a priori knowledge needed to design antisense RNA oligos for specific cleavage.

Techniques that are able to systematically assess mRNA translation have lagged behind compared to mRNA measuring techniques. As mentioned before, polysome profiling in combination with high throughput mRNA analysis can provide a useful estimate of global protein synthesis, but lacks accuracy and is unable to resolve where ribosome binding is actually occurring.

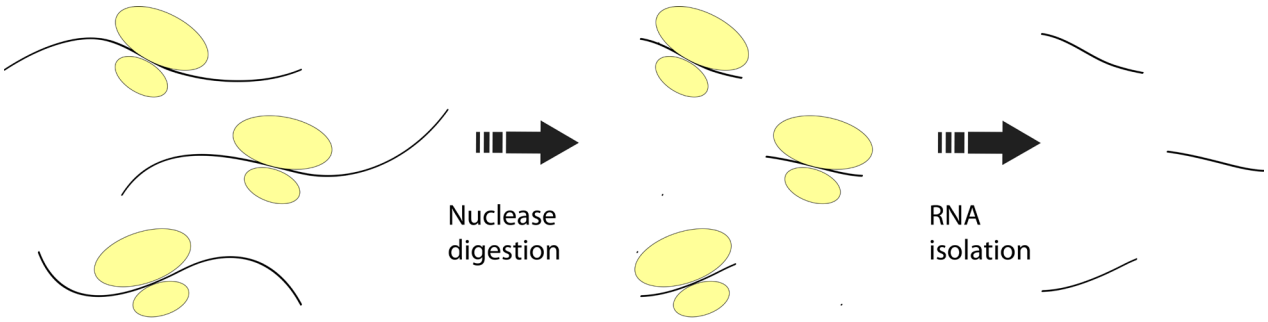

Figure 4, ribosome footprinting procedure
Polysome isolation yields ribosome-mRNA complexes (left), nuclease digestion only affects mRNA that is not protected by a bound ribosome (middle) and subsequent RNA isolation allows for interrogation of the identity of the protected fragments (right).

However, the position of a ribosome can be accurately determined by taking advantage of the fact that a ribosome protects a 'footprint' of approximately 30 nucleotides on the mRNA from nuclease digestion (Steitz, 1969). Combining sucrose gradient sedimentation to obtain polysomes and nuclease digestion to digest non-ribosome bound RNA yields a library of ribosome occupancy on the transcriptome (Figure 4). The combination of ribosome footprint isolation and next generation RNA sequencing allowes for genome-wide analysis of the translational state with single nucleotide resolution (Ingolia et al 2009; Ingolia et al 2011).
The advantage of ribosome footprinting over other methods is the generation of a transcriptome wide ribosome binding map without the need for a priori knowledge. However, neither ribosome footprinting nor other methods have a direct way of measuring ribosome occupancy in vivo, in addition the measurements are performed on a population level, dismissing single molecule variation in ribosome binding.

Translational regulation
Compared to transcriptional regulation, translational regulation results in faster changes in protein concentrations and allows for highly coordinated spatial and temporal patterns of protein concentrations, supplying the cell with a more direct way to change protein synthesis. There are two forms of translational regulation, one leading to a global regulation of protein synthesis contributing to regulation of metabolism and cell growth and a second form, where translation of a specific mRNA or a subset of mRNAs is regulated. Translation of the majority of mRNA requires the m7G cap and regulating cap dependent translational initiation is an extensively used mechanism in regulating global translation. The m7G cap and factors binding the poly A tail have shown to interact and mediate circularization of the mRNA (Kuersten and Goodwin, 2007) (Figure 5), a vital part of the translation process. The poly A binding protein (PABP) is able to increase mRNA binding to the small ribosomal subunit partly by facilitating eIF4F binding to the m7G cap (Kahvejian et al 2005). PABP has also been shown to stimulate 60S subunit joining (Kahvejian et al 2005), indicating a direct role for the 3'UTR in translational stimulation. The looped structure created by protein interactions between the 3 and 5' could facilitate ribosome recycling by reinitiating terminated ribosomes from the 3' to the 5 'end.
Most regulation of the m7G cap is directed at the eIF factors associated with the cap. Binding of translation initiation factor eIF4F to the m7G cap can be prevented by the eIF4E homolog 4E-HP. 4E-HP is part of a family of eIF4E binding proteins that achieve repression of initiation by preventing the interaction between eIF4G and eIF4E and subsequently disrupting the eIF4F complex.
One of the prime mediators of global translational regulation is the mammalian target of rapamycin protein (mTOR) (Holz et al 2005). mTOR is a serine/ threonine kinase that is able to phosphorylate 4E-HP and subsequently activate it (Sonenberg and Hinnebusch, 2009). The mTOR kinase responds to external signals and the energy status of the cell and is pivotal in regulating a multitude of cellular processes including, cell growth, proliferation and motility, transcription and translation. Apart from regulation via mTOR, global translation regulation via eIF phosphorylation is also influenced via the Ras-MAPK pathway (Holz et al 2005). Both MAPK and mTOR signaling pathways are tightly linked to cell growth and proliferation and provide the logical link between regulating protein synthesis for biomass and cell growth and proliferation.

Specific translational regulation
There are instances where translation has to be regulated in a specific manner, resulting only in different translational events for specific transcripts. Specific regulation can be quantitative, leading to changes in protein levels or qualitative, generating different protein isoforms. Only a few systems of mRNA

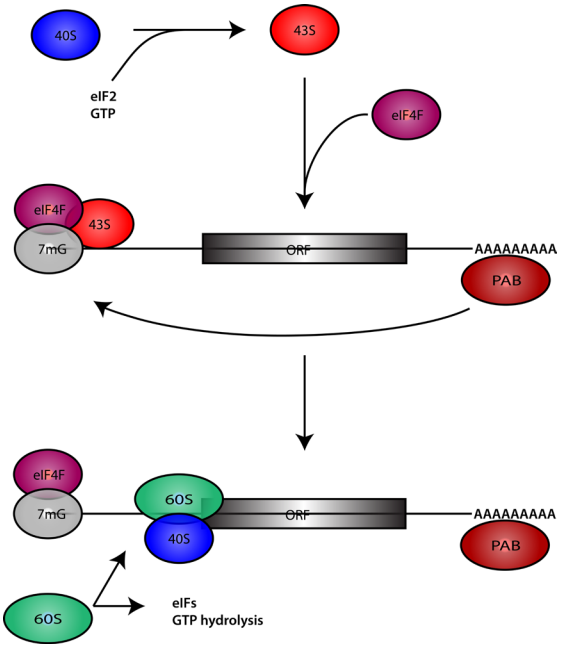

Figure 5, mRNA circularization and initiation of translation. *The 40S subunit binds translation initiation factors to create the pre initiation 43S complex. Circularisation of the mRNA by binding of PAB to the 7mG cap facilitates binding of eIF4F that recruits the 43 S subunit that scans the 5'end of the mRNA for the initiator codon and subsequently releases the initiator factors to start the elongation process.*
Figure adapted from Kuersten and Goodwin 2007

specific translational control have been elucidated on the molecular level, although it has become clear that multiple mechanisms have evolved to inhibit translation at multiple steps. (Gebanner and Hentze 2004). Translational control has shown to be an important factor in development, with special emphasis on development of oocytes and the early embryo, this is mainly due to the absence of transcription during these stages. The earliest discoveries in translational regulation were made in Drosophila melanogaster. In Drosophila, translational control is essential for the establishment of body axes and proper expression of sex chromosomes. In contrast to mammalian dose compensation of sex chromosomes, where one of the two X chromosomes is inactivated in females, dose compensation in drosophila males is achieved by up-regulation of genes on the single X-chromosome. A complex of male sex lethal proteins (MSL) coat the male X-chromosome and up-regulates transcription by 2-fold for several genes on the X-chromosome.

In female flies however, upregulation of X-linked genes is prevented due to translational repression of MSL by the female sex lethal protein (SXL). SXL has a dual function in repressing the MSL mRNA, it binds the 3'UTR regulatory region on the MSL mRNA and recruits the corepressor UNR to adjacent binding sites where it blocks recruitment of the 43S ribosomal subunit to the 5'end of the mRNA (Abaza et al, 2006; Beckman et al 2005; Duncan et al, 2006; Duncan et al 2009). Ribosomes that escape the first level of regulation and initiate on the mRNA encounter SXL proteins bound on the 5' end, resulting in stalling and destabilizing the ribosomal subunit (Beckmann et al 2005). Additionally to containing SLX binding sites, the 5'UTR of the MSL mRNA contains upstream open reading frames (uORFs) that also participate in regulating translation. Binding of SLX downstream of these uORFs has a strong inhibitory effect on translation of the major reading frame. This is achieved by increasing the initiation of scanning ribosomes at the uORFs and thereby preventing translation from occurring on the downstream open reading frame (Medenback et al 2011).

Apart from regulation of sex chromosomes, translational regulation also determines the anterior and posterior (A-P) axis during early embryogenesis in the drosophila embryo. Maternal contributions of axis determining transcripts are translationally inactive in oocytes and become activated upon fertilization. This translational activation is performed in a precise spatial pattern that relies on translational repression. Formation of the anterior and posterior axis depends on 2 pathways of translational control of Caudal and Hunchback mRNA.

Caudal mRNA is found throughout the embryo, yet proper localization of the protein is essential for anterior development. This is achieved by translational repression of the mRNA by the anterior protein bicoid, which is localized to the anterior cortex. Bicoid protein binds a cis acting element on the 3´UTR of the bicoid mRNA which allowes, after circularization of the mRNA for bicoid protein to compete for elF4 binding sites on the 5´UTR and inhibit translation. So although caudal is ubiquitously expressed in the embryo, localized bicoid protein can create a caudal protein gradient by specifically inhibiting caudal mRNA translation. (Figure 6)

The posterior end of the embryo is regulated in a slightly more complex manner. Posterior development is dependent on the repression of Hunchback mRNA on the posterior end and is achieved by the posterior recruitment of silencing factors Nanos, Brain tumor and Pumilio. Of these three factors, only Nanos is enriched on the posterior end and therefor creates the specific local repression on hunchback. Although Nanos mRNA is enriched on the posterior end, considerable mRNA levels are detectable throughout the embryo, Nanos itself therefor has to be a target of translational repression. In order for the specific localization of Nanos to occur, the Smaug protein inhibits Nanos mRNA translation throughout the embryo in a similar manner as hunchback is inhibited. Although Nanos translation is repressed by the uniformly distributed Smaug, the abundant amount of Nanos on the posterior end is able to

overcome repression and create a high local concentration of Nanos (Figure 6).

A similar inhibition strategy of silencing maternally contributed mRNA is used in vertebrate development. Translational control of maternally inherited mRNA is a key feature of oocyte development. These mRNA are stored in the oocyte in an inactive state and are translated upon exogenous signals. A highly used model is Xenopus leavis oocytes that contain inactive mRNAs which are activated upon oocyte maturation and the subsequent meiosis. Inactive mRNAs MOS and cyclins are pivotal for the oocyte maturation process and their translational regulation is critical as their premature translation would lead to premature oocyte maturation and terminated development (de Moor and richter 1999). These mRNAs harbor specific sequences that allow their translation to be prevented until the moment of maturation. Mutagenesis of putative regulatory regions in the 3ÚTR revealed a role for the cytoplasmic poly A element (CPE) protein in regulating translation of these specific mRNAs (de MOOR and richter). It is unlikely that this factor alone has a role in specific translational repression, as it is a general translation factor involved in binding/regulating the 3'poly A tail. The specific role of CPE in translation could be mediated by binding to an additional factor. Co immune precipitation studies resulted in the discovery of the protein Maskin, that binds both CPE and the cap binding translation initiating factor elF4 silencing translation by competition (Stebbins-Boaz 1999).

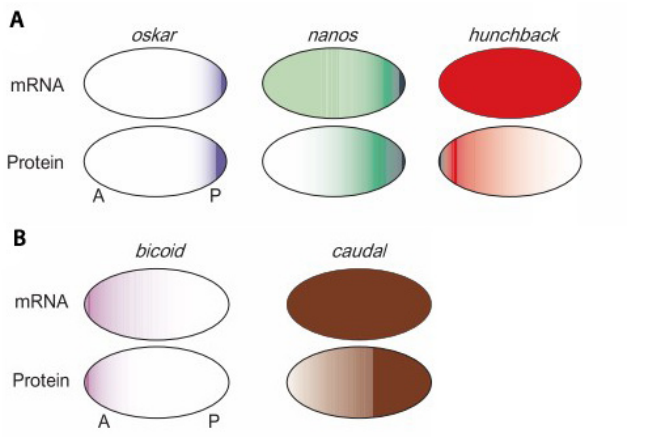

Figure 6, mRNA and protein distribution in the Drosophila embryo.
Posterioir development (A) is dependant on selective translation of hunchback via localized oskar mRNA (blue) that in turn facilitates localized Nanos protein (green).
Anterior development (B) is dependent on selective translation of ubiquitously transcribed caudal (grown) via localized bicoid mRNA and protein (purple). Adapted from Kuersten and Goodwin.

Small RNAs in translational regulation

Micro RNAs (miRNAs) are small 22nt RNA molecules that regulate gene expression at the post transcriptional level. They are transcribed by RNA polymerase II and are also capped with a m7G cap and polyadenylated (Kim, 2005). Most miRNAs are transcribed in clusters of multiple miRNAs usually consisting of a miRNA family (Bartel, 2004). The resulting transcript contains immature pre-miRNAs that are subsequently processed by Drosha and Dicer to generate mature double stranded miRNAs. One of the two miRNA strands is loaded into the RISC complex, generating a miRISC complex that is able to specifically recognize and regulate mRNAs. Most miRNA target sequences reside in the 3'UTR of the mRNA and direct the miRISC complex to those regions. The miRISC complex contains an endonuclease that can cut the target mRNA strand when the mRNA:miRNA sequence complementary is perfect. However, most miRNA do not have a perfectly complementary 'seed' sequence as it is called and most likely rely on translational repression to influence gene expression. The exact mechanism of how miRNAs effect translation is still highly controversial with discordant results showing how miRNAs regulate translational initiation (Humphreys et al, 2005; Wakiyama et al, 2007; Ding and Grosshans, 2009) or regulate events post initiation (Maroney et al, 2006; Nottrott et al, 2006; Peterson et al, 2006). There are currently three models that explain how miRNAs repress translational initiation (Figure 7). The first model revolves around competition between miRISC and eIF4E, both eLF4E and a human component of the RISC complex (Ago2) show similarities in binding the methylated cap (Kiriakidou, 2007), providing the RISC complex with a mechanism for competition. Mutations in the Ago2 binding site and providing excess eIF4E both impair the ability of Ago2 to repress translation (Mathonnet et al, 2007), hinting that Ago2 competes for cap binding. Opposing evidence challenges these claims by showing that mutations in the Ago1 protein also represses translational inhibition, but does not influence binding to m7G molecules in vitro (Eulalio et al, 2008).

The second model links miRISC mediated repression to deadenylation of the poly A tail. Deadenylation prevents the PABP1 protein from binding to the 3'end of the mRNA and therefor inhibits circularization, repressing translation. Many mRNAs targeted by miRNA are deadenylated in vivo (Behm-Ansmant et al, 2006; Giraldez et al, 2006; Wu et al, 2006), yet also non-polyadenylated mRNAs are silenced by miRNAs (Pillai et al, 2005; Wu et al, 2006; Eulalio et al, 2008). An additional effect of deadenylation is the recruitment of exonucleases and subsequent degradation of the mRNA. In what sense this is mediated by the inhibition

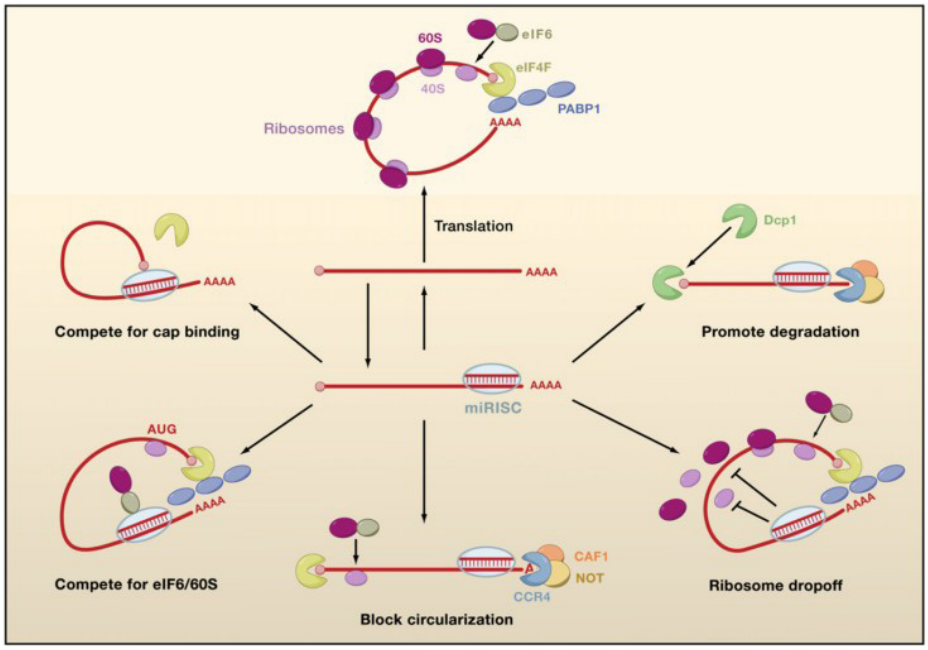

Figure 7, Different mechanisms of miRSC mediated repression.
Non repressed mRNAs recruit ribosomal subunits and create a circularized structure to enhance translation (top). Binding of the miRISC complex to the 3'UTR of a mRNA (middle) can lead to translational repression via competition for cap binding proteins (top left), competition for eIF6 and 60S formation (bottom left), blocking circularization of the mRNA by regulating the poly A tail and inducing premature ribosome dropoff (bottom right). miRISC can also lead to mRNA degradation by the Argonaut endonuclease activity or effecting the poly A tail and m7G cap (top right). Figure adapted from Carthew RW and Sontheimer EJ 2009)

of translation or if it is an independent effect seems to differ for different mRNAs. But it has been shown that in the absence of translation machinery, miRNA mediated degradation is still present in vitro (Wakiyama et al, 2007).

The third model proposes that miRISC is able to inhibit assembly of the ribosome by blocking 60S and 40S assembly. Human Ago2 has been shown to associate with 60S subunits and eIF6 in vitro (Chendrimada et al 2007). eIF6 is an initiation factor involved in maturation of the 60S complex and prevents their association with the 40S ribosomal subunit at the initiation step. In human and *C. elegans* cells inhibition of eIF6 recues miRNA mediated translation inhibition, but surprisingly silencing is unaffected in drosophila cells. (Eulalio et al 2008).

Although the general consensus identifies translation initiation as the the preferred target for translational control (Gebauer and Hentze, 2004; Gingras et al., 1999; Richter and Sonenberg, 2005), Peterson et al (2006) shows a possible mechanism of translational repression during the elongation step. First, by performing pulse labeling experiments it was shown that repression occurs before the completion of a full length polypetide chain, showing that repression occurs on polysomes undergoing an active peptidyl transferase reaction. Second, they suggest that binding of the argonaut subunit causes ribosomes to disassociate more rapidly.

Ribosome in translational regulation

These mechanisms of translational repression and regulation rely on additional factors that prevent the ribosome from binding or assembling. But the ribosome itself is also involved in specific mRNA translation, even though the ribosome historically has been viewed as having a constitutive rather than regulatory function.

As mentioned before, the eukaryotic ribosome consist of a small (40S) and a large (60S) subunit together with rRNAs and 79 core proteins, but in addition to the core make up, ribosomes can vary in their protein compostition. Developmental regulated synthesis of cell type specific eukaryotic ribosomes using different protein compositions was proposed almost 30 years ago, based on observations by 2D gel analysis, of different ribosomes purified from vegetative amoebae and differentiated Dictyostellium spores (Ramagopal and Ennis 1981). Despite this early hypothesis substantial functional evidence for specialization of RPs was still lacking.

The yeast Saccharomyces cerevisiae genome contains many duplicated ribosomal genes that have been reported to be functionally redundant. The initial hypothesis to explain the multiple RP copies in yeast revolved around adjusting the amount of RP expression to match the rRNA synthesis (Otha, 1988). These paralogs however have recently been shown to to confer specific translation for localized mRNAs (Kolili et al, 2007). Most of the evidence for RP redundancy has come from studies showing that absence of RP paralogs does not lead to reduced fitness. That measuring only this parameter is flawed was shown by the deletion of RP Rps27a (while retaining the wild type copy of Rps27b) had no effect on growth rate, yet showed ribosomal assembly defects and rRNA processing deficiencies (Biallien et al 1997).Deletions of up to 11 RP paralogs show specific defects in bud site selection caused by misregulation of budding factor Ash1. Ash1 protein exclusively localizes to the daughter cell in cell divisions where it is involved in the suppression of mating type switching. Protein localization is achieved by the localization of ASH1 mRNA to the bud tip site via a complex mechanism using active translation and translational repression involving RP paralogs (Beach et al 1999).

Absence of these paralogs also effect transcription of a wide range of processes like sexual reproduction and amine metabolism, but also increases sensitiity to a variety of antibiotics, indicating a wider role for specific regulation by RPs (Komili et al, 2007; Parenteau et al 2011). Further differences are revealed by the different cellular localization of the paralogs, paralogs showing differences in function also revealed localization differences. Despite all these different functions and characteristics of RP paralogs, they look almost identical, leaving little evidence for their different mechanisms of action.

One relatively special characteristic of RP genes in yeast is the presence of intronic sequences which are relatively rare in yeast genes. Deletions of these intronic sequences surprisingly alter the transcription either by induction or repression and not only of the gene itself but also of the other RP copy with each copy showing a distinct intron dependant regulatory mechanism. In addition, in wild type cells around 70% of the RP genes are asymmetrically transcribed. One possible mechanism of intron dependent gene regulation has been shown in a few RP genes and involves the binding of the RP to the intron to impair splicing and effect transcription (Vilardell and Warner 1994, 1997). This does explain the effect on transcription on RP genes caused by the intron deletion in the paralog copy, yet no further experiments have been performed to elucidate the exact mechanism of action.

RP specialization studies in yeat have yielded some results indicating specific functions for the RP copies, yet in mammals it has not been explored extensively and only a few examples are known. But regulation by tissue specific expression of RPs in multicellular organisms such as mammals allowes for a potential easier differential regulation of ribosome expression and possible translational regulation.

Immune response and translational regulation.

Induction of the immune response has to be regulated in a tight manner. The oxidatative properties that are pivotal to fight off infective agents also cause extensive tissue damage if left unchecked (Fox et al, 2000; Mukhopadhyay et al 1997). Ceruloplasmin (CP) is a factor involved in inflammatory responses and is rapidly expressed in monocytes after stimulation with cytokine interferon gamma. Upon this stimulation, CP mRNA levels increase substantially, but protein synthesis halts after 16 hours post stimulation by a mechanism of translational repression (Mazumder and Fox, 1999; Mazumder et al, 1997). The specific translational repression of CP mRNA involves the binding of an interferon gamma activated inhibitor of translation (GAIT) complex to a regulatory element on the CP mRNA 3'UTR (Sampath et al., 2003). The GAIT repressor complex is composed of 4 proteins, a NS1-associated protein-1, glyceraldehyde 3-phosphate dehydrogenase, glutamyl-prolyl-tRNA synthetase (GluProRS) and ribosomal protein RPL13a (Mazumder et al., 2003a; Sampath et al., 2004). Ribosomal protein RPL13a is phosphorylated and subsequently released from the ribosome upon interferon gamma stimulation. In addition, the release and occupation in the GAIT complex is highly correlated with CP mRNA translation inhibition. After phosphorylation and release, the ribosomal subunit RPL13a is able to bind to the 3'UTR of the CP mRNA. Immunoprecipitation experiments show that RPL13a binds to the elongation initiation factor eIF4G on the 5'm7G cap (Kapasi et al, 2007). There it competes for binding to eIF3, a critical scaffolding initiation factor that controls the assembly of the 40S subunit on the mRNA. Although this was one of the first pieces of evidence showing that ribosomal proteins can have a role in regulating gene expression, ribosomal protein RPL13a exerts its effect without being part of the ribosomal complex.

Embryonic developement and ribosome specificity

The process of development is also a tightly regulated process with requirements for sharp temporal and spatial gene expression. It might therefor not be hard to imagine that a RP has been shown to play arole in tissue paterning in the developing mouse embryo (Kondrashov et al 2011). Early forward genetic screens identified a mouse with specific skeletal patterning defects and homeotic transformations (Deol, 1961; Morgan, 1950). The phenotype and skeletal defects of the mouse lead to name the mutant Tail short (Ts). Characterizations of the mutation lead to detection of a deletion in the Rpl38 gene, a ribosomal protein (RP) component of the large ribosomal subunit. Using sucrose gradients to assess ribosome association with mRNAs as a measure for translation rate identified a subset of Hox genes that were decreased in ribosome association. This possible misregulation of Hox gene expression is in line with the phenotypic effects associated with Ts mutant, as Hox genes are key regulators of tissue patterning and morphology along the axial skeleton (Pourquie, 2009; Deschamps and van Nes, 2005; Wellik, 2009). Hox gene transcription was unaffected nor were global translation rates changed, indicating specific translational regulation by the RPL38 protein. But does RPL38 act as a subunit of the ribosome or does it have an independent function such as RPL13a repression of CP mRNA? Sucrose gradients were used to separate ribosome complexes from ribosome free cytosol. Exposing the obtained fractions to western blot analysis showed exclusive RPL38 presence in the ribosome fraction, indicating that RPL exerts its function as part of the ribosome. However, the exact mechanism of how RPL38 specifically regulates the translation of the subset of Hox genes has not been explained.

Further studies have to be performed to assess whether the affected Hox genes harbor a specific sequence that allows recognition by RPL38 or if RPL38 acts in a different manner. Also the position of RPL38 in the ribosome is of importance to be able to recognize the Hox gene transcripts, research will have to be performed to accurately determine RPL38s position in the ribosome.

The tissue specific effect of RPL38 indicates either a specific localized gain of function or a specific local expression.

Microdissection of E11.5 mouse embryo's and expression microarray analysis revealed that RPL38 mRNA peaks in the developing somites, vertebrae precursors and within the neural tube, explaining the specific effects in these tissues.

Looking at the expression levels of all RPs in different tissues revealed staggering distinct expression levels in the different tissues. This indicates that different RPs might have different functions specific to the tissues in which they are expressed. An interesting observation is that all RPs are expressed at a highly level in mouse embryonic stem (ES) cells, possibly indicating a requirement for specific translational regulation or alternatively having the right RPs when differentiating into the different germ layers.

Stem cells, differentiation and translation

Embryonic stem cells are harvested from the inner cell mass of the embryo (Evans, M. J. & Kaufman, 1981) and have a capacity to differentiate in all cells of the adult body, both in vivo and in vitro (Martin, G. R , 1981; Ying, Q. L. et al. 2008). Differentiation of ES cells involves rapid changes in transcription to facilitate the transfer from one cellular identity to the multitude of different adult cells (Dvash et al, 2004; Gunji et al 2004; Pritsker et al 2006). The processes underlying the transcriptional changes are well studied, but little is known about translational control during differentiation. In Sampath et al, genome wide translational regulation is analyzed during embryoid body (EB) differention of ES cells. ES cells can differentiate into a variety of adult cell types such as neuronal cell and cardiomyocites by forming embryoid bodies. Embryoid bodies are sphere like cell aggregates consisting of differentiated cells that are induced by removing differentiation inhibitory signals from ES cell cultures. Global transcript levels are elevated during differentiation, but to measure translational changes, mRNAs associated with ribosomes are isolated at the start and after 5 days of differentiation and interrogated using microarray analysis. Using this method, Sampath et al assayed changes in ribosome loading on a genome wide level as indicators of translation efficiency of mRNAs. Transcripts from the EB showed a general increase in ribosome loading compared to the ES cells. Undifferentiated ES cells are found to have a low number of polysomes (Garcia-Sanz et al, 1998; Grolleau et al, 2002), indicating inefficient loading of ribosomes. This could be a priming mechanism for ES to have sufficient ribosomes available when transcripts need to be translated quickly at the onset of differentiation. Most of the genes that changed the amount of ribosome loading did this in co-occurrence with an increase in mRNA, showing a likely increase in protein synthesis due to an increase in transcription. However 2% of the genes showed no increase in mRNA abundance but did show changes in the translational state, indicating a form of translational regulation. Of the genes that show increased translation in the absence of an increase in transcription, are two genes involved in neuronal development.

The mRNA of these two genes, ATF5 and DCC have a substantial number of ribosomes bound in the differentiated state, but are very poorly translated in undifferentiated ES cells, suggesting either repression in the ES cell state or an active form of translation during differentiation. Wnt1 however, showed decreased translational efficiency during differentiation with unchanged transcriptional levels. Wnt1 is an activator of the Wnt pathway, leading to activation of Beta catenin and an array of downstream targets. Wnt signaling has shown to be involved in development of the neural tube (Alvarez-Medina, 2008; Ulloa, 2009), cancer (Taketo, 2004) and maintance of pluripotency (Pereira et al, 2006; Hochedlinger et al, 2005; ten Berge et al, 2011). In ES cells, wnt1 mRNA is associated ribosomes and is properly expressed, but upon differentiation the wnt1 mRNA loses ribosomal association and expression is diminished. One possible reason for the translational silencing of wnt1 instead of regulating transcription is the possibility of fast reactivation of the wnt pathway after differentiation.

To assess if these mRNA are influenced the by global changes in translation mediated by mTOR, all mTOR mediated translation was inhibited using rapamycin, a well characterized inhibitor of mTOR. Translation of ATF5 and DCC was not affected by rapamycin treatment, showing that the translational changes are not caused by a global effect on protein synthesis and are likely the effect of specific translational regulation.

The most likely reason for translational regulation during EB differentiation is the more direct response of altered protein levels that bypass alteration of the transcriptional intermediate. This can lead to faster responses to mediate cell fate decisions or allow for a temporary silencing of mRNA so that they can be quickly translated if necessary. The observation that mES cells have a low number of active ribosomes and a large pool of unused ribosomes is highly inconsistent compared to other cell types with high numbers of unused ribosomes if cell cycle state is taken into account. Resting T cells are non-cycling cells with high numbers of unused ribosomes, upon T cell

activation, they rapidly transit into a highly proliferative state that is accompanied by a high number of polysomes. Mouse ES cells, are already highly proliferative, showing among the highest cell division rates measured, yet they still show low numbers of polysomes, normally associated with a quiescent state. My interpretation is that mES cells, like resting T cells are waiting for an activating signal to differentiate to a different cell state and in order to facilitate this transition, fast alterations of protein levels have to be mediated. The large pool of unused ribosomes can be a mean to quickly translate the newly synthesised mRNAs associated with the new cell state.

Unexplored avenues

As reported in Kondrashov et al, RPs are highy transcriped in mES cells, but information about the mRNA levels are obtained from a population of mES cells, even though it is reported in the same paper that these RPs are probably very heterogeneously expressed. These RPs are expressed in different parts of the developing embryo and are likely to have specific roles in the development of specific tissues, and as all these RPs are present in mES cells they could provide the mES cells with different RPs that facilitate the transition into different lineages upon differentiation. It would be very interesting to look at the mRNA abundance of the different RPs in both mES cells and the blastocyst using a single molecule RNA FISH approach. Single molecule FISH is a method developed by Arjun Raj in 2008 and involves hybridization of approximately 40-50 fluorophore coupled 20 nucleotide DNA oligo's to a mRNA molecule of choice. This results in diffraction limited fluorescent spots marking individual mRNA molecules allowing a quantitative analysis of mRNA abundance in individual cells. Using single molecule FISH for different mRNAs encoding the RPs can give us information if these RPS are indeed present in a subpopulation of mES cells or if they are ubiquitously expressed. If some RPs are expressed only in a subpopulation, their potential function in differentiation can be assessed by depleting these RPs using RNAi, differentiating the mES cells and scoring for absence or skewed differentiation potential. Screening all different RPs might not be viable, therefor selecting RPs based on their differential expression in the three germ layers might limit the potential candidates. Unfortunately, there is no transcription profiling data available generated from isolation of the three mouse germ layers, despite this genetic perturbations have generated skewed differentiation in mES cells that allow for transcriptional profiling of different germ layers. For example Mitsui et al, 2003 has shown that a Nanog knockdown resulted in endoderm differentiation, Oct4 knockdown was associated with trophectoderm lineage shown in Niwa et al, 2000 and Sherwood et al, 2007 constructed a dataset comprised of gene expression analysis of definitive and visceral endoderm.

Some RPs show a mES specific expression pattern based on the microarray data from Kondrashov et al. they could potentially exert a specific repressive role to maintain the pluripotent mES cell state or facilitate efficient translation of pluripotent factors in the polysome depleted mES cells, both roles would lead to loss of the pluripotent state in absence of the RPs responsible.

In addition, RPs come in two flavors, RPs associated with the small subunit of the ribosome and with the large subunit of the ribosome. As both RPs are heterogeneously expressed, the option exists that they act in a modular fashion, both having a separate function that in combination results in a specific function. To include this possibility, expression patterns of small and large RPs should not be judged separately, but have to be assessed for similar expression profiles to identify these possible combinations.

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