Chapter I: Introduction

1.1. Synthetic biology and metabolic engineering in *Saccharomyces cerevisiae*

Synthetic biology is an emerging field that joins biology and engineering to design and build new biological systems exhibiting desired functions, such as the biosynthesis of drugs and biofuels in microorganisms and genetic therapies that can target diseased cells in humans[1-4]. Synthetic biologists have focused on the development and application of genetic tools and engineering principles to design and implement synthetic gene networks and the rewiring or reprogramming of endogenous cellular networks[5]. Developed genetic regulatory tools function in the cellular environment to control transcriptional, posttranscriptional, and posttranslational processes. Precise levels of gene expression are critical for the proper functioning of genetic networks[6-8]. As complexity increases with the size of engineered networks, there is a growing need for control elements that allow for the fine-tuning of the levels of protein components in the network[9]. There is a particular need for the development of genetic regulatory tools that function in eukaryotes, as the majority of devices to date have been built in prokaryotes.

Metabolic engineering is defined as the redirection of cellular metabolism for the production of valuable chemicals and the removal of harmful or toxic compounds from the environment[10]. Research in this area often involves the implementation of gene expression tools to precisely control enzyme levels and thus regulate flux through natural or heterologous pathways[11-13]. While synthetic chemistry has traditionally been the main method used to synthesize chemicals for a wide variety of industries, many chemicals,

particularly ones with multiple chiral carbon centers, have proven extremely difficult to synthesize through these traditional methods[14]. Metabolic engineering addresses these challenges by utilizing enzymes to perform chemical conversions, which generally exhibit stereospecificity, thereby resulting in the efficient production of chiral products. Enzymatic reactions performed inside cells offer several advantages over *in vitro* based systems in that cells can be used to generate and replenish the desired enzymes and necessary cofactors from inexpensive starting materials and provide appropriate precursor chemicals[15]. However, the redirection and construction of cellular metabolic networks is not as straightforward as cloning the genes that encode the appropriate enzymes into the cell. Cellular productivity can be negatively impacted by metabolic burden associated with enzyme overexpression[16-17], the accumulation of cytotoxic intermediates[12, 18-19], and the redirection of cellular resources from central metabolism[20-22]. The tuning of enzymes levels has been found to be crucial for optimizing metabolic flux to alleviate these detrimental issues and achieve the desired function, namely increased product yield[12, 16, 23-24].

There are many examples of plants and other higher-level organisms that naturally produce chemicals that are of interest to various industries[25-27]. In particular, many plant species produce compounds with diverse pharmacological activities that are of interest as drug molecules[25]. These compounds have been traditionally extracted from their natural hosts. However, higher-order eukaryotic cells have very long doubling times and, due to differentiation, not every cell necessarily produces the product of interest. For example, natural products of interest in plants have been found to amass at low quantities and extraction procedures can be difficult due to the production of other chemically

similar compounds and the use of toxic solvents[28-29]. In addition, there are additional costs associated with the land and resources (including manpower) required to grow plants. It is desirable to transfer the ability to make these chemicals into organisms that grow more rapidly on inexpensive energy sources to lower the cost of these compounds. The construction of a biosynthetic network begins with the selection of the appropriate organism that naturally produces required intermediates or demonstrates similar chemistries[30]. A common tactic is to reconstitute the system in common host organisms used in industrial fermentation applications: a bacterium, *Escherichia coli*; and a eukaryotic microorganism, *Saccharomyces cerevisiae*.

Although *E. coli* is robust and fast growing, there are limitations in its ability to effectively express enzymes from eukaryotic host organisms. Many of these problems arise from differences in the protein expression pathways between bacteria and eukaryotic organisms. For instance, posttranslational processes such as glycosylation and the localization of enzymes to intracellular membranes are present in eukaryotes but not in prokaryotes. Therefore, *S. cerevisiae* can overcome these deficiencies present in *E. coli*, while having advantages over other eukaryotic cell lines due to its small fully-sequenced genome, fast doubling time, ability to grow in inexpensive chemically defined medium, and ease of scale-up to fermentation vessels similar to those used to grow *E. coli*[31-32]. Additional advantages of *S. cerevisiae* are associated with the accumulated knowledge of the organism's genetics, physiology, and biochemistry, its classification as GRAS (generally regarded as safe) by the U.S. Food and Drug Administration (FDA), and its tolerance at low pH levels and high concentrations of sugar and ethanol[33].

Enzyme levels are typically regulated by controlling the copy number of heterologous genes, transcription efficiency, translation efficiency, transcript abundance, and protein abundance[33]. In *S. cerevisiae*, very few genetic tools exist to control transcript levels and the translation of transcripts. The majority of genetic tools developed to date have focused on the incorporation of different endogenous promoter systems or the re-engineering of promoters to modulate the transcriptional output or the response to factors of transcriptional activation[34-38]. Posttranscriptional elements have the advantage of being coupled to any promoter of choice, providing for enhanced control strategies. Internal ribosome entry sites (IRESes) and AU-rich elements (AREs) have demonstrated the ability to modulate gene expression in yeast, while more recently, antisense- and ribozyme-based riboswitches have shown the ability to enhance or repress gene expression due to presence of a small molecule effector[39-43].

In the following sections of the Introduction, a detailed explanation of eukaryotic posttranscriptional mechanisms will be provided. Numerous RNA-based elements that regulate or bypass these mechanisms will be described. Finally, two specific regulatory elements, Rnt1p hairpin substrates and IRESes, will be described, including their function in *S. cerevisiaie*.

1.2. Common pathways of transcriptional decay and translation in yeast

The cellular processes of transcription, translation, and transcript turnover are common across all eukaryotes and prokaryotes. However, the eukaryotic gene expression pathway is more complex and contains intermediate steps between transcription and translation that provide further mechanisms of control such as splicing, transcript editing,

and nuclear export. In eukaryotes, a mature transcript is formed through a series of coupled processing events (Figure 1.1). Initially, a pre-messenger RNA (pre-mRNA) is transcribed from a gene by RNA polymerase II. The first processing step in the production of a translationally-competent transcript is the addition of a 5' cap, which contains a methylated guanine nucleotide[44]. The purpose of the cap is to protect the transcript from degradation by 5' to 3' exonucleases and to stimulate the initiation of translation[45]. The next processing step is the removal of introns, intervening noncoding sequences found within the coding region, to form the mature transcript through a process called splicing[44]. The final step before nuclear export to the cytoplasm is a 3' end modification in which a poly(A) tail is added. The transcript is then exported to the cytoplasm where it undergoes cytoplasmic decay or translation to produce protein molecules. Cells control the level of proteins by regulating each one of these steps, from the chromatin remodeling necessary for transcription of many genes to posttranslational protein stability.

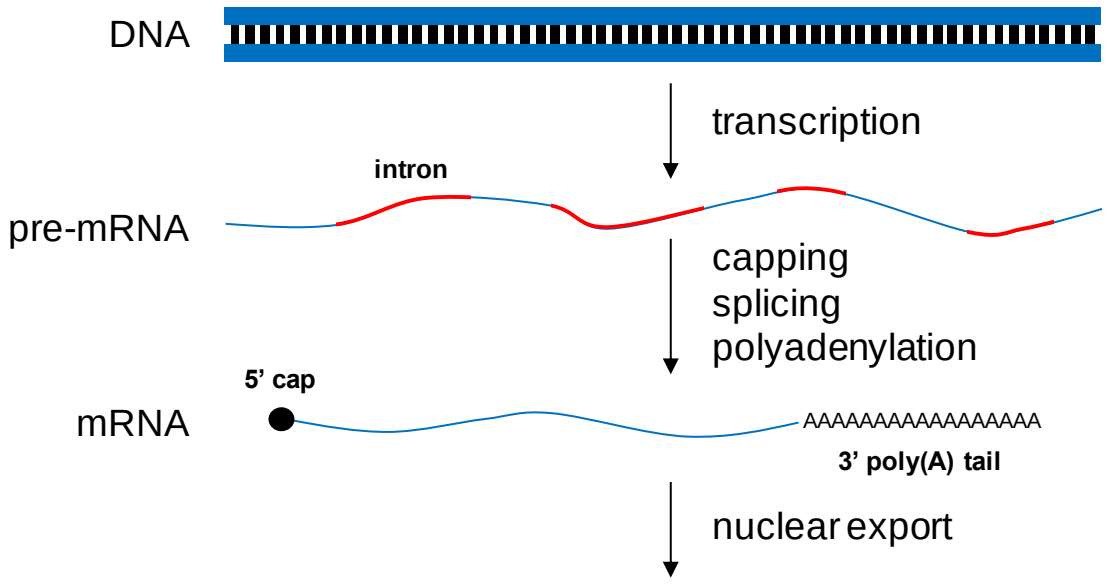

Figure 1.1. Maturation of eukaryotic transcripts after transcription.

1.2.1. Deadenylation-dependent decapping pathway of transcript degradation

There are several mechanisms by which transcripts are degraded, including the deadenylation-dependent decapping pathway, the deadenylation-independent decapping pathway, and the endoribonucleolytic cleavage pathway. In *S. cerevisiae*, the most common degradation pathway is deadenylation-dependent decapping.

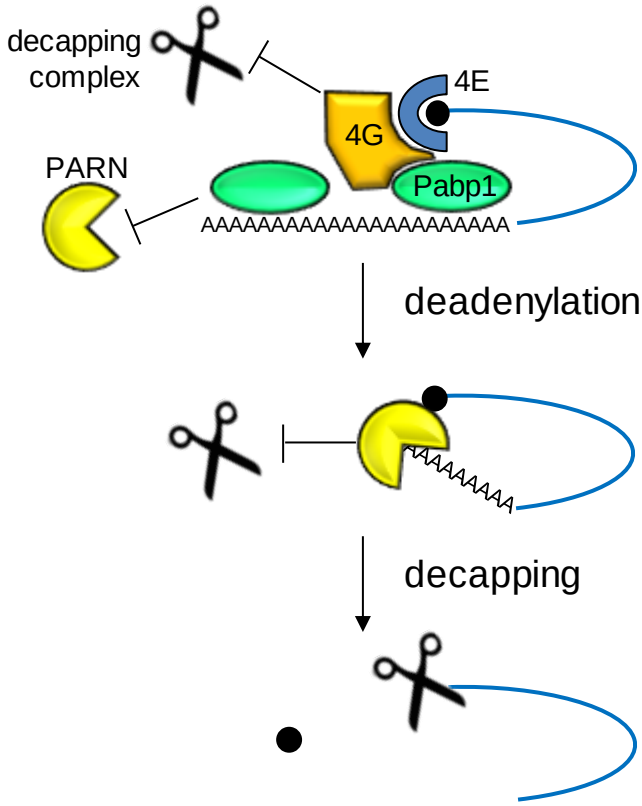

Figure 1.2. The deadenylation-dependent decapping pathway of transcript degradation in eukaryotes. 4E denotes eIF4E and 4G denotes eIF4G. Adapted from Wilusz et al. (2001)[46].

Transcripts are present in a circular conformation due to the interaction of the cap-binding protein, eukaryotic transcription factor (eIF)4E, on the 5' cap and the poly(A) binding protein (Pab1p) on the 3' poly(A) tail mediated through binding to eIF4G, a scaffolding protein (Figure 1.2). This circularization promotes translation and prevents the activity of decapping and deadenylation enzymes[46]. The interaction of Pab1p

with the poly(A) tail inhibits deadenylation[47]. When the poly(A) ribonuclease (PARN) binds to the 5' cap, it disrupts the cap's interaction with eIF4E causing a displacement of Pab1p, which allows deadenylation to occur[46, 48]. Deadenylation is the rate-limiting step in transcript decay[49]. While there are several different deadenylases that could be functioning[50], the predominant form in yeast is Ccr4p[51]. Once deadenylation is completed, PARN no longer stays associated with the cap and the decapping complex of Dcp1 and Dcp2 cleaves off the cap allowing a 5' to 3' exonuclease (Xrn1p) to rapidly degrade the rest of the transcript[46-47, 52]. 3' to 5' exonucleolytic activity does occur after deadenylation, but it tends to be slower than the activity of Xrn1p[49, 53].

1.2.2. Cap-dependent translation initiation

Translation initiation begins when the 40S small ribosomal subunit associates with two eIFs, eIF2 and eIF3, and the initiator methionine tRNA to form the 43S preinitiation complex (Figure 1.3)[54]. eIF2 must also be bound by guanosine 5'-triphosphate (GTP) in order for it to associate with the 40S ribosome. On the transcript, the 5' cap is bound by a cap-binding protein complex, eIF4F, which consists of three subunits: eIF4A, an RNA helicase; eIF4E, the actual cap-binding protein; and eIF4G, a scaffolding protein[55-56]. The transcript is initially in a closed, circular form due to eIF4G's interactions with both eIF4E at the 5' cap and Pab1p at the 3' poly(A) tail. The 43S complex binds eIF4F to form the 48S complex and scanning of the transcript begins for the initiating AUG start codon[57]. The scanning by the complex for AUG is caused by more initiation factors powered by ATP. Once the start codon is located, eIF1 and eIF1A bind to stabilize the binding of the 48S complex to the transcript[56]. eIF5 then stimulates

the hydrolysis of GTP on eIF2 followed by the association of the large 60S ribosomal subunit with the 48S complex to form the complete 80S ribosome[44, 56]. The complex is then in a form where translation can initiate and proceeds to the elongation stage.

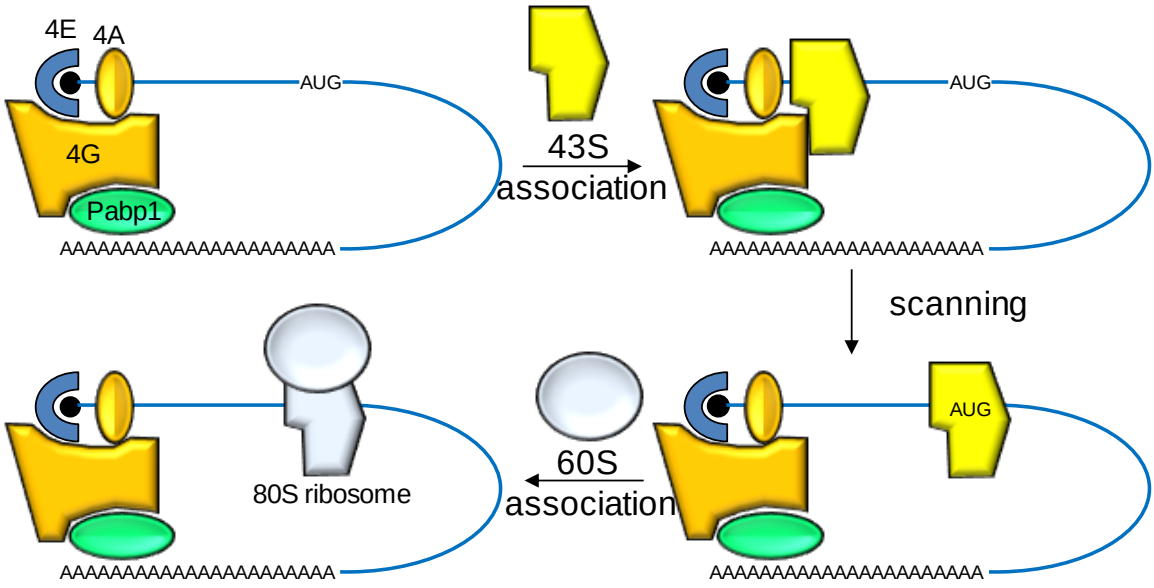

Figure 1.3. The mechanism of eukaryotic cap-dependent translation. 4A denotes eIF4A; 4E denotes eIF4E; and 4G denotes eIF4G. Adapted from Klann and Dever (2004)[58].

1.3. Posttranscriptional regulation through transcript stability and translation

1.3.1. Control of transcript decay

As discussed in Section 1.2.1, the deadenylation-dependent decapping pathway is the primary mechanism by which eukaryotic transcripts are degraded. Briefly, transcripts contain a 3' poly(A) tail whose interactions with proteins inhibit decapping. Once the tail is removed, decapping proceeds and the transcript is degraded 5' to 3' by an exonuclease. Eukaryotes have evolved additional non-coding elements in their genetic untranslated regions (UTRs) and sometimes in the actual coding regions by which this pathway can be

attenuated or bypassed[52]. These elements include deadenylation-independent decapping elements, transcript stability elements, and elements conveying endonucleolytic activity.

In the deadenylation-independent decapping pathway, transcript degradation proceeds without the removal of the 3' poly(A) tail. This pathway proceeds through the recruitment of elements that enhance decapping. The *RPS28B* transcript in *S. cerevisiae* contains a stem-loop structure within its 3' UTR and encodes for a protein, Rps28B, that binds directly to that stem-loop[59]. The Rps28B protein product recruits a decapping-enhancing protein, Edc3, and Edc3, in turn, recruits several other factors that lead to the decapping of the transcript. The *EDC1* transcript in *S. cerevisiae* encodes for a decapping-enhancing protein, Edc1, though it is unknown if Edc1 plays a role in *EDC1* degradation[52]. *EDC1* contains a stretch of uridine nucleotides that interacts with the poly(A) tail inhibiting deadenylation[60]. The decapping of the transcript is caused by several protein factors including those associated with deadenylation.

Transcript stability elements are located at multiple positions on the transcript, but primarily in the 3' UTR[52]. The largest class of elements that has been examined is the ARE in the 3' UTR. AREs are identified by a consensus AUUUA pentamer, but its activity is dependent on the context and number of those pentamers[52]. AREs can destabilize the transcript through the interaction of the sequence itself or ARE-binding proteins with the transcript decay protein complex[61-62]. AREs can also stabilize transcripts, where proposed mechanisms are based on competition with destabilizing factors or inhibition of deadenylation-dependent decapping decay, such as through strengthening the interaction between PABP and the poly(A) tail[52]. In addition, the PUF family of proteins binds to UG-rich sequences found in the 3' UTR of transcripts.

Destabilization by PUF binding is due to recruitment of deadenylases[63]. As another example, the proteins αCP1 and αCP2 are responsible for the stabilization of several genes through interactions with pyrimidine-rich elements in the 3' UTR. It is believed that the observed transcript stabilization is due to interactions with PABP that protect the poly(A) tail from deadenylases[64].

Endoribonucleolytic decay can also be described as deadenylation-independent and decapping-independent decay. Internal cleavage of the transcript results in the generation of two RNA fragments with unprotected ends (Figure 1.4)[52]. The 3' fragment is susceptible to exonucleolytic decay by Xrn1p in the 5' to 3' direction, while the 5' fragment is degraded in the same manner once the cap is removed or by 3' to 5' exonucleases. In eukaryotes containing the RNA interference (RNAi) pathway, gene expression is modulated through directed endonucleolytic cleavage, referred to as "Slicer" activity, of the target transcript[65]. Cleavage is mediated through components of the RNA-induced silencing complex (RISC), which contains the RNase III enzyme variant Dicer[66-68]. MicroRNAs (miRNAs) or small interfering RNAs (siRNAs) are loaded onto RISC and direct the complex to the transcript through perfect or nearly perfect (some mismatches allowed) base-pairing between the transcript and the miRNA/siRNA[69-71]. There are numerous endoribonucleases that regulate expression levels in eukaryotes, although for many of the enzymes, such as PRM1, IRE1, and RNase MRP, *cis*-acting consensus binding regions have not yet been determined[72-74]. As an alternative to the endonucleolytic cleavage caused by *trans* factors, transcript degradation in a diverse group of eukaryotes can also be mediated through *cis* self-cleaving catalytic RNA structures called ribozymes[75]. The RNase III variant Drosha and the *S. cerevisiae*-specific

RNase III variant Rnt1p has been shown to cleave transcripts containing stem loop structures[76-77]. Rnt1p specifically processes transcripts containing hairpins with AGNN tetraloops[78] and is explained in further detail in Section 1.3.3.

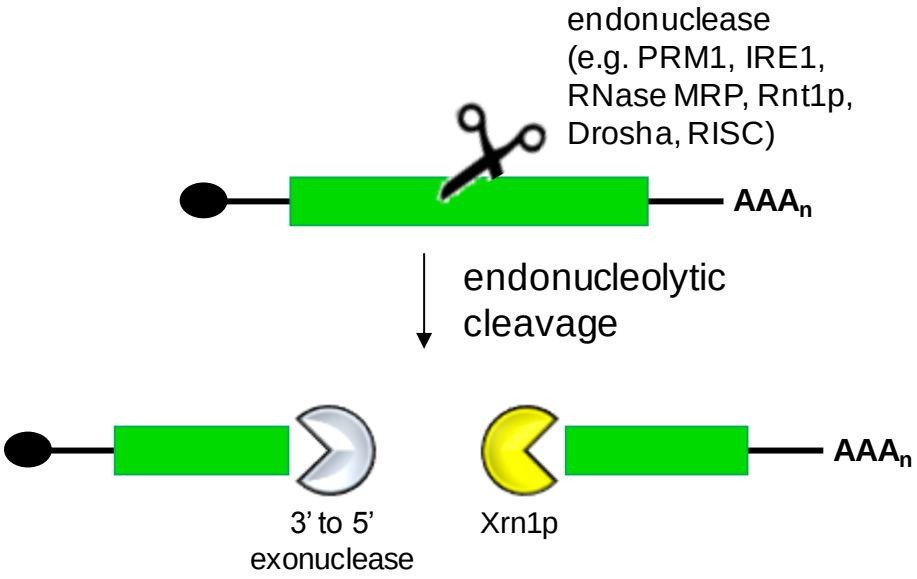

Figure 1.4. The processing of eukaryotic transcripts following endonucleolytic cleavage is independent of the 5' cap and the 3' poly(A) tail. Scissors denote the endonuclease. Adapted from Garneau et al. (2007)[52].

1.3.2. Control of the initiation of translation

As discussed in Section 1.2.2, the initiation of translation in eukaryotes is mediated through protein interactions at the 5' cap between eIFs and the small ribosomal subunit. The preinitiation complex scans the transcripts for the AUG start codon, where the large ribosomal subunit binds and translation begins. The majority of translational control is due to interference with the normal processes of the ribosome and the eIFs (Figure 1.5)[79]. Cap-independent translation by IRESes is described in its own section (1.3.4). The majority of these elements are located in the 5' UTR.

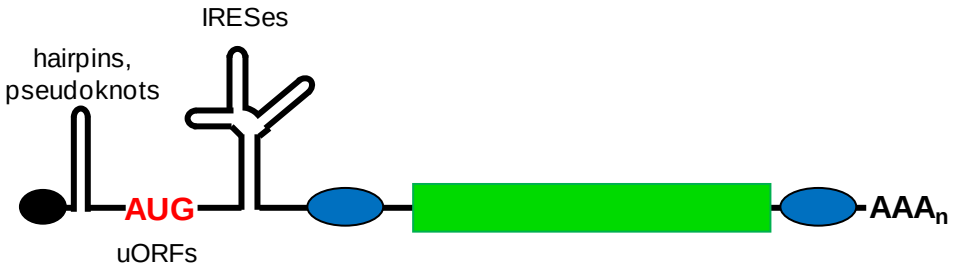

Figure 1.5. Genetic elements that affect the initation of translation in eukaryotes. The blue ovals in the 5' and 3' UTRs represent binding sites for protein factors that typically inhibit translation. Adapted from Gebauer and Hentze (2004)[79].

Translational repression for the ferritin transcript is mediated through a stem-loop structure called an iron-responsive element (IRE) located 40 nucleotides from the cap in the 5' UTR[80-81]. Iron regulatory proteins (IRPs) bind the IRE blocking the recruitment of the preinitiation complex to the 5' cap due to steric hinderance[82]. Steric hinderance is also observed with secondary and tertiary structures in the 5' UTR, such as RNA hairpins and pseudoknots[79]. Transcripts containing a U-rich sequence known as a cytoplasmic polyadenylational element (CPE) in the 3' UTR interact with the CPE-binding protein (CPEB)[83]. CPEB represses translation by associating with another protein, Maskin, that contains an eIF4E-binding domain, which inhibits eIF4E's interaction with eIF4G[84]. Translational repression can also interfere with the ribosome after it has been bound to the cap. The sex-lethal protein (Sxl) binds to U-rich sites on the *msl-2* transcript located in both the 5' and 3' UTR[85]. The binding of Sxl interferes with ribosomal scanning. Another cap-independent method interferes with the association of the large ribosomal submit in *LOX3* transcripts[86]. Here, two proteins, hnRNP K and hnRNP E, bind a differentiation-control element (DICE), which is a repeated CU-rich element, in the 3' UTR and block formation of the 80S ribosome. In the previous section, we discussed endonucleolytic cleavage mediated by the interaction of siRNA and miRNA to RISC.

The miRNA silencing pathway is also known to repress translation through direct or indirect interference with eIFs[65, 87].

1.3.3. RNA processing by the RNase III enzyme Rnt1p

The RNase III family is a class of enzymes that cleaves double-stranded RNA (dsRNA)[88]. Dicer is an RNase III enzyme in humans and other eukaryotes that cleaves dsRNA into 21–23 nt fragments referred to as siRNAs that go on to induce gene silencing though the RNAi pathway[89]. Drosha, another eukaryotic RNase III involved in the RNAi pathway, is involved in the processing of miRNA from long dsRNA transcripts referred to as primary (pri-)miRNA[90]. Rnt1p was discovered in *S. cerevisiae* due to similarities to the *E. coli* RNase III[91] and has been shown to cleave cellular ribosomal RNA (rRNA) precursors, small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and messenger RNA (mRNA)[91-94]. This protein is localized to the nucleus[95] and contains two domains: an RNase III domain and a dsRNA-binding domain (dsRBD)[96].

The RNA hairpin substrates of Rnt1p contain a consensus AGNN tetraloop with a cleavage site 14–16 base-pairs (bp) from the tetraloop[78]. The AGNN tetraloop forms a predetermined fold that is recognized by the dsRBD[88, 97]. The dsRNA region of Rnt1p substrates has an effect on the binding affinity and cleavage rate with this enzyme. The base-pairs immediately below the tetraloop can impact Rnt1p binding, while sequences near the cleavage site influence the cleavage rate[98]. These observations led to the definition of three regions on Rnt1p substrates: the initial binding and positioning box (IBPB) which consists of the tetraloop; the binding stability box (BSB) which is the base-paired region immediately adjacent to the tetraloop; and the cleavage efficiency box

(CEB) which is the region containing and surrounding the cleavage site[98]. An unique feature of Rnt1p is that it uses the tetraloop as its primary binding site, whereas for other RNase III enzymes it is the RNA helix[99].

Rnt1p is involved in the natural regulation of several genes in *S. cerevisiae*. Previously, transcripts had been discovered that undergo endonucleolytic cleavage but by factors other than Rnt1p[100-101]. The *RPS22B* and *RPL18A* transcripts contain intronic Rnt1p substrates that deplete unspliced transcripts as well as reducing levels of the mature transcripts[94]. The *MIG2* transcript contains an Rnt1p substrate in its coding region that increases the transcript's sensitivity to glucose-dependent degradation[77]. Several transcripts involved in iron uptake or iron mobilization contain Rnt1p substrates in the coding region that help avoid cytotoxicity to the cellular iron starvation reponse[102]. The diversity of structure and sequences in natural Rnt1p substrates, as well as the identification of critical regions, support that a set of engineered Rnt1p hairpins can be generated with differential activity.

1.3.4. Translation initiation mediated through internal ribosome entry sites

IRESes were initially discovered during the analysis of the 5' UTRs of picornaviral transcripts where it was determined that the transcripts lacked a 5' cap and translation continued in the absence of the cap-binding protein, eIF4F[103]. IRESes are critical elements for the translation of the genome of several viruses, including the Hepatitis A virus (HAV)[104], the Hepatitis C virus[105], the foot-and-mouth-disease virus (FMDV)[106], and the human immunodeficiency virus (HIV)[107]. Viral IRESes contain a diverse range of secondary and tertiary structures that mimic components of the

ribosomal machinery or mimic the interaction of such components with the 5' cap and other protein factors (Figure 1.6)[108-113]. IRESes illustrate the scaffolding power of RNA structures and the creative mechanisms by which viruses have evolved to essentially hijack the host-based expression machinery.

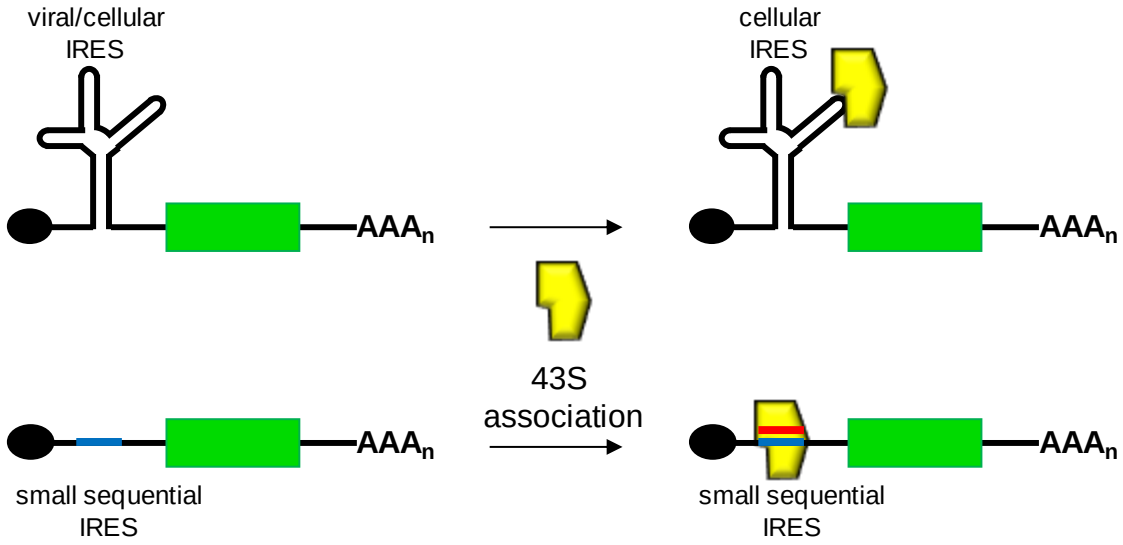

Figure 1.6. Simplified schematic of the interactions with structural and sequential IRESes with the translational machinery. Red line denotes the sequences on the 18S rRNA complementary to the IRES sequence (blue line).

Cellular IRESes were first discovered when researchers observed that immunoglobulin heavy chain binding protein (BiP) continued being expressed after cap-dependent translation had been shut down due to poliovirus infection[109]. Many cellular IRESes characterized thus far contain a Y-shaped stem-loop structure upstream of the initiation codon; however, the activity of these IRESes may not necessarily depend on that secondary structure[114-115]. In a mouse cell line, deletional studies of the structured 5' UTR of the Gtx protein, which demonstrated IRES activity, identified a 9 nucleotide (nt) module that retained the ability to internally initiate translation[114]. When multiple modules of the 9-nt module were placed in tandem, a synergistic effect was observed as

overall IRES activity increased. This 9-nt segment was determined to be completely complementary to a segment of the 18S ribosomal RNA, a critical component of the ribosomal machinery (Figure 1.6)[116]. In *S. cerevisiae*, two IRES-containing 5' UTRs for the *YAP1* and *p150* genes were also found to contain several regions of complementarity to 18S rRNA[39]. These studies have demonstrated that cellular IRESes contain regions that directly base-pair to the 18S rRNA. This mechanism of translation initiation by cellular IRESes in eukaryotes suggest that their function is analogous to Shine-Dalgarno sequences in prokaryotes, which initiate translation through direct base-pairing with the prokaryotic analogue of the 18S rRNA, the 16S rRNA[117]. Based on this observation, a short segment of nucleotides in the intercistronic region (IR) of a yeast and mammalian dicistronic vector were randomized and screened for IRES activity by expression of the second cistron[118-119]. The resultant IRESes demonstrated complementarity to the 18S rRNA.

Prokaryotic genes are typically expressed from operons, where multiple coding regions are located on one transcript under the control of a single promoter. Each coding region contains a Shine-Dalgarno sequence upstream of its start codon in order to initiate translation of each gene. Viruses are also known to produce multicistronic transcripts or genomes. For example, the entire positive-strand genome of HCV is contained on a single piece of RNA[120]. The entire genome is translated through an IRES at the 5' end. The resultant polyprotein is then processed by a series of proteases and peptidases to create each individual protein product. HIV translation is similar to HCV except that its IRES can also cause translation initation at multiple start codons resulting in different protein products[121]. In a manner akin to prokaryotic operons, multicistronic transcripts

can be generated through the introduction of an IRES element before each gene. Retroviral mulitcistronic vectors had been developed in mammalian systems where multiple viral IRESes were incorporated[122-123]. Recently, a dicistronic reporter construct had been characterized in *S. cerevisiae* where the *YAP1* and *p150* IRES were placed in the IR to alter the ratio of expression between two genes[124]. Since only the *p150* IRES worked in this system, the work highlights the need for additional IRESes to be discovered or engineered in yeast to increase the ability to tune gene expression through this method.

1.4. Interrelationship among the thesis projects

Chapter I provides an overview of the field of synthetic biology and metabolic engineering and gives an in-depth examination of the cellular processes of transcript translation initiation and decay and the RNA elements that control these processes. Chapter II describes the development of a library of RNA hairpins that regulate posttranscriptional decay to attenuate gene expression due to the endonucleolytic processing of the hairpins by the *S. cerevisiae* RNase III Rnt1p. The library is based on the randomization of nucleotides associated with controlling the cleavage rate by the enzyme. Chapter III describes a second library of Rnt1p-cleaved hairpins based on the randomization of nucleotides associated with the binding of Rnt1p to the hairpin. In addition, the two library elements are integrated combinatorially to extend the accessible levels of gene expression. Chapter IV describes the integration of the small molecule-responsive aptamers into Rnt1p substrates to achieve ligand-controlled cleavage. The engineered riboswitches function through direct inhibition of Rnt1p activity by ligand

binding in proximity of the cleavage sites and switching dynamics are altered through incorporation of additional aptamers and Rnt1p-based modules, as well as the construction of multiple switch devices in tandem. Chapter V describes a strategy to select for a library of small sequential IRES elements with various strengths to initiate translation at physiological conditions. These elements will aid in the development of yeast 'operons' or multicistronic vectors where relative gene expression levels can be controlled. These research projects collectively demonstrate the capacity of utilizing RNA-based control elements to predictably tune gene expression levels in *S. cerevisiae*.

References

1. Chen, Y.Y., Jensen, M.C. & Smolke, C.D. Genetic control of mammalian T-cell proliferation with synthetic RNA regulatory systems. *Proc Natl Acad Sci U S A* **107**, 8531-8536 (2010).
2. Culler, S.J., Hoff, K.G. & Smolke, C.D. Reprogramming cellular behavior with RNA controllers responsive to endogenous proteins. *Science* **330**, 1251-1255 (2010).
3. Lee, S.K., Chou, H., Ham, T.S., Lee, T.S. & Keasling, J.D. Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. *Curr Opin Biotechnol* **19**, 556-563 (2008).
4. Lee, S.Y., Kim, H.U., Park, J.H., Park, J.M. & Kim, T.Y. Metabolic engineering of microorganisms: general strategies and drug production. *Drug Discov Today* **14**, 78-88 (2009).

5. Andrianantoandro, E., Basu, S., Karig, D.K. & Weiss, R. Synthetic biology: new engineering rules for an emerging discipline. *Mol Syst Biol* **2**, 2006 0028 (2006).

6. Elowitz, M.B. & Leibler, S. A synthetic oscillatory network of transcriptional regulators. *Nature* **403**, 335-338 (2000).

7. Gardner, T.S., Cantor, C.R. & Collins, J.J. Construction of a genetic toggle switch in Escherichia coli. *Nature* **403**, 339-342 (2000).

8. Basu, S., Mehreja, R., Thiberge, S., Chen, M.T. & Weiss, R. Spatiotemporal control of gene expression with pulse-generating networks. *Proc Natl Acad Sci U S A* **101**, 6355-6360 (2004).

9. Khalil, A.S. & Collins, J.J. Synthetic biology: applications come of age. *Nat Rev Genet* **11**, 367-379 (2010).

10. Smolke, C., Martin, V. & Keasling, J. in Protein expression technologies: current status and future trends. (ed. F. Baneyx) (Horizon Bioscience, Wymondham, Norfolk; 2004).

11. Pitera, D.J., Paddon, C.J., Newman, J.D. & Keasling, J.D. Balancing a heterologous mevalonate pathway for improved isoprenoid production in Escherichia coli. *Metab Eng* **9**, 193-207 (2007).

12. Pfleger, B.F., Pitera, D.J., Smolke, C.D. & Keasling, J.D. Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. *Nat Biotechnol* **24**, 1027-1032 (2006).

13. Peralta-Yahya, P.P. & Keasling, J.D. Advanced biofuel production in microbes. *Biotechnol J* **5**, 147-162 (2010).

14. Hughes, E.H. & Shanks, J.V. Metabolic engineering of plants for alkaloid production. *Metab Eng* **4**, 41-48 (2002).

15. Smolke, C.D., Martin, V.J. & Keasling, J.D. in Protein Expression Technologies: Current Status and Future Trends. (ed. F. Baneyx) (Horizon Scientific Press, U.K.; 2004).

16. Jin, Y.S., Ni, H., Laplaza, J.M. & Jeffries, T.W. Optimal growth and ethanol production from xylose by recombinant Saccharomyces cerevisiae require moderate D-xylulokinase activity. *Appl Environ Microbiol* **69**, 495-503 (2003).

17. Jones, K.L., Kim, S.W. & Keasling, J.D. Low-copy plasmids can perform as well as or better than high-copy plasmids for metabolic engineering of bacteria. *Metab Eng* **2**, 328-338 (2000).

18. Zhu, M.M., Lawman, P.D. & Cameron, D.C. Improving 1,3-propanediol production from glycerol in a metabolically engineered Escherichia coli by reducing accumulation of sn-glycerol-3-phosphate. *Biotechnol Prog* **18**, 694-699 (2002).

19. Zhu, M.M., Skraly, F.A. & Cameron, D.C. Accumulation of methylglyoxal in anaerobically grown Escherichia coli and its detoxification by expression of the Pseudomonas putida glyoxalase I gene. *Metab Eng* **3**, 218-225 (2001).

20. Alper, H., Jin, Y.S., Moxley, J.F. & Stephanopoulos, G. Identifying gene targets for the metabolic engineering of lycopene biosynthesis in Escherichia coli. *Metab Eng* **7**, 155-164 (2005).

21. Alper, H., Miyaoku, K. & Stephanopoulos, G. Construction of lycopene-overproducing E. coli strains by combining systematic and combinatorial gene knockout targets. *Nat Biotechnol* **23**, 612-616 (2005).

22. Paradise, E.M., Kirby, J., Chan, R. & Keasling, J.D. Redirection of flux through the FPP branch-point in Saccharomyces cerevisiae by down-regulating squalene synthase. *Biotechnol Bioeng* **100**, 371-378 (2008).

23. Dueber, J.E. et al. Synthetic protein scaffolds provide modular control over metabolic flux. *Nat Biotechnol* **27**, 753-759 (2009).

24. Alper, H., Fischer, C., Nevoigt, E. & Stephanopoulos, G. Tuning genetic control through promoter engineering. *Proc Natl Acad Sci U S A* **102**, 12678-12683 (2005).

25. Morant, M., Bak, S., Moller, B.L. & Werck-Reichhart, D. Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. *Curr Opin Biotechnol* **14**, 151-162 (2003).

26. Veen, M. & Lang, C. Production of lipid compounds in the yeast Saccharomyces cerevisiae. *Appl Microbiol Biotechnol* **63**, 635-646 (2004).

27. Hughes, E.H., Hong, S.B., Gibson, S.I., Shanks, J.V. & San, K.Y. Metabolic engineering of the indole pathway in Catharanthus roseus hairy roots and increased accumulation of tryptamine and serpentine. *Metab Eng* **6**, 268-276 (2004).

28. Chemler, J.A. & Koffas, M.A. Metabolic engineering for plant natural product biosynthesis in microbes. *Curr Opin Biotechnol* **19**, 597-605 (2008).

29. Silva, G.L., Lee, I. & Kinghorn, A.D. in Natural Products Isolation. (ed. R.J.P. Cannell) (Humana Press, Totowa, New Jersey; 1998).

30. Alper, H. & Stephanopoulos, G. Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? *Nat Rev Microbiol* **7**, 715-723 (2009).

31. Cereghino, G.P. & Cregg, J.M. Applications of yeast in biotechnology: protein production and genetic analysis. *Curr Opin Biotechnol* **10**, 422-427 (1999).

32. Gerngross, T.U. Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. *Nat Biotechnol* **22**, 1409-1414 (2004).

33. Nevoigt, E. Progress in metabolic engineering of Saccharomyces cerevisiae. *Microbiol Mol Biol Rev* **72**, 379-412 (2008).

34. Hawkins, K.M. & Smolke, C.D. The regulatory roles of the galactose permease and kinase in the induction response of the GAL network in Saccharomyces cerevisiae. *J Biol Chem* **281**, 13485-13492 (2006).

35. Nevoigt, E. et al. Engineering promoter regulation. *Biotechnol Bioeng* **96**, 550-558 (2007).

36. Nevoigt, E. et al. Engineering of promoter replacement cassettes for fine-tuning of gene expression in Saccharomyces cerevisiae. *Appl Environ Microbiol* **72**, 5266-5273 (2006).

37. Mumberg, D., Muller, R. & Funk, M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156**, 119-122 (1995).

38. Jeppsson, M., Johansson, B., Jensen, P.R., Hahn-Hagerdal, B. & Gorwa-Grauslund, M.F. The level of glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor sensitivity in recombinant Saccharomyces cerevisiae strains. *Yeast* **20**, 1263-1272 (2003).

39. Zhou, W., Edelman, G.M. & Mauro, V.P. Transcript leader regions of two Saccharomyces cerevisiae mRNAs contain internal ribosome entry sites that function in living cells. *Proc Natl Acad Sci U S A* **98**, 1531-1536 (2001).

40. Vasudevan, S. & Peltz, S.W. Regulated ARE-mediated mRNA decay in Saccharomyces cerevisiae. *Mol Cell* **7**, 1191-1200 (2001).

41. Lautz, T., Stahl, U. & Lang, C. The human c-fos and TNFalpha AU-rich elements show different effects on mRNA abundance and protein expression depending on the reporter in the yeast Pichia pastoris. *Yeast* **27**, 1-9 (2010).

42. Bayer, T.S. & Smolke, C.D. Programmable ligand-controlled riboregulators of eukaryotic gene expression. *Nat Biotechnol* **23**, 337-343 (2005).

43. Win, M.N. & Smolke, C.D. A modular and extensible RNA-based gene-regulatory platform for engineering cellular function. *Proc Natl Acad Sci U S A* **104**, 14283-14288 (2007).

44. Alberts, B. Molecular biology of the cell, Edn. 4th. (Garland Science, New York; 2002).

45. Day, D.A. & Tuite, M.F. Post-transcriptional gene regulatory mechanisms in eukaryotes: an overview. *J Endocrinol* **157**, 361-371 (1998).

46. Wilusz, C.J., Wormington, M. & Peltz, S.W. The cap-to-tail guide to mRNA turnover. *Nat Rev Mol Cell Biol* **2**, 237-246 (2001).

47. Tucker, M. & Parker, R. Mechanisms and control of mRNA decapping in Saccharomyces cerevisiae. *Annu Rev Biochem* **69**, 571-595 (2000).

48. Dehlin, E., Wormington, M., Korner, C.G. & Wahle, E. Cap-dependent deadenylation of mRNA. *Embo J* **19**, 1079-1086 (2000).

49. Jacobson, A. & Peltz, S.W. Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. *Annu Rev Biochem* **65**, 693-739 (1996).

50. Mitchell, P. & Tollervey, D. mRNA stability in eukaryotes. *Curr Opin Genet Dev* **10**, 193-198 (2000).

51. Tucker, M. et al. The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in Saccharomyces cerevisiae. *Cell* **104**, 377-386 (2001).

52. Garneau, N.L., Wilusz, J. & Wilusz, C.J. The highways and byways of mRNA decay. *Nat Rev Mol Cell Biol* **8**, 113-126 (2007).

53. Caponigro, G. & Parker, R. Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae. *Microbiol Rev* **60**, 233-249 (1996).

54. Huang, H. & Donahue, T.F. in mRNA Metabolism and Post-transcriptional Gene Regulation. (eds. J.B. Harford & D.R. Morris) (Wiley-Liss, New York; 1997).

55. Richter, J.D. & Sonenberg, N. Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* **433**, 477-480 (2005).

56. Weaver, R. Molecular biology, Edn. 2nd. (McGraw-Hill, New York; 2005).

57. Dever, T.E. Gene-specific regulation by general translation factors. *Cell* **108**, 545-556 (2002).

58. Klann, E. & Dever, T.E. Biochemical mechanisms for translational regulation in synaptic plasticity. *Nat Rev Neurosci* **5**, 931-942 (2004).

59. Badis, G., Saveanu, C., Fromont-Racine, M. & Jacquier, A. Targeted mRNA degradation by deadenylation-independent decapping. *Mol Cell* **15**, 5-15 (2004).

60. Muhlrad, D. & Parker, R. The yeast EDC1 mRNA undergoes deadenylation-independent decapping stimulated by Not2p, Not4p, and Not5p. *EMBO J* **24**, 1033-1045 (2005).

61. Anderson, J.R. et al. Sequence-specific RNA binding mediated by the RNase PH domain of components of the exosome. *RNA* **12**, 1810-1816 (2006).

62. Chen, C.Y. et al. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107**, 451-464 (2001).

63. Goldstrohm, A.C., Hook, B.A., Seay, D.J. & Wickens, M. PUF proteins bind Pop2p to regulate messenger RNAs. *Nat Struct Mol Biol* **13**, 533-539 (2006).

64. Wang, Z., Day, N., Trifillis, P. & Kiledjian, M. An mRNA stability complex functions with poly(A)-binding protein to stabilize mRNA in vitro. *Mol Cell Biol* **19**, 4552-4560 (1999).

65. Valencia-Sanchez, M.A., Liu, J., Hannon, G.J. & Parker, R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev* **20**, 515-524 (2006).

66. Liu, J. et al. Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**, 1437-1441 (2004).

67. Okamura, K., Ishizuka, A., Siomi, H. & Siomi, M.C. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev* **18**, 1655-1666 (2004).

68. Song, J.J., Smith, S.K., Hannon, G.J. & Joshua-Tor, L. Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* **305**, 1434-1437 (2004).

69. Guo, H.S., Xie, Q., Fei, J.F. & Chua, N.H. MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. *Plant Cell* **17**, 1376-1386 (2005).

70. Mallory, A.C. et al. MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region. *EMBO J* **23**, 3356-3364 (2004).

71. Yekta, S., Shih, I.H. & Bartel, D.P. MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304**, 594-596 (2004).

72. Gill, T., Cai, T., Aulds, J., Wierzbicki, S. & Schmitt, M.E. RNase MRP cleaves the CLB2 mRNA to promote cell cycle progression: novel method of mRNA degradation. *Mol Cell Biol* **24**, 945-953 (2004).

73. Hollien, J. & Weissman, J.S. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* **313**, 104-107 (2006).

74. Yang, F. & Schoenberg, D.R. Endonuclease-mediated mRNA decay involves the selective targeting of PMR1 to polyribosome-bound substrate mRNA. *Mol Cell* **14**, 435-445 (2004).

75. Serganov, A. & Patel, D.J. Ribozymes, riboswitches and beyond: regulation of gene expression without proteins. *Nat Rev Genet* **8**, 776-790 (2007).

76. Gregory, R.I., Chendrimada, T.P. & Shiekhattar, R. MicroRNA biogenesis: isolation and characterization of the microprocessor complex. *Methods Mol Biol* **342**, 33-47 (2006).

77. Ge, D., Lamontagne, B. & Elela, S.A. RNase III-mediated silencing of a glucose-dependent repressor in yeast. *Curr Biol* **15**, 140-145 (2005).

78. Chanfreau, G., Buckle, M. & Jacquier, A. Recognition of a conserved class of RNA tetraloops by Saccharomyces cerevisiae RNase III. *Proc Natl Acad Sci U S A* **97**, 3142-3147 (2000).

79. Gebauer, F. & Hentze, M.W. Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol* **5**, 827-835 (2004).

80. Gray, N.K. & Hentze, M.W. Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs. *EMBO J* **13**, 3882-3891 (1994).

81. Muckenthaler, M., Gray, N.K. & Hentze, M.W. IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4F. *Mol Cell* **2**, 383-388 (1998).

82. Stripecke, R., Oliveira, C.C., McCarthy, J.E. & Hentze, M.W. Proteins binding to 5' untranslated region sites: a general mechanism for translational regulation of mRNAs in human and yeast cells. *Mol Cell Biol* **14**, 5898-5909 (1994).

83. Mendez, R. & Richter, J.D. Translational control by CPEB: a means to the end. *Nat Rev Mol Cell Biol* **2**, 521-529 (2001).

84. Stebbins-Boaz, B., Cao, Q., de Moor, C.H., Mendez, R. & Richter, J.D. Maskin is a CPEB-associated factor that transiently interacts with elF-4E. *Mol Cell* **4**, 1017-1027 (1999).

85. Gebauer, F., Grskovic, M. & Hentze, M.W. Drosophila sex-lethal inhibits the stable association of the 40S ribosomal subunit with msl-2 mRNA. *Mol Cell* **11**, 1397-1404 (2003).

86. Ostareck, D.H., Ostareck-Lederer, A., Shatsky, I.N. & Hentze, M.W. Lipoxygenase mRNA silencing in erythroid differentiation: The 3'UTR regulatory complex controls 60S ribosomal subunit joining. *Cell* **104**, 281-290 (2001).

87. Humphreys, D.T., Westman, B.J., Martin, D.I. & Preiss, T. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc Natl Acad Sci U S A* **102**, 16961-16966 (2005).

88. Wu, H. et al. A novel family of RNA tetraloop structure forms the recognition site for Saccharomyces cerevisiae RNase III. *Embo J* **20**, 7240-7249 (2001).

89. Lingel, A. & Izaurralde, E. RNAi: finding the elusive endonuclease. *Rna* **10**, 1675-1679 (2004).

90. Lee, Y., Jeon, K., Lee, J.T., Kim, S. & Kim, V.N. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J* **21**, 4663-4670 (2002).

91. Elela, S.A., Igel, H. & Ares, M., Jr. RNase III cleaves eukaryotic preribosomal RNA at a U3 snoRNP-dependent site. *Cell* **85**, 115-124 (1996).

92. Chanfreau, G., Elela, S.A., Ares, M., Jr. & Guthrie, C. Alternative 3'-end processing of U5 snRNA by RNase III. *Genes Dev* **11**, 2741-2751 (1997).

93. Chanfreau, G., Rotondo, G., Legrain, P. & Jacquier, A. Processing of a dicistronic small nucleolar RNA precursor by the RNA endonuclease Rnt1. *Embo J* **17**, 3726-3737 (1998).

94. Danin-Kreiselman, M., Lee, C.Y. & Chanfreau, G. RNAse III-mediated degradation of unspliced pre-mRNAs and lariat introns. *Mol Cell* **11**, 1279-1289 (2003).

95. Catala, M., Lamontagne, B., Larose, S., Ghazal, G. & Elela, S.A. Cell cycle-dependent nuclear localization of yeast RNase III is required for efficient cell division. *Mol Biol Cell* **15**, 3015-3030 (2004).

96. Filippov, V., Solovyev, V., Filippova, M. & Gill, S.S. A novel type of RNase III family proteins in eukaryotes. *Gene* **245**, 213-221 (2000).

97. Wu, H., Henras, A., Chanfreau, G. & Feigon, J. Structural basis for recognition of the AGNN tetraloop RNA fold by the double-stranded RNA-binding domain of Rnt1p RNase III. *Proc Natl Acad Sci U S A* **101**, 8307-8312 (2004).

98. Lamontagne, B. et al. Sequence dependence of substrate recognition and cleavage by yeast RNase III. *J Mol Biol* **327**, 985-1000 (2003).

99. Lebars, I., Lamontagne, B., Yoshizawa, S., Aboul-Elela, S. & Fourmy, D. Solution structure of conserved AGNN tetraloops: insights into Rnt1p RNA processing. *EMBO J* **20**, 7250-7258 (2001).

100. Presutti, C., Ciafre, S.A. & Bozzoni, I. The ribosomal protein L2 in S. cerevisiae controls the level of accumulation of its own mRNA. *EMBO J* **10**, 2215-2221 (1991).

101. Vreken, P. & Raue, H.A. The rate-limiting step in yeast PGK1 mRNA degradation is an endonucleolytic cleavage in the 3'-terminal part of the coding region. *Mol Cell Biol* **12**, 2986-2996 (1992).

102. Lee, A., Henras, A.K. & Chanfreau, G. Multiple RNA surveillance pathways limit aberrant expression of iron uptake mRNAs and prevent iron toxicity in S. cerevisiae. *Mol Cell* **19**, 39-51 (2005).

103. Nomoto, A., Kitamura, N., Golini, F. & Wimmer, E. The 5'-terminal structures of poliovirion RNA and poliovirus mRNA differ only in the genome-linked protein VPg. *Proc Natl Acad Sci U S A* **74**, 5345-5349 (1977).

104. Glass, M.J. & Summers, D.F. A cis-acting element within the hepatitis A virus 5'-non-coding region required for in vitro translation. *Virus Res* **26**, 15-31 (1992).

105. Tsukiyama-Kohara, K., Iizuka, N., Kohara, M. & Nomoto, A. Internal ribosome entry site within hepatitis C virus RNA. *J Virol* **66**, 1476-1483 (1992).

106. Kuhn, R., Luz, N. & Beck, E. Functional analysis of the internal translation initiation site of foot-and-mouth disease virus. *J Virol* **64**, 4625-4631 (1990).

107. Buck, C.B. et al. The human immunodeficiency virus type 1 gag gene encodes an internal ribosome entry site. *J Virol* **75**, 181-191 (2001).

108. Kieft, J.S. Viral IRES RNA structures and ribosome interactions. *Trends Biochem Sci* **33**, 274-283 (2008).

109. Hellen, C.U. & Sarnow, P. Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev* **15**, 1593-1612 (2001).

110. Kaminski, A. & Jackson, R.J. The polypyrimidine tract binding protein (PTB) requirement for internal initiation of translation of cardiovirus RNAs is conditional rather than absolute. *Rna* **4**, 626-638 (1998).

111. Pilipenko, E.V. et al. A cell cycle-dependent protein serves as a template-specific translation initiation factor. *Genes Dev* **14**, 2028-2045 (2000).

112. Pestova, T.V., Shatsky, I.N., Fletcher, S.P., Jackson, R.J. & Hellen, C.U. A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. *Genes Dev* **12**, 67-83 (1998).

113. Martinez-Salas, E., Ramos, R., Lafuente, E. & Lopez de Quinto, S. Functional interactions in internal translation initiation directed by viral and cellular IRES elements. *J Gen Virol* **82**, 973-984 (2001).

114. Chappell, S.A., Edelman, G.M. & Mauro, V.P. A 9-nt segment of a cellular mRNA can function as an internal ribosome entry site (IRES) and when present in linked multiple copies greatly enhances IRES activity. *Proc Natl Acad Sci U S A* **97**, 1536-1541 (2000).

115. Yang, Q. & Sarnow, P. Location of the internal ribosome entry site in the 5' non-coding region of the immunoglobulin heavy-chain binding protein (BiP) mRNA: evidence for specific RNA-protein interactions. *Nucleic Acids Res* **25**, 2800-2807 (1997).

116. Hu, M.C., Tranque, P., Edelman, G.M. & Mauro, V.P. rRNA-complementarity in the 5' untranslated region of mRNA specifying the Gtx homeodomain protein: evidence that base- pairing to 18S rRNA affects translational efficiency. *Proc Natl Acad Sci U S A* **96**, 1339-1344 (1999).

117. Shine, J. & Dalgarno, L. The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proc Natl Acad Sci U S A* **71**, 1342-1346 (1974).

118. Owens, G.C., Chappell, S.A., Mauro, V.P. & Edelman, G.M. Identification of two short internal ribosome entry sites selected from libraries of random oligonucleotides. *Proc Natl Acad Sci U S A* **98**, 1471-1476 (2001).

119. Zhou, W., Edelman, G.M. & Mauro, V.P. Isolation and identification of short nucleotide sequences that affect translation initiation in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **100**, 4457-4462 (2003).

120. Moradpour, D., Penin, F. & Rice, C.M. Replication of hepatitis C virus. *Nat Rev Microbiol* **5**, 453-463 (2007).

121. Ricci, E.P., Rifo, R.S., Herbreteau, C.H., Decimo, D. & Ohlmann, T. Lentiviral RNAs can use different mechanisms for translation initiation. *Biochem Soc T* **36**, 690-693 (2008).

122. De Felipe, P. & Izquierdo, M. Tricistronic and tetracistronic retroviral vectors for gene transfer. *Hum Gene Ther* **11**, 1921-1931 (2000).

123. de Felipe, P. & Izquierdo, M. Construction and characterization of pentacistronic retrovirus vectors. *J Gen Virol* **84**, 1281-1285 (2003).

124. Edwards, S.R. & Wandless, T.J. Dicistronic regulation of fluorescent proteins in the budding yeast Saccharomyces cerevisiae. *Yeast* **27**, 229-236 (2010).