Uncovering a novel function of the exonuclease Xrn1 in viral and cellular mRNA translation

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SUMMARY

The highly conserved exonuclease Xrn1 is central in messenger RNA (mRNA) decay and also acts as a transcriptional activator. By using a model system that allows the replication of brome mosaic virus (BMV) in *Saccharomyces cerevisiae*, here we show that Xrn1 promotes translation of BMV RNA in a manner dependent on the highly structured 5' untranslated region (UTR) and the coding sequence (CDS). Polysome profiling analyses indicate that Xrn1 favours viral RNA translation initiation and interacts physically with the translation machinery.

As found for the function of Xrn1 in transcription, the exonuclease activity of Xrn1 is required for its role in viral RNA translation. However, this requirement is not due to the exonuclease activity *per se*, since the nuclear paralog of Xrn1 (Rat1) complements BMV RNA decay and cell growth but not BMV RNA translation when expressed in the cytoplasm of *xrn1Δ* cells. The role of Xrn1 in viral RNA translation is independent from its function in transcription because an Xrn1ΔNLS mutant unable to shuttle to the nucleus is still able to promote BMV RNA2 translation. Importantly, ribosome profiling analyses reveal that Xrn1 acts as a translational activator of a specific subset of cellular mRNAs enriched for functions related to glycosylation and membrane transport proteins. As described for BMV RNA, these cellular mRNAs depend on the exonuclease activity of Xrn1 for translational activation and contain long and structured 5'UTR. Together, our results reveal a new cross-talk between mRNA degradation and translation, and uncover an unexpected function of the exonuclease Xrn1 in viral and cellular translational control.

RESUM

Xrn1 és una exonucleasa molt conservada entre espècies i que té un paper cabdal en la degradació de l'ARN missatger (ARNm) i la regulació de la transcripció. Emprant un sistema model basat en el virus del mosaic del brom (BMV) i el llevat *Saccharomyces cerevisiae*, en aquest treball es demostra que Xrn1 promou la traducció de l'ARN viral. Aquesta funció en traducció està lligada a la regió no traduïda a l'extrem 5' i a la seqüència codificant. A més, mitjançant l'anàlisi amb perfils de polisomes s'observa que Xrn1 assisteix la iniciació de la traducció de l'ARN viral i que interacciona directament amb la maquinària de traducció.

Tenint en compte que Xrn1 participa en la degradació i la transcripció d'ARNm, es van crear diferents mutants per tal d'analitzar la relació entre aquestes funcions i el rol de Xrn1 en traducció. En primer lloc, es va observar que l'activitat exonucleasa pròpia de Xrn1 és necessària per tal que Xrn1 actuï en traducció. Expressant la exonucleasa nuclear (Rat1) al citoplasma de cèl·lules *xrn1Δ*, s'aconsegueix compensar els defectes en degradació de l'ARN i en la taxa de creixement. En canvi, no es rescaten els defectes en la traducció de l'ARN viral. En segon lloc, utilitzant un mutant de Xrn1 que no pot ser importat al nucli (Xrn1ΔNLS) es va concloure que la funció de Xrn1 en transcripció és independent de la funció en traducció. Tot seguit, mitjançant estudis amb perfil de ribosomes, es va constatar que Xrn1 també actua com a activador de la traducció d'un subgrup d'ARNm cel·lulars. Aquests, codifiquen per funcions relacionades amb la glucosilació i estan enriquits en proteïnes del reticle endoplasmàtic. Tal i com s'observa per l'ARN de BMV, aquests ARNm cel·lulars depenen de l'activitat exonucleasa de Xrn1 per a la seva traducció i tenen una elevada estructura secundària a la regió no traduïda de l'extrem 5'.

En conjunt, els nostres resultats descriuen un nou exemple de la comunicació existent entre la degradació i la traducció de l'ARNm i revelen que Xrn1 té una funció inesperada en el control de la traducció de l'ARNm viral i cel·lular.

PREFACE

The classical view of gene expression considered its different steps as single and isolated processes. However, research in the last decades has revealed the existence of a complex cross-talk between them. Therefore, by coupling subsequent steps of gene expression, a single interconnected system is formed. Multiple evidences bridge nuclear and cytoplasmic events in yeast and higher eukaryotes. For instance, the RNA polymerase II subunits Rpb4 and Rpb7 shuttle together with nascent mRNAs from the nucleus to the cytoplasm, linking mRNA transcription, translation and degradation. The idea of nuclear events affecting cytoplasmic processes or vice versa is not new. However, recent findings describing the exonuclease Xrn1 as a key regulator of transcription have added an extra layer of control in cellular homeostasis.

Decay factors are not only involved in mRNA degradation and transcription but they are also linked to translation. There are many examples that illustrate this interconnection, such as the translational repression carried out by decapping activators or the fact that mRNA degradation can take place co-translationally. In spite of all these studies, there are questions regarding the role of mRNA decay factors in translation control that remain to be answered. In this thesis we have explored the role of the exonuclease Xrn1 in promoting viral RNA translation and we have demonstrated that this function extends to cellular mRNAs. These results reveal a new layer of cellular control and further support the idea of gene expression as a whole single system.

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INTRODUCTION

1. An introduction to mRNA decay

The degradation of cellular RNAs plays a crucial role in regulating gene expression and cellular homeostasis, since it controls mRNA steady-state levels modulating them according to stimuli (Parker, 2012). In addition, mRNA decay monitors the quality of the mRNA along its life cycle. Although all RNA molecules are subject to degradation, here we will focus on messenger RNAs (mRNAs). We will review the current findings related to general mRNA decay and specialized mRNA degradation.

1.1. General mRNA decay pathways

There are two general pathways responsible for mRNA decay in the cytoplasm: the decapping-mediated 5'-3' degradation and the exosome 3'-5' degradation (Figure I1). Both require the deadenylation or shortening of the poly(A) tail at the 3´end of mRNAs to initiate the decay process (Garneau et al., 2007; Parker, 2012) . This is due to the fact that the complex formed between the poly(A) tail and the poly(A) binding protein (Pab1) stabilizes the transcript and directs translation initiation through the

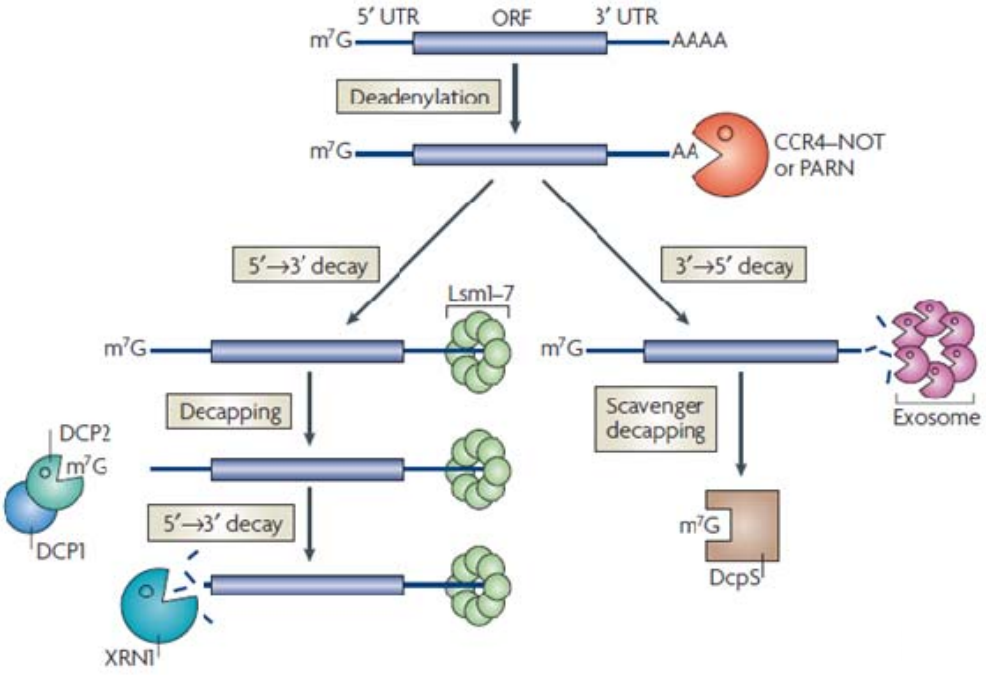


Figure I1. General mRNA decay pathways. After deadenylation, mRNAs in the cytoplasm can be degraded by two major pathways: the decapping-dependent 5'-3' pathway mediated by the exonuclease Xrn1 or the 3'-5' pathway mediated by the exosome. Figure adapted from (Garneau et al., 2007).

interaction with the cap binding complex (Coller et al., 1998; Tarun and Sachs, 1996). The process of deadenylation is catalyzed by the Ccr4/Pop2/Not and the Pan2/Pan3 complexes, which are conserved in eukaryotes (Goldstrohm and Wickens, 2008; Wolf and Passmore, 2014). Deadenylation is modulated by translation *per se*, by RNA binding proteins or by stress. Once deadenylation has occurred mRNA undergoes degradation via either the 5'-3' or the 3'-5' pathway (Parker, 2012).

1.1.1. The 5'-3' deadenylation-dependent decay pathway

The 5'-3' deadenylation-dependent decay pathway is the major mRNA decay pathway in eukaroytes and its components are evolutionary conserved (Muhlrad et al., 1995; Parker, 2012). Dcp2 and Xrn1 are the two key enzymes in this pathway. Dcp2 is the decapping enzyme and its activity is enhanced by Dcp1 by promoting a conformational change in Dcp2 required for activation. This complex is responsible for cleaving the cap structure and releasing a m^7GDP and a 5' monophospate mRNA (She et al., 2008). Xrn1 (eXoRiboNuclease I or Kem1 in yeast) is the 5'-3' exonuclease and it is responsible for the degradation of mRNA. Xrn1 is not only involved in general 5'-3' mRNA decay but also in specialized decay pathways (Jinek et al., 2011). Besides the enzymes, there are other factors named decapping activators that assist and enhance decapping. These include Lsm1-7, Pat1, Edc3, Scd6 and Dhh1 conserved from yeast to humans and the yeast Edc1 and Edc2 (Bouveret et al., 2000; Coller and Parker, 2005; Dunckley et al., 2001; Fischer and Weis, 2002; Steiger et al., 2003).

The 5´-3´ deadenylation-dependent mRNA decay pathway consists of three steps: (I) translational repression, (II) decapping and (III) Xrn1-dependent 5'-3' degradation (Figure I2). Multiple evidences support that translation repression takes place prior to decapping, as translation and degradation of mRNAs are interconnected processes in dynamic competition. First, the same cap structure that is bound by the cap-binding complex (eIF4E and eIF4G) for translation initiation to occur must become accessible and exposed to the decapping machinery (Parker, 2012). Second, multiple decapping activators inhibit translation at different steps by using distinct mechanisms. For instance, Scd6, Pat1 and Dhh1 inhibit translation initiation before the formation

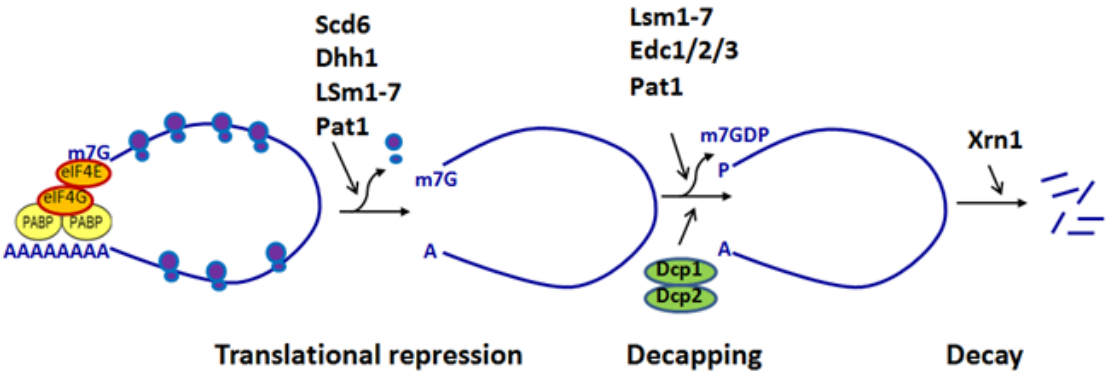


Figure I2. Deadenylation-dependent 5'-3' mRNA decay pathway. Once deadeynlation has occurred, the mRNP is remodelled and translation initation factors are exchanged for decapping activators and translational repressors. Next, decapping is carried out by Dcp1/Dcp2. Finally, Xrn1 degrades the mRNA in a 5' to 3' direction.

of the 48S pre-initiation complex (Coller and Parker, 2005; Nissan et al., 2010; Rajyaguru et al., 2012). Dhh1 also hinders translation elongation by slowing down the ribosome (Sweet et al., 2012). The competition between translation initiation factors and decapping activators shapes the composition of messenger ribonucleoproteins (mRNP) and ultimately determines the mRNA fate. Interestingly, recent results indicate that there is a widespread co-translational mRNA decay during the last round of translation both in yeast and plants (Hu et al., 2009; Merret et al., 2015; Pelechano et al., 2015). Notably, mRNA decay factors have been detected in polysomes in yeast and humans (Lubas et al., 2013; Mangus and Jacobson, 1999; Wang et al., 2002).

Decapping is a key control step of mRNA decay and is carried out by the holoenzyme Dcp1/Dcp2. The regulation of the decapping process takes place through two distinct mechanisms: *cis*-acting factors and *trans*-acting factors. *Cis*-acting factors are sequences inside the mRNAs that modulate translation and decay rates. Given the inverse correlation between translation and decay, sequences that promote translation inhibit decapping and vice versa (Schwartz and Parker, 1999). For instance, the poly(A) tail enhances translation while hindering decapping. In contrast, an unfavourable sequence context at the start codon or an AU-rich element (ARE) present in the 3' untranslated region (UTR) enhance decapping (LaGrandeur and Parker, 1999; Vasudevan et al., 2001). *Trans*-acting factors comprise multiple proteins that bind to the mRNA directly or indirectly and activate or inhibit decapping. These

include the decapping activators Lsm1-7, Pat1, Edc1-3, Scd6 and Dhh1. After mRNA deadenylation, the Pat1/Lsm1-7 complex binds to the oligoadenylated 3' end of the mRNA (Bouveret et al., 2000; Tharun and Parker, 2001). Pat1 acts as a scaffold for formation of larger decapping complexes, which include Edc3, Dcp1 and Dcp2 or Scd6 and Dhh1. Decapping activators favour decapping either by repressing translation or by directly enhancing the activity of the holoenzyme Dcp1/Dcp2. For instance, Pat1, Scd6 and Dhh1 repress translation initiation. In addition, Edc1-3, Scd6 and Pat1 are capable of directly interacting with Dcp2 and increasing its catalytic activity *in vitro* (Fromm et al., 2012; Nissan et al., 2010). Once the mRNA is decapped, it is recognized by the exonuclease Xrn1 and degraded processively in a 5' to 3' direction. The 5'-3' decay factors form a complex network of interactions that enables a tight control of translational repression, decapping and degradation (Figure I3). The dynamic rearrangement of mRNA binding partners is possible through the modular organization of decapping factors. In their structure, they have globular domains that are connected to each other through disordered regions.

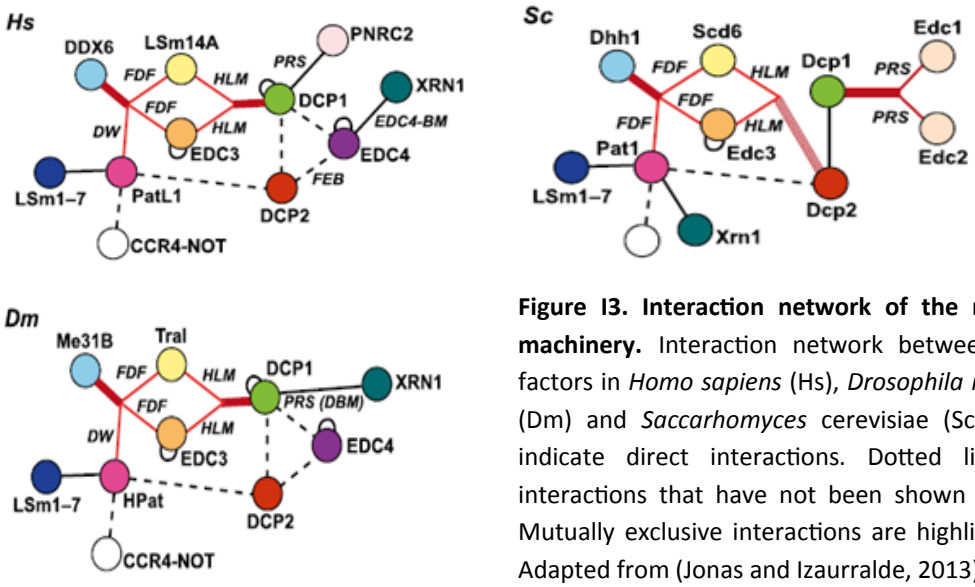


Figure I3. Interaction network of the mRNA decay machinery. Interaction network between decapping factors in *Homo sapiens* (Hs), *Drosophila melanogaster* (Dm) and *Saccarhomyces* cerevisiae (Sc). Solid lines indicate direct interactions. Dotted lines indicate interactions that have not been shown to be direct. Mutually exclusive interactions are highlighted in red. Adapted from (Jonas and Izaurralde, 2013).

These disordered regions have two main functions: (I) they contain short linear motifs (SLiMs) that mediate the interaction between decapping factors, and (II) they direct the formation of large mRNPs reversible aggregates such as processing bodies (PBs). SLiMs provide low affinity yet specific interactions that allow transient binding. Interestingly, although the core decapping pathway is conserved from yeast to humans, a certain rewiring of their interactions has occurred during evolution. The remodelling of this interaction network throughout evolution suggests the emergence of novel functions for these complexes (reviewed in (Jonas and Izaurralde, 2013)).

1.1.2. The 3'-5' mRNA degradation

The 3'-5' mRNA degradation is the other major decay pathway in the cytoplasm (Anderson and Parker, 1998). Following deadenylation, the exosome and its cofactors bind to the 3' end of some mRNAs and degrade them in a 3' to 5' direction. The exosome is formed by six RNAsePH domain proteins with no catalytic activity, three RNA binding subunits and one catalytic subunit (Dis3/Rrp44) with exonuclease and endonuclease activities (Lykke-Andersen et al., 2011). The cytoplasmic cofactors Ski2/3/8 are also required for 3'-5' mRNA decay. They target the exosome to its substrates, positioning them towards the active site of Dis3/Rrp44 (Bonneau et al., 2009). The fact that mutations in the exosome components are synthetic lethal with mutations in the 5'-3' exonuclease Xrn1 reflects the importance of these two pathways in mRNA and cellular homeostasis (Johnson and Kolodner, 1995).

1.2. Specialized mRNA decay

The presence of aberrant mRNAs leads to the production of protein products that might be deleterious. The cell has evolved specialized co-translational mRNA decay to ensure that these aberrant mRNAs are recognized and degraded. A key feature of these mRNAs is that they cause ribosome stalling during translation. Ribosome stalling events provide a very good opportunity for detection and degradation of aberrant mRNAs and recycling of the translation components (Lykke-Andersen and

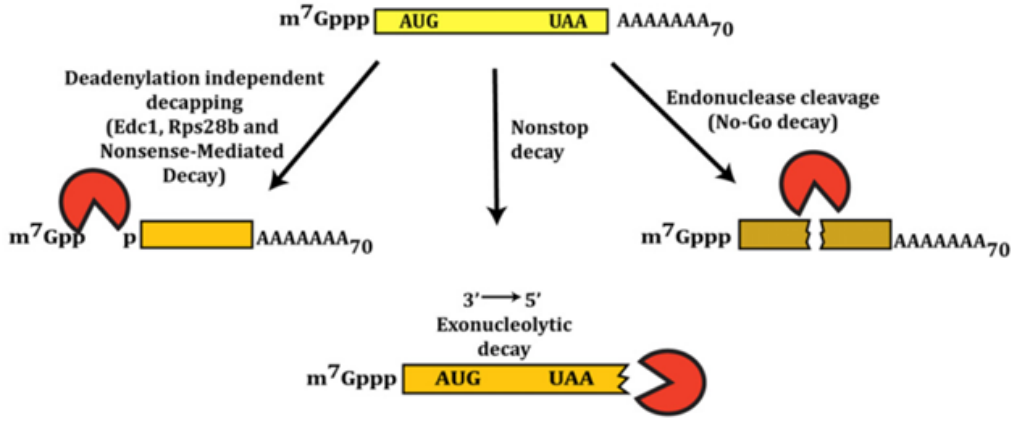


Figure I4. Specialized mRNA decay. Specialized mRNA degradation pathways monitor the quality of mRNA and degrade transcripts that are aberrant or are trapped in aberrant translation processes. From left to right: Nonsense-Mediated Decay (NMD) targets mRNAs with a premature stop codon; Non-stop Decay (NSD) targets mRNAs without termination codon; No-Go decay (NGD) targets mRNAs with a strong stall during translation. Adapted from (Parker, 2012).

Bennett, 2014). There are three pathways conserved in eukaryotes that recognize and degrade aberrant mRNAs: Nonsense-mediated decay (NMD), Non-stop decay (NSD) and No-go decay (NGD) (Figure I4). These three pathways are conserved from yeast to mammals; however they are more extensively studied in yeast.

Non-sense mediated decay (NMD) targets mRNAs with anomalous translation termination (Isken and Maquat, 2007; Parker, 2012). This event is caused by multiple situations, such as abnormal 3'UTR contexts that are not favourable for translation termination, premature termination codons, errors in transcription, or frame-shifting events, among others (Amrani et al., 2004; Belew et al., 2011). In yeast, aberrant termination is detected by the Upf1 protein through the interaction with eRF1 and eRF3 in the terminating complex bound to the ribosome. Upf2 and Upf3 proteins then interact with Upf1 leading to a rapid deadenylation, decapping and Xrn1-dependent degradation of the NMD mRNA substrate (Baker and Parker, 2004). Alternatively, these substrates can undergo decapping-independent decay by the exosome and the Ski complex (Mitchell and Tollervey, 2003).

Non-stop decay (NSD) detects mRNAs without a termination codon. The lack of a stop codon is caused by premature polyadenylation, mRNA truncation or mutations (Frischmeyer et al., 2002; van Hoof et al., 2002). In this pathway, the exosome

cofactor Ski7 detects a stalled ribosome in the 3' end. This leads to the subsequent degradation of the mRNA by the exosome and Ski2/3/7 in a 3' to 5' direction. The Dom34/Hbs1 complex is responsible for the release of the stalled ribosome and promotes upstream endonucleolytic cleavage. NSD substrates can also undergo decapping and subsequent 5'-3' degradation by the exonulcease Xrn1 when Ski7 is not present (Inada and Aiba, 2005).

No-go decay (NGD) drives degradation of mRNAs with a strong stall during translation, caused by stable stem-loop structures, polybasic tracts that strongly interact with the ribosome exit tunnel, rare codons or depurination sites (Doma and Parker, 2006; Harigaya and Parker, 2010). The detection of stalled ribosomes leads to an endonucleolytic cleavage in the upstream vicinity. The Dom34/Hbs1 complex is responsible for the release of the stalled ribosome, but whether it directly stimulates endonucleolytic cleavage or not remains to be clarified (Lykke-Andersen and Bennett, 2014). Although the Dom34/Hbs1 complex stimulates NGD, it is not essential for NGD to take place (Doma and Parker, 2006; Passos et al., 2009).

1.3. The 5'- 3' exonuclease Xrn1

1.3.1. The Xrn1 protein and its exonuclease activity

The mRNA decay pathways reviewed until now employ different mechanisms and factors to achieve a common goal: mRNA degradation. In spite of their differences, they all direct RNA molecules towards endo- and exonucleolytic events. Xrn1 plays a prominent role in all cytoplasmic 5' to 3' mRNA degradation processes. Xrn1 also directs maturation of ribosomal RNAs (rRNAs), degradation of long non-coding RNAs such as XUTs (Xrn1-sensitive unstable transcripts) and destruction of aberrant splicing intermediates (Geerlings et al., 2000; Hilleren and Parker, 2003; Parker, 2012; van Dijk et al., 2011). Besides, Xrn1 controls the basepairing occurring between convergent transcripts (Sinturel et al., 2015). Although the functions of Xrn1 mentioned so far are linked to its exonuclease activity, Xrn1 plays a role in meiosis and in karyogamy in an exonuclease-independent manner (Kim et al., 2004; Solinger et al., 1999).

Xrn1 is a protein of 175 kDa with an N-terminal exonuclease domain responsible for the degradation of decapped mRNAs (Johnson and Kolodner, 1991). It is highly conserved across species (Nagarajan et al., 2013), displaying the highest conservation in the N-terminal domain, especially around the exonuclease catalytic site (Figure I5). Crystallographic studies with the paralogs of Xrn1 in *Drosophila melanogaster* (PACMAN) and *Kluyveromyces lactis* elucidated the mechanism of its exonuclease activity. Xrn1 has a deep basic pocket that selects RNAs with a 5' phosphate while m^7G capped RNAs or triphosphorylated RNAs are sterically excluded. After a round of hydrolysis, the next nucleotide is recognized and translocated, allowing a processive degradation and an ATP-independent unwinding of substrate structures (Chang et al., 2011; Jinek et al., 2011). The Xrn1 C-terminal domain is intrinsically disordered and much less conserved than the rest of the protein. Nevertheless, it stabilizes the exonuclease domain and enhances its activity. In yeast, an Xrn1 mutant lacking the C-terminal end (residues 1206-1528) displays defective exonuclease activity and is incapable of rescuing the slow growth defect in *xrn1Δ*. Interestingly, the overexpression of this C-terminal extension leads to growth inhibition phenotypes in yeast (Chang et al., 2011; Page et al., 1998).

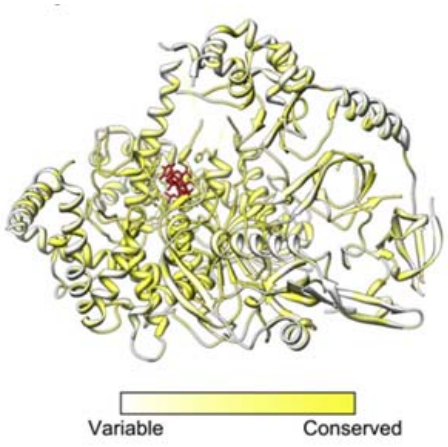


Figure I5. Xrn1 is highly conserved among eukaryotes. This 3D structure depicts the conservation of residues between 195 eukaryote XRN1s. In *red* it can be observed three nucleotides of uncapped RNA bound in the active site. Conservation is higher around the catalytic site and in certain regions of the C-terminal extension. Adapted from (Nagarajan et al., 2013).

1.3.2. Xrn1 and its interacting partners

Xrn1 interacts with multiple proteins of the mRNA decay pathway (Figure I3). These interactions have been rewired throughout evolution. In *S. cerevisiae*, Xrn1 interacts with Pat1/Lsm1-7 complex *in vivo* (Bouveret et al., 2000). Biochemical studies with

recombinant proteins reported a direct interaction between the C-terminal domain of Pat1 and Xrn1 (Nissan et al., 2010). The scavenger enzyme Dcs1 acts as a cofactor of Xrn1 by directly activating it *in vitro* and *in vivo* (Sinturel et al., 2012). In *D. melanogaster*, Pacman (Xrn1 homolog) interacts directly with Dcp1 through a proline-rich sequence. This sequence is conserved in yeast, but no direct interaction has been reported between Xrn1 and Dcp1 in *S. cerevisiae* (Braun et al., 2012). XRN1 interacts with the Pat1L and Edc4 in an RNA-independent manner in humans, and with Upf3 protein from NMD in monkey COS cells (Lejeune et al., 2003; Ozgur et al., 2010).

1.3.3. Rat1: the nuclear paralog of Xrn1

Xrn1 has an essential nuclear paralog named Rat1 (XRN2 in humans) that functions in rRNA processing, transcription termination and telomere length maintenance (Geerlings et al., 2000; Luo et al., 2006; Maicher et al., 2012). Rat1 shares the conserved N-terminal exonuclease domain but lacks the C-terminal extension present in Xrn1 (Xiang et al., 2009). When Rat1 is expressed in the cytoplasm by mutation of the nuclear localisation signal (Rat1ΔNLS), it can suppress the lethality of the *xrn1Δski2Δ* double mutant. Importantly, Rat1ΔNLS suppresses many of the phenotypes observed in *xrn1Δ* such as the impaired general mRNA degradation, sensitivity to a microtubule-destabilizing agent (benomyl) or sporulation defects. Inversely, Xrn1 restores a temperature sensitive mutant *rat1-1* when expressed in the nucleus. Thus, Rat1 and Xrn1 are functionally interchangeable proteins (Johnson, 1997; Sinturel et al., 2012).

1.3.4. Xrn1 as a transcriptional regulator

Xrn1 functions as a transcriptional regulator in *S. cerevisiae*. It buffers mRNA levels by acting both in transcription and mRNA degradation. Together with other mRNA decay factors, Xrn1 can shuttle to the nucleus by a mechanism dependent on its exonuclease activity and participate in transcription (Haimovich et al., 2013). Notably, a recent study characterized in detail a set of genes called “Xrn1 synthegradosome”

that are targeted by Xrn1 for degradation and transcription. These genes are highly enriched for translation factors and ribosome biogenesis functions (Medina et al., 2014). A parallel study also observed a general compensation of mRNA levels by buffering transcription and mRNA degradation. However, in this case the authors proposed that Xrn1 was essential for buffering to occur. They did not observe a direct function of Xrn1 in transcription, but an indirect effect mediated by the transcriptional repressor Nrg1 (Sun et al., 2013).

In conclusion, Xrn1 acts as a master regulator of RNA homeostasis, since it connects both mRNA decay and transcription. Interestingly, there are other examples that reflect the interconnection existing between the different stages of gene expression. For instance, RNA Pol II subunits Rpb4 and Rpb7 bind the nascent mRNA and shuttle between the nucleus and the cytoplasm, participating in mRNA transcription, export, decay and translation through the interaction with eIF3 in *S. cerevisiae* (Harel-Sharvit et al., 2010). In this context, it remains to be elucidated whether Xrn1 is also implicated with other key cellular processes such as translation.

1.3.5. Xrn1 mutants: phenotypes

Unicellular and pluricellular organisms lacking Xrn1 show pleiotropic phenotypes. In *S. cerevisiae* they include slow growth rate, defective karyogamy, hypersensitivity to benomyl, defects in philamentous growth and spore lethality (Kim and Kim, 2002; Larimer et al., 1992; Solinger et al., 1999; Tishkoff et al., 1991). In higher eukaryotes, the effect of Xrn1 on development has been addressed. Mutations in XRN1 result in diverse development defects by deregulating the gene expression program. Accordingly, *xrn-1* is required for ventral enclosure in *Caenorhabditis elegans* (Newbury and Woollard, 2004). In line with this, *pcm* (Pacman) mutants in *D. melanogaster* have abnormal epithelial sheet sealing and reduced male and female fertility (reviewed in (Jones et al., 2012)).

2. An introduction to translation

Translation consumes up to 50% of the cell energy depending on the organism (Holcik and Sonenberg, 2005). This is mainly due to the massive amount of resources devoted to the biogenesis of ribosomes. In *S. cerevisiae* 60% of total transcription can be attributed to rRNAs (ribosomal RNAs), whereas 50% of RNA Pol II transcription corresponds to ribosomal proteins (Rudra and Warner, 2004). Therefore translation must be regulated and monitored to optimize cellular energy consumption. In this section we will briefly review translation and its regulation, from ribosome biogenesis to initiation, elongation, termination and quality control.

2.1. Ribosome biogenesis

The synthesis of ribosomes requires the coordination of many cellular processes that lead to the production of equimolar amounts of 79 ribosomal proteins and the ribosomal RNAs (rRNAs) in yeast. The coordination of three RNA polymerases is needed to produce rRNAs (RNA Pol I), mRNAs encoding for ribosomal proteins (RNA Pol II) and the 5S rRNA (RNA Pol III). Almost 200 proteins play a role in the processing of rRNA and the assembly of ribosomal subunits (Baserga, 2013; Rudra and Warner, 2004). Transcription of ribosomal genes is regulated through many cellular pathways that sense the growth state of the cell and integrate stress responses. Amino acids availability and active TOR (target of rapamycin) or PKA (protein kinase A) pathways enhance ribosome biosynthesis. In contrast, stresses of different kinds or the inactivation of TOR or PKA lead to a repression of ribosome biosynthesis (Rudra and Warner, 2004).

2.2. Translation initiation

During translation initiation the 40S and 60S subunits of the ribosome are assembled together with the initiator methionyl-tRNA (Met·tRNAi) to produce an elongation-competent ribosome positioned in the AUG start codon. It is a coordinated process assisted by many factors that can be summarized in five steps. (i) Formation of the

ternary complex, (ii) association of the ternary complex to the 40S subunit to form the 43S PIC (pre-initiation complex), (iii) recruitment of the PIC to the 5' end of the mRNA, (iv) scanning of the 5'UTR to find the start codon and (v) 60S subunit joining to form the 80S initiation complex (Aitken and Lorsch, 2012; Hinnebusch, 2014; Holcik and Sonenberg, 2005).

Regulation during translation initiation enables the cell to elicit an immediate response to physiological changes. Translation is globally reduced in response to different kinds of stress. This is mainly achieved through the phosphorylation of eIF2. The kinase Gcn2 is responsible for this in *S. cerevisiae*. In vertebrates there are three additional kinases (PKR, HRI and PERK) that are activated upon stress. These include amino acid availability, viral infection, osmotic shock, heat shock and ER (Endoplasmic Reticulum) stress, among others. A general reduction of the cap-dependent translation leads to the enhanced selective translation of a subset of mRNAs related to stress response. Actually, some viruses employ this strategy to favour the translation of their genome through IRES (internal ribosome entry site) (Holcik and Sonenberg, 2005; Jiang et al., 2001; Zhan, 2004).

2.3. Translation elongation

Translation elongation has been historically considered to be much simpler than initiation, but a growing number of examples indicate that elongation is also regulated (Figure I6). The process of elongation is assisted by elongation factors and consists of three steps: (i) recognition between the mRNA codon and an aminoacylated cognate tRNA, (ii) peptidyl transfer reaction and (iii) ribosome translocation. This process is repeated until the ribosome reaches a termination codon (reviewed in (Richter and Coller, 2015)). It is worth noting that yeast require an additional eEF3 for translation elongation for which no homolog has been found in higher eukaryotes or bacteria (Dever and Green, 2012).

There are many examples where translation elongation is regulated. For instance, during the translation of ER proteins, ribosomes pause in the first codons due to the interaction of the signal recognition particle (SRP) with the nascent chain and the

ribosome A-site. This allows translationally paused ribosomes to localize onto ER membranes and resume translation when translocation can take place. Elongation can also be regulated to allow for proper protein folding, change of reading frame or recruitment of regulatory factors (Gutierrez et al., 2013; Halic et al., 2004; Ketteler, 2012; Zhang et al., 2009).

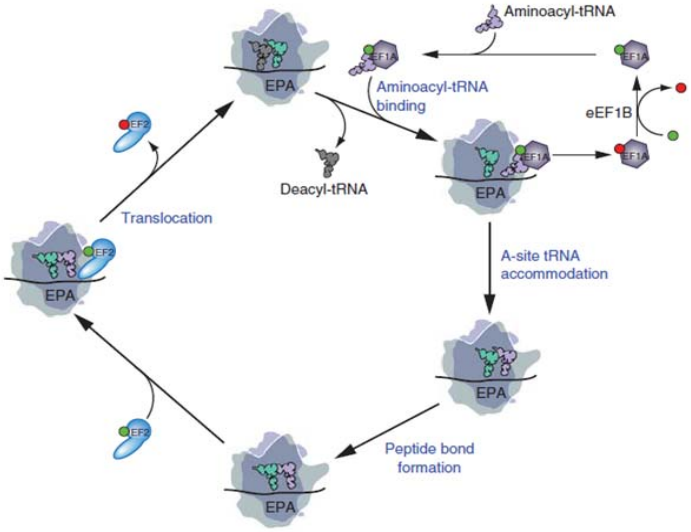


Figure I6. Schematic of translation elongation in eukaryotes. EF1A assists the recognition between the mRNA codon and an aminoacylated cognate tRNA. After tRNA is accommodated in the A-site, the peptidyl transfer reaction takes places and a new peptide bond is formed. Next, EF2 assists in the translocation of tRNA and mRNA through the ribosome. Adapted from (Dever and Green, 2012).

2.4. Translation termination and recycling

Translation termination occurs when a ribosome reaches a termination codon that enters the A-site. The distance to the poly(A) is important because it contributes to the peptide release (Fatscher et al., 2014). The process of translation termination is directed by eukaryotic release factor 1 (eRF1). This factor is a tRNA-shaped protein that recognizes the stop codon in the A-site and, together with other factors, catalyzes the peptide release and the dissociation of ribosomal subunits. In some cases, however, the recycling of ribosomal subunits is not complete and the 40S subunit can scan along the 3'UTR and be easily transferred to the 5'UTR to reinitiate translation of the same transcript (reviewed in (Dever and Green, 2012)).

2.5. Translation quality control

Translation is an essential process in the cell. As such, there are mechanisms that monitor translation to avoid the generation of aberrant products with potentially toxic effects. Errors during translation can come from defects in the ribosome, defects in the mRNA (section 1.2) or problems detected in the nascent polypeptide (Lykke-Andersen and Bennett, 2014). Nascent polypeptides are monitored via two different pathways. The first one is termed co-translational quality control and it checks the folding state of the nascent chain through specialized chaperones. If the peptide cannot be folded properly, it is targeted for destruction. The second one is called ribosome-associated quality control (RQC) and it senses the translational state. If ribosomes stall during translation, the translating mRNA and the associated nascent chain are degraded (Brandman and Hegde, 2016). This is independent of the folding of the nascent chain but it is related to the translation machinery *per se*. The RQC complex was first described while performing a genome-wide screening to find activators and repressors of Hsf1 (heat shock factor 1). Interestingly, Brandman and co-workers observed that a complex formed by Ltn1, Cdc48, Tae2 and Ydr333C associates to the ribosome and is responsible for both degrading stalled polypeptides and signalling stress to Hsf1 through a newly described pathway (Brandman et al., 2012).

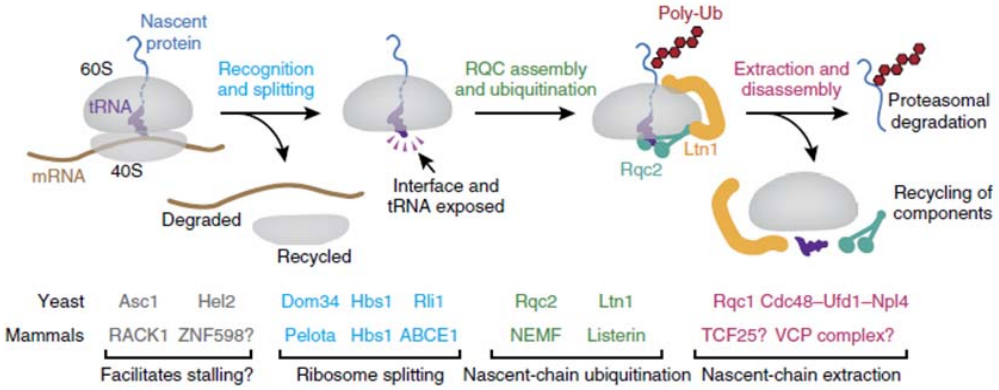


Figure I7. Schematic of the ribosome-associated quality control (RQC) pathway in eukaryotes. Dom34/Hbs1 and Rli1 are responsible for recognizing strong stalling events and splitting the ribosome subunits. The 60S subunit in complex with the peptydyl-tRNA is exposed and recognized by Rqc2 (Tae2) and Ltn1, which elongate the nascent chain with (CAT) tails and ubiquitylate it, respectively. Rqc1 and Cdc48 are recruited, the nascent chain is extracted and degraded and the RQC components and the 60S subunit are recycled. The homologous yeast and mammalian factors are shown below. Question marks indicate factors still to be validated. Adapted from (Brandman and Hedge, 2016).

Ribosomes can stall during elongation as a consequence of stress or due to a programmed regulation. Upon stress, the stalled ribosome is split by Dom34/Hbs1 complex and the 60S subunit bound to the peptidyl-tRNA is recognized by Tae2 and Ltn1 proteins (Figure I7). Tae2 elongates the polypeptide with (CAT) tails (C-terminal Alanine or Theronine) independently of the 40S subunit or the mRNA. This signal leads to the induction of an Hsf1-mediated stress response. In parallel, the E3 ligase Ltn1 ubiquitylates the stalled polypeptide. This event leads to the recruitment of Cdc48, which extracts the incomplete protein product from the 60S subunit and targets it for proteasomal degradation (Bengtson and Joazeiro, 2010; Brandman et al., 2012; Lykke-Andersen and Bennett, 2014; Lyumkis et al., 2014; Shen et al., 2015; Shoemaker and Green, 2012; Tsuboi et al., 2012). Thus, the RQC enables the detection and degradation of stalled polypeptides during translation. The rapid response to stalling events is energy-saving and might prevent undesired effects of the truncated products.

3. Interplay between mRNA decay machinery and (+)RNA viruses

Viruses are intracellular parasites that completely depend on the cellular machinery to multiply. Elucidating the mechanisms by which viruses use host factors is not only important from the virology point of view, but it can also uncover novel cellular functions. In fact, fundamental post-trancriptional processes such as splicing and capping and have been first identified through viral studies and then proved to be essential in cells (Cullen, 2009). Positive-strand RNA [(+)RNA] viruses are characterized by having mRNA-like genomes formed by single-stranded RNA molecules with positive sense polarity. As such, they can be directly translated by ribosomes when they enter the cell (Ahlquist, 2006). Therefore, they are a very powerful tool to study mRNA translation and degradation from both viral and cellular perspectives. In this section we will review (+)RNA viruses and their interplay with the mRNA decay machinery.

3.1. Positive strand RNA viruses

(+)RNA viruses include one third of all virus genera and many established and emergent human pathogens with clinical, social and economical importance (Marston et al., 2014). Among them we find the families of *Coronoviridae* (SARS-CoV), *Picornaviridae* (Poliovirus), *Flaviviridae* (hepatits C virus, Dengue virus, West Nile virus and Zika virus) and *Togaviridae* (Rubella virus) (Ahlquist et al., 2003). Hepatitis C virus (HCV), for instance, infects chronically around 130-150 million people worldwide (Scheel and Rice, 2013). In contrast, Zika virus (ZIKV) is an emergent virus that is now spreading in South America with a major outbreak in Brazil (Calvet et al., 2016).

In spite of their differences in host-range, genome organization and virion morphology, all the members of (+)RNA viruses share the same strategy to replicate their genome. Upon infection, the viral genome acts as an mRNA since it is directly translated and viral replication proteins are synthesized (Figure I8). Next, when sufficient viral proteins have been produced, viral RNA translation must be stopped and the viral genome is recruited to act as template for replication in membrane-associated complexes. Different viruses target different membranes, such as ER, mitochondria, endosomes or chloroplasts (Ahlquist, 2006). Membrane-associated replication provides protection for the viral RNA against the cellular decay machinery. In addition, it increases the local concentration of replication factors and facilitates the coordination of the different steps of the viral life cycle (Paul et al., 2013). During replication, the (+)RNA strand genome is copied to a complementary (-)RNA strand replication intermediate. These intermediates are used as a template to generate new copies of the (+)-strand viral genome. In turn, these engage in subsequent rounds of translation and replication. Finally, progeny RNAs assemble with structural proteins to form viral particles that are released to produce new infections. Thus, in early infection (+)RNA genomes serve both as messenger RNAs and as templates for replication. Taking into account that replication and translation cannot take place simultaneously, the switch between translation and recruitment/replication has to be tightly regulated. This regulation is carried out by both viral and cellular host factors.

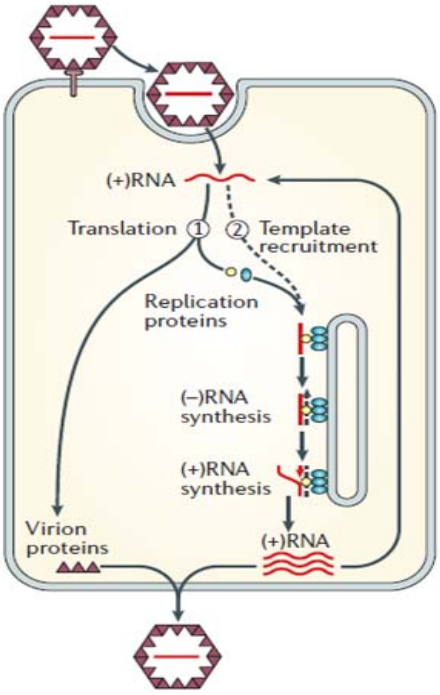


Figure I8. (+)RNA viruses life cycle. Once (+)RNA genome is released into the cytoplasm it is directly translated. After translation of viral proteins, the viral genome is used as a template for viral replication in membrane-bound complexes. New genomes are then encapsidated and released from the cell. Adapted from (Ahlquist, 2006).

3.2. Host factors and (+)RNA viruses

The identification of host factors required by (+)RNA viruses has experienced a revolution over the past decades due to the implementation of genome-wide strategies. The first genome-wide screenings were carried carried out in *S. cerevisiae* model systems that allow the replication of higher eukaryote viruses. The tractability of yeast genetics allowed the identification of hundreds of cellular factors with a role in the replication of brome mosaic virus (BMV) or tomato bushy stunt virus (TBSV) (Jiang et al., 2006; Kushner et al., 2003). The development of siRNA screening allowed parallel studies in human cells. Currently, hundreds of host factors have been described to affect the replication of the human immunodeficiency virus (HIV), hepatitis C virus (HCV), Dengue virus (DENV) and West Nile virus (WNV) (reviewed in (Nagy and Pogany, 2011)). The study and characterization of these host factors is not only crucial to better understand the biology of viruses and the host cell, but it can also provide novel targets for anti-viral therapies (Ma-Lauer et al., 2012). Given the genomic stability of the host, therapies directed to host factors are predicted to develop less resistances (Ruiz and Russell, 2012).

3.3. (+)RNA viruses and the cellular mRNA decay machinery

The genome of (+)RNA viruses is formed by RNA molecules acting as mRNAs. Therefore, it is not surprising that viruses have evolved different strategies to interact with the mRNA decay machinery. Some (+)RNA viruses protect their RNA by promoting degradation of decay factors, while others are able to hijack the cellular mRNA decay for their own benefit (Dickson and Wilusz, 2011). Poliovirus (PV) infection, for instance, leads to an increased degradation of Xrn1, Dcp1a and Pan3, which results in the dispersion of processing bodies (PBs) (Dougherty et al., 2011). In contrast, HCV inhibits Xrn1 by stalling it during viral RNA degradation. This inhibition leads to a disregulation of the cellular mRNA homeostasis, which is associated to the upregulation of oncogenes and angiogenic factors (Moon et al., 2015). Interestingly, human pathogens such as the flavivirus WNV or DENV hijack Xrn1 during 5' to 3' viral RNA degradation. By following this strategy Xrn1 is not only inhibited, but also used by the virus to produce a sfRNAs (subgenomic flavivirus RNA) (Charley and Wilusz, 2015; Pijlman et al., 2008). Importantly, these sfRNAs are required for efficient viral replication and pathogenesis (Chapman et al., 2014).

Another group of (+)RNA viruses hijack decapping factors to promote viral RNA translation and replication. Studies with BMV in *S. cerevisiae* revealed that the decapping activators Lsm1-7, Pat1 and Dhh1 are required for translation of the viral genome and its subsequent recruitment to membrane-associated replication complexes (Mas et al., 2006; Alves-Rodrigues et al., 2007). Lsm1-7/Pat1 complex binds the viral *cis*-acting sequences that regulate translation and replication and interact with the recruitment protein 1a (Galão et al., 2010; Scheller et al., 2009). Interestingly, Lsm1-7/Pat1 assists viral translation and recruitment by different mechanisms (Jungfleisch et al., 2015). The ability of Lsm1-7/Pat1 complex to bind the viral RNA is essential for its positive role in viral RNA translation. In contrast, this ability is not required for the function of Lsm1-7/Pat1 in the recruitment of viral RNA genomes. The role of Dhh1 as an activator of BMV RNA translation is linked to the presence of secondary structures in the UTRs and the coding sequence (CDS) (Jungfleisch et al, submitted). All these decapping activators accumulate in PBs

together with BMV RNA. This localization depends on *cis*-acting BMV RNA elements that also direct replication (Decker and Parker, 2012; Beckham et al., 2007). Remarkably, the use of decapping activators by (+)RNA viruses is evolutionary conserved. The human homologs of Lsm1-7, Pat1 and Dhh1, named Lsm1-7, Pat1L and Rck/p54, are positive regulators of HCV RNA translation and replication (Scheller and Díez, 2009; Scheller et al., 2009). Moreover, WNV sequesters Lsm1, Rck/p54 and Xrn1 to replication sites (Chahar et al., 2013) and DENV RNA binds Rck/p54 to promote viral RNA replication (Ward et al., 2011). Strikingly, the bacterial homolog of Lsm1, Hfq, is required for the replication of the (+)strand phage Qβ (Kajitani et al., 1994). The conserved use of these proteins in viruses of different kingdoms highlights the robustness of the strategy employed by (+)RNA viruses to translate and replicate.

4. The BMV/yeast system

4.1. Yeast as a model host for viral studies

Saccharomyces cerevisiae is a very powerful platform for the elucidation of general cellular processes in eukaryotes and for the study of viral replication. This is due to the fact that *S. cerevisiae* is easy to manipulate and grow and there exist many powerful tools that allow functional analysis. While plants and animals have large genomes with functional duplication, *S. cerevisiae* has a smaller genome comprising approximately 6000 genes with little redundancy. About 60% of genes are functionally annotated, 40% of yeast proteins are conserved with humans and 30% of human disease genes have yeast homologs (Foury, 1997). In addition, the data banks for *S. cerevisiae* are very well annotated and complete. There are many tools to study *S. cerevisiae*, such as (i) gene-deletion collections of non-essential genes, (ii) a down-regulatable essential gene collection, (iii) gene expression collections with tags fused to all ORFs that allow the study of subcellular localization or the purification of the protein of interest, (iv) DNA microarrays chips and (v) protoarrays (reviewed in (Nagy and Pogany, 2011)). These characteristics have made yeast a key tool for the discovery of cellular regulators of viral life cycle.

There are many viruses that can replicate in yeast. These include RNA and DNA viruses that infect plants (BMV), animals (*Flock House virus* (FHV)) and humans (*Human* Papillomavirus (HPV)) (reviewed in (Alves-Rodrigues et al., 2006)). All these systems share some common features: (i) the RNA-dependent RNA polymerase and other trans-acting elements are expressed from a plasmid in trans, (ii) the genomic viral RNA conserves the wild-type UTRs and is either expressed from a plasmid or electroporated, (iii) the use of a reporter to measure replication. Viral studies in yeast have pioneered the discovery of host factors hijacked by viruses and thus have greatly contributed to the study of virus-host interactions.

4.2. Brome mosaic virus

BMV is a plant (+)RNA virus and a member of the *Bromoviridae* family and the alphavirus-like superfamily. It was isolated from bromegrass (*Bromus inermis*) and it also infects many crop cereals. Due to its simplicity, BMV has been a fruitful model to study (+)RNA virus translation and replication in eukaryotes (Noueiry and Ahlquist, 2003). As an example, BMV was the first RNA virus from which infectious cDNA clones were generated (Ahlquist et al., 1984) and the first virus from higher eukaryotes to be replicated in *S. cerevisiae* (Kao and Sivakumaran, 2000).

BMV has a small segmented genome (Figure I9), with two monocistronic RNAs (RNA1 and RNA2) and one bicistronic RNA (RNA3) (Ahlquist, 1992). The three genomic RNAs are 5' capped but they do not contain a poly(A) tail. Instead, the 3'UTR forms a tRNA-like structure (Drehler et al., 1984; Rietveld et al., 1983). BMV RNA1 (3.2 kb) encodes for protein 1a, which is a multifunctional protein of 109 kDa. The N-terminal domain is responsible for RNA capping *in vivo* and has GTP binding activity. The C-terminal corresponds to the helicase homology domain (Kong et al., 1999). Protein 1a localizes to the ER membranes and induces the formation of invaginations and spherule compartments where replication occurs. In addition protein 1a directs itself, the viral RNAs and protein 2a to the ER membrane-associated replication complexes (Schwartz et al., 2002). BMV RNA2 (2.9 kb) codifies for protein 2a, which is a 94 kDa RNA-dependent RNA polymerase. Both 1a and 2a proteins are essential for

replication to take place. BMV RNA3 (2.1 kb) codifies for the 32 kDa cell-to-cell movement protein (protein 3a) in the 5' region and the coat protein (CP, 20 kDa) in the 3' region. Protein 3a is directly translated from RNA3 and is required for the spread of the infection in the natural host. CP is translated from the subgenomic RNA4 (0.9 kb), which is only synthesized from the negative-strand RNA3 used as the replication intermediate. Both 3a protein and CP are only required for virus infectivity in plants but not for viral replication (Janda and Ahlquist, 1998; Sacher and Ahlquist, 1989).

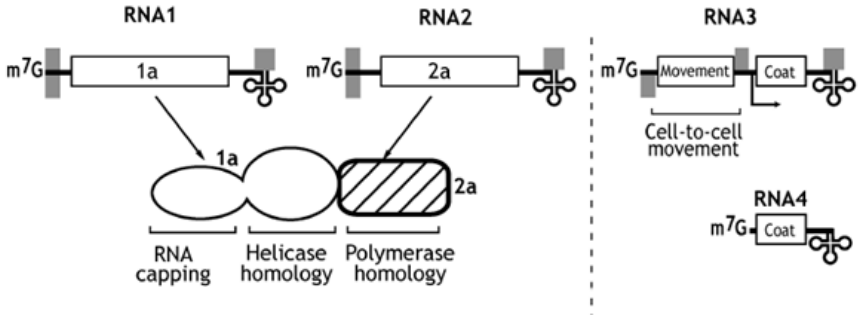


Figure I9. Schematic of BMV genome. BMV RNAs have a cap structure (m7G) and a tRNA-like structure instead of a poly(A) tail at the 3'end (cloverleaf). The ORFs are depicted with open boxes and the solid black lines represent non-coding regions. The bent arrow in RNA3 indicates the start site for the subgenomic RNA4. The replication elements are shown as grey boxes. Proteins 1a and 2a (replication factors) are shown below RNA1 and RNA2. Adapted from (Noueiry and Ahlquist, 2003).

BMV RNAs contain overlapping cis-acting signals that control replication and translation (reviewed in (Sullivan and Ahlquist, 1997)). On one hand, the 5'UTR confers different translational efficiencies to each BMV RNA. For instance, a 5'UTR element in RNA2 represses the translation of protein 2a and regulates the relative abundance of 2a and 1a proteins (Noueiry et al., 2000). In addition, the 5'UTRs of RNA1 and RNA2 also contain conserved elements that are important for replication and recruitment. In RNA3, this element is localized in the intergenic region (IGR). The 3'UTR in all BMV RNAs is highly conserved, both in terms of sequence and structure. The 3' tRNA-like structure is recognized and completed by the host tRNA-nucleotidyl transferase by addition of a 3'-CCA_{OH} and charged *in vivo* with Tyrosine (Noueiry and Ahlquist, 2003). Although BMV 3'UTR stimulates translation, it is not a key determinant in balancing the relative levels of BMV proteins (Neeleman et al., 2004).

The BMV RNA 3' tRNA-like ends are important for replication, since they contain a promoter for the negative-strands synthesis, and for encapsidation (Chapman and Kao, 1999; Choi and Rao, 2003). In turn, the 3' ends of the negative-strand contain sequences that promote the synthesis of genomic RNAs. RNA3 negative-strand has an intergenic region that promotes synthesis of subgenomic RNA4.

4.3. Brome mosaic virus replication in *S. cerevisiae*

BMV can direct viral replication, subgenomic RNA synthesis and encapsidation in *S. cerevisiae*. Importantly, the BMV/yeast system recapitulates all features of BMV replication in the plant host. These include the dependence on proteins 1a and 2a for replication, the excess of positive- to negative-strand RNA and the formation of replication complexes associated to ER membranes (Restrepo-Hartwig and Ahlquist, 1996; Sullivan and Ahlquist, 1999). Thus, *S. cerevisiae* has all the host factors that BMV RNA requires for translation and replication.

The segmented nature of BMV genome enables the study of the different steps of the viral life cycle separately (Figure I10). To study translation, BMV RNA2 or RNA3 are expressed from plasmids that transcribe the viral RNA genomes harbouring the natural UTRs. Translation can be assessed by measuring viral protein levels relative to the amount of viral RNA detected. To study the recruitment of the viral RNAs to membrane-associated complexes, BMV RNA3 harbouring natural UTRs is expressed from a plasmid either alone or together with protein 1a, which is the only viral protein responsible for the selection of the template and the formation of the replication complex. The comparison of RNA3 levels with and without 1a co-expression enables assessment of the recruitment process. Upon recognition by 1a protein, viral RNA3 is recruited to spherules formed in the ER membranes. This confers stability to the viral RNA, since it is spatially protected from the host nucleases (Janda and Ahlquist, 1998).

To study full replication, 1a and 2a proteins are expressed in *trans* together with wild-type RNA3. In order to uncouple replication from translation or recruitment, RNA1 and RNA2 are devoid of the *cis*-acting regulatory elements and their expression is

driven by cellular promoters. Simultaneously, a wild-type RNA3 is introduced to the cell either by electroporation or via *in vivo* transcription from a plasmid (Ishikawa et al., 1997). This strategy allows for monitoring solely the replication of viral RNA3 and the replication-dependent production of sgRNA4. Actually, by replacing the CP coding sequence by a reporter gene high throughput studies were conducted to identify host factors affecting viral replication (Kushner et al., 2003). Finally, encapsidation is studied by providing CP RNA4 in *trans* and expressing BMV RNAs from a plasmid (Krol et al., 1999).

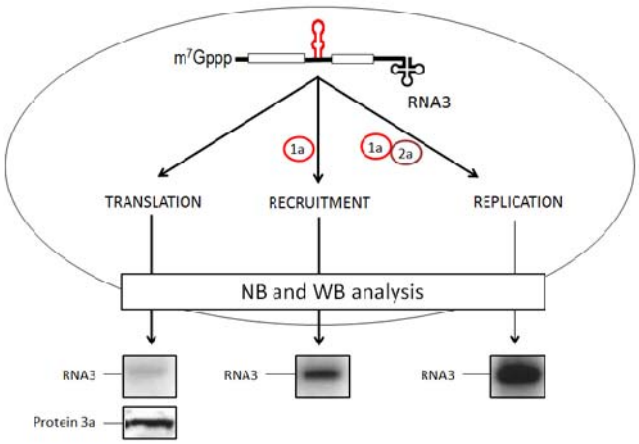


Figure I10. Experimental strategy to dissect the different steps of BMV replication in *S. cerevisiae*. BMV RNA3 harbouring natural UTRs is expressed from a plasmid. The viral RNA can be monitored for translation, recruitment or replication. (I) To study translation, RNA2 or RNA3 and the viral proteins 2a and 3a can be directly quantified by Northern blot (NB) and Western blot (WB) analysis. (II) If viral protein 1a is coexpressed in *trans*, recruitment of RNA3 takes place and an increase in the total amount of RNA3 is observed. (III) If proteins 1a and 2a are co-expressed in *trans*, RNA3 is recruited to membrane-bound replication complexes and replication occurs.

OBJECTIVES

The exonuclease Xrn1 maintains homeostatic mRNA levels by regulating both mRNA degradation and transcription. During preliminary studies in our lab we found that Xrn1 was required for BMV RNA translation. This intriguing observation connected Xrn1 to translation and suggested a novel role for this multifunctional protein. Given that many cellular processes have been first identified in virus research, we wondered whether Xrn1 would be exerting a similar role in the translation of cellular transcripts. Taking into account the high connectivity between different stages of gene expression and the precedents implicating mRNA decay factors in translation, we decided to investigate whether and how Xrn1 controls translation of viral and cellular mRNAs.

The specific aims of this PhD thesis are:

1) To elucidate the molecular mechanism underlying the role of Xrn1 in viral RNA translation.

2) To investigate whether Xrn1 exerts translational control on cellular mRNAs.

MATERIALS AND METHODS

1. Yeast strains and plasmids

Table M1. Yeast strains

Yeast strain	Genotype	Source
BY4742 wild-type	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf
xrn1Δ	*BY4742; MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YGL173c::KanMX4*	Euroscarf
Xrn1-GFP	*BY4741; MATa leu2Δ0 met15Δ0 ura3Δ0 his3Δ1 XRN1-GFP (HIS)*	(Huh et al., 2003)
ltn1Δ	*BY4742; MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YMR247C::HIS*	This study
ltn1Δxrn1Δ	*BY4742; MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YGL173c::KanMX4 YMR247C::HIS*	This study
YPH500 wild-type	*MATα ura3-52 lys2-801 ade2-101 trpΔ63 his3-Δ200 leu2-Δ1*	(Sikorski and Hieter, 1989)
xrn1Δ	*YPH500; MATα ura3-52 lys2-801 ade2-101 trpΔ63 his3-Δ200 leu2-Δ1 YGL173C::URA3*	(Larimer and Stevens, 1990)
BY25598	*W303-1a; MATa ura3-1::ADH1-OsTIR1-9Myc(URA3) ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	(Nishimura and Kanemaki, 2014)
Xrn1-AID	*BY25598; MATa ura3-1::ADH1-OsTIR1-9Myc(URA3) ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100 XRN1-AID (HIS)*	This study
BY4741 wild-type	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
Xrn1 (D208A)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 xrn1D208A*	This study
Xrn1 (ΔNLS)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Xrn1(ΔNLS)*	This study

Table M2. Plasmids

Plasmid	Description	Source
pB2NR3	wild-type BMV RNA2 under a GAL1 promoter (LEU)	(Chen et al., 2001)
pB3RQ39	wild-type BMV RNA3 under a GAL1 promoter (URA)	(Ishikawa et al., 1997)
pB4MK2	wild-type RNA4 under a GAL1 promoter (URA)	(Noueiry et al., 2003)
pJJ-16	RNA2-Renilla Luciferase with viral UTRs (LEU)	Unpublished data
pB2YT5-2	GAL1 5'UTR/2a ORF/ADH1 terminator (LEU)	(Ahola et al., 2000)
pAON_883	BMV 5' UTR/ 2a ORF/ADH1 terminator (LEU)	(Noueiry et al., 2000)
pAON_884	GAL1 5' UTR/ 2a ORF/BMV 3'UTR (LEU)	(Noueiry et al., 2000)
pAON60	BMV 5' UTR/ GFP ORF/BMV 3'UTR (LEU)	(Noueiry et al., 2003)
pAON64	GAL1 5'UTR/GFP ORF/ADH1 terminator (LEU)	(Noueiry et al., 2003)
YCpLac111	Cloning vector used as empty plasmid with LEU marker	(Gietz and Sugino, 1988)
YCpLac33	Cloning vector used as empty plasmid with URA marker	(Gietz and Sugino, 1988)
pBBM1	Xrn1 expressed from its own promoter (URA)	This study
pBBM2	D208A Xrn1 expressed from its own promoter (URA)	This study
pBBM3	Rat1ΔNLS expressed from its own promoter (URA)	This study. Derived from pAJ228
pAJ37	Xrn1 expressed from its own promoter (LEU)	(Johnson and Kolodner, 1991)
pAJ37 E176G	E176G Xrn1 expressed from its own promoter (LEU)	(Johnson and Kolodner, 1991)
pAJ228	Rat1ΔNLS expressed from its own promoter (LEU)	(Johnson, 1997)
pWDH245	Xrn1 expressed from GAL10 promoter (URA)	(Solinger et al., 1999)
pB2NR2	wild-type BMV RNA2 under a GAL1 promoter (TRP)	(Chen et al., 2001)
pMC1	Xrn1 expressed from its own promoter (LEU)	M. Choder Lab (unpublished)
pMC2	Xrn1ΔNLS expressed from its own promoter (LEU)	M. Choder Lab (unpublished)

2. Yeast cultures

Yeast cells were grown in synthetic complete medium (SC) at 30ºC. Galactose (2%) was used as carbon source and it also served as inductor for *GAL1*-directed viral RNA expression. After transformation, three colonies for each condition were selected and streaked on a selective media plate. Cells were grown over-day in selective liquid media and diluted to grow overnight. Next day, they were diluted and grown until the doubling time between triplicates was similar and an OD_{600} of ~0.6 was reached.

3. BMV RNA translation assay

To evaluate BMV RNA translation, yeast cells were transformed with the corresponding plasmids and grown as specified in the previous section. Two OD units (Optical Density units) were harvested for protein extraction and three OD units for total RNA extraction. Total protein was extracted from equivalent number of cells and loaded on an SDS-Page gel to be separated according to their molecular weight. Next, samples were immunoblotted as previously described (Ishikawa et al., 1997). Antibodies against 2a protein, 3a protein (Noueiry and Ahlquist, 2003), CP (Loewe, 07160S/500), GFP (Ahola et al., 2000), PGK (Molecular Probes) and Xrn1 (gift from Arlen Johnson) were used. Detection of 2a protein was done with FUJIFILM Luminiscent Image Analyzer LAS-1000. For the rest of proteins, the infrared imaging system Odyssey (LI-COR Biosciences) was used.

Total RNA from yeast cells was isolated by a hot-phenol method, concentration was measured with nanodrop and 3 µg of total RNA were loaded on denaturing gels for Northern Blot analysis, as described before (Janda and Ahlquist, 1998). MAXIscript *in vitro* transcription kit (Ambion) was used to generate probes that specifically detect RNA2, RNA3, sgRNA4, GFP RNA and 18S RNA. The generation of these probes by *in vitro* transcription was based on previously described plasmids (Alves-Rodrigues et al., 2007; Ishikawa et al., 1997; Noueiry et al., 2003). Northern Blots were developped by exposure to PhophorImager screens and imaging on a Typhoon 8600 (Amersham). Quantification was carried out by measuring band intensity using the ImageQuant software (Molecular Dynamics).

4. Protein turnover assay

Yeast cells transformed with a plasmid encoding for RNA2-Rluc reporter (pJJ-16) were grown as previously described. When they reached an OD_{600} of 0.5, protein synthesis was stopped with 300 µg/ml cycloheximide. Renilla Luciferase activity assay (Dual-Glo®, Promega) was used following the protocol provided by the manufacturer. Samples were collected at different intervals during three hours by directly transferring 10 µl of culture to 100 µl of 1x Passive Lysis Buffer. Only 10 µl of the lysate were used (the rest was stored at -80ºC) and 200 µl of LARII-StopGlo solution (1:1) was subsequently added. FB12 Luminometer was employed to read Luciferase activity, with 5 sec of equilibration time and 5 sec of measurement time. The values obtained were corrected by the corresponding OD_{600} and were represented relatively to the first time-point (t=0). This protocol was adapted from (Preissler et al., 2015).

5. Polysome profiling

Cultures were grown from OD_{600}=0.02 to an OD_{600}=0.5 in YPD media (Formedium) at 30ºC. In order to stabilize elongating ribosomes, cells were treated with cycloheximide (CHX, 100 µg/ml final concentration) during 1 min with manual shaking at room temperature. Cells were quickly harvested with a vaccum filtration system, scraped out of the filter and immediately frozen in liquid nitrogen with 500 µl of lysis buffer (20 mM Tris-HCl (pH=7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X100, 0.5 mM DTT, 100 µg/ml CHX). Cells were lysed with the Freezer/Mill (SPEX SamplePrep) with two cycles of 2 min at 5 cps with a 2 min cooling-down step in between. Cell lysates were thawn at 30ºC for 1 min and centrifuged at 3000 g and 4ºC for 3 min. The soluble fraction was transferred to new tubes and centrifuged at 10000 g and 4ºC for 5 minutes. After quantification, aliquots of 12 UA_{260} were made and stored at -80ºC. Linear gradients of 10%-50% sucrose were prepared in 50 mM Tris-HCl (pH=7.5), 50 mM NH_4Cl, 12 mM $MgCl_2$, 0.5 mM DTT, 100 µg/ml CHX. The Gradient Master (Biocomp) was used for making the gradients in 14x89 mm polyallomer tubes (331372, Beckman Coulter). One aliquot of 12 UA_{260} was loaded on each gradient and centrifuged in a Beckman SW41 rotor at 35000 rpm and 4ºC for 3 h. Gradients were

fractionated with fraction collector Model 2128 (Biorad). These fractions were used for hot-phenol RNA extraction or TCA protein precipitation and analyzed by Northern blot or Western blot, respectively.

6. Co-immunoprecipitation

Yeast cells carrying a genomic Xrn1-GFP fusion (50 ml culture) were grown in exponential phase until an OD_{600}~0.6 was reached. They were harvested by centrifugation (5 min, 3000 rpm, 20ºC) and lysed by vortexing with glass beads (4 cycles of 30 sec and 3 cycles of 1 min) in lysis buffer (50 mM Tris-HCl (pH=7.5), 150 mM NaCl, 0.1% NP-40, 1 mM EDTA and protease inhibitors). After recovering the soluble fraction, total protein amount was measured by Pierce TM BCA Protein Assay Kit (ThermoFisher). As a control for the input sample 100 µg of total protein were kept. For the immunoprecipitation 3 mg of total protein were used. Protein extracts were incubated with 15 µl of GFP-trap_A beads (Chromotek) for 1 h at 4ºC shaking in a rotating mixer. After one hour, samples were measured in the nanodrop and 1.12U RNAseI/10 AU_{260} were added in the RNAse-treated samples. These were incubated for 1h at 22ºC with inversion mixing every 10 minutes. Untreated samples were kept at 4ºC with rotating mixing. Three washes with 500 µl of dilution buffer (10 mM Tris-HCl (pH=7.5), 150 mM NaCl, 0.5 mM EDTA, 1mM PMSF, Protease inhibitor Cocktail) were performed and beads were pelleted in-between by centrifugation (2500 g, 2 min, 4ºC). Beads were resuspended in 20 µl of dilution buffer and 10 µl of 3x loading dye were added. Samples were eluted from the beads by boiling at 95ºC for 5 min.

7. RNA stability assay

Yeast cells transformed with the desired plasmid were grown in selective SC media with 2% galactose, as described before. Cultures of 50 ml were inoculated and were grown until they reached exponential growth and an OD_{600} of 0.7. Three OD units were harvested by centrifugation (4000 rpm, 4ºC, 3 min) and frozen directly in liquid nitrogen. The rest of the yeast culture was centrifuged simultaneously and the media was exchanged with new pre-warmed media containing 2% glucose, in order to shut

off the transcription of BMV RNA2. Samples of 3 OD units were harvested at different time-points (15, 25 and 60 min) and frozen. Total RNA extraction and Northern blot analysis were performed as described in section 3.

8. Translation assay upon Xrn1 auxin-induced degradation

A yeast strain with an integrated TIR1 was used for the generation of a genomic fusion of Xrn1 to an Auxin Induced Degron (AID). Together with TIR1, this fusion enabled the quick degradation of Xrn1 protein upon addition of auxin, taking advantage of a protein degradation pathway in plants (Nishimura and Kanemaki, 2014).

Yeast cells transformed with BMV RNA2-Rluc plasmid were grown in SC media with 2% raffinose until they reached exponential phase and an OD_{600}~0.5 in 50 ml cultures. Galactose (2%) and Auxin (500 µM) were added to induce BMV RNA2-Rluc expression and deplete Xrn1, respectively. Samples were taken at different time-points for OD measurement, Luciferase activity assay and total RNA extraction. Luciferase values were measured as described in section 4. BMV RNA2-Rluc was quantified by reverse transcription quantitative PCR using TaqMan probes and the qScript XLT One-Step RT-qPCR ToughMix (Quanta Biosciences).

9. Ribosome profiling

Yeast cells were grown, harvested and lysed as described in section 5. In the case of ribosome profiling, 10 OD260 units of lysates were treated with 112.5 U of RNaseI (Ambion) for 60 min at 22ºC and 1400 rpm in the Thermomixer. RNaseI activity was stopped by addition of 100 U of SUPERaseIn (Ambion) and digested extracts were loaded in 7%-47% sucrose gradients. The preparation of gradients followed the same protocol as in section 5, but in this case SUPERaseIn was added to the gradients as well (10 U/ml). Ultracentrifugation was performed for 3h at 35000 rpm and 4ºC in a TH-641 rotor (Thermo Scientific). The fractionation of gradients was performed with a Biocomp Instruments Gradient Station (Teledyne Isco) at a rate of 0.75 ml/min.

Monosomal fractions corresponding to digested polysomes were collected, SDS to 1% was added to stop any possible RNAse activity and samples were flash-frozen in liquid nitrogen and stored at -80ºC. RNA was isolated from monosomal fractions using the hot acid phenol method. Ribosome-Protected Fragments (RPFs) were isolated by running 15% polyacrylamide, 8M urea, 1X TBE gels and isolating RNA fragments of 28-32 nucleotides (nt). For RNA-seq, 150 µl of the same lysate were used for total RNA extraction with the hot acid-phenol protocol and subsequent TURBO DNase treatment (Ambion). Total RNA was quantified and 100 µg were used for two rounds of purification with the Poly(A)Purist MAG kit (Ambion). Next, the purified mRNA was fragmented by alkalyne hydrolysis in 50 mM sodium bicarbonate (pH=9.2) and 1 mM EDTA for 20 min at 95ºC. The RNA was purified by ethanol precipitation and fragments of 50-80 nt were selected on a 15% polyacrylamide, 8M urea, 1×TBE gel. The protocol described in (Ingolia et al., 2012) was used to prepare sequencing libraries for both RPFs and fragmented RNA, with minor modifications. The ligation of the 3'-adapter was performed for 4 h at 22ºC with 200,000 U of T4 RNA ligase 2 (truncated, NEB), 25% PEG 8000 and 10 U of SUPERase In.

10. Data analysis

Ribosome profiling

Sequencing data was processed using Bowtie 1.0.0 (Langmead et al., 2009). Ribosomal RNA (rRNA) was removed from the sequencing data and the 3' linker sequence was trimmed. Next, the remaining reads were aligned to the reference. This reference was obtained from the Saccharomyces Genome Database and contained verified or uncharacterized ORFs and 18 nt of their 5' and 3' UTRs (5906 sequences). Only reads mapping to the sense strand and containing no more than one mismatch were considered. Reads mapping within the first 15 codons were removed in order to avoid artefacts close to the initiation site due to the use of cycloheximide, as described before (Nedialkova and Leidel, 2015). Per-gene counts were obtained and differences in gene-level mRNA abundance were tested with DESeq

(Anders and Huber, 2012). Statistical significance was defined by a Benjamini-corrected *p*-value of less than 0.01. To study translational control, DESeq2 package was used. First, mRNA levels were tested for log2(foldchange) to be lower than 0.433 with an adjusted significant *p*-value< 0.1. Next, ribosome occupancy changes were tested for significant differences with an adjusted *p*-value< 0.1 and no fold-change threshold. This way we could define a group of genes with significantly constant mRNA levels and significant RPFs changes. To study ribosome occupancy along CDS, reads were centred and read counts per codon were normalized by gene length and library size (observed/expected). Next, a meta-gene was created by re-scaling all RNAs into 20 bins to obtain a smooth profile and then testing for significant differences. For visualization, the observed/expected values were averaged per bin for each of the groups. A Wilcoxon-Mann-Whitney test was used to detect significant differences in all analyses.

Analysis of PARS scores

Nucleotide resolution PARS scores for yeast mRNAs were obtained from GitHub (https://github.com/abelew/prfdb/tree/master/pars/sce_Score.tab) and further processed using the statistical programming language R. Yeast transcripts were divided in three regions: 5'UTR, CDS and 3'UTR. PARS scores were averaged separately for each region to compare increased or decreased RNA structure between the different groups defined (activated, repressed, not affected). PARS scores as well as feature lengths were statistically tested. A Wilcoxon-Mann-Whitney test was used to detect significant differences in all analyses.

To identify potentially interesting structural differences between the defined groups (activated, repressed, not affected), a meta-gene for each group of RNAs was created by scaling their sequence features to a common length. Ten bins for both the 5' and the 3' UTR as well as eighty bins for CDS were used, the signal was averaged of all nucleotides assigned to the same bin and tested for significant differences between the bins of the different groups.

Other analyses

Biovenn was generally used to overlap our datasets and compare the different groups under study (Hulsen et al., 2008). The functional annotation tool of the FUNSPEC bioinformatics web server (http://funspec.med.utoronto.ca/) was used to analyse Gene Ontology (GO) term enrichment.

RESULTS

1. Xrn1 controls translation of BMV RNA2 through its 5'UTR and CDS

We have previously shown that the decapping activators Lsm1-7, Pat1 and Dhh1 promote translation of BMV RNAs (Alves-Rodrigues et al., 2007; Mas et al., 2006; Noueiry et al., 2003). To test whether the exonuclease Xrn1 also displays this positive role in translation we used the BMV/yeast system.

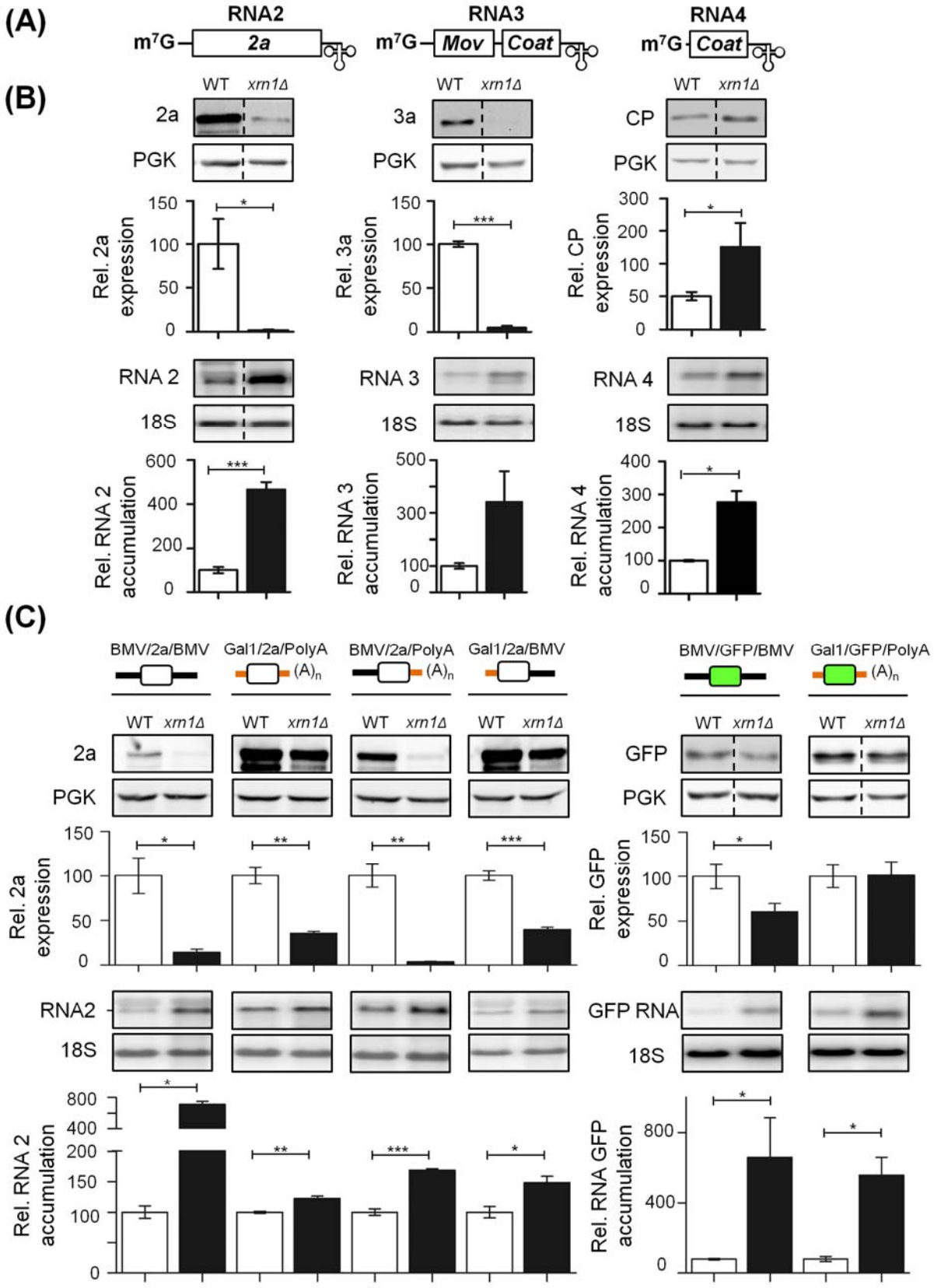
1.1. Xrn1 is required for translation of BMV RNA2 and RNA3, but not of sgRNA4

We transformed wild-type (wt) yeast and an isogenic *xrn1* deletion strain (*xrn1Δ*) with plasmids expressing BMV RNA2, RNA3 or sgRNA4 from the *GAL1* promoter. In this system the transcribed BMV RNAs harbour their natural untranslated regions (UTR) (Figure 1A). We did not include RNA1 in this study as the encoded 1a protein recruits the RNA out of the translation machinery into the viral replication process, and thus it is not possible to discriminate effects in translation from effects in recruitment. Cells were grown to log phase and total RNA and protein were purified. Xrn1 depletion resulted in a dramatic reduction of 2a and 3a protein expression while expression of the coat protein was increased (Figure 1B). The steady-state levels of the three viral RNAs were increased, indicating that Xrn1 degrades BMV RNAs. The Xrn1-dependent reduction in 2a and 3a expression in spite of the overaccumulation of the corresponding viral RNAs, indicates that Xrn1 plays a role in the translation of these mRNAs. To rule out a putative effect of Xrn1 on protein stability, we performed a protein degradation assay using a BMV RNA2-Rluc reporter whose translation still depends on Xrn1 (Figure S1). No difference was observed in the degradation kinetics of 2a-Rluc protein obtained from wt and *xrn1Δ* cells. Thus, as found for a specific group of decapping activators (Alves-Rodrigues et al., 2007; Noueiry et al., 2003), Xrn1 drives translation of genomic RNA2 and RNA3 but not of subgenomic RNA4. As Xrn1 degrades both the genomic and subgenomic BMV RNAs, the degradation of the viral RNAs *per se* cannot explain the positive effect of Xrn1 in translation.

1.2. The 5'UTR and CDS of BMV RNA2 confer dependence on Xrn1 for translation

The 5' and 3' viral UTRs are structured sequences with overlapping *cis*-acting signals essential for the translation and replication of the viral RNA (Sullivan and Ahlquist, 1997). Given that BMV RNA2, RNA3 and sgRNA4 share a highly conserved 3'UTR, Xrn1 -dependence for translation might be linked to sequences located at the 5'UTR or the coding sequence (CDS). To test this, we focused on BMV RNA2 because the *cis*-acting sequences directing its translation have been extensively studied (Noueiry et al., 2000). We used a set of RNA2 constructs that either maintain the natural viral UTRs or replace them by the 5'UTR of the *GAL1* mRNA and/or by the 3'UTR of the *ADH1* mRNA (Figure 1C) (Noueiry et al., 2000). When the two viral UTRs were replaced, 2a protein levels increased both in wt and *xrn1Δ* cells, indicating that the viral UTRs are less favourable for 2a expression. Xrn1-dependence for translation, defined as the ratio of 2a expression in wt compared to *xrn1Δ*, was reduced from 7-fold to 2.5-fold. Next, we examined the 5'UTR and the 3'UTR separately to uncouple their effects. When the viral 3'UTR was replaced, the absolute expression of 2a protein was also increased but Xrn1-dependence was similar to that obtained with the natural BMV RNA2. In contrast, when solely the viral 5'UTR was replaced, we detected both a global increase in 2a levels and a drop in Xrn1-dependence to 2.8-fold.

Figure 1. Xrn1 controls translation of BMV RNA2 through its 5'UTR and CDS.
(A) Schematic of BMV (brome mosaic virus) RNA2, RNA3 and RNA4. Black solid lines represent UTRs (untranslated regions) and boxes depict CDSs (Coding Sequence). tRNA-like 3' ends are shown with a cloverleaf. All the experiments where BMV is involved are based on plasmids encoding for the viral RNAs, which are transformed to wild-type and yeast mutant strains. In all BMV experiments following, such transformations will not be specified in the manuscript any more. **(B)** Western blot and Northern blot analysis showing steady-state levels of viral proteins (2a, 3a and CP) and viral RNAs (RNA2, RNA3 and RNA4) in wild-type (WT) and *xrn1Δ* strains. Dotted lines represent a separation of the shown samples in the same membrane. PGK protein and 18S RNA were used as loading controls for Western and Northern blot, respectively. All Western and Northern blot in this manuscript followed the same controls. Quantification of protein expression (normalized with PGK) and RNA accumulation (normalized with 18S) relative to wild-type levels are represented with a graph below the blots. The graph depicts the mean and the standard error of the mean (SEM), which were calculated from at least three different experiments. Significance was calculated with a t-student test and it is indicated by asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). **(C)** Mapping of RNA2 elements that confer on Xrn1 for translation. From top to bottom: schematic diagrams of BMV RNA2 constructs, Western/Northern blot analysis and quantification in wt and *xrn1Δ* strains. The histograms are all relative to wild-type yeast (100%) for all combinations. Concerning the diagrams; black solid bars represent viral UTRs, orange solid bars represent Gal1 mRNA 5' and ADH1 3' UTRs, the white box represents 2a CDS and the green box the GFP CDS.


(A)
RNA2
m7G
2a
RNA3
m7G
Mov
Coat
RNA4
m7G
Coat
(B)
WT
xrn1Δ
2a
PGK
Rel. 2a expression
3a
Rel. 3a expression
CP
Rel. CP expression
RNA 2
18S
Rel. RNA 2 accumulation
RNA 3
Rel. RNA 3 accumulation
RNA 4
Rel. RNA 4 accumulation
(C)
BMV/2a/BMV
Gal1/2a/PolyA
(A)n
BMV/2a/PolyA
Gal1/2a/BMV
BMV/GFP/BMV
Gal1/GFP/PolyA
GFP
Rel. GFP expression
RNA2
GFP RNA
Rel. RNA 2 accumulation
Rel. RNA GFP accumulation

The RNA steady-state levels of all the constructs were increased in *xrn1Δ* cells, indicating that Xrn1 drives their degradation. These results indicate that the 5'UTR is a key *cis*-acting determinant in conferring dependence on Xrn1 for translation.

Noteworthy, Xrn1-dependence was not completely abrogated even when both viral UTRs were replaced, suggesting that the CDS was also contributing to it. To explore this possibility, we generated two constructs containing the GFP (Green Fluorescent Protein) CDS and either cellular or viral UTRs (Figure 1C). When GFP was fused to cellular UTRs, GFP expression was equivalent in wt and *xrn1Δ.* However, when the cellular UTRs were replaced by the viral UTRs, a significant 2-fold difference in GFP expression was observed between wt and *xrn1Δ*. As a similar increase in GFP RNA levels was detected in both constructs upon Xrn1 deletion, differences in 2a expression levels correspond to changes in translation rather than in mRNA stability. All together, these data show that both the 5'UTR and the 2a CDS confer dependence on Xrn1 for translation.

2. Xrn1 promotes BMV RNA2 translation initiation and interacts with ribosomal proteins

Next, by polysome profiling we investigated at which step of translation Xrn1 was exerting its positive role. In agreement with previous studies, global translation was only mildly affected in *xrn1Δ* (Figure 2A)(Larimer et al., 1992). Northern Blot analyses of RNA2 distribution along the gradient showed that Xrn1 depletion shifted RNA2 from heavy polysomal to monosomal and free subunits fractions (Figure 2B). To assure that the observed changes were related to differences in RNA2 association to ribosomes, we fractionated parallel samples in the presence of EDTA (Figure S2A). In accordance with a specific association with ribosomes, EDTA treatment shifted BMV RNA2 from heavy polysomes to lighter fractions. These results suggest that Xrn1 is required for efficient translation initiation of BMV RNA2. However, we cannot rule out that defects in translation elongation are also taking place.

To explore further the implication of Xrn1 in translation initiation, we examined the interaction of Xrn1 with the translation machinery by two different approaches. First, we analysed the association of Xrn1 to ribosomes by polysome profiling. After gradient fractionation and TCA precipitation we observed an Xrn1 enrichment in fractions corresponding to the free 40S and 60S subunits (Figure 2C) but not in monosomal (80S) or polysomal fractions (LP and HP). We performed the same experiment with a fully functional Xrn1-GFP fusion, obtaining similar results (Figure S2B). Second, we carried out immunoprecipitation assays using Xrn1-GFP to detect putative interactions with ribosomal proteins (Figure 2D). PGK was used as a negative control and Pat1, which is known to interact with Xrn1, as a positive control. Xrn1 co-immunoprecipitated with Rpl17 and Rps26, which are two ribosomal proteins from the large (60S) and the small (40S) subunits. The signal corresponding to Rpl17 and Rps26 was enriched 5-fold and 3-fold in comparison to background. Upon RNAseI treatment, this enrichment was further increased to 28-fold and 7-fold, respectively, suggesting a rearrangement of the interactions after RNA digestion. In addition, Xrn1 co-imunprecipitated with eIF4E independently of RNA with a 3-fold enrichment

over background. Taken together, these results show an interaction of the exonuclease Xrn1 with the translation machinery, supporting a putative role of Xrn1 in translation control.

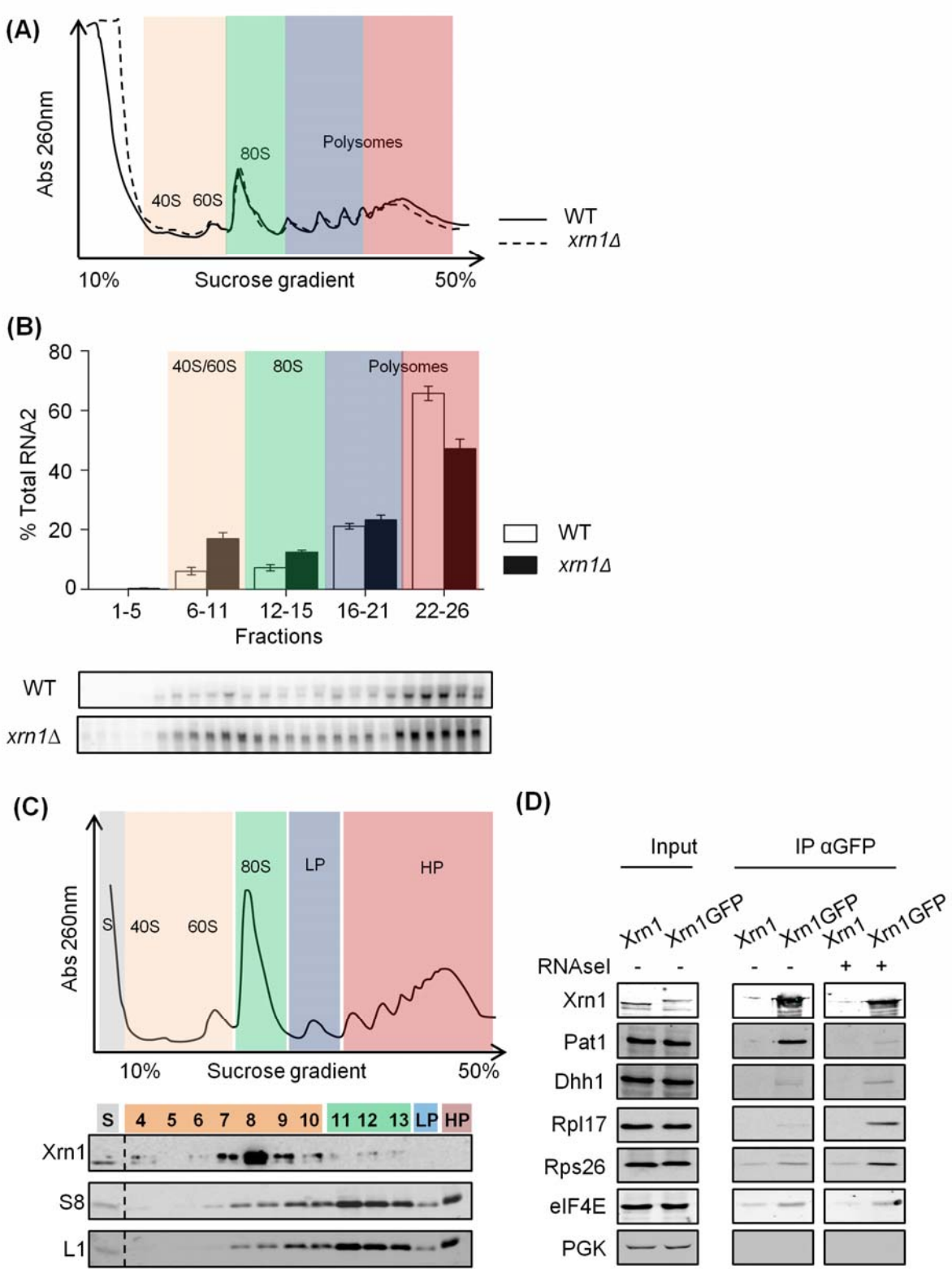


Figure 2. Xrn1 promotes BMV RNA2 translation initiation.
(A) UV absorbance at 260 nm corresponding to the polysome profile of wt and *xrn1Δ* strains after sedimentation on a 10 to 50% (wt/vol) sucrose gradient. **(B)** Quantification of the BMV RNA2 present in each fraction relative to the total amount of BMV RNA2 in the whole gradient, grouped in fractions as specified. Error bars depict the SEM calculated from three different experiments. Below are representative Northern blots. **(C)** UV absorbance at 260nm corresponding to the polysome profile on a 10 to 50% (wt/vol) sucrose gradient of a wt yeast. Protein was TCA precipitated from each fraction and analyzed by Western blot using antibodies directed against Xrn1, S8 (small ribosomal subunit) and L1 (large ribosomal subunit). S: Soluble fraction, single fractions analyzed (4 to 10), 80S monosomal fractions (11-13), LP: light polysomal fractions, HP: heavy polysomal fractions. **(D)** Western blot corresponding to the immunoprecipitation of Xrn1 and Xrn1-GFP cell lysates with GFPtrap ChromoTek beads. Leftmost column corresponds to the input samples and the middle and rightmost columns correspond to the immunprecipitates. RNAse treatment is indicated with a (+) symbol. All proteins detected by Western blot are indicated.

In polysome profiling analysis we observed a strong signal of BMV RNA2 in polysomal fractions of *xrn1Δ* cells. Taking into account that 2a protein stability is not affected upon Xrn1 depletion (Figure S1), we sought to determine whether other processes contribute to the dramatic decrease in 2a protein production. One possibility is that ribosomes stall during BMV RNA2 translation, activating the Ribosome Quality Control (RQC) pathway. RQC detects stalled ribosomes and orchestrates the recycling of the translation machinery and the degradation of the nascent polypeptides. Ltn1 is an RQC E3 ubiquitin ligase that targets stalled peptides for proteasomal degradation. When Ltn1 is mutated, there is an increase in the accumulation of arrested polypeptides and full-length arrested proteins (Bengtson and Joazeiro, 2010). The presence of a polybasic tract in the protein sequence causes ribosome stalling (Brandman and Hegde, 2016). Indeed, following previously published criteria we found a polybasic tract close to the C-terminal end of the 2a protein (Figure 3A) (Brandman et al., 2012). Next, we examined whether stalling on BMV RNA2 occurred in *ltn1Δ* and *ltn1Δxrn1Δ* mutant cells. Ltn1 depletion increased protein 2a accumulation 2-fold both in the presence or absence of Xrn1 (Figure 3B), indicating that RQC modulates 2a expression. Intriguingly, when Ltn1 was depleted in *xrn1Δ* cells RNA2 steady-state levels decreased while 2a expression increased, suggesting that translation elongation is affected in *xrn1Δ* and thus that Xrn1 is required to overcome ribosome stalling during translation elongation.

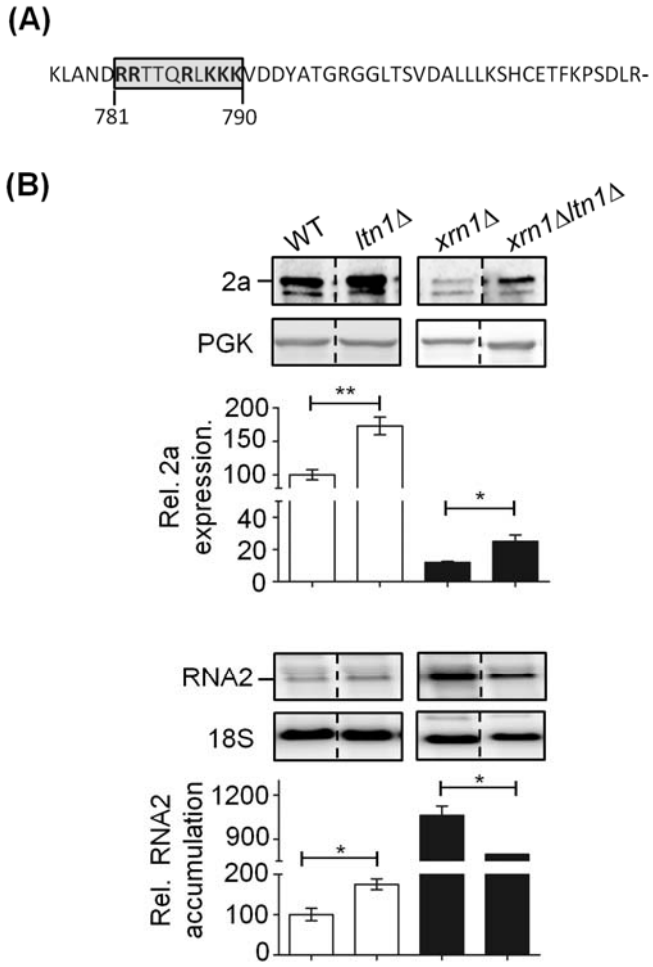
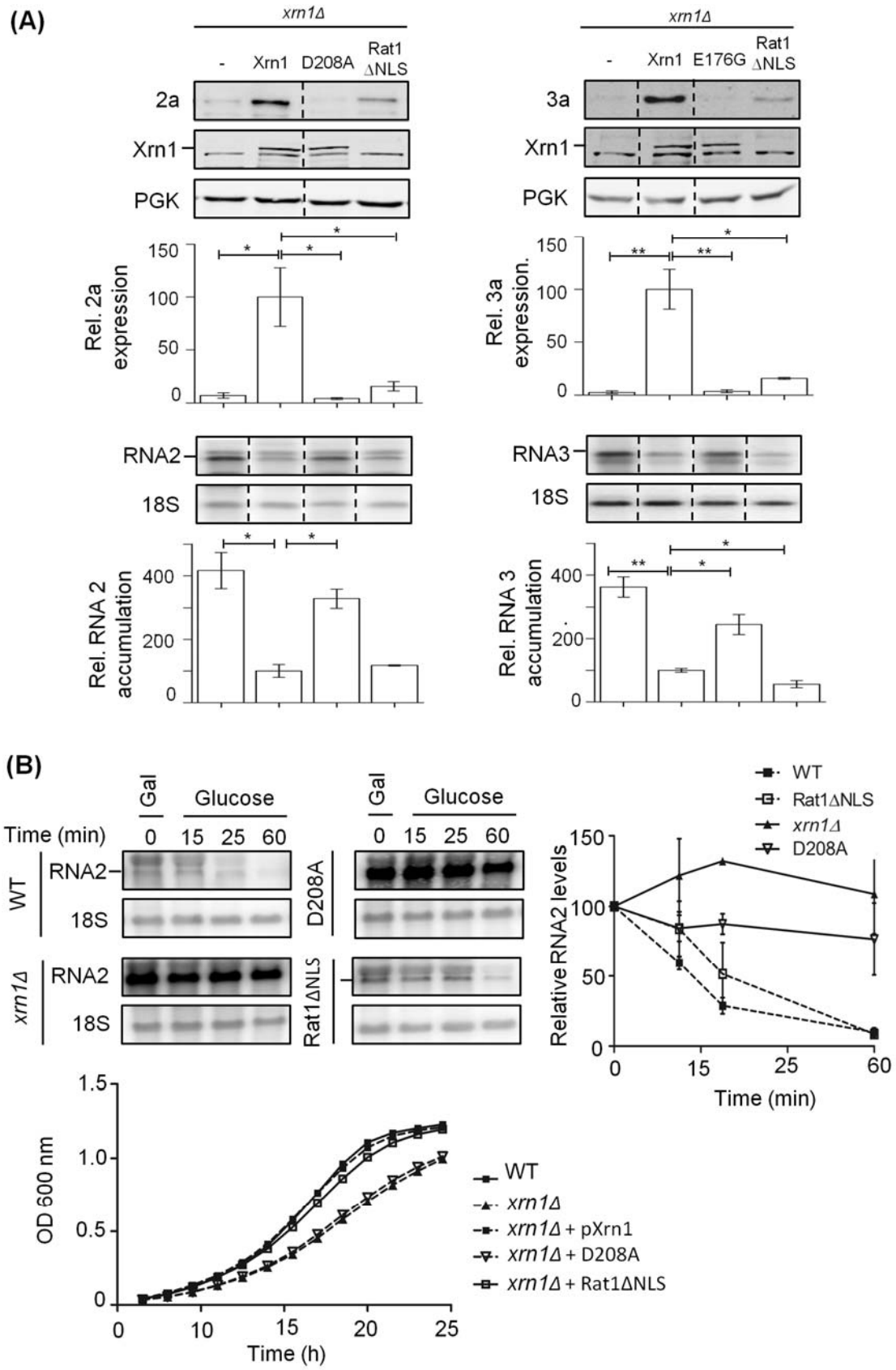


Figure 3. Xrn1 is required to overcome ribosome stalling during translation elongation.
(A) Polybasic tract (shaded in grey) localized at the C-terminal end of viral protein 2a. Positive residues are marked in bold and residue number indicated below. – indicates termination codon. **(B)** Western blot and Northern blot analysis showing steady-state levels of viral 2a protein and BMV RNA2 in wt, *ltn1Δ, xrn1Δ* and *xrn1Δltn1Δ* strains. Quantifications are all relative to the wt yeast strain (100%).

3. Xrn1 exonuclease activity but not its nuclear localization is required for BMV RNA2 translation

Xrn1 regulates mRNA levels by controlling both mRNA degradation and transcription. We used different mutants to test whether any of these functions was related to the role of Xrn1 in stimulating BMV RNA2 translation. To abrogate Xrn1 exonuclease activity we introduced either D208A or E176G point mutations, which completely abolish exonuclease activity (Solinger et al., 1999). To abrogate the function of Xrn1 in transcription, we used an Xrn1 with a mutated NLS that can no longer shuttle to the nucleus (kindly provided by Mordechai Choder, unpublished).

By transforming *xrn1Δ* cells with a plasmid encoding for wt Xrn1, we rescued the expression of the viral proteins 2a and 3a and the accumulation of viral RNA was reduced to wt levels (Figure 4A). In contrast, when cells were transformed with a catalytically dead Xrn1 mutant, D208A or E176G, expression of 2a and 3a was completely abolished and viral RNA levels were increased. These differences were not related to an altered abundance of the mutant Xrn1 (Figure 4A), suggesting that the exonuclease activity of Xrn1 is linked to its function in translation. To investigate whether any exonuclease activity *per se* or the specific exonuclease activity associated to Xrn1 is required for viral RNA translation, we made use of a Rat1 mutant. The 5'-3' exonuclease Rat1 is the nuclear paralog of Xrn1. It suppresses mutant phenotypes of *xrn1Δ* cells when restricted to the cytoplasm by deletion of the nuclear localization signal (Rat1ΔNLS) (Johnson, 1997). In line with this, Rat1ΔNLS could direct viral RNA2 degradation and suppress the growth defect associated to *xrn1Δ* (Figure 4B). However, Rat1ΔNLS could not rescue the translation of BMV RNA2 or RNA3, since levels of proteins 2a and 3a were only marginally increased. Thus, Rat1ΔNLS functionally replaces Xrn1 in BMV RNA2 degradation and growth, but not in viral RNA translation. This indicates that specific features of Xrn1 and its exonuclease activity are needed for viral RNA translation.


(A)
xrn1Δ
-
Xrn1
D208A
Rat1 ΔNLS
2a
Xrn1
PGK
Rel. 2a expression
RNA2
18S
Rel. RNA 2 accumulation
xrn1Δ
-
Xrn1
E176G
Rat1 ΔNLS
3a
Xrn1
PGK
Rel. 3a expression.
RNA3
18S
Rel. RNA 3 accumulation
(B)
Gal
Glucose
Time (min)
0
15
25
60
WT
RNA2
18S
D208A
xrn1Δ
RNA2
18S
Rat1ΔNLS
WT
Rat1ΔNLS
xrn1Δ
D208A
Relative RNA2 levels
Time (min)
OD 600 nm
Time (h)
WT
xrn1Δ
xrn1Δ + pXrn1
xrn1Δ + D208A
xrn1Δ + Rat1ΔNLS

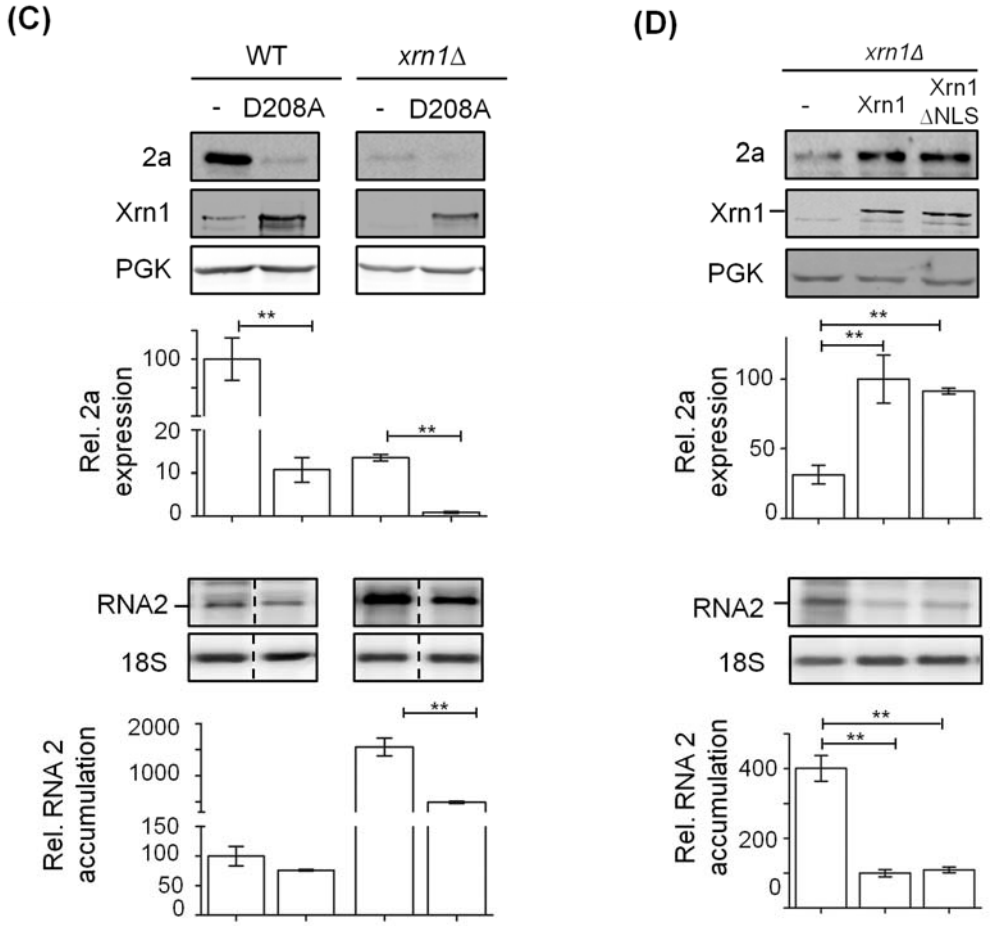


Figure 4. Xrn1 exonuclease activity is required for BMV RNA2 translation.
(A) Western blot and Northern blot analysis showing steady-state levels of viral proteins (2a and 3a) and viral RNAs (RNA2 and RNA3) in *xrn1Δ* cells transformed with either an empty plasmid (-), a plasmid encoding for wild-type Xrn1 (Xrn1), a mutant Xrn1 with impaired exonuclease actvity (D208A or E176G) or the nuclear exonuclease paralog expressed in the cytoplasm (Rat1ΔNLS). Quantifications are all relative to *xrn1Δ* transformed with wt Xrn1 plasmid. **(B)** Top: BMV RNA2 decay analysis in wt cells and *xrn1Δ* cells transformed with the plasmids detailed in figure 4A. Cells were grown in galactose (BMV RNA2 inducer) and the transcription of BMV RNA2 was shut off by addition of glucose (2%). Samples were taken at different time points after glucose addition. Northern blot analysis allowed measurement of total BMV RNA2 in each sample. The graph depicts the quantification of BMV RNA2 in each sample relative to the first time point (t=0). Error bars show the SEM. Bottom: growth curves at 30ºC for wt and *xrn1Δ* cells transformed with the aforementioned plasmids. **(C)** Western blot and Northern blot analysis showing steady-state levels of viral 2a protein and BMV RNA2 in wt or *xrn1Δ* cells transformed with either an empty plasmid (-) or a plasmid encoding for the exonuclease defective Xrn1 (D208A) under a galactose promoter. **(D)** Western blot and Northern blot analysis showing steady -state levels of viral 2a protein and BMV RNA2 in *xrn1Δ* cells transformed with either an empty plasmid (-), a plasmid encoding for wild-type Xrn1 (Xrn1) or a mutant Xrn1 defective for nuclear localization (Xrn1ΔNLS).

The catalytically dead Xrn1 mutant D208A binds decapped mRNAs but cannot degrade them as it gets trapped in an RNA-bound state (Jinek et al., 2011). We reasoned that if the link between viral translation and Xrn1 exonuclease activity was related to Xrn1 being sequestered in this bound state, providing extra copies of Xrn1 D208A would stimulate viral RNA translation. To test this hypothesis we overexpressed Xrn1 D208A both in wt and *xrn1Δ* and examined the effect in viral RNA translation (Figure 4C). In wt, overexpression of Xrn1 D208A reduced 2a protein expression without significantly altering BMV RNA2 levels. In *xrn1Δ* cells, overexpression of Xrn1 D208A did not recover 2a expression and decreased RNA2 levels, probably due to the deleterious effect of D208A in transcription (Haimovich et al., 2013). Importantly, this reduction in viral RNA2 did not account for the drop in 2a accumulation. Thus, overexpression of a catalytically dead Xrn1 mutant exerts a dominant negative effect in BMV RNA2 translation.

To uncouple the function of Xrn1 in transcription and translation we used an Xrn1 with a mutated NLS. Expression of Xrn1ΔNLS in *xrn1Δ* recovered 2a expression and RNA2 accumulation (Figure 4D). Importantly, Xrn1ΔNLS exhibited similar BMV RNA2 degradation kinetics when compared to wt Xrn1 and was expressed at equivalent levels (Figure S3 and Figure 4D). Therefore, the function of Xrn1 in stimulating RNA2 translation is independent of its function in cellular transcription.

4. Xrn1 knock down results in immediate defects in viral RNA translation

Deletion of a single gene in yeast can lead to the selection of adaptive secondary mutations (Teng et al., 2013). Therefore, stably deleting Xrn1 might cause compensatory events that could result in false positives. To overcome this potential limitation, we used a knock down system that is based on a protein degradation pathway in plants (Nishimura and Kanemaki, 2014). By fusing an auxin-inducible degron (AID) to Xrn1 we generated a fully functional Xrn1-AID capable of stimulating viral RNA2 translation (Figure S4). Wild-type cells carrying a genomic Xrn1-AID fusion in the endogenous locus were grown in raffinose to logarithmic phase. Addition of galactose and auxin resulted in simultaneous induction of BMV RNA2-Rluc transcription and depletion of Xrn1, respectively (Figure 5A and 5B). Samples were collected at different time-points and luciferase activity and BMV RNA2-Rluc RNA levels were measured. Xrn1 protein levels decreased upon addition of auxin and could no longer be detected after 35 minutes (Figure 5B). Notably, global translation was not affected upon Xrn1 depletion as the ratio of polysomes to monosmes remained unchanged 50 minutes after auxin addition (Figure 5C). This is in contrast to the mild decrease in global translation observed for the stable *xrn1Δ* mutant (Figure 2A). Expression of 2a-Rluc protein was lower in auxin-treated cells compared to the untreated control, as soon as luciferase signal could be detected (25 minutes). This difference became more pronounced at later time-points, with a 2-, 3- and 4-fold decrease at 35, 50 and 150 minutes, respectively (Figure 5D). RNA2-Rluc levels were equivalent in both conditions up until 50 minutes after auxin addition. In contrast, a clear overaccumulation of RNA2-Rluc RNA could be observed after 150 minutes (Figure 5E). Thus, defects in BMV 2a-Rluc expression were detectable earlier than defects in BMV RNA2-Rluc accumulation. Together, these results indicate that decreased viral translation in Xrn1 depleted cells is immediate and unlikely to be caused by secondary adaptive effects.

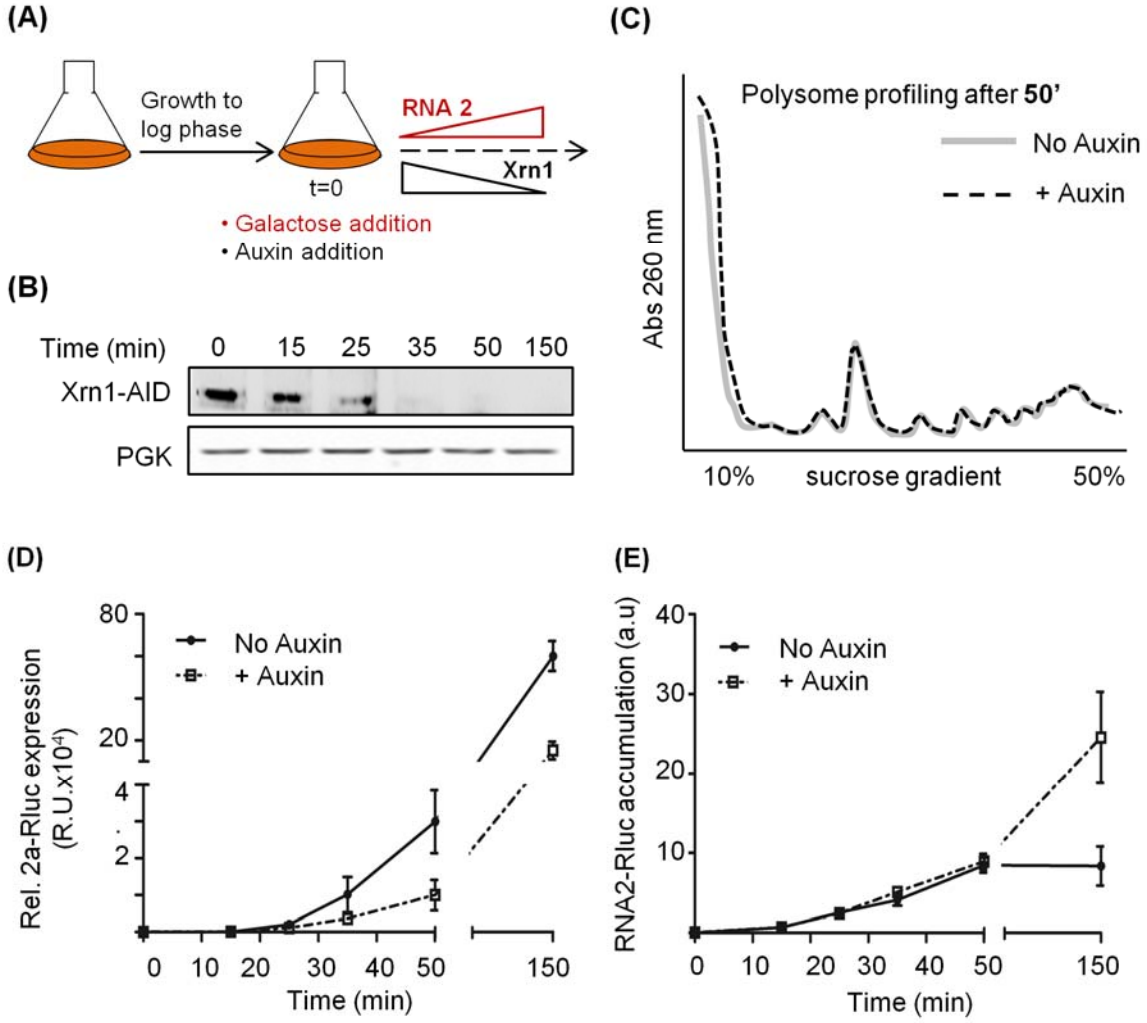


Figure 5. Xrn1 knock down leads to an immediate defect in viral translation.
(A) Scheme of the experimental set up to deplete Xrn1 and express viral 2a-Rluc simultaneously. The yeast strain used contains a genomic fusion of an auxin inducible degron (AID) to the C-terminus of Xrn1. Cells were grown in raffinose (2%) as carbon source until they reached log phase and an OD_{600} of 0.5. Galactose (2%) was added to induce 2a-Rluc expression and auxin (500 µM) was added to target Xrn1-AID protein for immediate proteasomal degradation. Samples were collected at different time points to evaluate 2a-Rluc expression and translation. **(B)** Western Blot analysis showing the depletion of Xrn1-AID upon addition of auxin to the media. **(C)** UV absorbance at 260 nm corresponding to the polysome profiles of Xrn1-AID strain without auxin addition (No Auxin), or with the addition of auxin during 50 minutes (+ Auxin). These two profiles are representative of a total of three experiments. **(D)** Relative 2a-Rluc expression (Relative units (R.U.); Light units/OD at 600 nm). **(E)** Relative 2a-Rluc RNA accumulation (arbitrary units (a.u)). Error bars depict the SEM calculated from triplicates.

5. Xrn1 acts as a translational regulator of cellular transcripts

Using the BMV/yeast system, we found that Xrn1 has an unexpected role in the stimulation of viral RNA translation. Taking into account that viruses hijack existing cellular pathways for their own benefit, we wondered whether Xrn1 may also regulate the translation of cellular transcripts. To address this question we used the Xrn1-AID degron system and studied translational changes with ribosome profiling. This method is based on the isolation and deep sequencing of ribosome-protected fragments (RPFs) and parallel transcriptome analysis (Ingolia et al., 2009). The combination of ribosome profiling and rapid Xrn1-AID depletion allowed following genome-wide translation regulation while avoiding potential adaptive effects. Ribosome profiling was performed on samples after 30 and 45 minutes of auxin treatment (Figure 6A). As before, global translation was not affected and Xrn1 accumulation was reduced to less than 10% (Figure 5C and 6A). Similarity plots showed good clustering between replicates (Fig S5A).

We first examined how Xrn1 depletion affects cellular mRNA levels. We overlapped our 30 and 45 minutes data to select those mRNAs with consistent behaviour in our two time-points. Next, we divided the data into three different groups. mRNAs that were (i) significantly unchanged, (ii) significantly decreased or (iii) significantly increased in Xrn1-depleted cells in comparison to untreated controls (Figure 6B). Downregulated mRNAs (332) were functionally enriched with gene ontology (GO) terms related to ribosome biogenesis and rRNA processing (p-value<10^{-14}). These data are consistent with the known role of Xrn1 in the transcription and mRNA degradation of genes implicated in ribosome biogenesis (Medina et al., 2014). Upregulated mRNAs (267) were functionally enriched for transcription-related functions (p-value<10^{-6}).

Next, we focused on mRNAs with translational regulation upon Xrn1 depletion. For this purpose, we selected genes whose mRNA levels remained unchanged but had significant changes in ribosome occupancy measured by RPFs (Figure 6C). We identified 152 mRNAs that are activated by Xrn1 and 78 that are repressed by Xrn1. Activated mRNAs were significantly enriched for functions related to protein

glycosylation and transport from ER to Golgi (Figure 6D, top), as well as ER- and Golgi-resident proteins (Figure 6D, bottom). Repressed mRNAs were enriched for functions related to protein unfolding, protein transport and aerobic respiration. The mitochondrial inner membrane and the ER were the most highly enriched cellular compartments (Figure 6E).

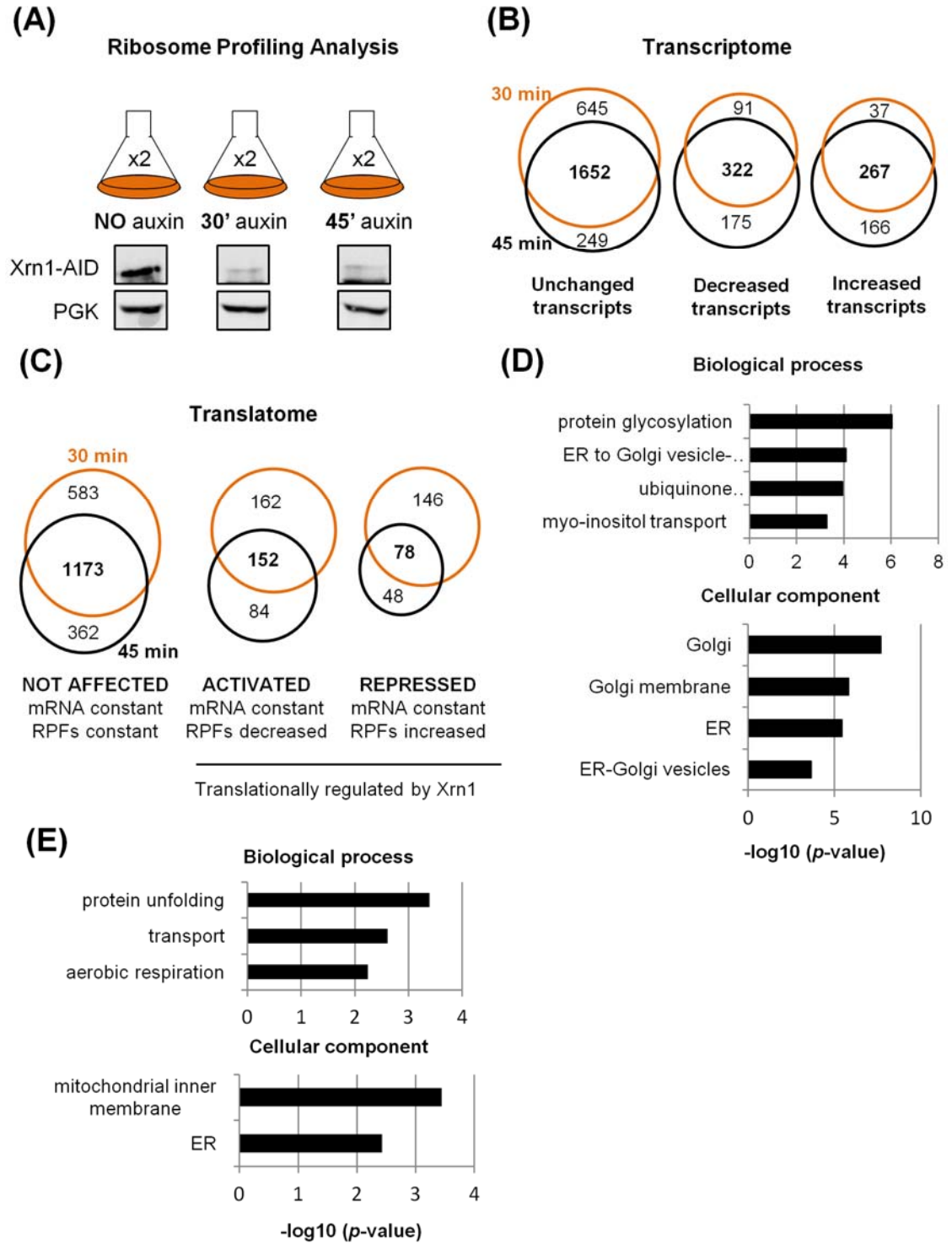


Figure 6. Xrn1 acts as a translational regulator of cellular mRNAs.
(A) Experimental set up of the samples used for ribosome profiling. Two duplicates for each condition (no auxin, 30 and 45 min with auxin) were included. Western Blot analysis showed Xrn1 depletion after auxin addition. **(B)** Analysis of the results from RNAseq. Common genes were considered for analysis. **(C)** Analysis of the results from ribosome profiling. From the group of common genes with significantly unchanged mRNA levels (defined in Figure 6B) we analyzed changes in RPFs. We considered Activated and Repressed groups as the ones being translationally regulated by Xrn1. **(D)** Gene ontology (GO) analysis of Activated genes. Top: Biological Process GO-terms. Bottom: Cellular compartment. **(E)** GO analysis of Repressed genes. Top: biological process. Bottom: cellular compartment.

To gain insight into the function of Xrn1 in translational control, we investigated whether mRNAs regulated by Xrn1 shared common physical properties. First, we calculated the average length of the 5'UTR, CDS and 3'UTR for mRNAs translationally activated (A), repressed (R) or not affected (NA) by Xrn1 (Figure 7A). Xrn1-activated mRNAs had significantly longer 5'UTRs (122 nt) and CDS (1885 nt) when compared to repressed (75 and 1185 nt) or not affected mRNAs (80 nt and 1460 nt). In contrast, Xrn1-repressed mRNAs had longer 3'UTR (176 nt) when compared to activated (128 nt) and not affected groups (121 nt). Second, given that the Xrn1-dependence of BMV RNA2 for efficient translation is mainly linked to its highly structured 5'UTR and the CDS (Figure 1C), we sought to determine whether cellular transcripts translationally regulated by Xrn1 also shared this feature. We used previously published datasets of genome-wide RNA secondary structure obtained by PARS (Parallel Analysis of RNA structure) to analyze the RNA structure profile in the 5'UTR, CDS and 3'UTR (Kertesz et al., 2010). PARS is based on the deep sequencing of RNA fragments obtained by RNA digestion with structure-specific enzymes. Thus, it provides experimental *in vitro* data on RNA secondary structure at single nulecotide resolution. Interestingly, while there were no significant differences in the 3'UTR, cellular transcripts activated by Xrn1 had a significantly higher PARS score (0.20) in the 5'UTR compared to repressed (0.05) and not affected mRNAs (0.077) (Figure 7A). We also observed a higher PARS score in the CDS in Xrn1-activated (0.4) and repressed mRNAs (0.44) in comparison to the unchanged ones (0.27), although this effect was not as pronounced as for the 5' UTR.

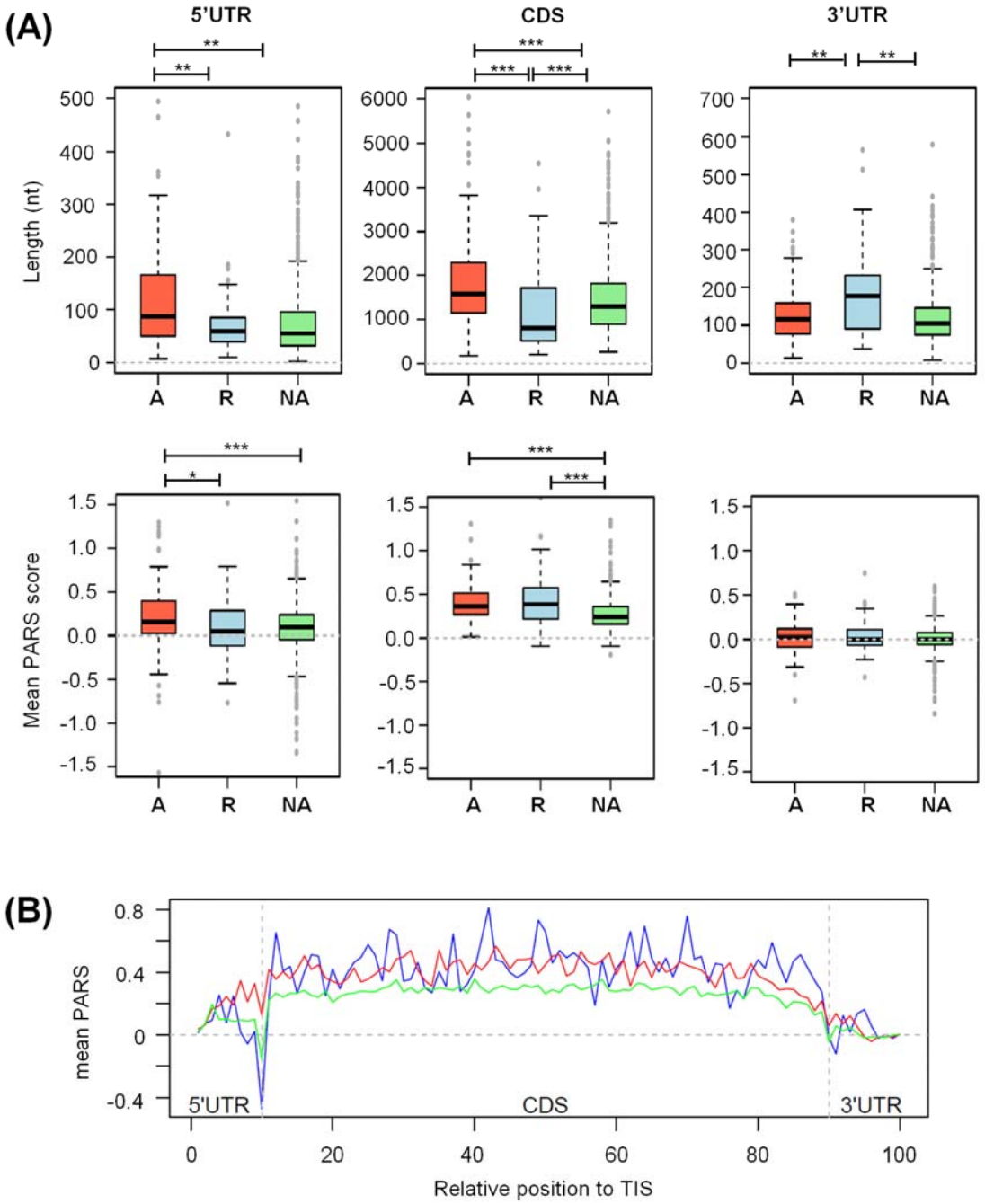


Figure 7. Xrn1 activates translation of mRNAs with highly structured 5'UTR and CDS.
(A) Box-plot depicting the mean length (top) or the mean PARS score (bottom) of the 5'UTR, CDS and 3'UTR for Activated (A), Repressed (R) and Not affected (NA) genes. Statistical significance was calculated with the Wilcoxon-test (* p<0.05, ** p<0.01, *** p<0.001) **(B)** Meta-gene analysis of the PARS scores for Activated (red), Repressed (blue) and Not Affected (green) mRNAs. Dotted lines separate the three different regions: 5'UTR, CDS and 3'UTR. The x-axis represents the relative position to the transcription initiation site.

Next, we analyzed the PARS score distribution along a virtual metagene for each of the three mRNA groups. Xrn1-repressed and not affected mRNAs display a significant drop in mean PARS score around the translation initiation site. The fact that this drop is not present in Xrn1-activated transcripts suggests a more structured translation initiation context for this group of genes (Figure 7B). These results demonstrate that highly structured 5'UTRs are a common feature between viral and cellular transcripts dependent on Xrn1 for translation. This suggests a common Xrn1-dependent mechanism for translational regulation of cellular and viral RNAs.

As BMV RNA2 translation initiation is less efficient in *xrn1Δ*, we asked whether the changes observed in ribosome occupancy in activated transcripts were due to changes in initiation and/or changes during elongation. A discrete ribosome pausing increases the likelihood of capturing footprints by sequencing. Therefore, defects in elongation might result in a peak in ribosome density. In contrast, defects in translation initiation result in lower ribosome occupancy all along the CDS. To this end, we examined the relative RPF distribution along the CDS of the genes translationally activated by Xrn1. Notably, we detected a general reduction of footprint density rather than pic and a downstream sudden drop in ribosome occupancy (Figure S5B). This shows that Xrn1 is required for efficient translation initiation of a specific subset of transcripts.

The role of Xrn1 in the activation of viral RNA translation is linked to its exonuclease activity and is independent of its function in cellular transcription. To test whether Xrn1 exerts cellular translational control through a similar mechanism, we performed ribosome profiling on stable genomic Xrn1 mutants targeting either the exonuclease activity (D208A) or the function in transcription (Xrn1ΔNLS). We followed the same approach to analyze the data and define the group of translationally activated mRNAs as before. Next, we compared the results previously obtained with the Xrn1 degron system to the data generated with the stable Xrn1 mutants (Figure 8). Xrn1-activated mRNAs highly overlap with the group of mRNAs dependent on the exonuclease activity of Xrn1 for translational activation. One-third of the transcripts (47/152) identified as translationally activated by Xrn1 with the degron approach are present

in the same group for the exonuclease defective mutant (D208A). These mRNAs are enriched for protein glycosylation functions and 50% of them belong to the secretory pathway. Importantly, when analyzing solely the transcripts dependent on the exonuclease activity for activation, we also observe an enrichment for glycosylation functions and for secretome proteins. Oppositely, no overlap was found with the transcripts that depend on the function of Xrn1 in transcription, which were enriched for ribosome biogenesis functions. These data indicate that translational activation of cellular mRNAs mediated by Xrn1 is dependent on Xrn1 exonuclease activity and independent of Xrn1 function in transcription. Thus, viral and cellular mRNAs are translationally activated by Xrn1 through similar mechanisms.

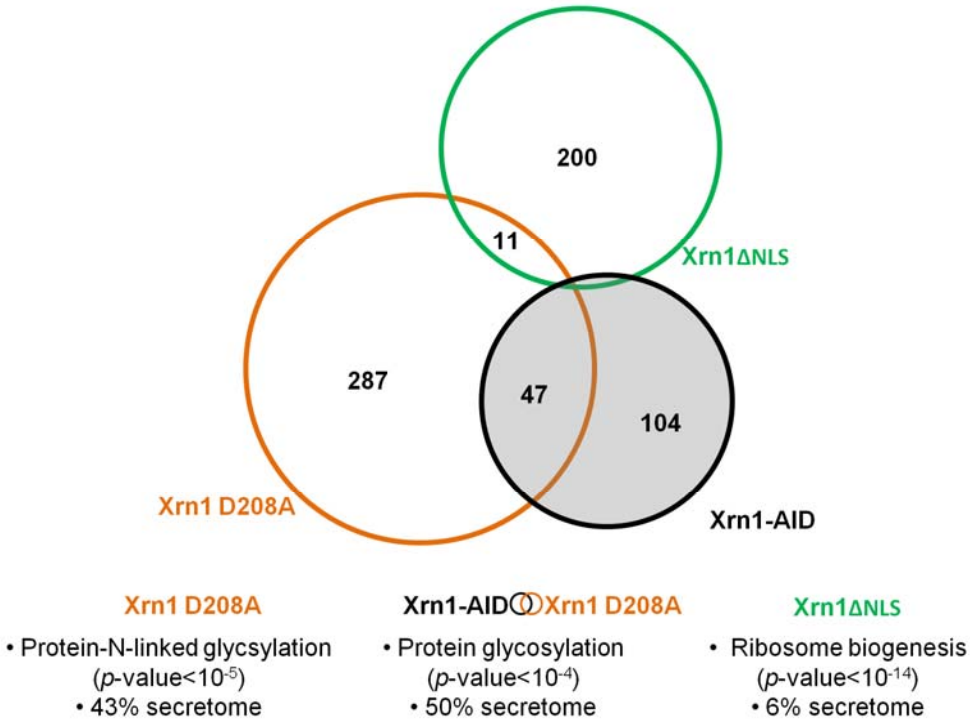


Figure 8. Xrn1-dependent translational activation is linked to its exonuclease activity.
Top: Venn diagram depecting the overlap between activated mRNAs defined in the Xrn1-AID system (Figure 6) and the equivalent groups of mRNAs found for Xrn1 D208A mutant and Xrn1ΔNLS mutant, by ribosome profiling. Below: The most significant enriched GO-term and the percentage of mRNAs comprised in the secretome are indicated.

SUPPLEMENTAL FIGURES

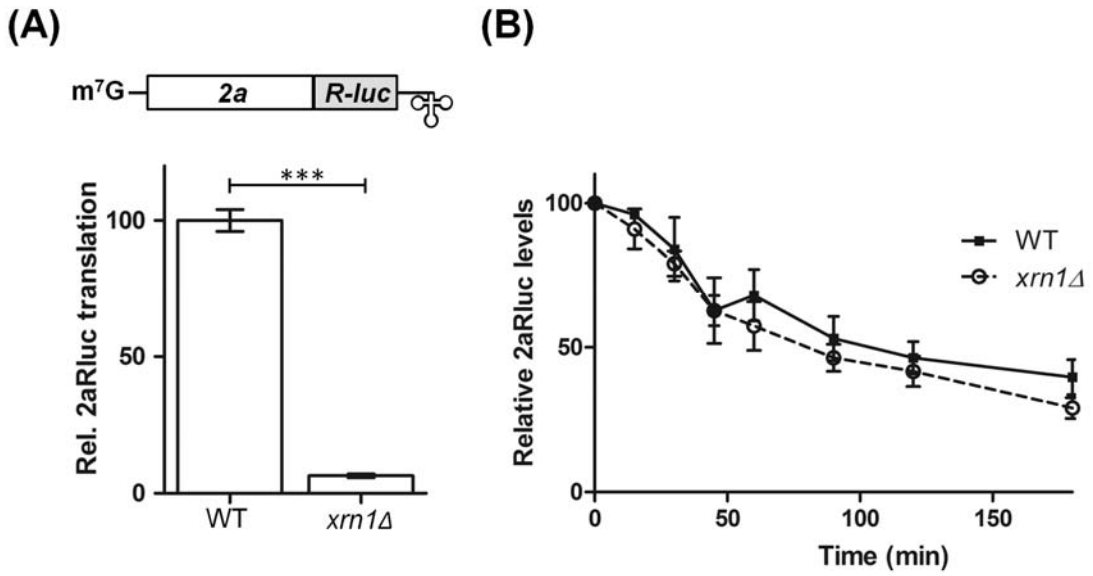


Figure S1. The stability of BMV 2a protein is not affected in *xrn1Δ*.
(A) Top; schematic of the reporter construct for BMV RNA2. This construct (2a-Rluc) maintains the viral 5'UTR and 3'UTR and incorporates Renilla Luciferase (Rluc) in frame to the carboxy terminal of the 2a protein. Bottom; histogram showing the translation of 2a-Rluc (Light units/OD at 600 nm normalized by RNA2 accumulation measured by quantitative PCR) in wild-type (WT) and *xrn1Δ* strains. **(B)** 2a-Rluc protein turnover in wt and *xrn1Δ* strains. Cells were grown in log phase and the translation elongation inhibitor cycloheximide was added at a final concentration of 300 µg/ml. Samples were collected at different time points and measured for Rluc activity. The graph shows the normalized amount of 2a-Rluc (light units/OD at 600 nm) relative to the first time point. Error bars depict SEM.

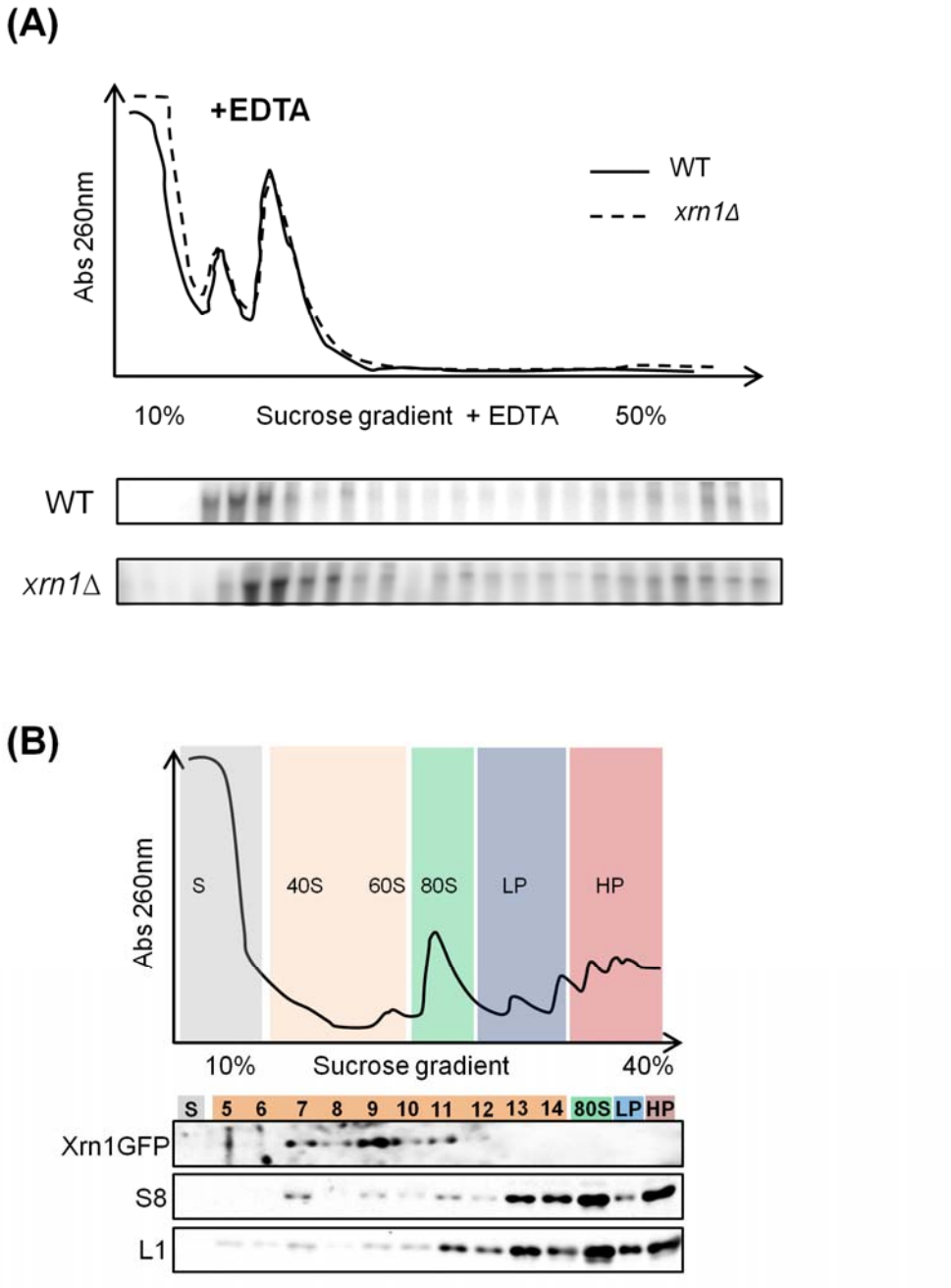


Figure S2. RNA2 found in polysomal fractions is associated to ribosomes.
(A) Top; UV absorbance at 260 nm corresponding to the polysome profile of wt and *xrn1Δ* strains after sedimentation on a 10 to 50% (wt/vol) sucrose gradient with 15 mM EDTA. Bottom; representative Northern blots corresponding to the BMV RNA2 present in each fraction. **(B)** UV absorbance at 260 nm corresponding to the polysome profile on a 10 to 40% (wt/vol) sucrose gradient of a wt yeast. Protein was TCA precipitated from each fraction and analyzed by Western blot using antibodies directed against GFP, S8 (small ribosomal subunit) and L1 (large ribosomal subunit). S: Soluble fraction, single fractions analyzed (5 to 14), 80S: monosomal fractions, LP: light polysomal fractions, HP: heavy polysomal fractions.

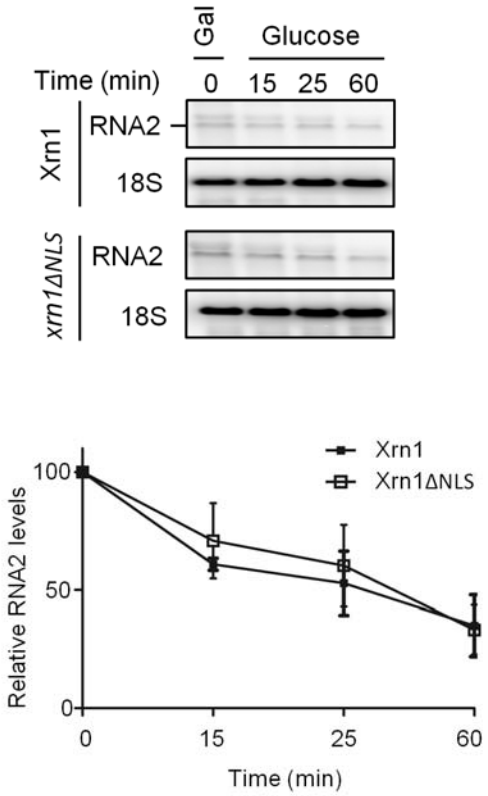


Figure S3. Xrn1ΔNLS displays wild-type degradation kinetics of BMV RNA2
BMV RNA2 decay analysis in *xrn1Δ* cells transformed with wild-type Xrn1 or Xrn1ΔNLS. Top: Northern blot analysis. Bottom: quantification of BMV RNA2 relative to the first time-point (t=0).

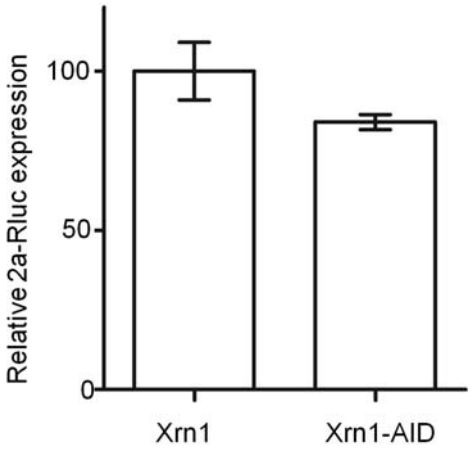


Figure S4. Fusion of AID to the C-ter end of Xrn1 does not affect its functionality
Histogram depicts 2a-Rluc expression (light units/OD at 600 nm) in wt yeast cells and the isogenic strain with Xrn1 CDS fused to an auxin inducible degron (AID) in its C-terminus.

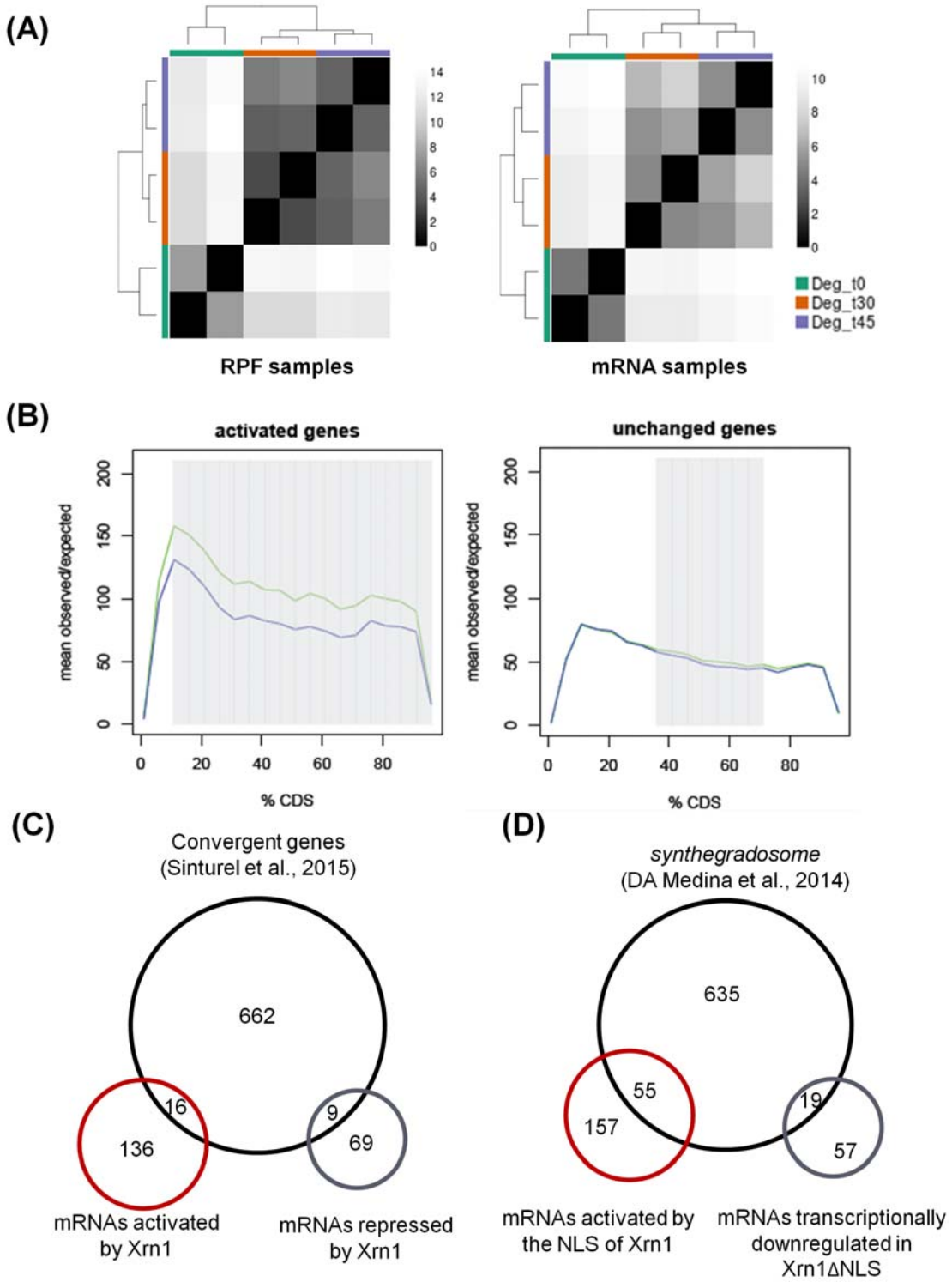


Figure S5. Supplementary analysis for Ribosome Profiling experiment.
(A) Similarity measurement between duplicates in RNAseq and RPF libraries for the three timepoints (0, 30 and 45 minutes) **(B)** Metagene analysis of the RPF coverage for Activated and Not affected mRNAs. Green: no treatment, Blue: treatment with auxin for 30 minutes. Grey shades indicate statistically significant different between the two curves in that particular window. **(C)** Overlap of Activated and Repressed genes with the convergent genes forming hybrids, identified in the study by (Sinturel et al., 2015). **(D)** Overlap of the the synthegradon genes with genes translationally activated by the NLS of Xrn1 or transcriptionally downregulated in Xrn1ΔNLS.

DISCUSSION

By using the BMV/yeast system we show that Xrn1 promotes the translation of BMV RNA. Our data indicate that the 5'UTR and the CDS confer dependence on Xrn1 for translation. Multiple evidences support that Xrn1 promotes translation initiation of BMV RNA. First, by polysome profiling we observe a shift of BMV RNA2 from polysomal to monosomal and free subunits fractions. Second, Xrn1 is found in fractions corresponding to free 40S and 60S subunits. Third, Xrn1 co-immunoprecipitates with ribosomal proteins and eIF4E. Interestingly, by ribosome profiling we uncover that Xrn1 promotes translation of cellular transcripts enriched for secretome proteins. As for BMV RNA, these mRNAs contain highly structured 5'UTRs. Similarly to the role of Xrn1 in transcription, the Xrn1-dependent translation of viral and cellular mRNAs requires the exonuclease activity of Xrn1. Taken together, our data indicate that viral RNAs have hijacked a translational control mechanism in which Xrn1 promotes translation of highly structured mRNAs.

Our work shows for the first time that the exonuclease Xrn1 is required for the translation of viral RNAs. The mRNA decay factors Pat1, Lsm1-7 or Dhh1 and their human counterparts have been described to participate in the translation of BMV and higher eukaryote viruses such as HCV, DENV or WNV (Alves-Rodrigues et al., 2007; Chahar et al., 2013; Scheller and Díez, 2009; Ward et al., 2011). However, a role of an exonuclease in enhancing viral translation had never been reported before. Noteworthy, although translation of Vaccinia virus (VacV) is reduced upon Xrn1 depletion, this is related to a general translational shutoff caused by the increased formation of viral dsRNA. In contrast, the role of Xrn1 is constricted to BMV RNA translation, since previous studies demonstrated that Xrn1 does not affect either replication or recruitment of the viral RNA to the replication complex (Ahola et al., 2000). Xrn1 promotes translation of BMV RNA2 and RNA3 but not of sgRNA4 (Figure 1B). Interestingly, previous studies reported different translation rates of CP in comparison to the non-structural proteins 2a and 3a in a wheat germ cell-free system. In conditions that are limiting for translation initiation, translation was more efficient for sgRNA4 than for BMV RNA2 or RNA3 (Chroboczek et al., 1980). These differences in translational efficiency are important for the viral life cycle, since they

regulate the temporal production of viral proteins. During the first steps of an infection a limited amount of 2a polymerase needs to be produced. After several rounds of replication, the translation of viral non-structural proteins is shifted to the production of CP from sgRNA4, so that the genomic RNA can be encapsidated and virions produced (Sullivan and Ahlquist, 1997). The fact that RNA3 and sgRNA4 share the same 3'UTR links this difference in translation rate to the short unstructured 5'UTR of sgRNA4 in contrast to the longer highly structured 5'UTRs of RNA2 and RNA3. The presence of *cis*-acting elements such as mRNA structures in the 5'UTR have been long known to influence translation initiation (Gebauer et al., 2012). Accordingly, we proved that the 5' UTR confers dependence on Xrn1 for translation (Figure 1C).

Previously it was reported that Xrn1 is present in polysomes and associates to unassembled ribosomal subunits (Covarrubias et al., 2011; Lubas et al., 2013; Mangus and Jacobson, 1999). However, these interactions were connected to the role of Xrn1 in co-translational mRNA decay. In our hands, Xrn1 localizes preferentially in the fractions corresponding to the free 40S and 60S subunits. Moreover, Xrn1 co-immunoprecipitates with ribosomal proteins and eIF4E independently of mRNA, suggesting a direct role of Xrn1 in translation (Figure 2). Interestingly, previous studies demonstrated that Xrn1 and eIF4E are genetically linked (Brown et al., 2000). A single point mutation in eIF4E that abrogates interaction with eIF4G is synthetic lethal with Xrn1 disruption. In addition, mutants of the nuclear guanylyltransferase Ceg1 are also synthetic lethal with *xrn1Δ*. Thus, Xrn1 deletion enhances the requirement for eIF4E/eIF4G interaction and 5' capping. In line with this, we showed that Xrn1 plays a role in the translation initiation of viral BMV RNA2 and cellular mRNAs with highly structured 5'UTRs. Taken together, our biochemical and functional data suggest a direct implication of Xrn1 in cap-dependent translation.

Xrn1 mediates translational activation of viral and cellular mRNAs through similar mechanisms. This is supported by four different observations. First, viral and cellular mRNAs that are translationally activated by Xrn1 share similar RNA features. The 5'UTR of BMV RNA2 is highly structured (Noueiry et al., 2000) and transfers

dependence on Xrn1 for translation (Figure 1C). The cellular mRNAs activated by Xrn1 have higher PARS score at the 5´UTRs than those mRNAs that are not translationally activated by Xrn1 (Figure 7A). Second, both viral and cellular mRNAs require Xrn1 for efficient translation initiation. In viral RNA translation, depletion of Xrn1 leads to a shift of BMV RNA2 from polysomal towards monosomal fractions. Equally, Xrn1 depletion leads to a clear decrease in ribosome occupancy all along the CDS for Xrn1-activated mRNAs, suggesting a defect in translation initiation. Third, the exonuclease activity of Xrn1 is required for the translational activation of both BMV RNA2 and cellular mRNAs. A single point mutation abrogating Xrn1 exonuclease activity decreases BMV RNA2 translation and reduces ribosome occupancy in one-third of Xrn1-activated cellular transcripts (Figure 4 and Figure 8). Fourth, cellular mRNAs translationally activated by Xrn1 are enriched in ER proteins. Although there is no evidence that BMV 2a and 3a proteins are translated in association with the ER, it is intriguing that Xrn1 is needed for the translation of viral RNAs that are recruited to the ER to serve as templates for replication (BMV RNA2 and RNA3), but not for sgRNA4, which is not replicated. Given that viral replication occurs in spherules formed in the ER, it is tempting to speculate that viruses have hijacked a mechanism for translating viral proteins in close proximity to their function in replication.

The exonuclease activity of Xrn1 is required for the role of Xrn1 in viral and cellular mRNA translation. However, whether this is an indirect or a direct effect has not been elucidated. An indirect effect of Xrn1 in translation could be explained by three different situations. First, the depletion of Xrn1 might lead to the perturbation of mRNA homeostasis and the generation of a hostile cellular environment with too many mRNAs outcompeting highly structured mRNAs for the translation machinery. This would be supported by Sun and co-workers, who described an increase of mRNA levels upon Xrn1 depletion (Sun et al., 2013). However, others have demonstrated that mRNA levels remain constant for most transcripts when Xrn1 is mutated (Haimovich et al., 2013; Medina et al., 2014). Second, the depletion of Xrn1 leads to an increase in the formation of RNA duplexes between convergent genes (Sinturel et al., 2015). A recent work in *S. cerevisiae* demonstrated that hundreds of pairs exist *in*

vivo. By studying the particular case of *POR1-OCA2* mRNAs pair in *xrn1Δ* cells, they could show that an increase in *OCA2* mRNA levels leads to increased base-pairing with *POR1* mRNA, activation of NGD and reduced expression of POR1. Consequently, Xrn1 affects translation of convergent cellular genes by controlling base-pairing events between convergent genes. However, in our ribosome profiling data-set we did not observe enrichment for convergent genes, indicating that the effect we observe in translation upon Xrn1 depletion is not mediated by this indirect mechanism (Figure S5C). Third, Xrn1 depletion could be affecting the mRNA abundance or the expression of a number of factors that are directly implicated in the translation of BMV RNA2 and certain cellular mRNAs.

A direct effect of Xrn1 in translation is supported by the following evidences. First, we observe a physical association of Xrn1 with ribosomes and eIF4E in an RNA-independent manner (Figure 2D). Second, the nuclear exonuclease paralog cannot rescue viral RNA translation when expressed in the cytoplasm (Rat1ΔNLS) of *xrn1Δ* cells, but it rescues BMV RNA2 decay and normal growth rate (Figure 4). The fact that Rat1ΔNLS can complement mRNA decay in *xrn1Δ* suggests that protein-protein interactions in the cytoplasm are not crucial for the function of Xrn1 in mRNA decay (Johnson, 1997). Conversely, it suggests that the role of Xrn1 in translation requires both the exonuclease activity and the specific interaction with cytoplasmic factors. This hypothesis is supported by the fact that Xrn1 protein contains a C-terminal domain that is not present in Rat1. This domain recapitulates the features required for acting as an interaction platform, since it is highly disordered and includes short linear motifs (SLiMs) that have the potential to bind interacting partners in yeast (unpublished observation). Such motifs have been described in the C-terminal domain of Xrn1 in *Homo sapiens* and *Drosophila melanogaster* as well (Jonas and Izaurralde, 2013). Third, upon depletion of Xrn1 with the auxin-induced degron system we observe defects in 2a-Rluc expression prior to defects in RNA2-Rluc accumulation. Fourth, widespread co-translational decay by Xrn1 is more frequent in genes related to vacuole transport in yeast and the endomembrane system in *A. thaliana* (Merret et al., 2015; Pelechano et al., 2015). This observation provides a link between the role of

Xrn1 in co-translational mRNA degradation and translational activation of mRNAs targeted to the ER for translation. An interesting area for future research is determining how these two processes are mechanistically coupled.

Xrn1 and Dhh1 regulate the translation of similar mRNA subsets with overlapping features. Dhh1 is a decapping activator that plays a role in translational repression, both in initiation and elongation (Nissan et al., 2010; Sweet et al., 2012). As found for Xrn1, Dhh1 promotes translation of BMV RNA2 and RNA3, but not of sgRNA4. Although this dependence is mediated by a structured 5'UTR, the function of Dhh1 in translation also requires the 3'UTR and a region inside the CDS. Actually, Jungfleisch and co-workers (submitted work) defined a region close to the AUG that folds into a stem-loop and confers dependence on Dhh1. The function of Dhh1 in the translational activation of cellular mRNAs shares some of its features with Xrn1. First, Dhh1-activated mRNAs have highly structured UTRs and CDS. Second, these mRNAs encode for secretome proteins. Third, Dhh1-activated mRNAs include one third of the Xrn1(D208A)-activated mRNAs. However, Dhh1 controls the translational activation of more than 400 genes that are not common with Xrn1. The fact that Xrn1 is translationally downregulated in *dhh1Δ* could explain the existing overlap between the two datasets. Thus, Dhh1 and Xrn1 both contribute to the translation of proteins of the secretome but their targets are not completely overlapping.

In our study we also identified a subset of mRNAs that are repressed by Xrn1. These were enriched in protein unfolding, protein transport and aerobic respiration GO-terms (Figure 6E). Although no hallmarks for stress were found in Xrn1-depleted samples (unpublished observation), these results suggest an indirect mechanism by which Xrn1 depletion leads to the translational upregulation of the machinery to assist folding and degrade proteins. Given that some of these mRNAs (13 out of 78) are part of the secretome, it is also possible that Xrn1 exerts a direct effect in their translation at the ER. Further experiments will be needed to better understand this question.

Xrn1 acts as a cellular sensor that connects mRNA decay, transcription and translation. Xrn1 plays a key role in the regulation of mRNA decay and transcription.

Xrn1 affects synthesis and degradation of most mRNAs, but it regulates more strongly a group of transcripts related to ribosome biogenesis and translation, named "synthegradosome" (Haimovich et al., 2013; Medina et al., 2014). Depletion of Xrn1 by the auxin-induced degron system leads to a transcriptional downregulation of ribosome biogenesis mRNAs. These mRNAs comprise one-third of the "synthegradosome", suggesting that upon depletion of Xrn1 in the cytoplasm, the function of Xrn1 in the nucleus is compromised while mRNA degradation is still taking place. The fact that mutating the NLS in Xrn1 leads to reduced translation of ribosomal proteins, hints the possibility that Xrn1 is regulating a subset of transcripts (Figure S5D) during three different stages: transcription, degradation and translation. Interestingly, a recent report showed a correlation of transcription rate (TR) and decay rate (DR) with growth rate to maintain constant mRNA concentrations for most cellular transcripts (García-Martínez et al., 2015). However, there are gene subsets that uncouple mRNA degradation and transcription to regulate their relative abundance. For instance, genes related to translation and ribosome biogenesis increase their concentration by increasing their TR in high growth rate conditions. This enables the cell to meet the demand for more translational machinery.

Several observations suggest that Xrn1 acts as a sensor of the translational state of the cell. On one hand, by studying the interplay between RQC and BMV RNA2 translation we noticed that Xrn1 depletion might be involved in increased ribosome stalling, presumably due to a polybasic tract close to the C-terminal end. In line with this, Xrn1 downregulation provides a worse cellular context for the translation of a polybasic reporter, as shown in a genome-wide study (Brandman et al., 2012). Strikingly, Xrn1 itself contains a polybasic tract in its coding sequence that is conserved from yeast to higher eukaryotes. Therefore, it could be that upon translational stress Xrn1 expression might be translationally upregulated due a saturation of the RQC system. Whether this is really the case and the possible implications on cellular metabolism are questions that still need to be explored. Second, Xrn1 controls the translational activation of secretome proteins. ER proteins contain hydrophobic domains and have a strong tendency to aggregate. Therefore,

translation of mRNAs encoding for ER proteins must take place when the cell is capable of folding them properly, avoiding toxic effects. Actually, most ER proteins are translated while being translocated into the ER (Ast et al., 2013). The fact that these mRNAs are co-translationally degraded by Xrn1 more frequently (Pelechano et al., 2015) suggest a role of Xrn1 as a guard, ensuring translation takes place only when mRNA degradation is not impaired.

Collectively, our results demonstrate that Xrn1 plays a key role in translational regulation of viral and cellular mRNAs. The fact that Xrn1 functions in mRNA transcription, degradation and translation reinforces the central position of this protein as a major coordinator of gene expression. Key issues for future work include elucidating the role of Xrn1 under translation stress conditions and the better understanding of the molecular mechanism driving Xrn1-mediated translational activation.

CONCLUSIONS

From the results presented in thesis, the following conclusions can be drawn:

Xrn1 functions in viral RNA translation:

- BMV RNA2 and RNA3 but not sgRNA4 depend on Xrn1 for translation.
- The 5'UTR and the CDS of BMV RNA2 confer dependence on Xrn1 for translation.
- Xrn1 promotes viral RNA translation initiation and interacts with components of the cellular translational machinery.
- The positive function of Xrn1 in viral RNA translation requires its specific exonuclease activity but not its function in transcription.
- The dependence on Xrn1 for viral RNA translation is immediate and unlikely to be caused by adaptive mutations.

Xrn1 functions in cellular mRNA translation:

- Xrn1 is required for the translation initiation of a group of mRNAs with longer and highly structured 5'UTRs that are enriched for ER and Golgi proteins.
- Xrn1 represses the translation of a group of mRNAs enriched for respiration, protein folding and transcription.
- The role of Xrn1 in cellular mRNA translation activation, as in BMV RNA translation, requires its specific exonuclease activity but not its function in transcription.
- Xrn1 not only regulates mRNA transcription and decay but also mRNA translation. Therefore, Xrn1 is a coordinator of multiple stages of gene expression.

ANNEXES

LIST OF ABBREVIATIONS

AID	auxin-induced degron
ARE	AU-rich element
BMV	brome mosaic virus
(CAT)	C-terminal Alanine or Threonine
CDS	coding sequence
CP	coat protein
CHX	cycloheximide
DENV	Dengue virus
ER	endoplasmic reticulum
FHV	Flock House virus
GFP	Green fluorescent protein
HCV	hepatitis C virus
HPV	Human Papillomavirus
Hsf1	heat shock factor 1
IRES	internal ribosome entry site
mRNA	messenger RNA
mRNPs	messenger ribonucleoproteins
NGD	No-go decay
NLS	nuclear localization sequence
NMD	Nonsense-mediated decay
NSD	Non-stop decay
ORF	open reading frame
PARS	Parallel Analysis of RNA Structure
PBs	Processing bodies
PGK	Phosphoglycerate kinase
PKA	protein kinase A
(+)RNA	positive-strand RNA
PV	Poliovirus
Rluc	Renilla luciferase
RNA	Ribonucleic acid
RPF	Ribosome protected fragment
RQC	ribosome-associated quality control
rRNA	ribosomal RNA

SARS-CoV	SARS coronavirus
sfRNA	subgenomic flavivirus RNA
sgRNA4	subgenomic RNA4
SLIMs	short linear motifs
SRP	signal recognition particle
TBSV	tomato bushy stunt virus
TCA	trichloroacetic acid
TOR	target of rapamycin
tRNA	transfer RNA
UTR	untranslated region
WNV	West Nile virus
wt	wild-type
XUTs	Xrn1-sensitive unstable transcripts
ZIKV	Zika virus

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