Investigation of NMD Factors in *Schizosaccharomyces pombe*

Kim Suzanne Piechocki

Supervised by Dr. Saverio Brogna

This thesis is submitted for the degree of

Master of Philosophy

School of Biosciences

2013

SUMMARY

Nonsense Mediated mRNA Decay (NMD) is a translation-coupled mRNA surveillance mechanism which targets aberrant mRNAs harbouring premature termination codons (PTCs). This class of mutations would otherwise result in the translation of truncated proteins that could potentially be detrimental to the cell. Intriguingly, NMD is linked to pre-mRNA splicing: in mammalian cells PTCs induce strong NMD only in the presence of a downstream intron. In these systems, the link between pre-mRNA splicing and NMD is thought to be mediated by the Exon-Junction-Complex (EJC), which is a multiprotein complex that marks splice junctions.

In fission yeast *Schizosaccharomyces pombe*, pre-mRNA splicing can also enhance NMD however this mechanism does not require the EJC. To gain further insight into the mechanisms of NMD I undertook a genome-wide screen to identify and characterize additional factors that may be involved in this process in fission yeast. In particular, I hoped to identify genes required for the link between splicing and NMD. The screen consisted of crossing a GFP reporter strain subject to NMD with haploid deletion mutants of 3,308 non-essential genes in the *S. pombe*.

Analysis of 2790 mutants identified 18 candidates. These were further characterised by northern blot analysis. From these 18 strains, 7 mutants look like genuine NMD mutants resembling the phenotype of known NMD genes. 5 genes have either direct or indirect links with splicing factors or the exosome-associated TRAMP complex. Future work will need to elucidate the molecular mechanism of the NMD phenotype and possible involvement of these genes in splicing, NMD and the TRAMP-mediated mRNA surveillance processes.

ACKNOWLEDGEMENTS

I wish to acknowledge the support obtained from the School of Biosciences at the University of Birmingham where over the last six years I have obtained my undergraduate degree, gained laboratory employment experience and studied for this Masters degree. It will always be an invaluable period of my life for which I am very grateful.

In particular, I wish to thank Dr Saverio Brogna for the opportunity and for his support during my time in his laboratory where I worked as a research technician during the writing of this thesis.

Other key people who provided support and friendship are Dr Jikai Wen who explained the basic techniques in yeast cell culture and molecular biology and helped me with every hurdle encountered.

I would also like to thank Preethi Ramanathan who taught me molecular techniques and introduced me to polysome profiling, Tina McLeod for her friendship, keeping me sane and assisting in lab duties. Emanuela Zaharieva and Min Li for their help during the chaos that prevailed during printing and binding of this thesis. All the members of the Brogna lab, both past and present; Gavin Ross, Sandip De, Jiannan Guo, Akilu Abdullahi, Jianming Wang, Subhendu Roy Choudhury and everyone on the 6th floor for their friendship and for making this time memorable!

Last but not least, my friends from beyond the university, my Family; mom, dad and of course; my boyfriend of eleven years, Jonathan Forty- for all their love, support, and tolerance of me during the difficult times.

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ABREVIATIONS AND ANNOTATIONS

AS	Alternative Splicing
APS	Ammonium persulphate
Dd	Double distilled
EJC	Exon Junction Complex
LB	Lysogeny Broth
NMD	Nonsense Mediated mRNA Decay
Nmt	No message thiamine
NZY	NZ Amine Yeast extract
O/N	Overnight
ORF	Open Reading Frame
PEG	Polyethylene glycol
PTC	Premature Termination Codon
Rt	Room Temperature
SDS	Sodium dodecyl sulphate
SMG	Suppressor with morphological effect on genitalia
St.	Sterilised
UPF	Up-frameshift

CHAPTER I: INTRODUCTION

1.1 Eukaryotic Gene Expression and Conservation of Genetic Fidelity

Eukaryotic gene expression as defined by the Central Dogma of molecular biology proposed by Francis Crick in 1958 (Crick 1970) refers to the process of decoding of the genetic information into proteins.

The general consensus is that transcription and translation are compartmentalised, with DNA being transcribed into a precursor messenger RNA (mRNA) intermediate within the nucleus by RNA polymerase II. During which it undergoes modification by the addition of a 5'-cap structure, 3'-end processing and polyadenylation to add the poly (A) tail, and removal of introns by the process of pre-mRNA splicing. The 5' and 3' modification are important for mRNA stability and have a role in translation initiation and termination. The mature mRNA is exported to the cytoplasm whereby it can sequentially bind the 40S and 60S ribosomal subunits and undergo translation by an elongation-competent 80S ribosome docked at the translation initiation codon. While translation is understood to be confined to the cytoplasm, it should be noted that a number of early studies concluded that this might happen also in the nucleus (Allfrey et al., 1957). In later studies, it has again been reported that amino acid incorporation can be visualised in the nucleus of permeabilised HeLa cells (Iborra 2001). In parallel, it has been reported that many ribosomal proteins and some translation factors are recruited to transcription sites at polytene chromosomes in Drosophila (Brogna, et al., 2002). The study in Drosophila also reported evidence of direct radioactive amino acid incorporation at transcription of intact cells sites and at the nucleolus. Most recently, visualisation of nascent polypeptide chains, also reveal their presence in the nucleus and nucleolus (David et al., 2012). Finally, using a novel technique to visualise the interaction between ribosomal

subunits, the Brogna lab has also found compelling evidence of functional nuclear 80S in Drosophila (manuscript submitted).

1.2 Translation

Translation initiation is a two-step process involving a minimum of nine eukaryotic initiation factors (eIFs) (Jackson 2010). The 40S ribosomal subunit, in conjunction with other factors, is understood to scan the 5'-3' mRNA (as a 43S pre-initiation complex) to locate the start codon. The initiator Methionyl-transfer RNA (Met- tRNA) composes part of the 40S subunit by binding to the ribosomal peptidyl site (P site). Once this charged tRNA anticodon binds with the complementary AUG initiation codon, the 48S initiation complex is formed (Majumdar 2007). The second step in translation initiation is the binding of the 60S subunit to the 48S complex. This is achieved by the displacement of eIFs in the 48S initiation complex, or more specifically, that eIF5 and eIF5b hydrolyses and displaces eIF2-GTP, allowing 80S formation (Jackson 2010).

Translation elongation may proceed in the presence of a complimenting amino acid containing tRNA (aminoacyl-tRNA), eukaryotic elongation factor eEF-1 and GTP to form a complex that can enter the vacant acceptor Site (A site) of the ribosome. Proofreading ensures that the correct anticodon is matched to the codon occupying the A site. Amino acids are coupled to their corresponding tRNA via an ester bond, which is catalysed by aminoacyl-tRNA synthatase. The accuracy of this coupling is critical as the ribosome cannot distinguish between amino acids, only the tRNA anticodon. The ribosome subsequently catalyses the formation of a peptide bond between the new amino acid and the growing polypeptide in the P site (Jørgensen 2006; Capa 1998). It is understood that eIF-2 is responsible for the conformational changes in the ribosome, which possibly induces the mRNA movement by one codon sequentially, there is a co-ordinated movement of the tRNA's, with the peptidyl-

free tRNA displaced to the exit site (E site), allowing a new peptidyl-tRNA to translocate from the A site to the P site (Jørgensen 2006). This process is repeated until a termination codon is encountered. The three possible termination codons that will be encountered do not have a corresponding anticodon. Instead a eukaryotic release factors eRF-1 and eRF-3, a GTP binding protein, form a complex with the ribosome and catalyses the cleavage of the ester bond between the peptide and the final tRNA, releasing the newly synthesised polypeptide from the ribosome. Following the release of additional factors, the ribosome is recycled for another round of translation.

1.3 Nonsense Mediated mRNA Decay

The preservation of cellular function and therefore cell viability depends on genome integrity and its correct expression. It is the inevitable, that with such regulatory complexity, this fundamental system will be prone to high rates of error. Such fidelity is maintained by numerous decay pathways in order to safeguard the cell and these can initiate in both the nucleus and the cytoplasm to tightly regulate both transcription and translation. The most studied of these quality control decay mechanisms is the translation-coupled Nonsense Mediated Decay (NMD) (Jacobson 2007; Schweingruber et al., 2013).

NMD is a quality control mechanism which is involved in the regulation of gene expression, specifically reducing the expression of aberrant mRNAs, which would otherwise result in the translation of truncated proteins that could potentially be detrimental to the cell. This surveillance mechanism has several different substrates, the most apparent being mRNAs that have acquired nonsense mutations. These can either be generated by point mutations or a frame-shift caused by deletions or insertions during DNA replication, transcription or as consequence of inefficient pre-mRNA processing, particularly during splicing (Amrani et al.,

2006; Wen and Brogna, 2010). Nonsense mutations are changes in a triplet of bases that result in a premature termination codon (PTC) that constitutes either, UAG, UAA or UGA.

A class of NMD substrates are aberrant mRNAs containing extended 3'UTRs, as a consequence of a mutation in the poly (A) site, can result in the use of cryptic polyadenylation sites further downstream as 3' end formation sites.

1.4 *Trans*-acting factors involved in NMD

NMD is not a passive mechanism but requires a number of specific and conserved protein factors in order to distinguish between premature termination codons (PTCs) and normal stop codons (Amrani N 2006; Brogna S, Wen J 2009). NMD requires active translation and specific *trans*-acting factors, which include the core machinery composed of the Up-frameshift (UPF) proteins, UPF1,-2 and -3. These factors were first identified in a genetic screen in *S. cerevisiae* (Culbertson 1980), and mutations or deletion of any of the *UPF1-3* genes have been seen to abolish NMD. UPF1 is an RNA binding protein with ATP dependant helicase activity (Cheng, Z et al 2007). It is understood that UPF1 forms a trimeric complex, with UPF2 bridging the interaction of UPF1 and -3. The UPF's are essential for NMD in most cells (Amrani, N 2006; Cheng, Z et al., 2007) and it is believed that it is this complex which links premature translation termination to mRNA degradation (Schweingruber et al., 2013).

Other known NMD factors include the SMG (suppressor with morphological effect on genitalia) proteins in higher eukaryotes. These are important for NMD in *Caenorhabditis elegans* and other higher eukaryotes; *smg2*, *-3* and *-4* are homologs of *UPF1*, *-2* and *-3* respectively) (Conti & Izaurralde 2005). SMG1 is a protein kinase responsible for phosphorylating UPF1. In its phosphorylated state, UPF1 can bind specifically to the

prematurely terminating ribosome, this association is regulated by Smg 5 and -7 through dephosphorylation (Conti & Izaurralde 2005; Page et al., 1999). The interaction of UPF1 with UPF2 and the release factors induces mRNA decapping (Amrani et al., 2006), which triggers mRNA instability. The mRNA decay involves both exonucleolytic degradation directed from the ends of the nascent mRNA and endonucleolytic degradation in the vicinity of the PTC (Gatfield D & Izaurralde E 2004). The latter was understood to be the preferential process of degradation in *Drosophila melanogaster* while in both yeast and human cell, exonucleolytic degradation was thought to be favoured. Interestingly however Smg6 has since been identified as an endonuclease and implicated in the degradation of nonsense transcripts in both human and *D. melanogaster* cells (Eberle et al., 2009; Huntzinger et al., 2008).

1.5 NMD mechanism: how PTCs are distinguished from normal stop codons

Although the mechanism of differentiating PTCs from normal stop codons is not yet fully understood, several models have been proposed. Of the numerous models aiming to address the underlying mechanisms of NMD, conflicting evidence shows the inadequacies of all current explanations, or at least support the concept of species-specific divergence of what was once a conserved process. Initially, in mammalian cells a splicing dependent model based on the discovery of the EJC has been proposed; this defines the distance of the PTC from a splice site as a determining factor in PTC recognition. In support of this model, it was observed that in naturally intronless transcripts, the mRNA is not subject to NMD, but following the artificial insertion of an intron downstream of a normal termination codon, the wild-type stop codon is read as a PTC, resulting in reduced mRNA (Zhang 1998).

However this does not fully explain all the observation and it has been in proposed that a key feature is, like in budding yeast, the distance between the PTC and the poly (A) tail.

Furthermore, a recent characterisation of the NMD phenomenon in *S. pombe* suggests that neither model can explain NMD in this organism. Below I give a brief outline of the current models.

1.6 The EJC model of NMD

In mammalian cells, the link between splicing and NMD is thought to be mediated by the exon junction complex (EJC), a multi-protein complex (Wen and Brogna 2008; le Hir 2000a/b). In human cells a minimum of five EJC components are required for NMD induction, these include; RNPS1, the heterodimer MAGOH:Y14, in addition to Barentsz and EIF4A111. The EJC is deposited by the spliceosome 20-24nt upstream of a splice junction and marks these boundaries as exon-exon junctions (Le Hir et al., 2001).

As normal termination codons tend to reside within the terminal exon, it is thought that the EJC would be displaced by the translating ribosome, thus UPF1 is not activated and the mRNA will escape NMD. A stop codon situated >50-55 nucleotides upstream of the last splicing induced exon-exon junction, has been observed to trigger NMD (Izaurralde et al., 2007; Lejeune and Maquat 2005), and is thought to be discriminated by the positional information provided by the EJC. During the pioneer round of translation, the first ribosome to encounter the PTC in this vicinity will initiate mRNA decay by the SURF complex (consisting of smg-1-upf1-eRF1-eRF3) (Kashima et al., 2006). The concept of a pioneer round of translation is that NMD occurs preferentially during the first translation round while the 5' cap of the mRNA is still associated to the cap binding complex (CBC). The pioneer round is postulated to involve loading of one or more ribosomes and is biochemically distinguishable for the subsequent cycles of steady-state translation (Maquat 2010). The concept that NMD is restricted to the first translation cycle, remain however, controversial in

mammalian systems and there is no support for it in yeast (Amarani 2004; Keperwasser et al., 2004).

1.7 NMD polarity effect and the *Faux* 3' UTR model

Studies in initially in *S. cerevisiae*, but later also in *Drosophila* and mammalian cells, suggest that the distance between a stop codon and the 3'UTR is the key determinant in PTC recognition, and the *Faux* 3' UTR was proposed. This model posits that normal translation to proceed the Poly (A) Binding Protein (PABP) is required to interact with the terminating ribosome and directly with eRF3, facilitating ribosome release from the mRNA. Should a termination codon occur 'abnormally' upstream, the increased distance reduces this interaction, allowing the core NMD factors, UPF proteins to instead interact with the terminating ribosome, thus facilitating NMD.

In support of the above model, the artificial tethering of the poly (A) binding protein (pabp1) in yeast to a region downstream of the PTC leads to 'normal' translation termination without inducing NMD (Amrani 2004). pabp1 is a defining feature of the 3'UTR and possibly mimics the spatial arrangement between the normal termination codon and 3'UTR and suggests that pabp1 acts independently of 3' end cleavage and polyadenylation. By interacting with eRF3 the terminated ribosome is released from the mRNA as in the normal instance of termination (Izaurralde 2007). Consisting with this model is the observation that strong NMD is typically produced only by PTC located at the beginning of the coding region while those located further downstream are often immune. This polarity effect has been first detected in yeast, both *S. cerevisiae* and *S. pombe*, and in *D. melanogaster* (reviewed in Brogna & Wen, 2009; Wen and Brogna, 2010). But there are indications that it may also be occurring in mammalian cells (reviewed in Schweingruber et al., 2013). A strong indication that an abnormal long distance between the PTC and the poly (A) tail is a key NMD trigger in mammalian cells is

the observation that artificial folding of the poly (A) tail next to the PTC inhibits NMD (Eberle et al., 2008; Eberle et al., 2009).

1.8 Project aims

To gain further insight into the mechanisms of NMD, I will execute a genome wide screen of all non-essential genes in *S. pombe*, a powerful model organism allowing advanced molecular genetic manipulation, to identify and characterize any additional factors that may be involved in NMD in fission yeast. The screen will consist of crossing a reporter strain expressing Green Fluorescent Protein (GFP) with the total 3,308 haploid deletion mutants from the *S. pombe* deletion library developed by Bioneer and KRIBB in Korea, representing 92.8% genome coverage.

The reporter consists of a GFP ORF containing an intron insertion in the 3' UTR, immediately after the normal termination codon, subsequently rendering this construct an NMD substrate. The expectation is that in the absence of any gene required for NMD, there will be a reduction or abolishment of the NMD mechanism, and the mRNA substrate will escape degradation. Thus any potential NMD factor can be screened based on an increase in the level of GFP expressed in relation to wild-type, and potentially comparative levels to those produced in the UPF1Δ strain. The mRNA levels will be quantified by Northern Blot analysis.

While the screen will be unbiased, potentially it could identify genes involved in both splicing independent NMD, and splicing dependent NMD, the expectation for the latter is as a consequence of current literature, supporting splicing enhanced NMD (Wen J, Brogna S 2010).

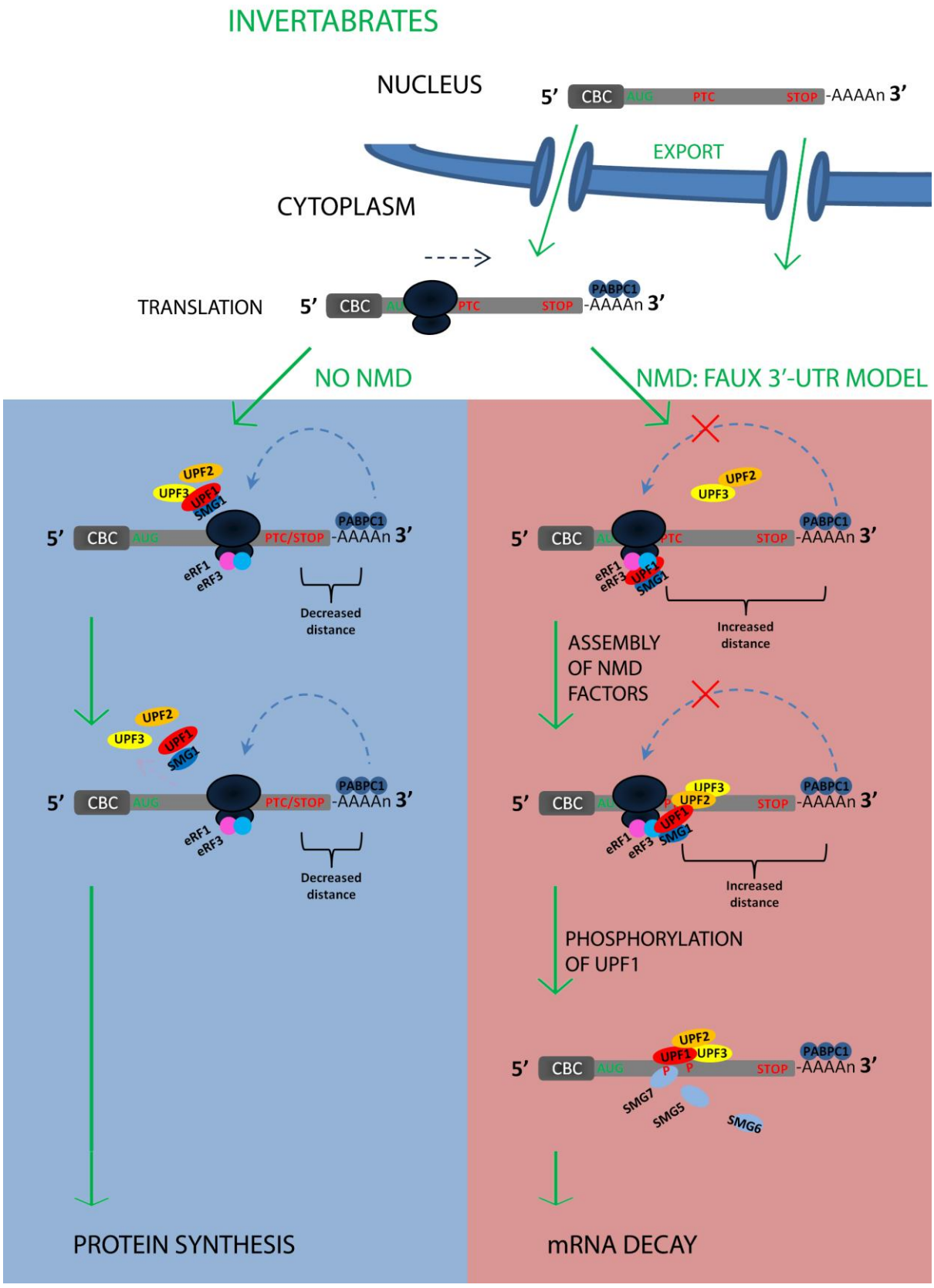


Figure 1:A. Schematic of PTC differentiation in invertebrate and yeast.

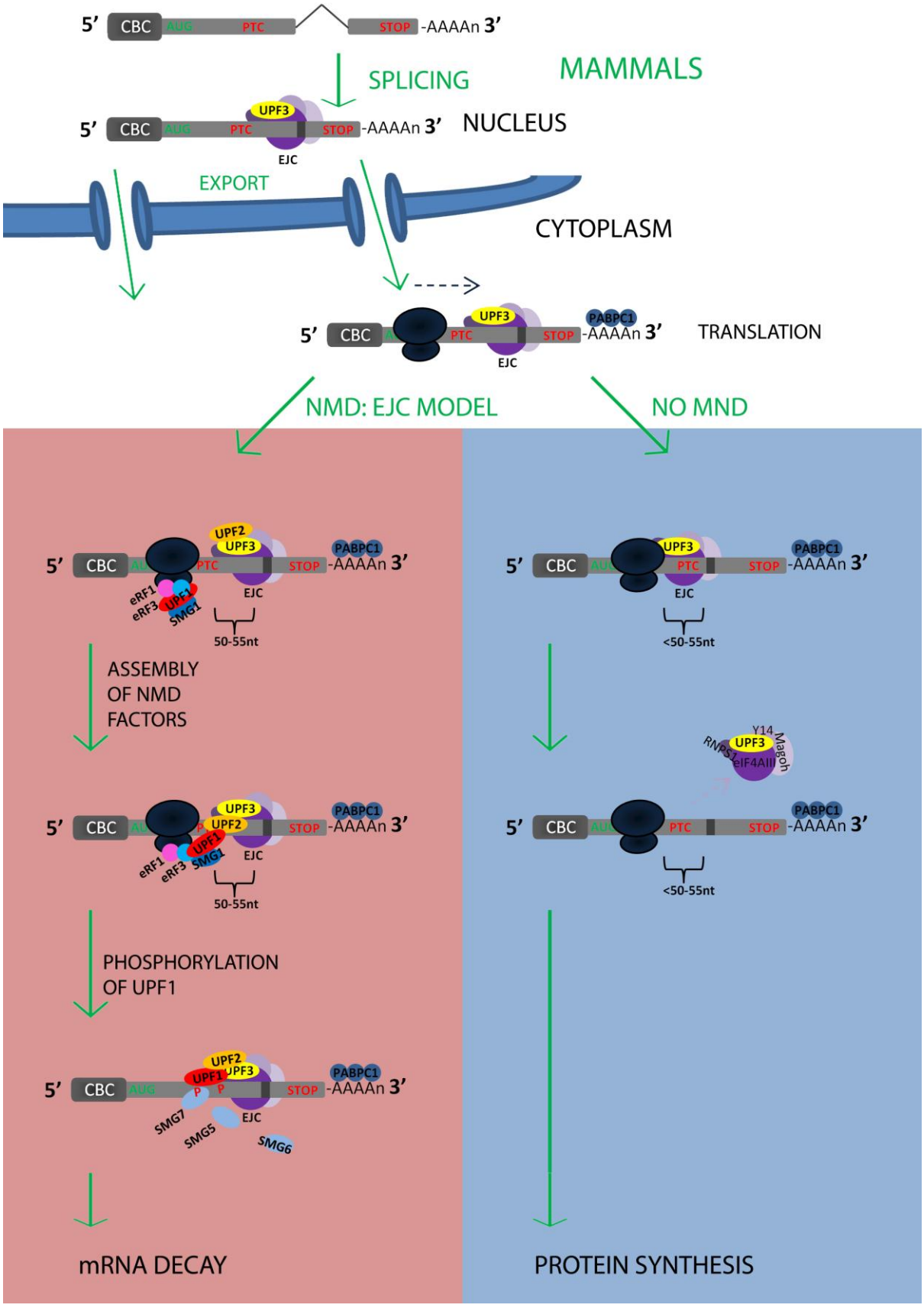


Figure 1:B. Schematic of PTC differentiation in mammalian cells.

Figure 1. Schematic of NMD substrate recognition models.

A) In yeast and Drosophila: **The Faux 3'UTR Model** Splicing independent model postulates that, NMD is determined by the distance between the PTC and the 3' poly (A) tail. Increased distance reduces the interaction of the terminating ribosome and poly (A) binding protein (PABP), allowing NMD factors to instead bind. In the case of normal termination, or if a PTC resides close to the 3'end, then NMD is not initiated and protein synthesis ensues. **B) In mammalian cells: The EJC Model** Splicing dependent NMD model whereby NMD induction is dependent on the PTC residing upstream of the last occurring intron and is facilitated by the recruitment of the EJC complex to the terminating ribosome during pre-mRNA splicing facilitating NMD. During normal termination or in the instance when a PTC resides <50-55 nucleotides upstream of the last exon-exon junction, the EJC is displaced by the translating ribosome and NMD is not initiated. Protein synthesis proceeds.

CHAPTER II: MATERIALS AND METHODS

2.1 Molecular Cloning and Bacterial Cell culture

Refer to Appendix 1 for LB broth and LB agar recipe.

Transformants were grown on either inverted 9cm LB agar plates in 37°C incubator (LEEC Incubator) or as liquid culture, overnight in rotating 37°C incubator (Innova 4000 Incubator Shaker) at 225 RPM.

2.1.1 Rubidium Chloride chemi-competent cells

E.coli (XL1-Blue) cells were rendered chemi-competent by the RB Cl method.

1. XL1-Blue cells were inoculated in 5ml of LB media and grown overnight at 37°C in a rotating incubator.
2. 5ml of overnight culture was further Inoculated into 100ml LB + 1ml 1M $MgCl_2$ + 1ml 1M $MgSO_4$ and incubated at 37°C for ~3-4 hours until OD_{600} reached 0.5-0.6
3. The flask was placed on ice for 15 minutes.
4. The sample was transferred to a pre-cooled micro-centrifuge tube and centrifuged at 4°C, 2-4000 RPM for 10 minutes to pellet cells.
5. The media was discarded and the pellet re-suspended (on ice) in 33ml of cold RF1 buffer (Refer to Appendix 2 for recipes for Buffers)
6. The cells were incubated on ice for 1 hour.

7. After incubation, the cells were centrifuged at 4°C, 2-4000 RPM for 15 minutes to pellet.

8. The RF1 buffer was removed and the pellet was re-suspended in 8ml of cold RF2 buffer.

9. The cells were incubated on ice for 15 minutes.

10. 100-200µl aliquots were distributed into 1.5ml micro-centrifuge tubes and stored at -80°C

11. To confirm transformation efficiency in the newly competent cells, 50µl of cells were transformed with plasmid of known concentration i.e. 1µl of Puc19 (100pg/µl). After transformation, 450µl of NZY media was added to cells and 50µl (equivalent of 10pg/µl) of the total volume was spread on selective media. (Refer to Bacterial transformation protocol below). The number of colonies were counted and efficiency was calculated by applying details to the transformation efficiency calculator: http://www.sciencegateway.org/tools/transform.htm

2.1.2 Bacterial Transformation (XL1-Blue)

1. ≥0.1ng (generally 5ng) of DNA was mixed with 10-50µl of competent cells ($x10^6$) and incubated for 15 minutes on ice.

2. The cells were then heat shocked for 60 seconds in a 42°C water-bath and cooled on ice for a further 2 minutes.

3. 40-450µl of NZY media (refer to appendix 1 for recipe) was added and the cells were allowed to recover for 45 minutes at 37°C

4. After incubation the cells were spread on LB plate containing 100µg/ml of Ampicillin and incubated overnight at 37°C

2.1.3 Large-scale preparation of plasmid (Midi prep)

1. One colony of transformed *E.coli* cells were picked from agar and inoculated into 1ml of LB broth with 100µg/ml of Ampicillin. This was cultured in a 37°C rotating incubator overnight.

2. 300µl of culture was transferred to 50ml of LB broth with 100µg/ml of Ampicillin and further incubated at 37°C for 3 hours or until OD_{650} 0.8 was achieved.

3. Plasmid DNA was extracted using a commercial Hi pure plasmid filter midi prep kit (Invitrogen). The purified Plasmid DNA was re-suspended in 200µl of TE buffer (PH8).

4. The DNA concentration was confirmed by either gel electrophoresis or by nanodrop spectrometer (ND-1000).

2.1.4 Plasmids and Primers

Selective primers used in this study were provided by a former PHD student (Wen, J 2010). Others were designed for this study using pombase and NCBI.

The GFP 3'ivs and GFPivs reporter were cloned by digestion and purification of pREP GFP 3'ivs or pREP GFPivs (provided by former PHD student (Wen, J 2010)) and ligated into pDUAL backbone (Wen, J 2010). The pDUAL based constructs would allow for digestion and integration into *S. pombe* for either stable or episomal expression.

For a list of plasmids and primers used, refer to Appendix 3.

2.1.5 Standard PCR

Buffer (5x)	1x
DNTPs (25mM each)	200µM
Primers (10µM)	0.2 µM (0.1-0.5µM)
Enzyme (5U/µM)	1U/µl
MgCl2* (25mM)	1.5mM
Template	10-20ng
St. ddH_2O	made up volume (total mix: 20-50µl per reaction)

*$MgCl_2$ was only used in conjunction with GoTaq polymerase when GoTaq Flexi buffer was used (Standard Go Taq and Phusion buffer already contains $MgCl_2$).

The DNA polymerase that was routinely used was either GoTaq for checking or Phusion High Fidelity (FINNZYME) for cloning.

For a list of primers used, refer to Appendix 3.

PCR program:

25-30 cycles

Initialisation	95°C	5min
Denaturation	95°C	30sec
Annealing	-	30sec
Extension	72°C	-
Final Extension	72°C	5min
Final hold	4°C	∞

While certain parameters remain constant, Annealing temperature will vary, depending on the melting temperature (T_m) of the primers and the polymerase used (Phusion tends to be double that of GoTaq). Also the extension time varies depending on template size (i.e. GoTaq = 1kb/min). Cycle number tends to be 25 for checking or 30 for other applications.

The PCR reaction was run in a thermal cycler (2720/Applied Biosystems) and the amplified product confirmed by agarose gel electrophoresis.

2.1.6 Colony PCR

A fresh colony was picked and directly re-suspended in 25µl of PCR mix. Refer to standard PCR for details. 35 cycles was applied, in comparison to the standard PCR protocol and the initialisation step was increased to 10 minutes. This substituted the normal colony preparation

step (re-suspending the colony in water and heating to 95°C for 10 minutes prior to adding to PCR mix).

The above protocol is not very efficient, for some applications it was necessary to treat the cell with Zymolase enzyme to ensure the cell wall is broken.

1. Zymolase (10,000U/ml) was prepared in 1U/µl of 0.1M Sodium Phosphate Buffer (PH 7.4) and mixed thoroughly.

   e.g. 1µl Zymolase in 9µl Buffer

2. A fresh colony was selected and re-suspended in the 10µl Zymolase/Buffer mix.

3. The sample was incubated at 37°C for 20 minutes

4. DdH_2O was added to dilute the sample (~1:5) e.g. 40µl added to 10µl sample

5. 5µl of diluted sample can be used per reaction as template DNA.

2.1.7 Purification (PEG8000)

1. As DNA is >300bp, an equal volume of PEG solution was added (if <300bp then 3x volume added) and sample was vortexed prior to centrifugation, 13000 RPM, RT for 30mins (Eppendorf Centrifuge 5415R).

2. The PEG was removed and the DNA was washed in 600µl of 70% ethanol (tube was inverted 3-4x).

3. The sample was then centrifuged for 10 minutes, RT, 13000 RPM prior to removing the ethanol. This washing step was repeated for a second time.

4. After the ethanol was completely removed, the DNA was re-suspended in 16µl of either TE buffer or st.ddH_2O.

5. 1µl was run on a standard agarose gel to confirm quality and concentration of the DNA purified.

2.1.8 Agarose Gel Electrophoresis (DNA)

1. A 1% gel was prepared by adding 40ml of 1x TAE buffer to 0.4g agarose.

2. This was heated until the agarose had fully dissolved and 2.5µl of ethidium bromide was added

3. The solution was poured into gel cast/cradle with the addition of 10 well comb before the gel was left to set.

4. Once the gel had set, the tank was filled to maximum line with 1x TAE buffer

5. <20µl of sample and 2.5µl of 100bp or 1kb ladder was loaded onto gel and left to run at 90V for 30 minutes until sample reaches the bottom of the gel.

6. The DNA/RNA was visualised using the chemi-doc (GeneGenius BioImaging System).

2.1.9 Digestion

1. Reporter DNA was digested in a total reaction mix of 50µl, consisting of 15µl of ~ 0.5µg DNA, 1µl of SACII, 5µl of Buffer 4 and the volume made up with 29µl ddH_2O. The digestion mix was incubated at 37°C for 1 hour 30 minutes. The digestion efficiency was confirmed by running 1µl on an agarose gel.

Note: Some restriction enzymes require heat inactivation to dissociate from the DNA. The tubes containing digestion mix are kept at 65°C for 20 minutes, and transferred to ice for two minutes prior to de-phosphorylation.

2.1.10 De-phosphorylation of DNA

De-phosphorylation of the 5' end phosphate group of the plasmid will prevent re-circularisation, providing the efficiency of the digestion is satisfactory.

2. The 50µl volume of digested plasmid was mixed with 5µl of 10x buffer and 1µl of Antarctic Phosphatase.

3. The DNA/enzyme mix was incubated at 37°C for 60 minutes

4. After de-phosphorylation of the plasmid, the Antarctic phosphatase was heat inactivated to dissociate it from the DNA. The mix was incubated at 65°C for 20 minutes and kept on ice for a further 2 minutes.

2.1.11 Gel Purification

1. 50µl of DNA was loaded onto a 1% agarose gel and run for 30-40min at 90mV (refer to section: 2.1.8).

2. DNA band was confirmed by size and the concentration noted prior to excision from gel using a clean blade and visualised by UV.

3. The excised DNA is transferred to a clean, pre-weighed micro-centrifuge tube and re-weighed to obtain weight of sample.

4. DNA is purified by the Fermentas Silica Bead DNA Extraction Kit.

2.1.12 Ligation

1. The ligation reaction was set up to a total reaction volume of 10µl, with a 1:10 molar ratio of vector:insert. T4 DNA ligase buffer was added 1:10 with T4 ligase added last in a 1:20 ratio and incubated overnight at 18°C.

2.2 Yeast Culture (*S. pombe*) and Integration of Reporters

Refer to Appendix 1 for EMM or YES liquid media and agar recipes.

Wild-type strains were grown on inverted 9cm YES agar plates at 32°C, while transformants were grown either on EMM or YES agar plates in 25-32°C incubator (LMS Incubator) for 2-3 days or as liquid culture, overnight in rotating 25-32°C incubator, 220 RPM (Innova 44 Incubator Shaker Series).

2.2.1 Strains

See Appendix 3 for a list of strains used in this study.

S. pombe Deletion Mutant Library (M3030H)

The *S.pombe* Haploid Deletion Mutant Library was developed by Bioneer and KRIBB in Korea. The 3308 mutant strains were generated from the wild type strain SP286, by using PCR fragment-targeted mutagenesis to knockout most of the nonessential genes in the *S. pombe* genome. The haploid deletion library represents 92.8% coverage of nonessential genes in the genome.

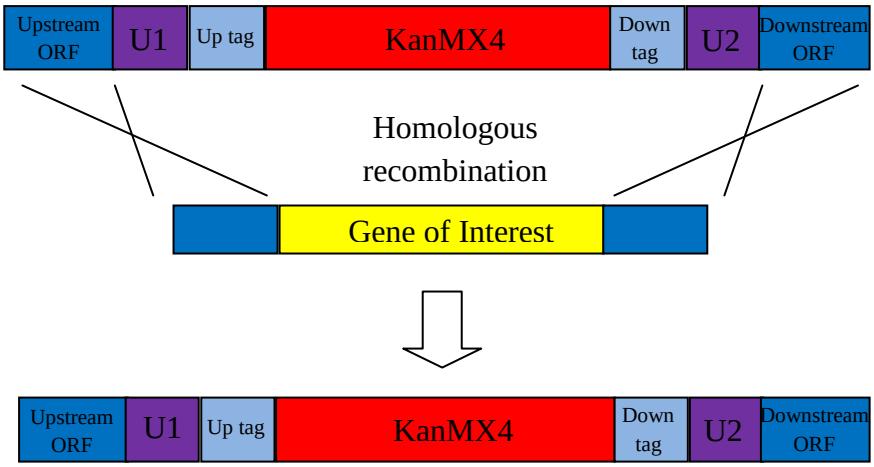


Figure 2: Schematic of PCR fragment-targeted mutagenesis. The *S. pombe* Deletion Mutant Library was generated with gene knockout by KanMX4.

2.2.2 Integration of reporters into *S.pombe*

In preparation for the Preliminary screen, GFP 3'ivs and GFPivs was integrated into SPJK001, a modified wild-type *S. pombe* (h+) strain, using SACII digestion and PEG purification. GFP3'ivs and GFPivs was also integrated into wild-type (039), (H-) strain in preparation for screening the deletion mutant library.

2.2.3 Yeast Transformation

The strain used for transformation was inoculated primarily as pre-culture (in 1ml YES, 33°C O/N) and re- inoculated (100-200µl) in 5-8ml of YES media and grown O/N at 33°C in a rotating incubator (Innova44 Incubator Shaker System) until OD_{650} of 0.3-0.6 was reached (cell density of $1x10^7$ was confirmed by cell counting using Haemocytometer).

The cells were rendered competent by LiAC method

1. Cells were harvested by centrifugation, 3000RPM, 21°C for 4 minutes. All media was subsequently removed and the pellet was washed with LiAc by adding 500µl of 0.1M (mixed gently).

2. Cells were centrifuged 3000RPM, 21°C for 4 minutes and LiAc removed before the cell pellet was re-suspended in LiAc (100µl per cell pellet derived from 1ml of culture).

3. 1-10µg DNA was added to 100µl of LiAc treated cells ($1x10^8$), 1µg for plasmid transformation, 10µg for integration). In addition, 2 µl of single-stranded carrier DNA (10mg/ml) was added and mixed gently.

4. The transformation mix was kept at RT for 10 minutes before adding PEG3350 (260µl of 50%, bringing final to 35%).

5. The transformation mix was kept at 30°C for 1hour (mixing after 30minutes to resuspend cells) and heat shocked for 30minutes in 42°C water bath.

6. Cells were then centrifuged at 5000RPM, 21°C for 2min to pellet the cells and PEG was removed.

7. The pellet was re-suspended in 1ml st.dd water and centrifuged for a further 2min. The water was poured off and the cells re-suspended in the remaining water (~100µl).

8. The 100µl of transformed cells were spread on an appropriate selection plate and incubated at 33°C for 5-6 days or until colonies are established.

2.3 Pilot Screen: Cross and Random Spore analysis

In order to screen the haploid mutant library for potential NMD mutants it was essential to quickly and efficiently insert the GFP reporter construct into the 3308 strains in the library in preparation for screening. On a smaller scale, yeast transformation would have been the quicker option, however this was not plausible with such a large number of strains. The pilot screen was important for trouble shooting any unforeseeable complications that may have arisen during the large scale screen. To optimize this process, four strains were crossed on agar mating media, two reporter strains; SPJK001 (h+) integrated with GFP 3'ivs, and GFPivs (selection by leu-), and two mutant strains; SPJK030 (h-) with *UPF1*Δ, and SPJK031 (h-) with *UPF2*Δ, knocked out by KanMX6 cassette pertaining G418 resistance. However the methods used in the preliminary or pilot screen would also not be feasible on a large scale. To increase the efficiency for screening of the entire library, the protocol required optimisation (2.4 Large Scale Screening of Haploid Deletion Mutant Library).

2.3.1 Cross (Pilot)

1. Using a sterile toothpick, a visible quantity of the first strain (i.e. mutant strain) was scraped and transferred to a small region on the mating plate (SPAS) ~5mm diameter.

2. Using a new toothpick, an equal amount of the second strain (reporter strain) was transferred to the same region and gently mixed.

3. 5µl of st.ddH_2O was added to the patch, mixed and streaked out.

4. Plates were Incubated (LMS Incubator) at 25°C for 3-4 days or until colonies were visible.

2.3.2 Random spore analysis (Pilot)

1. When colonies were visible, 3 colonies were scratched and re-suspended in 20µl of st.ddH_2O.

2. 5µl was diluted ¼ with st.ddH_2O

3. The percentage of spores present were estimated by counting the cell number (total of vegetative cells and asci) on Haemocytometer. Then calculated by the total number per 15µl (remaining from dilution). Example: 20,000 cells present with ~20% of these being asci.

4. The number of spores plated, were calculated based on the percentage of spores present in the remaining 15µl diluted culture. For example: If 15µl contained ~4000 asci, the culture would give rise to ~16000 spores. If we assume that only half may grow (due to heat treatment), and that of the remaining 8000 spores only ¼ will be correct then we would expect to give rise to 2000 potentially correct colonies. 1000

colonies is sufficient, therefore 7.5µl would be required (volume could be made up to 100µl with dd H_2O for ease of spreading).

5. 1ml of culture was heated at 55°C for 30min to destroy vegetative cells
6. The remaining spores were Spread on selective media (YES +G418 plate) and incubated at 32°C for 3-4 days or until colonies had grown.
7. Once cells had grown, colonies were replica plated on EMM Leu- and incubated for a further 2 days.
8. To confirm haplotype, cells were checked by colony PCR and verified by fluorescence microscopy to confirm reporter was working correctly within the strain.

2.4 Large Scale Screen of Haploid Deletion Mutant Library

As above mentioned the protocol used for the pilot screen required modification to improve efficiency and handling of large numbers of yeast strains. During mating, the reporter strain SPJK039 (h-) containing GFP 3'ivs and mutants from the Bioneer library (M3030H) (h+) required two different methods for plating. The reporter strain would need to cover a large surface area to allow for mating of multiple mutant strains on a single plate. While these numerous mutants would need to cover a smaller surface area to maximise the number of strains plated, while minimising the risk of cross-contamination. It is also important that these strains make sufficient contact with the reporter strain, therefore the liquid:colony ratio for cell number was optimised, specifically the volume of reporter to be plated. During the process of random spore analysis it became apparent that heating individual strains in water to kill the vegetative cells would be too laborious during the large scale mating, instead the

heat shock process was optimised for strains while still cultured on agar mating plates prior to replicating onto selective media.

2.4.1 Large Scale Cross

1. The reporter strain was grown in 1ml of overnight culture to OD_{650} of 1.0. 150µl of culture was spread on SPAS agar plate using 20 glass beads and allowed to part-dry.
2. The mutant strains were transferred (48 strains per 9cm agar plate) from the same array on YES+ G418 stock plates (cultured from 96 well glycerol stock plates) to the SPAS mating plate using a 48 pin multi-blot replicator. On contact with agar/reporter strain, the replicator pin was rotated very slowly within the defined region.
3. Plates were Incubated (LMS Incubator) at 25°C for 3-4 days or until spores develop.

2.4.2 Large scale Random Spore Analysis

1. After 3-4 days, one mutant per plate was checked under the microscope to confirm sporulation.
2. If asci were present, plates were heat shocked at 75°C for 40minutes to destroy vegetative cells.
3. Once plates had cooled to RT, spores were transferred to selective media (YES+G418 initially) by replica plating by velveteen squares and incubated at 32°C for 3-4 days or until colonies were visible.
4. Colonies were replica plated on EMM Leu- plate for further 3 days
5. The strains were screened by fluorescence microscopy (see section: 2.5 Microscopy).

2.5 Microscopy

2.5.1 Pilot Microscopy

1. The *S.pombe* cells containing the reporter constructs were grown in 1ml EMM leu- media, 32°C O/N in a rotating incubator (Innova44 Incubator Shaker System) until OD_{600} of 0.8-1.0 was reached.

2. 200µl of overnight culture was centrifuged (Eppendorf centrifuge 5415 R), 2000 RPM for 1 minute to pellet the cells.

3. After discarding the media, the pellet was washed by re-suspending in 200µl of ddH_2O.

4. Further centrifugation was required at 2000RPM for 1 minute to pellet the cells.

5. After discarding the water, the pellet was re-suspended in 20µl of ddH_2O.

6. 3µl of the cell suspension was transferred to a slide and cover-slip ready for viewing under Nikon Ti Eclipse fluorescence microscope.

7. DIC images were taken for each and GFP was visualised by FITC, with an exposure time of 500ms for integrated strains or 90ms for episomal expression. The Gain used was set at 100. 40X objective was used for initial screening and optimisation.

   Note: this protocol was adapted for use in the final round of screening (for 100X images of 18 strains), as OD_{600} 0.2-0.3 was used for RNA extraction, the culture for these images was also grown to this OD.

2.5.2 Microscopy for Large Scale Screening

As with any mutant strain there will be additional phenotypes associated with the particular deletion, such as growth defects. Due to the large number of strains being handled it was not possible to adjust for this time factor. Ideally, and time permitting, the library would have been re-screened, taking into account the longer culture time required for these sick strains.

1. The strains were transferred by scraping a visible amount from agar plates containing the progeny of the crossed mutants with the reporter strain and directly re-suspended in 50µl of selective media (PMG) in 96 well Corning plates. These plates were black polystyrene/clear non-binding surface for microscopy.

2. DIC images were taken for each strain and GFP was visualised by FITC, with an exposure time of 500ms for integrated strains or 90ms for episomal expression. The Gain used was set at 100. The objective used with these plates was 20X during screening**.**

2.6 Verification of Mutants and Molecular Analysis of Protein and RNA Levels

To confirm that the strains provided by BIONEER were correct, any putative strains identified in the screen should be confirmed by PCR using primer specific for each mutant.

The control strains used for this study, wild-type and UPF1Δ containing the reporters were confirmed by western blot analysis to compliment the fluorescence microscopy, although this method would not necessarily be used for quantification purposes.

For verification and quantification that the gene deleted, was exhibiting reduced NMD, in those putative mutants identified in the screen, northern blot analysis was performed to confirm the effect on RNA levels.

2.6.1 Genomic Extraction

1ml of pre-culture was inoculated into 3ml YES and grown overnight. The Genomic DNA was extracted using the Qiagen: Puregene yeast/bact Kit B and the concentration confirmed by agarose-gel electrophoresis.

2.6.2 Protein extraction (for *S.pombe*)

1. 1ml of overnight culture was grown at 37°C in either EMM leu- or YES
2. The overnight culture was centrifuged at RT, 3000 RPM for 3 minutes to pellet the cells.
3. The media was removed prior to washing the cells by re-suspending pellet in 500µl ddH_2O and centrifuged for a further 3 minutes to pellet cells
4. The water was removed and the pellet re-suspended in 300µl of fresh ddH_2O. 130µl of 1M NaOH was added to make a final concentration of 0.35M.
5. The sample was incubated at RT for 5-10 minutes prior to centrifugation at 4°C, 8000 RPM for 3 minutes.
6. The NaOH was removed and the pellet re-suspended in 50µl of 1x SDS loading buffer containing 1:25 B-mecaptoethanol and 1:1000 PMSF
7. The sample was boiled for 6 minutes, then cool on ice for 2 minutes before centrifugation at 4°C 13,000 RPM for 2 minutes
8. The sample was loaded onto a polyacrylamide gel.

2.6.3 Sodium-dodecyl-sulfate Polyacrylamide Gel Electrophoresis

1. The resolving and stacking gels were prepared without the addition of TEMED to the latter until ready to pour (see appendix 2).

2. The apparatus was set up as per manufacturer's instructions (BioRad) and 500ml 1x SDS-running buffer prepared

3. 10-20µl of samples and 7µl of marker were loaded onto gel and run for initial 5 minutes at 50V until samples pass through the stacking gel, then for further 2 hours at 90V.

4. When samples had resolved the gel was removed from the apparatus and over laid on 2x moistened filter paper. Nitrocellulose membrane was added to the stack with the addition of 2x filter papers and placed between sponges prior to inserting into cassette for protein transfer.

5. The cassette was inserted into transfer tank with 500ml cold transfer buffer and run at 350mA for two hours.

6. After transfer, the membrane was incubated in blocking solution, at RT for 30 minutes with gentle agitation.

7. Subsequently, the blocking solution was replaced with primary antibody (either monoclonal or polyclonal anti GFP) 1:2000 in TBST and incubated at 4°C overnight with agitation.

8. The excess antibody was removed by washing the membrane 2x in 15ml TBST briefly, followed by 3x at 10 minute intervals.

9. After washes, the membrane was incubated in secondary antibody (1:10,000) in TBST for 1 hour at room temperature with gently agitation (either anti-mouse or anti-goat depending on whether monoclonal or polyclonal 1° antibody was used respectively).

10. Washing step 8 was repeated.

11. TBST was removed and the membrane incubated in 1ml ECL substrate (500µl of each; luminol and pico stable peroxide per membrane) at RT for 5 minutes.

12. The membrane was wrapped in saran wrap and exposed using chemi-doc (G.BOX Syngene).

2.6.4 RNA Extraction

1. Yeast cells were cultured in 10ml volume of selective media to OD_{600} =0.3-0.8 (OD 0.3 appears optimal for expression of this reporter).
2. cultured cells were centrifuged for 5 minutes at 4.5K RPM in tabletop centrifuge at 4°C to pellet cells.
3. The supernatant was discarded and pellet re-suspended in 1ml of cold water prior to transfering to 1.5ml micro-centrifuge tube. Cells were centrifuged for 10 seconds at 4°C to pellet.
4. The supernatant was removed and cells re-suspended in 600µl of TES solution. 600µl of acid phenol was added and vortexed vigorously (10 sec).
5. Samples were incubated for 40 minutes at 65°C, with vigorous vortexing every 10 minutes.
6. Samples were cooled on ice for 5 minutes and centrifuged for 5 minutes, 13.2K RPM at 4°C

7. The aqueous phase (top layer) was transferred to a clean micro-centrifuge tube containing 600µl of pre-cooled acid phenol and vortexed vigorously. Step 6 was repeated.
8. The aqueous phase was transferred to clean micro-centrifuge tube containing 600µl of pre-cooled chloroform. The samples were vortexed vigorously and micro-centrifuged for 5 minutes, 13.2K RPM, at 4°C.
9. The aqueous phase was transferred to a clean micro-centrifuge tube containing 1/10 the volume of pre-cooled sodium acetate PH 5.3 (i.e. 36µl to 360µl aqueous phase), and 1ml cold 100% ethanol to precipitate for 20-30 minutes at -20°C.
10. The samples were micro-centrifuged for 5 minutes, 13.2K RPM at 4°C and the supernatant removed.
11. The RNA was washed in 1ml cold 70% ethanol, by vortexing briefly. Step 10 was repeated.
12. RNA was air-dried to ensure no residual ethanol remained. 30-50µl of DEPC-treated water was added and samples kept on ice for 15 minutes prior to re-suspending.
13. The concentration was determined by spectrophotometry by measuring the A_{260} and A_{280}.

2.6.5 Northern Blot Analysis

1. 1.2% formaldehyde gel (120ml volume) was prepared by dissolving agarose in DEPC-treated H_2O firstly, and allowed to cool prior to adding additional components.

Agarose	1.44g
DEPC H_2O	85ml
Formaldehyde	23ml
10x MOPS	12ml

Running Buffer: 1X MOPS

2. RNA samples were prepared as follows;

RNA sample	10µg of RNA (unless conc is low, if require >8µl then only use 5µg)
Formaldehyde	5.5µl
Formamide	15µl (or ~50% of total)
10x MOPS	3µl (1X)

3. Samples were incubated at 65°C for 15 minutes then ice for 5 minutes
4. The gel was pre-run for 5 minutes at 80V
5. 1µl of loading buffer was added per sample and loaded on formaldehyde gel. This was run at 80V for 2 hours and 30 minutes (3-4 volts/cm)
6. For blot transfer, the excess gel was trimmed and washed 2X with DEPC-treated H_2O for 20 minutes followed by 1X with 20X SSC for 30 minutes.
7. The overnight blot was set up with 20X SSC to allow RNA to transfer to HYBOND membrane by capillary action. Blot consists of;

   1X HYBOND 12x12.5cm

   1x Whatmann filter paper bridge 23x12cm

   6x Whatmann filter paper 11.5x12cm

   5x sheets of white paper roll (folded to ~11.5x12cm)

   Green paper towel cut in half (3x thickness of white roll stack)
8. For UV crosslinking, membrane was crosslinked 1x at 120J
9. Membrane was subsequently washed 1X with DEPC-treated H_2O for 5 minutes prior to methylene blue staining to check RNA and washed after with DEPC-treated H_2O until excess stain was removed.

10. For pre-hybridisation, the membrane was incubated at 68°C (Stuart Scientific hybridization oven/shaker SI2OH) for 3-4 hours with 30ml hybsol containing 250µg/ml heparin and 100µg/ml SSDNA.
11. For overnight hybridisation at 68°C, a leucine (control) or GFP probe was prepared as follows;

Probe	10-20ng/µl
5X labelling buffer (Promega)	5µl
DEPC H_2O	≤21µl

12. The above mix was boiled for 3 minutes and cooled on ice for 5 minutes prior to adding the following;

dNTPs (C-) (Bioline 25µM each)	1µl
Klenow	0.5µl
P^{32}dCTP (Perkin Elmer 250µCi)	2.5µl

13. The above mix was incubated at RT for 2-4 hours.
14. After 4 hours the prepared probe was purified by column equilibration in a pre-prepared screw cap column containing G50/STE. The probe was centrifuged for 6 minutes at 5000 RPM in a bench top centrifuge (Eppendorf centrifuge 5415D) and the purified probe collected in a clean micro-centrifuge tube.
15. After confirming level of radioactivity (cps) the purified probe was boiled for 5 minutes and cooled on ice for a further 5 minutes.
16. After re-confirming radioactivity the purified probe was added to hybridisation buffer, consisting of 20ml hybsol, 250µg/ml heparin and 100µg/ml SSDNA
17. The pre-hybridisation buffer was replaced with the above hybridisation buffer containing the probe and incubated overnight with the membrane at 68°C.

18. After hybridisation, the membrane was washed at 68°C with 4x with 100ml 2X SSC and 0.1% SDS, for 2, 5, 30 and 30 minute washes respectively.
19. The membrane was washed for a final time in 100ml 0.2X SSC and 0.1% SDS for 30 minutes at 68°C before confirming radioactivity. Generally the cps for GFP was high enough for 4 hour exposure (50+), however the leucine control was much weaker (20-30 cps) and required overnight exposure.
20. The film was developed using a BIO-RAD molecular imager.
21. To strip the membrane (for re-hybridisation with a second probe), the membrane was boiled for 5 minutes in 0.1% SDS and left to cool to RT. The radioactivity was confirmed, if lower than 5 cps then pre-hybrdisation (step 10) was initiated.

2.7 Functional analysis of putative mutants

2.7.1 Pombase

Pombase is a resource (www.pombase.org), offering structural and functional information of *S. pombe* genes. Using the systemic ID provided by BIONEER with the M3030H library, of the putative mutants identified from the screen, it was possible to search the database to confirm gene name (if characterised), functional and localisation information. Prior to pombase, GeneDB was used as reference (www.old.genedb.org/genedb/pombe/).

2.7.2 BioGrid

Biogrid (www.thebiogrid.org) is a repository for interaction datasets and provides protein and genetic interactions published in the current literature for a number of model organisms.

2.7.3 STRING

STRING database (www.string-db.org) offers both known and predicted protein interactions through direct or indirect associations, obtained from the literature (textmining and

databases), coexpression, genetic fusion and neighbourhood experiments, it also provides levels of confidence and summary networks.

CHAPTER III: RESULTS

3.1 NMD Reporter Constructs

Before performing the screen, I had to construct and test a number of GFP reporter constructs with or without NMD features (Fig. 3). The key feature of these constructs was the presence of an intron in the 3' UTR, located just downstream of the GFP normal stop codon. Two standard *S. pombe* expression vectors were used: plasmids pREP and pDUAL (Maundrell K 1993; Matsuyama, A *et al.*, 2004).

Both plasmids allow either episomal expression or integration into the genome by homologous recombination. As detailed below, two versions of these reporters were generated.

3.1.1 GFP-3'ivs and GFP-ivs

One of the reporters used was GFP-3'ivs (Fig. 3: C); this contains a 370 bp long intron in the 3' UTR, immediately after the normal termination codon, subsequently rendering its mRNA an NMD substrate (Wen and Brogna, 2010). When GFP-3'ivs is expressed in wild-type cells, GFP levels are very low (Fig. 4: A, right panel). In UPF1/2Δ strains however, the transcript level is rescued and GFP expression increased (Fig. 4: A, left panels). The level of GFP in the NMD mutants is similar to that of the control (GFP-ivs, data not shown) that does have the intron situated in the middle of the coding reading, position 110; this construct does not trigger NMD, in neither wild-type nor in UPF deletion strains (Wen and Brogna, 2010). As a second GFP reporter lacking both PTC and intron produced similar results (data not shown).

3.1.2 GFP147-3'ivs and GFP147

Because GFP-3'ivs, started giving inconsistent results – it was extensively characterised, subsequently re-cloned and partially sequenced, but still, for unknown reasons, produced inconsistencies. As a result, a second set of reporters was used in the later stages and for the re-screening of putative mutants. These second reporters were GFP147-3'ivs and GFP147 (Fig. 3: E and D respectively) which are identical to GFP-3'ivs except for the addition of a spacer 147 bp in length, positioned immediately after the normal termination codon. These reporters produce the same expression phenotype as their predecessors, and were used for the late stage of the screening and for the Northern blot analysis of the putative mutants.

Figure 3: Schematic of Reporters

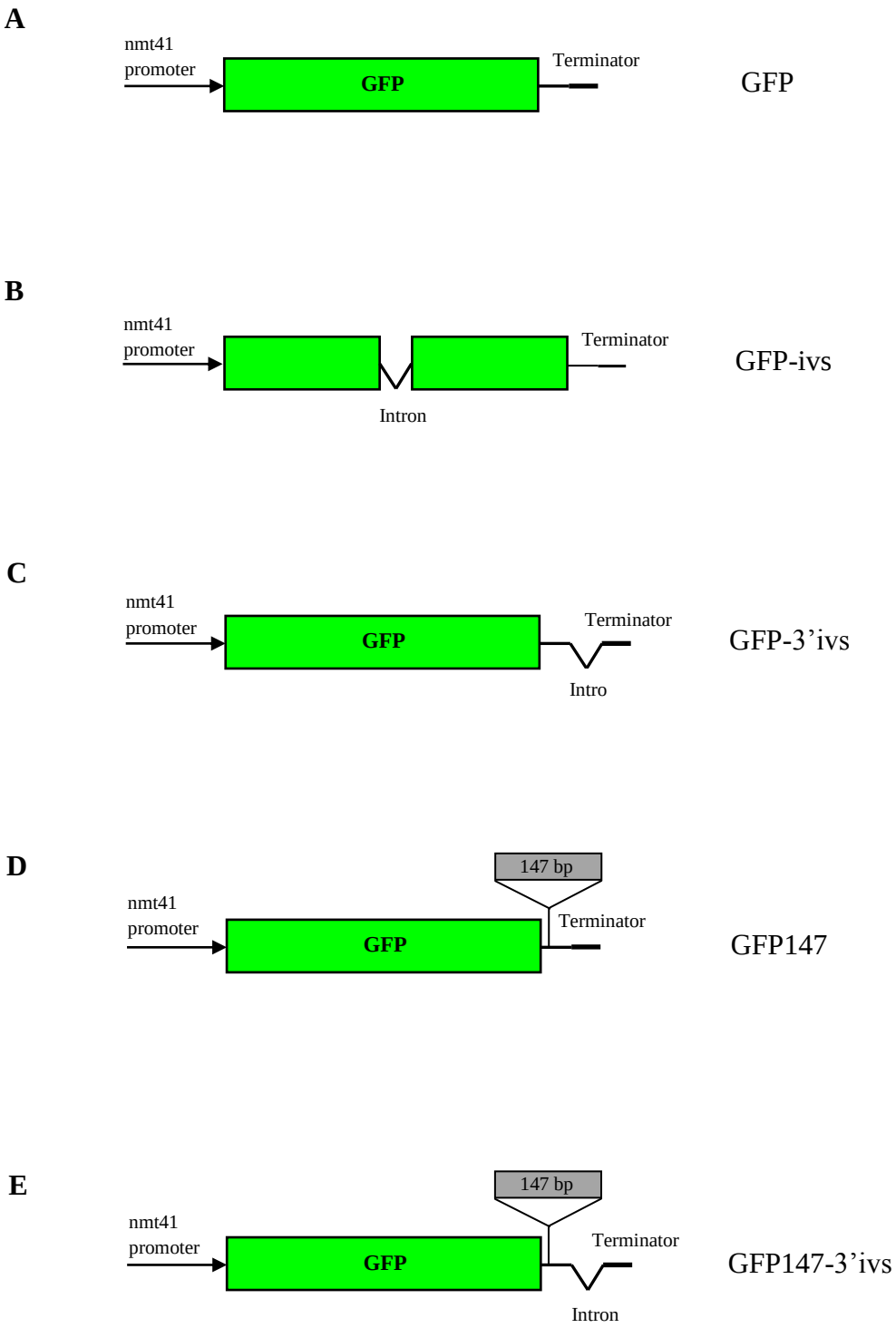


Figure 3: Schematic of NMD reporters and controls

A) Schematic of the GFP control reporter containing GFP ORF, under the control of nmt41 promoter and terminator. **B)** GFP-ivs construct, based on the same reporter as GFP, containing an intron, measuring 370bp in length, at position n110. This is not subject to NMD **C)** GFP-3'ivs, contains an NMD feature consisting of a 370bp intron insertion in the 3' UTR immediately after the normal termination codon. Due to the position of the intron insertion, the termination codon will be interpreted as a premature termination codon. **D)** GFP147, based GFP reporter with additional 147bp spacer inserted immediately after the normal termination codon. **E**) GFP147-3'ivs, based on GFP-3'ivs reporter, with a 147bp spacer inserted immediately after the normal termination codon and upstream of 3' intron.

3.2 Preliminary Large Scale Screen of Haploid Mutant Library

As previously mentioned, when integrated into wild-type *S. pombe* cells, the GFP-3'ivs reporter produces a very low level fluorescents, however, when integrated into an NMD mutant strain, the level of GFP mRNA and the subsequent protein expression are restored to control levels comparable to GFP reporters not subjected to NMD. The expectation is that in the absence of a gene required for NMD, there will be an increase in the level of GFP expressed in relation to wild-type, and potentially comparative levels to those produced in the UPF1Δ strain.

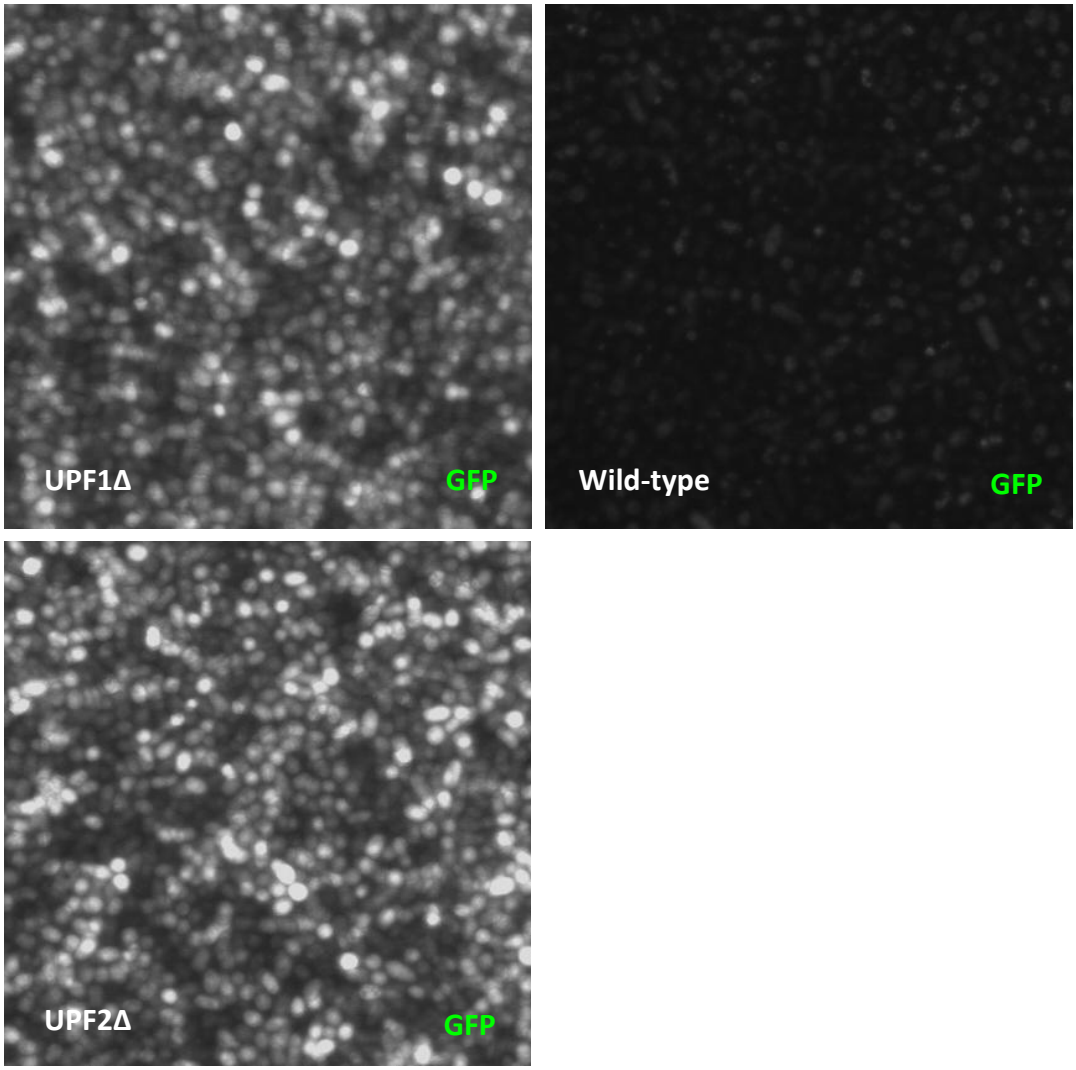
For the screen, the reporter construct GFP-3'ivs was firstly integrated into wild-type (SPJK039 h-) cells which were subsequently mated with mutants derived from the haploid mutant library (Bioneer, M3030H h+ Version 3). The progeny were selected for leucine prototrophy and G418 resistance to confirm integration of construct into Δ mutant strain prior to screening. Due to time limitation, of the total 3308 non-essential mutants from the deletion mutant library, 2790 strains have been screened using the integrated GFP-3'ivs reporter for increased GFP fluorescence in comparison to wild-type, and comparable to a known NMD factor, UPF1Δ.

Following two rounds of screening, eighteen putative mutants were identified (Fig. 4, panels A to R show pictures taken in under the same conditions). In these strains GFP fluorescence was consistently higher than that in wild-type. In some of the strains (panels A, B, G, H, I, J, Q, and R) fluorescence appears only marginally higher than wild-type in the pictures yet the difference was apparent under the microscope and so were included for further analysis. Some of the strains show high level of fluorescence only in a fraction of cells, with >90% of cells having no GFP expression and <10% exhibiting very strong GFP. We cannot rule out a cross

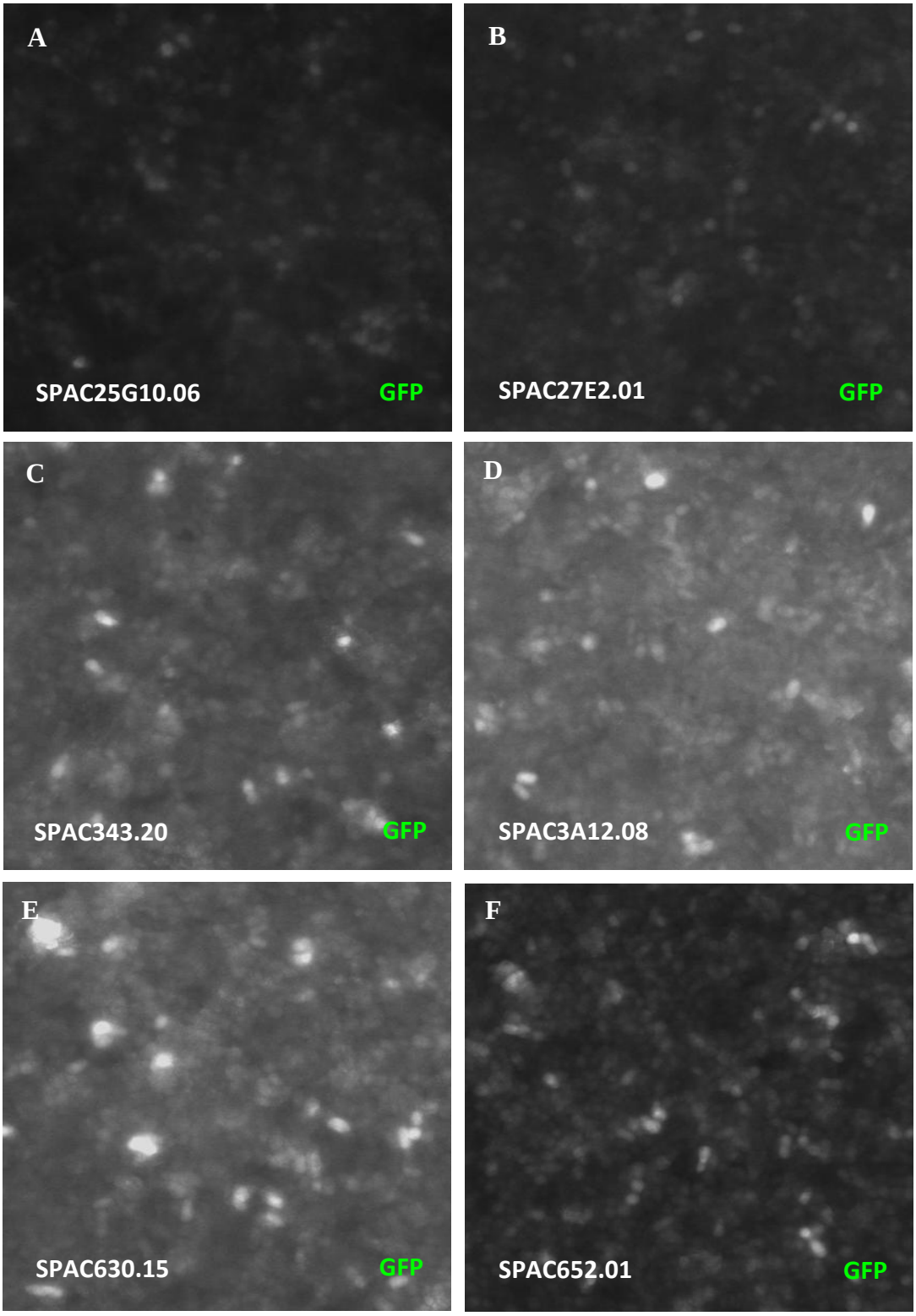
contamination; these strains will need to be confirmed by PCR to eliminate this possibility. While screening, the level of GFP was noted for both the cytoplasm and the nucleus, with the former less intense. Also the level of background GFP was taken into account, and confounding variables such as cell number were addressed, after optimisation this variable was dismissed as having a very insignificant effect on the level of background GFP observed.

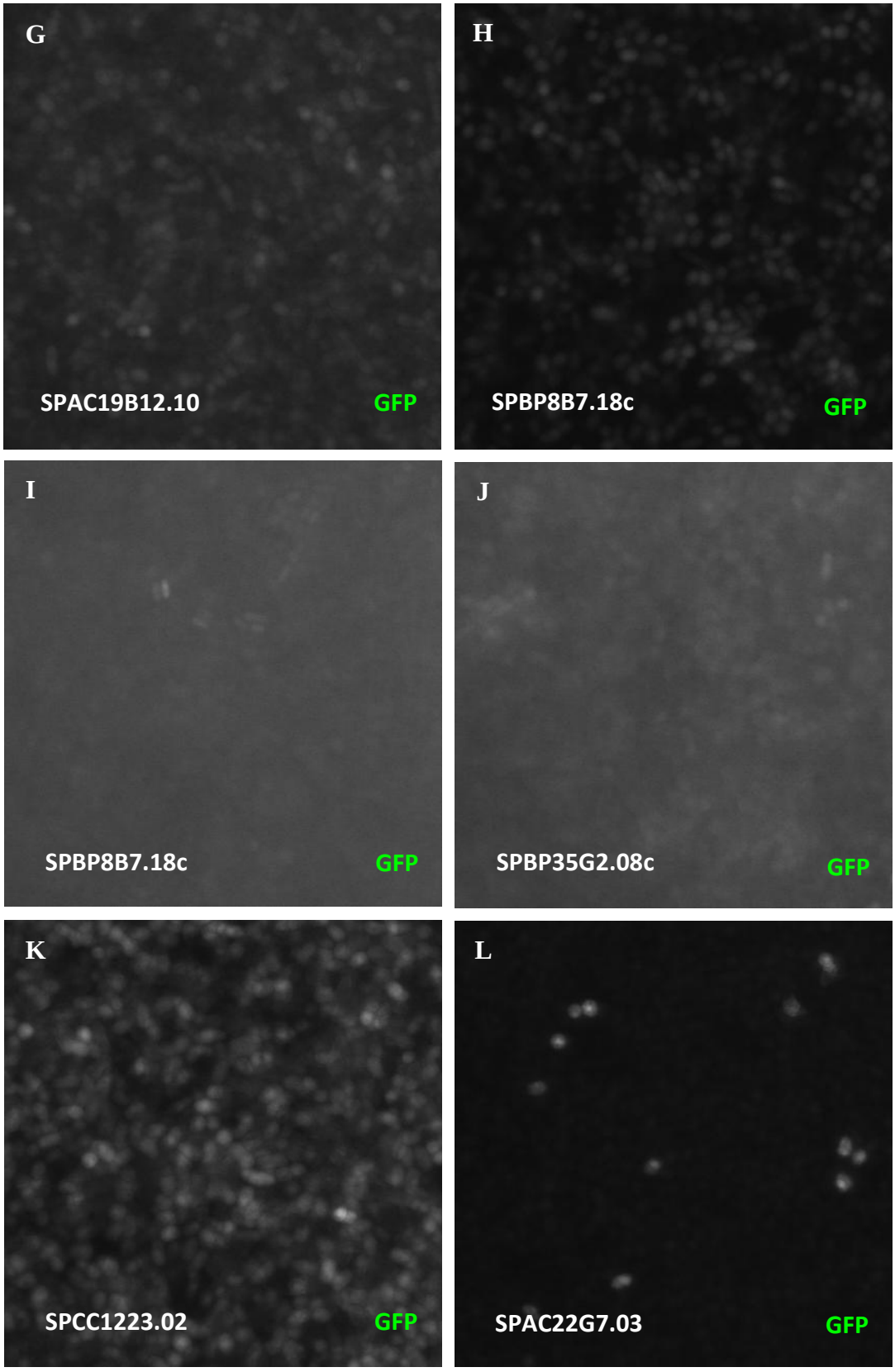
Figure 4: GFP-3'ivs integration

A) GFP expression in wild-type and UPF1/2Δ strain



B) Screening of Haploid Deletion Mutant Library




G
SPAC19B12.10
GFP
H
SPBP8B7.18c
GFP
I
SPBP8B7.18c
GFP
J
SPBP35G2.08c
GFP
K
SPCC1223.02
GFP
L
SPAC22G7.03
GFP

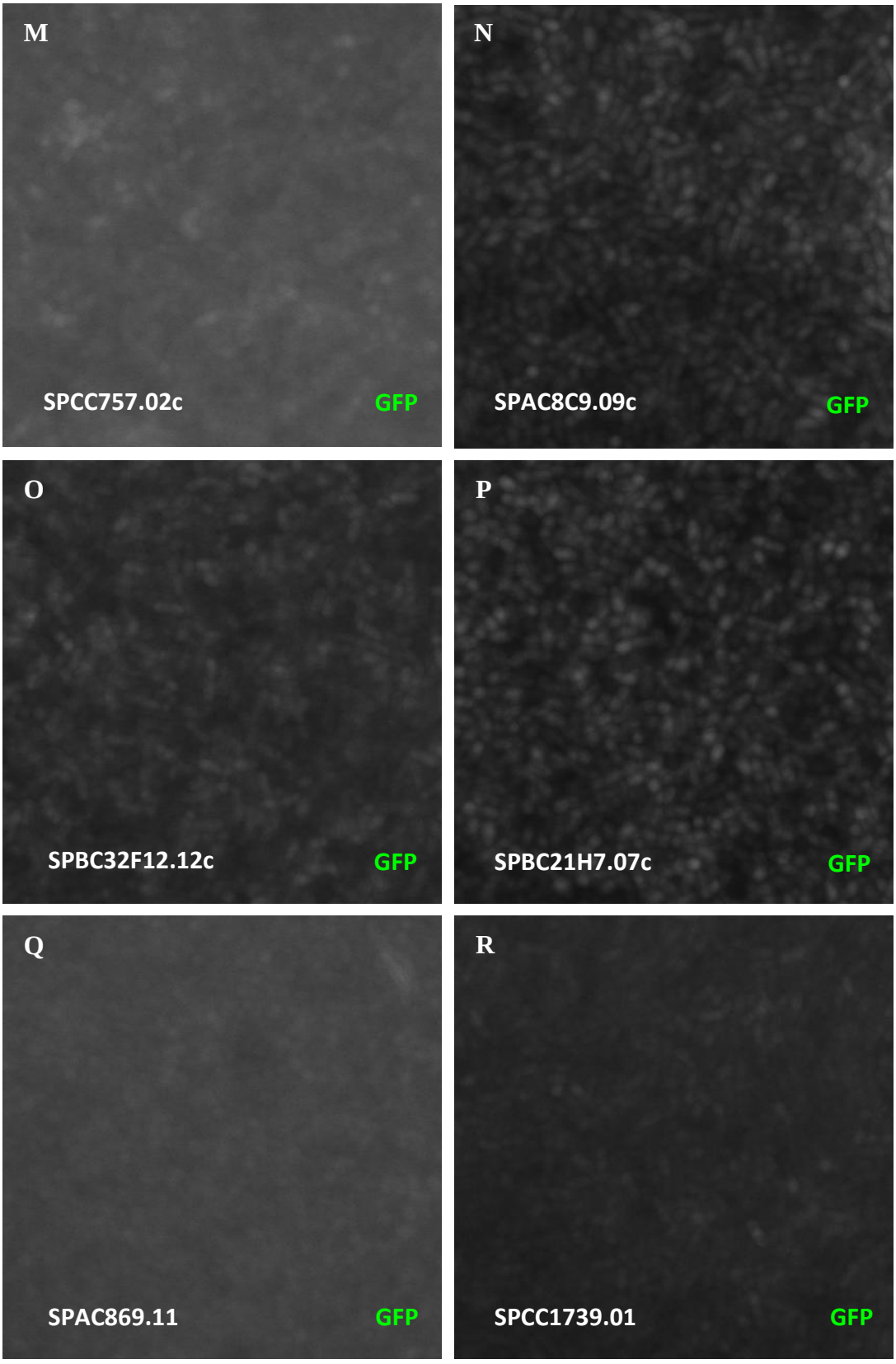


Figure 4: Mutant strains showing enhanced expression of GFP3'ivs

A) GFP expression in wild-type and UPF1/2Δ strain. GFP expression in the wild-type *S. pombe* cells integrated with GFP-3'ivs reporter show little fluorescence due to the NMD feature of the construct. In both positive controls; UPF1Δ and UPF2Δ strains, the GFP levels are restored due to the abolishment of NMD. **B)** Screening of haploid deletion mutant library, all strains pictured, show a level of GFP higher than that of wild-type, however lower when compared to both positive controls.

3.3 Secondary Screen of Putative Mutants using GFP147-3'ivs

Following the initial screen, we thought to further test the putative mutants with a secondary screen in which I used the GFP147-3'ivs NMD reporter, which as described above behaves similarly to the GFP3'ivs used in the initial screen. Thus any potential NMD factor can be screened based on an increase in the level of GFP expressed in relation to levels in the GFP147 control strains that does not carry a 3'UTR intron.

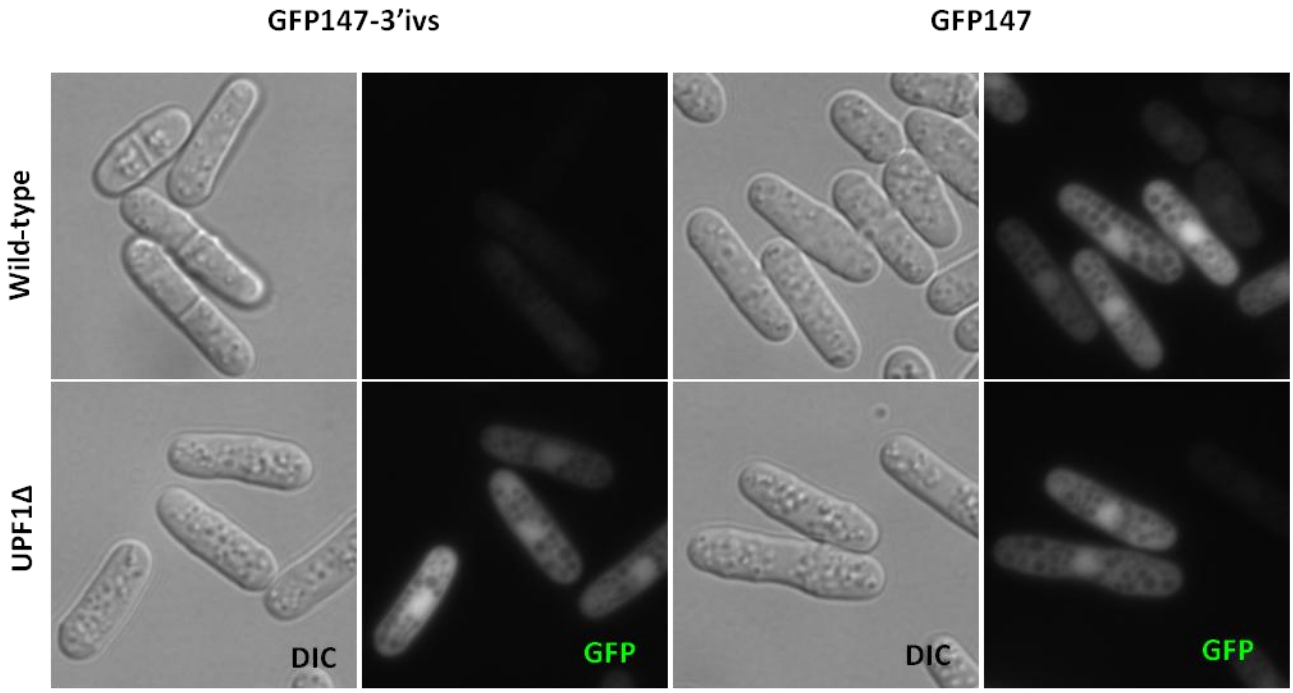
This time the GFP reporter expressed episomally with a pREP-based vector (Maundrell 1993). The advantage of using plasmid expression vector is the increase in quantity of mRNA produced (and therefore GFP protein) which is more abundant than the single copy-integrated GFP-3'ivs, this is advantageous for both microscopy and northern blot analysis. However, there are two significant drawbacks, firstly, despite this construct containing an NMD feature, it is a less preferential substrate due to the presence of a spacer which extends the distance of the intron from the stop codon, as a result the mRNA levels are higher than those produced in wild-type strains with GFP-3'ivs (Wen and Brogna, 2010). Secondly, unlike its integrated counterpart, copy number is an additional variable of episomal expression of the GFP147-3'ivs construct, making it slightly more difficult to judge the levels of GFP between strains, by microscopy. It can however be easily be quantified by Northern blot analysis, using the plasmid leucine marker to account for plasmid copy number variation.

Of the 18 putative strains re-screened with GFP147-3'ivs, 9 strains expressed levels of GFP comparable to the same strains expressing GFP147 (Fig. 5:B). These 9 strains are shown in panels: B, G, H, I, K, L, O, P, and R. The strains were selected based on whether the nucleus (a easy to distinguish feature) exhibited strong GFP fluorescence, in addition to a less intense, cytoplasmic fluorescence, which was comparable to that of the GFP147 equivalent. This

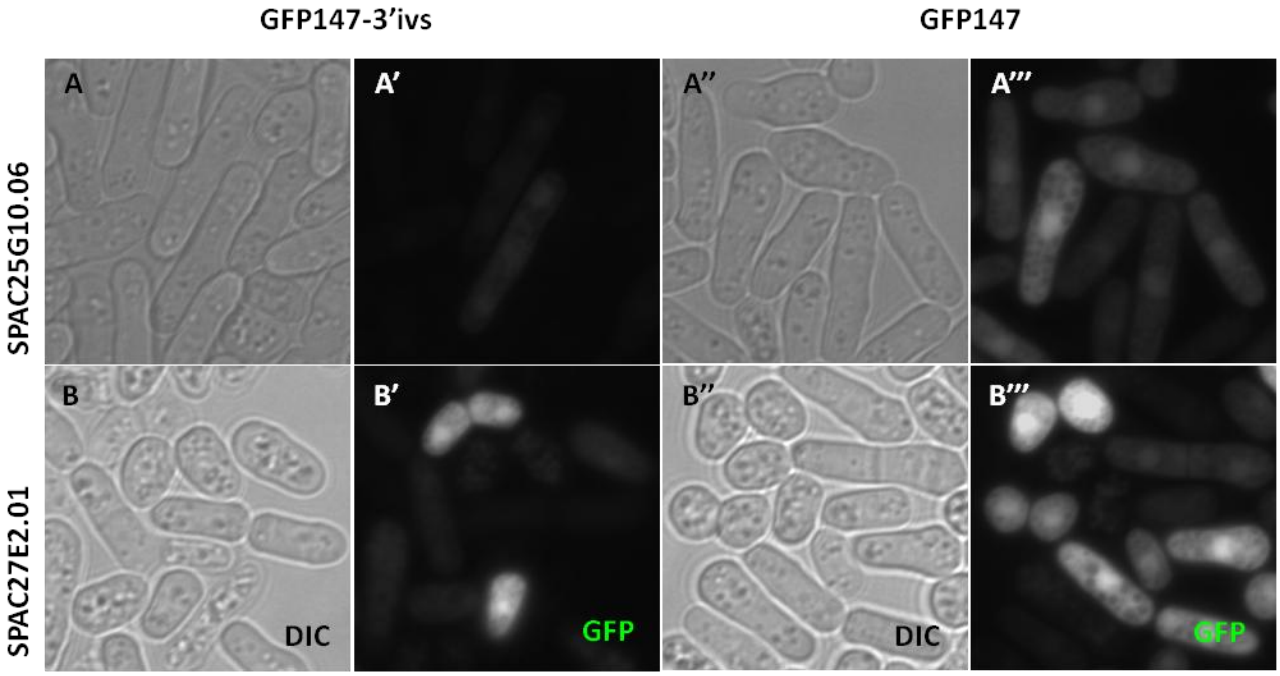
method of screening was difficult due large cell-to-cell variation in fluorescence in some strains. For instance, Fig. 5:B, C, D, and E had comparable levels of GFP, however about 10% of the cells show much higher fluorescence. It is not clear why there is such large difference in GFP levels. One possibility could be due to copy number, as previously mentioned, some cells, despite being a potential candidate for further study, simply does not have sufficient copies of the plasmid, thus some cells express lower levels of GFP. Alternatively these strains may be valid, however due to the stage in their cell cycle or other confounding variable, only a small proportion of cells in the correct stage, appear positive. As such these negative strains were not immediately discounted, instead all 18 strains were examined by northern blot analysis to confirm.

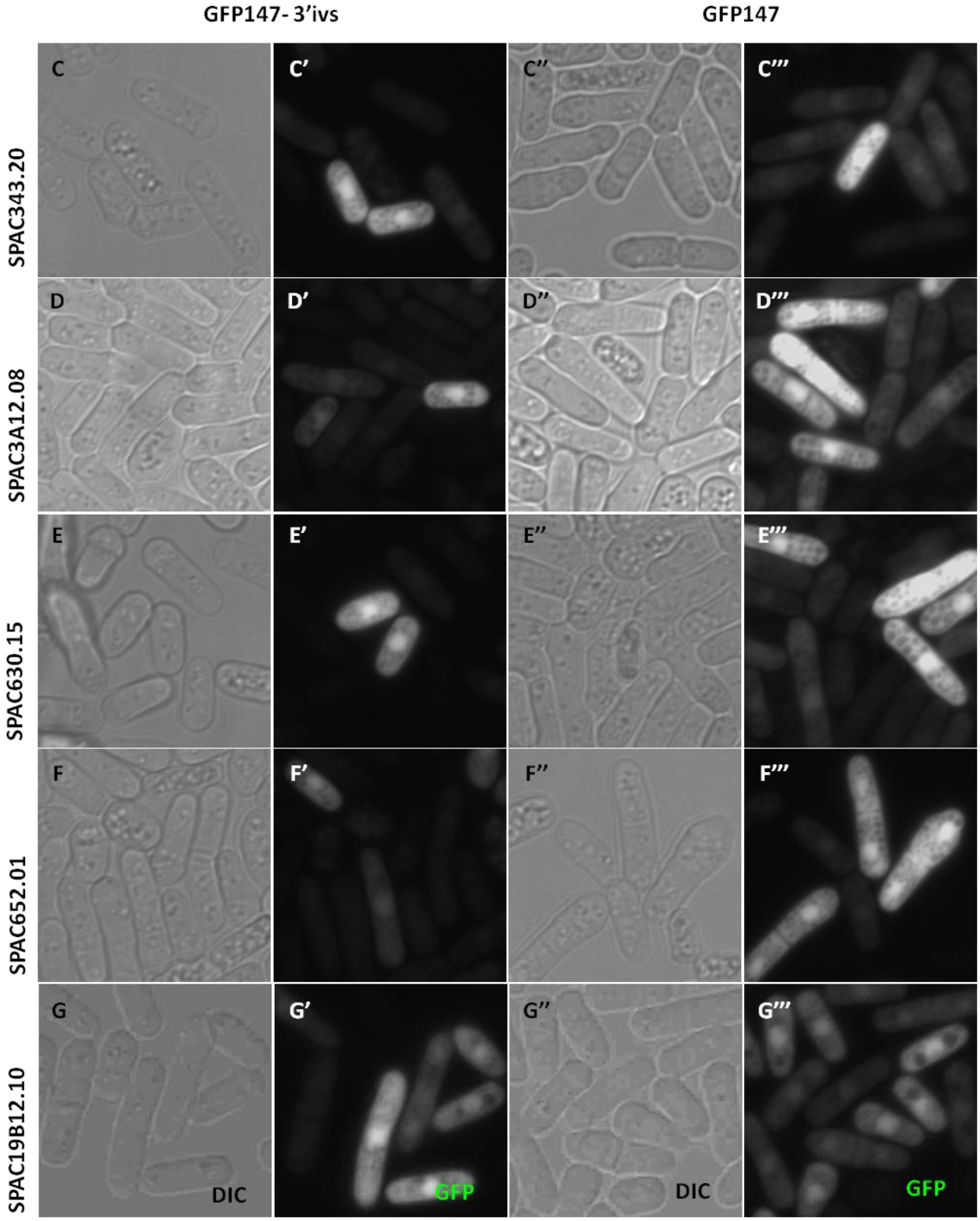
Figure 5: Episomal expression of GFP147-3'ivs

A) Wild-type and UPF1Δ controls



B) Screening of putative mutants from Haploid Deletion Mutant Library

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SPAC3A12.08
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SPAC652.01
SPAC19B12.10
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C'
C''
C'''
D
D'
D''
D'''
E
E'
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F
F'
F''
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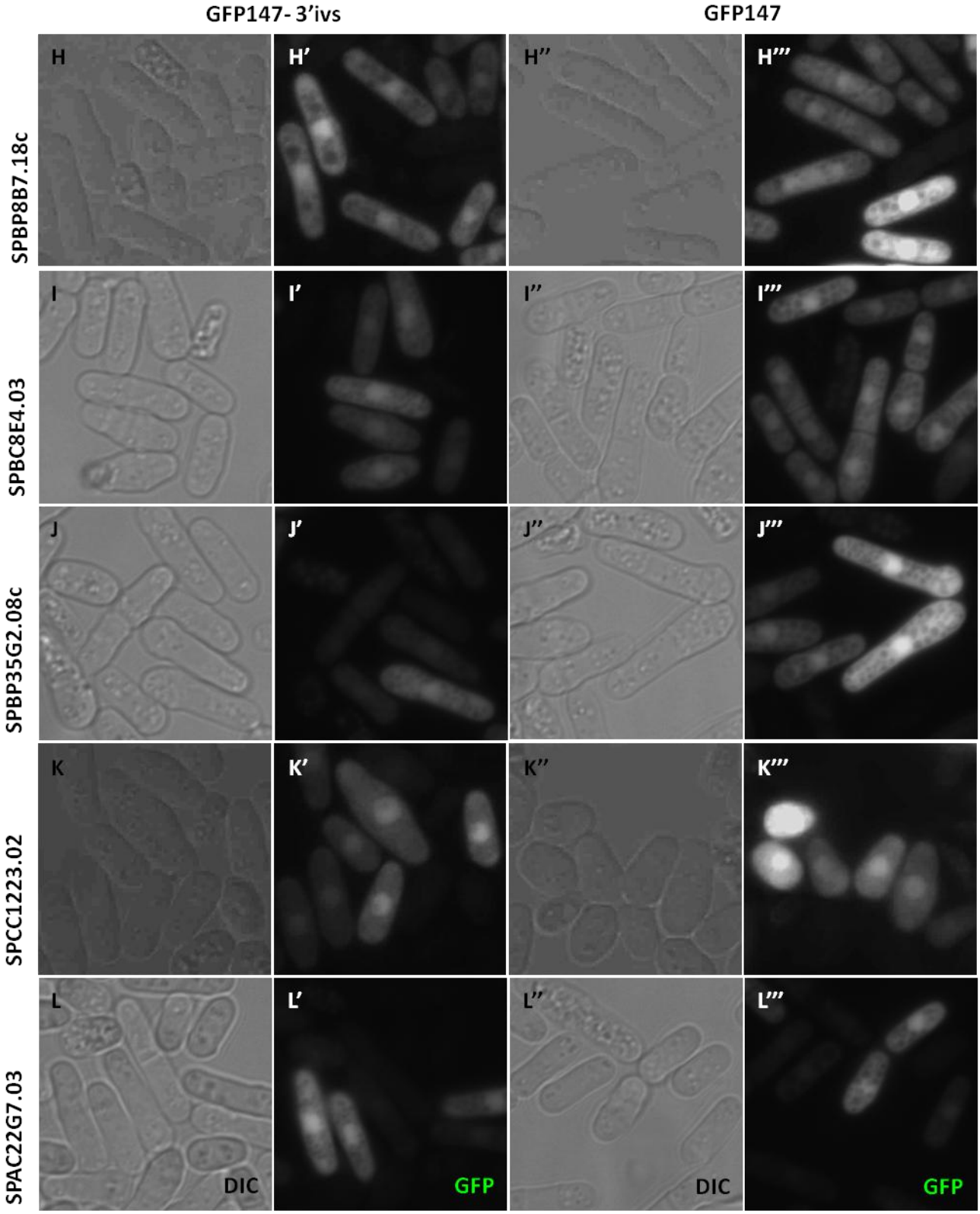
G
G'
G''
G'''
DIC
GFP
DIC
GFP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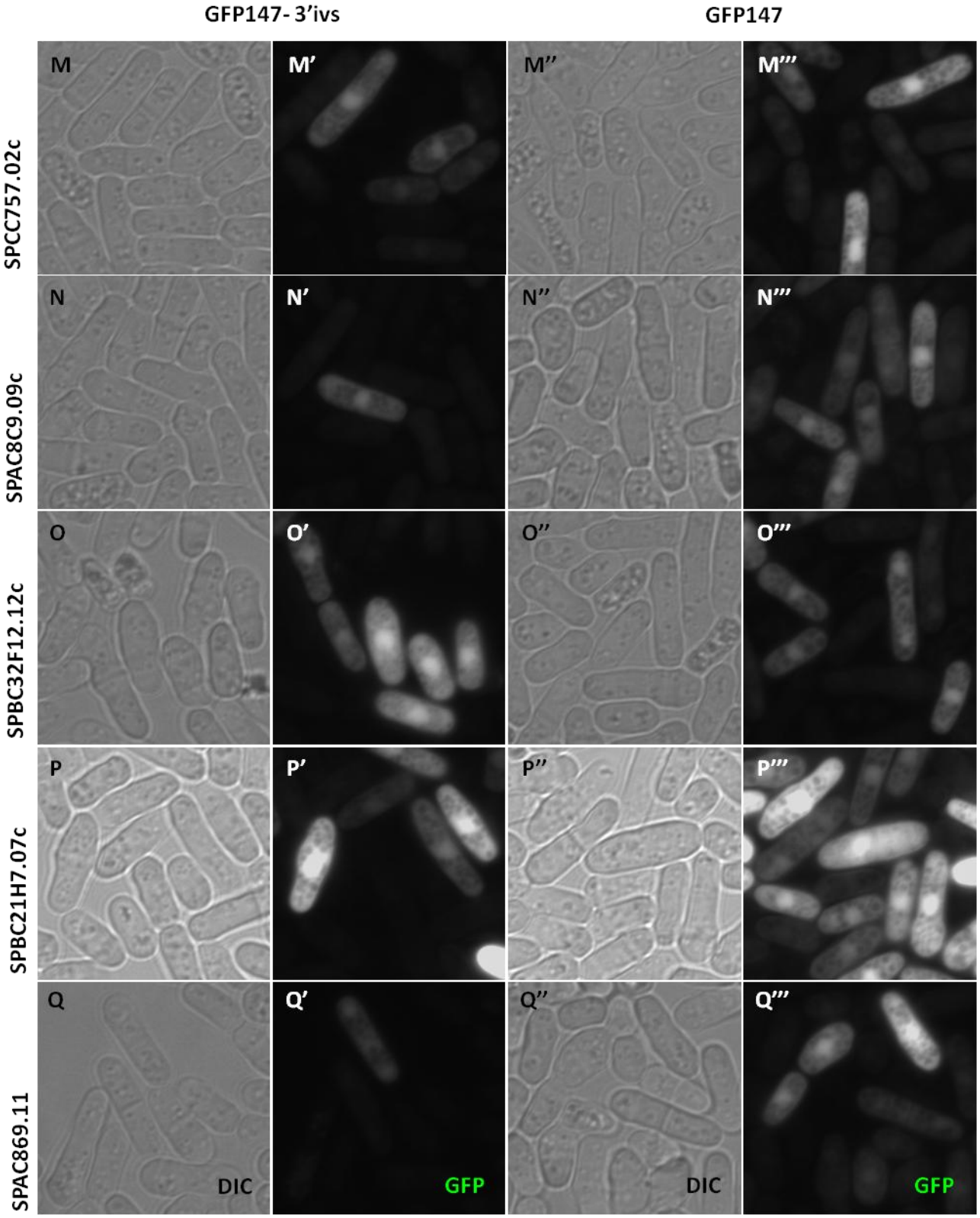
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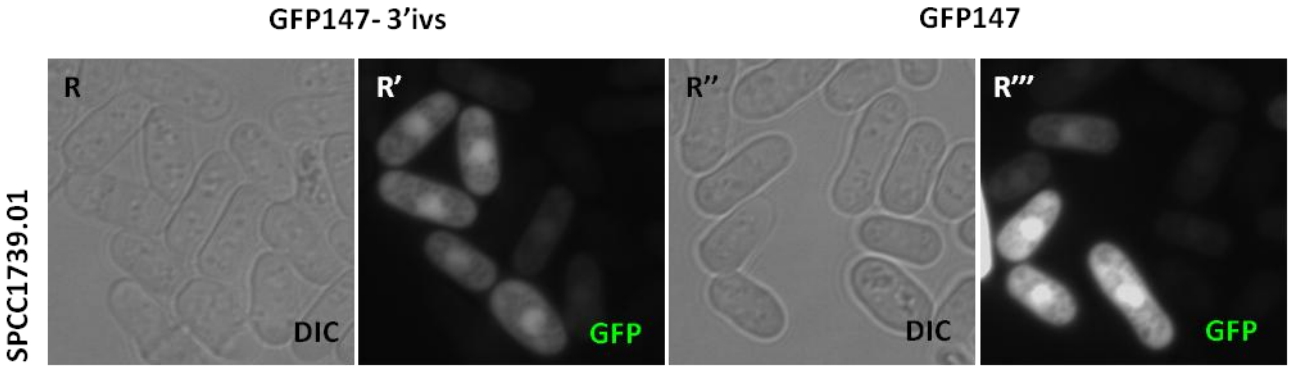


Figure 5: Secondary screen confirmation of putative NMD mutants.

A) GFP fluorescence in wild-type and UPF1Δ controls. Episomal expression of GFP in the wild-type *S. pombe* cells from GFP147-3'ivs reporter, show little fluorescence due to NMD. In UPF1Δ strain, the GFP levels are restored due to the abolishment of NMD. **B)** Screening of putative mutants from haploid deletion mutant library. The second column (X'), shows FITC images for 18 putative mutants transformed with GFP147-3'ivs reporter and the first (X) the corresponding DIC image. The fourth column shows FITC images of strains containing GFP147, and the third, DIC. Of the 18 strains pictured (A-R), 9 mutants show a level of GFP comparable (GFP147-3'ivs: X') to the control reporter within the same strain.

3.4 Quantification of putative mutants by northern blot analysis

As previously mentioned, unlike its integrated counterpart, copy number is an additional variable of episomal expression of the GFP147-3'ivs construct, making it slightly more difficult to compare by fluorescence microscopy the levels of GFP between strains.

As such the sub-library of putative candidates selected by first round of screening (initially by GFP-3'ivs), were analysed by Northern blot analysis to provide quantification of the levels of mRNA. Total-RNA was extracted by hot phenol method from strains episomally expressing GFP147-3'ivs. The strains were derived from primary cultures of those used previously for screening by microscopy and grown to OD_{600} 0.3. This OD is lower than typically used, however at higher OD there tends to be more variation in RNA obtained from the same cultures (based on observations in control strains; wild-type and UPF1Δ). The level of the leucine mRNA was used to correct for plasmid copy number variation.

Of the eighteen putative strains analysed, seven strains showed restored levels of RNA (50% of GFP147 control) in both rounds of Northern blot (Fig. 6). The quantifications given are (%) averages based on data from both experiments, thus some of the values may not best represent the band pictured. Due to time constraints it was not possible for a third repeat, as such, no statistical analyses could be performed.

Three strains showed increased mRNA level for only one round: SPAC27E2.01, SPAC652.01 and SPAC869.11 (Fig.6: lanes 8, 16 and 42) and have been excluded as putative NMD mutants in this study. The reason for this variation is unknown, however one possibility could be due to difficulties with quantification of data from the second round of Northern. This is because there are two bands present following hybridisation with the leucine probe (refer to

Fig.6); the lower band is approximately ¼ the intensity of the higher band it is possible that the additional band correspond to residual GFP. As a result, subtracting the background for quantification was more difficult. Subsequently, normalization and quantification for difficult bands were based on peak data, opposed to the more representative trace data normally used. This could account for some variation in the values between blots for the same strains.

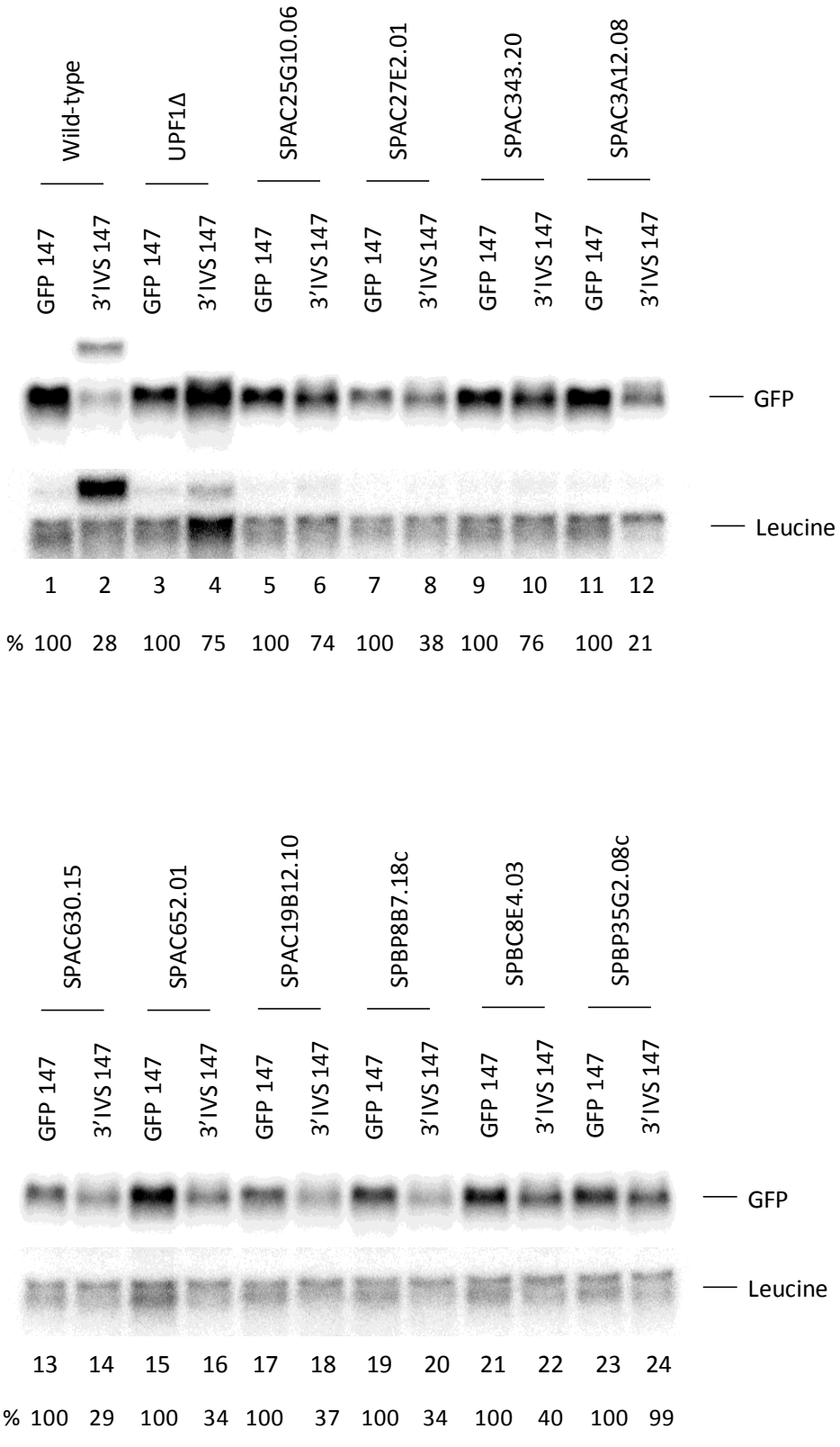
A third repeat would have been beneficial to establish the significance of the data obtained, and they may be worth re-visiting for any future work, particularly for strain SPAC27E2.01 (Fig. 5B and Fig. 6, lane 8), as this tested positive by all microscopy based screens, and confirmed by one round of Northern blot analysis.

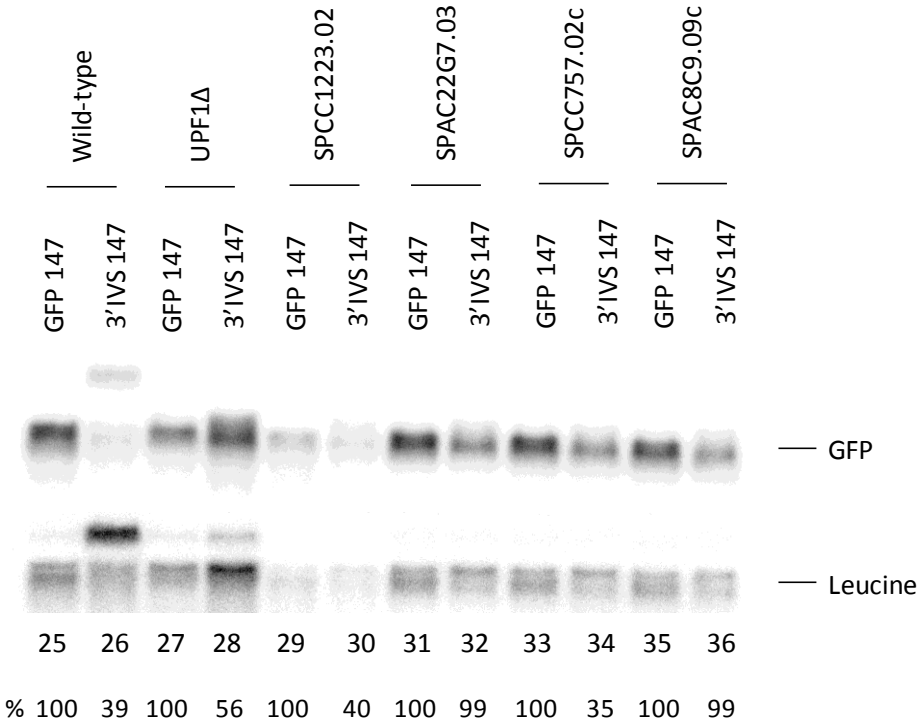
Eight strains; SPAC3A12.08 (Fig. 6, lane 12), SPAC630.15 (14), SPAC19B12.10 (18), SPBP8B7.18c (20), SPBC8E4.03 (22), SPCC1223.02 (30), SPCC757.02c (34), and SPCC1739.01 (44) exhibited levels of RNA less than 50% of control in both rounds of Northern. These were 21%, 29%, 37%, 34%, 40%, 40%, 35% and 31% respectively, and as such have been discounted. Some of these strains, however, show an apparent GFP increase phenotype - SPAC19B12.10 (Fig. 5B: G), SPBP8B7.18c (H), SPBC8E4.03 (I), SPCC1223.02 (K), and SPCC1739.01 (R), suggesting that GFP protein levels are similar to that of the NMD-insensitive GFP147 control.

Thus, seven strains were confidently identified as NMD mutants in both rounds of Northern blot: SPAC25G10.06 (Fig. 6, lane 6), SPAC343.20 (10), SPBP35G2.08c (24), SPAC22G7.03 (32), SPAC8C9.09c (36), SPBC32F12.12c (38) and SPBC21H7.07c (40). These showed levels of GFP mRNA similar to that of GFP147 and UPF1 deletion: mRNA levels were 74%, 76%, 99%, 99%, 99%, 63%, and 59% respectively. Of these, three strains - SPAC22G7.03 (Fig. 5B: L), SPBC32F12.12c (O) and SPBC21H7.07c (P) - were consistently positive

throughout all rounds of screening and concurrently had restored levels of RNA and protein from the NMD substrate. In conclusion, these seven strains resulted in suppression of NMD. Whether, this is due to abolishment of NMD or some indirect effect needs to be investigated.

Figure: 6. Quantification of putative NMD mutants by Northern blot.




Wild-type
UPF1Δ
SPCC1223.02
SPAC22G7.03
SPCC757.02c
SPAC8C9.09c
GFP 147
3'IVS 147
GFP
Leucine
25 26 27 28 29 30 31 32 33 34 35 36
% 100 39 100 56 100 40 100 99 100 35 100 99

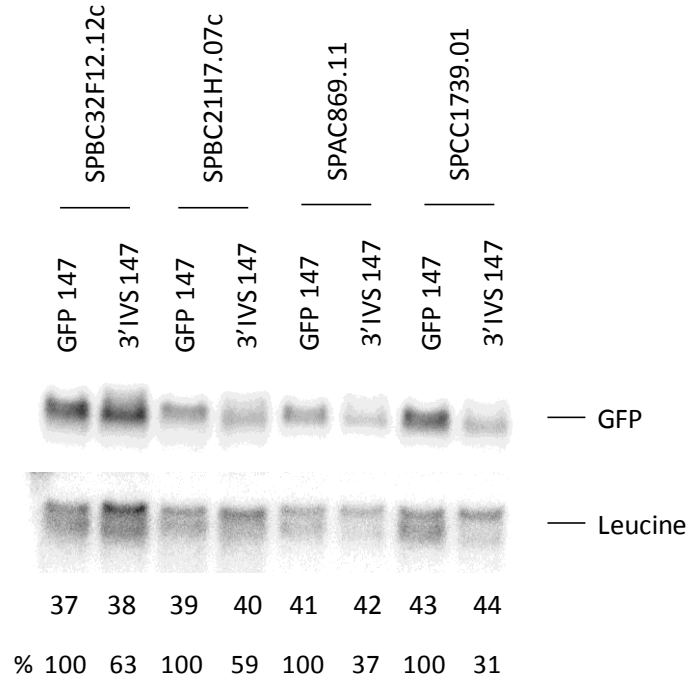

SPBC32F12.12c
SPBC21H7.07c
SPAC869.11
SPCC1739.01
GFP 147
3'IVS 147
GFP
Leucine
37 38 39 40 41 42 43 44
% 100 63 100 59 100 37 100 31

Figure 6: Quantification of putative mutants by northern blot.

Northern blot analysis of GFP mRNA in the putative NMD mutants identified in the microscopy screen. GFP bands were normalised against leucine to correct for plasmid copy number variation. The quantifications given are (%) averages based on data from two independent RNA samples. The quantification was done using the QuantityOne software of images acquired with a Bio-Rad phosphorimager.

Figure 7: Table of putative mutants; function, localisation and summary of results.

M-3030H position	Systemic ID	Figure reference (microscopy)	Figure reference (northern blot)	Name/Gene synonyms	Gene description	Localisation	Positive by microscopy GFP-3'ivs	Positive by microscopy GFP147-3'ivs	Positive by northern blot 1 analysis	Positive by northern blot 2 analysis
V3-P01-49	SPAC25G10.06	A	5,6	*rps2801/rps28-1*	40S ribosomal protein S28 (predicted)	Cytosol	+	-	+	+
V3-P01-50	SPAC27E2.01	B	7,8		Alpha-amylase homolog (predicted) [glycosyl hydrolase family 13 protein]	Nucleus, cytosol, cellsurface	+	+	-	+
V3-P01-61	SPAC343.20	C	9,10		Dubious	Nucleus, cytosol	+	-	+	+
V3-P01-62	SPAC3A12.08	D	11,12		Conserved fungal protein	Mitochondria, membrane	+	-	-	-
V3-P01-73	SPAC630.15	E	13,14	*Mug177*	Sequence orphan	Cytosol	+	-	-	-
V3-P01-74	SPAC652.01	F	15,16		BC10 family protein	Membrane	+	-	+	-
V3-P04-30	SPAC19B12.10	G	17,18	*Sst2*	Human AMSH/STAMBP protein homolog, ubiquitin specific-protease	Endosome, transport vesicle, cell division site	+	+	-	-
V3-P11-73	SPBP8B7.18c	H	19,20		TENA/THI family protein [Transcriptional activator]	Cytosol	+	+	-	-
V3-P13-89	SPBC8E4.03	I	21,22		Agmatinase 2 (predicted)	Fungal type vacuole, endoplasmic reticulum	+	+	-	-
V3-P13-91	SPBP35G2.08c	J	23,24	*air1*	Zinc knuckle TRAMP complex subunit Air1	Nucleus, nucleolus, tramp complex	+	-	+	+
V3-P14-05	SPCC1223.02	K	29,30	*nmt1/thi3*	No message in thiamine Nmt1	Nucleus, cytosol	+	+	-	-
V3-P16-57	SPAC22G7.03	L	31,32		Sequence orphan	Nucleus, cytosol	+	+	+	+
V3-P23-89	SPCC757.02c	M	33,34		Epimarase (predicted)	Unknown	+	-	-	-
V3-P24-08	SPAC8C9.09c	N	35,36	*mug129*	Sequence orphan	Unknown	+	-	+	+
V3-P24-20	SPBC32F12.12c	O	37,38		Golgi membrane protein involved in vesicle-mediated transport (predicted)	Golgi apparatus, golgi membrane	+	+	+	+
V3-P24-48	SPBC21H7.07c	P	39,40	*his5*	Imidazoleglycerol-phosphate dehydratase His5	Nucleus, cytosol	+	+	+	+
V3-P28-67	SPAC869.11	Q	41,42		Cationic amino acid transporter Cat1	Cytoplasm and plasma membrane	+	-	-	+
V3-28-93	SPCC1739.01	R	43,44		Zf-CCCH type zinc finger protein	Cytosol	+	+	-	-

CHAPTER IV: DISCUSSION

4.1 Putative NMD Mutants Identified in the Screen

Despite the vast amount of research into the process of NMD, our understanding of this mechanism is still not satisfactory. It is possible that different mechanisms have arisen across species, particularly following the observation that splicing-dependent NMD is independent of the EJC in *S. pombe* (Wen and Brogna, 2010). This highlights the fact that the EJC model cannot provide an explanation for this nucleus-cytoplasmic link in all systems where such link has been reported. This study aimed to identify any additional factors that may exist in *S. pombe* in an attempt to unveil the mechanisms behind these observations.

I have screened 2790 deletion mutants for increased GFP expression of an NMD sensitive reporter. Following two rounds of screening seven strains identified were: SPAC25G10.06 (Fig. 5B:A/Fig.6 lane 6), SPAC343.20 (C/10), SPBP35G2.08c (J/24), SPAC22G7.03 (L/32), SPAC8C9.09c (N/36), SPBC32F12.12c (O/38) and SPBC21H7.07c (P/40). Here I provide additional information about these genes and some preliminary bioinformatics analysis.

4.1.1 SPAC25G10.06 (rps2801/rps28-1)

A ribosomal protein was identified during the screen, which due to the central role of the ribosome in NMD induction, it would be expected that some ribosomal proteins (RPs) would be involved in this process, particularly with the recruitment of NMD factors to the terminating ribosome. This gene, *rps2801/rps28-1*, encodes for the ribosomal protein S28, which is a structural constituent of the small 40S subunit of the ribosome (Pombase). Interestingly, the human homolog of S28 is RPS23 associates with HNRNPU (heterogenous nuclear ribonuclearprotein U) or scaffold attachment factor A. HNRNPU is a spliceosome component and part of the coding region determinant (CRD)-mediated complex that was seen

to promote MYC mRNA stabilisation (Hüttelmaier 2009). It should however be noted that there are no known HNRNPU homologues in yeast, although it would be interesting to investigate further whether the link between splicing and NMD in yeast is facilitated by other components that are mediated through RPS28, and may have a similar role to HNRNPU. Using the String database (www.string-db.org) I find evidence of interactions between S28 and snu13 (SPAC607.03c) in *S. pombe*; this is a U3 snoRNP-associated protein that is associated with the U4/U6-U5 snRNP tri-snRNP (Stevens and Abelson, 1999).

4.1.2 SPBP35G2.08c (air1)

The zinc knuckle protein, air1, is a component of the TRAMP complex (Trf4p/air2p/mtr4p polyadenylation complex) which is a multi-protein complex consisting of air1p or air2p, an RNA helicase (mtr4p), and a poly (A) polymerase (either trf4p or trf5p) (Corbett et al, 2011). This complex operates in the nucleus and nucleolus and is implicated in nuclear RNA processing and degradation (Biogrid). Substrates include aberrant pre-mRNA, rRNA, impaired tRNA methyltransferase, snoRNA, telomeric and cryptic unstable transcripts (CUTs) (Corbett, A et al 2011). Air1/2p is a critical component of the TRAMP complex that has a role in bridging mtr4p by its N-terminal domain to trf4p by its ZnK5 domain (Vanacova, S et al 2012). In *S. pombe*, air1 interacts with cid14 SPAC12G12.13c, a poly (A) polymerase that is a functional ortholog of trf4/5 in *S. cerevisiae* suggesting that a TRAMP-like complex does exist in *S. pombe* (Biogrid, Keller 2010). It has been observed that cid14 co-purifies with a number of RPs, more specifically, interacting with the 60S ribosome subunits and associated assembly factors. As such it was inferred that cid14 may interact with ribosomal proteins during the assembly of the large subunit of the ribosome (Keller 2010).

The role of air1/2 in mRNA surveillance may not be limited to TRAMP-exosome mediated degradation. UPF1 interacts with air2p and mtr4p in *S. cerevisiae* (STRING). Although there is no known interaction in *S. pombe*, Air1 and UPF1 are present in both species. The

information available for *S. cerevisiae,* does suggest a link between the TRAMP and NMD pathway. The TRAMP complex, however, is believed to work in the nucleus while NMD is thought to be a cytoplasmic process. Yet, the observation that splicing is linked to NMD in an EJC-independent manner does however leave open the possibility that perhaps NMD can occur in the nucleus. This view is supported by the observation that UPF1 and other ribosome related factors bind with chromatin-associated RNA in *S. pombe* (De et al, 2011 and Brogna lab unpublished).

4.1.3 SPBC32F12.12c (Golgi membrane protein)

SPBC32F12.12c was identified as positive in all of my GFP microscopy screens and NMD suppression was confirmed by the Northern blot analysis. The gene encodes a Golgi membrane protein, involved in vesicle-mediated transport of secretary proteins to either vacuole/lysosome or the cell surface (Yoda et al., 2005). Bioinformatic analysis using Biogrid shows interaction with ARP42 SPAC23D3.09, also known as SWI/SNF, a nucleosome remodelling complex that allows transcription factors to access the chromatin, thus facilitating transcription. This complex has been implicated in a much wider set of cellular processes, other than gene expression, including centromere function and chromosome stability (Snyder 2011). ARP42 has UPF1, UPF3, air1 and mrt4 as interaction partners (Biogrid), indirectly linking the Golgi membrane protein identified in this screen to both the TRAMP complex and NMD factors.

4.1.4 SPAC22G7.03 (a sequence orphan)

This gene has no apparent orthologs. The protein orphan is localised in both the nucleus and the cytosol. While there is little information available for this gene, as such, no explanation can currently be offered, and further investigation and characterisation is required. Having

searched for interaction partners in Pombase and Biogrid, SPAC195.06c, a spliceosomal complex unit was identified which may offer some insight, although with little more information, an in-depth hypothesis cannot be provided. In addition, par1, a protein phosphatase regulatory subunit and hrr1, a helicase required for RNAi-mediated heterochromatin assembly were also listed as genetic interaction partners.

4.1.5 SPAC8C9.09c (*mug129*)

Mug129 is also a sequence orphan with no apparent orthologs. It does appear to have genetic interactions with the ski complex subunit Rec14, which assists the exosome in 3' end degradation of mRNAs and Arg1 (Argonaute), some of these proteins have endonuclease activity against mRNAs.

4.2 Other putative NMD mutants

These include SPAC343.20 (listed as dubious), which is believed to have a role in the cellular response to stress and currently there is no interaction data available for this strain in Biogrid. Additionally, SPBC21H7.07c (*his5*) an imidazoleglycerol-phosphate dehydratase, which is responsible for histidine metabolism, does not appear to have any interactions with factors involved in splicing nor decay pathways.

4.3 Positive hits discounted after further analysis

Eleven strains have been discounted following Northern blot analysis which did not indicate NMD suppression. These include: SPCC1223.02 (*nmt1/thi3*) a 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase and is understood to be involved in thiamine biosynthesis. It was identified as a fully repressible gene and has since been utilised as a tool through its use as an inducible expression system (Maundrell 1990). SPBC8E4.03

(Agmatinase 2) is implicated in urea and cellular amino acid metabolism. SPAC27E2.01 (Alpha-amylase homolog) involved in cellular and extracellular polysaccharide metabolism. In addition; SPAC3A12.08 (Conserved fungal protein), SPAC630.15 (*Mug177*), SPAC652.01 (BC10 family protein), SPAC19B12.10 (*Sst2*), SPBP8B7.18c (TENA/THI family protein), SPCC757.02c (Epimarase), SPAC869.11 (Cat1) and SPCC1739.01 (Zf-CCCH type zinc finger protein), were all excluded.

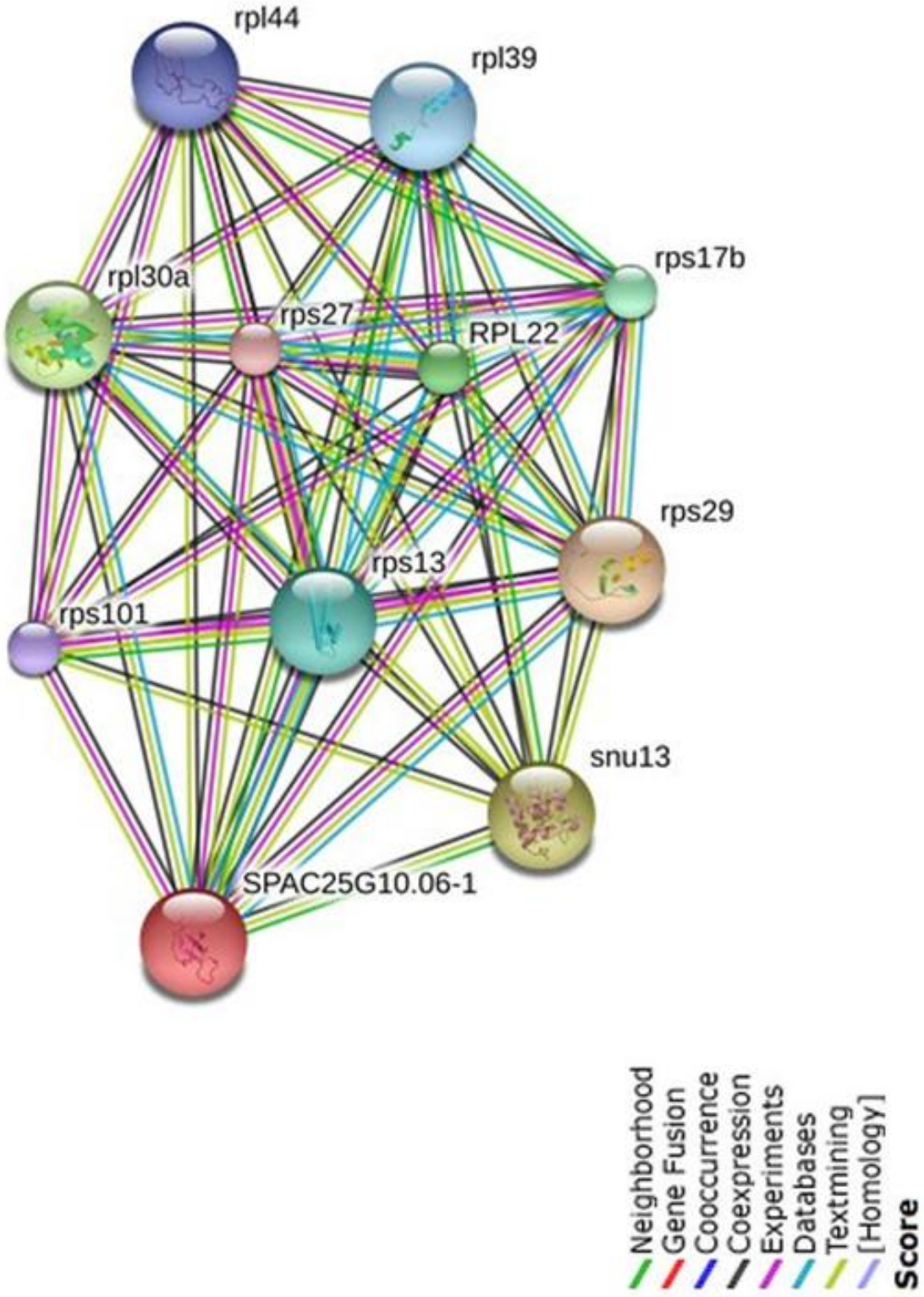


Figure 8:A. Protein interaction network for SPAC25G10.06 (rps2801/rps28-1) in *S.pombe*.

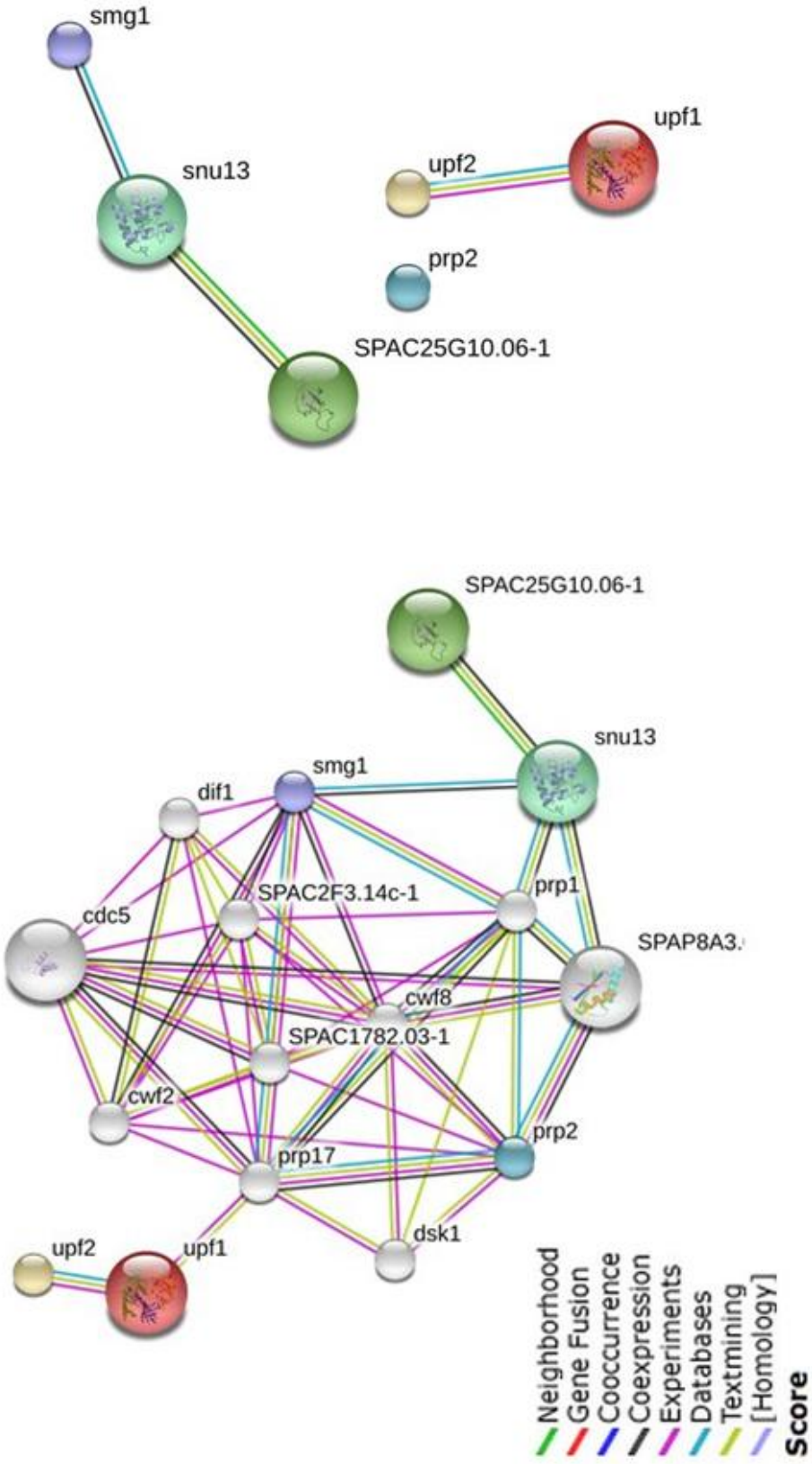


Figure 8:B. Protein interaction network for SPAC25G10.06 (rps2801/rps28-1), SPAC607.03c (snu13), SPBC146.07 (prp2), Smg1, Upf1 and UPF2 (top) and addition of 10 nodes (bottom), in *S.pombe*.

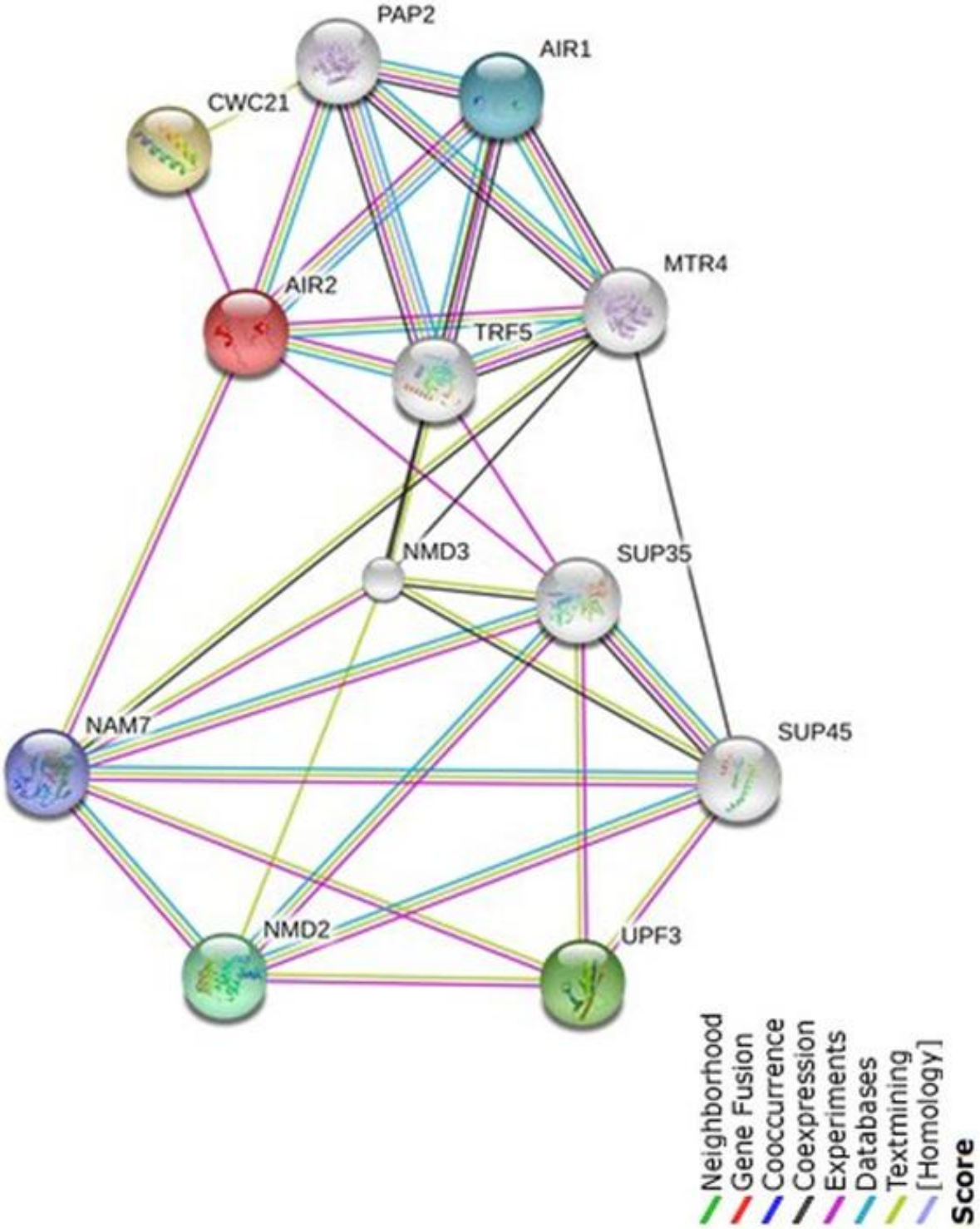


Figure 8:C. Protein interaction network for NAM7, NMD2, UPF3, air1, air2, and cwc21 in *S.cerevisiae*.

4.4 Problems with this Screening System

As with any mutant strain there will be additional phenotypes associated with a particular deletion. In UPF1Δ strains, the cells are more elongated and tend to grow slower than wild-type. Growth defects were very apparent while working with the deletion mutant library, with a number of strains that failed to grow. Additionally some mutants may be temperature sensitive, as such it is essential for these factors to be considered for any future work to eliminate the likely-hood of missing potential candidates, based on their disadvantaged phenotypes.

4.4 Conclusions

Due to time constraints it was not possible to screen all sets within the library and as such there may be additional NMD genes. Furthermore I did not screen essential genes. The screen I have described here in detail was based on splicing-dependent reporters, however, it will be informative to assay whether the mutants I have identified are able to suppress splicing-independent NMD; for example in GFP-N6, a nonsense mutation introduced in the GFP coding region at residue 6, results in very strong NMD in wild-type cells (Wen and Brogna, 2010). The prediction is that some of the mutants identified suppress only NMD of splicing-dependent reporters. In future studies it will be necessary to further characterise the protein identified so to test for any direct or indirect binding with know NMD and mRNA decay factors. Finally, in view that some of the proteins I have identified that are known to be involved in nuclear processes, such as TRAMP nuclear mRNA surveillance, future studies should directly assess whether NMD is directly linked to nuclear events.

REFERENCES

1. Allfrey V.G, Mirsky A.E, and Osawa S. Protein synthesis in isolated cell nuclei. *J Gen Physiol*. **40(3):** 451–490 (1957).

2. Amrani, N et al. A faux 3'-UTR promotes aberrant termination and triggers nonsense-mediated mRNA decay. *Nature* **432**: 112-118 (2004).

3. Amrani, N. Sachs, M.S. & Jacobson, A. Early nonsense: mRNA decay solves a Translational problem. *Nat. Rev. Mol. Cell Biol.* **7**: 415–425 (2006).

4. Blencowe, B. J. Alternative splicing: new insights from global analyses. *Cell*. **126**: 37-47 (2006).

5. Brogna S, Wen J. Nonsense-mediated mRNA decay (NMD) mechanisms. *Nat Struct Mol Biol*. **2:** 107-13 (2009).

6. Brogna, S., Sato, T.A., and Rosbash, M. Ribosome components are associated with sites of transcription. *Mol Cell* **10**: 93-104 (2002).

7. Capa, L et al. Translation Elongation Factor 2 Is Part of the Target for a New Family of Antifungals. *Antimicrob Agents Chemother.* **10**: 2694-9. (1998).

8. Cheng, Z et al. Structural and functional insights into the human Upf1 helicase core. *EMBO J* 26: 253-264 (2007).

9. Conti, E. & Izaurralde, E. Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. *Curr. Opin. Cell Biol.* **17**: 316–325 (2005).

10. Corbett AH., et al. Air1 Zinc Knuckles 4 and 5 and a Conserved IWR*X*Y Motif Are Critical for the Function and Integrity of the Trf4/5-Air1/2-Mtr4 Polyadenylation (TRAMP) RNA Quality Control Complex. *J Bio chem*. **286**: 37429-37445 (2011).

11. Culbertson MR, Underbrink KM, Fink GR. Frameshift suppression Saccharomyces cerevisiae. II. Genetic properties of group II suppressors. *Genetics*. **4**: 833-53 (1980).

12. Crick, F. Central Dogma of Molecular Biology *Nature* **227**: 561 - 563 (1970).

13. David, A et al. Nuclear translation visualized by ribosome-bound nascent chain puromycylation. *J Cell Biol*. **1**: 45-57 (2012).

14. David, A et al. RNA binding targets aminoacyl-tRNA synthetases to translating ribosomes. *J Biol Chem*. **23**: 20688-700 (2011).

15. De, S. Are ribosomal proteins present at transcription sites on or off ribosomal subunits? *Biochem. Soc. Trans*. **38**: 1543-1547 (2010).

16. Doma, M. K. & Parker, R. Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation. *Nature* **440**, 561–564 (2006).

17. Eberle AB, Stalder L, Mathys H, Orozco RZ, Mühlemann O. Posttranscriptional gene regulation by spatial rearrangement of the 3' untranslated region. *PLoS Biol*. **6(4)**: 0849-59 (2008).

18. Eberle AB, Lykke-Andersen S, Mühlemann O, Jensen TH. SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nat Struct Mol Biol.* **16(1)**: 49-55 (2009).

19. Frischmeyer, P. A. *et al.* An mRNA surveillance mechanism that eliminates transcripts lacking termination codons. *Science* **295**, 2258–2261(2002).

20. Huang T. Pre-spliceosome formation in S.pombe requires a stable complex of SF1±U2AF59±U2AF. *EMBO J*. **21**(**20)**: 5516-5526 (2002).

21. Huntzinger, E., Kashima, I., Fauser, M., Sauliere, J., and Izaurralde, E. SMG6 is the catalytic endonuclease that cleaves mRNAs containing nonsense codons in metazoan. *RNA* **14**: 2609-17 (2008).

22. Hodgkin, J., Papp, A., Pulak, R., Ambros, V. & Anderson, P. A new kind of informational suppression in the nematode *Caenorhabditis elegans. Genetics* **123**: 301–313 (1989).

23. Holbrook JA et al. Nonsense-mediated decay approaches the clinic. *Nature Genetics* **36** (8): 801-808 (2004).

24. Iborra, F. J., Jackson, D. A. and Cook, P. R. Coupled transcription and translation within nuclei of mammalian cells. *Science* **293**: 1139-1142 (2001).

25. Isken, O. *et al.* Upf1 phosphorylation triggers translational repression during nonsense-mediated mRNA decay. *Cell* **133**: 314–327 (2008).

26. Izaurralde E et al.mRNA quality control: An ancient machinery recognizes and degrades mRNAs with nonsense codons. *MPI for Developmental Biology*. **35** (2007).

27. Jackson, R.J et al. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol* **11**: 113-127 (2010).

28. Jacobson, A. & Izaurralde, E. in *Translational Control in Biology and Medicine* (eds Mathews, M.B., Sonenberg, N. & Hershey, J.W.B.) 659–691 (Cold Spring Harbor Laboratory Press, 2007).

29. Jørgensen. R, Merrill. A.R, and Andersen. G.R. The life and death of translation elongation factor 2. *Biochem Soc Trans*. **34**: 1-6 (2006).

30. Keller C., et al. Proteomic and functional analysis of the noncanonical poly(A) polymerase Cid14. *RNA*.**16(6)**: 1124–1129 (2010).

31. Kuperwasser, N *et al*. Nonsense mediated decay does not occur within the yeast nucleus. *RNA* **10**: 1907-15 (2004).

32. Leeds P, Peltz S.W, Jacobson A, Culbertson MR. The product of the yeast UPF1 gene is required for rapid turnover of mRNAs containing a premature translational termination codon. *Genes Dev*. **12A**: 2303-14 (1991).

33. Le Hir, H., Gatfield. D., Izaurralde, E & Moore, M.J. The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. *EMBO J*. **20**: 4987-4997 (2001).

34. Le Hir, H., Izaurralde, E., Maquat, L.E., and Moore, M.J. The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. *Embo J.* **19**: 6860-9 (2000a).

35. Le Hir, H., Moore, M.J., and Maquat, L.E. Pre-mRNA splicing alters mRNP composition: evidence for stable association of proteins at exon-exon junctions. *Genes Dev* **14:** 1098-108 (2000b).

36. Lejeune, F and Maquat, L.E. Mechanistic Links between nonsence-mediated mRNA decay and pre-mRNA splicing in mammalian cells. *Curr. Opin. Cell Biol* **17**: 309-315 (2005).

37. Majumdar R, Chaudhuri J, Maitra U. Reconstitution of mammalian 48S ribosomal translation initiation complex. *Methods Enzymol*. **430**: 179-208 (2007).

38. Maquat. L.E, Tarn. W.Y and Isken. O. The Pioneer Round of Translation: Features and Functions. *Cell*. **142(3)**: 368-74 (2010).

39. Maundrell K. nmtl of Fission Yeast. *J Bio Chem*. **265 (19)**: 10857-10864 (1990).

40. Maundrell K. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene*. **123(1)**: 127-130 (1993).

41. Matsuyama, A *et al*. pDUAL, a multipurpose, multicopy vector capable of chromosomal integration in fission yeast. *Yeast* **21** 1289-305 (2004).

42. Mitrovich QM, Anderson P. Unproductively spliced ribosomal protein mRNAs are natural targets of mRNA surveillance in C. elegans. *Genes Dev*. **17**: 2173-84 (2000).

43. Muhlrad D, Parker R. Aberrant mRNAs with extended 3' UTRs are substrates for rapid degradation by mRNA surveillance. *RNA*. **10**: 1299-307 (1999).

44. Nagy, E. & Maquat, L.E. A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. *Trends Biochem. Sci.* **23**, 198–199 (1998).

45. Nam K, Lee G, Trambley J, Devine S.E and Boeke J.D. Severe Growth Defect in a Schizosaccharomyces pombe Mutant Defective in Intron Lariat Degradation. *Mol Cell Biol.* **17 (2)**: 808-818 (1997).

46. Oliveira CC, McCarthy JE. The relationship between eukaryotic translation and mRNA stability. A short upstream open reading frame strongly inhibits translational initiation and greatly accelerates mRNA degradation in the yeast Saccharomyces cerevisiae. *J Biol Chem*. **15**: 8936-43 (1995).

47. Page, M.F., Carr, B., Anders, K.R., Grimson, A. & Anderson, P. SMG-2 is a phosphorylated protein required for mRNA surveillance in *Caenorhabditis Elegans* and related to Upf1p of yeast. *Mol. Cell. Biol.* **19**: 5943–5951 (1999).

48. Schweingruber C, Rufener SC, Zünd D, Yamashita A, Mühlemann O. Nonsense-mediated mRNA decay - Mechanisms of substrate mRNA recognition and degradation in mammalian cells. *Biochim Biophys Acta*. (2013).

49. Snyder M., et al. Diverse Roles and Interactions of the SWI/SNF Chromatin Remodeling Complex Revealed Using Global Approaches. *Plos genetics* **7(3)** (2011).

50. Stevens SW and Abelson J. Purification of the yeast U4/U6·U5 small nuclear ribonucleoprotein particle and identification of its proteins. *Proc Natl Acad Sci U S A.* **96(13)**: 7226–7231 (1999).

51. Vanacova S., et al. Air2p is critical for the assembly and RNA-binding of the TRAMP complex and the KOW domain of Mtr4p is crucial for exosome activation. *Nucleic Acids Research* 1–15 (2012).

52. Wen J, Brogna S. Nonsense-mediated mRNA decay. *Biochem Soc Trans*. **36**: 514-6 (2008).

53. Wen J, Brogna S. Splicing-dependent NMD does not require the EJC in *Schizosaccharomyces Pombe*. *EMBO J*. **29**: 1537-1551 (2010).

54. Weidensdorfer D., et al. Control of c-myc mRNA stability by IGF2BP1-associated cytoplasmic RNPs. *RNA* **15**:104-115 (2009).

55. Yoda K., et al. Immunoisolaton of the Yeast Golgi Subcompartments and Characterization of a Novel Membrane Protein, Svp26, Discovered in the Sed5-Containing Compartments. *Mol Cell Biol*. **25(17)**: 7696–7710 (2005).

56. Zhang. J, Sun. X.L, Qian. Y.M, LaDuca. J.P. & Maquat. L.E. At least one intron is required for the nonsense-mediated decay of triosephosphate isomerase mRNA: a possible link between nuclear splicing and cytoplasmic translation. *Mol. Cell. Biol.* **18**: 5272–5283 (1998).

Website:

57. http://www-bcf.usc.edu/~forsburg/vectors.html#tag

58. www.pombase.org

59. http://www.sciencegateway.org/tools/transform.htm

60. www.string-db.org

61. www.thebiogrid.org

Other:

62. Wen, J. Characterization of Nonsense Mediated mRNA Decay in *Schizosaccharomyces Pombe* (PhD thesis), (2010).

63. De, S. Tracking translation factors in fission yeast nuclei (PhD thesis), (2011).

APPENDIXES

Appendix 1: Recipes for *E. coli* and *S. pombe* media

LB Broth 1L

NaCl	10g
Bacto-tryptene (Peptone)	10g
Yeast extract	5g
dd H_2O	1L

Adjust PH to 7.5 (with NaOH)

Autoclave for 30 minutes at 121°C

LB-Agar (plates) 1L

Same as for LB Broth (see above)

Bacto Agar (prior to Autoclaving)	20g

YES Media (Arg+) 1L

Yeast Extract	5g
Sp complete supplements	1g

Arginine	0.112g
Dd H_2O	1L

Autoclave for 30 minutes at 121°C

YES-Agar (Plates) 1L

Same as for YES Media (see above)

Bacto Agar (Prior to autoclaving)	20g

EMM Media (Leu- Arg+) 1L

Pottassium Hydrogen Phthallate	3g
Di-Sodium Hydrogen Orphaphosphate-2-hydrate	2.2g
Ammonium Chloride	5g
Sp Supplements (Leu- Ura-)	0.17g
Uracil	0.112g
Arginine	0.225g
dd H_2O	929ml

Autoclave for 30 minutes at 121°C

Glucose (40% stock)	50ml
Salt (50X stock)	20ml
Vitamins (1000X stock)	1ml
Minerals (10,000X stock)	0.1ml

EMM-Agar (Leu-) plates 1L

Same as for EMM (see above)

Bacto Agar (prior to autoclaving)	20g

PMG- selection Media (Leu-/G418+) 1L

Potassium Hydrogen Phthalate

Sodium Hydrogen Orphophosphate di-hydrate

L- Glutamic Acid Mono Sodium Salt

SP Supplements (Leu- Ura-)	0.17g
Uracil	0.112g
Arginine	0.112g
dd H_2O	924ml

PMG- Agar (Leu-/G418+) 1L

Same as for PMG (see above)

Bacto Agar (prior to autoclaving)	20g

SPAS-Agar mating plates (1L)

1%	Glucose	10g
7.3mM	KH_2PO_4	1g
	SP supplements (complete)	45mg
	Arginine	0.112g
	Bacto Agar	10g

Autoclave for 30 minutes at 121°C

	Vitamins 1000X	1ml

Appendix 2: Solutions

RF1 (250ml)

100mM	RbCl	3g
50mM	$MnCl_2$ x $4H_2O$	2.475g
80mM	KAc	1.96g
10mM	$CaCl_2$ x $2H_2O$	0.368g
15%	Glycerol (100%)	37.5ml

Adjust PH to 5.8 (with 0.2M Acetic Acid)

Filter Sterilise

RF2 (250ml)

10mM	MOPS	0.523g
10mM	RbCl	0.302g
75mM	$CaCl_2$ x $2H_2O$	2.76g
15%	Glycerol (100%)	37.5ml

Adjust PH to 6.8 (with NaOH)

Filter Sterilise

Stock solutions:

Vitamins 1000X (100ml)

4.2mM	Pantothenic acid	0.1g
81.2mM	nicotinic acid	1g
55.5mM	Inositol	1g
40.8uM	Biotin	1mg

Minerals 10,000X (100ml)

80.9mM	Boric acid	0.5g
23.7mM	$MnSO_4$	0.4g
13.9mM	$ZnSO_4.7H_2O$	0.4g
7.4mM	$FeCl_2.6H_2O$	0.2g
2.47mM	Molybdic acid	40mg
6.02mM	Kl	0.1g
1.6mM	$CuSO_4.5H_2O$	40mg
47.6mM	Citric acid	1g

Salt 50X (500ml)

0.26M	$MgCl_2.6H_2O$	26.25g
4.99mM	$CaCl_2.2H_2O$	0.3675g
0.67M	KCl	25g
14.1mM	Na_2SO_4	1g

PEG (8000) 30ml

NaAC	1.47g
$MgCl_2.6H_2O$	0.036g
PEG8000	7.86g
ddH_2O	≤ 30ml

PEG (3350) 50% 100ml

Peg 3350	50g
ddH_2O	100ml

LiAc stock (0.3M)

9.18g LiAc di-hydrate

300ml ddH2O

Autoclave

LiAc/TE 50ml

0.1M LiAc	16.6ml of 0.3M
20mM Tris HCl	1ml of 1M
2mM EDTA	200µl of 0.5M
St.ddH_2O	32.2ml

Filter Sterilise

Resolving gel: 10% for 20ml

ddH_2O	7.9ml
30% Acrylamide	6.7ml
1.5M Tris (PH 8.8)	5ml
10% SDS	200µl
10% APS	200µl
TEMED (to be added only on pouring gel)	16µl

Stacking gel: 5% for 6ml

ddH_2O	4.1ml
30% Acrylamide	1ml
1.5M Tris (PH 6.8)	670 µl
10% SDS	60µl
10% APS	60µl
TEMED (to be added only on pouring gel)	6µl

TAE (50X) 1L

Tris Base	242g
Glacial Acetic Acid	57.1ml
0.5M EDTA (PH 8.0)	100ml
ddH_2O	≤ 1L

Working concentration= 1X TAE

20X SSC 1L

3M NaCl	175.32g
300mM Na Citrate	88.23g
DEPC- H_2O	≤1L

Adjust to PH 7 with NaOH

Hybsol 1L

1.5X SSPE	75ml of 20X
7% SDS	70g
10% PEG 8000	100g
DEPC-H_2O	≤1L

10X MOPS Running Buffer 1L

0.2M MOPS	41.2g
80mM NaAc	26.7ml of 3M
10mM EDTA	20ml of 0.5M
DEPC-H_2O	≤1L

Adjust PH 7

Appendix 3: Plasmids, Primers and Strains

Plasmids

Plasmid name	Marker in yeast	Resistance in *E.Coli*	Labelled	Description
pDUAL-GFPivs	leu1/ura	amp	p106	GFPivs ORF cloned by BamH1
pDUAL-GFP3'ivs			p152	
pDUAL-M.N6			p131	
pDUAL-M.GFP			p138-2	

Primers

Primer Name	**Sequence**	**Annotation**
PMP	ACGGTAGTCATCGGTCTTCC	sexual determinant (haplotype in *S.pombe*)
PMM	TACGTTCAGTAGACGTAGTG	sexual determinant (haplotype in *S.pombe*)
PMT	AGAAGAGAGAGTAGTTGAAG	sexual determinant (haplotype in *S.pombe*)
SSP41	GGAATCCTGGCATATCATCA	sequencing primer for pREP41
W196	GGTCTGCTAGTTGAACGCT	GFP.Q2.Rev

Strains

py114	SPJK001	h+ ade6-210 arg3D his3D leu-32 ura4D18	Janet F. Partridge janet.partrigde@stjude.org
py115	SPJK002	h- ade6-210 arg3D his3D leu-32 ura4D18	Janet F. Partridge janet.partrigde@stjude.org
MR3567	SPJK030	h- Upf1::KanMX6, leu1-32 ura4D18	Mol Cell Biol.2006 September; 26 (17): 6347-6356
MR3569	SPJK031	h- Upf2::KanMX6, leu1-32 ura4D18	Mol Cell Biol.2006 September; 26 (17): 6347-6356
MR3570	SPJK032	h- Upf1::KanMX6/Upf2::KanMX6, leu1-32 ura4D18	Mol Cell Biol.2006 September; 26 (17): 6347-6356
M3030H	SP286 library	h+/h+ ::KanMX6, ade6-M210/ade6-M216 leu1-32/leu1-32 ura4D18/ura4-D18	Bioneer