

Gene Expression & Regulation in the Yeast Metabolic Cycle



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Abstract

Biological oscillations such as the circadian rhythm are central to physiology of organisms ranging from humans to bacteria. By coupling cellular processes to a periodic oscillator or “clock”, cells can pro-actively coordinate their behaviour with periodic changes in their environment such as cycles of the Earth and the Moon. Defects in these processes can lead to diseases ranging from diabetes to cancer and improving these processes has the potential to enhance yields from crops and bioreactors. As such, a thorough understanding of the mechanisms underlying biological rhythms is critical for medical, agricultural and industrial applications.

The yeast metabolic cycle is a model ultradian rhythm that can be induced in prototrophic *S. cerevisiae* cells under nutrient-limiting conditions. However, very little is known about either the role of post-transcriptional gene-regulation in maintenance of metabolic cycling or the extent to which protein levels cycle. In order to better understand these processes, this study examines the effects of disruption of RNA degradation pathways on transcript cycling and uses polysome profiling, RiboSeq & proteomics to assess the impact of cycling transcripts on the proteome of the yeast metabolic cycle. We find that low rates of protein turnover effectively suppress transcriptional cycling at the protein level and argue that the role of cycling mRNA levels in the yeast metabolic cycle is to control steady-state rather than dynamic protein levels, coupling gene expression to growth rate and nutrient availability.

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List of Abbreviations

A

A - amplitude ($\frac{1}{2}\log_2(x + y)$); A_{256} - absorbance at 256 nm; A_{595} - absorbance at 595 nm; aatRNA - aminoacylated tRNA; A-site - tRNA acceptor site; Acetyl-CoA - Acetyl-coenzyme A; AID - auxin inducible degradation (protein domain); ADP - adenosine diphosphate; AMP - adenosine monophosphate; ANOVA - analysis of variance test; ATP - adenosine triphosphate; AUG - Start codon (adenine-uracil-guanine)

B

BCA assay - bicinchonic acid; BMAL - brain and muscle ARNT-like 1; BSA - bovine serum albumin

C

cAMP - cyclic adenosine monophosphate; CCR4-NOT - carbon catabolite repression - negative regulator of transcription deadenylase complex; cDNA - DNA generated through reverse-transcription; cDTA - comparative dynamic transcriptome analysis; CI - confidence interval; CK1 - casein kinase 1; CLOCK - circadian locomotor output cycles kaput; ClonNAT - nourseothricin; CRY1/2 - cryptochrome 1/2; CUT - cryptic unstable transcript

D

DMSO - dimethyl sulphoxide; DNA - deoxyribonucleic acid; dO_2 - dissolved oxygen; $dO_2\%$ - percentage dissolved oxygen; DTT - dithiothreitol

E

E-site - exit site; EDTA - ethylenediaminetetraacetic acid; eEF1A - eukaryotic elongation factor 1A; eEF2 - eukaryotic elongation factor 2; EGO-complex - complex containing Gtr1p, Gtr2p, Meh1p & Slm4p; eIF1 - eukaryotic initiation factor 1; eIF1a - eukaryotic initiation factor 1a; eIF2a - eukaryotic initiation factor 2a; eIF2b - eukaryotic initiation factor 2b; eIF3 - eukaryotic initiation factor 3; eIF4A - eukaryotic initiation factor 4A; eIF4B - eukaryotic initiation factor 4B; eIF4E - eukaryotic initi-

ation factor 4E; eIF4F - eukaryotic initiation factor 4F (cap-binding complex); eIF4G - eukaryotic initiation factor 4G

F

“free-mRNPs” - untranslated messenger ribonucleoprotein complexes from the lightest fractions of a sucrose gradient

G

G418 - geneticin; GEF - guanine exchange factor; GO - gene ontology; GPCR - G-protein coupled receptor; GSK3 - glycogen synthase kinase 3; GTP - guanosine triphosphate

H

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; HOC - high oxygen consumption phase of the YMC; HRP - horseradish peroxidase; H3 - Nucleosome subunit histone H3; H3K4me_{1/2} - mono/dimethylation of Histone H3 lysine 4; H3K4me₄ - trimethylation of Histone H3 lysine 4

I

IAA - indole-3-acetic acid; ISWIa/b - “imitation SWI” nucleosome remodelling complex

K

KAT - lysine acetyltransferase; KDAC - lysine deacetylase complex

L

LOC - low oxygen consumption phase of the YMC

M

M - magnitude ($\log_2(x - y)$); mRNA - messenger RNA; mRNP - messenger ribonucleoprotein; mTORC - mechanistic target of rapamycin complex; Mw - molecular mass

N

NAD⁺ - nicotinamide adenine dinucleotide; NADH - reduced nicotinamide adenine dinucleotide; NADP⁺ - nicotinamide adenine dinucleotide phosphate; NADPH - re-

duced nicotinamide adenine dinucleotide phosphate; ncRNA - “non-coding” RNA; NET-Seq - native elongating transcript sequencing; NMD - nonsense-mediated decay; NMP - nucleotide monophosphate; NTP - nucleotide triphosphate

O

OD₆₀₀ - optical density at 600 nm; O-GlcNAcyl - O-linked N-acetylglucose; ORF - open reading frame; OsTIR1 - F-box protein “transport inhibitor 1” from *Oryza sativa*; OX - “Oxidative” phase of the YMC

P

P-bodies - processing bodies; P-site - peptidyltransferase site; padj - adjusted p-value; PAGE - polyacrylamide gel electrophoresis; PAN2/3 - PolyA-specific ribonuclease 2/3 deadenylase complex; PBS - phosphate-buffered saline; PCR - polymerase chain reaction; pCUP - promotor of *CUP1*; PER1/2 - period circadian homologue 1/2; PFK1 - phosphofructokinase-1; PFK2 - phosphofructokinase-2; PKA - protein kinase A; PolyA+ - possessing a polyadenylate tail; poly-A - polyadenylate; PP2A - protein phosphatase 2A; “PPP” motif - triproline motif; PTC - peptidyltransferase centre

R

RB - “Reductive Building” phase of the YMC; RC - “Reductive Charging” phase of the YMC; REV-ERB α - “nuclear receptor subfamily 1, group D, member 1”; REV-ERB β - “nuclear receptor subfamily 1, group D, member 2”; RiBi - ribosome biogenesis gene; RiboSeq - ribosome profiling; RNA - ribonucleic acid; RNA-Seq - RNA-sequencing; RNAPII - RNA polymerase II; RNase - ribonuclease; RNase I - ribonuclease I; RNase PH - ribonuclease PH; RNP - ribonucleoprotein; RO - ribosome occupancy; RP - ribosomal protein; rpf - ribosome-protected fragment; RPG - ribosomal protein gene; rRNA - ribosomal RNA; RSC - “remodelling the structure of chromatin” nucleosome remodelling complex

S

S. cerevisiae - *Saccharomyces cerevisiae*; *S. pombe* - *Schizosaccharomyces pombe*;

SDS - sodium dodecylsulphate; SET1C - H3K4 methyltransferase complex containing Set1p; STAR - spliced transcripts alignment to a reference; StDev - standard deviation; SUT - stable unannotated transcript; SWATH-MS - sequential window acquisition of all theoretical mass spectra; SWI/SNF - “switching defective/sucrose non-fermentable” nucleosome-remodelling complex

T

T-test - Student’s T-test; Taq - *Thermus aquaticus* heat-stable DNA polymerase; TET-off - tetracycline-off promotor; TF - transcription factor; TIF-Seq - transcript isoform sequencing; TMT - tandem mass tag; TRAMP - Trf4p - Air2p - Mtr4p polyadenylation complex; Tris - tris(hydroxymethyl)aminomethane; Trp - tryptophan; tRNA - transfer RNA; tRNA^{meti} - initiator methionyl-tRNA; $t_{\frac{1}{2}}$ - half-life

U

UGRR - universal growth-rate response; UTR - untranslated region; U-test - Wilcoxon’s rank-sum test

X

XUT - Xrn1p-unstable transcript

Y

YMC - yeast metabolic cycle; YPD - yeast extract, peptone, dextrose media; YPDA - yeast extract, peptone, dextrose agar media

0-9

4E-BP - eIF4E-binding protein; 40S - small ribosomal subunit; 43S - preinitiation complex of the small ribosomal subunit, eIF1, eIF1a & eIF3; 48S - preinitiation complex of the 43S, cap-binding complex and mRNA; 60S - large ribosomal subunit, 7meG - 7-methylguanosine; 80S - complex of large and small ribosomal subunits along with associated factors; 9xmyc - nine copies of the myc epitope tag;

Chapter 1

Introduction

1.1 Biological rhythms and the Yeast Metabolic Cycle

1.1.1 What is a biological rhythm?

Life, as we know it, exists on planet Earth. Due to the orbits and axial rotation of the Earth and the Moon, all organisms living on or near the surface of the planet, are exposed to cyclical changes in environmental conditions. These can range from light levels, temperature and humidity, to the behaviour of other nearby organisms but regardless, it means that there is a strong selective advantage to being able to predict and these changes and react accordingly (Vaze & Sharma (2013); Dodd et al. (2005)). To this end, organisms have evolved a series of semi-autonomous oscillators that act as internal timekeepers. These are known as biological rhythms.

Reflecting the different sources of oscillations in the environment, several different kinds of biological rhythms exist. The most well known of these is the circadian rhythm. Almost universally conserved within the tree of life, circadian rhythms are found all the way from cyanobacteria and archaea to man (Pattanayak & Rust (2014); Edgar et al. (2012)). These rhythms allow organisms to predict the changing conditions of the day/night cycle and thus be primed for the appropriate metabolism and behaviour for that time of day. For most organisms studied, the natural period of

the circadian rhythm is close to 24 hours and any gradual deviation from the phase of the cycle is prevented by continual entrainment by light or other stimuli (Wright et al. (2001)).

Several other entrained rhythms are also important for certain species. For example, circannual rhythms whose periods are approximately 1 year, are of great importance to organisms with strong seasonal behaviour such as the golden-mantled ground squirrel and Arctic red knot, which adhere to annual patterns of hibernation and migration respectively (Mrosovsky et al. (1976); Karagicheva et al. (2016)). Likewise, organisms that are exposed to the extremes of temperature and salinity of the shoreline often exhibit circatidal rhythms of approximately 12.4 hours, allowing them to adapt to their extreme environment (Schnytzer et al. (2018); Chabot et al. (2016)).

It is easy to understand the selective advantage of synchronizing cellular functions with a changing extrinsic environment. However, interestingly, diverse organisms also possess apparently non-entrained rhythms. Prime examples of these include some mammalian oestrous and menstrual cycles as well as the yeast metabolic cycle (Downey (1980); Clark et al. (2003); Kaspar von Meyenburg (1973)), the latter of which will be the subject of this study. The existence of these rhythms demonstrates that temporal separation of biological processes can have inherent physiological advantages, even in the absence of a changing external environment. For example, antagonistic metabolic reactions that occur during cell growth and division may need to be temporally separated to maximize their efficiency and prevent futile cycling of metabolites (Tu et al. (2007)). This concept is known as temporal compartmentalization.

1.1.2 The Yeast Metabolic Cycle: A model ultradian rhythm.

The yeast metabolic cycle is a readily-induced and highly tractable eukaryotic ultradian rhythm. When prototrophic *S. cerevisiae* cells are grown to high density in a chemostat and fed limiting concentrations of glucose, cyclic changes in dissolved oxygen concentration ($\text{dO}_2\%$) are observed in the chamber (Kaspar von Meyenburg (1973); Chen & McDonald (1990); Chen et al. (1990); Borzani et al. (1977)). Under cycling conditions, the yeast metabolic cycle is a master regulator of cellular physiology, coordinating oscillations in intracellular and extracellular metabolites; levels of histone modifications; expression of over 60 % of cellular mRNAs as well as synchronization of the cell cycle (Chen & McDonald (1990); Chen et al. (1990); Klevecz et al. (2004); Tu et al. (2007, 2005); Kuang et al. (2014); Machné & Murray (2012)).

As a non-entrained biological rhythm, the period of the YMC is not constrained by the period of an extrinsic oscillation, meaning it can exhibit a large range of periods from 40 minutes to greater than 4 hours (Tu et al. (2007); Sohn et al. (2000); Burnetti et al. (2016)). However, duration of the YMC is not arbitrary and cycle periods are determined by a number of factors including genetic background; feeding rate and media composition and pH (Chen & McDonald (1990); Chen et al. (1990); Tu et al. (2007); Sohn et al. (2000); Cai et al. (2011); Burnetti et al. (2016)). The metabolic cycle integrates these diverse cues to compute a period which is then maintained throughout the culture, indefinitely.

The yeast metabolic cycle has been divided into three stages based on the transcripts that are expressed and the cell cycle stages present during different portions of the $\text{dO}_2\%$ trace (Tu et al. (2005)). The ground state of the cycle is known as the reductive charging or RC phase. This phase is characterized by relatively low rates of oxygen-consumption and expression of transcripts associated with quiescence and

proteostatic quality control. After RC phase, cells begin to consume larger amounts of oxygen and enter the oxidative (OX) phase. In this phase, levels of metabolic intermediates such as acetyl-CoA and NADPH rise along with histone acetylation levels and expression of translation-associated genes (Tu et al. (2005, 2007); Kuang et al. (2014)). Finally, the cell continues to consume large amounts of oxygen throughout the rather misleadingly-named reductive-building (RB) phase (Burnetti et al. (2016)). This phase is associated with expression of cell-cycle genes and mitochondrial components as well as the initiation of DNA replication and the cell-cycle in a subset of cells (Tu et al. (2005); Kuang et al. (2014)).

Based on the ability of the YMC to coordinate diverse cellular processes, it is clear that it is central to *S. cerevisiae* biology. However, despite several models being put forward, its specific purpose, beyond the general concept of temporal compartmentalization (Tu et al. (2005, 2007)), remains under debate. Several more specific models have been put forward with varying degrees of rigour. An early proposition by Tu et al. (2005) was that the cycle protects genome integrity by minimizing production of reactive oxygen species during RB phase, when DNA replication occurs. However, this model is questionable as RB phase is characterized by low $dO_2\%$ and by extension relatively high rates of oxygen-consumption (Burnetti et al. (2016)).

More recently, Kuang et al. (2014) used high temporal-resolution RNA-Seq data to propose that the YMC acts to coordinate assembly of complex macromolecules and organelles. Observing sequential expression of ribosome biogenesis genes (RiBis) and ribosomal protein genes they argue that this implies a “just in time” model where a pulse of RiBi expression primes the cell to assemble ribosomes using the ribosomal protein genes that follow it. These ribosomes are in turn used to drive accumulation of biomass and cell division during RB phase. This model is consistent with much of

the literature although some of the results from this study call it into question.

Finally, in their critique of the Tu et al. (2005) model, Burnetti et al. (2016) propose that the YMC exists instead to couple the start of the cell-cycle to the breakdown of storage carbohydrates such as trehalose and glycogen, ensuring that cells generate enough energy to progress through the cell cycle. This may also explain why cells enter a premature OX phase when treated with a pulse of accessible carbon (Cai et al. (2011)). Such a model explains why individual cells cycle quite well. However, it does not explain why the entire population should synchronize.

How individual *S. cerevisiae* cells communicate with one another to maintain synchrony is poorly understood, although several compounds including hydrogen sulphide, carbon monoxide, acetaldehyde and various carbon sources have all been proposed to act as synchronization signals (Sohn et al. (2000); Tu & McKnight (2009); Cai et al. (2011); Murray et al. (2003)). However, demonstrating causality for this role is challenging. Addition of an extrinsic pulse of a compound can have complex and indirect effects on cells and correlation of metabolite levels in the media and out-gas with specific phases of the cycle cannot be assumed to reflect causality. Theoretically, the best way to prove that a metabolite is necessary for synchronization would be to remove it from the media and show that cycling cannot proceed. However, the selective removal of metabolites from the media of cycling cells has not been achieved to date and would pose a formidable technical challenge.

Likewise, although much is known about the behaviour of cycling cells at the metabolite and RNA levels, the nature of the YMC clock itself and how it is regulated remains very much up for debate. To date, two competing models have been proposed. Based on the highly coordinated cycling of mRNA levels during the YMC,

several studies have proposed a transcription-driven cycle where sequential activation of transcription factors leads to subsequent upregulation of their targets at the mRNA, and by extension, protein levels (Kuang et al. (2014); Tu et al. (2005); Rao & Pellegrini (2011); Kuang et al. (2017)). They claim that these changes in protein levels lead to remodelling of cell structure and metabolic pathways explaining the coordination of metabolism and the cell-cycle. Thus, they argue that the YMC is fundamentally a cycle of gene expression with impacts on the metabolome.

Others however, support the opposite viewpoint. They point out the conserved changes in modifications of proteins such as peroxiredoxins between circadian rhythms and the yeast metabolic cycle as well as the dependency of both cycles on kinase activities such as those of CK1 & GSK (Causton et al. (2015)). The conservation of these features they argue points to a common origin of both cycles and a common biological clock that is based on cycling of signalling cascades and cellular redox state.

1.1.3 How does the YMC relate to other biological rhythms?

Much like the YMC, circadian rhythms comprise of coordinated oscillations in transcript levels, metabolism and the cell cycle. The classical model for the core circadian clock in eukaryotes is a delayed negative-feedback loop of transcription factor activity involving the transcriptional activators BMAL & CLOCK and the repressors PER1/2 & CRY1/2 (Reviewed in Takahashi (2016), Figure 1.1). This is bolstered by an auxiliary loop comprising of REV-ERB α and REV-ERB β . Collectively, these interlocking feedback loops generate a robustly oscillating transcriptional circuit which in turn coordinates the circadian expression of thousands of CLOCK-BMAL & PER-CRY targets genome-wide.

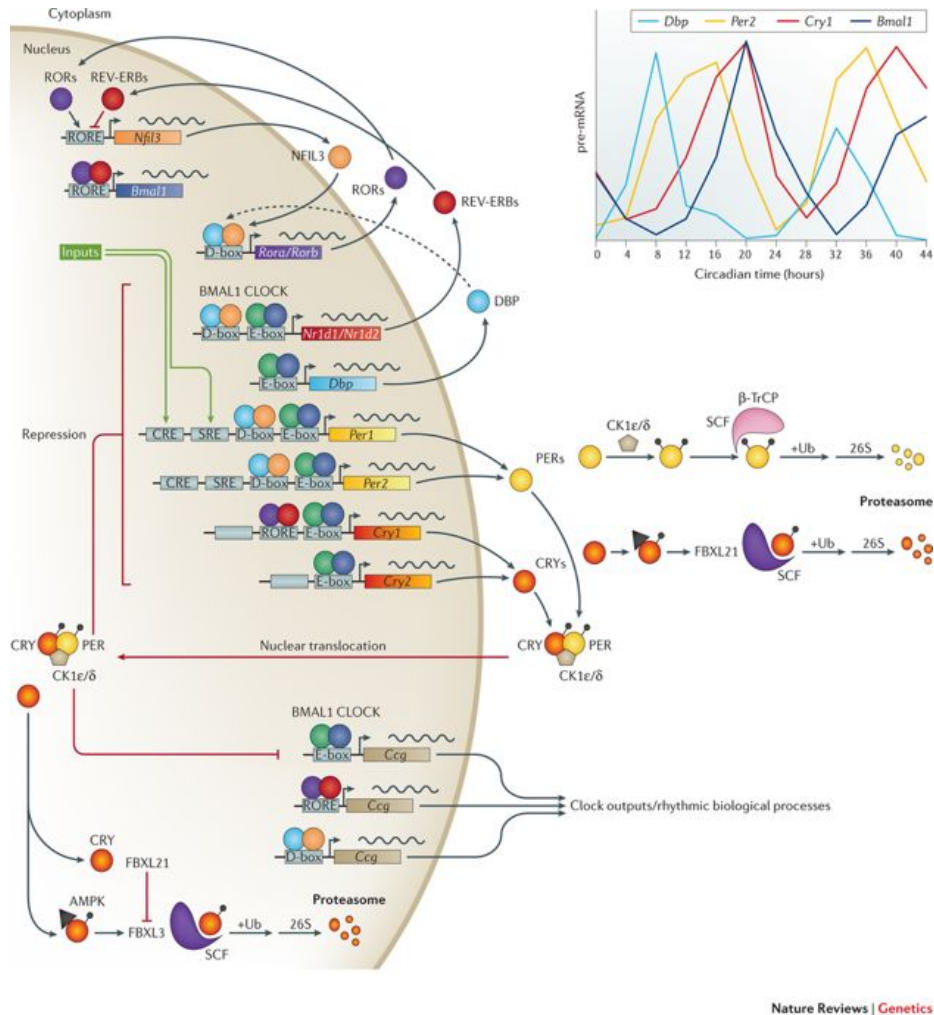


Figure 1.1: Schematic of the transcriptional components of the mammalian circadian clock (Extract from Takahashi (2016)).

During the daytime the transcriptional activator CLOCK-BMAL accumulates, activating transcription of the repressors *Per* & *Cry* by late afternoon/evening. PER-CRY interacts with CK1 δ/ϵ during the night and these translocate to the nucleus to repress transcription of *Clock* & *Bmal*. Degradation of CLOCK and BMAL protein overnight deactivates *Per* & *Cry* transcription resetting the cycle.

An auxiliary loop involving activation of the repressors REV-ERB α and REV-ERB β by CLOCK-BMAL also acts to repress *Clock* & *Bmal* expression overnight.

However, this oscillating transcription factor circuit cannot be the entire story as anucleate cells such as erythrocytes are able to maintain circadian oscillations in the absence of transcription (O'Neill & Reddy (2011)). Rhythmic fluctuations in the overoxidation of peroxiredoxins persisted for several days in the absence of any external stimuli and were found to be entrainable and temperature-compensated, key features of circadian rhythm markers. Furthermore, these oscillations have also been observed in nucleated cells from a variety of organisms suggesting that they are a conserved marker of circadian clocks rather than an adaptation of erythrocytes to overcome the limitations that arise from lacking a nucleus (O'Neill & Reddy (2011); Causton et al. (2015)). It may well be that the circadian rhythm is fundamentally regulated by metabolism with a transcriptional circuit overlaid in certain systems to expand its functionality and robustness.

This duality is reminiscent of the two conflicting models for regulation of the YMC (Kuang et al. (2014); Tu et al. (2005); Rao & Pellegrini (2011); Kuang et al. (2017); Causton et al. (2015)). As discussed already, the parallels between the two rhythms run deep. In fact, it has been proposed that the circadian clock is fundamentally a metabolic cycle with additional timekeeping functions (Klevecz & Li (2007); Tu & McKnight (2006)). This is an interesting proposition given that *S. cerevisiae* lacks orthologues of proteins such as BMAL, and to-date a set of master transcription factors for the YMC have not been agreed upon (Rao & Pellegrini (2011); Kuang et al. (2017)). In contrast, both systems exhibit oscillations in peroxiredoxin over-oxidation and the activity of kinases such as CK1 & GSK3 suggesting that oscillations in redox state and post-translational modifications might be the more fundamental oscillators (Causton et al. (2015); O'Neill & Reddy (2011)).

These similarities mean that, aside from the differences in entrainment and pe-

riod, the yeast metabolic cycle provides an excellent model for studying mammalian circadian rhythms. The ease of synchronization and genetic manipulation, as well as the ability to easily generate large amounts of biomass, allow scientists to ask questions of the yeast metabolic cycle that would be challenging to achieve in mammalian systems, such as deletion and disruption of central components of gene expression pathways. Thus, the yeast metabolic cycle allows us to directly investigate the relative contribution of transcriptional circuits to metabolic cycling.

1.2 Regulation of gene expression in *S. cerevisiae*.

1.2.1 Gene expression: From DNA to protein.

In order to assess the importance of different aspects of gene expression to metabolic cycling, we must first discuss the mechanics and regulation of these processes.

The genome of most eukaryotes comprises of a small number DNA polymers called chromosomes, divided into units known as genes which encode the information required for the organism to survive and reproduce (Hartwell (1970)). The interpretation of this information is known as gene expression. Most, though not all, eukaryotic genes ultimately encode proteins via an RNA intermediary known as mRNA (Crick (1970); van Dijk et al. (2011); Xu et al. (2009)). Thus, for most genes, the eventual output of gene expression is dynamic or steady-state changes in the levels of the corresponding protein.

This process occurs via a number of stages (Figure 1.2). First of all, the mRNA molecule encoding the protein (Aka the transcript) is synthesized from nucleotide triphosphates (NTPs) through a process known as transcription. During transcription the 5'-end of the nascent transcript is capped with ⁷meG (7-methylguanosine)

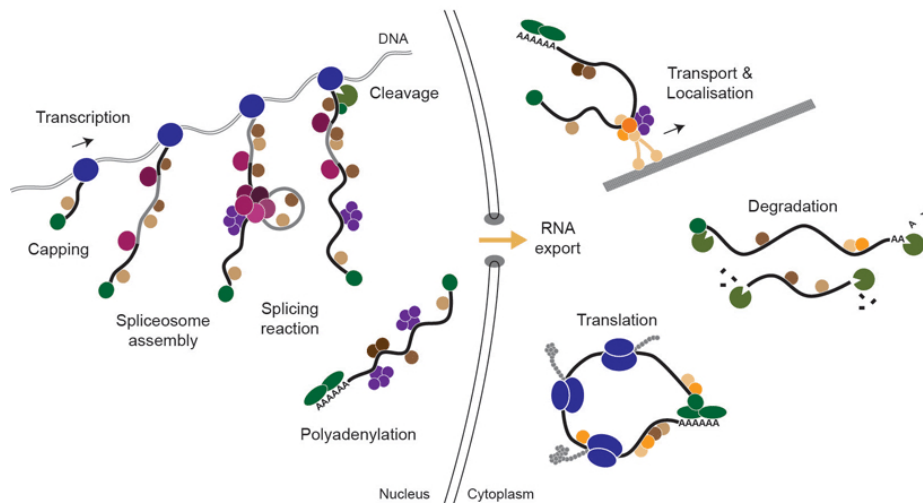


Figure 1.2: Overview of eukaryotic gene expression. (Extract from https://www.bmls.de/Computational_RNA_Biology/aboutus.html).

This diagram summarizes the key stages in eukaryotic gene expression, from mRNA synthesis and processing through to RNA degradation and translation. In the nucleus, DNA is transcribed by RNAPII, generating premRNA. Whilst in the nucleus, the premRNA undergoes extensive maturation and processing including, capping, splicing, cleavage and polyadenylation. Following processing, mRNAs are exported to the cytoplasm where they are translated to produce proteins. At the end of their lifespans, both mRNAs and proteins are degraded via their own dedicated pathways.

and internal, non-coding sequences known as introns are removed through splicing (Reviewed in Lee & Tarn (2013)). During termination of transcription, the nascent transcript is cleaved and poly-adenylated resulting in a mature mRNA which is subsequently exported from the nucleus to the cytoplasm. If any of the stages of mRNA synthesis and processing fail, or an RNA is not destined for the cytoplasm, it can be degraded at this stage by the nuclear exosome (Reviewed in Parker (2012)).

Once in the cytoplasm, mRNAs can have a number of fates (Figure 1.2). Most notably, mRNAs will normally be translated. This is a process where the nucleotide sequence of the mRNA is interpreted at a ratio of 3:1 to produce the protein product of the gene (Reviewed in Dever et al. (2016)). Alternatively, mRNAs can be localized to specific compartments of the cell by motor proteins or sequestered in translationally incompetent granules such as P-bodies and stress-granules (Reviewed in Singer-

Krüger & Jansen (2014) & Decker & Parker (2012)). Finally, once an mRNA is no longer needed it will be deadenylated and degraded by one of two cytoplasmic pathways (Reviewed in Parker (2012)).

Completing the cycle, the final stage of gene expression is protein turnover. Once a protein is no longer required by the cell, either because the gene is no longer to be expressed or the protein has accumulated damage, it will be marked for proteasomal degradation by K48-polyubiquitylation (Reviewed in Finley et al. (2012)). Alternatively, proteins and even entire organelles can be turned over in bulk by a process known as autophagy, where a portion of the cell is enclosed by a membrane known as an autophagosome, which in turn fuses with the proteolytic environment of the vacuole (Reviewed in Reggiori & Klionsky (2013)).

Of the many stages of gene expression, only some are critical for comprehension of this study. The following sections will briefly touch on transcription and chromatin before delving into detail on RNA degradation and translation. For more information on the other stages of gene expression, please consult the following reviews of the literature (Lee & Tarn (2013); Reggiori & Klionsky (2013); Singer-Krüger & Jansen (2014); Finley et al. (2012)).

1.2.2 Transcription and chromatin.

Transcription in eukaryotes is a complex and tightly regulated process which can be broken down into three main stages, initiation, elongation and termination (Reviewed in Kaplan (2013)). Naturally, transcription begins with initiation. During this stage, a plethora of sequence-specific and general transcription factors assemble on a region to the 5'-end of the gene known as the promoter. Here they remodel the chromatin

and recruit the RNAPII holoenzyme, loading it onto the template strand of the DNA (reviewed in Hahn & Young (2011)). In higher eukaryotes such as mammals, this is often promoted by interaction with a distant region of the same DNA molecule known as an enhancer, which promotes pre-initiation complex assembly (Reviewed in Bulger & Groudine (2011)). Once stably loaded, RNAPII forms an elongation complex that proceeds down the gene, creating an RNA polymer that is the reverse complement of the template strand and remodelling chromatin as it travels. This is known as the elongation phase. It is during elongation when much of the processing of transcripts occurs, particularly capping and splicing (Reviewed in Lee & Tarn (2013)). The termination phase involves the 3'-end processing of the transcript and disassembly of the elongation complex so that it can be recycled for future rounds of transcription (Reviewed in Kaplan (2013)).

Both elongation and termination of transcription are critical processes in the field of gene expression. However, for this study I will be limiting my discussion to regulation of the initiation phase of transcription and particularly the roles that sequence-specific transcription factors and chromatin modifications at the promoter have to play in this.

Sequence-specific transcription factors such as *GAL4* & *ADR1* are central to understanding many of the models for how biological rhythms such as the YMC and the circadian rhythm are regulated (Rao & Pellegrini (2011); Kuang et al. (2017, 2014); Tu et al. (2005); Takahashi (2016)). They are small proteins that are rich in the basic amino acids arginine and lysine making them highly positively charged. This gives them a high affinity for DNA, allowing them to recognise and bind to specific DNA sequences. Once bound, transcription factors signal whether a region of DNA such as a gene is to be transcribed or silenced by recruiting other proteins known as

co-activators and co-repressors. These proteins remodel the chromatin and recruit components of the pre-initiation complex whose assembly is the central event of transcription initiation (Reviewed in Hahn & Young (2011)).

In this way, transcription factors provide sequence specificity to the process of initiation, allowing the activation of individual genes to be controlled by the presence and accessibility of transcription factor binding sites in their promoters. This also allows them to be arranged into logical circuits, controlling one another's sequential activation and repression. It is this feature that has allowed the evolution of cyclic feedback loops such as the CLOCK-BMAL/PER-CRY circuit in mammalian circadian rhythms (Takahashi (2016)).

When discussing the initiation of transcription, especially in the context of metabolism, it is critical to consider the chromatin environment of genes. In eukaryotic cells, chromatin is the primary mechanism of packaging DNA (Reviewed in Rando & Winston (2012)). Higher-order chromatin structures are a hot topic for research at the moment, involving looping of genes and clustering of transcription units in space (Reviewed in Li & Reinberg (2011)). However, at its most basic, chromatin is composed of a DNA duplex wrapped around an octameric histone core. This coiling of DNA allows the 3 m long human genome to fit inside a 6 μm diameter cell nucleus. However, it also condenses the molecule, rendering transcription factor and pre-initiation complex binding sites inaccessible. As such it has powerful effects on the regulation of gene expression, both preventing initiation from spurious sites within genes and controlling the accessibility of genuine gene promoters (Youdell et al. (2008) & reviewed in Rando & Winston (2012)).

The accessibility of chromatin can be regulated either by changing the position or

the properties of the nucleosomes that comprise it. Large protein complexes such as SWI/SNF, ISWIa/b and RSC can physically move nucleosomes along the DNA via ATP hydrolysis (Reviewed in Rando & Winston (2012)). This can expose or occlude transcription factor binding sites, facilitating further regulation of the gene or even open and close the nucleosome-depleted regions where pre-initiation complex assembly occurs. Thus, translocation of nucleosomes is a versatile and effective method of gene activation and repression.

However, the most interesting mechanism of regulation of transcription by chromatin with regards to this study is through histone modification. This is because of its tight links to metabolism. A plethora of histone modifications exist across eukaryota (Reviewed in Britton et al. (2011)). This discussion will mostly focus on lysine acetylation as this is the best studied in *S. cerevisiae* but the principle of coupling of modifications to core metabolite levels extends to many other modifications including ADP-ribosylation, O-GlcNAcylation and methylation.

Lysine acetylation is a highly abundant modification that has been described on 6 different lysine residues on histone H3 alone (Reviewed in Rando & Winston (2012)). Acetylation has dramatic effects on the chemistry of lysine residues, neutralizing their positive charge and converting them into neutral, hydrophilic amino acids. In the case of histones, this reduces their electrostatic interactions with, and by extension the strength of their binding to, DNA, facilitating their movement along the polymer (Reviewed in Fan et al. (2015)). Acetyllysine residues can also specifically recruit proteins containing bromodomains, such as the SWI/SNF and RSC chromatin remodelling complexes (Reviewed in Rando & Winston (2012)). The affinity of individual bromodomains for acetyllysine is low. However, complexes often contain proteins with multiple bromodomains or multiple bromodomain-containing proteins.

By acting in concert, it is thought that these can integrate cues from several residues to generate a more precise and nuanced output.

The link between lysine-acetylation and metabolism arises through its deposition and removal (Reviewed in Fan et al. (2015)). Lysines are acetylated through the activity of proteins known as KATs (lysine acetyl transferases - also known as HATs - histone acetyltransferases). These proteins utilize the core metabolite Acetyl-CoA as a substrate, transferring its acetyl-group onto lysine residues of various proteins including histones. As a result, KAT activity, and by extension rates of acetylation, are dependant on the synthesis and consumption of acetyl-CoA. Similarly, some classes of lysine deacetylase complexes (KDACs - also known as HDACs - Histone Deacetylase complexes), particularly the sirtuins, require NAD^+ as a substrate, coupling their activity directly to cellular redox state. Thus, levels and distributions of lysine acetylation couple regulation of protein activity to cellular metabolism and in the case of histone acetylation, provide a direct line of communication to gene expression.

The importance of this to the yeast metabolic cycle can clearly be seen in the work by Cai et al. (2011) & Kuang et al. (2014). Together they observe a global pulse of histone acetylation in OX phase, particularly focused around the promoters of OX phase genes. This correlates both with the peak of intracellular acetyl-CoA and expression of these mRNAs, pointing towards a signalling cascade where the metabolic changes that occur in OX phase drive acetylation and activation of OX phase promoters. This paradigm presents a useful lens through which to observe gene expression and metabolism and inspires speculation about the links between other histone modifications and metabolism.

1.2.3 Nuclear and cytoplasmic RNA degradation

The steady-state levels of transcripts are determined by the balance between rates of transcription and RNA degradation, giving both processes great potential to determine mRNA levels (Shalem et al. (2008)). Furthermore, due to mistakes during RNA synthesis or accumulation of damage over time, mRNAs can become defective and non-functional. As such, RNA degradation is crucial for regulation of gene expression and quality control of the transcriptome.

For the purposes of this study, turnover and regulation of intact, functional, mRNAs in the cytoplasm is of primary interest. This occurs via two partially overlapping pathways, 5'->3' degradation by Xrn1p and 3'->5' by the cytoplasmic exosome (Figure 1.3, Reviewed in Parker (2012)). Although they differ in the mechanics of RNA degradation, the initial targeting of transcripts to each pathway is common.

With few exceptions, the first step of RNA degradation is deadenylation of the transcript. Deadenylation is performed by two separate complexes, CCR4-NOT & PAN2/3 which differ in their responses to Pab1p, and as a result their activities (Reviewed in Parker (2012)). *S.cerevisiae* mRNAs are synthesized with a poly-A tail of around 90 nt in length but upon reaching the cytoplasm this is rapidly trimmed by Pan2p to around 65 nts in length. Further deadenylation is then blocked by Pab1p binding until it dissociates from the poly-A tail, which may occur in response to a number of different cues. Pab1p dissociation allows Ccr4p to degrade the remainder of the tail to a length of 10-12 nts, at which length the Lsm1-7/Pat1p complex can bind. This is the primary process that stimulates decapping.

Binding of Pab1p to the poly-A tail acts as the primary regulator of deadenylation (Reviewed in Parker (2012)). The association of Pab1p itself to the tail is stimulated

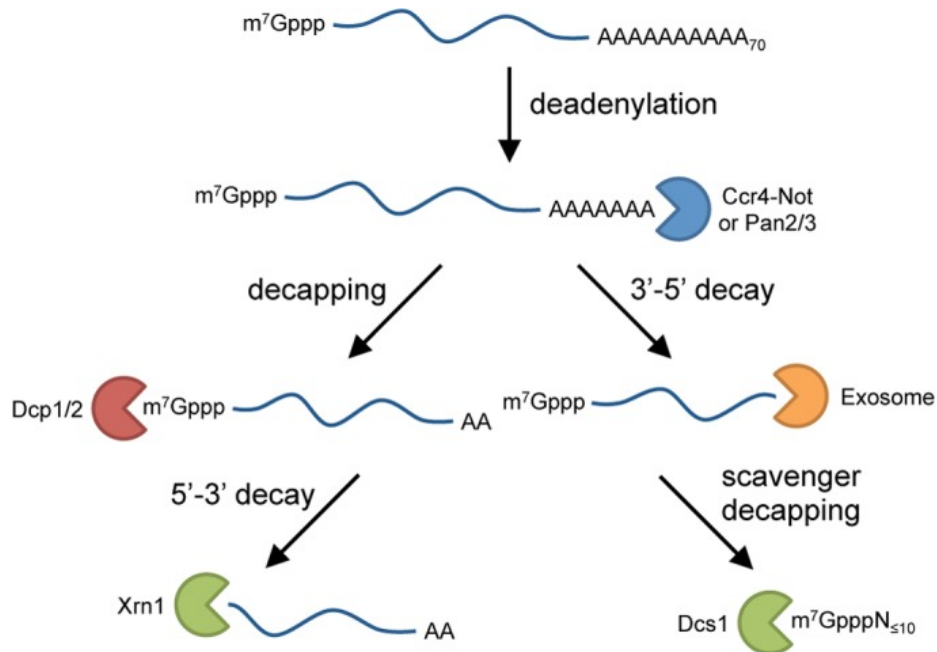


Figure 1.3: Major pathways of eukaryotic mRNA degradation. (Extract from Braun & Young (2014)).

The initial step of mRNA decay is common to both 5'→3' and 3'→5' degradation pathways. Degradation begins with trimming of the 3' polyA tail to 10-12 nts in length. This process is stimulated by binding of RNA-binding proteins such as the PUF-family to the 3'-UTR of the transcript. Following deadenylation, the mRNA can either be immediately degraded from the 3'-end by the cytoplasmic exosome, or more commonly, it will be decapped and degraded 5'→3' by Xrn1p.

in a large part by translation initiation. This can be observed in mRNAs whose translation is defective, such as those with poor AUG contexts. As a result of their reduced translation initiation rates, these transcripts show higher rates of deadenylation and are consequently less stable. Thus, a major determinant of mRNA stability is the efficiency of translation. However, mRNAs can also contain specific regulatory motifs that recruit RNA-binding proteins which either promote or repress deadenylation. Over 10 % of the transcriptome is regulated by members of the Puf protein family whose six members bind to mRNAs and promote deadenylation through repression of translation and direct recruitment of CCR4-NOT which occurs via its Pop2p subunit.

Once a transcript has been deadenylated, its fate branches and it can be degraded by one of two pathways. The simpler, and yet less common pathway is to be degraded

from the 3'-end by the cytoplasmic exosome (Reviewed in Parker (2012)). The exosome is a complex composed of a hexameric ring of catalytically inactive members of the RNasePH family flanked by three small RNA binding proteins at one end and the catalytic subunit Dis3p, which possesses both endonuclease and 3'->5' exonuclease activities, at the other end. In the cytoplasm, an adaptor protein, Ski7p, is also present with the core complex and acts as a docking point for the Ski2/3/8-complex. The Ski2/3/8-complex in turn is required for 3'->5' degradation of cytoplasmic mRNAs. Although targetting of mRNAs to the exosome is poorly understood, it is thought that they are recruited to it by the Ski2/3/8-complex and the Ski2p helicase domain acts to funnel mRNAs into the exosome core.

The nuclear exosome possesses an additional 3'->5' exonuclease activity in the form of Rrp6p (Reviewed in Parker (2012)). The exact function of this nuclease is unclear but it is required for the repression of an important class of non-coding RNAs known as cryptic unstable transcripts (CUTs) and may also be involved in nuclear surveillance and the degradation of highly structured RNA sequences (Xu et al. (2009); Schneider & Tollervey (2013)). Substrate specificity for the nuclear exosome appears to be determined in a large part by the TRAMP complex which contains RNA helicase and oligo-adenylation activity allowing it to supply the core exosome with a substrate not unlike that proposed to be generated by the Ski2/3/8-complex.

Although exosome-mediated degradation of mRNAs seems to be the more direct pathway, most deadenylated transcripts in *S. cerevisiae* are in fact degraded from the 5'-end by Xrn1p (Reviewed in Parker (2012)). Unlike the exosome, Xrn1p is a single-subunit 5'->3' exonuclease which is capable of processive degradation of any RNA with a 5'-monophosphate. As such, regulation of Xrn1p is mediated by two known mechanisms. Primarily, Xrn1p activity is limited by regulation of the decapping com-

plex that it is often found associated with. Since Xrn1p requires a 5'-monophosphate for its activity, it can only degrade decapped transcripts meaning that mRNAs with a 5'-cap are protected from degradation. However, once decapping has occurred, transcripts appear to be freely degraded by Xrn1p.

However, Xrn1p catalytic activity can also be regulated in a more sophisticated manner. The C-terminal domain of Xrn1p is thought to be autoinhibitory and limit Xrn1p activity in high-glucose conditions. Braun et al. (2014) showed that phosphorylation of the inhibitory C-terminus of Xrn1p by Snf1p relieves glucose-inhibition of mRNA decay. They further speculate that this activity may feed back and affect decapping. Thus, Xrn1p substrate-specificity may be influenced by intracellular phosphorylation cascades.

One of the most puzzling features of *XRN1* is the unique phenotype of *xrn1Δ* mutant strains. Numerous studies have demonstrated that *xrn1Δ* cells exhibit a 1.9-fold global upregulation of mRNAs (Sun et al. (2013); Malabat et al. (2015); Woloszczuk et al. (2016)). Sun et al. (2013) show that *XRN1* is uniquely required for buffering of gene expression. Using cDTA analysis, they demonstrate that mutations that affect the rates of mRNA degradation normally have a proportionate effect on mRNA synthesis and vice versa, which prevents global changes in protein levels from occurring. However, in *xrn1Δ*, this relationship breaks down and a reduction in mRNA degradation rates leads to an increase in mRNA synthesis, explaining how global mRNA levels increase in the *xrn1Δ* mutant.

This was a remarkable conclusion. However, they were unable to provide a satisfactory model for how this occurs. They propose a set of nested feedback loops where Xrn1p autoregulates degradation of its own transcript and regulates transcrip-

tion by degrading the transcripts of the transcriptional repressor *NRG1* (Sun et al. (2013)). This mechanism however, does not fit their data as it means that *XRN1* acts indirectly as a transcriptional activator when their cDTA analysis shows that it is a repressor, rendering their model internally inconsistent.

Numerous other studies have also tried to explain buffering of gene expression by *XRN1*. The Morillon lab has been particularly prolific in this regard, proposing two conflicting models for the mechanism by which this occurs. In their first model, van Dijk et al. (2011) invoke a class of *S. cerevisiae* “non-coding” RNAs they call Xrn1p-unstable transcripts (XUTs). These are a class of ncRNA similar to the SUTs described by Xu et al. (2009) (Woloszczuk et al. (2016)) which have nuclear and cytoplasmic components and are uniquely sensitive to Xrn1p (van Dijk et al. (2011)). They propose that XUTs shuttle between the nucleus and the cytoplasm, sampling Xrn1p activity. Inside the nucleus, they bind to their sense transcript pair and repress its transcription in an H3K4me_{1/2}-dependant manner. However, it has since been shown that H3K4-methylation status is not generally instructive for transcription in *S. cerevisiae* which casts doubt over their model (Howe et al. (2017); Venkatasubrahmanyam et al. (2007); Carvin & Kladde (2004); Rando & Winston (2012)).

Their second model proposes that cytoplasmic hybridization of overlapping 3'-UTRs is normally resolved by Xrn1p. In *xrn1Δ* cells, these duplexes accumulate triggering NoGo-decay of transcripts (Sinturel et al. (2015)). However, Woloszczuk et al. (2016) have shown that of the 1500 transcripts with overlapping 3'-UTRs in the genome, only two show reductions in mRNA levels in *xrn1Δ*. Therefore, NoGo-decay cannot be occurring at an appreciable rate and this model cannot be correct.

A fourth model proposed by Haimovich et al. (2013) states that *XRN1* acts as a transcriptional activator of genes whose transcripts it has just degraded. They claim it does this by physically entering the nucleus, binding to promoters and activating transcription. Again however, this model is inconsistent with subsequent discoveries by Woloszczuk et al. (2016) who show that *xrn1* Δ cells exhibit rapid induction of *GAL*-genes, a result that is inconsistent with a global transcriptional activator.

Finally, Braun & Young (2014) aim to link buffering of steady-state gene expression to Snf1-signalling during stress-responses. They argue that the global changes in *xrn1* Δ cells might reflect a breakdown of the coupling of transcription and degradation that would otherwise allow for rapid, coordinated responses to changes in gene-expression. They do not propose a specific mechanism for this but much like the work of Sun et al. (2013), it could potentially function through transcription factor mRNA stability.

To date, no mechanism for Xrn1-mediated buffering can be agreed upon. However, its importance to the regulation of gene expression is clear. By connecting rates of RNA synthesis to rates of degradation, *XRN1* generates a feedback loop that is vital for transcriptome homeostasis, regardless of mechanism. In a rapidly fluctuating system such as the YMC, this property may allow tight and efficient coordination of mRNA expression programs, explaining the rapid transitions that occur throughout the cycle.

1.2.4 Translation and its regulation

Although the processes that control levels of mRNA within cells are of vital importance to gene expression, for the majority of genes, their final activity is determined by the levels of their proteins. As such, rates of translation are a critical determinant

of the final levels of a gene product.

The process of translation can be divided into four main stages; initiation, elongation, termination and ribosome recycling (Dever et al. (2016)). Of these, initiation and elongation will be the primary focus of this discussion.

Translation is an ancient process and one that is conserved from bacteria to humans. However, it is at the initiation stage where it shows greatest complexity and divergence (Reviewed in Dever et al. (2016), Figure 1.4). Translation initiation begins with the formation of a 43S pre-initiation complex composed of the small ribosomal subunit bound to eIF1, eIF1a & eIF3. This complex then recruits the initiator-methionyl-tRNA ($\text{tRNA}^{\text{meti}}$) bound to eIF2a-GTP and subsequently binds to the cap-binding complex eIF4F, making its first association with the transcript to be translated. eIF4F is a multi-subunit complex composed of eIF4E - the cap-binding protein; eIF4A - a helicase thought to prepare the transcript for 43S-association; eIF4B and the scaffold protein eIF4G.

Having bound to the cap and formed the 48S pre-initiation complex, the ribosome proceeds to scan the 5'-UTR of the transcript (Reviewed in Dever et al. (2016)). In higher eukaryotes, this stage is a source of much regulation as the choice of initiator AUG is dependant on the sequence context and secondary structure surrounding it (Reviewed in Kozak (2002)). However, in *S. cerevisiae* 5'-UTRs are generally short and unstructured so scanning is highly processive, and with a few exceptions such as the translationally-regulated gene *GCN4* (as discussed on page 26), initiation of the main ORF occurs at the first AUG (Reviewed in Dever et al. (2016)).

Upon selection of the initiator AUG, the GTP molecule bound to eIF2a is hy-

drolyzed. This results in conformational changes that position the (tRNA^{meti}) anticodon to base-pair with the AUG in the future peptidyl-transferase site (P-site) and dissociate many of the existing factors from the complex (Reviewed in Dever et al. (2016)). Subsequently, the eIF5-GTP-60S complex is recruited, binds to the 48S and the GTP bound to eIF5 is hydrolyzed. This causes further conformational rearrangements which dissociate the remaining initiation factors and lock together the 40S & 60S subunits forming an elongation-competent 80S complex.

Upon completion of translation initiation, the ribosome travels processively towards the 3'-end of the transcript, decoding the nucleotide sequence to extend the nascent peptide chain. This stage is known as elongation (Reviewed in Dever et al. (2016)). The elongation stage of translation is inherently cyclical meaning that we can begin our description at any point. However, assuming that we are following directly from the formation of the 80S complex at the end of initiation, elongation proceeds as follows:

Aminoacyl-tRNAs (aatRNA) in complex with the GTP-binding protein eEF1A sample the mRNA codon in the acceptor site (A-site) adjacent to the P-site. If the codon is recognized, GTP hydrolysis occurs causing dissociation of eEF1A-GDP and loading of the aatRNA into the A-site. This brings the amino acid into proximity with the nascent peptide in the peptidyltransferase center (PTC), leading to spontaneous transfer of the nascent chain onto the aatRNA in the A-site. This in turn causes conformational changes in the ribosome which result in it adopting a “ratcheted” conformation where the acceptor ends of the tRNAs are in the P & E(exit)-sites but the anticodons and mRNA remain in the A & P-sites (Reviewed in Dever et al. (2016)).

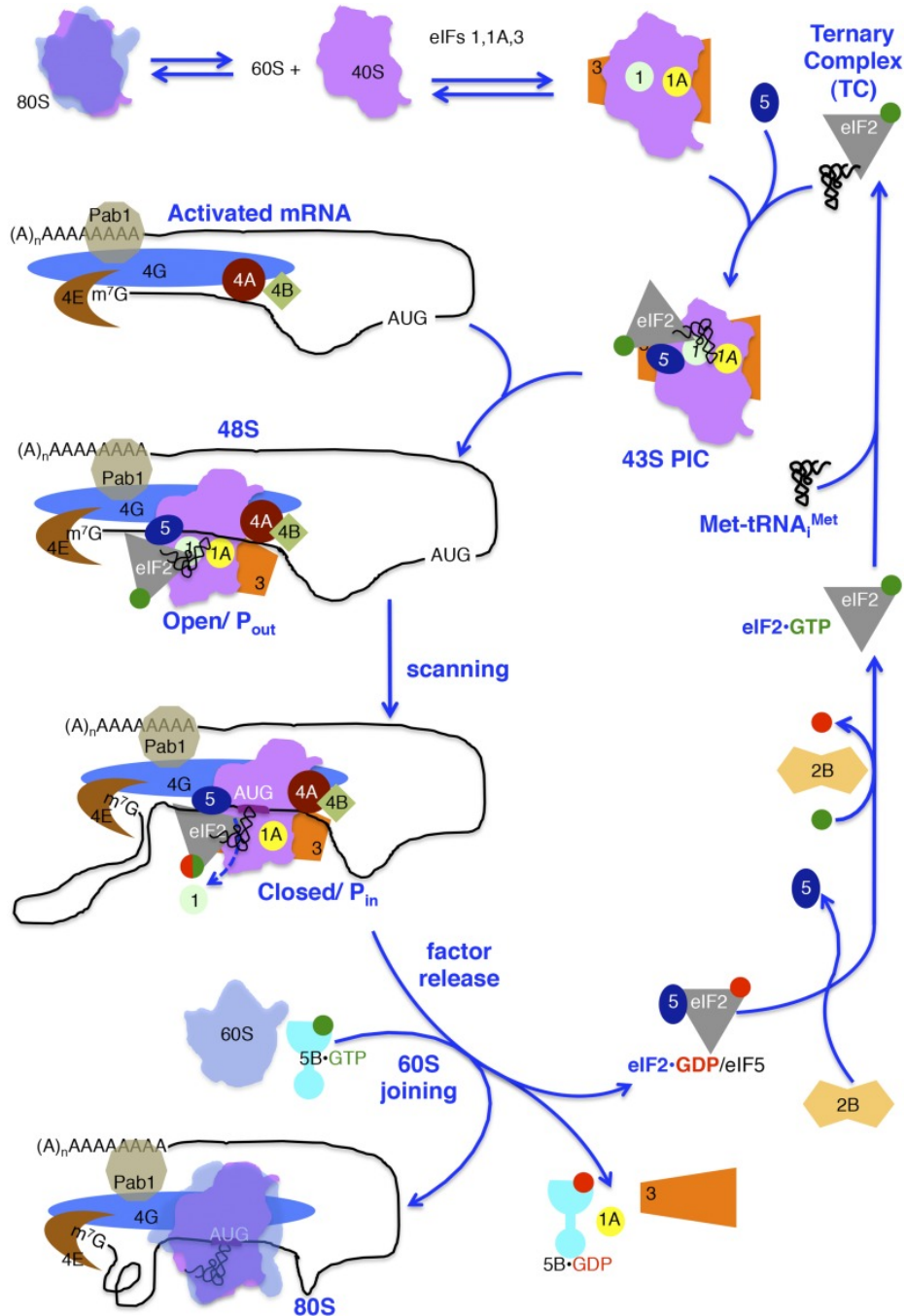


Figure 1.4: Translation initiation in *S. cerevisiae*. (Extract from Dever et al. (2016)).

Diagram summarizes the main steps of translation initiation, from 43S formation; through cap-binding; scanning and AUG selection to 60S loading and 80S formation.

The ratcheted state is resolved by a second round of GTP hydrolysis. eEF2-GTP binds to the ribosome in this conformation, activating its GTPase domain. The resulting GTP hydrolysis drives conformational changes that translocate the tRNA anticodons into the P & E sites, converting the ribosome back to its original state. Following this, eEF2-GDP and the exhausted tRNA in the E-site dissociate from the complex, returning the ribosome to its original conformation, translocated 3 nts downstream. Elongation continues in this manner until a “STOP” codon enters the A site of the ribosome, at which point termination and eventually ribosome recycling occur (Reviewed in Dever et al. (2016)).

Discussing translation from a step-wise, reductionist angle is important to appreciate its mechanistic details but to understand its regulation, we must consider it within the wider context of the cell. In log-phase, *S.cerevisiae* cells divide approximately every 2 hours. This requires synthesis of a phenomenal amount of biomass and therefore, extensive biosynthetic capacity. As testimony to this, modelling by Shah et al. (2013) demonstrates using experimentally-derived parameters that the limiting factor for translation in log-phase is the availability of ribosomes. This is particularly interesting as it calls into question the historical notion of a large pool of non-translating 80S monosomes, especially in light of the recent publication by Heyer & Moore (2016) demonstrating translation by monosomes. It appears that in log-phase, *S. cerevisiae* cells are utilizing their ribosomal pools to full capacity and growing as fast as their protein-synthetic machinery will allow them to.

In times of stress however, the situation is profoundly different. Due to its ability to directly and immediately affect protein abundances, translation is one of the fastest modes of gene expression regulation to be effective during stress (Reviewed in Liu & Qian (2014)). As such, it is responsible for some of the earliest changes in

gene output in response to changing environmental conditions. Although there are a multitude of mechanisms by which stress can modulate translation at every stage, two that are of particular interest are discussed below.

Arguably the best-studied mechanism of translational regulation in *S. cerevisiae* is the response to amino acid starvation (Reviewed in Dever et al. (2016)). Depletion of intracellular methionine pools leads to accumulation of uncharged methionyl-tRNAs. These bind to the kinase Gcn2p leading to its activation. Gcn2p then phosphorylates numerous targets including eIF2a (Dever et al. (1992)). Phospho-eIF2a-GDP cannot be recycled by its guanine exchange factor (GEF) eIF2b and in fact binds to it preventing it from recycling other eIF2a-GDP molecules. This leads to rapid translational inactivation of most mRNAs (Reviewed in Dever et al. (2016)). However, a minority of mRNAs escape and are actually more highly translated. Notable among these is the transcription factor *GCN4*, which is involved in the transcriptional response to starvation. *GCN4* contains a number of upstream open reading frames (uORFs). These are short open reading frames that are upstream of the main ORF and thus initiated in its place, translationally repressing it (Reviewed in Dever et al. (2016)). However, when levels of eIF2a-GTP are low, the ribosome can bypass some of these uORFs and re-initiate at the AUG of the main ORF allowing *GCN4* to be expressed (Dever et al. (1992)).

Another kinase that's involved in translational repression due to stress is *RCK2*. Rck2p is activated through phosphorylation by the Hog-signalling pathway in response to osmotic stress. Phospho-Rck2p then proceeds to phosphorylate a number of targets including the translation elongation factor eEF2 to adapt the cell to high osmolarity conditions (Teige et al. (2001)). Phospho-eEF2 is inactive and therefore Rck2p activity slows translation elongation by depleting the pool of active eEF2.

Protein synthesis is a costly process, occupying a large part of cellular biomass and energy reserves (Shah et al. (2013); Lynch & Marinov (2015)). Therefore, under the low-energy conditions of the yeast metabolic cycle, regulation of translation is critical to ensure that cells make efficient use of the nutrients that they have available.

1.3 Using Next-Generation Sequencing techniques to study post-translational regulation of gene expression

1.3.1 Principles of RNA-Sequencing

Over the past 20 years, the development of RNA sequencing (RNA-Seq) technologies has revolutionized our ability to study gene expression from transcription through to translation. Although myriad techniques are currently available for applying RNA-Seq to different applications, the fundamental principles are common between them (Reviewed in Boone et al. (2018)). Essentially, RNA is extracted from either intact cells or a sub-fraction thereof; it is reverse-transcribed; DNA adaptors are ligated to make it compatible with a sequencing platform; the resulting cDNA is amplified by PCR and the completed library is loaded onto a sequencing platform for data-acquisition and base-calling.

This study has used two different methods for library preparation depending on the level of resolution required to answer a particular question. Many RNA-Seq experiments are primarily interested in quantifying changes in the abundance of RNA molecules by generating and comparing counts tables. For this purpose we have used the QuantSeq 3' mRNA-Seq for Ion Torrent kit (Lexogen) which uses the poly-A tail of transcripts as a reverse-transcription primer (Figure 1.5, As used in Efremova

et al. (2017); Songserm et al. (2018)). This has the advantage of efficiently selecting for poly-adenylated transcripts such as mRNAs, SUTs, XUTs & CUTs, as well as identifying the locations of the 3'-ends of transcripts. However, care must be taken in analyzing QuantSeq data as poly-A priming necessarily generates a substantial 3'-bias. To account for this, counts tables generated from QuantSeq datasets in this study used a restricted and uniform window flanking the 3'-end of the annotated transcript (-100bp - +400bp).

For routine analysis, gene-level counts tables are usually sufficient. However, often more information can be gleaned by taking a base-pair resolution view of the RNA. For example, one might be interested in the location of a particular RNA-binding complex on a transcript. In our case, RiboSeq experiments were performed using the Ingolia et al. (2012) (Figure 1.6) protocol with some minor adjustments as described in the Materials and Methods (Section 2.8). This method involves fragmentation of RNA followed by ligation of a linker to the 3'-end, reverse transcription, circularization and finally amplification.

One of the big challenges in the analysis of RNA-Seq data is finding an appropriate normalization strategy, as the depth of sequencing of a library is arbitrary and does not usually reflect the global levels of RNA in the input material. A commonly employed solution is simply to make the assumption that most mRNAs do not change in expression between datasets and so the global library sizes should be equal (Chen et al. (2015)). This assumption is widely used in the literature and provides the basis for differential expression analysis packages such as DESeq2 and EdgeR (Love et al. (2014); Robinson et al. (2010)).

However, it is not always a reasonable assumption to make. Cells can vary dramat-

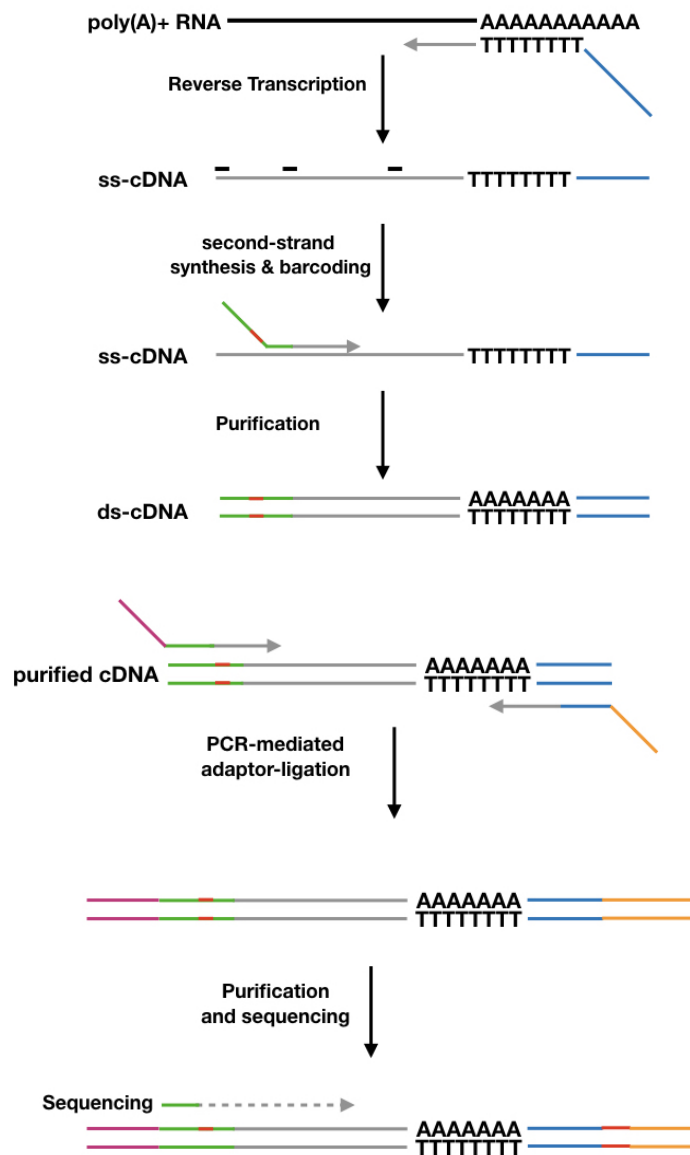


Figure 1.5: Schematic of Lexogen QuantSeq library preparation.

Lexogen Quantseq libraries are prepared from PolyA+ RNA (black) as follows:

Reverse-transcription from an oligo-dT containing primer (blue) generates antisense cDNA of the 3'-end of transcripts (grey).

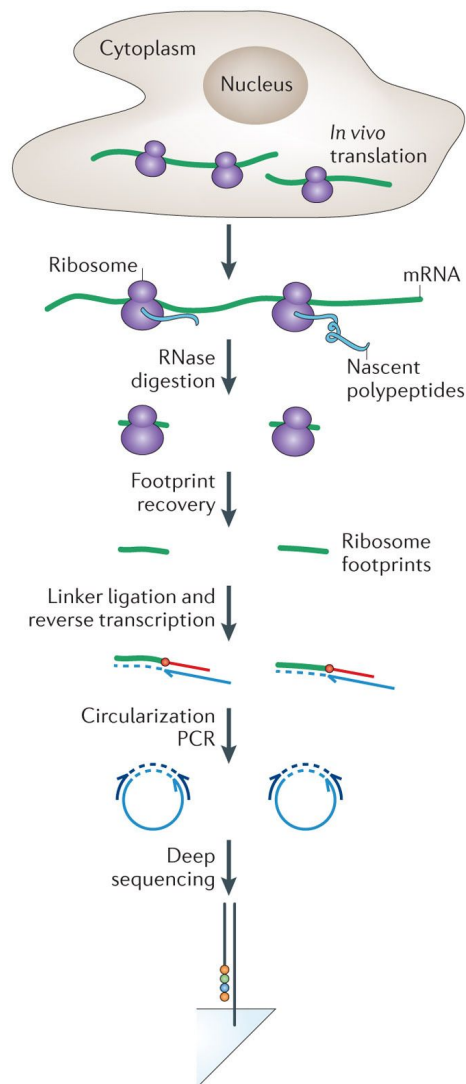
The RNA template is degraded by alkaline hydrolysis.

Second-strand synthesis is performed by random priming (green). During this step, an in-line barcode is incorporated into the sequence to facilitate sample multiplexing.

ds-cDNA is purified using magnetic beads.

The library is amplified by PCR and sequencing adaptors are incorporated in the process.

The library is purified using magnetic beads then sequenced using the Ion Proton system (ThermoFisher).



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Figure 1.6: Schematic of RiboSeq library preparation. (Extract from Ingolia (2014)).

RiboSeq libraries are prepared from RNaseI-digested cell lysate as follows:

Individual ribosomes containing ribosome-protected mRNA fragments (rpf) are purified using sucrose-gradient fractionation; sucrose cushioning or size-exclusion chromatography.

RNA is extracted and rpfs are isolated based on their characteristic size (27-30 bp) by Tris-Borate Urea polyacrylamide gel electrophoresis.

A DNA linker is ligated to the 3'-end of each rpf and used as a primer for reverse-transcription.

The resulting single-stranded DNA is circularized and amplified by PCR to generate the RiboSeq library.

ically in their size and concentrations of macromolecules meaning that global changes in RNA levels can and will occur under certain conditions (Chen et al. (2015)). A key example of this is the global upregulation of mRNA in *xrn1* Δ cells that was previously discussed (Sun et al. (2013); Malabat et al. (2015); Woloszczuk et al. (2016)). In order to detect such global changes, an internal standard whose levels are unchanged between samples must be added (Chen et al. (2015)). This may be a series of commercially available, defined RNA sequences or, more reliably, a fixed number of cells from another organism such as *Schizosaccharomyces pombe* or *Drosophila* S2 cells (Taruttis et al. (2017)). With external standards incorporated into the library, the true library sizes can be calculated, allowing global changes to be detected. In this study, many libraries have been calibrated with fixed numbers of *S. pombe* cells. Where this has occurred it is referred to as calibration.

1.3.2 Applications of RNA-Sequencing in the study of translation.

In order to understand the role of gene expression in the yeast metabolic cycle, this study has measured translation using RNA-Seq in several ways, employing both of the library preps described above.

Ever since the earliest experiments studying translation, a principle method for isolating translating ribosomes and their associated mRNAs has been sucrose gradient fractionation (Spyrides & Lipmann (1962); Penman et al. (1963)). The process by which sucrose gradient fractionation is performed is described in detail in Section 2.6 but briefly, cells are lysed in the presence of cycloheximide and Mg^{2+} ions, which stabilize the interactions between ribosomes and the mRNAs that they translate. The lysate is then loaded onto the top of a gradient from low to high sucrose concentration and centrifuged at high velocity. This separates macromolecules based on their molecular weight and since the bulk of the mass of a translated mRNP originates

from ribosomes, it effectively separates mRNAs based on the number of ribosomes bound to them. Since the turn of the millennium, fractions from these gradients have been analyzed using microarrays and various RNA-Seq methodologies to measure the rates of translation within cells (Arava et al. (2003); Arribere & Gilbert (2013); Sterne-Weiler et al. (2013); Floor & Doudna (2016)). In this study we follow this pedigree by applying the Lexogen QuantSeq protocol to gradient-fractionated polysomes, monosomes, untranslated fractions that we refer to as “free-mRNPs” and their inputs. We refer to this technique as polysome profiling.

More recently, the Weissman lab have developed an extremely powerful technique for capturing the locations of individual ribosomes on a transcript (Ingolia et al. (2009, 2011, 2012, 2013, 2014)). This technique goes by two names in the literature, ribosome profiling and RiboSeq. To avoid confusion with polysome profiling, I will be using the latter terminology whilst discussing this study. The basic principle of RiboSeq is RNase-protection followed by size-selection (Ingolia et al. (2009, 2012, 2013)). As in polysome profiling, cells are lysed in the presence of cycloheximide and Mg^{2+} ions to stabilize ribosome-mRNA interactions and lock ribosomes in place. Next, cell lysate is incubated with RNaseI which degrades most of the mRNA in the cell, leaving only 27-30 bp “footprints” where the ribosomes are bound. These are known as ribosome-protected fragments (rpf). Whilst still associated with the rpfs, ribosomes are then isolated by sucrose gradient centrifugation, sucrose cushioning or size-exclusion chromatography and subsequently RNA fragments of 27-30 bp in size are extracted. Stranded RNA-Seq libraries are prepared from these fragments and sequenced providing a map of the locations of all ribosomes in the cell.

A limitation of both of these techniques is that although they accurately report the locations of ribosomes at their respective resolutions, they give no information

as to their velocities (Arava et al. (2003); Arribere & Gilbert (2013); Sterne-Weiler et al. (2013); Floor & Doudna (2016); Ingolia et al. (2009, 2012, 2013)). Thus a stalled ribosome gives much the same signal as one that is elongating efficiently. To overcome this issue, a modified form of RiboSeq has recently been developed by Immagina Biotech known as RiboLace. This method employs a puromycin analogue to specifically pull-down elongating ribosomes, excluding stalled ribosomes from the library and also allowing the use of much smaller amounts of input material (Clamer et al. (2017)). Unfortunately, this technique only became available in recent months so it was not possible to incorporate it into this study. However, future high-resolution studies on translation should aim to incorporate this technique into their workflow.

1.3.3 Analysis of RNA-Sequencing datasets

RNA-Seq experiments produce extremely large datasets whose analysis requires careful consideration. Depending on the experimental design, different strategies can be employed to identify statistically robust effects.

The most common experimental design for an RNA-Seq experiment is a straightforward comparison between two conditions. This kind of experiment has numerous applications, from comparing the phenotypes of a wild-type and mutant strain, to assessing the effect of a drug on a population after a single, optimized incubation period. The straightforward nature of this design lends itself to many different forms of analysis.

The most direct question to ask from such data is simply which genes are differentially expressed between two conditions. Several statistical packages have been developed with this in mind. The most famous of these are DESeq2 and EdgeR which both employ a negative-binomial distribution to determine which genes deviate sig-

nificantly in their expression patterns from the transcriptome as a whole (Love et al. (2014); Robinson et al. (2010)). This allows researchers to identify a statistically robust set of genes whose expression is dependant on the conditions that they are testing. However, as mentioned above, these packages are limited by their dependence on the assumption that the expression of most genes remains constant between conditions. This may not always hold. As of now, this study is unaware of a package that can quantify differential expression between conditions whilst accounting for global changes in gene expression but development of such a tool would be of great usefulness to the field.

An alternative way to analyze RNA-Seq datasets is to consider groups of genes with similar properties. Since a differential expression analysis will normally produce a large number of significantly changing genes. This method has the advantage of focussing one's analysis onto groups of genes that are co-regulated or exhibit consistent behaviour and it can be done in a number of ways. Firstly, groups of genes that exhibit common properties and behaviour can be identified through gene ontology (GO) analysis. This utilizes statistical packages to trawl databases of gene descriptors known as GO-terms and identify ones that are significantly enriched in one condition over another. One such package, known as GORILLA, is available online and offers two straightforward inputs, either a target list derived from significantly upregulated genes in a differential expression analysis along with a background set, or a single ranked list of genes (Eden et al. (2009)). From these, GORILLA outputs any GO-terms that have been identified as enriched in one condition over another by a pre-determined value of significance. Thus it identifies sets of genes that are likely to be differentially regulated between conditions. This is the package that has been used for GO analysis throughout this study.

Alternatively, if one already has a set of genes in mind, either based on prior analysis or results from the literature, one way to assess their behaviour is through violin plots. Violin plots present the distribution of a set of data points as a 2-dimensional shape whose width in the x-axis at a particular value of y reflects the density of data points in that region (Hintze & Nelson (1998)). In many ways they behave similarly to box and whisker plots, although they provide additional information, including whether the distribution is unimodal or not. This is an important consideration when treating related but distinct variables such as members of a GO-term as a single group. For statistical analysis, it is often useful to present Tukey's box and whisker plot on top of a violin plot (Tukey (1977)). This allows researchers to assess whether a difference in medians between groups is statistically significant by displaying the median, upper and lower quartiles and importantly, the 95 % confidence limits of each group which are represented by the notches surrounding the median. Significant changes between pairs of violins can also be identified using the non-parametric U-test (also known as Wilcoxon's rank-sum test) or by using ANOVA (analysis of variance) to compare multiple populations in parallel. Elegant examples of a hybrid violin/box and whisker plots can be found in Figures 3.12C & 3.16B.

With extreme care, tentative analyses occasionally can be performed on $n = 1$ datasets. These should generally be considered as preliminary studies to be followed up with additional replicates or complimentary techniques. However, some cautious interpretation is necessarily required to make preliminary studies worthwhile. When analysing such data, it is important to account for the expression levels of individual genes as well as the degree to which they are differentially expressed as lowly-expressed genes will be more prone to statistical noise. For this purpose, MA-plots, also known as Bland-Altman plots, can be especially useful (Bland & Altman (1986); Tukey (1977)). MA-plots display the magnitude (M) of differential expression, calculated

as the $\log_2$ fold-change in expression, against the amplitude (A) of expression levels, calculated as the mean of $\log_2$ expression in each condition. As more highly-expressed genes are less sensitive to random variation in counting, “A” gives an approximation of reliability for a degree of differential expression “M”. However, MA-plots cannot adequately account for biological variation between datasets as they contain no replicate information and so can never replace experimental replication and validation. For examples of MA-plots in this study, please refer to Figure 3.9.

An alternative experimental design is to take samples from a population over time and measure how variables interact with one another across the resulting time series. This method is particularly useful for studying time-resolved phenomena such as biological rhythms. By correlating the behaviour of a gene over the course of a cycle in two different strains or cellular fractions we can determine how predictive the behaviour under one condition is for the behaviour under another, i.e. how sensitive a gene is to perturbation in this way. One can even calculate confidence intervals for this value to determine whether a correlation is truly above or below a specific threshold for dysregulation (Fisher (1915)). In this way researchers can quickly identify genes whose expression patterns are perturbed by a treatment or mutation which can then be assessed in more detail. Correlation analyses such as these have been performed on time-resolved datasets in Figures 3.5, 4.6 & elsewhere.

All of the analysis techniques above have been utilized in this study as and when appropriate. It is hoped that this brief overview will give most readers enough familiarity to interpret the data that are presented.

1.4 Regulation of cellular metabolism by modulation of enzymatic activity.

1.4.1 Post-translational modification of proteins as a means of modulating activity

Up to this point, discussion of signalling and regulatory mechanisms in this study has been focussed primarily on control of gene expression. However, it is also common for cell signalling to directly affect the activity of existing proteins, rather than impacting on protein abundance (Reviewed in Conrad et al. (2014)).

This has a number of advantages, chiefly it is faster and more economical. Rather than discharging up to hundreds of aaRNAs and hydrolysing over twice as many molecules of GTP to synthesize a protein over a period of 1-2 min, post-translational modification of existing proteins can alter their activities over similar ranges at a fraction of the cost (Lynch & Marinov (2015); de Assis et al. (2013); Dever et al. (2016)). Phosphorylation for example can immediately increase the *in vitro* activity of pyruvate decarboxylase 1.6-fold for the cost of a single ATP molecule per copy of the enzyme (de Assis et al. (2013)). Therefore, in many cases where rapid and reversible changes in protein activity are required, evolution may have selected for regulation through modulation of protein activity rather than abundance.

Amongst the great many post-translational modifications that exist in *S. cerevisiae*, protein-phosphorylation is arguably the best studied (Reviewed in Conrad et al. (2014)). Therefore, much of this discussion will be focussed upon it, although the arguments made can apply equally well to many other modifications.

During the yeast metabolic cycle, rapid and extensive remodelling of the metabolome occurs (Tu et al. (2007)). These changes could be explained by modulation of the ac-

tivities of key metabolic enzymes rather than their synthesis and degradation. Several well studied phosphorylation cascades in *S. cerevisiae* both respond to nutrient levels and affect the activities of metabolic enzymes (Reviewed in Conrad et al. (2014)). For example, the protein kinase A pathway in *S. cerevisiae* is primarily regulated by cAMP levels, which are themselves determined by extracellular and intracellular glucose concentrations (Rolland et al. (2000)). In turn, many of the targets of protein kinase A are enzymes involved in glycolysis such as phosphofructokinase-2, fructose-1,6-bisphosphatase & pyruvate kinase (Reviewed in Conrad et al. (2014)). Likewise, signalling by the yeast AMP-activated kinase Snf1p, requires the presence of phosphorylated T210 in its activation loop. Removal of this mark is inhibited by the regulatory subunit Snf4p in the presence of ADP or AMP allowing Snf1-activity to reflect cellular energy charge (Reviewed in Conrad et al. (2014)). Snf1-signalling influences many stages of gene expression, including transcription and RNA degradation but it also targets many metabolic enzymes directly. Braun et al. (2014) identify 26 glycolytic enzymes whose phosphorylation status is dependent on Snf1p activity. One of these, Pfk2p, is a subunit of Phosphofructokinase-1 (PFK1), the rate-limiting enzyme in glycolysis, and also appears in three independent phosphoproteomics datasets, implying that it is one of Snf1p's most robust targets (Ptacek et al. (2005); Zhang et al. (2011); Bodenmiller et al. (2010)).

Whereas PKA and Snf1-signalling primarily transmit information regarding carbon sources, the TOR pathway is the primary sensor of intracellular nitrogen levels (Reviewed in Conrad et al. (2014)). Through an poorly understood mechanism, the EGO-complex, tethered to the vacuolar membrane, senses amino acid levels and in response its Gtr1p-subunit binds GTP. Gtr1p-GTP then interacts with mTORC1 (Mechanistic Target of Rapamycin Complex 1), activating it. Active mTORC1 elicits its activities through two downstream effectors Sch9p & Tap42p. Phosphorylation

of Sch9p is activatory and once active, phospho-Sch9p targets proteins involved in diverse processes from translation to autophagy, promoting cell growth at the expense of longevity (Reviewed in Conrad et al. (2014)). In contrast, Tap24p is an inhibitory regulator of PP2A family phosphatases. Upon phosphorylation of Tap24p, it binds to these proteins preventing their activity and suppressing stress-response pathways such as the cell wall integrity pathway (Reviewed in Conrad et al. (2014)).

The links between mTORC1 activity and autophagy/the endomembrane system appear to be the most critical to its role in metabolism as inhibition of mTORC1 results in changes to the metabolome reflective of a disruption to vesicular transport (Mülleder et al. (2016)). This suggests that the role of the Tor-pathway in metabolism might be less direct. Rather than activating metabolic enzymes directly to drive flux through specific pathways, perhaps Tor-signalling achieves its effects through modulation of the recycling of cellular components and providing substrates for metabolic processes.

Protein phosphorylation is by no means the only way to modulate enzymatic activity. Hundreds of other post-translational modifications have been observed *in vivo* and each one has the potential to transform a protein's functionality (Olsen & Mann (2013); Britton et al. (2011)). Many of these modifications such as acetylation, methylation, glutaminylation, O-GlcNAcylation, ADP-ribosylation along with many others are generated from substrates that are core metabolites (Sakabe et al. (2010); Messner et al. (2010); Jank et al. (2017); Fan et al. (2015)). This provides a direct link between substrate availability and metabolic activity, facilitating direct modulation of cell signalling by metabolism. This is best exemplified by the well-studied post-translational modification lysine acetylation.

As discussed in subsection 1.2.2, since acetyllysines are generated using acetyl-CoA and may be removed through consumption of NAD^+ , levels of lysine acetylation integrate information about carbon metabolism and cellular redox state (Reviewed in Fan et al. (2015)). As one of the main targets of lysine acetylation are histones, this provides a clear route for signalling from metabolism to gene expression via chromatin. However, thousands of other acetylation targets exist in *S.cerevisiae* (Henriksen et al. (2012)). For example, 37 of the 57 enzymes involved in glycolysis & gluconeogenesis and 22 of the 31 enzymes involved in small amino acid biosynthesis are known to be acetylated, providing astonishing regulatory potential over these pathways. Interestingly, the products of these pathways tend to be those that peak during OX phase of the YMC, the period when acetyl-CoA levels and histone acetylation levels also peak (Tu et al. (2007); Cai et al. (2011); Kuang et al. (2014)).

It should come as no surprise that cells are capable of regulating their metabolism through post-translational modifications and signalling cascades. However, the yeast metabolic cycle has been relatively understudied at this level, as investigations to date have focussed on gene expression and metabolism itself. Although this study only briefly touches on the role of post-translational modifications in the yeast metabolic cycle, investigation of these, in particular lysine acetylation, should be a priority for future research.

1.4.2 Allosteric modulation of glycolytic enzymes

Beyond post-translational modifications, control of metabolism can be even more direct. The inputs, intermediates and end products of metabolic pathways can feed back upon the activities of the enzymes that process them by binding to sites on these enzymes distinct from the active site and modulating their activities. This effect is

known as allostery (Motlagh et al. (2014)). Allostery is found throughout protein biology and forms a critical component of metabolic auto-regulation. Although, it has been observed in proteins of diverse origins, two of the best studied examples come from the glycolytic pathway.

The rate-limiting enzyme complex in glycolysis is PFK1 (phosphofructokinase-1). It catalyzes the first committed step in the glycolytic pathway, the conversion of fructose-6-phosphate + ATP to fructose-1,6-bisphosphate + ADP (Reviewed in Piškur & Compagno (2014)). Due to its limiting activity within the pathway, it is the node at which the most flux control can be exerted. Consequently, it is the subject of extensive allosteric modulation (Reviewed in Piškur & Compagno (2014)). First of all, PFK1 activity is closely dependent on the energy charge of cells. Specifically, its activity depends on the $\frac{AMP}{ATP}$ ratio. When cells are replete with energy, ATP levels are high allowing ATP to bind to the C-terminal domain of PFK1 and inhibit it, limiting flux through glycolysis to a rate proportionate to the energy charge of the cell. However, when cellular energy levels fall, AMP accumulates. Accumulation of AMP occurs much more rapidly than depletion of ATP as the $\frac{AMP}{ATP}$ ratio is usually incredibly small. AMP competes with ATP for binding to PFK1 and converts the enzyme to its active conformation. This allows greater flux through the pathway, regenerating ATP levels in the process, providing an autoregulatory loop that ensures glycolytic flux matches cellular energy requirements.

However, eukaryotic PFK1 also exhibits a more sensitive mode of allostery in the form of activation by fructose-2,6-bisphosphate (Reviewed in Piškur & Compagno (2014)). Fructose-2,6-bisphosphate is a potent activator of PFK1 and inhibitor of fructose-1,6-bisphosphatase, the enzyme responsible for the antagonistic reaction of fructose-1,6-bisphosphate hydrolysis. Regulation by fructose-2,6-bisphosphate is

thought to exist to supercede regulation by $\frac{AMP}{ATP}$ in response to intracellular signalling, providing eukaryotes with additional regulatory flexibility. PFK2 (phosphofructokinase-2), the enzyme responsible for production and degradation of fructose-2,6-bisphosphate, is regulated through phosphorylation by protein kinase A which shifts the balance of its opposing reactions towards accumulation of fructose-2,6-bisphosphate and stimulation of glycolysis (Yamashoji & Hess (1984)). In this way, allosteric regulation by fructose-2,6-bisphosphate allows communication between cell signalling pathways and metabolism.

A second example of allostery in glycolysis is provided by the final enzyme in the pathway, pyruvate kinase (Reviewed in Piškur & Compagno (2014)). This enzyme converts phosphoenolpyruvate + ADP to pyruvate + ATP, another irreversible reaction. Unlike eukaryotic PFK1 which integrates protein kinase A signalling into its allosteric regulation through fructose-2,6-bisphosphate, pyruvate kinase is primarily regulated by metabolites at either end of the glycolytic pathway. In mammalian cells, much like PFK1, pyruvate kinase is inhibited by ATP ensuring that glycolysis does not occur at a rate that exceeds the energy requirements of the cell (Carbonell et al. (1973)). Conversely, in both yeast and mammals, it is activated by fructose-1,6-bisphosphate, the product of PFK1 (Piškur & Compagno (2014); Carbonell et al. (1973)). By coupling the activity of this enzyme to upstream and downstream metabolic flux, allostery ensures that the beginning and end of glycolysis proceed at similar rates. This prevents accumulation or depletion of glycolytic intermediates, avoiding build-up of toxic metabolite levels on the one hand and loss of flux control through substrate depletion on the other.

Allostery provides a vital mechanism by which metabolism can be coordinated and autoregulated as well as providing opportunities for crosstalk between cell signalling

pathways and metabolism. As such it is likely to be central to nuances of temporal coordination of metabolism in biological rhythms. Likewise, when core metabolites are dysregulated, allostery is likely to result in cascading of this dysfunction across the metabolome with unpredictable consequences.

1.5 Competing models for YMC regulation

It is clear from the examples presented above that eukaryotic cells employ many diverse mechanisms to regulate their metabolism depending on the magnitude and timescale of the required change. Both regulation of protein levels and modulation of protein activity have the potential to regulate biological rhythms but determining whether either process or both is responsible for regulation is non-trivial. Both mechanisms have been shown to be regulated across circadian rhythms and metabolic cycles and disruption of either process leads to defects in both rhythms (Kuang et al. (2017, 2014); Causton et al. (2015); Edgar et al. (2012)). This makes discovery of the true identity of the underlying biological clock deeply challenging.

Regarding circadian rhythms, over the course of many years a compelling argument has been built up for a cycle driven by interlocking transcription factor feedback loops (Reviewed in Takahashi (2016), Figure 1.1). However, new evidence shows that circadian rhythms can be maintained in erythrocytes, cells which are utterly devoid of transcription (O'Neill & Reddy (2011)). Furthermore the cycles observed in erythrocytes, detected through cycling of peroxiredoxin oxidation states, are conserved across all three domains of life, far beyond the conservation of any individual transcription factor circuit (Edgar et al. (2012)).

Oscillations in peroxiredoxin over-oxidation have also been observed in the yeast

metabolic cycle which has led to the proposition that peroxiredoxin cycling is a conserved marker of all biological clocks (Causton et al. (2015)). However, although the yeast metabolic cycle is affected by the loss of peroxiredoxins, cycling is not fully dependant on them in the same way that it depends on the KAT *GCN5* and the pentose phosphate pathway enzyme *ZWF1* (Tu et al. (2007); Kuang et al. (2014)). Therefore, in the yeast metabolic cycle there must at least be parallel cycling processes able to partially compensate for the loss of peroxiredoxins. This is not to say however, that *GCN5* & *ZWF1* are necessarily key clock components either, interpretation of the loss of metabolic cycling should be done cautiously. A gene may be required for metabolic cycling for indirect and even trivial reasons. For example auxotrophic strains are believed not to undergo synchronized cycles because they fail to reach the required OD₆₀₀ for metabolic cycling (Tu et al. (2005); Alam et al. (2016), Mellor Lab - Unpublished observations). Alternatively, it may be that strains that apparently do not cycle still do so at the single-cell level but have lost the ability to synchronize across the culture. The many potential explanations for defects in the yeast metabolic cycle make interpretations of cycling phenotypes complicated.

The incredible complexity and interdependence of biological oscillators makes disentangling the core mechanics of them complicated. However, if we hope to understand these ancient systems then this is exactly what we must do. The scope for regulation in biological systems is vast and so far most research on the yeast metabolic cycle has been focussed on either end of the regulatory cascade from transcription to metabolism. Perhaps the answers we seek lie somewhere in between?

1.6 Aims and outcomes of this study.

This study aims to further our understanding of the role of gene expression in regulation of biological rhythms and to develop the yeast metabolic cycle as a model for control of gene expression under nutrient-limiting conditions. This has been achieved by assessing the effects of disruption of major cellular RNA degradation pathways on metabolic cycling alongside investigation of the behaviours of the proteome and translatoe under the conditions of the yeast metabolic cycle.

In studying these processes we find that the proteome of cycling cells remains largely constant throughout the cycle and does not reflect the observed oscillations in total and translated mRNA levels. In light of exceptionally low levels of translation during the YMC, we propose that the absence of cycling at the proteome level is a result of low protein turnover rates, which minimize cellular energy demands, and that the YMC acts to determine steady-state rather than dynamic protein levels.

In parallel, we glean insights into the mechanism behind loss of buffering in *xrn1Δ* cells and how it is tolerated through limitation of the rates of translation.

Specifically, this study asks the following questions:

1. How is metabolic cycling affected by deletion of components of the 3'→5' & 5'→3' RNA degradation pathways?
2. What impact does loss of 5'→3' RNA degradation have on cycling transcript levels?
3. How do *xrn1Δ* strains tolerate global transcript accumulation?

4. What is the effect of loss of 5'→3' RNA degradation on translation?
5. To what extent is cycling of transcripts reflected at the protein level?
6. What role does translation play in determining steady-state and dynamic protein levels during the yeast metabolic cycle?

We hope you find the conclusions interesting.

Chapter 2

Materials and Methods.

2.1 Strains and Transformations.

S. cerevisiae strains used for this study were derived from two backgrounds. For batch culture analysis, strains were derived from *BY4741* (Euroscarf). For experiments on the yeast metabolic cycle, strains were derived from the prototrophic background *CENPK113-7D* used in Tu et al. (2005) (Euroscarf). Strains derived from both backgrounds are listed in Table 2.1.

Deletion and tagging strains were generated according to previously described molecular genetics techniques (Longtine et al. (1998)). Briefly, primers containing 40 bp homology arms flanking the DNA sequence to be deleted were used to amplify the selection marker (and tag where appropriate) from pFA6a-KANMX6, pFA6a-hphMX6, pFA6a-NATMX6 & pFA6a-3xFLAG-KANMX6. The resulting cassettes were transformed into chemically competent *S.cerevisiae* cells according the lithium acetate procedure (Gietz et al. (1992)) and transformed cells were plated onto YPDA+G418, YPDA+nourseothricin or YPDA+hygromycin as appropriate. Successful transformants were verified by diagnostic PCR using Taq polymerase.

To insert the OsTIR1 E3-ligase into *CENPK113-7D*, the strain was first rendered auxotrophic for uracil by insertion of a hphMX6 cassette between positions 688-764

Strain	Parent	Genotype	Origin
BY4741	S288C	<i>MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0</i>	Euroscarf
<i>BY4741 xrn1Δ</i>	<i>BY4741</i>	<i>xrn1Δ::KANMX6</i>	Euroscarf
<i>BY4741 rrp6Δ</i>	<i>BY4741</i>	<i>rrp6Δ::KANMX6</i>	Euroscarf
<i>BY4741 rpl39Δ</i>	<i>BY4741</i>	<i>rpl39Δ::KANMX6</i>	Euroscarf
<i>BY4741 bem1Δ</i>	<i>BY4741</i>	<i>bem1Δ::KANMX6</i>	Euroscarf
<i>CENPK113-7D</i>	<i>ENY.WA-1A</i> & <i>MC996A</i>	<i>MATα URA3 HIS3 LEU2 TRP1 MAL2-8c SUC2</i>	Euroscarf
<i>CENPK113-7D rpb3-FLAG</i>	<i>CENPK113-7D</i>	<i>rpb3-3xFLAG::KANMX6</i>	Xi. S
<i>CENPK113-7D xrn1Δ</i>	<i>CENPK113-7D</i>	<i>xrn1Δ::KANMX6</i>	This study
<i>CENPK113-7D rrp6Δ</i>	<i>CENPK113-7D</i>	<i>rrp6Δ::KANMX6</i>	Townley. A
<i>CENPK113-7D ura3Δ(688-764)::hphMX6</i>	<i>CENPK113-7D</i>	<i>ura3Δ(688-764)</i>	Townley. A
<i>CENPK113-7D OsTIR1</i>	<i>CENPK113-7D ura3Δ(688-764)::hphMX6</i>	<i>URA3-pADH1-OsTIR1-ura3Δ(688-764)::hphMX6</i>	Townley. A
<i>CENPK113-7D OsTIR1 ski7Δ</i>	<i>URA3-pADH1-OsTIR1-ura3Δ(688-764)::hphMX6</i>	<i>URA3-pADH1-OsTIR1-ura3Δ(688-764)::hphMX6 ski7Δ::NATMX6</i>	This study
<i>S. pombe</i>		<i>u+; ura4Δ18; leu1-32; ade6-M216</i>	Wittman. S

Table 2.1: Genotypes of strains used in this study.

of the *URA3* gene. A DNA cassette containing pADH1-OsTIR1 was generated by digestion of pMHK728 (Nishimura & Kanemaki (2014)) with *Stu*I (1.5 hours, 37 °C) followed by gel extraction. This was transformed into *CENPK113-7D ura3Δ(688-764)* to restore uracil prototrophy and successful transformants were verified by diagnostic PCR.

2.2 Yeast media & growth conditions.

2.2.1 Growth on solid media.

S. cerevisiae strains were streaked onto YPDA (1 % Yeast Extract (Difco), 2 % Bactopeptone, 2 % glucose, 2 % agar (Difco)) from glycerol stocks and incubated at 30 °C until visible colonies emerged.

2.2.2 Batch culture.

Colonies were picked from YPDA plates, resuspended in 5 ml YPD (1 % Yeast Extract (Difco), 2 % Bactopeptone, 2 % glucose) and grown overnight. The following day, fresh flasks of YPD were inoculated at $OD_{600} = 0.2$ and cultured at 30 °C with shaking (160 rpm) to $OD_{600} = 0.6$. *S. pombe* was treated in the same manner except grown in YES media (0.5 % Yeast Extract, 3 % glucose, 0.0225 % adenine, 0.0225 % histidine, 0.0225 % leucine, 0.0225 % uracil, 0.0225 % lysine hydrochloride).

2.2.3 Chemostat culture.

Colonies were picked from YPDA plates, resuspended in 10 ml YPD and grown overnight. The following day, this culture was inoculated into 1.1 l of pH 3.5 YMC media (5 g/L $(NH_4)_2SO_4$; 2 g/L KH_2PO_4 ; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 0.1 g/L $CaCl_2 \cdot 2H_2O$;

0.02 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.01 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.005 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.001 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; 1 g/L yeast extract; 10 g/L glucose; 0.5 mL/L 70% H_2SO_4 ; and 0.5 mL/L Antifoam 204 (Sigma)) in a Bioflo320 system (Eppendorf) (30 °C, 1000 rpm, 1 l/min air). Cultures were grown at constant pH and aeration to maximal density ($\text{OD}_{600} = 16.0$) then starved for >4 hours. After starvation the culture was fed under chemostatic conditions at a dilution rate of 0.09 /hr to induce metabolic cycling.

For most applications, 1 ml samples were harvested from the chamber via the sampling port. However, for RiboSeq experiments, 50 ml of cells was aspirated via the sampling port using a syringe and harvested by vacuum filtration.

2.3 Logarithmic growth rate analysis.

Logarithmic growth curves were recorded using a Bioscreen C system (GrowthCurvesUSA). Briefly, cultures grown overnight in YPD were diluted to $\text{OD}_{600} = 0.2$ in the media conditions described in the figure legend. 200 μl of culture was added to each well of a honeycomb microplate and cultures were grown in the Bioscreen C at 30 °C with shaking. OD_{600} measurements were made every 20 minutes.

2.4 Coomassie gels and western blotting.

2.4.1 Puromycin incorporation assays.

Unless otherwise indicated, cultures to be treated with puromycin were harvested and resuspended in 1 ml conditioned media to which was added SDS (0.003 %) & puromycin (400 $\mu\text{g}/\text{ml}$). Puromycylation reactions were allowed to proceed for 5 minutes after which time cells were harvested by centrifugation (16,100 rcf, 15 seconds), supernatant was removed and cells were flash-frozen and stored at -20 °C. Control samples either additionally contained 100 $\mu\text{g}/\text{ml}$ cycloheximide or omitted puromycin

as indicated.

2.4.2 Whole cell extracts and SDS-PAGE analysis.

Whole cell extracts were prepared from cell pellets by vortexing (3 min) in 60 μ l 1 M DTT, 300 μ l 8 M urea, 240 μ l 2x loading buffer (100 mM Tris-HCl pH 6.8, 20 % glycerol, 4 % SDS) & 100 μ l acid-washed glass beads. Cells were then boiled for 5 min and protein concentrations were tested with 1:1 Bradford Reagent:water at A_{595} .

An appropriate volume of each sample was loaded and run on a 15 % polyacrylamide gel and the gel was either stained with Coomassie Reagent or processed for Western Blotting (below).

2.4.3 Western blotting.

Samples were transferred from the polyacrylamide gel onto nitrocellulose membranes using a semi-dry transfer (15 V, 45 minutes). Membranes were reversibly stained with PonceauS and imaged using white light photography before destaining and blocking with TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % Tween-20) +5 % BSA for 1 hour. Membranes were then treated with primary antibody (mouse anti-puromycin clone 12D10 (Millipore) - 1:5000) in TBS-T +2.5 % BSA for 1 hour, washed for 3x10 min in TBS-T then treated with HRP-conjugated anti-mouse secondary antibody (1:4000) in TBS-T +2.5 % BSA for 45 min. Membranes were washed for an additional 3x10 min in TBS-T and visualized by chemiluminescence assay (Pierce) using x-ray film.

2.5 Mass spectrometry.

2.5.1 Proteomic mass spectra - Sample lysis and preparation.

Cultures for proteomics analysis by mass spectrometry were harvested and pellets were flash-frozen in $N_{2(l)}$. Pellets were thawed and washed in 1x PBS (2x3 min, 2300 rcf, 4 °C). Supernatant was removed and pellets were resuspended in 1 ml Proteome Lysis Buffer (50 mM Tris-HCl pH 8.0, 8 M Urea, 75 mM NaCl, 100 mM sodium butyrate, 1x cOmplete protease inhibitor cocktail (+EDTA) Roche, 1x PhosSTOP Roche (TMT-labelling only)) with 300 μ l acid-washed glass beads. Mechanical lysis was performed using a MagNA Lyzer (Roche - 8 cycles, 6000 rpm, 1 min on 2 min off, 4 °C) and protein concentrations were measured using the Pierce Microplate BCA Assay (ThermoFisher).

2.5.2 Unlabelled mass spectra - Generation and analysis.

For label-free peptide mass-spectra, protein extracts generated above were sent to the Mohammed Lab for tryptic digest, extraction, mass spectrometry and preliminary data analysis.

Further data analysis was performed using custom R scripts using normalized intensity values.

2.5.3 TMT-labelled mass spectra - Generation and analysis.

For label-free peptide mass-spectra, protein extracts generated above were sent to the Kessler Lab for tryptic digest, extraction, TMT-labelling, mass spectrometry and preliminary data analysis.

Further data analysis was performed using custom R scripts by Charlotte George.

2.5.4 Metabolite mass spectra - Sample preparation and analysis.

Cultures for metabolomic analysis were harvested, washed in 1 ml deionized H₂O, supernatant was removed and pellets were flash-frozen in N_{2(l)}.

Frozen pellets were sent to the McCullagh Lab for extraction in 50 % methanol and mass-spectrometric analysis.

2.6 Sucrose gradient fractionation.

Cells for sucrose-gradient fractionation were harvested and pellets immediately re-suspended in 400 μ l Buffer A (20 mM HEPES, 50 mM KCl, 10 mM MgCl₂, 1 % Triton-X100, 100 μ g/ml cycloheximide, 1x cOmplete Protease inhibitors (-EDTA) Roche) with 500 μ l acid-washed glass beads. Mechanical lysis was performed using a MagNA Lyzer (Roche - 8 cycles, 6000 rpm, 1 min on 2 min off, 4 °C). Lysates were clarified (16,100 rcf, 10 min, 4 °C) and total protein concentrations were measured using the Pierce Microplate BCA Assay (ThermoFisher). 2 mg of lysate was then loaded onto the top of 12 ml 7-47 % gradients of sucrose in Buffer B (20 mM HEPES, 50 mM KCl, 10 mM MgCl₂, 100 μ g/ml cycloheximide). Gradients were centrifuged at 18,800 rcf, 4 °C for 2 hours in a Beckman SW41Ti swinging bucket rotor then fractionated using a Biocomp Gradient Station. Fractions were pooled to create separate “free mRNP”, monosomal & polysomal fractions for RNA extraction. For calibrated QuantSeq experiments, the stated mass of purified *S. pombe* mRNA was added to the fraction pools at this stage.

No freeze thaw cycles were permitted at any stage of this procedure prior to pooling of fractions.

2.7 QuantSeq 3'-end PolyA+ Sequencing (Lexogen).

2.7.1 Library preparation.

RNA was extracted from samples using a standard hot acid-phenol extraction or in the case of YMC sucrose gradient fractions, a miRNEasy kit (Qiagen).

Libraries were generated from 1 μ g purified RNA according to the QuantSeq library prep protocol (Lexogen), quantified using an Agilent Bioanalyser 2100, diluted to 200 pM, pooled and loaded onto an Ion PITM Chip (ThermoFisher) using the Ion Torrent Chef (ThermoFisher) then sequenced using the Ion PI Hi-Q sequencing 200 Kit on the Ion Proton Sequencer (ThermoFisher).

2.7.2 Alignment and counts table generation.

.fastq files generated by the Ion Proton Sequencer (ThermoFisher) were aligned and processed using the following “Bash” script which was generated for this study. All required software was installed prior to running the commands:

The script performs sequential STAR alignment; extraction of uniquely-aligning reads and conversion to .bed format for a set of files described in by the barcodes argument.

```
# Set the Shebang.  
#!/bin/bash
```

```

#Set arguments.
input_prefix=$1
output_folder=$2
barcodes=$3
STARfiles=$4

#Safety check (this script burns its inputs as you run it.)
echo "WARNING! HAVE YOU BACKED UP YOUR .FASTQ FILES ON THE HARD DRIVES?
THIS SCRIPT WILL BURN INPUT FILES AS IT RUNS TO SAVE MEMORY. MAKE
SURE THESE AREN'T YOUR ONLY COPIES!"
echo -n "Have you backed up your input files?"
read emergencybrake

if [ $emergencybrake = "Yes" ]
then

# Command line variables will be called as "input_prefix", "
output_folder", "barcodes", "STARfiles"

echo "Input location and prefix: $input_prefix"
echo "Output location: $output_folder"
echo "barcodes file: $barcodes"
echo "STAR indices etc: $STARfiles"
echo ""
echo -n "Are these correct and do you want to proceed?"
read file_check

echo "Ok then, let's go!"
echo ""
if [ $file_check = "Yes" ]
then

#Making directories and internal variables for loop.
mkdir -p $output_folder
barpos=1
barnum=$(wc -l < $barcodes) #Get number of rows in barcodes file ("<"
ensures only the number and not the file name are printed).
echo "Barcode file row: $barpos"
echo "Total Barcode file rows: $barnum"
until [ $barpos -gt $barnum ]
do
#Setting barcode and sample name based on barcodes file.
barcode=$(awk -v variable=$barpos 'BEGIN { FS = "[ \\t]+" } ; NR ==
variable {print $1}' $barcodes) #Have to define barpos as "
variable" to get it to work in awk. NR means row number
sample=$(awk -v variable=$barpos 'BEGIN { FS = "[ \\t]+" } ; NR ==
variable {print $2}' $barcodes)

#Creating input and output file names to run in STAR.
input_file="${input_prefix}_${barcode}_${sample}.fastq"
output_file="${output_folder}/${sample}_${barcode}_"

#Run STAR.
echo "Running STAR on $sample"

```



```

STAR --runThreadN 8 --genomeDir $STARfiles --readFilesIn $input_file --
    outFilterType BySJout --outFilterMultimapNmax 20 --
    alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --
    outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.08 --
    alignIntronMin 13 --alignIntronMax 2482 --outFileNamePrefix
    $output_file

#Delete .fastq file to make space for new files.
rm $input_file

#Extract unique reads.
echo "extracting unique reads."
mkdir -p "${output_folder}/Unique"
unique_file="${output_folder}/Unique/Unique-${sample}-${barcode}"
samtools view -Sbq 20 "${output_file}Aligned.out.sam" > "$unique_file.
bam"

#Delete STAR output to make space (Keeps log information)
rm "${output_file}Aligned.out.sam"

#Make .bed file
echo "Converting to .bed format"
bedtools bamtobed -i "$unique_file.bam" > "$unique_file.bed"

#Delete unique-reads.bam
rm "$unique_file.bam"

echo "Finished processing $sample"
#Move to next barcode row.
let "barpos=barpos+1"
done
fi
fi

```

The output .bed files were used to generate counts tables using a custom R script. Windows for counts tables spanned 400 bp upstream of, to 100 bp downstream of, the 3'-ends of the TIF-Seq annotations from Pelechano et al. (2013).

2.7.3 Differential expression analysis.

For direct comparison between two datasets, differential expression analysis was performed using a custom R script employing the DESeq2 package for statistical analysis (Love et al. (2014)).

For comparison of differential ribosome occupancies, a package was required that could account for changes in the input mRNA libraries. Therefore, analysis was performed using a separate custom R script based around the Xtail package which was designed for this purpose (Xiao et al. (2016)).

2.7.4 Gene ontology analysis.

Gene ontology analysis was performed using the GORILLA webpage developed by Eden et al. (2009). Inputs consisted of a list of genes significantly upregulated according to DESeq2 or Xtail as appropriate. These were compared to a background set of all genes passed through the DESeq2/Xtail pipeline. Analyses were performed for Process, Function and Component GO-terms and significantly-enriched terms are presented in tabular format.

2.8 RiboSeq

2.8.1 Library preparation.

RiboSeq libraries were prepared from approximately 0.6 g of flash frozen *S. cerevisiae* cells harvested by vacuum filtration combined with flash frozen, vacuum-filtered *S. pombe* cells grown under similar conditions at ratio of 1:5. Cells were cryogenically lysed using a Retsch Mixer Mill (6 cycles, 3 min on, 3 min off, 15 Hz) and resuspended in RiboSeq lysis Buffer (20 mM Tris-HCl pH 8.0, 140 mM KCl, 1.5 mM MgCl₂, 100 µg/ml cycloheximide, 1 % Triton-X100).

Library preparation was performed according to the Nature Protocols paper Ingolia et al. (2012) with the following modification. In place of monosome extraction by sucrose cushioning or sucrose gradient fractionation, monosomes were extracted by

size-exclusion chromatography using S-400 columns (GE Healthcare). This has been shown to give similar performance to sucrose cushioning (Aeschmann et al. (2015)). Small RNA sequencing was performed on a NextSeq500 platform (Illumina) by the Harvard Medical School Biopolymers Facility.

2.8.2 Alignment and Counts table generation.

Alignment of RiboSeq libraries was performed using the following generalized commands in the Linux Terminal. All required software was installed prior to running the commands:

Remove Adaptors:

```
cutadapt -q 20 -a ATCTCGIATGCCGTCTTCTGCTTG -m 20 -o [output_file_name]  
XXX.fastq.bz2 [input_file_name]AAA.fastq.bz2
```

Remove sequences that align to rDNA:

```
bowtie2 -t --un-bz2 rDNA_Alignments/[output_file_name]YYY_no-ribosomals.  
fastq.bz2 -x Indexing_files_SC_and_SP/rRNA_SC_and_SP -U [  
input_file_name]XXX.fastq.bz2 -S /rDNA_alignments/[output_file_name  
]YYY_ribosomals.bam
```

Align remaining reads to genome using Tophat:

```
tophat -N 1 -I 2000 -g 5 -G Indexing_files_SC_and_SP/sc_and_sp.gtf --no-  
novel-juncs -o Alignments/tophat-out_[output_folder]ZZZ/  
Indexing_files_SC_and_SP/SC_and_SP rDNA_Alignments/[input_file]  
YYY_no-ribosomals.fastq.bz2
```

Extract unique reads:

```
samtools view -bq 49 Alignments/tophat-out_[input_folder_]ZZZ/  
accepted_hits.bam > Uniquely_aligned_reads/[output_file_name]UUU.bam
```

Counts tables, metagenes and heatmaps were generated from the resulting .bam files using custom R scripts. Transcript and open reading frame annotation data was obtained from .gff files available at “<https://www.ensembl.org/>”.

Chapter 3

What is the role of RNA turnover in the Yeast Metabolic Cycle

3.1 Results

3.1.1 Phenotypic consequences of disrupting RNA turnover on metabolic cycling

It is well established that transcript levels cycle across the YMC (Tu et al. (2005); Kuang et al. (2014); Machné & Murray (2012)). However, it is still unclear what the significance of cycling transcripts is and how transcript cycling is achieved. Steady-state RNA levels are determined by their relative rates of synthesis and degradation. Therefore, changes in transcript levels can be achieved through the regulation of either process (Shalem et al. (2008)). To date however, studies have primarily focussed on chromatin and transcription (Tu et al. (2005); Kuang et al. (2014, 2017); Rao & Pellegrini (2011); Machné & Murray (2012)). In order to try to determine the relative importance of RNA degradation during the yeast metabolic cycle, deletions of the key RNA degradation enzymes *XRN1*, *RRP6* & *SKI7* were created and their phenotypes under cycling conditions were assessed (Figure 3.1).

Unexpectedly, despite the creation of severe disruptions to RNA degradation, including the complete abolition of cytoplasmic 5'->3' exonuclease activity through deletion of *XRN1*, only very mild effects on the YMC were observed. All the mutants

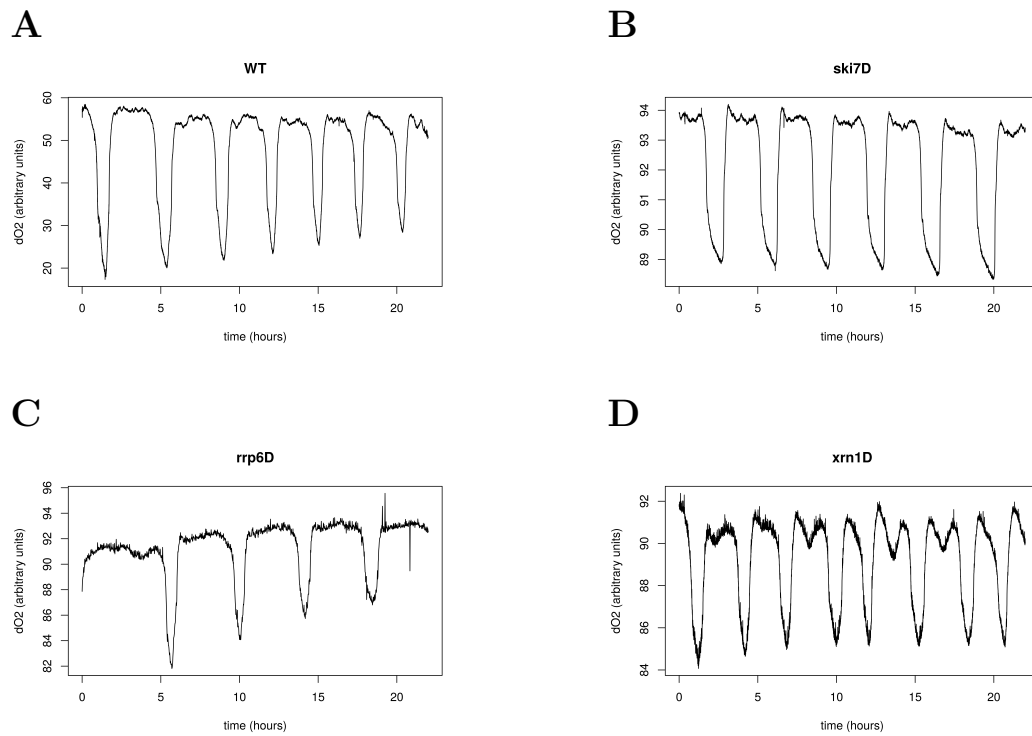


Figure 3.1: Deletion of key RNA degradation factors has little effect on metabolic cycling.

A-D - Representative dO₂% traces for RNA decay mutants in the YMC. dO₂% traces for *CENPK113-7D rpb3-FLAG* (A), *CENPK113-7D ski7Δ* (B), *CENPK113-7D rrp6Δ* (C) & *CENPK113-7D xrn1Δ* (D) over a 22 hour period with a feeding rate of 1.5 ml/min. (A n = 15, B n = 1, C n = 2, D n = 3)

tested maintained robust and stable metabolic cycles, although *xrn1* Δ (Figure 3.1D) exhibited an unusual cycle shape and *rrp6* Δ (Figure 3.1C) had an increased cycle period (from 3.25 hour to 4.25). *ski7* Δ exhibited a completely wild-type metabolic cycle over the period of observation (Figure 3.1A & B).

Although the observed phenotypes are mild, they hint at potentially interesting biology. The *xrn1* Δ metabolic cycle is characterized by a dip in dO₂% concentrations in mid-RC phase which could be interpreted as an aborted attempt to enter a premature OX phase. In contrast, the longer period of the *rrp6* Δ strain is indicative of difficulties in entering OX phase (or HOC). However, compared to some of the phenotypes that have been published in the past, both of these are relatively mild (Kuang et al. (2017); Cai et al. (2011); Kuang et al. (2014); Causton et al. (2015)) meaning that any defects in metabolic cycling in *rrp6* Δ & *xrn1* Δ cells are relatively mild, although some of these could be due to loss of synchronization, rather than loss of metabolic cycling *per-se*.

Despite the central positioning of the deleted factors in RNA degradation pathways, it was proposed that the mutations that were made were not severe enough to disrupt global RNA turnover. For example, it is possible that the two major cytoplasmic RNA turnover pathways are redundant in the YMC, a scenario which could be investigated by subjecting cells deficient for both *XRN1* & *SKI7* activity to metabolic cycling conditions. Alternatively, it could be that core exosome activity is critical for metabolic cycling but peripheral subunits such as *RRP6* & *SKI7* are not. Examining this possibility would require disruption of *DIS3* catalytic activity. Unfortunately, both the *xrn1* Δ *ski7* Δ double mutant strain and the *dis3* Δ strain are inviable. Therefore, the auxin degon system was utilized as a means to transiently deplete these factors from cycling cells.

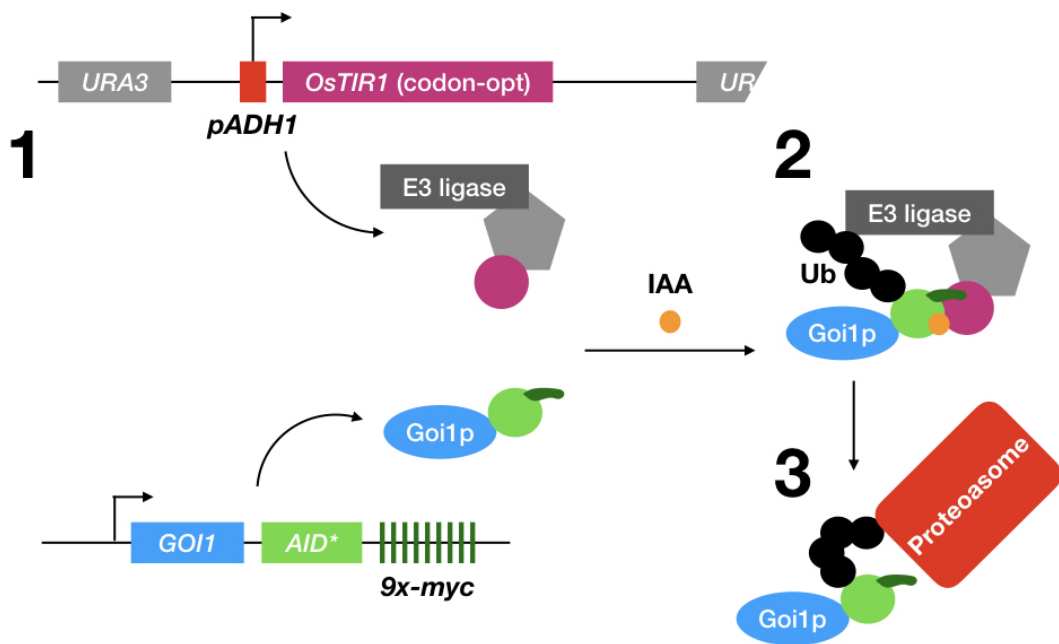


Figure 3.2: Description of the auxin degron system in *S. cerevisiae*.

1 Background strains compatible with the auxin degron system have a cassette encoding the F-box protein Tir1p from *Oryza sativa* under an *ADH1* promotor inserted into the *URA3* locus. Tir1p is expressed at high levels in these cells and is incorporated into endogenous E3-ligases.

1, 2&3 The protein to be depleted is tagged with a truncated AID-domain from *A. thaliana* by genomic insertion (**1**) and upon addition of IAA, the AID domain binds to Tir1p resulting in ubiquitylation and degradation of the target protein (**2&3**). Additional tags such as 9xmyc can be attached to the protein of interest along with AID to facilitate monitoring of degradation rates and efficiencies.

The auxin degron system developed by Nishimura & Kanemaki (Nishimura & Kanemaki (2014); Nishimura et al. (2009)) and developed further by Morawska & Ulrich (2013) uses components of a plant hormone signalling pathway to rapidly deplete tagged proteins from the cell upon addition of indole-3-acetic acid (IAA) (Figure 3.2). Since the YMC is exquisitely sensitive to changes in media composition, the first concern when using it was to ensure that any changes in cycling caused by IAA itself were characterized and accounted for. Therefore, IAA was added to the chamber and feed bottle of cycling *CENPK113-7D OsTIR1* cells to a concentration of 500 μ M, as was used in Nishimura et al. (2009), and the effect on the dO_2 % trace was recorded

(Figure 3.3A).

Upon IAA addition, cells in RC phase immediately induced a premature OX phase and during continuous feeding with IAA-supplemented media, cells persistently exhibited a shortened metabolic cycle (Figure 3.3A). Therefore, it appears that IAA promotes OX phase induction in cycling cells. Unfortunately, this limits the applicability of the auxin degreen system in the YMC as it means that small changes in cycling phenotype due to protein depletion are indistinguishable from the effect of IAA on the background strain. However, since our IAA stock solution was dissolved in 1 M NaOH, it remained possible that the sudden pH change caused by addition of the vehicle was the source of the observed OX phase induction. If this were the case it might be possible to prevent wild-type cells from responding to treatment by changing the vehicle. To investigate this, equal volumes of 1 M NaOH to those used in Figure 3.3A were added to the chamber and feed bottle of cycling *CENPK113-7D* *OsTIR1* cells (Figure 3.3B). An equivalent concentration of NaOH alone was unable to induce premature OX phases or shorten the period of the metabolic cycle arguing that these events are caused by IAA itself.

Intrigued by the effect of IAA on the wild-type metabolic cycle, we decided to investigate it further. As well as its role as a plant hormone, IAA is notable in biology as an important tryptophan degradation product (Rao et al. (2010)). Therefore, an intriguing mechanism for the effect of IAA on cycling might be that it mimics a high rate of cellular tryptophan catabolism or high levels of tryptophan itself. If this were the case then one might expect addition of a pulse of tryptophan to the media to mimic that of IAA. However, the effect of addition of tryptophan to cycling cells is very different to that of IAA (Figure 3.3C). Addition of tryptophan seems to cause abortion of the subsequent cycle in mid-OX phase, although later cycles are

unaffected. This could imply that aromatic amino acids are repressive to the high oxygen consumption phases of the YMC. Consistent with this, Tu et al. (2007) show that tryptophan levels peak in RC phase, positively associating this amino acid with low oxygen consumption.

Since the effect of IAA on cycling cells appears to be separate from general tryptophan metabolism, alternative mechanisms of action must be considered. The most commonly reported class of OX phase inducing compounds are *S. cerevisiae* carbon sources such as ethanol and acetic acid (Cai et al. (2011); Kuang et al. (2017)). Therefore, given the structural similarity between IAA and acetic acid, a simple explanation for premature OX phase induction might be that cells can utilize IAA as a carbon source in the same way that they would glucose or ethanol. If IAA could be metabolized efficiently in this way then it would be sufficient to drive high oxygen consumption rates in starved cells. However, this appears not to be the case (Figure 3.3D) so it must be concluded that IAA is not an efficient carbon source for *S. cerevisiae*. Therefore, treating cells with IAA is not simply accelerating the YMC by increasing the effective feeding rate.

Recent studies have demonstrated that *S. cerevisiae* both synthesizes and responds to IAA (Prusty et al. (2004); Rao et al. (2010); Liu et al. (2016)). Since the two most likely indirect explanations for the effect of IAA on metabolic cycling were ruled out, the possibility that IAA is a YMC regulatory signalling molecule warranted investigation. For IAA to be an endogenous signalling molecule under YMC conditions it must be present at detectable levels in the culture media and its levels might also be expected to fluctuate as the YMC progresses. Preliminary data appears to be consistent with this narrative (Figure 3.3E). IAA is detectable above background levels in media taken from cycling cells and levels appear to peak just prior to the initiation

of OX phase.

The possibility that IAA acts as a signal for triggering OX phase is tantalizing and certainly warrants investigating further. However, it is problematic for experiments that use the auxin degron to study the YMC. Despite this, attempts were made to generate a *dis3-AID*-9xmyc* strain and an *ski7Δ xrn1-AID*-9xmyc* strain to determine whether either loss of core exosome activity or simultaneous loss of 5'→3' and 3'→5' exonuclease activity would induce cycling defects large enough to overcome the changes caused by IAA itself. However, neither strain could be generated within the duration of this study. It is not uncommon for the fusion of large tags to proteins to cause partial or complete loss-of-function which might explain why these strains could not be made. Therefore, future studies might consider using alternative genetic tools to induce the desired mutations. For example, inducible promoters such as pCUP or TET-off. These would have the downsides of over-expressing the gene of interest when the promoter is active and slower response times as they regulate expression at the level of mRNA rather than protein. However, they should not affect the protein sequence avoiding the risk of loss of protein function. Furthermore, they are induced by alternative molecules to IAA, and so could be screened to find one that does not affect metabolic cycling in WT cells.

3.1.2 The buffering defects of *xrn1Δ* cells extend to the YMC.

Although this study was unable to abolish all RNA degradation pathways during the YMC, the differences in phenotype that were observed when disrupting individual degradation pathways provide exciting avenues of study. In particular, the phenotype of *xrn1Δ* cells during the YMC was intriguing. *XRN1* has been the subject of a surge in interest in recent years due to its role in buffering of gene expression (Sun

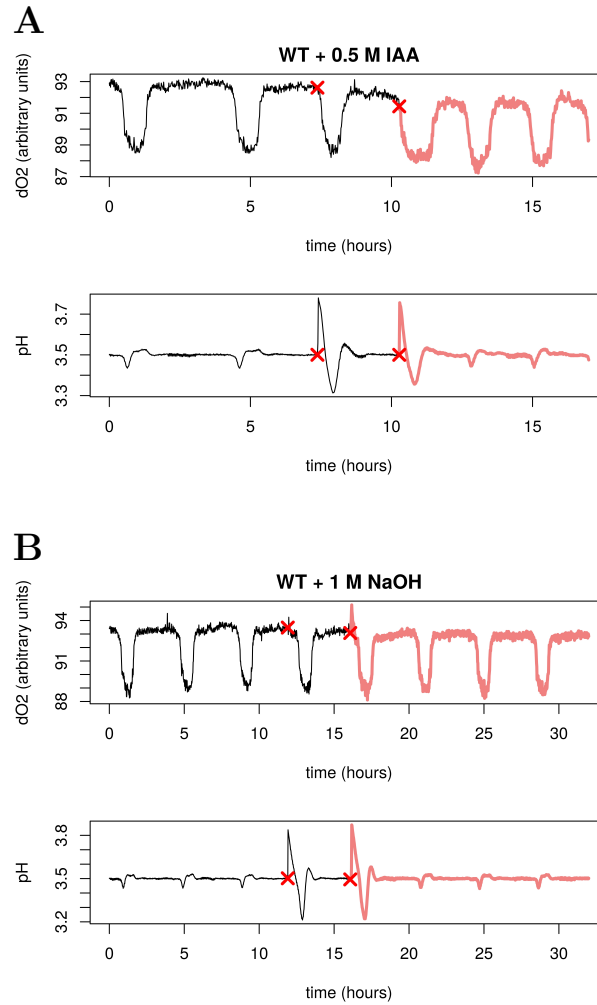


Figure 3.3: IAA disrupts metabolic cycling in non AID-tagged strains

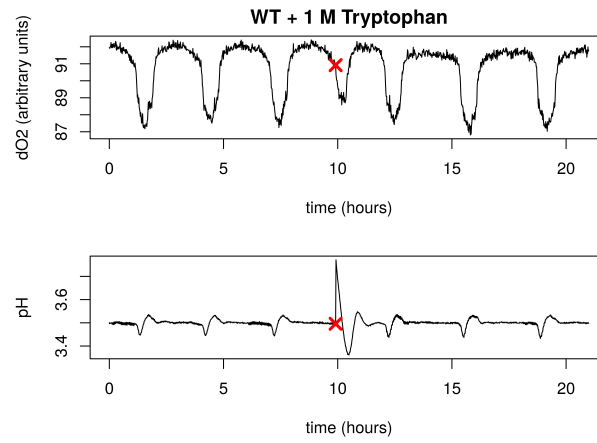
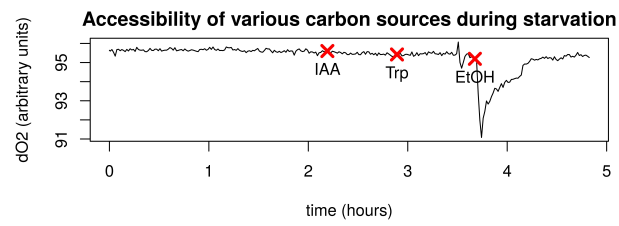
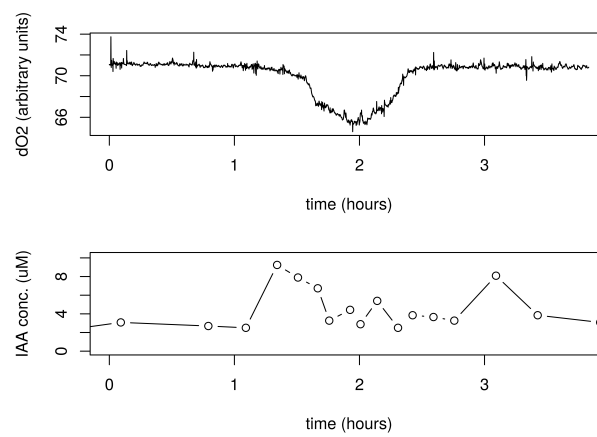
A - 500 μ M IAA causes premature induction of OX phase in untaged cells. dO₂% trace of *CENPK113-7D OsTIR1* cells in a 1.1 l YMC culture. The red crosses indicate time points where 1ml of 500 mM IAA in 1 M NaOH was added directly to the chamber. The coral lines indicate where the feed media was supplemented with 500 μ M IAA (n = 1).

B - Premature OX phase induction is not caused by 1 mM NaOH dO₂% trace of *CENPK113-7D OsTIR1* cells in a 1.1 l YMC culture. The red crosses indicate time points where 1 ml of 1 M NaOH was added directly to the chamber. The coral lines indicate where the feed media was supplemented with 1 mM NaOH (n = 1).

C - Premature OX phase induction is not triggered by metabolism of tryptophan. dO₂% trace of *CENPK113-7D OsTIR1* cells in a 1.1 l YMC culture. The red cross indicates where 1ml of 500 mM tryptophan in 1 M NaOH was added directly to the chamber (n = 1).

D - IAA and tryptophan cannot be used as carbon sources by chemostat-grown *S. cerevisiae*. Feeding of cycling *CENPK113-7D OsTIR1* cells was halted. The culture was allowed to consume all available carbon and reach dO₂-saturation. Then 1 ml of either 500 mM IAA, tryptophan or ethanol respectively, all in 1 M NaOH, were separately added to the media at the times indicated. Only ethanol was able to induce O₂-consumption (n = 1).

E - IAA is produced at low levels during the YMC. 1 ml samples of YMC media were harvested from cycling *CENPK113-7D rpb3-FLAG* cells. The media was clarified by centrifugation (16,100 rcf, 15 sec, 4 °C) and IAA levels were quantified using Salkowski's reagent. Sterile, unconditioned YMC media was also measured to provide a background absorbance. Background-subtracted IAA concentrations were calculated and are plotted alongside the dO₂% trace over the sampling period (n = 1).

C**D****E**

et al. (2013); Sinturel et al. (2015); Haimovich et al. (2013); van Dijk et al. (2011); Braun et al. (2014)). Several studies have found that total mRNA levels in *xrn1Δ* cells are elevated approximately 1.9-fold relative to wild-type (Sun et al. (2013); Malabat et al. (2015); Woloszczuk et al. (2016)) demonstrating a failure to couple mRNA synthesis and decay rates. The Cramer lab show that this phenotype is unique to *xrn1Δ* (Sun et al. (2013)). Whereas the deletion of most decay factors leads to reduction of both mRNA decay and synthesis rates, *xrn1Δ* exhibits increased synthesis rates of most mRNAs when deleted.

However, in the *xrn1Δ* mutant, not all mRNAs are stabilized equally. Previous work in the Mellor lab has shown that genes involved in transcription, chromatin remodelling and the cell cycle are preferentially stabilized in *xrn1Δ*, whereas ribosomal protein gene expression remains completely unaffected (Woloszczuk et al. (2016) & Figure 3.4). Since the ribosomal protein genes comprise a large fraction of the OX phase gene cluster, this begged the question of whether different YMC phases would be differentially affected by the loss of *XRN1*. To investigate this, 1 ml samples were taken from a cycling *xrn1Δ* culture, spiked with a fixed volume of *S. pombe* cell suspension, processed for QuantSeq and sequenced on an Ion Proton (ThermoFisher). The *xrn1Δ* cycle was compared to an existing QuantSeq dataset from wild-type cells (Shidong Xi - unpublished data). The time points taken for analysis are displayed in Figure 3.5A.

Deletion of *XRN1* has a number of effects on the YMC. The most immediately obvious is that, as in log-phase, *xrn1Δ* cells have a global increase in mRNA & ncRNA levels (Figure 3.5B - “All genes” & “non-coding transcripts”). However, the behaviours of individual clusters are also interesting. The OX genes seem to be the best coordinated between *xrn1Δ* & wild-type. They show only a small global increase

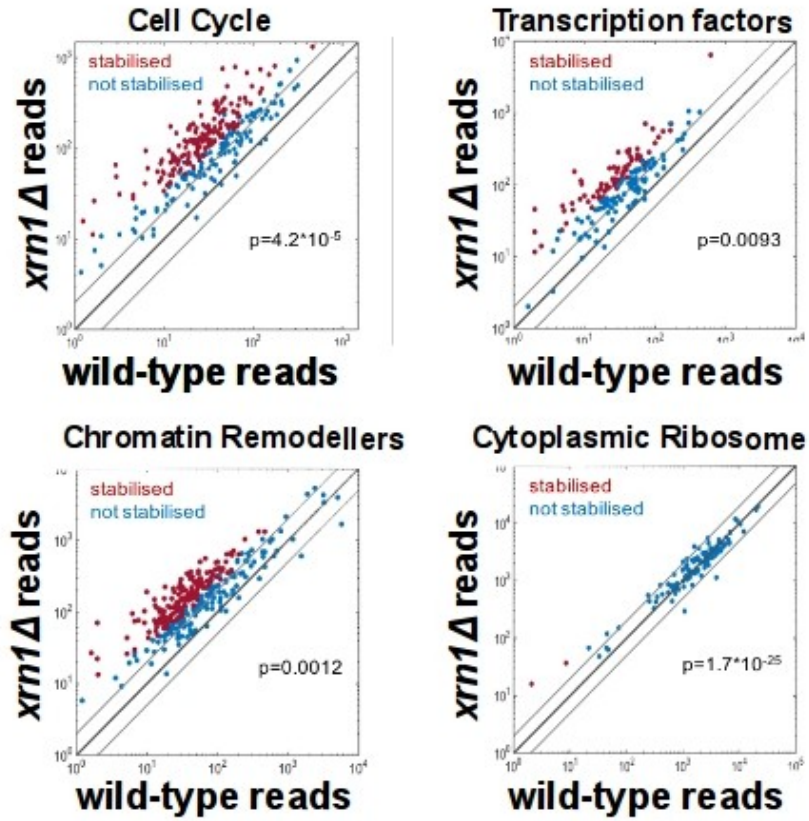


Figure 3.4: Preferential stabilization of transcription and cell-cycle associated genes in *xrn1*Δ.

(Extract from Woloszczuk et al. (2016)). *xrn1*Δ preferentially stabilizes genes associated with transcription and the cell cycle whereas ribosomal protein genes resist stabilization. Log-phase *BY4741* & *xrn1*Δ cultures were mixed with log-phase *S. pombe* cells at a ratio of 2.5:1 OD₆₀₀ and pelleted (2300 rcf, 3 min). RNA was extracted using the miRNEasy kit (Qiagen) and libraries were generated using the QuantSeq kit (Lexogen). RNA reads are plotted against one another for four classes of genes and genes which are significantly stabilized are plotted in red. p-values were calculated using Student's T-test and indicate the probability that the fold-changes for each group of genes is no different to the fold-changes for all genes.

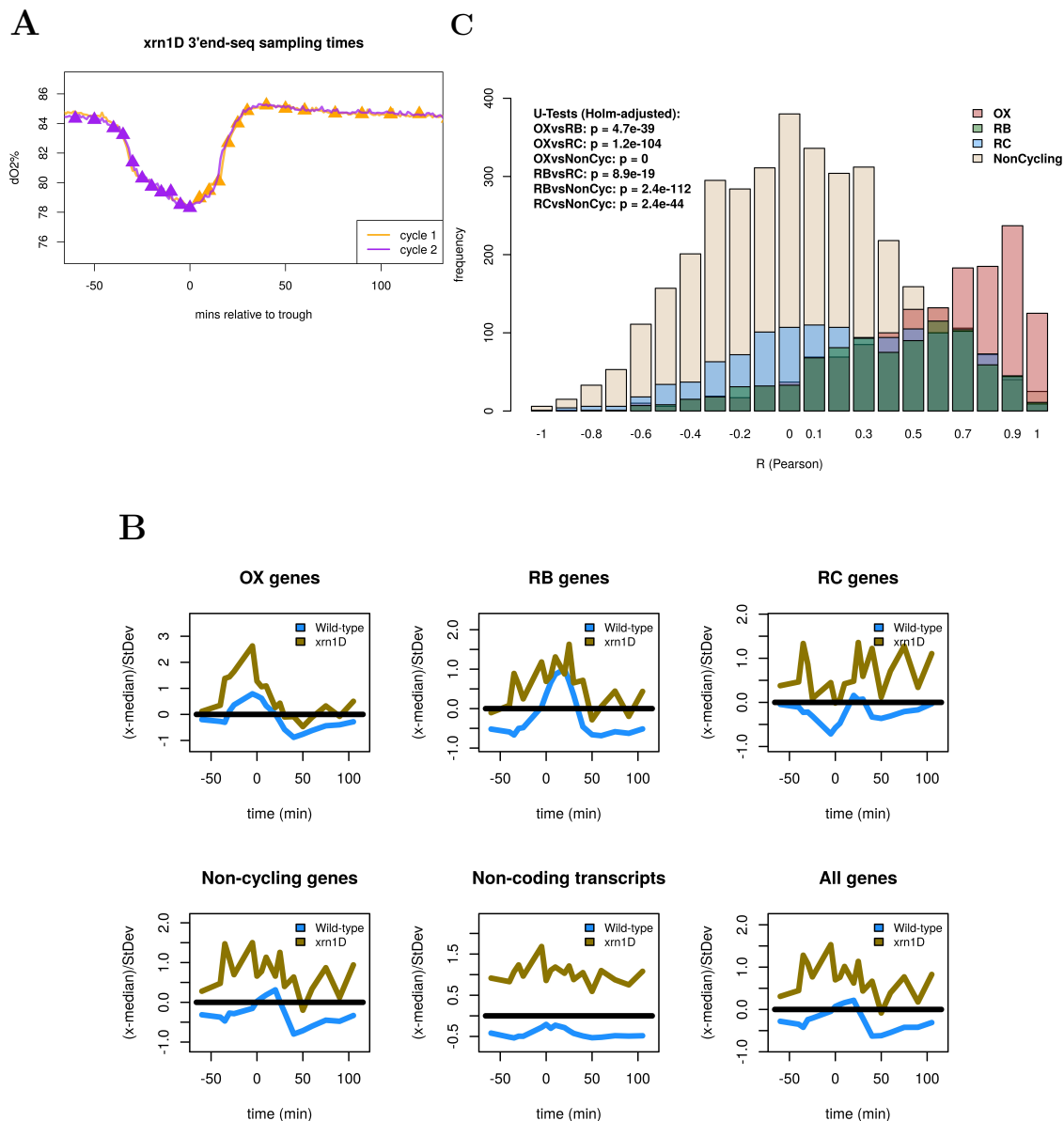


Figure 3.5: *xrn1*Δ cells exhibit dysregulation of RB & RC gene clusters.

A - Time points used for *xrn1*Δ QuantSeq. 1ml samples were taken from two consecutive *CENPK113-7D xrn1*Δ cycles and pellets were flash-frozen. Upon thawing, they were spiked with an equal volume of *S. pombe* cells, lysed immediately in phenol-chloroform and processed for QuantSeq.

B - Metaplots comparing *xrn1*Δ with wild-type across the YMC. Genes were divided into groups based on the three major YMC clusters identified by the Boeke lab (Kuang et al. (2014)) and coding potential. Calibrated counts for *xrn1*Δ and wild-type (Shidong Xi - unpublished data) were normalized to a common median (0) and standard deviation (1), then metaplots were created for each gene class and plotted over time. Times points are plotted in minutes relative to the point of lowest dO₂%.

C - OX genes correlate well with wild-type but many RB and RC genes are dysregulated. *xrn1*Δ calibrated counts were correlated with a wild-type QuantSeq dataset (Shidong Xi - Unpublished data) on a gene-by-gene basis and correlation coefficients were divided into 21 equally sized bins. The frequency of genes in each bin was then plotted as a histogram for each of the three YMC clusters and non-cycling genes. Differences between mean correlation coefficients were calculated using U-Tests adjusted for multiple testing by Holm's method.

in expression and the shape of the two curves throughout the cycle remains largely similar (Figure 3.5B). For the other clusters, the shapes of the curves are less similar. Correlation analysis allows quantification of how well expression of each gene is maintained in the *xrn1* Δ metabolic cycle (Figure 3.5C). Unsurprisingly, most genes show a positive correlation between wild-type and *xrn1* Δ . However, the magnitude of this correlation varies across a large range depending on the gene studied. OX genes correlate very well with wild-type, exhibiting a mean correlation coefficient that was extremely significantly higher than 0.50 ($p = 1.03 \times 10^{-50}$ - Wilcoxon signed-rank). In contrast, genes from the other classes generally exhibited correlation coefficients significantly lower than 0.50 ($R < 0.5$, $p = 0.025$ RB genes, $p = 3.96 \times 10^{-53}$ RC genes, $p < 10^{-54}$ non-cycling genes - Wilcoxon signed-rank). Excitingly, this is completely consistent with the behaviour of *xrn1* Δ in log-phase where ribosomal protein genes are notable in their escape from stabilization which otherwise affects the majority of genes to a greater or lesser extent.

This tendency demonstrates that proper regulation of the majority of RB/RC genes is not important for metabolic cycling as extreme disruption of their expression profiles, to the point that less than 25 % of the variance in their expression in *xrn1* Δ is explained by their expression in the wild-type ($R^2 < 0.25$), does not affect cycle period. One interpretation of this finding is that the OX phase gene expression program is the only one required for cycle maintenance and RB and RC gene classes exist either to boost cycle efficiency in a minor way or simply as an off-target consequence of OX phase expression. Alternatively, RB & RC profiles might reflect the default cellular expression profile which is then suppressed upon induction of OX phase.

However, both of these models assume that the only regulation of gene expression occurs at the level on transcripts. Instead, it could be that the YMC is regulated at

the level of gene expression but the additional transcripts in *xrn1* Δ are phenotypically silent. For example, they might not be efficiently translated. In this way, the cell could tolerate dysregulation of mRNA levels whilst still being sensitive to the protein products. Alternatively, it could be that regulation of the YMC occurs independently from dynamic changes in gene expression altogether.

3.1.3 Assessing the importance of different transcription factors for metabolic cycling

Intrigued by the fact that dysregulation of many RB & RC genes seemed to be well tolerated in *xrn1* Δ , we decided to question the widely-supported transcriptional regulation model. In this model, the YMC is driven by sequential expression of transcription factors which coordinate mRNA expression programs. These in turn define and determine the behaviour of each YMC phase.

Rao & Pelligrini computationally define a set of transcription factors which, based on their peaks of expression, are likely to regulate the YMC (Rao & Pellegrini (2011)). Kuang et al. (2017) have also implicated transcription factors based on their peaks of expression and the phenotypes of their deletions, claiming that *MSN2* & *4* are important for controlling the RC/OX transition. To determine the relative contributions of these transcription factors to metabolic cycling, we examined the strength of their correlations and the shapes of their expression profiles between *xrn1* Δ & wild-type cells.

The selected transcription factors exhibited a wide range of correlation coefficients between *xrn1* Δ and wild-type. By far the most highly correlated factor was *ADR1* which exhibits a correlation very close to 1.0 and 95 % confidence intervals greater than 0.50 (Figure 3.6A). In fact it is one of the best correlated transcripts in the

A

Gene	Pearson's R	+95%CI	-95%CI	rank	percentile
ADR1	0.95	0.98	0.86	19	0.26
GCN4	0.72	0.89	0.39	1075	14.98
HAP3	0.7	0.88	0.34	1236	17.22
BAS1	0.69	0.87	0.33	1319	18.38
GAL3	0.52	0.8	0.08	2344	32.66
SWI6	0.49	0.78	0.03	2558	35.65
HAP4	0.43	0.75	-0.04	2943	41.01
ARO80	0.43	0.74	-0.05	2986	41.61
SPT2	0.29	0.67	-0.21	4013	55.92
ACE2	0.23	0.63	-0.26	4453	62.05
MSN2	-0.07	0.41	-0.52	6353	88.53
MSN4	-0.21	0.28	-0.62	6823	95.08

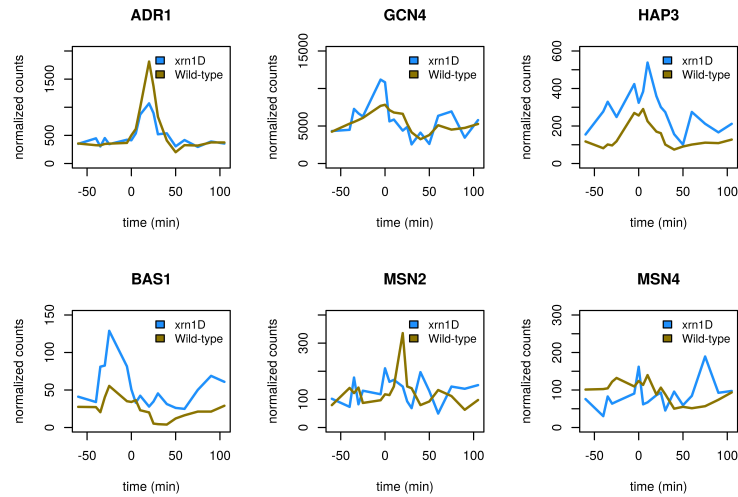
B

Figure 3.6: Large variability in expression levels and conservation of expression profile for transcription factors in *xrn1Δ* & wild-type metabolic cycles.

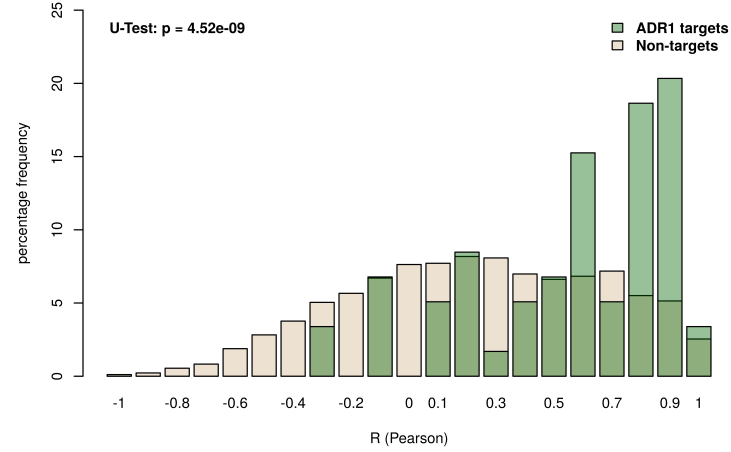
A - Exceptional correlation of *ADR1* expression between *xrn1Δ* & wild-type across the YMC. *xrn1Δ* calibrated counts were correlated with a wild-type QuantSeq dataset (Shidong Xi - Unpublished data) on a gene-by-gene basis and Pearson correlation coefficients, alongside their 95 % confidence intervals were tabulated for several transcription factors implicated in YMC regulation by Rao & Pellegrini (2011) and Kuang et al. (2017).

B - Expression of outlying transcription factors across the Yeast Metabolic Cycle. Calibrated counts for *xrn1Δ* & wild-type are plotted across the YMC for select transcription factors from (A) with particularly high or low correlation coefficients.

genome. This suggests that it is tightly regulated in an *XRN1*-independent manner across the YMC. *ADR1* is also very highly expressed during the YMC by transcription factor standards, and its expression peaks sharply at the RB/RC boundary (Figure 3.6B). This could be a sign that it is important for switching off high oxygen consumption metabolism and entering the low oxygen consumption state, a role that would fit with its importance in glucose repression (Young et al. (2003)). Several other factors, notably *GCN4*, *HAP3* & *BAS1*, also showed very high correlation, indicative of tight regulation throughout the YMC (Figure 3.6A & B). Although their correlation coefficients could not be confidently determined to be greater than 0.50. Interestingly, *MSN2* & *MSN4* are completely deregulated in *xrn1Δ* cells, exhibiting noisy expression and no correlation with wild-type expression patterns (Figure 3.6A & B). This raises serious questions about the Kuang model where these factors take on the role of master regulators of OX phase induction (Kuang et al. (2017)).

The exceptional correlation between *ADR1* expression in *xrn1Δ* & wild-type cells caught our interest, as unlike most of the highly correlated genes which peak in OX phase, *ADR1* peaks at the RB/RC boundary (Figure 3.5C & 3.6B). Based on the high correlation of *ADR1* itself, we speculated that *ADR1* targets might also be well correlated between strains due to their common regulation by *ADR1*. Indeed, this is what we see (Figure 3.7A). With a few exceptions, *ADR1* target genes are highly correlated between *xrn1Δ* & wild-type, indicating that they are tightly and consistently regulated across the YMC (mean Pearson's R >0.50, p = 0.00697 Wilcoxon signed-rank). What was less expected was that, although most genes were correlated with themselves, they did not all peak at the same time as one another. Even more unexpectedly, many *ADR1* targets, including its canonical target *ADH2*, exhibited completely different phasing to *ADR1*, peaking shortly after *ADR1* peaks but with their expression persisting throughout RC & early-OX and only beginning to drop

A



B

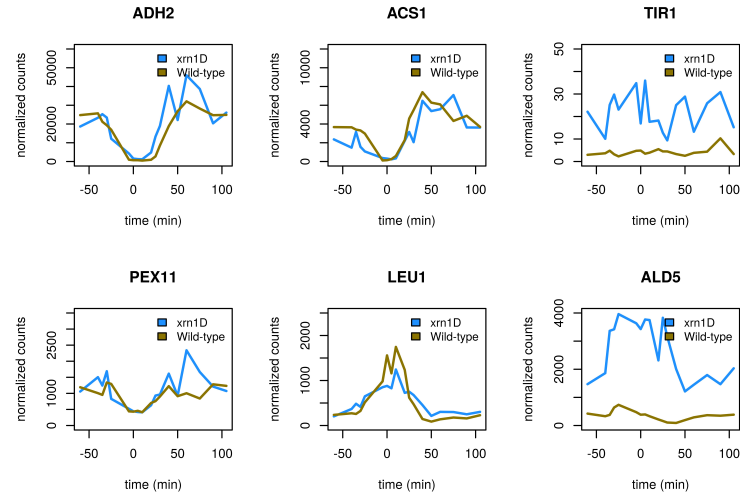


Figure 3.7: Expression of most *ADR1* targets is well-conserved between *xrn1Δ* & wild-type metabolic cycles.

A - High correlation of *ADR1* target gene expression between *xrn1Δ* & wild-type across the YMC. *xrn1Δ* calibrated counts were correlated with a wild-type QuantSeq dataset (Shidong Xi - Unpublished data) on a gene-by-gene basis and correlation coefficients were divided into 21 equally sized bins. The frequency of genes in each bin was then plotted as a histogram for *ADR1* target and *ADR1* non-target genes as defined by Young et al. (2003). The difference in mean correlation coefficients was calculated using a U-Test.

B - Expression of *ADR1* targets across the Yeast Metabolic Cycle. Calibrated counts for *xrn1Δ* & wild-type are plotted across the YMC for select *ADR1* target genes (Young et al. (2003)).

shortly before RB phase. This is likely to be due to persistence of DNA-binding of Adr1p long after the peak of its transcript disappears. Other *ADR1* targets such as *LEU1* exhibit completely different phasing however, peaking in RB phase, before and during the peak of *ADR1* transcript expression. The regulation of these transcripts must be more complicated. Perhaps under certain promoter environments Adr1p can act as a repressor? This would be an exciting and controversial finding but would require a lot of validation. Alternatively, other transcription factors, or transcription factor independent changes in the local chromatin environment could be what are responsible for determining gene activation and repression on these targets.

3.1.4 The *xrn1* Δ proteome shows few differences to wild-type.

In order to gain insight into how *xrn1* Δ cells can tolerate global mRNA dysregulation, relative changes in protein abundance between *xrn1* Δ and wild-type were assessed by mass-spectrometry (Table A.1). Surprisingly, only 243 proteins showed significant changes in abundance between *xrn1* Δ & wild-type and only 44 showed changes that matched the observed change in transcript levels.

Interestingly, ribosomal proteins stand out as the one class of proteins which are consistently down-regulated in *xrn1* Δ cells (Table 3.1). This is distinctly reminiscent of their behaviour at the transcript level where they stand out from other genes as not being stabilized (Woloszczuk et al. (2016) & Figure 3.4). Since the proteomics data isn't internally calibrated it may be that, like the mRNA levels, total protein concentrations in *xrn1* Δ cells are higher than wild-type, and again, only expression of the ribosomal proteins is still properly regulated. Alternatively, total protein levels could be assumed to be invariant between *xrn1* Δ & wild-type in which case competition with the upregulated majority of the mRNA pool might deprive ribosomal protein

Annotation Cluster 1 Category	Enrichment Score: 13.796497642403255 Term	Count	%	PValue	Bonferroni	Benjamini	FDR
SP_PIR_KEYWORDS	protein biosynthesis	53	4	1.98E-25	4.44E-23	4.44E-23	2.53E-22
GOTERM_CC_FAT	GO:0022626~cytosolic ribosome	39	3	2.77E-21	7.77E-19	7.77E-19	3.66E-18
SP_PIR_KEYWORDS	ribosome	38	3	1.18E-20	2.65E-18	1.33E-18	1.51E-17
GOTERM_CC_FAT	GO:0033279~ribosomal subunit	43	3	4.25E-19	1.19E-16	5.97E-17	5.62E-16
SP_PIR_KEYWORDS	ribosomal protein	40	3	6.37E-19	1.43E-16	4.76E-17	8.14E-16
GOTERM_CC_FAT	GO:0005840~ribosome	52	4	2.07E-18	5.82E-16	1.94E-16	2.74E-15
GOTERM_CC_FAT	GO:0044445~cytosolic part	40	3	8.93E-18	2.51E-15	6.27E-16	1.18E-14
SP_PIR_KEYWORDS	cytosol	24	2	2.81E-16	7.46E-14	1.49E-14	4.22E-13
SP_PIR_KEYWORDS	ribonucleoprotein	44	3	3.73E-16	7.46E-14	1.24E-14	4.22E-13
GOTERM_MF_FAT	GO:0003735~structural constituent of ribosome	40	3	1.73E-14	6.03E-12	6.03E-12	2.37E-11
GOTERM_CC_FAT	GO:0005829~cytosol	54	4	3.51E-14	9.86E-12	1.97E-12	4.64E-11
GOTERM_CC_FAT	GO:0030529~ribonucleoprotein complex	64	5	1.57E-13	4.41E-11	7.35E-12	2.08E-10
GOTERM_BP_FAT	GO:0006412~translation	75	6	1.59E-13	1.6E-10	5.33E-11	2.52E-10
KEGG_PATHWAY	see03010:Ribosome	34	3	2.98E-13	1.73E-11	1.73E-11	2.97E-10
GOTERM_CC_FAT	GO:0022625~cytosolic large ribosomal subunit	21	2	2.34E-11	6.56E-09	9.37E-10	3.09E-08
GOTERM_CC_FAT	GO:0015934~large ribosomal subunit	25	2	6.41E-11	1.8E-08	2.25E-09	8.49E-08
GOTERM_MF_FAT	GO:0005198~structural molecule activity	46	4	2.21E-10	7.7E-08	3.85E-08	3.03E-07
GOTERM_CC_FAT	GO:0022627~cytosolic small ribosomal subunit	16	1	1.85E-09	5.21E-07	5.78E-08	2.45E-06
Annotation Cluster 2 Category	Enrichment Score: 13.003607240900195 Term	Count	%	PValue	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0010608~posttranscriptional regulation of gene expression	38	3	3.76E-14	3.78E-11	3.78E-11	5.95E-11
GOTERM_BP_FAT	GO:0006417~regulation of translation	36	3	1.52E-13	1.53E-10	7.64E-11	2.4E-10
GOTERM_BP_FAT	GO:0032268~regulation of cellular protein metabolic process	38	3	1.7E-13	1.71E-10	4.28E-11	2.7E-10

Table 3.1: Genes with translation-associated GO terms whose products are strongly depleted from the *xrn1Δ* proteome.

Gene ontology analysis was performed comparing the *xrn1Δ* and wild-type proteomes. GO terms that were enriched with a p-value of less than 10^{-9} are displayed with relevant statistics.

transcripts of ribosomes leading to a reduction in their expression (Shah et al. (2013)).

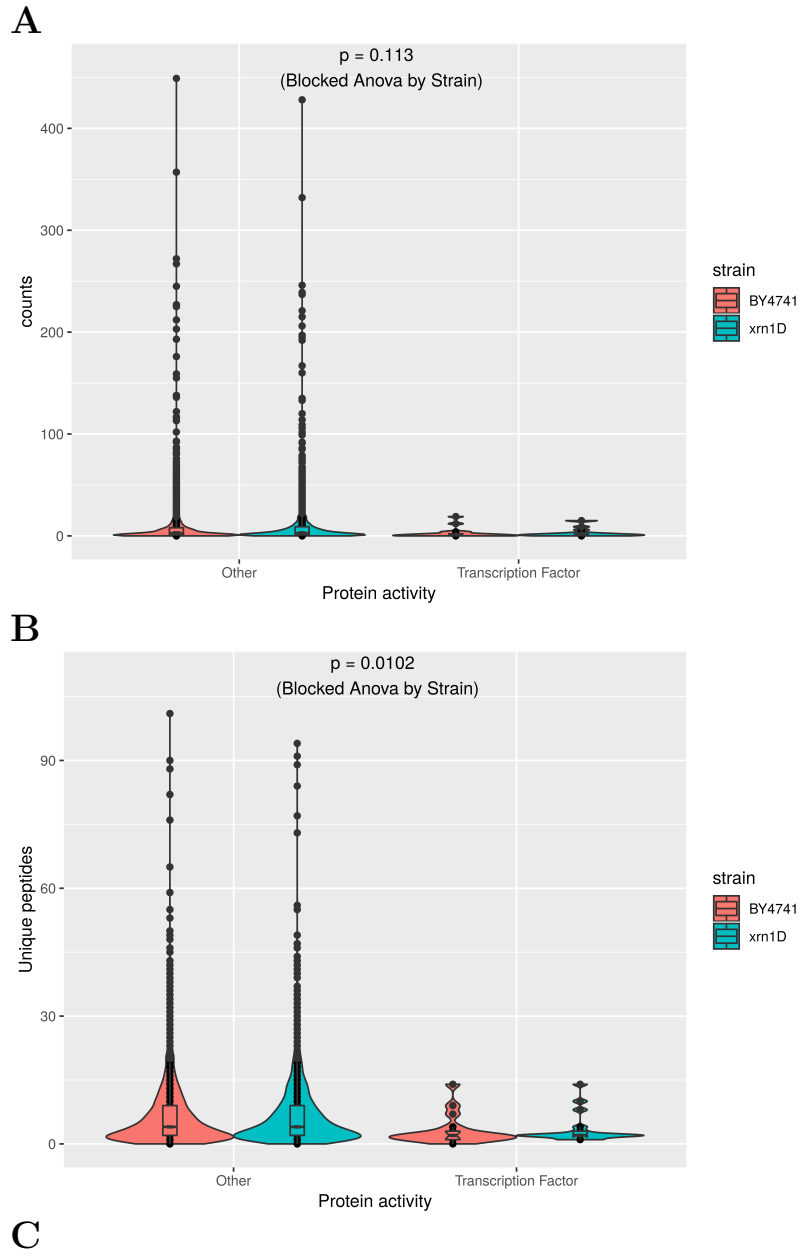
Unfortunately however, the proteomics data is unable to tell us much about some of the other classes of proteins that were identified in (Woloszczuk et al. (2016)). Transcription factors in particular, are generally difficult to detect in proteomics datasets. This is partly because of the high dynamic range of proteomics data, which due to their low abundance, means that they will only ever comprise a very small fraction of the detected counts. This limitation can be seen in the *xrn1Δ* data. Only 17 transcription factors were detected in the dataset and generally only one or two unique peptides were detectable at fairly low count numbers for those that were (Figure 3.8A & B). This substantially limits the power of mass-spectrometry to detect changes in the abundance of these proteins. Therefore, despite not detecting any changes in transcription factor expression in the proteomics dataset, these proteins may still be changing in expression in *xrn1Δ*.

However, a novel data-independent acquisition methodology known as SWATH-MS has recently been published which promises to detect low-abundance peptides such as those from transcription factors more robustly (Ludwig et al. (2018)). This may allow future studies to better quantify transcription factor levels in *xrn1* Δ cells.

3.1.5 RiboSeq reveals no increase in translation of mRNAs which are stabilized in *xrn1* Δ .

Since changes in transcription factor abundance could not be detected directly, we next tried to measure their rates of translation using RiboSeq. RiboSeq is a highly sensitive RNA-sequencing technique which utilizes RNase-digestion and size-selection to specifically sequence 27-30 bp mRNA fragments known as ribosome-protected fragments or rpfs, which are protected by the footprint of the ribosome. This provides single base-pair resolution data on the position and number of translating ribosomes in a cell. To measure any changes in the rpf levels of ribosomal proteins and transcription factors in *xrn1* Δ , calibrated RiboSeq libraries were prepared and sequenced.

Interestingly, although a wide range of changes in rpf levels appear to be detectable in an *xrn1* Δ /wild-type MA-plot (See subsection 1.3.3 for a discussion of MA-plot interpretation), there is no global increase in read counts (Figure 3.9A). Instead, translation of individual mRNAs seems to change whilst leaving the total number of engaged ribosomes approximately constant. This suggests that some factor other than mRNA levels, such as ribosome or Pab1p, is limiting for translation availability (Shah et al. (2013); Sachs & Davis (1989); Costello et al. (2015)), allowing cells to tolerate the additional transcripts in *xrn1* Δ through suppression of translation. Supporting this, none of the classes of genes that were previously identified as being the most stabilized in *xrn1* Δ (Woloszczuk et al. (2016)), particularly stand



Strain	Protein category	median counts	median unique peptides
<i>BY4741</i>	Transcription Factor	1 ± 0.76	2 ± 0.77
	Other	3 ± 0.23	4 ± 0.19
<i>xrn1D</i>	Transcription factor	1 ± 0.77	2 ± 1.77
	Other	3 ± 0.20	4 ± 0.20

Figure 3.8: Transcription factor abundances are at the limit of detection in proteomics datasets.

A-C Median values for counts (**A**) & unique peptides (**B**) were calculated for each protein detected in the mass-spectrum. Proteins were then separated into two groups based on membership of the "DNA-binding transcription factor" GO term and violin plots of these values for *xrn1D* & *BY4741* were generated. Blocked ANOVAs grouped by strain were calculated for unique peptides and counts. For an explanation of violin plots and their interpretation, please consult subsection 1.3.3. The medians $\pm 95\%$ confidence intervals of each group of proteins are summarized in a table (**C**).

out as being more stabilized than the bulk of the transcriptome (Figure 3.9A). Genes annotated as encoding transcription factors, chromatin remodelling proteins and cell cycle proteins are scattered across a range of $\log_2$ fold-changes, rather than showing a trend towards the upper parts of the MA-plot distribution.

Interestingly, as was seen at the mRNA level, the ribosomal proteins stand out as being very tightly coordinated, clustering tightly at the bottom right of the distribution, showing no change in translation (Figure 3.9A). Since the ribosomal protein gene mRNAs themselves are unaffected by *xrn1* Δ , this is the expected result in the absence of any regulation by *XRN1* at the level of translation.

Measuring association of ribosomes with RNAs in the form of rpfs allows questions to be asked about the translational status of more unusual classes of transcript. Over the past decade, a flurry of techniques for measuring the nascent transcriptome have been developed and applied to various organisms revealing that large swathes of the genome which have classically been thought of as silent are in fact transcriptionally active (Schwalb et al. (2016); Churchman & Weissman (2012); Core et al. (2008)). Since most of these newly discovered transcripts are highly unstable it has been assumed that they lack coding-potential. However, in mammalian cells, it appears that many of these lncRNAs are in fact translated (Ingolia et al. (2014)).

Stabilization of several classes of *S. cerevisiae* ncRNAs occurs in *xrn1* Δ strains, most notably XUTs (Woloszczuk et al. (2016); van Dijk et al. (2011)). Few ncRNAs could be detected at high levels in either the *xrn1* Δ or *BY4741* RiboSeq datasets, although a small number were observable (Figure 3.9B). Interestingly, as was the case for the most stabilized coding transcripts, those ncRNAs that were sufficiently highly expressed to be quantified showed little tendency to be upregulated in *xrn1* Δ (Figure

3.9B). This further supports the idea that those RNAs that are stabilized in *xrn1* Δ are not necessarily competent for translation.

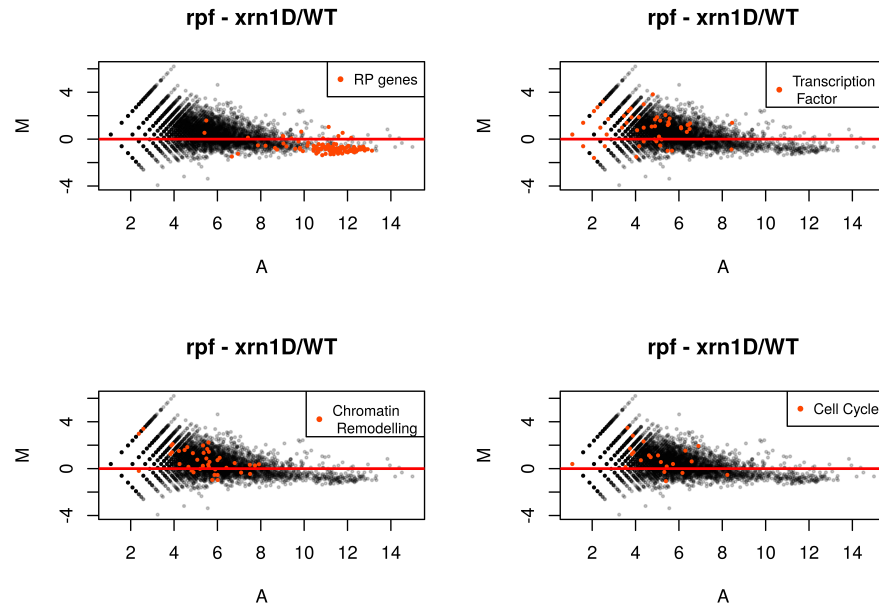
Up to here, discussion of the RiboSeq data has been restricted to the whole-gene level. However, one of the key advantages of RiboSeq over other methods for measuring translation is its ability to provide a high-resolution view of cellular translation. To exploit this, rpf reads were mapped to their peptidyltransferase sites (P-sites) and metagenes were plotted over the 5' and 3' ends of all annotated open-reading frames (Figure 3.10A - C).

Both strains show the strong 3 nt periodicity characteristic of RiboSeq data, confirming that true rpfs predominate in the data, and therefore the data reflects the position of translating ribosomes (Figure 3.10A). There are a number of intriguing differences between the strains though. First of all, the *xrn1* Δ metagene shows a lower amplitude of phasing caused by an accumulation of out-of-phase reads. These could potentially originate from ribosomes that are trapped in unusual conformations or have undergone frame-shifting, although in the case of frame-shifting, one might expect a gradual loss-of phasing as the ribosome travels along a transcript, a feature which is not observed.

Another interesting feature of the *xrn1* Δ metagene is the size of the peak over the start codon (AUG). It is much larger than that of *BY4741* (Figure 3.10B). This suggests that the ribosome is dwelling somewhat longer at the AUG, a behaviour that points to a mild defect in one of the late stages of translation initiation after scanning and START site selection.

Metagenes which average across many transcripts can mask the nuances of gene expression by merging profiles which have very different behaviours. For example,

A



B

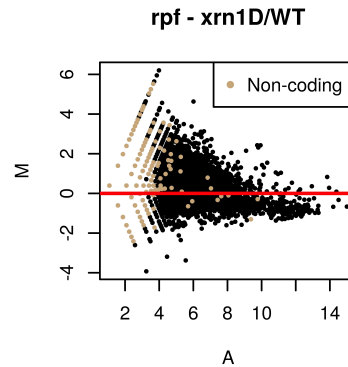


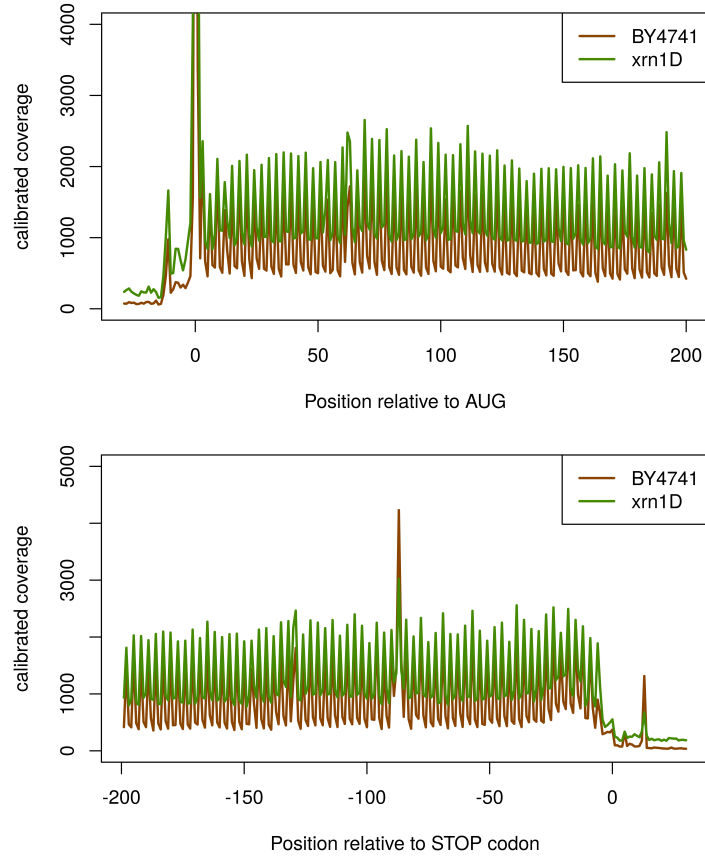
Figure 3.9: Classes of genes stabilized in *xrn1Δ* are not unusually highly translated.

A series of MA plots demonstrating the changes in expression of various classes of gene between *xrn1Δ* & *BY4741*. For a detailed description of MA-plots and their interpretation, please consult subsection 1.3.3 but in brief, MA plots compare $\log_2$ fold-change in expression of each gene in *xrn1Δ* over *BY4741* (M) with the mean $\log_2$ expression (A) in both conditions.

A - MA plots for ribosome-protected fragments in several classes of coding genes. Counts tables were generated from the *xrn1Δ* & *BY4741* ribosome-protected fragment libraries and calibrated counts were plotted as MA plots. Various classes of genes of interest to this study were highlighted.

B - MA plots for ribosome-protected fragments aligning to “non-coding” RNAs. Counts tables were generated from the *xrn1Δ* & *BY4741* ribosome-protected fragment libraries and calibrated counts were plotted as MA plots and transcripts annotated as “SUTs”, “XUTs” & “CUTs” were highlighted.

A



B

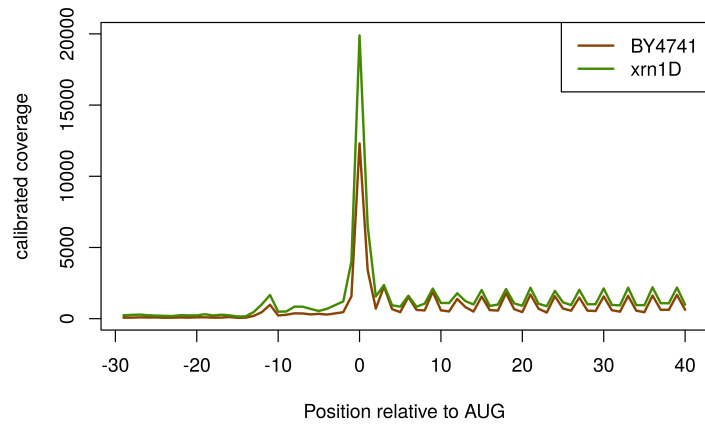


Figure 3.10: *xrn1*Δ cells show mild defects in translation initiation and elongation.

A&B - Metagenes of rpf distribution around AUG and STOP codons. Rpf coverage flanking AUG and STOP codons was calculated for each transcript, summed, scaled to a calibration factor based on overall *S. pombe* counts (**A**) and plotted as a metagene. A narrower window flanking the AUG was also plotted to aid visualization of the distribution around this codon (**B**).

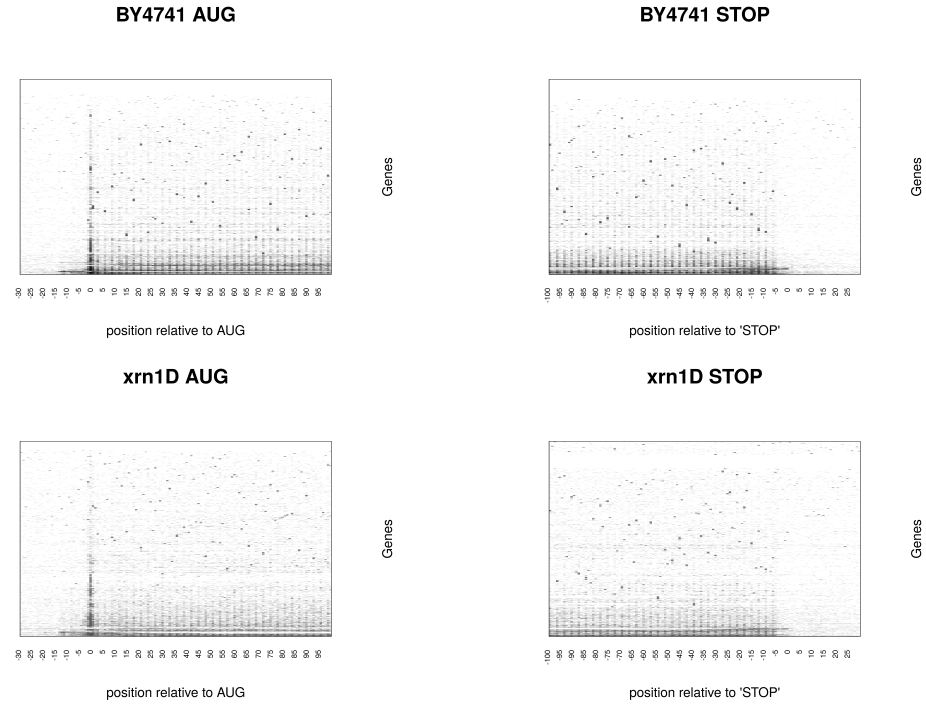


Figure 3.11: Reduced phasing in *xrn1*Δ is not restricted to a minority of ORFs.

Heatmaps of rpf distribution around START (AUG) and STOP codons. Rpf coverage flanking START and STOP codons was calculated for each transcript, scaled to a calibration factor based on overall *S. pombe* counts and plotted as heatmaps.

the reduction in phasing of the *xrn1*Δ metagene could be attributed to different subsets of transcripts having different 5' offsets. In order to get a more detailed view of coverage across wild-type and *xrn1*Δ open-reading frames, heatmaps of coverage flanking the AUG and STOP codons were plotted (Figure 3.11). These plots show that although there is a large dynamic range of gene expression, the AUG peak and 3 nt periodicity are maintained across all transcripts that have sufficient coverage for them to be detectable and the reduction in phasing in *xrn1*Δ cells is not due to overlaying of two sub-populations of transcripts with different 5' offsets.

3.1.6 Polysome profiling confirms that the additional poly-A RNA molecules in *xrn1* Δ are not translated.

The changes in rpf levels observed in the RiboSeq data suggested that *xrn1* Δ cells are able to tolerate the additional transcripts present within them by suppressing their translation. However, it remained unclear whether this suppression occurs by sequestering the additional RNAs in translationally incompetent states or by a general dampening of translation of all copies of stabilized RNAs. Furthermore, with only a single replicate for the RiboSeq experiment, additional validation of it was important. Therefore, in order to measure the translational activity of mRNAs at the whole transcript level, polysome profiling of wild-type and *xrn1* Δ cells was performed.

The RNA absorbance profiles from the sucrose gradients show a small increase in polysomes in *xrn1* Δ but a larger increase in low Mw RNPs which are not being translated, consistent with the increase in total mRNA levels in *xrn1* Δ previously observed (Figure 3.12A).

To examine what proportion of transcripts from each gene are translated, calibrated QuantSeq libraries (Lexogen) were generated from 2 mg of the input lysate, the pooled monosomal fractions and the pooled polysomal fractions.

The input libraries showed a 2.1-fold increase in median mRNA levels in *xrn1* Δ from 327 ± 14 (95 % CI) in *BY4741* to 588 ± 21 (95 % CI) in *xrn1* Δ confirming the observations of Sun et al. (2013); Malabat et al. (2015); Woloszczuk et al. (2016) (Figure 3.12B). In contrast, although there was a median increase in levels in polysomal fractions, it was very modest in comparison (1.3-fold - Medians $\pm$ 95 % CI: *BY4741* 74 ± 3 , *xrn1* Δ 100 ± 4 - Figure 3.12C). Consequently, the ribosome occupancy, measured as the proportion of input in the polysomal fractions, was reduced to 75 % of

Gene category	Fraction	median fold-upregulation	95% CI
All Genes	Input	1.71	1.68-1.74
	Polysomal	1.27	1.25-1.29
Transcription Factors	Input	1.91	1.68-2.14
	Polysomal	1.43	1.22-1.64
Chromatin-Remodellers	Input	1.79	1.56-2.02
	Polysomal	1.35	1.20-1.50
Cell-cycle	Input	1.62	1.47-1.81
	Polysomal	1.25	0.95-1.55
Ribosomal Proteins	Input	0.88	0.84-0.92
	Polysomal	0.74	0.70-0.78
“ncRNA” (SUTs, XUTs & CUTs)	Input	2.79	2.67-2.90
	Polysomal	2.06	1.95-2.16

Table 3.2: Summary statistics for Figures 3.12 & 3.13.

The median values and 95 % confidence intervals for fold-upregulation of different classes of genes in input and polysomal libraries as plotted in Figures 3.12 & 3.13.

wild-type (Median $\pm$ 95 % CI: 24.1% $\pm$ 0.2) in *xrn1* Δ (Median $\pm$ 95 % CI: 17.5% $\pm$ 0.2) (Figure 3.12D).

The above data clearly show an overall decrease in the proportion of mRNAs translated in *xrn1* Δ . However, they do not show whether this is a general shunt of ribosome occupancy at all transcripts downwards or whether the changes in percentage ribosome occupancy apply most to those genes whose transcripts are most stabilized, i.e. whether those mRNAs that are most stabilized in *xrn1* Δ also show the greatest increase in translation.

Highlighting the classes of genes previously found to be the most stabilized in *xrn1* Δ shows some evidence of this. Transcription factors for example have a slightly higher median upregulation than the overall transcriptome in the polysomal fractions but not as high as they do in the input and not to the point of significance (Figure 3.13A, Table 3.2). Likewise, neither chromatin-remodellers, nor cell cycle genes are significantly upregulated in either fraction (Figure 3.13B & C, Table 3.2).

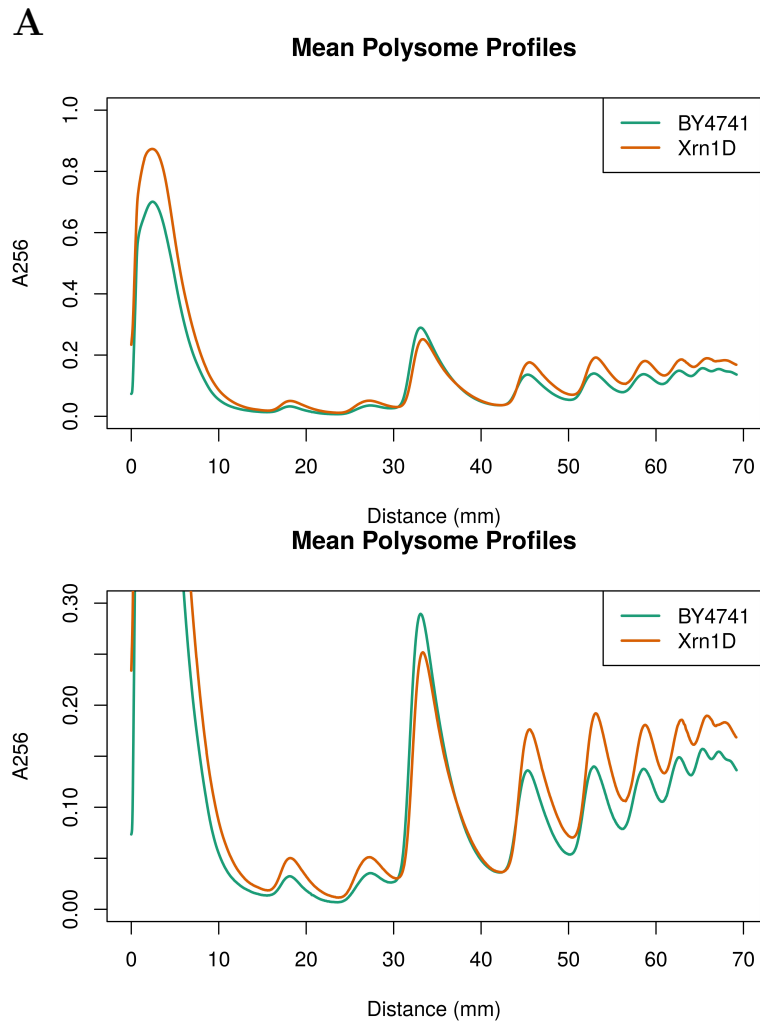


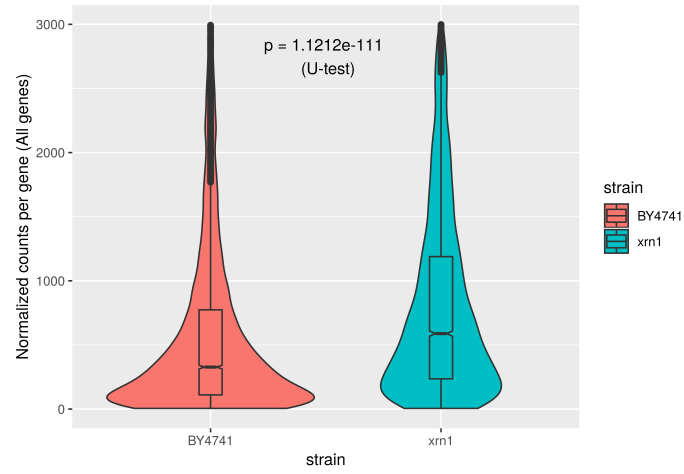
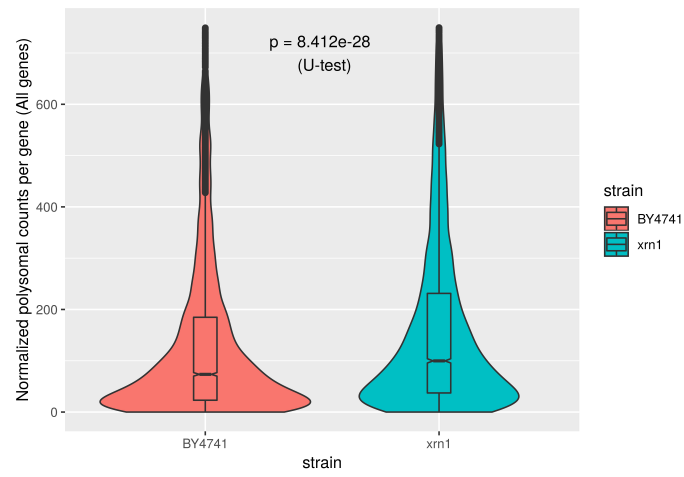
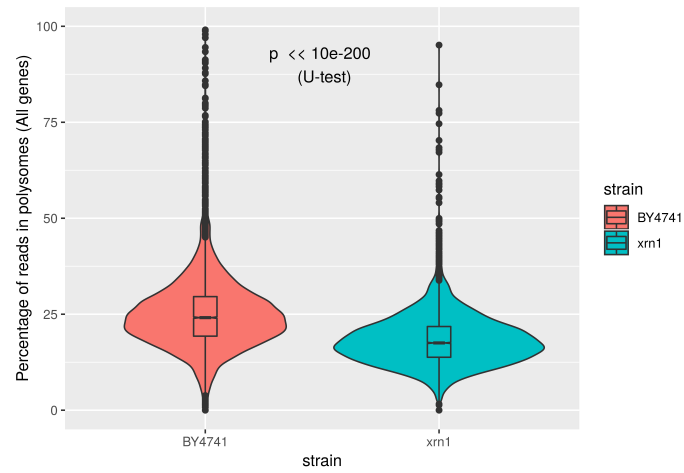
Figure 3.12: Polysome profiling shows that RNAs stabilized in *xrn1* Δ are generally not translated.

A - Absorbance traces from sucrose gradient fractionation of *xrn1* Δ and wild-type cell lysate. Log-phase *BY4741* & *xrn1* Δ cultures in YPD were harvested and cells were mechanically lysed according to the Materials and Methods in the presence of 100 μ g/ml cycloheximide & 20 mM $MgCl_2$ then 2 mg of lysate was separated by sucrose gradient centrifugation. Gradients were then fractionated using a Biocomp gradient profiler and A_{256} was recorded ($n = 3$).

B - Global increase in mRNA levels in *xrn1* Δ input lysate. 2 mg of the lysate loaded for sucrose fractionation in (A) was spiked with 6 μ g of *S. pombe* total RNA and processed for QuantSeq according to the Materials and Methods. Counts tables were generated and calibrated to the *S. pombe* library sizes factors calculated using DESeq2. Calibrated counts were then plotted as violin plots for both strains. ($n = 3$).

C - Modest global changes in RNA levels in polysomal fractions. Polysomal fractions from the sucrose gradients in A in (A) were pooled and spiked with 2 μ g of *S. pombe* total RNA and processed for QuantSeq according to the Materials and Methods. Counts tables were generated and calibrated to the *S. pombe* library sizes factors calculated using DESeq2. Calibrated counts were then plotted as violin plots for both strains ($n = 3$).

D - Global reduction in ribosome occupancy in *xrn1* Δ . Polysomal and input counts tables from (B&C) were calibrated to *S. pombe* library sizes factors calculated using DESeq2 and adjusted per μ g of *S. pombe* RNA. Percentage ribosome occupancy was calculated for each transcript as the percentage of calibrated reads in input lysate that were in polysomal fractions ($n = 3$).

B**C****D**

These results differ from the conclusions of Woloszczuk et al. (2016) as in our study, the classes of genes just listed weren't significantly upregulated in the input either. The reason for this difference is unclear but it could reflect the different lysis conditions used between the datasets. Mechanical lysis, as employed for polysome fractionation, is much slower than phenol-chloroform extraction and generally considered to be non-denaturing. As such, residual nuclease activity may remain in the cell lysate, leading to small changes in transcript enrichment.

In contrast, the ribosomal protein genes form a tight cluster that is slightly, but significantly, downregulated in *xrn1* Δ (Figure 3.13D, Table 3.2). As seen in previous datasets, ribosomal protein gene mRNAs are completely resistant to the stabilization through loss of *XRN1* and this persists at the level of translation (Woloszczuk et al. (2016); Sun et al. (2013); Malabat et al. (2015)).

One class of transcripts that stands out as being particularly highly upregulated are those generally considered in the literature to be “non-coding” such as SUTs, CUTs & XUTs (Xu et al. (2009); van Dijk et al. (2011)). These transcripts are highly significantly upregulated in *xrn1* Δ in both the polysomal and input libraries (Figure 3.13E, Table 3.2). Unexpectedly, this shows that in addition to being stabilized in *xrn1* Δ , many SUTs, XUTs & CUTs are also translated, a result which contrasts with the RiboSeq data where most “non-coding” RNAs were scattered throughout the lowly-expressed part of the plot (Figure 3.9B - $A < 5$). However, due to the low count numbers for “ncRNAs” in the RiboSeq data, changes in expression may not be detectable in that dataset. The purpose of translation of these RNAs is unclear. One possibility, as argued by Malabat et al. (2015), is that translation acts as a form of quality control, targeting these mRNAs for NMD. Alternatively, Ingolia et al. (2014) have shown that short ORFs as found on “ncRNAs” can encode immunogenic pep-

tides in mammals. *S. cerevisiae* “ncRNAs” might also produce functional peptides but confirmation of this would require a great deal of further investigation.

In order to get a more general picture of translational regulation in *xrn1Δ*, specific changes in percentage ribosome occupancy were identified using Xtail (Xiao et al. (2016)). Since statistical packages that measure differential expression usually make the assumption that most genes do not change in expression, uncalibrated counts had to be used for this analysis. Surprisingly, only 13 protein-coding genes were detected as being translationally repressed and just two were translationally activated *xrn1Δ* (Figure 3.14). However, a number of poorly-annotated transcripts and transcripts classically annotated as “non-coding” were found to be significantly changed. Including these hits brings the number of transcripts with differential ribosome-occupancy up to 27 downregulated & 5 upregulated transcripts. The most interesting of these hits is *GIP3*, which stands out as it regulates SNF1-signalling and may be associated with ribosomes (Pinsky et al. (2006); Fleischer et al. (2006)) which means it may provide a potential link between translation and metabolism in *xrn1Δ*. Gip3p was detected in the *xrn1Δ* proteome but only at very low levels which were not amenable to quantitation (data not shown).

3.1.7 Behaviour of translating monosomes in wild-type & *xrn1Δ* cells.

Although monosomes are classically considered to be translationally inactive, this assumption has recently been challenged by the Moore lab. Heyer & Moore (2016) demonstrated the existence of genuine rpfs that exhibit strong triplet periodicity and a pronounced 5' bias in RiboSeq libraries from gradient-fractionated monosomes. They go on to show that monosomes preferentially translate NMD targets; short ORFs such as ribosomal-protein genes and low-abundance regulatory proteins such as tran-

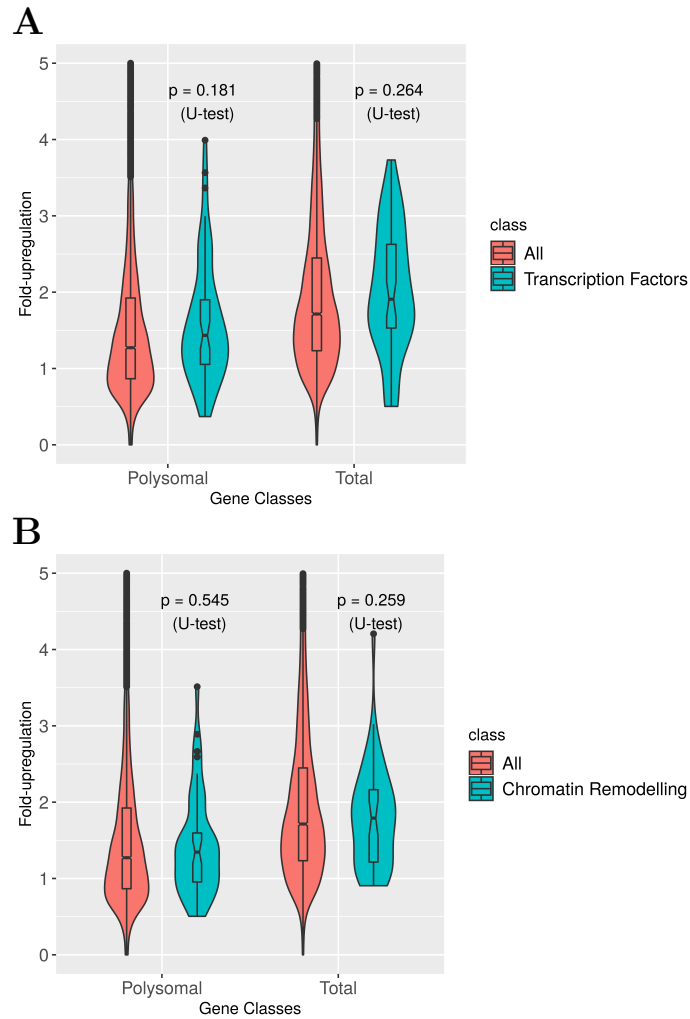
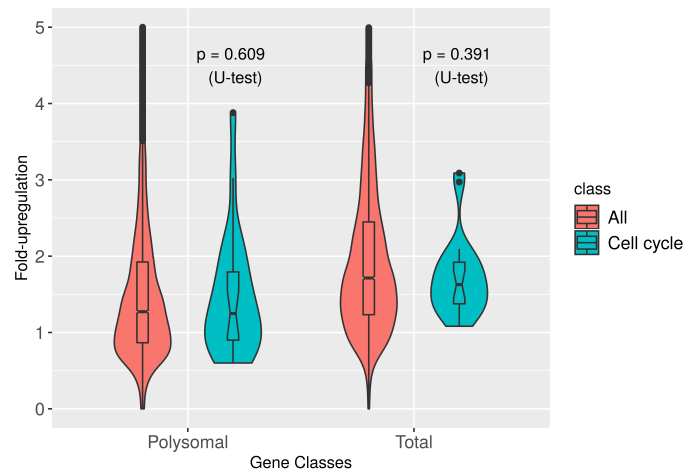
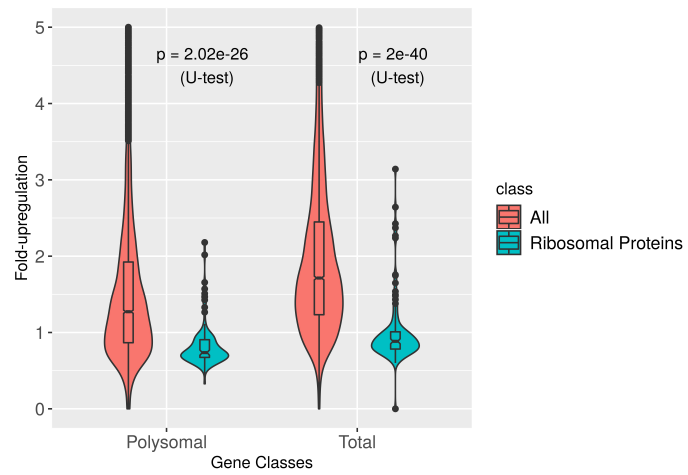
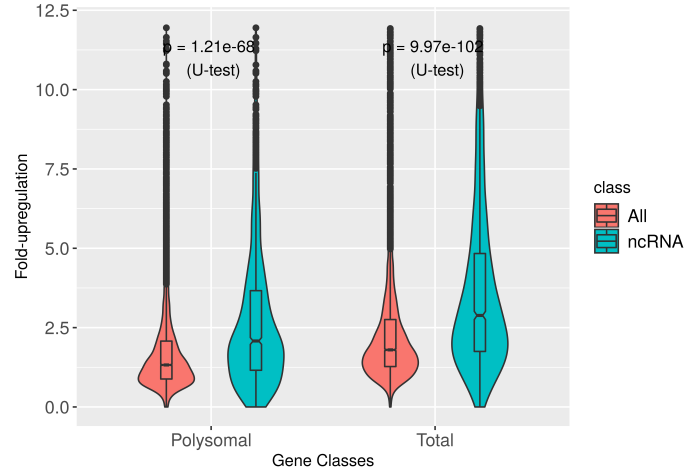


Figure 3.13: Fold-changes in stability and translation of different gene classes between *xrn1Δ* & *BY4741*.

A-E - Fold-upregulation of different classes of transcript in *xrn1Δ* input lysate and polysomal fractions. Fold-upregulation in input lysate (Total) and polysomal fractions was calculated for each transcript and these data are represented as violin plots for “All genes” (**A - E**), “Transcription factors” (**A**), “chromatin remodelling complexes” (**B**), “cell-cycle related genes” (**C**), “Ribosomal proteins” (**D**) & “non-coding” RNAs (SUTs, XUTs & CUTs) (**E**). Medians and 95 % confidence intervals for each set of genes can be found in Table 3.2 ($n = 3$).

C**D****E**

Systematic Names	Common Names	log ₂ fold-change RO	pvalue	pvalue (adjusted)
SUT054	NA	2.32	5.70E-06	5.53E-03
CUT793	NA	1.57	1.70E-06	2.36E-03
XUT_7R-374	NA	1.50	4.70E-05	1.77E-02
YJL152W		1.45	3.60E-05	1.60E-02
YPR158W	CUR1	1.14	1.90E-04	4.36E-02
YER009W	NTF2	-1.18	1.20E-05	8.77E-03
YLR046C		-1.19	2.10E-04	4.69E-02
YLR152C		-1.28	1.50E-04	4.34E-02
YER019W	ISC1	-1.30	3.80E-05	1.60E-02
YOR116C	RPO31	-1.39	2.70E-05	1.55E-02
YIR006C	PAN1	-1.41	1.30E-04	3.89E-02
YDR050C	TPI1	-1.49	7.90E-05	2.69E-02
YKL174C	TPO5	-1.51	1.30E-04	3.89E-02
SUT270	NA	-1.52	2.30E-04	4.76E-02
YJL101C	GSH1	-1.53	2.30E-05	1.44E-02
YGL140C		-1.56	1.80E-04	4.36E-02
YHR078W		-1.58	3.20E-05	1.57E-02
YGL176C		-1.60	1.60E-04	4.36E-02
YPL221W	FLC1	-1.61	4.70E-05	1.77E-02
SUT665	NA	-1.69	5.50E-05	1.98E-02
YDR504C	SPG3	-1.83	3.20E-05	1.57E-02
YPL137C	GIP3	-2.20	7.50E-07	1.28E-03
YKL220C	FRE2	-2.27	1.50E-05	1.01E-02
YJR020W		-2.54	1.70E-04	4.36E-02
YHL006W-A	NA	-2.95	1.80E-04	4.36E-02
XUT_7R-435	NA	-3.19	1.80E-04	4.36E-02
XUT_11R-39	NA	-3.74	2.00E-04	4.55E-02
XUT_9F-202	NA	-3.76	4.60E-06	5.23E-03
CUT707	NA	-5.67	2.30E-04	4.76E-02
XUT_2R-271	NA	-6.96	1.20E-05	8.77E-03
XUT_13F-315	NA	-7.28	6.80E-07	1.28E-03

Table 3.3: Transcripts identified as differentially-translated in *xrn1*Δ cells by Xtail.

Log₂ fold-changes in ribosome occupancy (RO) and adjusted p-values for differentially translated transcripts. All transcripts which Xtail identifies as differentially translated (adjusted p-value <0.05) are listed alongside their log₂ fold-change in RO.

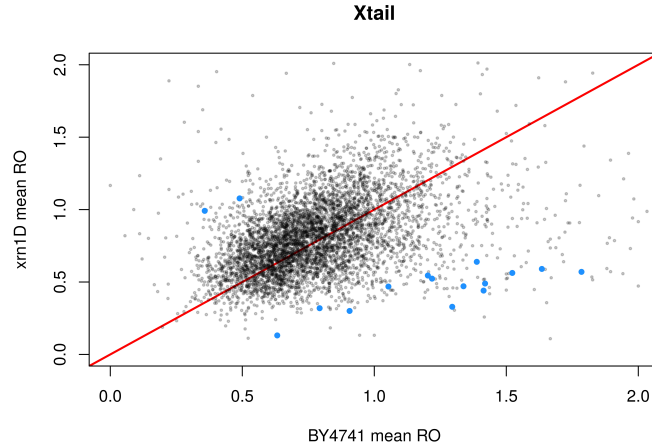


Figure 3.14: Very few protein-coding genes are differentially translated in *xrn1Δ* cells.

Xtail identifies only 15 differentially-translated protein-coding genes in *xrn1Δ*. Uncalibrated counts from input and polysomal libraries for *xrn1Δ* & *BY4741* were passed through the Xtail pipeline. Relative ribosome occupancy (RO - Polysomal counts/Input counts) in each strain is plotted for each gene and differentially-translated protein-coding genes are highlighted in blue ($p_{adj} < 0.05$) ($n = 3$).

scription factors. Ribosomal protein gene and transcription factor mRNAs stand out as two classes of transcripts that are most distinctly regulated in *xrn1Δ*. Likewise, the “ncRNAs” that are stabilized and translated in *xrn1Δ* are likely to be targets for NMD due to their short ORF length and so likely to be enriched in monosomes (Figure 3.13E, Table 3.2, Ingolia et al. (2014); Malabat et al. (2015)).

Based on these results, we were interested in detecting any differential translation of monosomes and polysomes within wild-type and between *xrn1Δ* and wild-type. To this end, calibrated QuantSeq libraries generated from monosomal sucrose gradient fractions from *xrn1Δ* & wild-type cell lysate were compared with the input and polysomal libraries (Subsection 3.1.6).

It was immediately apparent from sequencing the monosomal fractions that the overall levels of poly-A mRNA associated with monosomes are much lower than those

of polysomes. Only $3.03 \pm 0.04\%$ of the reads present in the wild-type input are found in monosomal fractions as opposed to around $24.1 \pm 0.2\%$ for polysomal fractions (Figure 3.15A). The picture is similar for *xrn1* Δ where $17.5 \pm 0.2\%$ of input is in the polysomal fractions but only $2.86 \pm 0.2\%$ is present in monosomal fractions. It appears that translation does occur through monosomes but only for a relatively small proportion of transcripts. This is consistent with the Heyer & Moore finding since they did not calibrate their data, meaning that their results apply to relative changes, as opposed to absolute ones (Heyer & Moore (2016)).

Before comparing the behaviour of monosomes and polysomes between *xrn1* Δ & wild-type, we wanted to further validate and expand upon the Heyer & Moore findings (Heyer & Moore (2016)). To this end, the DESeq2 pipeline was run to look for significant changes in relative mRNA abundance between *BY4741* monosomal and polysomal fractions. mRNAs that are specifically enriched in either monosomes or polysomes are listed in Table A.2. GO analysis of the enriched transcripts reveals two general categories that are enriched on monosomes (Table 3.4). Consistent with the findings of Heyer & Moore (Heyer & Moore (2016)), the terms most strongly associated with monosomes are all related to ribosomal integrity and primarily consist of the ribosomal protein genes. As short transcripts, they are naturally more likely to be enriched in monosomes (Heyer & Moore (2016)). However, we also detect enrichment for transcripts encoding mitochondrial outer-membrane proteins. It is unclear why these proteins would be preferentially translated by monosomes but it may be related to the fact that mRNAs for mitochondrial proteins are often localized to the surface of the mitochondrion where unusual translational dynamics may occur (Gadir et al. (2011)).

Heyer and Moore claim that short transcripts, specifically ones shorter than 590

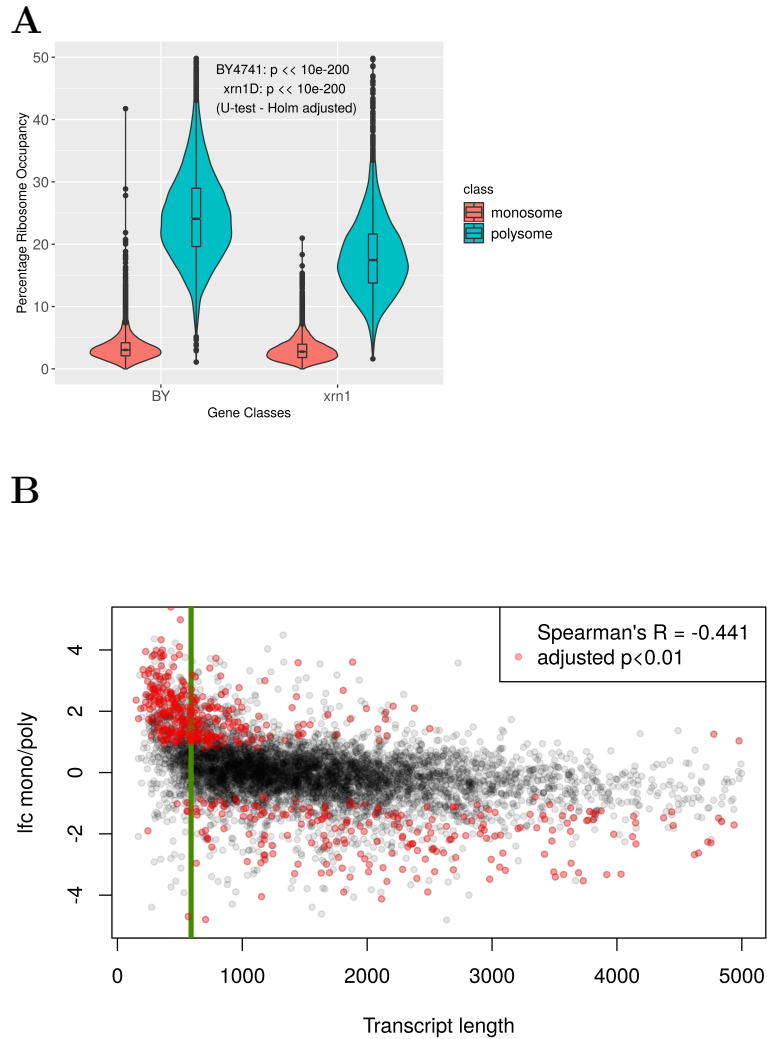
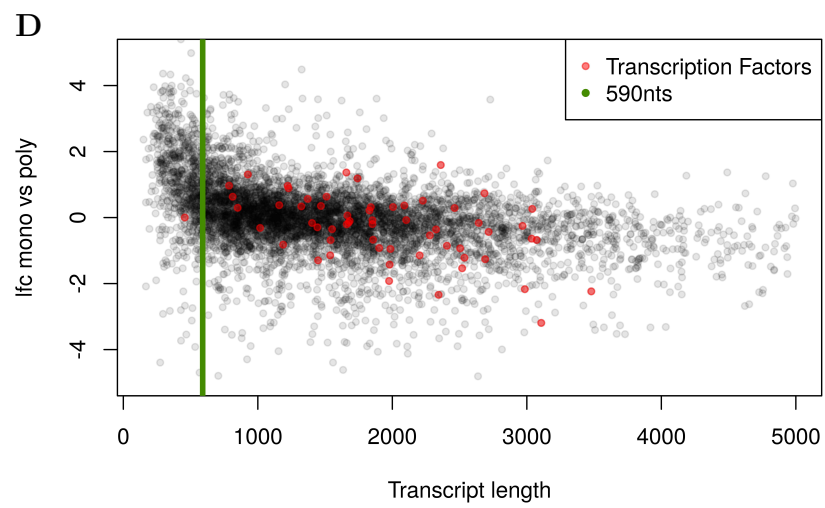
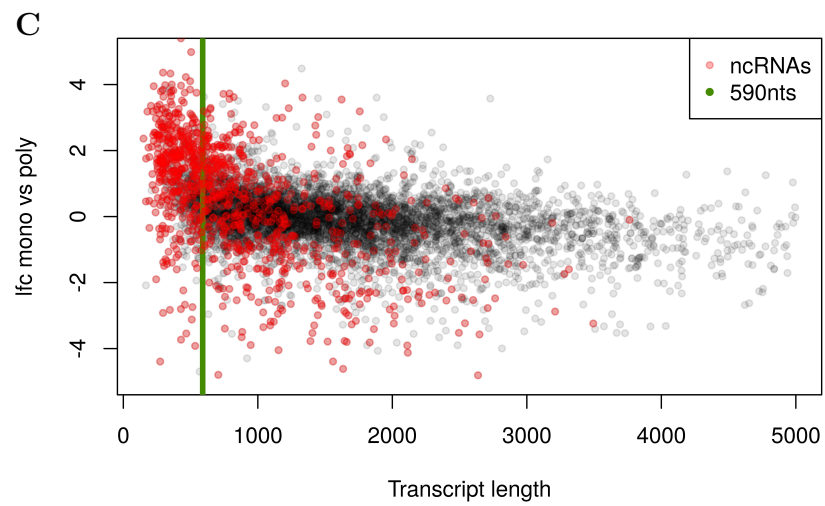


Figure 3.15: The distribution of monosomal poly-A RNAs is consistent with the work of the Moore lab (Heyer & Moore (2016)).

A - Percentage ribosome occupancy considering monosomal and polysomal translation units in *BY4741* & *xrn1*Δ cells. Polysomal and input counts tables from (Figure 3.12B & C) and monosomal libraries spiked with 200 ng *S. pombe* total RNA were calibrated to *S. pombe* library sizes factors calculated using DESeq2 and adjusted per μg of *S. pombe* RNA. Percentage ribosome occupancy was calculated for each transcript as the percentage of calibrated reads in input lysate that were in polysomal fractions ($n = 3$).

B - Anti-correlation between transcript length and enrichment in monosomes versus polysomes.. Log2 fold-enrichment of uncalibrated counts in monosomal fractions versus polysomal fractions from *BY4741* was plotted against transcript length. Transcripts that are significantly enriched in one fraction or the other (DESeq2: $\text{padj} < 0.01$) are highlighted in red and the threshold transcript length identified by Heyer & Moore of 590 nts is signified by a green vertical line (Heyer & Moore (2016)). ($n = 3$)

C & D - No association between monosomal translation and ncRNAs or transcription factors.. Data was plotted as in (B) except that either transcripts annotated as XUTs, SUTs and CUTs (C) or transcription factors (D) were highlighted in red in place the DESeq2 hits. ($n = 3$)



nts, are enriched in monosomes due to their elongation time being generally shorter than their initiation time (Heyer & Moore (2016)). To determine whether this trend is also present in our datasets, transcript length was plotted against $\log_2$ fold-enrichment in monosomes over polysomes (Figure 3.15B). We observe a strong negative correlation between transcript length and enrichment in monosomes (Spearman’s $R = -0.441$) and a particular tendency of very short transcripts (<590 nts) to be enriched in monosomes, confirming the findings of Heyer and Moore.

Heyer & Moore further propose that NMD targets and low-abundance regulatory proteins such as transcription factors are preferentially translated by monosomes. We suspected that “ncRNAs” containing putative short ORFs would be targeted for NMD and this, along with the effects of their short ORF directly would cause them to tend to be translated by monosomes. However, we see no association between status as a SUT, XUT or CUT and monosomal fraction enrichment (Figure 3.15C). Similarly, we see no association of transcription factors with being monosomally associated (Figure 3.15D). It may be that other NMD targets and regulatory proteins are more strongly associated with monosomes. However, our analysis was restricted to these classes of genes due to their interesting behaviour in *xrn1* Δ mutants. Furthermore, for the ncRNA transcripts, it may also be that they are not expressed at sufficient numbers to detect their enrichment in the monosomal fraction in wild-type cells.

Overall however, and particularly for highly-expressed genes, our data corresponds well with that of the Moore lab (Heyer & Moore (2016), Figure 3.15). Therefore, we are confidently able to detect translation by monosomes at low levels in *S. cerevisiae*.

Ribosomal protein genes comprise a set of transcripts that are both tightly co-

GO Term	Cellular Component Description	P-value	FDR q-value	Enrichment	N	B	n	b
GO:0044391	ribosomal subunit	1.72E-09	1.80E-06	3.72	5173	218	172	27
GO:0005840	ribosome	2.06E-08	1.07E-05	3.07	5173	294	172	30
GO:0098798	mitochondrial protein complex	1.40E-07	4.88E-05	3.44	5173	201	172	23
GO:0015934	large ribosomal subunit	4.96E-06	1.29E-03	3.7	5173	130	172	16
GO Term	Cellular Process Description	P-value	FDR q-value	Enrichment	N	B	n	b
GO:0006518	peptide metabolic process	7.46E-07	3.83E-03	2.62	5173	345	172	30
GO:0006412	translation	8.57E-07	2.20E-03	2.72	5173	310	172	28
GO:0043043	peptide biosynthetic process	1.04E-06	1.79E-03	2.69	5173	313	172	28
GO:0043604	amide biosynthetic process	2.07E-06	2.66E-03	2.49	5173	362	172	30
GO:0002181	cytoplasmic translation	4.48E-06	4.60E-03	3.73	5173	129	172	16
GO:0043603	cellular amide metabolic process	6.83E-06	5.86E-03	2.27	5173	424	172	32
GO:0070096	mitochondrial outer membrane translocase complex assembly	8.44E-06	6.20E-03	15.04	5173	10	172	5
GO Term	Cellular Function Description	P-value	FDR q-value	Enrichment	N	B	n	b
GO:0003735	structural constituent of ribosome	1.17E-10	2.85E-07	4.19	5173	194	172	27
GO:0005198	structural molecule activity	4.82E-06	5.88E-03	2.49	5173	338	172	28

Table 3.4: GO analysis of monosome vs polysome DESeq2 hits.

The GORILLA GO analysis pipeline (Eden et al. (2009)) was run on DESeq2 hits from Table A.2 with a GORILLA pvalue threshold of 10^{-5} . Terms significantly enriched in monosomes are displayed for Component, Process & Function. No GO-terms were found to be enriched for polysomes.

regulated in *xrn1Δ* cells and relatively enriched in monosomal fractions (Figure 3.5B & C, 3.9A, 3.13D, 3.4 & Woloszczuk et al. (2016); Heyer & Moore (2016)). Therefore, it was suggested that there may be a link between translation by monosomes and resistance to dysregulation in *xrn1Δ*. To investigate whether deletion of *XRN1* changes the distribution of transcripts between monosomes and polysomes, Xtail was applied to the *xrn1Δ* & *BY4741* monosomal libraries using their respective polysomal libraries as background sets (Table A.3). 120 genes showed significant shifts between the monosomal and polysomal fractions, with the majority (94) shifting towards to polysomal fraction, indicating an increase in the ratio of initiation to elongation. GO analysis of the transcripts that shift most towards polysomal translation in *xrn1Δ* shows an enrichment for genes involved in chromatin remodelling indicating that those chromatin-remodeller transcripts that are translated may have slower elongation rates (Figure 3.16, Table 3.5). In contrast, no GO-terms were identified for transcripts shifting from polysomal to monosomal enrichment in *xrn1Δ*. However, there is a significant upregulation of ribosomal protein genes in *xrn1Δ* monosomal fractions determined Wilcoxon’s signed-rank test (Figure 3.17). Coupled with the decrease in the polysomal fractions, this may reflect a decrease in translation initiation

GO Term	Description	P-value	FDR q-value	Enrichment	N	B	n	b
GO:0045798	negative regulation of chromatin assembly or disassembly	9.31E-07	4.79E-03	100.39	5120	3	51	3
GO:0031452	negative regulation of heterochromatin assembly	9.31E-07	2.39E-03	100.39	5120	3	51	3
GO:0010847	regulation of chromatin assembly	3.70E-06	6.34E-03	75.29	5120	4	51	3
GO:0001672	regulation of chromatin assembly or disassembly	3.70E-06	4.75E-03	75.29	5120	4	51	3
GO:0031445	regulation of heterochromatin assembly	3.70E-06	3.80E-03	75.29	5120	4	51	3

Table 3.5: GO terms associated with transcripts that move from monosomes to polysomes in *xrn1Δ*.

The GORILLA GO analysis pipeline (Eden et al. (2009)) was run on Xtail hits from Table A.3 with a GORILLA p-value threshold of 10^{-5} . Terms significantly enriched for transition from monosomes in *BY4741* to polysomes in *xrn1Δ* are displayed for Process. No GO terms were found to be enriched for transition from monosomes in *xrn1Δ* to polysomes in *BY4741*.

Fraction	median fold-upregulation	95% CI
Input	0.88	0.84-0.92
Polysomal	0.74	0.70-0.78
Monosomal	1.13	1.07-1.19

Table 3.6: Summary statistics for Figure 3.17.

Figures for the median values and 95 % confidence intervals of fold-upregulation ribosomal proteins in input, monosomal and polysomal libraries as plotted in Figure 3.17.

for these genes. These Figures are quantified in Table 3.6.

3.1.8 *xrn1Δ* cells exhibit reduced translation elongation.

A feature of the *xrn1Δ* RiboSeq data that was not expected can be seen by comparing its metagene with wild-type. *xrn1Δ* has a greater proportion of reads which are out-of-phase with the main open reading frame than wild-type (Figure 3.10A & 3.11). These reads might originate from ribosomes that were trapped in unusual conformations as a result of a defect in translation elongation. In support of this, a greater number of transcripts shift from monosomal fractions to polysomal fractions in *xrn1Δ* than the reverse (Table A.3). This is indicative of an increase in the ratio of initiation to elongation rates. Unfortunately, translation elongation rates of individual ribosomes are difficult to measure directly. However, since global ribosome

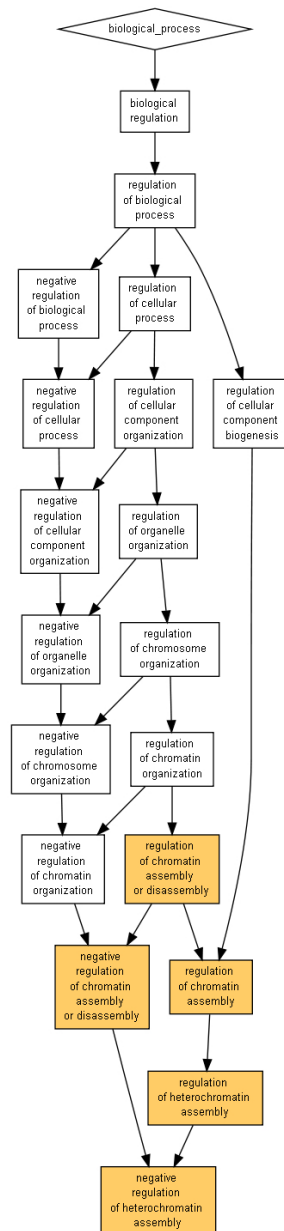


Figure 3.16: Translation of chromatin remodelling genes shifts into polysomes in *xrn1Δ*

GO enrichment of transcripts that shift to polysomes in *xrn1Δ*. Cartoon of interactions between GO-terms associated with transcripts that shift from monosomes to polysomes in *xrn1Δ*. Generated by GORILLA (Eden et al. (2009)). (n = 3).

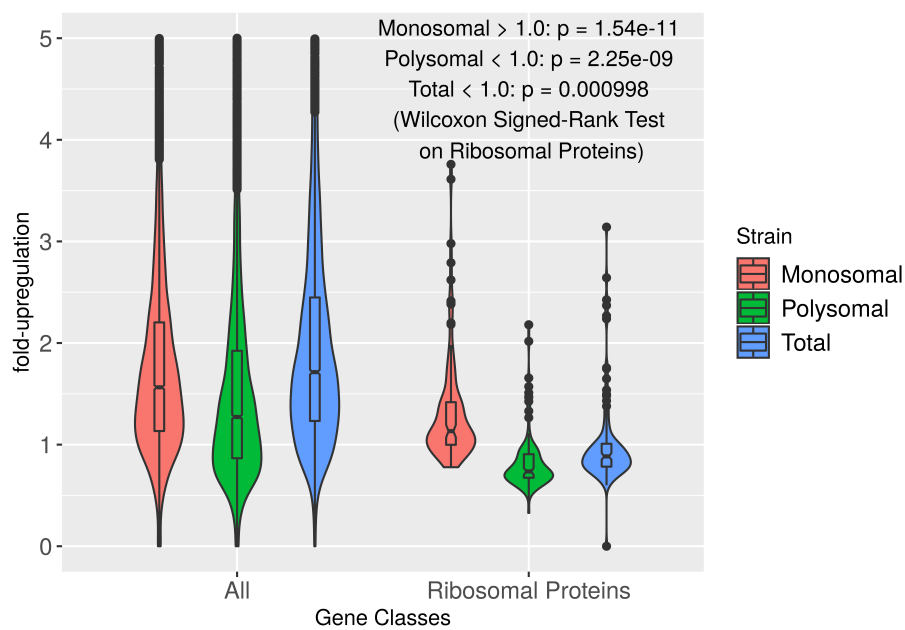


Figure 3.17: Ribosomal protein gene translation shifts into monosomes in *xrn1Δ*.

Ribosomal proteins show greater upregulation in monosomal fractions than polysomal. Fold-upregulation of all transcripts or only ribosomal proteins in *xrn1Δ* displayed as violin plots for input, monosomal and polysomal fractions. ($n = 3$)

occupancy levels are similar between *xrn1* Δ & wild-type, if global protein synthesis rates were lower in *xrn1* Δ , it would necessarily mean that translation elongation was reduced.

The antibiotic puromycin provides a useful way of labelling newly synthesized proteins and thereby measuring total protein synthesis rates. Puromycin is a tyrosyl-tRNA mimetic which can enter the active site of the ribosome and incorporate into nascent peptide chains. Puromycylated peptides can then be detected by western blotting or immunofluorescence microscopy using an anti-puromycin antibody. However, wild-type *S. cerevisiae* is highly drug-resistant and does not readily take up puromycin. Therefore, a method by which to sensitize cells to the drug was required.

In 2014, the Dudley lab presented a set of mutants that are naturally sensitive to puromycin due to deletion of the master transcription factors for drug-resistance *PDR1* Δ alongside an ergosterol biosynthesis gene *ERG6* (Cary et al. (2014)). However, the *erg6* Δ in particular leads to severe cell sickness. *erg6* Δ cells are permeable to sodium and lithium ions (Welihinda et al. (1994)) causing severe mortality during LiOAc transformations. They also exhibit defects in sexual conjugation and tryptophan metabolism (Cary et al. (2014); Gaber et al. (1989)). When combined these deficiencies are result in pleiotrophic phenotypes with the potential to confound the interpretation of experiments and make such strains highly unsuitable for genetic manipulation.

These problems could be overcome by finding a way to temporarily sensitize otherwise wild-type cells to puromycin. Previous studies have used very low concentrations of SDS (0.003 %) to facilitate uptake of Brefeldin A by *S. cerevisiae* (Pannunzio et al. (2004)). This seemed like a promising approach to sensitizing cells to puromycin as

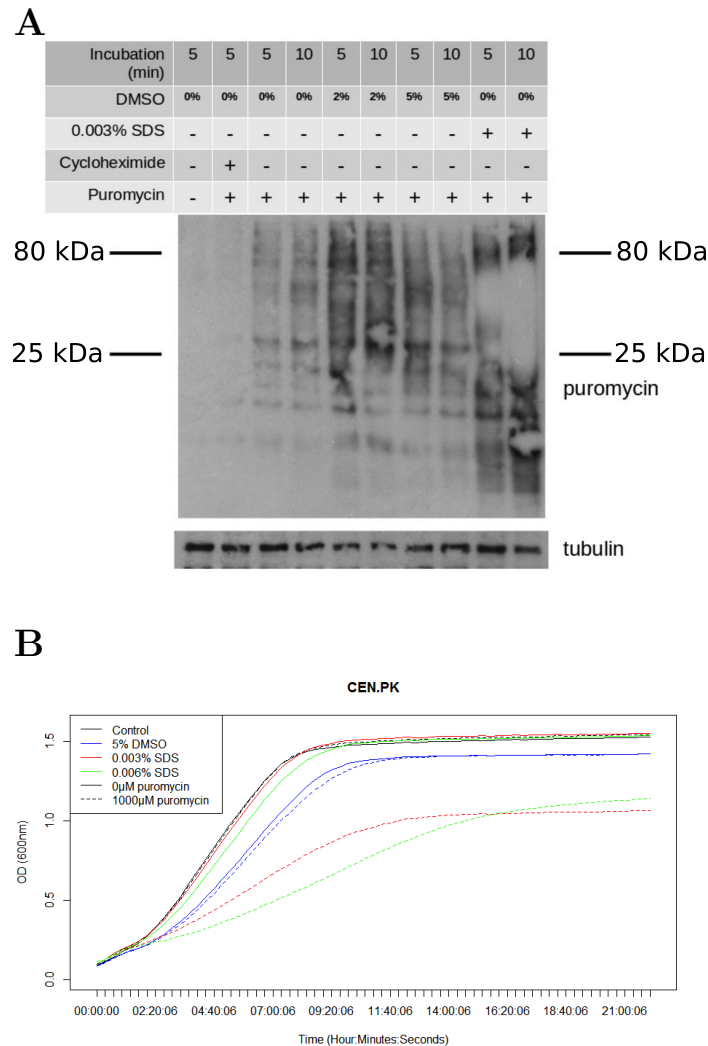


Figure 3.18: Anti-puromycin western blotting reveals a specific reduction in protein synthesis rates in *xrn1Δ*.

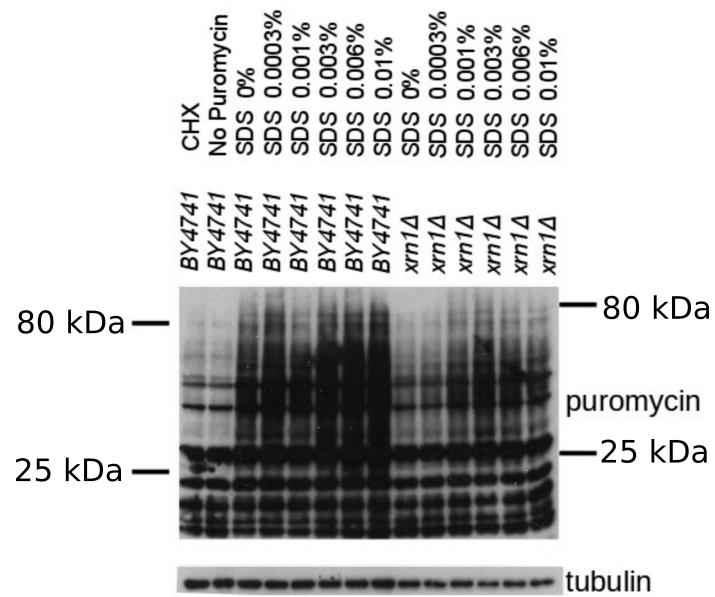
A - SDS and DMSO sensitize cells to puromycin. Log-phase cultures of *BY4741* in YPD were treated with 400 $\mu\text{g}/\text{ml}$ puromycin alongside a permeabilization agent (SDS or DMSO at the indicated concentrations) for the indicated times. Cultures were harvested and probed for puromycin and tubulin by western blotting. ($n = 1$)

B - 0.003 % SDS does not cause slow-growth in the absence of puromycin. A bioscreen growth assay was performed on *CENPK113-7D* cells grown in YPD supplemented with combinations of 400 $\mu\text{g}/\text{ml}$ puromycin, 5 % DMSO, 0.003 % SDS and 0.006 % SDS ($n = 5$).

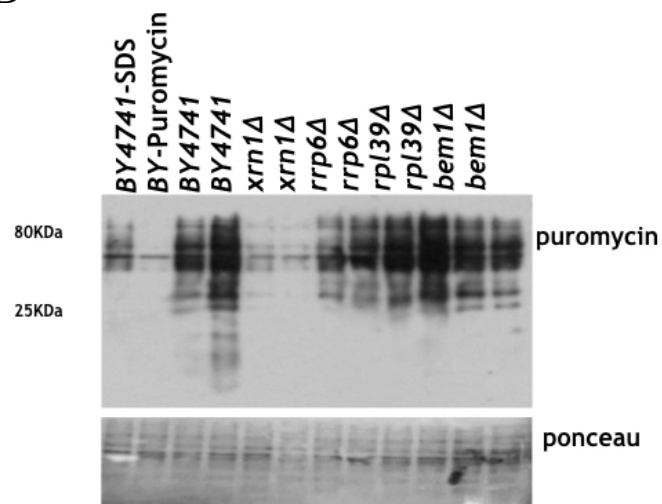
C - 0.003 % SDS fully permeabilizes wild-type & *xrn1Δ*. Log-phase cultures of *BY4741* and *xrn1Δ* in YPD were treated with 400 $\mu\text{g}/\text{ml}$ puromycin and varying concentrations of SDS for 5 min. Cultures were harvested and probed for puromycin and tubulin by western blotting. ($n = 2$, representative blot shown)

D - *xrn1Δ* cells have reduced translational output compared to other slow-growing mutants. The indicated strains were grown in YPD to mid-log phase, labelled for 5 min with 400 $\mu\text{g}/\text{ml}$ puromycin and 0.003 % SDS and analysed by anti-puromycin western blotting. ($n = 6$, representative blot shown)

C



D



well. Both 0.003 % SDS and 5 % DMSO were shown to cause efficient incorporation of puromycin into nascent peptides (Figure 3.18A & C). However, whereas cells grown in the presence of 0.003 % SDS displayed a puromycin-dependant slow-growth phenotype, those grown in the presence of 5 % DMSO exhibited a constitutive growth defect which wasn't enhanced by puromycin (Figure 3.18B). This shows that 0.003 % SDS alone doesn't stress cells but does robustly sensitize them to puromycin whereas 5 % DMSO induces a stress response but cannot permanently sensitize cells to puromycin. Therefore, 0.003 % SDS appears to be the superior permeabilization reagent and was chosen for further assays.

Applying this assay to *xrn1* Δ shows that puromycin incorporation rates are dramatically reduced in *xrn1* Δ relative to wild-type (Figure 3.18C & D). One explanation for this that was proposed was that the *xrn1* Δ strain might simply be more resistant to permeabilization by SDS. However, this appears not to be the case as increasing concentrations of SDS do not increase puromycin incorporation beyond 0.003 % and *xrn1* Δ incorporates less puromycin than wild-type at all SDS concentrations tested (Figure 3.18C). Therefore, it seems that *xrn1* Δ genuinely has a reduced global protein synthesis rate.

That said, there are many potential explanations for such a phenotype. For example, it could be interpreted simply as the result of the slow-growth phenotype of *xrn1* Δ , since slow-growing cells would not require such high rates of protein synthesis to generate biomass necessary for growth. However, other slow-growing mutants that affect RNA degradation (*rrp6* Δ), ribosome-integrity (*rpl39* Δ) & bud morphology (*bem1* Δ) have protein synthesis rates similar to wild-type, ruling out slow protein synthesis as a consequence of slow-growth and further suggesting that it is a defect that's fairly specific to *xrn1* Δ (Figure 3.18D). Since global ribosome:mRNA associ-

ation levels in *xrn1* Δ are similar to wild-type, this result confirms that *xrn1* Δ cells have a specific defect in translation elongation.

3.1.9 Elucidating the mechanism of slow translation elongation in *xrn1* Δ .

It follows that discovering the origin of this slow-elongation phenotype might inform on other aspects of *xrn1* Δ cell biology and perhaps provide insight into the role of *XRN1* in buffering of gene expression.

Broadly speaking, reduction of translation elongation rates in a mutant can be thought of as occurring in two main ways. Either as a regulated decrease in programmed translation rates, perhaps as part of a stress response, or a mechanistic defect in part of the translation reaction itself which causes output to decrease independently from other aspects of *xrn1* Δ cell biology. In the case of *xrn1* Δ , it is easy to imagine that either of these mechanisms might apply. Therefore, investigations into each of these possibilities are currently under way.

A frequently targeted regulatory pathway for translation elongation is phosphorylation of eEF2. Phosphorylation of eEF2 inactivates it, preventing translocation of the ribosome. The only known eEF2 kinase in *S. cerevisiae* is *RCK2* whose activity is responsible for translational repression in response to osmotic stress (Teige et al. (2001)). If *RCK2* signalling is upregulated in *xrn1* Δ then this could explain the observed slow elongation rates. In which case, deletion of *RCK2* should disrupt this signalling pathway and rescue *xrn1* Δ protein synthesis rates. However, this is not observed. *xrn1* Δ *rck2* Δ double mutants are unable to rescue *xrn1* Δ protein synthesis rates (Figure 3.19). Therefore, although it remains possible that alternative kinases might phosphorylate eEF2 in *xrn1* Δ , *RCK2*-signalling at least can be ruled out as

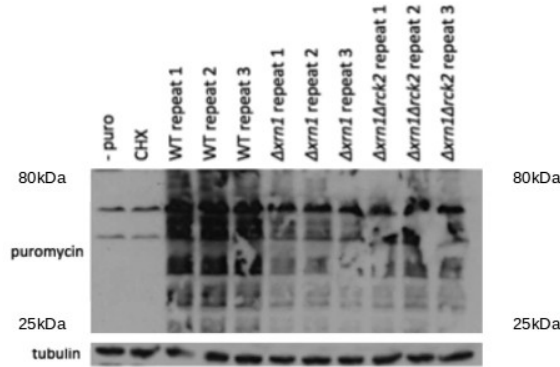


Figure 3.19: *RCK2*-signalling is not the source of slow translation elongation in *xrn1Δ*.

***rck2Δ* cannot rescue translational output in *xrn1Δ* cells.** *RCK2* was deleted in *BY4741* & *xrn1Δ* backgrounds and all four strains were grown in YPD to mid-log phase, labelled for 5 min with 400 μ g/ml puromycin and 0.003 % SDS and analysed by anti-puromycin western blotting. (n=3, representative blot shown)

the mechanism of translational repression in *xrn1Δ*.

Alternatively, the reduced protein synthesis rate in *xrn1Δ* could be the result of a mechanistic defect in translation. It is well established that *xrn1Δ* cells accumulate much higher concentrations of mRNA than wild-type (Sun et al. (2013); Malabat et al. (2015); Woloszczuk et al. (2016), Figure 3.12). This is normally considered from the perspective of the mRNA itself. However, RNA makes up a considerable proportion of cellular nucleobases. Therefore, without compensatory metabolic activities, accumulation of RNA molecules may sequester nucleotides such as GTP from the free NTP pools. Since translation elongation requires GTP hydrolysis (Reviewed in Dever et al. (2016)), sequestration of NTPs could potentially explain the slow protein synthesis in *xrn1Δ*.

A key assumption of this model is that NTP levels are actually lower in *xrn1Δ*. Wild-type and *xrn1Δ* metabolomes were generated to determine the validity of this

Nucleotide (mM)	<i>BY4741</i>	<i>xrn1Δ</i>	fold-change ($\frac{xrn1\Delta}{BY4741}$)
cAMP	0.2	0.05	0.25
CTP	0.5	5	10
ATP	15	60	4
UTP	20	50	2.5
GTP	15	70	4.67

Table 3.7: Depletion of GTP pools cannot explain slow translation elongation in *xrn1Δ*.

B - Nucleotide pools are larger in *xrn1Δ* cells. *BY4741* & *xrn1Δ* cells were grown to mid-log phase, pelleted (2,600 rcf, 4 min, 4 °C) and pellets were flash frozen. Metabolites were extracted using 50 % methanol and analyzed by mass spectrometry. (n = 3)

assumption. Contrary to expectations, NTP levels were actually much higher in *xrn1Δ* than the wild-type (Figure 3.7). In particular, levels of ATP & GTP, the two most important nucleotides for translation, increased by 4 & 4.67-fold respectively. This result rules out the possibility that NTP-depletion is the source of the *xrn1Δ* translational phenotype, although it reveals a new and strong phenotype of the *xrn1Δ* cells, that of NTP upregulation.

Although it is unclear why NTP levels are elevated in *xrn1Δ*, it provides an intriguing explanation for previous results from the Mellor lab (Woloszczuk et al. (2016)). Ronja Woloszczuk, found that *xrn1Δ* cells exhibit rapid transcriptional induction of GAL genes, along with a flatter NET-Seq profile than wild-type which may be interpreted as increased polymerase processivity (Woloszczuk et al. (2016)). Meanwhile, das Neves et al. (2010) have shown that the energy charge of cells directly influences their transcription rates. Since ATP levels are unexpectedly high in *xrn1Δ*, high energy charge could explain the rapid transcriptional responses displayed by *xrn1Δ*. This may be further exacerbated in the *GAL*-inductions performed by Woloszczuk et al. (2016) as high ATP levels have the potential to increase levels of phosphorylation of the transcription factor Gal4p which enhances its activatory capacity (reviewed in

Traven et al. (2006)).

Interestingly, the *xrn1* Δ strain also showed a four-fold reduction in cAMP levels in this experiment (Figure 3.7). This indicates that *xrn1* Δ cells sense lower glucose-availability than wild-type which raises the possibility that the slow growth phenotype of *xrn1* Δ is actually a programmed response to perceived nutrient-availability. This is discussed further in subsection 3.2.5 & 5.5.

3.2 Conclusions.

3.2.1 The importance of mRNA turnover in the Yeast Metabolic Cycle.

It is well established that the majority of the *S.cerevisiae* transcriptome changes in expression during the YMC (Kuang et al. (2014); Tu et al. (2005)). However, the function of these oscillations is poorly understood. Indeed, it has remained unclear whether the YMC is dependant on cycling of mRNA expression at all. To try to address this question directly, I investigated the role of RNA turnover in metabolic cycling.

The YMC is remarkably robust to perturbation of RNA degradation pathways, exhibiting only mild phenotypes in response to the deletion of catalytic components of the 5'->3' and 3'->5' degradation machinery (Figure 3.1). Since this study was unable to find a way to abolish all RNA degradation or completely abrogate exosome activity, RNA degradation cannot be completely ruled out as a means of regulating the YMC. However, it can be confidently said that the 5'->3' degradation pathway is largely redundant for metabolic cycling.

Furthermore, this study shows that toleration of loss of 5'->3' mRNA degradation in *xrn1Δ* by the YMC is not simply because *XRN1* has little activity in this system. All major classes of transcripts are over-expressed in cycling *xrn1Δ* cells, suggesting a global buffering defect as observed previously in log-phase (Sun et al. (2013); Sinturel et al. (2015); Haimovich et al. (2013); van Dijk et al. (2011); Braun et al. (2014); Woloszczuk et al. (2016)) In addition, with the exception of OX phase genes and ncRNAs, all classes of genes show some mis-regulation in time, replacing a coordinated oscillation in expression with much higher frequency expression noise (Figure 3.5B). This phenomenon is also replicated at the levels of individual genes since many RB, RC & non-cycling genes in *xrn1Δ* exhibit poor correlation with wild-type (Figure 3.5C).

Since cells can tolerate mis-expression of many RB & RC genes without loss of metabolic cycling, it implies that these genes are not important for metabolic cycling (Figure 3.5C). However, that isn't to say that all RB & RC genes are unimportant for metabolic cycling. for example, the transcription factor *ADR1* is amongst the most highly correlated of all genes between *xrn1Δ* & wild-type, exhibiting a sharp peak at the RB/RC boundary (Figure 3.6). Its target genes are also tightly correlated between *xrn1Δ* & wild-type indicating that *ADR1* is regulating these transcripts (Figure 3.7). Interestingly though, not all *ADR1* transcripts are co-regulated in time. Many, such as *ADH2* & *ASC1*, peak shortly after *ADR1* transcripts then continue to be expressed throughout RC, indicating that Adr1p is acting as a transcriptional activator of these genes and that it persists on chromatin until early OX phase (Figure 3.7B). Others however, such as *LEU1*, have the opposite pattern (Figure 3.7B). It is unclear what is happening at these genes, whether in certain promoter environments Adr1p can act as repressor or whether other transcription factors are also controlling the expression of these genes leading to different expression patterns. Discovering more about the

activity of *ADR1* across the YMC and elucidating its importance in metabolic cycling would provide an exciting avenue for future study.

Thus far, it is unclear to what extent gene expression controls cycling of metabolism and vice-versa. However, it certainly seems that many of the RB and RC transcripts are not required to cycle for metabolic cycling to be maintained (Figure 3.5C). This might mean that these genes aren't actually involved in establishment of YMC phases and they are simply cycling as a by-product of the cycling of other transcripts. However, an alternative interpretation might be that mis-expression of transcripts is suppressed at the level of translation. Under this scenario, it would not be the cycling of transcripts *per-se* that controls cycling of proteins throughout the YMC but rather cycling of translation and protein degradation rates. Investigations into this model are continued in Chapter 4.

3.2.2 *xrn1*Δ cells tolerate loss of mRNA buffering by limiting translation of stabilized mRNAs.

Loss of buffering of mRNA levels in log-phase and cycling *xrn1*Δ cells might be expected to have disruptive effects on the proteome of these cells. Surprisingly however, the dramatic changes to the transcriptome that were observed generally do not translate to changes in the *xrn1*Δ protein levels (Figure 3.8). This suggests that downstream, compensatory mechanisms must exist to suppress these additional transcripts.

Certainly, genome-wide this appears to be the case. The global increase in total mRNA expression does not result in an overall increase in mRNAs associated with polysomes indicating that total ribosome association with transcripts must be limited by something other than transcript abundance (Figure 3.12A - D).

However, on an individual gene basis, attenuation at the translational level is less consistent. Some of the classes of transcripts identified by Woloszczuk et al. (2016) such as chromatin-remodelling complexes and cell-cycle related genes appear to be well attenuated and have a similar distribution to the overall transcriptome of both *xrn1* Δ & wild-type at the level of translation (Figure 3.9A,3.13B & C). Other classes of transcript including those annotated as “ncRNAs” however, remain amongst the most highly upregulated transcripts within polysomal fractions, although since there is less of a global increase in polysomes, their fold-upregulation is lower than it is for the total cell extract (Figure 3.9A & B,3.13A & E). Therefore, it appears that the cell is able to adequately buffer translation of certain classes of *xrn1* Δ -stabilized transcripts but other, lowly expressed transcripts such as those annotated as “ncRNAs”, along with others are able to escape this suppression. It also seems that this mode of suppression acts at the level of individual mRNA molecules as a reduced proportion of input mRNAs are present in polysomes in *xrn1* Δ (Figure 3.12D).

Unfortunately, due to the low-abundance of proteins produced by escapee transcripts, it has not been possible to determine whether their protein abundances are increased as a result of their elevated translation. However, previous studies have demonstrated early induction of transcription in *xrn1* Δ strains during GAL-induction and release of glucose repression (Woloszczuk et al. (2016); Braun et al. (2014)). This could be interpreted as a result of greater levels of DNA-binding by transcription factors such as Gal4p & Adr1p which would be consistent with an increase in transcription factor abundances. Although it is unclear whether transcription factor levels or DNA-binding are in fact altered in *xrn1* Δ , if this is the case then it does not seem to cause significant defects in the yeast metabolic cycle.

Shah et al. (2013) produced a sophisticated computational model of translation in

S. cerevisiae, accounting for diffusion of mRNAs and ribosomes throughout the cell and considering their relative and absolute abundances. Comparing their model to RiboSeq data they show that in healthy, log-phase cells the rate-limiting factor for translation is ribosome availability. This could explain why in *xrn1* Δ cells, although global mRNA levels increase, levels of polysomal mRNA remain approximately the same. Since ribosomal proteins are amongst the few genes that aren't upregulated in *xrn1* Δ cells (Figure 3.1, 3.9A, 3.13D & Woloszczuk et al. (2016)), it stands to reason that the abundance of ribosomes themselves will be largely unchanged. If ribosome abundance is the limiting factor for translation as Shah *et al.*, propose, then this would necessarily prevent any global increase in ribosome-protected fragments from occurring. Why this phenomenon occurs at the transcript level, affecting the number of mRNAs translated rather than just the number of rpfs per mRNA is unclear (Figure 3.12) but it implies that translation of individual mRNAs exhibits strong positive feedback so that once an mRNA is translated it will be efficiently loaded with ribosomes whereas untranslated mRNAs are unavailable for ribosome loading. This could occur via a number of different mechanisms in cells.

One possibility is that ribosomes are efficiently recycled within closed-loop polysomes as proposed by the Greig lab (Rogers et al. (2017)). In this model, instead of being released to diffuse freely, terminating ribosomes are immediately reloaded onto the same transcript to begin a new cycle of translation, providing a means to ensure that translating mRNAs do so efficiently. However, the universality of the closed-loop model is questionable and several recent studies have presented evidence that it is likely to only apply to short transcripts such as ribosomal protein genes (Thompson & Gilbert (2017); Thompson et al. (2016); Costello et al. (2015)). Therefore, this mechanism can only explain positive feedback for a minority of transcripts.

Alternatively, transcript localization could be the determining factor for positive feedback of translation. Ribosome-associated mRNAs are excluded from subcellular compartments such as P-bodies and other cytoplasmic granules (Teixeira et al. (2005); Hoyle et al. (2007)). This in turn makes them more available for additional rounds of translation. In contrast, untranslated mRNAs have nothing stopping them from partitioning into compartments which are less competent for translation initiation. This automatic partitioning quickly separates mRNAs into translated and untranslated populations. In this model, escapee transcripts would exhibit less of an intrinsic tendency to partition into translationally-incompetent compartments meaning that excess transcripts would still be translated. This model can therefore explain all the translational behaviours observed for mRNAs in this study.

A similar model can be proposed which invokes changes in the composition of mRNPs, either through different subunit compositions or RNA/protein modifications. In this case, the act of translation would alter the mRNP to signal that it should be subject to further rounds of translation whereas a sustained period of no translation would reverse this signal marking an mRNA as untranslated. Potential candidates for such a process might include 4E-BP binding or poly-A tail length.

3.2.3 Ribosomal protein genes are exceptionally resistant to deletion of *xrn1*Δ.

One class of transcripts which has stood out throughout this analysis as being unusually resistant to dysregulation by loss of *xrn1*Δ is the ribosomal protein genes. As a major component of the OX phase gene expression profile, they are amongst the best correlated transcripts between the *xrn1*Δ and wild-type metabolic cycles (Figure 3.5B & C). Similarly, in log-phase they stand out as a tightly grouped cluster of genes which are neither stabilized at the transcript level, nor more highly translated than

they would be in wild-type (Figure 3.9A, 3.13D & Woloszczuk et al. (2016)).

How the transcripts of ribosomal protein genes escape stabilization in *xrn1* Δ is a question for future study. However, a simple explanation might be that they are simply not *xrn1* Δ substrates. One possibility is that they are able to escape Xrn1-mediated degradation due to their recently discovered unusual 5'-cap structure which is likely to prevent normal decapping (Walters et al. (2017)).

If they are indeed not degraded by the 5'->3' RNA-degradation pathway then they must instead degraded by the cytoplasmic exosome. In this study, attempts were made to generate a *dis3-AID*-9xmyc* strain which could have been used to determine this. However, it could not be made over the duration of this study. Future work on the degradation of ribosomal protein gene mRNAs should attempt to block exosome activity by other means, for example by using the exosome channel mutant identified by the Dziembowski lab (Drazkowska et al. (2013)).

3.2.4 *xrn1* Δ cells display defects in translation elongation for an as yet undetermined reason.

Although the RiboSeq and polysome profiling data indicate that there are similar overall levels of translating ribosomes on *xrn1* Δ and wild-type cells (Figure 3.12A - D), rates of puromycylation show that the total translational output of *xrn1* Δ cells is much lower than wild-type (Figure 3.18C & D). Since there are a similar number of ribosomes translating mRNAs but less protein is being produced, this must mean that *xrn1* Δ cells have severe defects in translation elongation. Furthermore, the general shift towards polysomal translation seen in the polysome profiling data (Table A.3) and the decrease in RiboSeq phasing (Figure 3.10A & 3.11) also point towards a reduced rate of translation elongation. Therefore, despite this being a rather unex-

pected finding, it seems to be a genuine and fairly specific feature of *xrn1* Δ cells.

Preliminary investigations into slow elongation in *xrn1* Δ have ruled out several models for how it may be occurring, although the actual mechanism remains elusive. The best-studied mechanism for physiological repression of translation elongation in yeast is by phosphorylation of threonine-57 of eEF2. This reduces its affinity for GTP and association with the ribosome thereby reducing the rate of ribosomal translocation. eEF2-T57 is phosphorylated in *S. cerevisiae* by a single kinase *RCK2* in response to stimuli such as osmotic stress (Teige et al. (2001)). This study shows that deletion of *RCK2* does not rescue protein synthesis rates in *xrn1* Δ (Figure 3.19A) ruling out *RCK2* signalling, and by extension eEF2 phosphorylation, as the cause of slow translation elongation in *xrn1* Δ .

Reduced translation elongation could also be due to deficiencies in the basal rates of the various elongation reactions. GTP hydrolysis provides the main energy source for translocation and tRNA loading, as well as being one of the four bases used as substrates for RNA synthesis. Since mRNA levels are greatly increased in *xrn1* Δ (Sun et al. (2013); Malabat et al. (2015); Woloszczuk et al. (2016), Figure 3.12B), this could effectively sequester cellular guanine bases, limiting GTP availability. However, this study finds that in fact, all NTP levels are greatly elevated in *xrn1* Δ cells, meaning that this explanation cannot be correct.

To date, the cause of slow translation elongation in *xrn1* Δ remains elusive but a number of intriguing possibilities are still to be investigated. Aside from NTPs, the main substrate that is consumed during translation elongation is charged tRNAs. One possibility is that the availability of charged tRNAs in *xrn1* Δ cells is reduced. This would most likely have to be through a decrease in the levels of tRNAs in general

as a decrease in the ratio of charged:uncharged tRNAs would lead to repression of translation initiation through eIF2a-phosphorylation by Gcn2p (Dever et al. (1992, 2016)). Therefore, it would be interesting to directly compare tRNA levels in *xrn1* Δ cells & wild-type in order to determine if their levels are enough to explain the reduction in elongation.

Alternatively, eEF1a, the GTPase responsible for tRNA loading, is also the target of numerous post-translational modifications including serine-phosphorylation, lysine methylation and glutamate glutaminylation. These modifications may be able to modulate its activity. Both methylation and glutaminylation are fairly static in *S. cerevisiae* and eEF1a in which all four methylatable lysines have been mutated to arginine exhibits no notable phenotypes in *in vitro* & *in vivo*. (Cavallius et al. (1997)). Similarly, mutational studies show that glutaminylation is not required for the normal functions of eEF1a either, as measured by growth under a variety of stress conditions (Jank et al. (2017)). As such, it seems unlikely that changes in one of these modifications is causing the dramatic phenotype seen in *xrn1* Δ , although since these studies did not measure puromycylation rates, it cannot be ruled out. In contrast, although eEF1a phosphorylation has not been highly studied in *S. cerevisiae*, in human cells, phosphorylation of S300 has clear effects on translation. If this modification is also present in *S. cerevisiae* it could provide a promising new avenue for future study.

Another translation elongation factor which could explain the defects in *xrn1* Δ is *HYP2* (eIF5 α). The role of *HYP2* in facilitating translation of “PPP” motifs is well studied but recently it has also been shown to be required for translation elongation in general (Schuller et al. (2017)). Hyp2p is usually considered to be a constitutively-expressed protein. However, this study finds both Hyp2p and Tef1p (eEF1a) to be

moderately down-regulated in *xrn1* Δ cells (Table A.1), raising the possibility that sub-optimal levels of one of these factors is what is limiting translation elongation in *xrn1* Δ cells.

More speculatively, this study finds a large reduction in translation of *GIP3* in *xrn1* Δ cells (Table 3.3). As a regulator of *GLC7*, *GIP3* has an important role in Snf1-signalling, a process known to be disrupted in *xrn1* Δ cells (Braun et al. (2014)). An intriguing possibility is that binding of Gip3p to the ribosome is required for efficient translation elongation. This could be an exciting avenue for future study.

3.2.5 Insights into buffering of mRNA levels by *XRN1*

One of the most enigmatic properties of *XRN1* is its role in the buffering of gene expression. Although many studies have shown that *XRN1* is required to couple RNA synthesis and degradation rates, no single unifying model for how this occurs has come to dominate (Sun et al. (2013); Sinturel et al. (2015); Haimovich et al. (2013); van Dijk et al. (2011); Braun et al. (2014)).

The Morillon lab alone has proposed several conflicting models for how *XRN1* activity could signal back to transcription. In their first paper, they identified a new class of antisense RNA in *S. cerevisiae* known as Xrn1-Unstable Transcripts or XUTs (van Dijk et al. (2011)). They propose that XUTs shuttle between the nucleus and the cytoplasm allowing them to sample Xrn1p activity. In the nucleus they interact with the promoter of their sense pair, silencing its transcription in an H3K4_{me1/2}-dependant manner.

This model is entirely dependent on inhibition of transcription of XUT sense-

pairs by H3K4_{me1/2} an observation that is not reproducible. *SPP1* is a subunit of SET1C required for deposition of the vast majority of H3K4_{me3} in *S. cerevisiae* but unnecessary for H3K4_{me1/2} deposition (Morillon et al. (2005); Schneider et al. (2005)). Consequently, *spp1*Δ cells exhibit a global reduction in H3K4_{me3} levels and increase in H3K4_{me1/2}, including at XUT sense-pairs. According to the Van Dijk model, *spp1*Δ cells should show widespread reduction in transcription and transcript levels. However, only 23 genes show a reduction in transcription in *spp1*Δ cells and in fact a greater number are up-regulated (57) (Howe et al. (2017)). Therefore, transcriptional repression by XUT-dependent H3K4_{me1/2} cannot explain the changes in transcription in *xrn1*Δ cells.

In their second paper, taking the *OCA2/POR2* locus as a model system, they propose a cytoplasmic mode of repression at the level of translation, where base pairing of sense and antisense transcripts leads to ribosome stalling and consequent no-go decay (Sinturel et al. (2015)). However, the *xrn1*Δ proteome shows no change in Oca2p levels meaning if *OCA2:POR1* hybridization is occurring, it is not repressive to Oca2p expression (Figure 3.8). Furthermore, Woloszczuk et al. (2016) show that of the 1500 transcripts with overlapping 3'-UTRs in *S. cerevisiae*, only two show reductions in mRNA levels in *xrn1*Δ meaning that they cannot be efficient targets of No-Go decay.

The Choder lab also proposes direct promoter interactions that signal from RNA degradation back to transcription. In their model, after degrading a transcript, Xrn1p itself enters the nucleus and locates the promoter of the transcript that it just degraded where it acts as an activatory transcription factor (Haimovich et al. (2013)). Haimovich et al., do not provide a detailed mechanism for promoter identification or Xrn1p transcriptional activity but demonstrate that it is dependant on Xrn1p cat-

alytic activity. However, Woloszczuk et al. (2016) observe accelerated induction of *GAL* genes during galactose induction in *xrn1Δ* cells, a result that is inconsistent with Xrn1p acting as a global transcriptional activator.

Through a large cDTA screen, Sun et al. (2013) presented a more indirect model for buffering by *XRN1*. They show that not only do *xrn1Δ* cells have a decreased mRNA degradation rate but they actually have an increased mRNA synthesis rate and the combination of these leads to an increase in total mRNA levels. This is in contrast to all the other RNA degradation and transcription mutants that they tested in which changes in degradation are matched by a changes in synthesis and vice versa, resulting in no overall changes in mRNA levels. They propose a simple feedback loop whereby Xrn1p degrades its own mRNA autoregulating 5'->3' overall mRNA degradation rates. On top of this, it feeds back to transcription by degrading transcription factor mRNAs such as *NRG1*. However, *NRG1* is a transcriptional repressor so its stabilization in *xrn1Δ* should reduce transcription rates. This conflicts with the *xrn1Δ* cDTA data which shows an increase in synthesis rates, rendering their model internally inconsistent. Furthermore, Woloszczuk et al. (2016) have observed delayed induction of ribosomal-protein genes in *xrn1Δ* showing that *XRN1* can act as a transcriptional activator under certain conditions. Indeed, it seems that the effects of *XRN1* on transcription are nuanced, gene and condition-specific and likely to be indirect.

Braun et al. (2014) propose that buffering by *XRN1* involves complex interactions with *SNF1*. Snf1p phosphorylates the C-terminus of Xrn1p de-repressing it and driving degradation of *ADH2* transcripts. Meanwhile, *XRN1* is required for the viability *reg1Δ* cells which possess constitutively-active Snf1p. They do not present a specific mechanism which would mediate these effects but their data is consistent

with an indirect role for *XRN1*. *xrn1Δ* might stabilize transcription factor mRNAs that are normally repressed in the presence of glucose such as *ADR1* (Young et al. (2003)). Indeed, Adr1p is significantly upregulated in *xrn1Δ* cells (Figure 3.8). This is consistent with previous data from the Mellor lab (Woloszczuk et al. (2016)) which shows an early induction of *GAL* genes in *xrn1Δ*.

To reconcile the many existing studies with one another and with our own data, we propose an indirect role for *XRN1* in transcription. In our model, deletion of *XRN1* stabilizes numerous activatory and repressive transcription factor mRNAs, increasing expression of their protein products. This leads to changes in the relative expression levels and induction kinetics of various different transcripts and explains the gene-specific changes in mRNA levels widely observed.

The low cAMP levels in *xrn1Δ* cells point to an additional potential mechanism of control. In *S.cerevisiae*, cAMP signalling integrates intracellular and extracellular glucose levels as cAMP synthesis by adenylate cyclase activity is dependant on both GPCR activation by extracellular glucose and intracellular hexokinase activity (Roland et al. (2000)). Therefore, reduced cAMP levels could indicate altered Hxk2p activity. Since Hxk2p activity is also repressive to Snf1p, this could explain both the reduced glucose repression, as this is mediated by *SNF1* and PKA signalling, and the requirement for *XRN1* in *reg1Δ* cells as Hxk2p activity (inferred from cAMP levels) might provide compensatory repression of Snf1p. These changes in intracellular signalling could enhance the rapid induction of *GAL* genes in *xrn1Δ* cells (Woloszczuk et al. (2016)) and potentially explain the well documented slow-growth phenotype.

Changes in transcription factor expression levels alone are unlikely to explain the global increase in transcription rates reported by Sun et al. (2013) and the

widely reported increase in global mRNA levels (Sun et al. (2013); Malabat et al. (2015); Woloszczuk et al. (2016), Figure 3.12B). Nonetheless, the results of the *xrn1* Δ metabolome provide a potential explanation for this long-standing, enigmatic phenomenon. *xrn1* Δ cells exhibit elevated NTP levels relative to wild-type which could potentially have dramatic, activatory, effects on transcription. Most straightforwardly, NTPs are the direct substrates for mRNA synthesis so increasing their concentration will increase the rate of substrate binding and catalysis by RNAPII. Although, since intracellular NTP concentrations lie within a range of 0.5 - 20 mM in wild-type, log-phase cells (Figure 3.19B), which is much greater than RNAPII K_M of 70 μ M, NTP availability is unlikely to be limiting for RNAPII transcription (Kaplan et al. (2008)). An alternative possibility is that elevated ATP concentrations might increase the activity of chromatin remodelling complexes which may lead to an increase in chromatin accessibility. If either of these changes are occurring, this could explain the increase in mRNA synthesis in *xrn1* Δ cells. Certainly, correlation between transcription rates and ATP levels have already been shown in mammalian cells so it would be reasonable for this to also occur in yeast (das Neves et al. (2010)).

3.2.6 Summary

xrn1 Δ cells are able to tolerate their additional transcripts by suppressing their translation. Therefore, the phenotypes exhibited by the *xrn1* Δ strain may be primarily of metabolic origin. Slow growth and loss of glucose repression could be the result of a reduction in cAMP signalling, indicative of a failure to absorb glucose or measure glucose availability. Likewise, the increase in transcript levels itself may be caused by an excess of NTPs, leading to chromatin decompaction and greater promoter accessibility. Coupled with impaired 5'->3' degradation, this provides one explanation for the global increase in transcript levels in *xrn1* Δ .

What causes these metabolic disturbances is unclear, although expression of Pfk1p, a subunit of phospho-fructokinase-1 (PFK1) which is the rate-limiting enzyme in glycolysis, is reduced in *xrn1* Δ cells (Figure 3.8). Furthermore, PFK1 is inhibited by ATP (Rodicio et al. (2000)). Since ATP levels are elevated in *xrn1* Δ , this could be having an inhibitory effect on glycolysis which would then perturb cAMP/PKA & Snf1 signalling leading to many of the observed phenotypes.

Inevitably, this leads to the question of what causes elevated NTP levels in *xrn1* Δ cells? One possibility is that it is a compensatory mechanism to deal with the metabolic consequences of impaired mRNA degradation. In the absence of *XRN1*, a major route for recycling of the nucleotides consumed to produce mRNA is closed off. *xrn1* Δ cells might be able to detect the resulting reduction in nucleotide turnover directly, possibly as a reduction in NMP levels (although it is not clear to what extent those are determined by mRNA degradation rates). In response, nucleotide biosynthesis pathways might be upregulated in order to increase *de novo* NTP biosynthesis and this may be what leads to elevated cellular NTP levels. This hypothesis is highly speculative but it may comprise an interesting avenue for future study.

Another question that arises from a metabolic explanation of the *xrn1* Δ phenotypes is why these disturbances do not lead to major defects in the yeast metabolic cycle? During the yeast metabolic cycle, cells are in a very different metabolic state than during log-phase. Extracellular glucose levels are undetectable (Chen & McDonald (1990)) and levels of diverse metabolites including glycolytic intermediates and ATP are low and fluctuate over time (Tu et al. (2007)). Such a fundamentally different metabolic state may prevent NTP levels from accumulating in *xrn1* Δ and disrupting cellular metabolism. Generation of an *xrn1* Δ metabolome across the YMC

and comparing it to wild-type should be a priority for future work.

The findings presented in this chapter have profound implications for how the YMC is regulated. Contrasting with the prevailing view in the literature (Tu et al. (2005); Kuang et al. (2014); Rao & Pellegrini (2011); Kuang et al. (2017)), we find that tight temporal regulation of transcript levels is not required for metabolic cycling (Figure 3.1, 3.5, 3.6 & 3.7). This calls into question the widely held assumption in the field that cycling of mRNA levels is transmitted to the protein level leading to dynamic changes in the proteome. These ideas are explored further in Chapter 4 where the impact of metabolic cycling on the proteome is investigated.

Chapter 4

Assessing the role of translation in regulation of the Yeast Metabolic Cycle.

4.1 Results

4.1.1 Cycling of mRNA levels during the YMC is not reflected at the level of the proteome.

During the YMC, levels of over 60 % of cellular mRNAs cycle (Tu et al. (2007)). It has often been assumed that cycling of mRNA levels during the YMC is what drives cycling of metabolism through changes in gene expression (Tu et al. (2005); Kuang et al. (2014)). However, this study challenges these assumptions, finding that severe disruption of mRNA turnover pathways has little to no effect on YMC establishment or maintenance, despite having profound effects on regulation of transcript levels (Figure 3.1, 3.5 & 3.6). This begs the question of whether cycling mRNA levels are actually causal for metabolic cycling. Cycling transcripts are most likely to exert phenotypic consequences via their protein products. Therefore, in order to determine whether the cycling of mRNA levels during the YMC is reflected at the protein level, a series of proteomics experiments were performed across the yeast metabolic cycle.

Although only a minority of cells divide during each metabolic cycle, those that do

are synchronized in the cell cycle (Chen & McDonald (1990); Burnetti et al. (2016); Slavov & Botstein (2011); Tu et al. (2005)). Therefore, it is expected that cell number and volume will oscillate with some degree of amplitude during the YMC, and in turn that total protein levels may not be constant during the YMC. As our proteomics experiments lack internal calibration, it was critical to ensure from the outset that this was not a major source of variation between time points. To ensure that total protein levels were not changing dramatically during the metabolic cycle, SDS-PAGE was performed on whole-cell extracts from 1 ml of cycling cells and gels were stained with Coomassie blue (Figure 4.1). Coomassie staining clearly demonstrates that global protein levels per unit volume remain constant during the YMC (ANOVA $p = 0.227$). Therefore, uncalibrated mass-spectra can be safely interpreted as reflecting absolute changes in protein levels.

With this knowledge in hand, a preliminary dataset of label-free mass-spectra across 6 time points, ranging from the RC/OX-transition to early RC, was generated in collaboration with the Mohammed lab (Figure 4.2). This range was chosen as it covers many of the most dramatic transitions at the metabolite and mRNA level, as well as the initiation of DNA replication (Tu et al. (2005, 2007); Kuang et al. (2014), Figure 3.5). It seemed likely that if any changes in the proteome occurred, they would be most dramatic there.

Comparison of the behaviours of genes categorized by the Boeke YMC clusters (Kuang et al. (2014)) shows that on a cluster level there is no relationship between changes in gene expression at the transcript level and changes at the protein level. In fact, there were no coordinated changes in protein levels of individual Boeke clusters whatsoever. Therefore, it appears that cycling mRNA levels during the YMC do not reflect cycling of gene expression at the protein level throughout OX & RB phase.

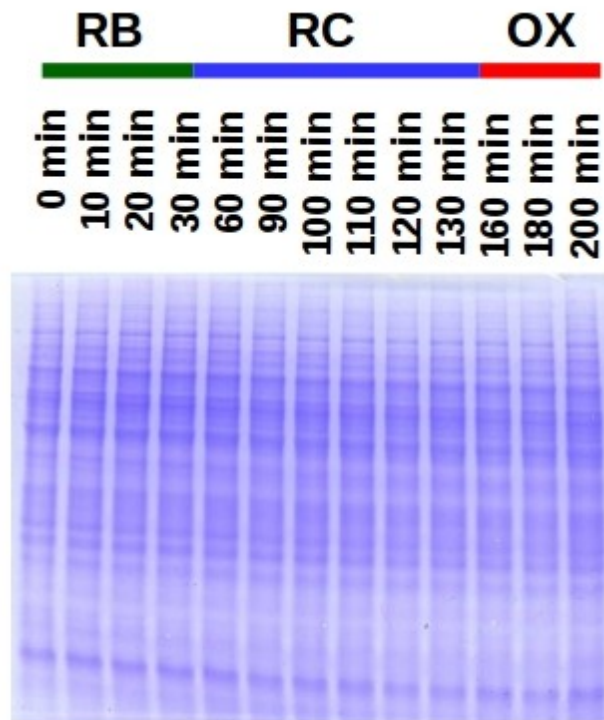


Figure 4.1: No global change in protein levels across the YMC.

Total protein levels do not fluctuate during the YMC. 1 ml samples from the cycling *CENPK113-7D rpb3-FLAG* cells were incubated with 0.003 % SDS and 400 $\mu\text{g}/\text{ml}$ puromycin for 5 min, harvested by centrifugation (16,100 rcf, 15 s) and frozen in $\text{N}_2(l)$. Whole-cell extracts were prepared, run on an SDS-PAGE gel and stained using Coomassie blue. Time points correspond to minutes relative to the point of lowest $\text{dO}_2\%$. Lane intensity was quantified using ImageJ and analyzed by ANOVA ($p = 0.227$, $n = 3$, representative gel shown. Experiment performed by Anna Townley).

This was a highly unexpected finding which has profound implications for regulation of the YMC. If cycling of mRNA levels is not affecting the protein complement of cells then in turn, it cannot be global changes in gene expression patterns that define YMC phases. Instead, phase-transitions must be regulated by modulation of the activity of pre-existing proteins, for example through post-translational modifications or allosteric binding of signalling molecules and metabolites such as cAMP or fructose-2,6-bisphosphate. Furthermore, decoupling of protein and transcript levels from one another requires extensive regulation of gene expression at the protein level,

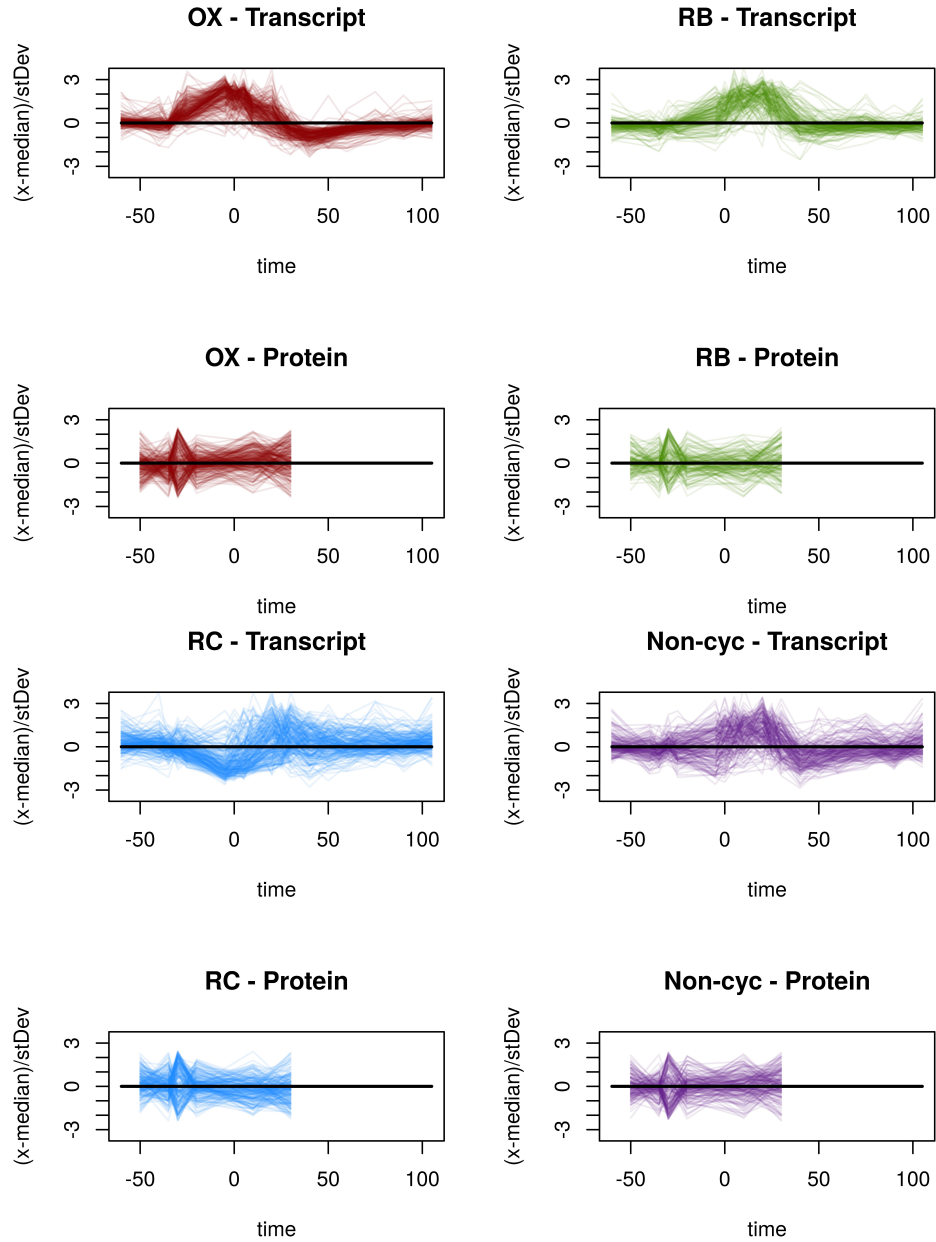


Figure 4.2: Protein levels do not cycle according the Boeke clusters (Kuang et al. (2014)).

Boeke YMC clusters do not cycle at the protein level. 1 ml samples were harvested from the YMC at the indicated timepoints relative to the point of lowest $dO_2\%$ in that cycle. Cells were then lysed and processed for label-free mass-spectrometry. Raw abundances for each protein were normalized by subtracting the median intensity over time and dividing by the standard deviation over time. Proteins were then split into YMC clusters & products of non-cycling genes and normalized expression at the RNA and protein level over time was plotted ($n = 1$).

either through global, or specific regulation of protein turnover.

However, the initial proteome only covered a fraction of the total metabolic cycle and was limited to a single biological replicate. In order to validate and expand upon it, a second, larger, tandem mass tag (TMT) labelled, dataset was also generated. The increase in temporal resolution and addition of TMT-labelling validate and strengthen the data from the initial proteome. Clustering proteins by cycling of their transcripts in the Boeke dataset (Kuang et al. (2014)) shows no consistent cycling of protein levels (Figure 4.3). Therefore, it is clear that cycling of mRNA levels during the YMC does not lead to dynamic changes in protein levels and YMC phases are not primarily defined by broad changes in their protein complement.

4.1.2 Global translation is low during the YMC but individual mRNAs do not appear to be translationally regulated.

Since cycling of YMC clusters at the transcript level does not appear to lead to cycling of the proteome, steady-state protein levels must be regulated at either the level of translation, protein degradation or both (Figure 4.4).

There are three general mechanisms by which this may occur. Firstly, total levels of translation of a gene may be unaffected by transcript cycling and remain constant throughout the YMC. For example, the additional transcripts produced may be retained in the nucleus or in cytoplasmic granules, leaving only a constant, basal, level of RNA available for translation (Figure 4.4A). This would mute cycling of gene expression upstream of protein synthesis leading to constant steady-state protein expression regardless of protein turnover rates.

Alternatively, genes could be translated at, or close to, rates that reflect their

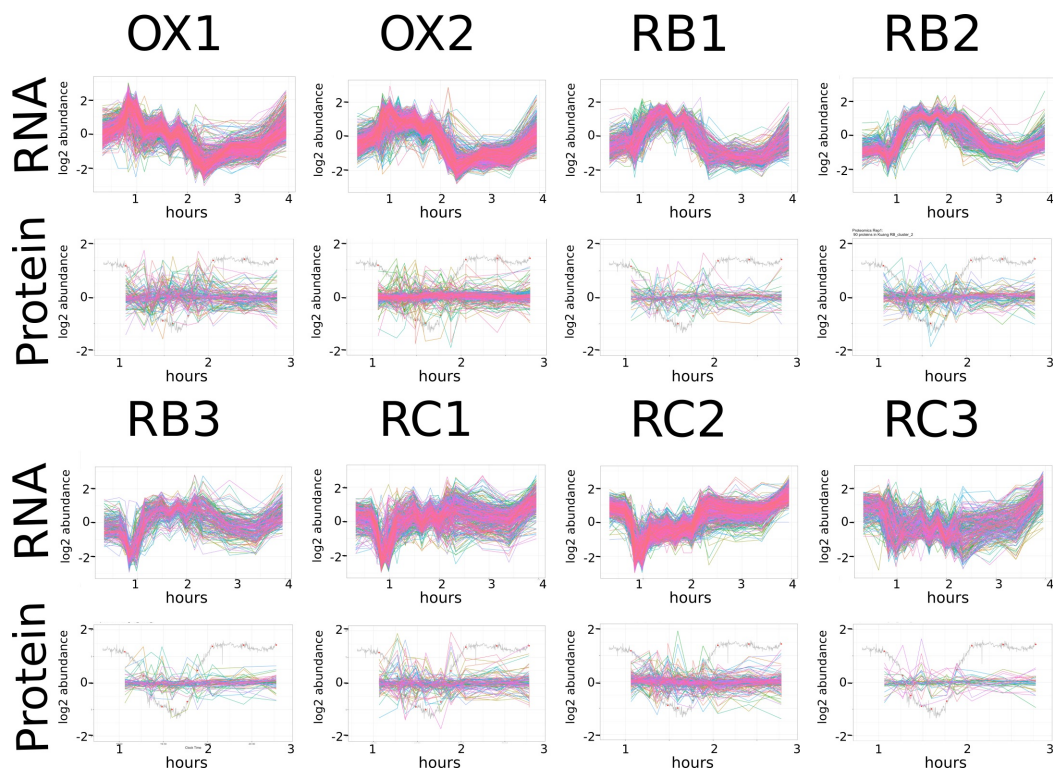
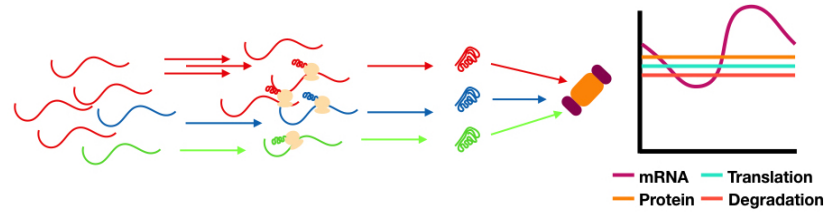


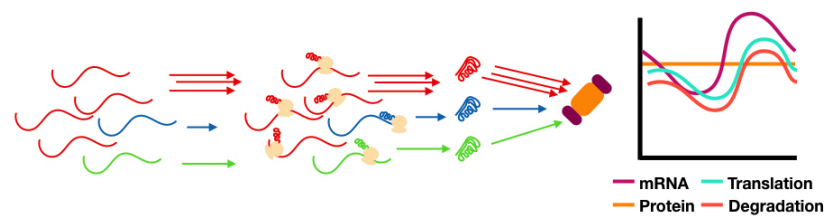
Figure 4.3: There is no association between cycling at the RNA level and cycling at the protein level.

TMT-labelled mass-spectra separated by the tight Boeke YMC clusters (Kuang et al. (2014)). 1 ml samples were harvested from the YMC at the indicated timepoints relative to the point of lowest $dO_2\%$ in that cycle. Cells were then lysed and processed for TMT-labelled mass-spectrometry. Raw abundances for each gene normalized to $\log_2$ -abundances and genes were split into YMC clusters & products of non-cycling genes. Normalized expression at the RNA and protein level over time was then plotted ($n = 1$). $dO_2\%$ levels are shown on the protein panels in grey (Data generated by Shidong Xi, analysis performed by Charlotte George)

A



B



C

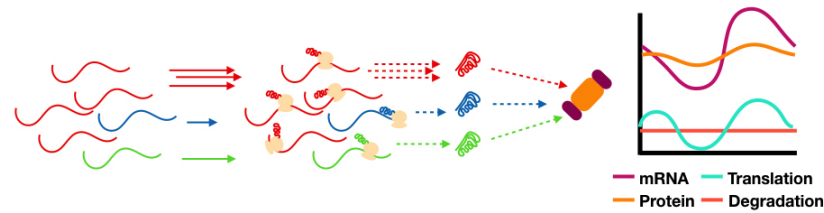


Figure 4.4: Models for the decoupling of mRNA and protein levels during the YMC.

Representation of the relative rates of processes that determine the steady-state protein levels of a model OX phase gene, during OX phase and throughout the YMC, according to three different models for the decoupling of mRNA and protein abundances. Additional arrows represent relative upregulation of a process and dotted arrows represent global downregulation of a process. Similar behaviour would be predicted for RB & RC genes but with their expression peaking at their respective phases of the cycle.

A - Translational suppression of cycling.. Cycling of mRNA levels is muted at the level of translation. Rates of protein synthesis remain constant meaning that protein levels do not cycle.

B - Suppression of cycling by regulated protein degradation. Translation is unregulated meaning that it cycles in synchrony with mRNA levels. As such, protein production rates oscillate throughout the YMC. However, changes in synthesis rates are matched by active degradation of proteins meaning steady-state levels remain constant.

C - Suppression of protein cycling by low protein turnover. There is no regulation of translation or protein degradation for individual genes. However, synthesis and degradation rates are globally very slow, meaning that only a small proportion of the proteome is turned over each cycle. Protein levels do not remain truly constant but the amplitude of their oscillation is too low to detect or to have a major physiological impact.

transcript abundances. Rates of protein synthesis for individual genes would then cycle in synchrony with mRNA levels. In this case, there are two potential explanations for why steady-state protein levels would be largely unaffected by cycling mRNA levels. One possibility is that the cell is somehow able to couple protein synthesis and degradation rates on a single-gene, or at least single YMC cluster, level (Figure 4.4B). In this case, when transcript levels, and by extension protein synthesis rates, rise, protein degradation rates would rise to an equal extent. This would mean that cycling of mRNA levels reflected cycling of protein turnover rates without affecting steady-state protein levels.

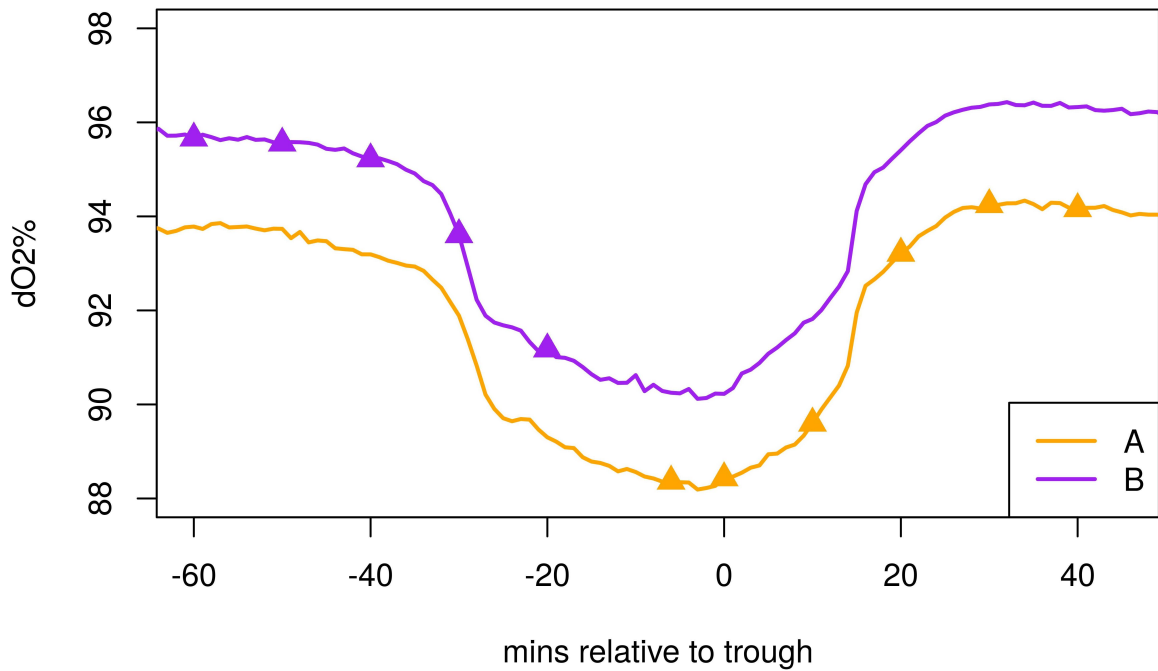
Finally, protein turnover rates could simply be exceptionally slow such that the $t_{\frac{1}{2}}$ of most proteins is much longer than YMC period (Figure 4.4C). In this model, steady-state protein levels are not truly constant across the cycle but the low synthesis and degradation rates simply mean that the amplitude of the oscillation is too small to detect or to have an impact on cellular physiology over short timescales. This model would allow cells to conserve energy under nutrient-limiting conditions by limiting protein turnover.

The first model can be distinguished from the second two by measuring levels of translation of individual genes across the YMC. To measure ribosome association with mRNAs genome-wide, polysome profiling was performed on 11 time points across two metabolic cycles as shown in Figure 4.5A.

From the A_{256} traces of the polysome gradients, it was immediately obvious that translation during the YMC is profoundly different to log-phase (Figure 4.5B). Whereas polysome profiles from log-phase cells contain large peaks for disomes and higher M_W polysomes, peaks corresponding to polysomes were virtually undetectable

A

YMC sampling times



B

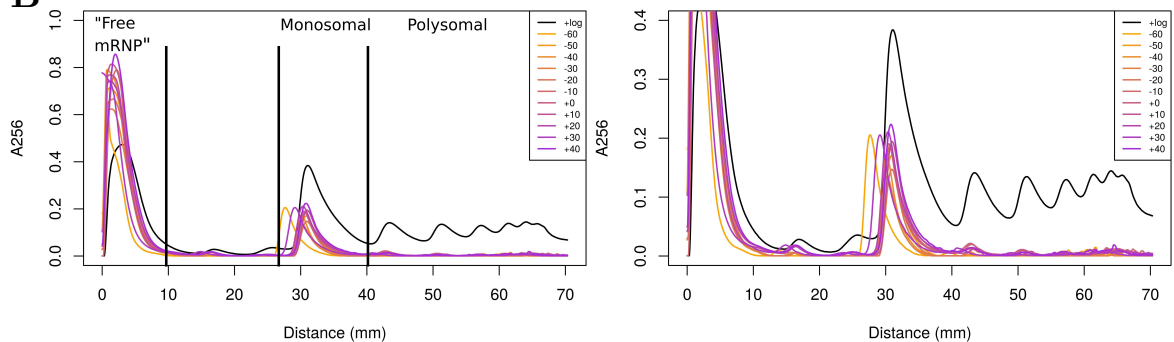


Figure 4.5: Virtual absence of polysomes throughout the YMC.

A - Sampling times for polysome fractionation across the YMC. 1 ml samples were taken from the YMC, lysed and processed for polysome fractionation at the times indicated. Samples were taken across two separate cycles (A & B) due to limited centrifuge capacity during processing. (n = 1)

B - Absorbance traces for polysome fractionation across the YMC. Absorbance traces were recorded during fractionation of the gradients in (A). Fractions were pooled as indicated to produce, "Free mRNP", monosomal & polysomal fractions. A log-phase sample was processed alongside these as a reference for log-phase polysome occupancy and to act as a positive control for polysome integrity. A₂₅₆ traces are shown at two different y-axis scales to allow for clear visualization of both "free-mRNP" (left) and polysomal (right) fractions.

in profiles from the YMC (Figure 3.12A & 4.5B). However, a log-phase sample processed in parallel to the YMC samples maintained normal polysome levels showing that this was not due to a general loss of polysome stability during sample processing. As such, it is reasonable to assume that these traces reflect the genuine translational status of cycling cells. Therefore, the translation initiation:elongation ratio must be far lower during the YMC than during log-phase as few, if any, transcripts are associated with more than one ribosome at a time which implies that elongation and termination of translation are completed before initiation of a second polypeptide chain can occur.

In order to measure levels of translation of individual genes, QuantSeq libraries were generated from monosomal and untranslated “free mRNP” (Figure 4.5B - Distance <10mm) fractions of the sucrose gradients. Unsurprisingly, given the A_{256} traces (Figure 4.5B), polysomal QuantSeq libraries could not be generated from YMC samples in this study.

Before assessing the behaviour of translation during the YMC, it was important to confirm that the behaviour of the mRNA pool in samples processed for gradient-fractionation was consistent with existing YMC datasets. In the absence of input libraries, the “free mRNP” libraries were used for this purpose. “Free mRNP” libraries from gradient-fractionated QuantSeq datasets across the YMC generally correlate well with their corresponding time points in existing YMC QuantSeq data (Figure 4.6A). Furthermore, individual YMC clusters show very similar profiles across the cycle between the “free mRNP” dataset and existing data (Figure 4.6B). Therefore, it appears that gradient-fractionation does not cause excessive biases to library-preparation that might distort YMC profiles.

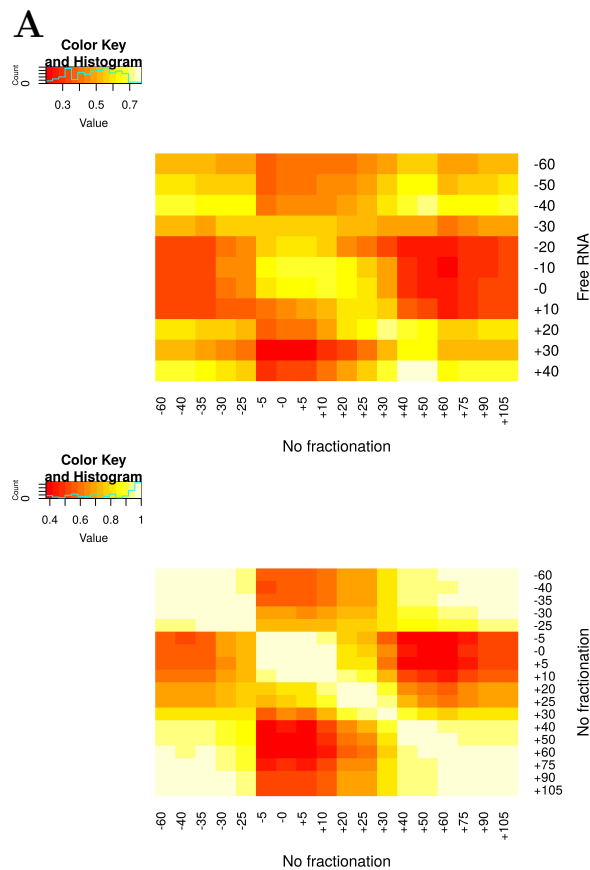


Figure 4.6: Translated and untranslated poly-A RNA levels broadly reflect one another throughout the YMC.

The fractions discussed in Figure 4.5 were spiked with 2 μg *S. pombe* RNA and RNA was extracted from the entire spiked fraction using a miRNEasy kit (Qiagen). Libraries were then prepared from purified RNA using the QuantSeq kit (Lexogen) and sequenced on an Ion Proton system. For the following analyses, counts were normalized to *S. cerevisiae* library size.

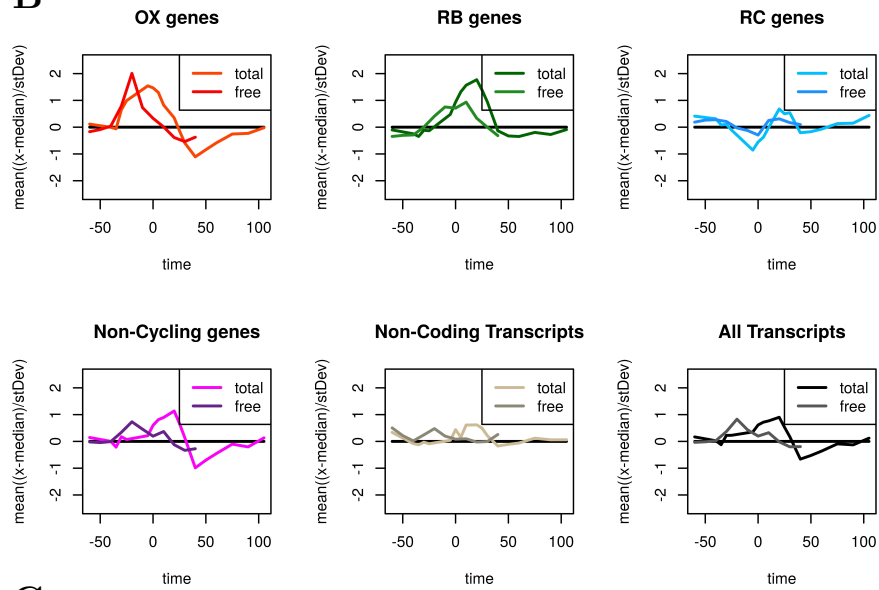
A - QuantSeq data from the free fractions correlates well with total RNA QuantSeq data from the same strain. Counts from free RNA fractions were correlated by timepoint with a total RNA QuantSeq dataset generated independently in the Mellor lab (Shidong Xi - Unpublished data) and plotted as a heatmap. For the total RNA QuantSeq data, cells from 1 ml of culture were spiked with a fixed volume of *S. pombe* cells and lysed immediately in phenol-chloroform.

B - Free poly-A RNA YMC clusters behave similarly to the wild-type total RNA QuantSeq data. Genes were divided into the major YMC clusters, and a “non-coding” category. For each gene, the median counts over time were subtracted and results were divided by their standard deviation. From the normalized data, metaplots were generated of the mean value for each time point. This was done for the free RNA dataset and the independent QuantSeq dataset (Shidong Xi - Unpublished data).

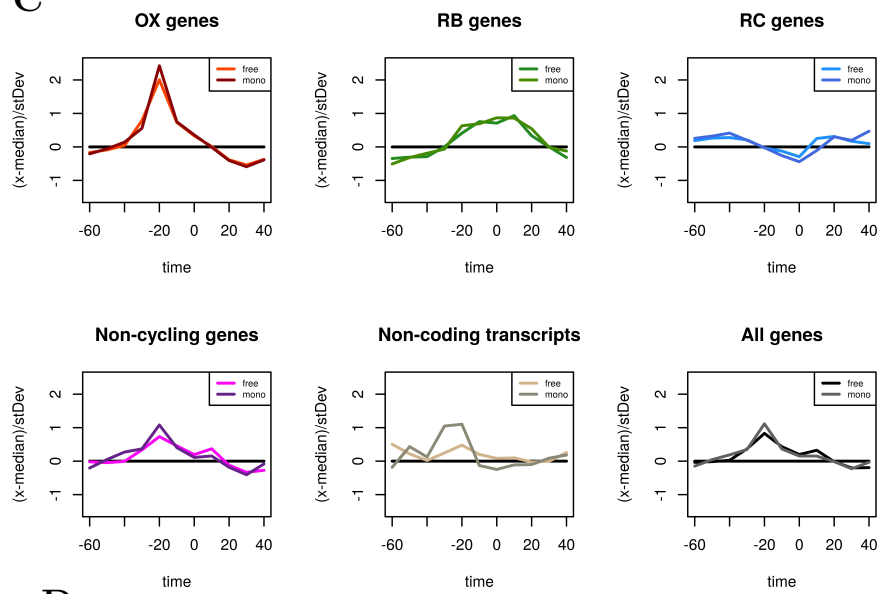
C - free and monosomal YMC clusters behave similarly. As in (B), genes were divided into the major YMC clusters, and a “non-coding” category. For each gene, the median counts over time were subtracted and results were divided by their standard deviation. From the normalized data, metaplots were generated of the mean value for each time point. This was done for the free and monosomal datasets.

D - Individual genes correlate over time between free and monosomal fractions and OX genes do so most strongly. Monosomal and free RNA datasets were correlated on a gene-by-gene basis and correlation coefficients were divided into 21 equally sized bins. The frequency of genes in each bin was then plotted as a histogram for each of the three YMC clusters and non-cycling genes. Differences between mean correlation coefficients were calculated using U-Tests adjusted for multiple testing by Holm’s method.

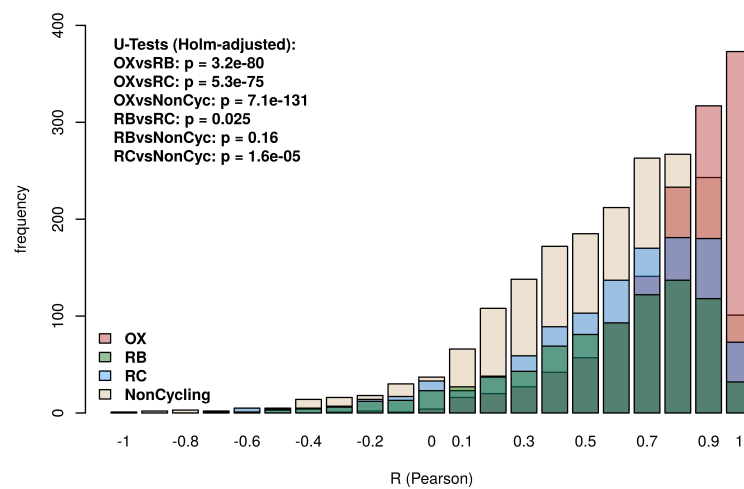
B



C



D



With this in mind, monosomal libraries and “free mRNP” libraries were then compared with one another. The YMC cluster profiles for “free mRNP” & monosomal fractions overlay almost perfectly (Figure 4.6C). Similarly, for the majority of individual RNAs, correlations between “free mRNP” & monosomal libraries are strikingly high, with mean correlation coefficients significantly above 0.50 according to Wilcoxon’s signed-rank test (OX: p-value = 9.04×10^{-203} , mean R = 0.81; RB: p-value = 4.12×10^{-50} , mean R = 0.64; RC: p-value = 2.48×10^{-84} , mean R = 0.66; NC: p-value = 4.90×10^{-100} , mean R = 0.67) and only a handful of genes exhibiting correlation coefficients of less than 0.0 (Figure 4.6D). This compellingly shows that for the vast majority of mRNAs, levels of ribosome-associated mRNA cycle in synchrony with the wider mRNA pool. Therefore, few genes are individually subjected to translational regulation that opposes changes at the transcript level. However, that is not to say that translation is unregulated during the YMC. Globally-speaking its behaviour is manifestly different during the YMC to during log-phase (Figure 4.5B), its just that regulation is applied uniformly and equally across transcript classes.

Based on this result, the first model for decoupling of transcript and protein levels seems unlikely to be true (Figure 4.4A). However, due to the unusual absence of polysomes from YMC sucrose-fractions, it was deemed important to validate this result using an independent method. As described in subsections 1.3.2 & 2.8.1, RiboSeq is a technique that sequences the footprints of individual ribosomes within cells, allowing it to provide a robust and high-resolution measurement of translation.

In order to validate that the monosomal QuantSeq libraries generated from the yeast metabolic cycle reflected genuine translation, three RiboSeq libraries from -30, -15 & +30 min relative to the point of lowest dO₂% (Figure 4.7A) were prepared and sequenced. Counts from ribosome-protected fragments correlated most strongly

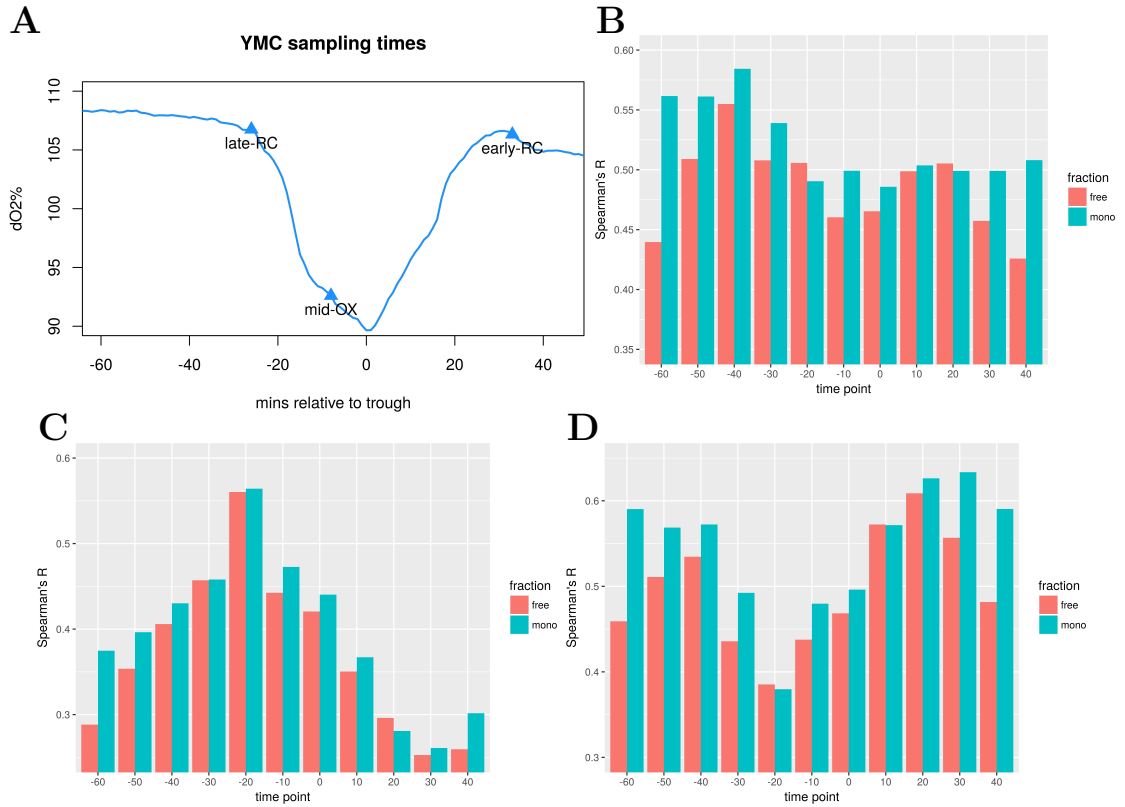


Figure 4.7: RiboSeq libraries validate monosomal translation during the YMC.

Three RiboSeq libraries were prepared from 50 ml of cycling *CENPK113-7D rpb3-FLAG* cells from 30 min before, 20 min before & 30 min after the point of lowest dO₂% in three separate cycles.

A - Sampling timepoints for RiboSeq. 50 ml samples were taken from a single cycle at the time points indicated. Cells were harvested by vacuum filtration and flash-frozen in N₂(l).

B-D - Correlations between RiboSeq libraries and polysome profiling libraries. Counts from “free mRNP” & monosomal fractions were correlated with ribosome-protected fragments from RiboSeq libraries from late RC phase (t= -30 min - B), mid OX phase (t= -15 min - C) & early RC phase (t= +30 min - D). Actual times are relative to the lowest point of dO₂% during the cycle in which the sample was harvested.

with the monosomal fractions corresponding to their nearest time points, demonstrating that monosomal fractions from YMC samples accurately represent levels of translation during the YMC (Figure 4.7). This provides powerful validation of the conclusions from Figure 4.6. Therefore, we can confirm that the similarities between monosomal and “free mRNP” libraries are not artefactual but in fact reflect a genuine absence of widespread transcript-specific translational regulation.

4.1.3 Global protein synthesis rates peak during RB and early RC phase of the YMC.

Although association of ribosomes with transcripts does not appear to be regulated across the YMC, as seen in our work on *xrn1* Δ , this does not necessarily mean that protein synthesis rates aren't changing (Figure 3.18). In the *xrn1* Δ mutant we saw a global reduction in protein synthesis rates with similar overall levels of polysomal mRNA. Since ribosome abundances are limiting for translation in log-phase (Shah et al. (2013)), this demonstrated a defect in translation elongation (Figure 3.18 & 3.12). To determine whether global initiation and elongation rates might be changing in concert to affect synthesis rates without affecting levels of monosomal mRNPs, global protein synthesis rates were measured across the YMC using the puromycin-incorporation assay developed in Subsection 3.1.8 (Figure 4.8).

Rates of puromycin incorporation remained low from mid-RC phase and throughout OX phase but peaked in RB phase and early RC (Figure 4.8). Therefore, protein synthesis rates are not constant through the YMC. Previous studies have predicted that protein synthesis rates would peak during RB phase due to inferred increases in abundance of ribosomes and translational components following their peak of mRNA expression in OX phase (Tu et al. (2005); Kuang et al. (2014)). However, an alternative explanation is that the dramatic changes in metabolism revealed by the dO₂% traces and published metabolome data (Tu et al. (2007), Figure 3.1A) provide higher levels of amino acids and NTPs during RB phase, accelerating enzymatic reactions that underlie translation. The lack of changes in expression of OX phase genes at the protein level favours the second explanation. However, it is possible that changes in a subset of OX phase proteins are being masked by the behaviour of the cluster as a whole. This inspired us to look a little deeper at changes in classes of genes that had previously shown interesting behaviours in the YMC & the *xrn1* Δ mutant (Figure

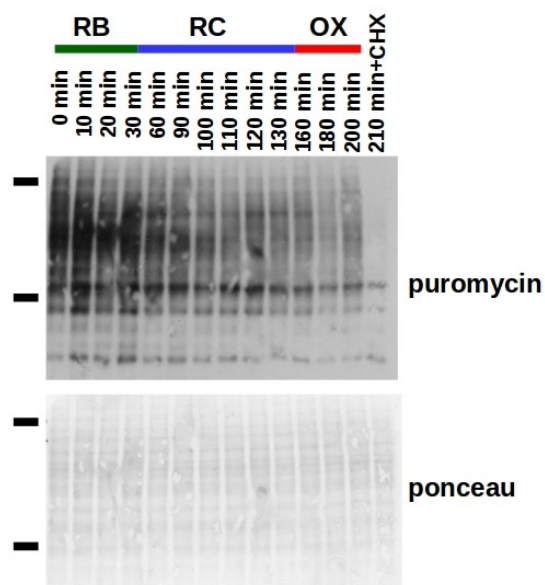


Figure 4.8: Protein turnover is low during the YMC.

Translational output in the YMC peaks during RB phase. 1 ml samples from the cycling *CENPK113-7D rpb3-FLAG* cells were incubated with 0.003 % SDS and 400 $\mu\text{g}/\text{ml}$ puromycin for 5 min, harvested by centrifugation (16,200 rcf, 15 s) and pellets were frozen in $\text{N}_2(l)$. Whole-cell extracts were prepared and analyzed by anti-puromycin western blotting. One sample per replicate was also treated with 100 $\mu\text{g}/\text{ml}$ cycloheximide as a negative control. (n = 4, representative blot shown)

3.5, 3.6, 3.7, 3.9, 3.13 & Woloszczuk et al. (2016)).

4.1.4 *ADR1* target genes and ribosomal protein genes do not cycle at the protein level.

Knowing that cycling of mRNAs in YMC clusters was reflected in their levels of translation (Figure 4.5 & 4.6) but not in the proteome inspired us to examine the possibility that specific classes of proteins, thought to be important for metabolic cycling, might deviate from their cluster and cycle at the protein level.

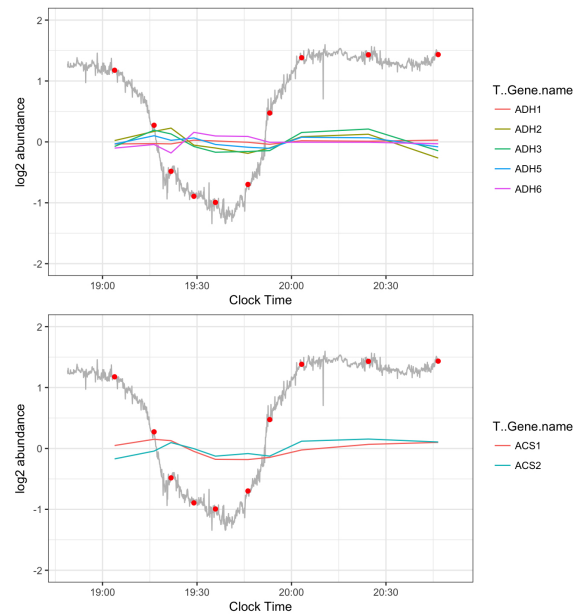
Based on the resistance of *ADR1* & its targets to dysregulation in *xrn1* Δ (Figure 3.6 & 3.7) we suspected that the tightly regulated *ADR1* axis, which is critical for glucose-repression (Young et al. (2003)), might escape the silencing effects of regulation through protein turnover and cycle at the protein level. In turn, this may re-open the idea of a gene-expression driven metabolic cycle.

However, this does not appear to be the case. Key *ADR1* targets such as *ADH2* & *ACS1* remain more-or-less constant at the protein level throughout the YMC (Figure 4.9A) which prevents changes in their abundance from driving metabolic switches.

On a similar vein, many studies have placed great emphasis on the fact that ribosomal protein genes comprise a major subclass of OX genes. They infer that coordinated synthesis of ribosomes in OX phase provides a surge of ribosome abundance which drives protein synthesis during RB phase (Tu et al. (2005); Kuang et al. (2014)). Consistently with this model, we found that rates of protein synthesis peak throughout RB phase and into early RC phase convincing us to interrogate this further (Figure 4.8).

However, with the exception of one component of the 40S subunit, it is clear

A



B

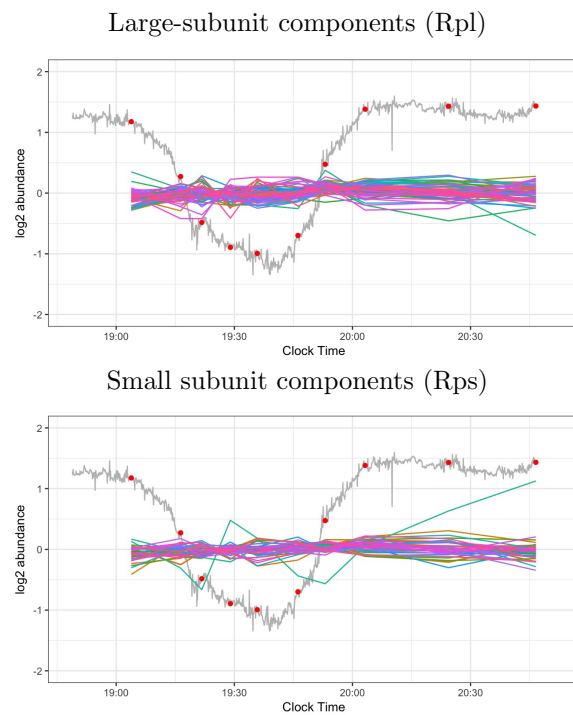


Figure 4.9: *ADR1* target proteins & ribosomal proteins do not cycle during the YMC.

Select protein families from the YMC proteome presented in (Figure 4.3.) plotted as log₂-abundances. dO₂% levels are plotted in grey (Data generated by Shidong Xi, analysis performed by Charlotte George)

A - Profiles of key *ADR1* target proteins and their family members across the YMC. Log₂-abundances of members of the Adh & Acs protein families across the YMC.

B - Profiles of ribosomal proteins across the YMC. Log₂-abundances of Rpl & Rps protein families across the YMC.

that ribosomal protein levels remain constant across the YMC (Figure 4.9B) and therefore there is no pulse of ribosome biogenesis in OX phase. As such, a more likely explanation for the increase in protein synthesis rates in RB is that the changes in metabolism, in particular the increases in amino acid and NTP levels (Tu et al. (2007)), allow the rates of tRNA charging, translation initiation and elongation to be accelerated.

At least for the classes of genes that we have focussed on, it does not appear to be the case that specific sets of cycling transcripts break the mould from the majority of their cluster and bleed through to cycle at the protein level. Therefore, cycling of ribosome abundances or *ADR1*-target protein levels cannot be what defines the behaviour of YMC phases. This solidifies the case against a cycle whose phase transitions and definitions are controlled by cycling of RNA and protein levels.

4.2 Conclusions

4.2.1 The importance of translational regulation during the YMC

Expression of over 60 % of cellular transcripts cycles during the YMC (Tu et al. (2005); Kuang et al. (2014)). This has long been assumed to reflect changes in the levels of their protein products, which in turn were predicted to regulate and define each phase of the YMC. For example, transcripts that are enriched in the OX phase are associated with ribosome-biogenesis and translation, leading many to propose that ribosome abundances increase in OX phase in preparation for growth and cell-division (Tu et al. (2005); Kuang et al. (2014)). However, expression of YMC cluster genes at the protein level does not support this (Figure 4.2 & 4.3). Likewise, ribosomal protein genes and *ADR1* targets show no evidence for cycling of protein

levels (Figure 4.9). Instead, the expression of most proteins during the YMC remains relatively constant. Somehow protein and transcript levels are disconnected during the YMC.

As sketched out in Figure 4.4, three models can be envisaged for how this might occur. First of all, protein synthesis rates could be decoupled from mRNA levels, effectively over-riding cycling of transcripts altogether (Figure 4.4A). In this model, translation is the critical stage of gene expression during the YMC and antagonistic regulation of translational efficiency (defined as the rate of protein synthesis per transcript) would ensure constant protein synthesis rates and constitutive protein levels. One way in which this could be achieved is by limiting the access of translational machinery to cycling transcripts. The additional mRNAs produced during expression peaks could be sequestered in the nucleus or cytoplasmic liquid droplets such as P-bodies and stress-granules for example. These components are known to be rapidly induced upon glucose deprivation (Teixeira et al. (2005); Hoyle et al. (2007)) and so are likely to be present and dynamic during the YMC.

However, we in fact know that levels of translated mRNA oscillate in synchrony with overall mRNA levels (Figure 4.6C & D). Therefore, an alternative mechanism must be invoked to decouple cycling protein synthesis rates from steady-state protein levels. Several models can be considered to explain this phenomenon. Firstly, one could envisage a model where changes in protein synthesis rates are perfectly matched by corresponding changes in degradation rates (Figure 4.4B). Therefore, when protein synthesis rates rise, protein turnover rises meaning overall levels of protein are unchanged. Similarly, when synthesis rates drop, degradation rates immediately fall to meet them, slowing protein turnover but maintaining steady-state levels. This model is reminiscent of the coupling of mRNA stability to transcription

via Rpb4/7p reported by the Choder lab, albeit at the level of protein, rather than mRNA, turnover (Shalem et al. (2011)). However, it is hard to imagine how protein synthesis and degradation could be coupled contemporaneously. To our knowledge, no study demonstrating such a link exists. It is possible that the stability of individual proteins could be pre-determined by post-translational modifications but the structural diversity of proteins in *S. cerevisiae* makes it the existence of a mechanism which would allow this to occur genome-wide improbable. The only reactive moieties that are more-or-less universally available for modification amongst proteins are the backbone amino and carboxy-terminii and although N-terminal acetylation is observed on 57 % of *S. cerevisiae* proteins (Arnesen et al. (2009)), to our knowledge there is no evidence linking its deposition to protein synthesis or degradation rates.

A much simpler explanation for the lack of oscillations in protein levels during the YMC is to simply state that protein turnover rates are much slower than the period of the YMC (Figure 4.4C). In this model, only a small proportion of the steady-state proteome is synthesized each cycle. As such, changes in the rates of protein synthesis during the cycle have only a minimal impact on steady-state levels. Therefore, although protein levels are not truly remaining constant during the YMC, the amplitude of their oscillations is sufficiently small as to go undetected using proteomics and to have a minimal impact on metabolism and other aspects of cellular physiology.

Given the dilution rate of 0.09 hr^{-1} and cycle period of 3.25 hr, in order to maintain a constant OD_{600} of 16.0, cells must replace 10 % of their biomass each cycle. Therefore, assuming an extreme scenario where a particular protein is only produced during a single YMC phase, the amplitude of the oscillation in protein levels could still be as low as 10 %. This is low enough to be likely to be missed in the mass-spectra. Furthermore, Lahtvee et al. (2017) demonstrated that in nutrient-limited conditions

similar to those of the YMC, median protein half-life was 17.2 hours. Therefore, turnover rates during the YMC are likely to be within the order of magnitude required for this model to be correct.

The low protein turnover rate model fits with several other findings from this study. Most directly, in Figure 4.5B we observe that levels of polysomes are extremely low during the YMC, pointing to low rates of global protein synthesis and translation primarily by monosomes. Additionally, in Chapter 3 we show that *xrn1* Δ mutants exhibit severe reductions in protein synthesis rates during log-phase growth (Figure 3.18C & D), a state where they show significant growth reduction relative to wild-type (Larimer & Stevens (1990)). In contrast, the changes in dO₂% trace of *xrn1* Δ during the YMC are very mild (Figure 3.1A & D). One possible explanation for this is that since wild-type translation is low during the YMC, it suppresses the slow-translation phenotype of *xrn1* Δ . Therefore, our data are consistent with slow protein synthesis during the YMC, and by extension with the slow-turnover model.

4.2.2 Protein levels do not cycle: The implications regarding regulation of the YMC

As extensively discussed, although mRNA levels cycle as a set of distinct clusters during the YMC, the vast majority of genes do not cycle at the protein level (Figure 4.2 & 4.3). This has profound implications when considering potential mechanisms of YMC regulation. In the past, many studies have attributed regulatory activity to cycling transcript levels and claimed that cycling of transcripts leads to cycling protein levels which in turn drive progression of the YMC (Tu et al. (2005); Kuang et al. (2014)). We now know this not to be the case. With a handful of exceptions, levels of proteins throughout the YMC stay largely constant (Figure 4.2 & 4.3). As such, although it remains possible that cycling of a small number of unidentified key regulatory pro-

teins provides the stimulus that drives metabolic cycling by shifting the balance of key metabolic pathways, the existence of a gene expression-driven metabolic cycle now seems unlikely. Therefore, we must consider alternative mechanisms for YMC regulation.

Recent work on circadian rhythms has begun to reveal that they may also not be regulated by transcription factors. Despite lacking a nucleus altogether, mammalian erythrocytes exhibit a circadian rhythm that can be observed through oscillations in the oxidation state of peroxiredoxins (O'Neill & Reddy (2011)), demonstrating that a perfectly functional circadian clock can exist in the absence of dynamic gene expression. Therefore, it is reasonable to suspect that the YMC may also be regulated independently of cycling gene expression.

As the name suggests, the YMC is principally defined by a cycle of metabolism, originally detected through cyclic changes in $\text{dO}_2\%$ in nitrogen-limited chemostat cultures (Kaspar von Meyenburg (1973)). Since then, numerous studies have demonstrated a deeply intertwined connection between metabolism and YMC maintenance and period, demonstrating oscillations in myriad metabolites and identifying multiple metabolites whose limitation induces cycling or whose addition drives phase-advancement. (Borzani et al. (1977); Chen et al. (1990); Chen & McDonald (1990); Tu et al. (2005); Machné & Murray (2012); Cai et al. (2011); Tu & McKnight (2009); Sohn et al. (2000)).

Metabolism is clearly critical to regulation of the YMC. Therefore, it seems reasonable to suspect that metabolites would be central to regulation of the YMC. In particular, specific core metabolites such as acetyl-CoA & NAD^+ have repeatedly been implicated in numerous aspects of the YMC (Tu et al. (2005); Cai et al. (2011)).

As a central metabolite in carbohydrate and lipid metabolism and a direct product of the Link Reaction between glycolysis and the citric acid cycle, acetyl-CoA is ideally positioned to act as a sensor for the metabolic status of cells. Furthermore, as the substrate used by KATs to acetylate proteins, it is also capable of integrating this information with protein activity.

A similar but antagonistic role can be attributed to NAD^+ . Utilizing NAD^+ as a substrate, sirtuins such as Sir2p deacetylate proteins generating O-acetyl-ribose and nicotinamide as products. Utilizing NAD^+ as a substrate allows sirtuin activity to be dependant on cellular redox balance measured as $\text{NAD}^+:\text{NADH}$ ratio. Thus there is a strong theoretical basis for intrinsic coupling of protein acetylation status to metabolic status.

Several lines of evidence also support protein acetylation as a key signal regulating the YMC, particularly the acetylation of histones. Cai et al. (2011) show that global levels of various histone acetylation marks cycle during the YMC and Kuang et al. (2014) demonstrate that acetylation levels change at the level of individual promoters. Furthermore, deletion of the major KAT *GCN5* and generation of non-acetylatable K $\rightarrow$ R mutations in histones abolishes metabolic cycling (Cai et al. (2011); Kuang et al. (2014)). Interestingly, the equivalent K $\rightarrow$ A mutants cycle normally, indicating that neutralization of the positive charge coating histones is part of what is required for metabolic cycling.

However, many other proteins are also acetylated in cells, in particular those involved in glycolysis and gluconeogenesis. It is likely that their acetylation patterns are also regulated during the YMC (Henriksen et al. (2012)). Thus there is the potential for an inherent feedback loop between changes in metabolism and changes in

protein activity which may be able to provide a simple and elegant oscillatory system with the added advantage of being much more energy-efficient than repeatedly synthesizing and degrading large portions of the proteome.

Of course, lysine acetylation is not the only post-translational modification likely to be involved in metabolic cycling. Dozens of dynamic post-translational modifications have been detected in *S. cerevisiae* and well studied modifications such as phosphorylation and ubiquitylation are well known to regulate carbon metabolism (Tripodi et al. (2015)). Indeed a number of key nutrient signalling pathways, including TOR-signalling and PKA-signalling, centre around protein kinase activity (Alarcon et al. (1999); Ptacek et al. (2005)) making cycles of protein phosphorylation promising candidates for YMC regulation. Furthermore, the cell cycle, which is partially coupled to the YMC (Burnetti et al. (2016); Tu et al. (2005); Slavov & Botstein (2011)), is primarily regulated through protein phosphorylation (Mendenhall & Hodge (1998)). Therefore, the two systems may well share common regulatory components.

We propose an inversion of the transcription-driven metabolic cycle. Rather than cycling mRNA and protein levels rewiring cellular metabolic pathways, we suggest that metabolic oscillations occur upon a landscape of constitutive protein levels. Pathways oscillate as a result of allosteric metabolic interactions and signalling through phosphorylation and acetylation cascades. These signalling pathways would drive cycling of transcription through changes in transcription factor activity (Treitel et al. (1998); Papamichos-Chronakis et al. (2004); Taylor & Young (1990)); promoter-localized acetylation status (Cai et al. (2011); Kuang et al. (2014)) & chromatin accessibility (Nocetti & Whitehouse (2016)). By inverting control of the YMC we are able to reconcile cycling of transcripts with their apparent lack of impact on YMC progression. Much more work will need to be done to validate and establish the de-

tails of this model but it appears to be a promising avenue for future study.

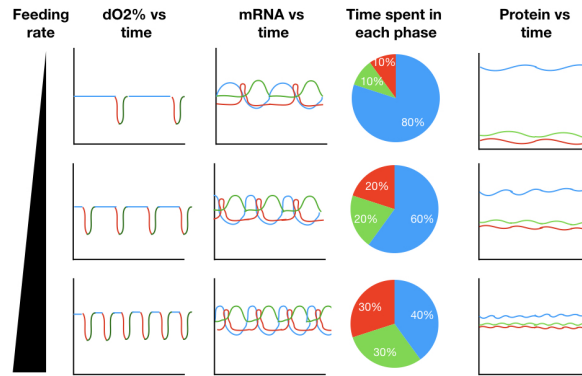
4.2.3 The function of cycling transcripts during the YMC.

The discovery that most proteins do not change in expression during the YMC was unexpected and profoundly informative. However, it raises a big question about the behaviour of cells during the YMC. If protein levels do not oscillate, why do transcript levels oscillate at all? Continual synthesis and degradation of mRNA has an associated energetic cost since RNA synthesis, splicing & export all require NTP hydrolysis (Lynch & Marinov (2015)). As such, unless it provides some selective advantage, under nutrient-limiting conditions, cycling of transcripts is expected to be an undesirable process.

Working under the assumptions of the third model for decoupling of mRNA levels from protein during the YMC (Figure 4.4C), we propose that cycling of mRNA levels is still able to regulate protein expression during the YMC. However, rather than tight coupling of mRNA and protein levels leading to dynamic oscillations in protein levels, coupling is drastically loosened by slow protein turnover. This allows transcript cycling to fine-tune steady-state protein expression to nutrient availability and growth rate (Figure 4.10).

The frequency of the YMC is tightly coupled to culture feeding rate and by extension growth rate. Therefore, since the length of the OX & RB phases remain relatively constant whilst RC is more variable (Burnetti et al. (2016); Slavov & Botstein (2011)), the higher the YMC frequency, the greater the proportion of time spent in OX/RB phases and by extension, the greater the OX & RB contribution to the time-averaged mRNA levels across the YMC. This means that even though the effect

A



B

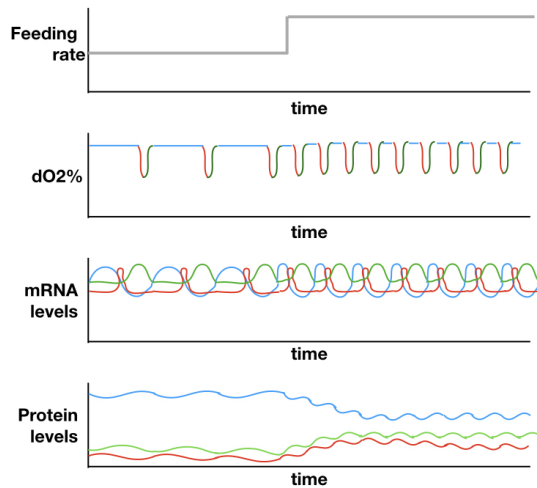


Figure 4.10: Cycling of transcripts during the YMC is proposed to couple steady-state protein levels to growth rate.

A - Proposed relationship between feeding rate and steady-state protein levels. The rate of feeding directly controls nutrient availability and growth rate. It also determines the period of the YMC. At higher feeding rates, the frequency of cycling is faster increasing the proportion of time cells spend in OX and RB phase. Over long time periods, this increases the number of OX and RB proteins produced relative to RC. Therefore, expression of growth-related proteins is directly coupled to growth rate with minimal energetic cost.

B - Adjustment of protein expression patterns in response to changing media conditions. Increasing the feeding rate of cultures causes them to cycle faster and spend more time in OX and RB. This gradually increases levels of OX and RB proteins to eventually equilibrate at a new steady-state expression level capable of driving faster growth rates.

of a single cycle on the proteome is small, over time the cellular protein levels tend towards a steady-state dictated by the YMC frequency (Figure 4.10A). Within the YMC-permissible range of feeding rates, when nutrient availability changes this model will automatically couple that information to expression of growth-related proteins. Increasing the feeding rate increases cycle frequency and consequently time spent in OX and RB phases. This leads to a gradual accumulation of OX & RB proteins up to the level dictated by the new cycle frequency. As such, expression of growth-related proteins is automatically adjusted to match nutrient availability (Figure 4.10B).

One of the biggest mysteries about the YMC has always been why cells synchronize their metabolic cycles. Cycling at the single-cell level can be justified by a number of competing models (Tu et al. (2005); Kuang et al. (2014); Tu et al. (2007)). However none has adequately explained why it would be beneficial for the entire population to cycle in synchrony. If the role of the YMC is to coordinate growth rate with long-term nutrient availability, then a simple explanation arises. Although the conditions in which *S. cerevisiae* grows in the wild are poorly understood, it is likely that nutrient availability in wild habitats is low and variable across space and time (Jouhten et al. (2016)). By synchronizing their metabolic cycles, *S. cerevisiae* cells may be able to effectively communicate local nutrient availability to one another within a colony. By integrating data over a larger area, cells would obtain a more reliable measurement of long-term nutrient availability and therefore more accurately couple their growth rate to environmental conditions. This kind of intra & even inter-colony communication has already been observed in laboratory conditions (Palková et al. (1997); Ohkuni et al. (1998)) albeit related to regulation of other processes, meaning this mechanism is not without precedent. However, establishing causality for any explanation of YMC synchronization is likely to require many years of research and therefore explanations for synchronization are likely to remain speculative for the foreseeable future.

4.2.4 Summary

In a break with the normal assumptions that are made when studying gene-expression, we find that during the YMC the large fluctuations of cycling transcripts generally have little-to-no effect on protein abundances across a single cycle (Figure 4.2 & 4.3). We also demonstrate that this is not due to ribosomes failing to associate with those transcripts causing them not to be translated. Instead, ribosome-association with transcripts cycles in-phase with levels of the transcripts themselves, indicating that if an mRNA is upregulated, so will its synthesis rates be (Figure 4.6 & 4.7). Furthermore, we find that global translation rates during the YMC are not constant. Rates of puromycin incorporation peak throughout RB & early RC phase, likely due to peaking levels of NTPs & complex amino acids such as tryptophan, phenylalanine & tyrosine (Tu et al. (2007)). Thus rates of protein synthesis are highly variable across the YMC and these data raise the question of how the abundances of proteins can remain largely constant throughout the metabolic cycle despite this fact.

We propose that this effect is due to the low rates of protein turnover observed during nutrient-limiting growth. Lahtvee et al. (2017) recently reported that half-lives of proteins in nutrient-limited *S. cerevisiae* range from 1.3 to 34.0 hours (1-99th percentiles) with a median of 17.2 hours. This means that for the majority of proteins, their half lives greatly exceed the period of the YMC under the conditions that we have been using (3.25 hours). Therefore, it stands to reason that during phases of the cycle where synthesis rates of an individual protein are low, only a small proportion of molecules would be degraded before being replaced during the next peak of synthesis of that protein. Thus, as described in Figure 4.4C, although protein levels would not be perfectly constant during the YMC, changes in the levels of most proteins would be of sufficiently low amplitude to remain undetected by mass-spectrometry and to have minimal impact on cellular physiology.

If protein turnover rates are sufficiently low as to prevent cycling of protein expression, this begs the question, what is the purpose of cycling at the mRNA level? We propose that rather than resulting in dynamic oscillations in protein expression, cycling of transcript levels couples steady-state protein abundances to the YMC frequency and by extension, nutrient availability and growth rate. As described in Figure 4.10, when nutrient availability is increased by increasing growth rate, this leads to an increase in YMC frequency. In turn, this means that cells spend a greater proportion of time in the OX & RB phases of the YMC, since these have a more fixed interval than RC phase (Burnetti et al. (2016); Slavov & Botstein (2011)). Since mRNA expression programs are coupled to specific YMC phases, this means that averaging over time, cells that cycle more rapidly exhibit a more OX/RB-like transcriptome. That is to say, a transcriptome that is associated with more rapid growth. Although, over the period of a single cycle, this has little effect on the proteome, as time progresses, OX & RB proteins accumulate to higher steady-state levels in more rapidly cycling cells. This allows the cells to increase their biosynthetic capacity and cell-division rate thereby intrinsically coupling long-term changes in the proteome to growth rate. Experimental verification of this will be a priority for future study.

To our knowledge, coupling of oscillatory signals to steady-state outputs has not been widely studied in the field of gene expression. However, it is likely to be a common occurrence in the biology of many organisms. For example in mammalian heart and neuronal cells the frequency of depolarization is proportional to the metabolic, and in one case mechanical, burden placed upon the cells. Therefore, it would be advantageous for cells to fine-tune changes in expression of respiratory components, along with various tissue-specific proteins such as cytoskeletal components, to the frequency of depolarization over time, without responding dramatically to any indi-

vidual depolarization event at the gene-expression level. As a slow-turnover model allows cells to do just that, it provides a potentially interesting mechanism of long-term adaptation and counterpoint to the rapid responses often focussed on in the field of gene-expression control. Much additional work will be needed to validate this model within YMC and to try to identify similar behaviour in other systems. This will provide many exciting avenues for future research.

Chapter 5

Conclusions

5.1 The Yeast Metabolic Cycle: A model for cyclic control of gene-expression.

The yeast metabolic cycle is a physiological state of prototrophic *S. cerevisiae* cells grown under nutrient-limiting conditions. This state is characterized by cyclic changes in $\text{dO}_2\%$ over time and synchronization of the cell-cycle within a population (Kaspar von Meyenburg (1973); Borzani et al. (1977); Chen et al. (1990); Chen & McDonald (1990)). These cyclic changes in gross cellular behaviour are associated with dramatic changes in transcript and metabolite levels as well as widespread changes in levels and distributions of histone modifications (Kuang et al. (2014); Tu et al. (2005, 2007)). This makes the YMC an ideal system for studying dynamic changes in gene expression.

These features also make the YMC an ideal model for other biological rhythms such as circadian and circatidal rhythms. In fact, it has previously been argued that the circadian rhythm is essentially a metabolic cycle with additional timekeeping behaviours (Tu & McKnight (2006); Mellor (2016); Klevecz & Li (2007); Causton et al. (2015)). Since disruption of the circadian rhythm in humans has been associated with diseases from cancer to diabetes, this makes the YMC a useful model to further understand these diseases (Ribas-Latre & Eckel-Mahan (2016)).

However, one of the main differences between the YMC and other biological oscillators is also one of its most intriguing and useful aspects. This is the way that during the YMC, cycling cells are able to generate fixed and robust period and phasing in the absence of any external entrainment. Rather than resetting the phase of the cycle according to the peaks and troughs of an external oscillator such as the daily, tidal or annual motions of celestial bodies, a cycling culture is able to maintain period and phase autonomously, determining its period based on steady-state media conditions (i.e. media composition and feeding rate) and maintaining an arbitrary phase across the entire culture (Kaspar von Meyenburg (1973); Borzani et al. (1977); Chen et al. (1990); Chen & McDonald (1990)). This provides a model system in which mRNA levels change dynamically within a constant external environment, making the YMC a unique model where one can observe induction and repression of gene-expression in the absence of a concomitant stress-response. In turn, this allows separation of the events required for the activation and repression of genes from those that facilitate stress tolerance. Thus the YMC provides a unique advantage compared other methods of inducing changes in gene-expression such as heat-shock and carbon-source switching, making it a powerful system in which to study gene-expression.

Using the YMC as a model, this study aimed to unravel the links between gene expression and metabolic oscillation, with a view to answering three main questions. First of all, what controls cycling of transcript levels during the YMC? In particular, what is the contribution of RNA degradation to oscillations in transcript levels? Several studies had already examined the roles of transcription factors and chromatin environments in controlling RNA synthesis across the cycle, but the role of degradation had been notably neglected (Cai et al. (2011); Kuang et al. (2014, 2017); Rao & Pellegrini (2011)). Secondly, what is the function of cycling of transcript levels during

the YMC? What impact do they have on the proteome and on metabolic cycling more generally? Finally, we hoped that by answering the first two of these questions, we would gain insights into what processes are responsible for regulation of metabolic cycle phase and period.

5.2 How is cycling of transcript levels regulated during the Yeast Metabolic Cycle?

It has been well established that during the YMC, over 60 % of genes cycle at the mRNA level (Tu et al. (2005); Kuang et al. (2014)). However, the mechanisms by which cells maintain precise and rapid control of transcript levels are not fully understood. This is despite much research having gone into understanding what controls activation of transcription and RNA synthesis during the YMC. Many models have been put forward in the past but generally mechanistic details are scarce and conclusions are open to interpretation. In contrast, to our knowledge, no work has been done on determining the role of RNA degradation in setting transcript levels during the YMC.

Our investigations into the role of RNA degradation in the YMC focussed primarily on the 5'->3' degradation pathway and specifically on the effects of deletion of *XRN1*. We find that loss of *XRN1* has dramatic effects on gene expression during the YMC, both globally and at specific loci. As has been well documented in log-phase (Malabat et al. (2015); Sun et al. (2013); Woloszczuk et al. (2016)), during the YMC global transcript levels are elevated in *xrn1* Δ (Figure 3.5B). Likewise, as in log-phase, different classes of transcripts show differing degrees of dysregulation (Figure 3.5C). In particular, the ribosomal protein genes, as part of the OX phase cluster, are amongst the best correlated between *xrn1* Δ & wild-type, with correlation coef-

ficients close to 1.0 (Figure 3.5B & C). This mirrors their resistance to stabilization in log-phase (Woloszczuk et al. (2016) & Figure 3.13D). In contrast, the majority of other genes, particularly those from RB & RC clusters, show a substantial degree of dysregulation in *xrn1* Δ , showing that 5'->3' RNA degradation is required for proper control of the levels of the majority of transcripts during the YMC.

Most of the OX phase YMC cluster, along with *ADR1* & its targets are able to escape from dysregulation in *xrn1* Δ , suggesting that there is something unique and common about the regulation of these genes (Figure 3.5B & C, 3.6, 3.7). This discovery implies that these transcripts are not targets of Xrn1p in wild-type cells as this is the simplest explanation for them being unaffected by its absence.

How they avoid degradation by Xrn1p is unclear. One likely explanation, at least in the case of the ribosomal protein genes is that they possess non-canonical 5' caps composed of NAD⁺ (Walters et al. (2017)). These are likely to be resistant to removal by decapping factors and since decapping is absolutely required for 5'->3' degradation, this would render such transcripts completely resistant to Xrn1p. They also provide an intriguing link between gene expression and metabolism as the availability of NAD⁺ is dependent on cellular redox-potential.

If, as expected, these transcripts resist mis-expression in *xrn1* Δ because they are not Xrn1p targets, they must be degraded by other means. The most likely candidate for this is the cytoplasmic exosome. It should be a priority for future studies in this area to validate or refute this model by measuring how well expression of these transcripts is maintained in cytoplasmic exosome mutants such as *ski7* Δ . If this model is borne out, it would go a long way towards identifying the factors that determine targetting of transcripts to different degradation pathways.

Comparing the changes in gene expression between *xrn1* Δ and wild-type also allows us to gain some insights into how transcription is regulated during the YMC. Transcription factor activity is one of the most important mechanisms of activation and repression of individual genes. As such, it is unsurprising that several studies have focussed primarily on identifying transcription factors whose levels oscillate in coordination with the YMC. Rao & Pellegrini (2011) present a shortlist of transcription factors that they find to be highly oscillatory during the YMC and present as likely candidates for regulators of the YMC. Kuang et al. (2017) performed a similar study in parallel, identifying the transcription factors *MSN2* & *MSN4* as being particularly important and justifying this based on the altered YMC period of *msn2* Δ & *msn4* Δ strains.

However, just because expression of a factor is required for metabolic cycling, doesn't mean that cycling of this factor's activity, let alone expression level, is what is important to maintain the YMC. For example, auxotrophic mutations abolish metabolic cycling by preventing cells from achieving OD₆₀₀ that is permissible for cycling (Alam et al. (2016); Tu et al. (2005), Mellor lab - Unpublished observations). Utilizing the profound dysregulation of gene expression in *xrn1* Δ during the YMC, we were able to determine whether cycling of several of these factors at the transcript level is required for metabolic cycling (Figure 3.6). By measuring the strength of the correlations in expression pattern between *xrn1* Δ & wild-type, we were able to rule out any requirement for cycling of levels of most of the transcription factors identified by Kuang et al. (2017) & Rao & Pellegrini (2011) for metabolic cycling, including *MSN2* & *4*.

Only four factors showed a Pearson's correlation between *xrn1* Δ & wild-type that

had 95 % confidence intervals greater than 0.30 (Figure 3.6A) and raising the threshold to $R > 0.50$, which this study considers to be a more robust threshold, produces only a single hit, the glucose-repressed transcription factor *ADR1*. Interestingly, *ADR1* is also amongst the best correlated RC genes between *xrn1Δ* & wild-type and many of its target genes, which seem to belong to a variety of different YMC clusters, also correlate very well between wild-type and *xrn1Δ* (Figure 3.7A & B). Although this does not prove that *ADR1* is required for transcriptional cycling, it does show that it is particularly resistant to loss of *XRN1*, indicating that it may possess common features with ribosomal protein genes.

Conflicting with the conclusions of Kuang et al. (2017), *MSN2* & *4* show the lowest correlations between *xrn1Δ* and wild-type of the transcription factors examined, and in fact both show negative correlations between the two strains (Figure 3.6). This does not necessarily mean that *MSN2* & *4* have no role in metabolic cycling as their activity may be regulated in other ways, for example through post-translational modifications, or their constitutive presence may be required for the cell to adopt a YMC-permissive state. In fact, we know that there is some requirement for these factors due to the phenotypes of their mutants (Kuang et al. (2017)). However, it certainly means that cycling of their expression levels is not required for regulation of the yeast metabolic cycle.

It is clear that 5'→3' RNA degradation is critical for proper expression of most mRNAs during the YMC (Figure 3.5B & C). However, unexpectedly, neither this pathway, nor by extension regulation of its dependant transcripts is required for metabolic cycling (Figure 3.1A & D). Indeed, none of the RNA degradation factors that we were able to test were required for metabolic cycling, although some had mild effects on dO₂% curve shape (*xrn1Δ*) and period (*rrp6Δ*) (Figure 3.1A, C & D).

Therefore, it is clear that the YMC is highly robust to loss of RNA degradation and perturbation of gene expression cycling. Given this, one has to wonder, what is the purpose of widespread cycling of mRNA levels?

5.3 What is the function of cycling transcripts during the Yeast Metabolic Cycle?

The widespread cycling of mRNA levels during the YMC has long been thought to be important for the regulation of the yeast metabolic cycle (Kuang et al. (2014); Tu et al. (2005); Kuang et al. (2017); Rao & Pellegrini (2011)). Tu et al. (2005) initially proposed the idea of temporal compartmentalization of gene expression, allowing isolation of antagonistic processes in time as well as space preventing the inefficiencies that result from futile cycling of metabolism. Kuang et al. (2014) go further, proposing a “Just in time” supply-chain model. In this model, tight temporal coordination of ribosome biogenesis factors and ribosomal protein genes facilitates efficient synthesis of ribosomes, increasing the translational capacity of cells from late OX phase onwards. They extend this model to argue that consecutive waves of gene expression are responsible for remodelling the structure and metabolism of cells thereby shaping and defining the phases of the YMC. Finally, following up on these studies, several labs have attempted to identify “master” transcription factors whose cycling expression coordinates the YMC as a whole (Rao & Pellegrini (2011); Kuang et al. (2017)).

However, little had been done in these studies to prove that cycling of transcript levels is actually required for metabolic cycling. We have shown that mis-expression of even the majority of cycling transcripts causes very few changes to the $\text{dO}_2\%$ trace of the YMC (Figure 3.1A & D, 3.5B & C), casting doubt upon the prevalence of temporal compartmentalization of gene expression at the protein level and the im-

portance of it for regulation of metabolic cycling.

However, by considering how rates of protein turnover are affected by limiting nutrient availability, this study has been able to resolve the apparent conflict described above. We show that despite rates of translation being coupled tightly to the oscillations in mRNA levels, steady-state protein levels remain constant throughout the YMC for almost all genes (Figure 4.1B, 4.3 & 4.6). We propose that, as has been seen in the proteomes of other nutrient-limited *S. cerevisiae* cultures, protein turnover rates are extremely low during the YMC (Lahtvee et al. (2017)). Under conditions where the half-life of a protein is much longer than the period of the YMC, only a small proportion of molecules of that protein will be synthesized and degraded each cycle. As such, even if the protein is only synthesized during one phase of the YMC, the oscillations in its levels will be small (Figure 4.4C). Although direct measurement of the turnover rates of proteins during the YMC has not been possible in the time available for this study, this model is supported by the low rates of translation observable by sucrose gradient fractionation (Figure 4.5B).

It is clear that the function of cycling for the majority of transcripts during the YMC is not to generate dynamic changes in protein levels. Therefore, an alternative explanation is required for this behaviour. An intriguing possibility arises from the uneven way that different phases are shortened and lengthened by changing YMC periods. As demonstrated by Burnetti et al. (2016) & Slavov & Botstein (2011), the variation in YMC period with changing feeding rates occurs primarily during the RC phase, with the durations of OX & RB phases remaining relatively constant. Therefore, as argued by Slavov & Botstein (2011), the relative length of time that cells spend in different YMC phases is proportional to the feeding rate. This in turn controls the relative length of time for which they express OX/RB vs RC transcripts.

They refer to this as the Universal Growth Rate Response (UGRR).

We propose that the function of cycling mRNA levels is to couple steady-state protein expression to growth rate via the UGRR. The period of the YMC is intrinsically coupled to growth rate, presumably through metabolic interactions. Therefore, by linking mRNA expression to specific YMC phases, a time-averaged transcriptome corresponding to the UGRR is established. In turn, the UGRR determines steady-state protein levels through low rates of translation and protein turnover (Figure 4.10A). This provides an inherent coupling of gene expression to nutrient availability for minimal energetic cost.

Further work will be required to validate this model. In particular, a large-scale mass-spectrometry experiment covering select YMC time points at a variety of feeding rates would allow determination of how strongly the YMC proteome is coupled to feeding rate and whether this linkage is stronger than the linkage to a particular YMC phase. This experiment will be a priority for future study.

5.4 What controls the timing and nature of metabolic oscillations?

Our research calls into question the notion that cycling gene expression controls the yeast metabolic cycle. Dynamic oscillations in protein levels are uncommon in the YMC, despite the widely reported oscillations in mRNA levels (Figure 4.1B, 4.3, Tu et al. (2005); Kuang et al. (2014)). Therefore, the oscillator that drives the YMC must exist at a different level.

This result was a little surprising but it mirrors trends that are emerging in re-

search into circadian rhythms. Despite lacking a nucleus altogether, mammalian erythrocytes exhibit a circadian rhythm that can be observed as oscillations in the oxidation state of peroxiredoxins (O'Neill & Reddy (2011)). This demonstrates that a perfectly functional circadian clock can exist in the absence of dynamic gene expression. Indeed, Edgar et al. (2012) present evidence that peroxiredoxins may comprise a universal marker of circadian rhythms, implying that they may be driven by redox cycles of metabolism. The notion that biological rhythms are driven by cycles of gene expression is very much under question.

In their work comparing the yeast metabolic cycle to circadian rhythms, Causton et al. (2015) show that peroxiredoxins also oscillate during the YMC. The *tsa1 Δ tsa2 Δ* double mutant exhibits a slightly shortened YMC period with a characteristic RC phase dip relative to wild-type. This phenotype is relatively mild and the authors do not claim that peroxiredoxin activity is solely responsible for timekeeping in the metabolic cycle. However, their work suggests that the underlying clock of the YMC might have redox cycles at its core.

When considering the connection between cycling of metabolism and gene expression, we were inspired by the work of Slavov & Botstein (2011) and Cai et al. (2011). We speculate that lysine acetylation might play a key role in coupling metabolic flux and redox balance to the UGRR. O₂ consumption rises during OX phase as glycogen and trehalose stores are broken down and funnelled into aerobic glycolysis (Xu & Tsurugi (2006); Burnetti et al. (2016)). As a result of this, levels of acetyl-CoA, the product of the Link reaction, rise as production rates temporarily exceed consumption through the citric acid cycle and fatty-acid biosynthesis (Tu et al. (2007)). In turn, increased acetyl-CoA availability causes global histone lysine acetylation levels to rise as observed by Cai et al. (2011). Conversely, during the reductive phases of

the YMC, NAD^+ levels are at their highest due to the reduced rates of glycolysis and this will stimulate histone deacetylation by sirtuin family HDACs such as Sir2p (Imai et al. (2000)).

We speculate that these changes to the epigenome are what drives the activation and repression of different gene clusters by remodelling chromatin to activate OX phase promoters and switch off those of RC phase. For example, this could explain the mechanism by which *ADR1* regulates its targets during the YMC. In both wild-type and *xrn1* Δ cells, *ADR1* mRNA is expressed briefly during RB phase (Figure 3.6) but transcripts belonging to many of its target genes remain present until the onset of OX phase (Figure 3.7). We interpret this as a result of Adr1p persisting at the promoters of its target genes after its mRNA has been degraded. Upon the induction of OX phase a signal must lead to dissociation of Adr1p from chromatin. Chromatin remodelling in response to histone hyperacetylation may provide this stimulus.

It is also likely that alongside the pulse of histone acetylation during the YMC, more widespread changes in lysine acetylation may occur. Previous studies have found that glycolytic and gluconeogenic enzymes are heavily acetylated in *S. cerevisiae* (Henriksen et al. (2012)). This may allow the same metabolite pulse to regulate OX/RB phase metabolism as well as gene-expression. As such, a priority for future research into regulation of the YMC should be to measure how the acetylome of cycling cells changes over time to assess how widespread changes to lysine acetylation are during the YMC and whether they are likely to modulate metabolism.

Another widespread post-translational modification that has been implicated in metabolic cycling is protein phosphorylation, particularly by the kinases CK1 δ & *RIM11* (Causton et al. (2015)). Members of the Mellor lab have performed a prelimi-

nary YMC phosphoproteome (Xi, George & Mellor - Unpublished results). Although quantification and interpretation have proved difficult with the current replicate number ($n = 1$), the data show tantalizing hints of rapid changes in protein phosphorylation status. With replication and validation, this dataset will provide many insights into the regulation of metabolic enzymes and nucleic acid binding-proteins by phosphorylation throughout the YMC informing research into regulation of metabolism and gene expression during the YMC.

One of the most interesting questions about the yeast metabolic cycle is how do cells communicate to coordinate their metabolism? *S. cerevisiae* cells exhibit cycling of specific transcripts and metabolites even in unsynchronized populations (Silverman et al. (2010); Dodd & Kralj (2017); Gustavsson et al. (2014); Papagiannakis et al. (2017)), leading many to believe that metabolic cycling at the single-cell level is a common behaviour of *S. cerevisiae*. What makes the population level YMC unique is that cycling cells are able to communicate and coordinate their metabolism to produce bulk oscillations in metabolites, dO_2 consumption and transcript levels.

Much work has been undertaken to try to identify the signal that synchronizes metabolism across the culture. Several metabolites have been proposed whose addition causes premature induction of OX phase including hydrogen sulphide (Sohn et al. (2000)), carbon monoxide (Tu & McKnight (2009)) & various carbon sources (Cai et al. (2011); Murray et al. (2003)). This study identifies an additional OX phase inducing and period-shortening molecule, indole-3-acetic acid (IAA) (Figure 3.3A). The effect of IAA on the YMC is specific to it compared to other indole-containing compounds (Figure 3.3C), and not the result of utilization as a carbon source (Figure 3.3D). This leads us to believe that there is a specific property of IAA which causes it to induce OX phase.

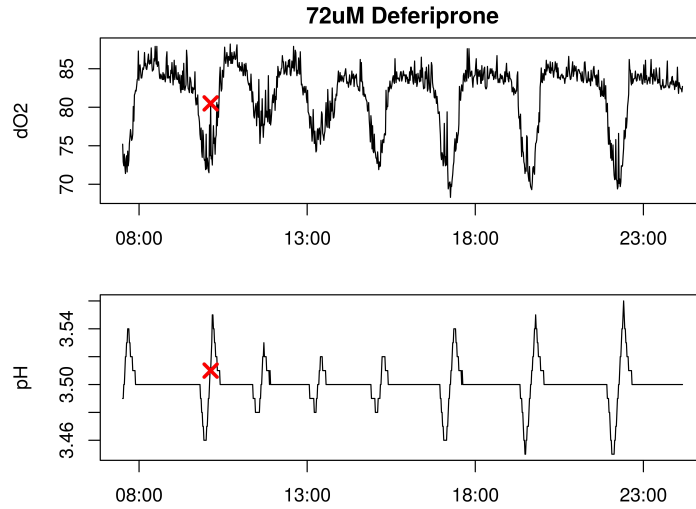


Figure 5.1: Reduction of Fe^{3+} ion availability shortens the yeast metabolic cycle.

Chelation of Fe^{3+} ions by deferiprone induces shortening of the YMC. dO₂% trace of *CENPK113-7D rpb3-FLAG* cells in a 1.1 l YMC culture. The red cross indicates the time when deferiprone was added to the chamber to a final concentration of 75 μM .

Interestingly, IAA is able to rapidly chelate and reduce Fe^{3+} ions (Kamnev (1998)). Similarly, under certain conditions Fe^{3+} can undergo redox reactions with hydrogen sulphide and carbon monoxide at 30 °C (Lin et al. (2005); Cherosky & Li (2013)). Therefore, a common mechanism of OX phase induction can be envisaged for each of these molecules. We propose that the signal for YMC synchronization is the redox state of the culture and that this is measured either through one of the many Fe^{3+} cofactors present in *S.cerevisiae* proteins such as haem or mitochondrial iron-sulphur clusters or the redox state of extracellular iron. As many of the proteins that utilize iron as a co-factor function in the mitochondrial electron transport chain, and respiratory metabolism more generally, this could provide a direct link between iron oxidation states and metabolic activity. In support of this, addition of the iron-chelator deferiprone to cycling cells generates a dO₂% trace that mimics the response to IAA (Figure 5.1).

A redox model for the role of iron in the YMC is also consistent with the conserved cycling of peroxiredoxin over-oxidation observed by (Causton et al. (2015)). The cycling phenotype of the *tsa1Δtsa2Δ* double mutant is remarkably similar to that of wild-type cells treated with IAA or deferiprone. If the role of Fe^{3+} during the YMC is linked to the regulation of respiratory metabolism, it is likely to affect the production of reactive oxygen species (ROS) which drive peroxiredoxin over-oxidation. This raises the possibility that the phenotypes of *tsa1Δtsa2Δ*, IAA & deferiprone may have a common origin, reflecting the behaviour of a redox-stressed metabolic cycle.

The link to redox cycles could also explain how sudden addition of carbon sources triggers OX phase induction. As the spike of available carbon is catabolized, it will generate cellular reducing equivalents such as NADH through glycolysis and the citric acid cycle and NADPH through the pentose-phosphate pathway. By increasing the cellular NADH/NAD⁺ ratio, Sir2p activity will be reduced and histone acetylation rates will rise, aided by elevated KAT activity as a result of increases in acetyl-CoA levels. Meanwhile, rising NADPH levels would promote recycling of peroxiredoxins and potentially also affect the redox state of cellular Fe-ions.

In conclusion, bringing together the latest literature along with the results from this study, we envisage a YMC model that is coordinated and synchronized by cellular redox state. Further research will be required to confirm that redox cycles are necessary and sufficient to drive metabolic cycling. Initial work on this should begin by measuring how the extracellular redox potential changes during the YMC. First of all, this would establish whether extracellular redox-potential changes throughout the cycle, an important check to ensure the fundamental assumptions of the model are valid. Secondly, this would inform on the range of redox potential spanned and the timing of the oscillations which in turn reflect the media component whose oxi-

dation state is changing and the point in the cycle that this corresponds with. This information could then be used to guide more detailed follow-up studies and since redox probes are commonly available for chemostat systems it would be relatively straightforward to perform.

5.5 Interactions between metabolism and gene-expression may explain the phenotypes of *xrn1* Δ cells.

S. cerevisiae cells naturally couple transcription to mRNA degradation to maintain constant steady-state mRNA levels and are able to maintain this effect despite serious mutations in either pathway (Sun et al. (2013)). This phenomenon is known as mRNA buffering. However, deletion of *XRN1* seems to abolish this effect leading to a global increase in transcript levels.

Despite suffering from some deleterious phenotypes, *xrn1* Δ cells are able to tolerate the additional mRNAs that they accumulate as a result of loss of buffering, maintaining viability and even a wild type YMC period (Figure 3.1A & D). However, until this study, little work had been done on the fates of these additional mRNAs and the mechanism by which cells can tolerate them.

This study shows that although some relative changes in protein expression occur in *xrn1* Δ , the phenotypic consequences of a global increase in mRNA levels are suppressed by limiting translation of these mRNAs (Figure 3.9, 3.12, A.1). We further show that this translational regulation occurs at the mRNA level through exclusion of additional mRNAs from the translatable pool (Figure 3.12). Based on the work of Shah et al. (2013), we propose that the exclusion of mRNAs from the translated pool in *xrn1* Δ is a result of log-phase translation being limited primarily by ribosome

availability. Since ribosomal-protein genes comprise the one class of genes that are not upregulated in *xrn1* Δ , it is likely that ribosomes themselves are expressed at similar levels to wild-type, imposing the same global constraint on wild-type and *xrn1* Δ cells (Figure 3.9A, 3.13D & Woloszczuk et al. (2016)). How this regulation occurs at the level individual mRNA molecules is unclear but it implies the existence of some form of positive feedback that ensures that translated mRNAs are preferentially targeted for future rounds of translation. Identifying the pathways responsible for this must unfortunately be left for future study.

Additionally and unexpectedly, we found that *xrn1* Δ cells show reduced protein synthesis rates, indicative of a defect in translation elongation (Figure 3.18C & D, 3.10A & 3.11). This defect does not seem to originate from a general slow-growth phenotype or from low NTP levels reducing the activity of the GTPases that are central to translation (Figure 3.18D & 3.19B). Likewise, we can confirm that it is not dependent on *RCK2* implying that it is not a result of eEF2-phosphorylation (Figure 3.19A). However, the origin of this defect remains elusive and further work will need to be done to determine its origin.

The other major question that arises from the loss of buffering in *xrn1* Δ is what is its cause? On this question, we have made substantial progress. Our key insight stems from studying the *xrn1* Δ metabolome. Against our expectations, *xrn1* Δ cells have greatly elevated NTP levels (Figure 3.19B). We propose that this is the origin of the failure of mRNA buffering and many of the other phenotypes of *xrn1* Δ cells. das Neves et al. (2010) show that mitochondrial activity and ATP levels strongly affect transcription rates in mammalian cells. Therefore, the increase in NTP levels observed in *xrn1* Δ is likely to result in increased rates of transcription (Figure 3.19B). Since Sun et al. (2013) showed using cDTA that an increase in transcription rates in

xrn1 Δ is the origin of the loss of buffering, this model can straightforwardly explain the loss of buffering in *xrn1* Δ .

Counter-intuitively, the elevated NTP concentrations in *xrn1* Δ can also explain the slow growth phenotype of this strain. ATP is a powerful inhibitor of PFK1 which catalyzes the rate-limiting step in glycolysis (Rodicio et al. (2000)). Therefore, the elevated NTP levels in *xrn1* Δ are likely to lead to reduced glycolytic flux. This in turn would reduce flux into the citric acid cycle, depriving cells of amino acid precursors and reducing equivalents in the form of NADH which would limit cell growth. Evidence for this can be seen in the reduction in cAMP levels in *xrn1* Δ cells. As a secondary messenger in the *S. cerevisiae* ras-signalling pathway, cAMP levels integrate extracellular glucose concentrations and intracellular Hxk2p activity (Rolland et al. (2000)). Therefore, a reduction in glycolytic flux due to PFK1 inhibition will result in a reduction in cAMP levels, as observed.

As yet it is not known whether the defects in translation elongation in *xrn1* Δ are also related to the elevated NTP levels. Although no mechanism for this comes to mind, it remains an intriguing possibility. Regardless, the next step in understanding the behaviour of *xrn1* Δ will be to try to establish causality for the elevated NTP levels in *xrn1* Δ slow-growth and transcription through chemical or genetic interventions. Rescuing the phenotypes of *xrn1* Δ by depleting NTP levels would be the ideal way to do this. However, drugs such as 6-azauracil and 2-deoxyglucose have indirect mechanisms of action that would complicate interpretation of their effects by interfering with glycolysis and transcription directly. Depending on the relative timing of NTP accumulation versus the other phenotypes of cells lacking *XRN1*, it may be possible to dissect these processes using an inducible knockout such as a degron. *xrn1-AID^{*}-9xmyc* strains have recently been generated in the lab for this purpose.

5.6 Concluding remarks.

For over four decades, researchers across the globe have been expanding our understanding of the yeast metabolic cycle. Following that pedigree, this study aimed to determine the role of post-transcriptional regulation of gene expression in control of the YMC. In doing so we have made some exciting discoveries.

We studied gene expression at three different levels, RNA degradation, translation and protein turnover. We find that although *XRN1*-mediated RNA degradation is required for the proper regulation of the majority of cycling transcripts, steady-state protein levels are primarily determined by slow protein-turnover rates which render the vast majority of proteins effectively constitutive.

Combining this discovery with insights from the literature, we determine that the function of transcript cycling is to couple the time-averaged transcriptome to nutrient availability, generating the UGRR and setting steady-state rather than dynamic protein levels. Thus, in line with trends developing in the field of circadian rhythms, we argue against a transcription factor driven metabolic cycle, favouring one defined by oscillations in cellular redox state, metabolite levels and protein modifications.

We also present a new angle on the puzzle of loss of transcript buffering in the *xrn1* Δ mutant, finding that this strain exhibits severe dysregulation of NTP levels and defects in translation elongation. These changes in metabolism and protein turnover go a long way to explaining the phenotypes of the *xrn1* Δ mutant, particularly regarding its increased mRNA synthesis rate and slow-growth phenotype. However, they raise a new question, what causes the elevated NTP levels in *xrn1* Δ ? This answer to

that, along with many other questions raised by this body of work will have to await future study.

Thank you for your time.

Appendix A

Appendix 1 - Supplementary Tables

Gene	Log ₂ fold-change (protein)	Overlap with RNAs
YGR169C-A	7.47	FALSE
SNZ1	6.31	TRUE
CDC36	5.38	FALSE
ADD66	5.36	TRUE
BUD32	5	FALSE
SPO14	4.76	TRUE
ADR1	4.64	TRUE
ESC1	4.58	TRUE
MRPL44	4.39	FALSE
ALY1	4.35	TRUE
EBS1	4.35	TRUE
MST1	4.07	FALSE
SOK1	4	FALSE
DSD1	3.88	FALSE
WTM2	3.84	FALSE
PAH1	3.78	FALSE
CDD1	3.76	FALSE
YOL086W-A	3.6	FALSE
ALY2	3.6	TRUE
MDV1	3.58	TRUE
OPY2	3.3	FALSE
YPL088W	3.3	FALSE
TDA2	3.24	FALSE
RSM24	3.19	FALSE
ACK1	3.17	FALSE
RIM20	2.94	FALSE
PRP45	2.79	FALSE
AIM17	1.57	FALSE
RGI1	1.55	TRUE
AIM37	1.4	FALSE
MBF1	1.2	FALSE
MRPL35	1.17	FALSE
MIA40	1.07	FALSE
SKY1	1.04	TRUE
COX9	1.02	FALSE
ALD5	0.95	TRUE
SDS23	0.95	FALSE
CYM1	0.95	FALSE
FCJ1	0.95	FALSE
UGP1	0.93	TRUE
PAM18	0.92	FALSE
DED1	0.9	FALSE

Table A.1: Few proteins change in abundance in the *xrn1* Δ proteome.

Gene	Log ₂ fold-change (protein)	Overlap with RNAs
MDM38	0.89	TRUE
MYO3	0.86	TRUE
MAS2	0.86	FALSE
YML6	0.85	TRUE
YPT52	0.81	TRUE
YDR333C	0.8	FALSE
COY1	0.79	FALSE
OXP1	0.78	FALSE
MLP1	0.76	TRUE
YPK1	0.74	TRUE
MRPL38	0.73	FALSE
RMD9	0.73	FALSE
NPL4	0.73	FALSE
TIM8	0.72	FALSE
AIM13	0.72	FALSE
TIM13	0.7	FALSE
TPK3	0.67	FALSE
NAM9	0.67	FALSE
YKL027W	0.66	FALSE
UBA1	0.65	FALSE
TIM9	0.63	FALSE
PAP1	0.62	TRUE
PIN4	0.62	TRUE
MON2	0.62	FALSE
DHH1	0.62	FALSE
MAS1	0.61	FALSE
ISD11	0.61	FALSE
MIR1	0.57	FALSE
ECM29	0.57	FALSE
ARI1	0.57	TRUE
YLR225C	0.56	FALSE
PPM1	0.56	FALSE
SWH1	0.56	TRUE
NAB6	0.56	TRUE
DEF1	0.55	TRUE
CAP1	0.55	FALSE
TOM40	0.54	FALSE
PBA1	0.54	FALSE
MSS116	0.51	FALSE
YME1	0.5	FALSE
RPN13	0.49	FALSE
HSP60	0.48	FALSE
HAT2	0.48	FALSE

Table A.1: Few proteins change in abundance in the *xrn1* Δ proteome.

Gene	Log ₂ fold-change (protein)	Overlap with RNAs
HSP10	0.47	FALSE
APL2	0.46	FALSE
TOM1	0.46	TRUE
TIM44	0.46	FALSE
PPZ1	0.46	FALSE
YTA12	0.44	TRUE
ATG21	0.44	FALSE
REH1	0.43	FALSE
PTH2	0.42	FALSE
TOM70	0.4	FALSE
PAN6	0.4	TRUE
BRO1	0.4	FALSE
YPR097W	0.39	FALSE
PUF4	0.38	TRUE
XPT1	0.38	FALSE
PHB2	0.37	FALSE
HMO1	0.37	TRUE
MMS2	0.37	FALSE
TRS33	0.37	TRUE
IMH1	0.35	TRUE
NUP145	0.33	TRUE
PCC1	0.33	FALSE
TMA108	0.31	FALSE
MET7	0.25	FALSE
SHE3	0.25	TRUE
CCA1	0.24	TRUE
OCA1	0.22	TRUE
KES1	0.19	FALSE
TIF5	0.17	FALSE
PAL1	-0.13	TRUE
PRD1	-0.17	FALSE
RPS29B	-0.2	FALSE
RPL33B	-0.23	FALSE
LSB3	-0.24	FALSE
RPS27B;RPS27A	-0.25	FALSE
TYS1	-0.25	FALSE
RPS9B	-0.25	FALSE
SUI2	-0.26	FALSE
ERG20	-0.27	FALSE
HYP2	-0.28	FALSE
GAR1	-0.28	FALSE
RPL3	-0.28	FALSE
RPS24B;RPS24A	-0.28	FALSE

Table A.1: Few proteins change in abundance in the *xrn1* Δ proteome.

Gene	Log ₂ fold-change (protein)	Overlap with RNAs
DPS1	-0.29	FALSE
RPL35B;RPL35A	-0.29	FALSE
RPL19B;RPL19A	-0.29	FALSE
SEC28	-0.29	FALSE
RPS19B;RPS19A	-0.29	FALSE
RPL26B	-0.29	FALSE
RPS20	-0.29	FALSE
LSG1	-0.29	FALSE
RPL21A	-0.3	FALSE
NHP2	-0.3	FALSE
RPL34A;RPL34B	-0.3	FALSE
SFB3	-0.3	FALSE
RPL30	-0.31	FALSE
RPL22A	-0.31	FALSE
RPS15	-0.31	FALSE
RPS4B;RPS4A	-0.32	FALSE
TEF1	-0.32	FALSE
YPT7	-0.32	FALSE
TDH3	-0.32	FALSE
SCD6	-0.33	FALSE
RPL25	-0.33	FALSE
RPL31B	-0.33	FALSE
RPS8B;RPS8A	-0.33	FALSE
ARB1	-0.34	FALSE
RPS18B;RPS18A	-0.34	FALSE
RPS26B;RPS26A	-0.34	FALSE
RPL2B;RPL2A	-0.34	FALSE
RPL16A	-0.34	FALSE
IDI1	-0.35	FALSE
RPL24B	-0.35	FALSE
RPL17B	-0.35	FALSE
RPL13A;RPL13B	-0.36	FALSE
YOR283W	-0.36	FALSE
PRO2	-0.37	FALSE
COP1	-0.37	TRUE
ASC1	-0.37	FALSE
ILV1	-0.38	FALSE
BNI5	-0.38	TRUE
PFK1	-0.39	FALSE
CYS3	-0.4	FALSE
ENO2	-0.4	FALSE
ADK1	-0.43	FALSE
ARF1	-0.43	FALSE

Table A.1: Few proteins change in abundance in the *xrn1* Δ proteome.

Gene	Log ₂ fold-change (protein)	Overlap with RNAs
TAN1	-0.43	FALSE
RPL8B	-0.43	FALSE
MNN9	-0.43	FALSE
IPP1	-0.44	FALSE
HOC1	-0.44	FALSE
SCP160	-0.44	FALSE
ALA1	-0.44	FALSE
SAS10	-0.45	FALSE
YRA1	-0.45	TRUE
CDC19	-0.46	FALSE
GRS1	-0.47	FALSE
ADE6	-0.47	FALSE
GRH1	-0.48	FALSE
RPB2	-0.48	FALSE
HOM6	-0.48	FALSE
ZUO1	-0.49	FALSE
ACT1	-0.51	FALSE
RPL6A	-0.51	FALSE
ERG6	-0.52	FALSE
MNN10	-0.52	FALSE
STM1	-0.52	FALSE
LYS12	-0.53	FALSE
MKT1	-0.53	FALSE
VTC4	-0.53	FALSE
SUB2	-0.55	FALSE
CDC21	-0.55	FALSE
DED81	-0.56	FALSE
FRS2	-0.56	FALSE
SPE3	-0.57	FALSE
KTR3	-0.57	FALSE
MYO2	-0.57	FALSE
KRS1	-0.57	TRUE
PAB1	-0.58	FALSE
RPO21	-0.58	FALSE
LCB1	-0.58	FALSE
EMP24	-0.59	FALSE
SSZ1	-0.59	FALSE
TUB2	-0.59	FALSE
TUB1	-0.59	TRUE
ADE5,7	-0.59	FALSE
EFT1	-0.6	FALSE
BFR1	-0.61	FALSE
WRS1	-0.61	FALSE

Table A.1: Few proteins change in abundance in the *xrn1* Δ proteome.

Gene	Log ₂ fold-change (protein)	Overlap with RNAs
MNN2	-0.61	TRUE
ADH1	-0.61	FALSE
CHD1	-0.61	FALSE
RRP5	-0.61	FALSE
HGH1	-0.62	FALSE
SEC53	-0.64	FALSE
GAS3	-0.64	FALSE
KRE2	-0.65	FALSE
VTC3	-0.66	FALSE
CWH41	-0.69	FALSE
ADO1	-0.69	FALSE
RAD23	-0.72	TRUE
GDA1	-0.73	FALSE
GPI16	-0.74	FALSE
KTR1	-0.75	FALSE
RTN1	-0.76	FALSE
ETT1	-0.77	FALSE
GPD2	-0.77	FALSE
CBR1	-0.78	FALSE
TPA1	-0.78	FALSE
GLN1	-0.78	FALSE
SHM1	-0.81	FALSE
NCP1	-0.84	FALSE
GLT1	-1.06	FALSE
GDH1	-1.06	FALSE
TCB2	-1.1	FALSE
KEX1	-4.53	FALSE
XRN1	-9.81	NA (Deletion)

Table A.1: Few proteins change in abundance in the *xrn1*Δ proteome.

Mass-spectrometric analysis of *xrn1*Δ proteome.

*xrn1*Δ & *BY4741* cells were grown to mid-log phase in YPD, total protein was extracted and protein extracts were subjected to label-free mass-spectrometry (materials and methods - n = 3). Log₂ fold-changes of genes whose proteins showed significant differences between *BY4741* & *xrn1*Δ are shown and whether they overlap with genes which change at the RNA level is reported.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
SUT537	16.80	-7.19	1.33	-5.42	0.00	0.00
YHR159W	10.58	-6.53	1.38	-4.73	0.00	0.00
XUT_13F-354	10.58	-6.51	1.40	-4.66	0.00	0.00
YCL021W-A	57.47	-4.73	0.60	-7.84	0.00	0.00
SUT709	11.40	-4.73	1.27	-3.72	0.00	0.00
SUT485	12.18	-4.19	1.06	-3.94	0.00	0.00
SUT138	11.27	-4.07	1.12	-3.63	0.00	0.00
YGL176C	31.53	-3.86	0.67	-5.80	0.00	0.00
YNR068C	18.97	-3.84	0.85	-4.52	0.00	0.00
YER038C	23.16	-3.53	0.70	-5.03	0.00	0.00
YPR115W	37.18	-3.49	0.60	-5.83	0.00	0.00
YOR129C	12.23	-3.47	0.96	-3.63	0.00	0.00
YDR076W	23.71	-3.41	0.70	-4.90	0.00	0.00
YCR107W	20.14	-3.36	0.78	-4.30	0.00	0.00
SUT435	33.64	-3.35	0.66	-5.07	0.00	0.00
SUT686	13.45	-3.33	0.96	-3.48	0.00	0.01
YMR316C-B	26.67	-3.31	0.67	-4.96	0.00	0.00
YKR050W	18.64	-3.30	0.76	-4.32	0.00	0.00
YPL137C	51.41	-3.30	0.53	-6.18	0.00	0.00
YKL038W	39.07	-3.29	0.60	-5.52	0.00	0.00
SUT569	36.09	-3.26	0.59	-5.57	0.00	0.00
YMR085W	28.13	-3.26	0.65	-5.02	0.00	0.00
SUT670	15.56	-3.26	0.83	-3.92	0.00	0.00
SUT379	45.55	-3.25	0.51	-6.38	0.00	0.00
SUT658	29.52	-3.21	0.62	-5.16	0.00	0.00
YER184C	15.06	-3.21	0.83	-3.87	0.00	0.00
YDR125C	12.57	-3.16	0.89	-3.55	0.00	0.01
YBR020W	12.82	-3.16	0.91	-3.47	0.00	0.01
YPL018W	62.08	-3.14	0.46	-6.76	0.00	0.00
YKL151C	163.90	-3.10	0.43	-7.24	0.00	0.00
YOR379C	19.34	-3.08	0.71	-4.34	0.00	0.00
SUT002	17.34	-3.02	0.76	-3.97	0.00	0.00
XUT_11F-209	12.82	-2.97	0.87	-3.41	0.00	0.01
YOR192C	27.42	-2.97	0.61	-4.85	0.00	0.00
YDR155C	87.49	-2.96	0.52	-5.74	0.00	0.00
XUT_4R-393	14.67	-2.95	0.88	-3.34	0.00	0.01
SUT336	17.74	-2.94	0.76	-3.86	0.00	0.00
SUT772	24.92	-2.91	0.65	-4.45	0.00	0.00
YER109C	49.56	-2.86	0.56	-5.12	0.00	0.00
SUT350	123.81	-2.83	0.41	-6.95	0.00	0.00
XUT_2R-85	17.50	-2.83	0.79	-3.59	0.00	0.00
YKL220C	15.36	-2.81	0.83	-3.36	0.00	0.01

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
SUT711	25.94	-2.80	0.66	-4.26	0.00	0.00
YJR020W	17.51	-2.72	0.79	-3.45	0.00	0.01
YPL076W	19.00	-2.69	0.77	-3.47	0.00	0.01
YMR096W	41.33	-2.68	0.50	-5.41	0.00	0.00
YDR293C	110.50	-2.67	0.41	-6.49	0.00	0.00
YIR020C	21.18	-2.66	0.64	-4.14	0.00	0.00
YJR107W	28.14	-2.64	0.61	-4.33	0.00	0.00
YBL075C	84.01	-2.63	0.41	-6.40	0.00	0.00
YDR443C	111.18	-2.62	0.39	-6.78	0.00	0.00
SUT510	22.83	-2.61	0.68	-3.82	0.00	0.00
YKL171W	23.38	-2.61	0.64	-4.11	0.00	0.00
YPR193C	47.87	-2.60	0.49	-5.29	0.00	0.00
YOR204W	317.31	-2.58	0.34	-7.66	0.00	0.00
SUT366	36.11	-2.52	0.57	-4.41	0.00	0.00
YLR234W	74.62	-2.52	0.42	-5.97	0.00	0.00
YDL172C	33.05	-2.47	0.62	-3.99	0.00	0.00
YJL077W-B	22.90	-2.45	0.64	-3.83	0.00	0.00
YLR434C	21.29	-2.43	0.65	-3.74	0.00	0.00
SUT629	36.98	-2.42	0.63	-3.83	0.00	0.00
YDL129W	20.92	-2.40	0.65	-3.68	0.00	0.00
YMR140W	63.69	-2.36	0.49	-4.86	0.00	0.00
YOL029C	64.77	-2.35	0.41	-5.70	0.00	0.00
SUT665	34.87	-2.35	0.61	-3.88	0.00	0.00
YDR363W	23.03	-2.32	0.64	-3.63	0.00	0.00
YHR080C	63.00	-2.32	0.46	-5.06	0.00	0.00
SUT745	38.84	-2.31	0.50	-4.63	0.00	0.00
YGR249W	49.53	-2.31	0.49	-4.74	0.00	0.00
YLR222C-A	32.30	-2.29	0.59	-3.89	0.00	0.00
YDR216W	32.00	-2.28	0.55	-4.15	0.00	0.00
YOR116C	301.94	-2.26	0.35	-6.39	0.00	0.00
YOR084W	29.67	-2.25	0.57	-3.94	0.00	0.00
YML032C	52.69	-2.24	0.44	-5.08	0.00	0.00
YPR104C	52.21	-2.22	0.43	-5.11	0.00	0.00
SUT342	20.26	-2.21	0.66	-3.36	0.00	0.01
YER135C	91.58	-2.21	0.42	-5.21	0.00	0.00
YDR488C	63.60	-2.20	0.43	-5.10	0.00	0.00
YOR071C	63.01	-2.18	0.50	-4.40	0.00	0.00
YFL057C	23.45	-2.18	0.63	-3.45	0.00	0.01
YJL019W	64.60	-2.18	0.47	-4.66	0.00	0.00
YBR212W	238.48	-2.17	0.43	-5.02	0.00	0.00
YDR433W	5545.82	-2.13	0.25	-8.42	0.00	0.00
YMR102C	65.97	-2.13	0.40	-5.29	0.00	0.00
YGL113W	37.38	-2.10	0.55	-3.83	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
YGL140C	118.31	-2.08	0.44	-4.71	0.00	0.00
YMR273C	23.37	-2.07	0.59	-3.48	0.00	0.01
YDR205W	108.45	-2.07	0.36	-5.67	0.00	0.00
YDL039C	56.38	-2.07	0.58	-3.56	0.00	0.01
YCR045W-A	67.22	-2.05	0.44	-4.64	0.00	0.00
YPL221W	189.70	-2.05	0.41	-4.96	0.00	0.00
SUT280	35.49	-2.05	0.55	-3.74	0.00	0.00
YNL245C	63.00	-2.04	0.42	-4.80	0.00	0.00
YLR385C	117.65	-2.03	0.39	-5.17	0.00	0.00
YGL212W	50.52	-2.03	0.47	-4.36	0.00	0.00
YML081W	118.78	-2.01	0.37	-5.43	0.00	0.00
YPR024W	62.37	-1.99	0.50	-3.97	0.00	0.00
YDR052C	30.27	-1.97	0.54	-3.63	0.00	0.00
YCL026C-B	23.45	-1.97	0.59	-3.36	0.00	0.01
YLR046C	59.25	-1.94	0.42	-4.66	0.00	0.00
YAL034C	76.92	-1.93	0.46	-4.17	0.00	0.00
YJR091C	48.78	-1.91	0.52	-3.69	0.00	0.00
YGR055W	289.26	-1.91	0.30	-6.43	0.00	0.00
CUT146	1008.59	-1.90	0.44	-4.32	0.00	0.00
YPL056C	35.63	-1.89	0.51	-3.69	0.00	0.00
YOR381W	32.17	-1.88	0.50	-3.73	0.00	0.00
YGL008C	2856.24	-1.88	0.30	-6.17	0.00	0.00
YDR050C	41986.79	-1.83	0.47	-3.90	0.00	0.00
YKL095W	63.87	-1.82	0.43	-4.26	0.00	0.00
YGR143W	42.85	-1.81	0.47	-3.87	0.00	0.00
YDR006C	46.98	-1.80	0.52	-3.46	0.00	0.01
YNL329C	98.28	-1.80	0.34	-5.22	0.00	0.00
YGL193C	44.30	-1.79	0.44	-4.05	0.00	0.00
YCL036W	42.87	-1.78	0.48	-3.70	0.00	0.00
YDL220C	48.08	-1.78	0.50	-3.54	0.00	0.01
YKL083W	132.74	-1.76	0.45	-3.88	0.00	0.00
YCL037C	149.53	-1.75	0.37	-4.71	0.00	0.00
YAL031C	49.12	-1.73	0.45	-3.89	0.00	0.00
YLR419W	34.00	-1.72	0.50	-3.42	0.00	0.01
YCR037C	94.46	-1.70	0.40	-4.28	0.00	0.00
YGL197W	44.25	-1.69	0.48	-3.56	0.00	0.01
YJR004C	48.99	-1.69	0.49	-3.44	0.00	0.01
YNL190W	1572.13	-1.68	0.42	-4.04	0.00	0.00
YLL051C	78.67	-1.66	0.41	-4.07	0.00	0.00
YHR033W	72.20	-1.65	0.43	-3.79	0.00	0.00
YPR022C	51.81	-1.64	0.42	-3.94	0.00	0.00
YHR082C	94.27	-1.63	0.45	-3.66	0.00	0.00
YAL011W	38.59	-1.62	0.47	-3.43	0.00	0.01

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
YGL256W	87.47	-1.62	0.39	-4.17	0.00	0.00
YOL004W	120.94	-1.60	0.37	-4.28	0.00	0.00
YDR475C	114.09	-1.57	0.35	-4.49	0.00	0.00
YER114C	96.51	-1.56	0.37	-4.18	0.00	0.00
YAR042W	109.16	-1.56	0.35	-4.47	0.00	0.00
YPR138C	44.76	-1.56	0.46	-3.40	0.00	0.01
YBR063C	54.51	-1.55	0.42	-3.65	0.00	0.00
YOR301W	52.24	-1.55	0.46	-3.35	0.00	0.01
YML100W	94.38	-1.54	0.39	-3.97	0.00	0.00
YPR008W	91.23	-1.54	0.36	-4.27	0.00	0.00
YAL020C	99.10	-1.53	0.36	-4.28	0.00	0.00
YIL112W	68.41	-1.52	0.40	-3.81	0.00	0.00
SUT717	206.32	-1.51	0.32	-4.67	0.00	0.00
YJL083W	63.59	-1.49	0.43	-3.45	0.00	0.01
YFL036W	51.03	-1.47	0.44	-3.37	0.00	0.01
YGR281W	77.07	-1.47	0.38	-3.85	0.00	0.00
YDL231C	56.59	-1.47	0.43	-3.43	0.00	0.01
YBR176W	79.12	-1.46	0.38	-3.80	0.00	0.00
YJL176C	84.57	-1.44	0.42	-3.48	0.00	0.01
YOR227W	70.18	-1.44	0.43	-3.34	0.00	0.01
YOL092W	440.77	-1.43	0.30	-4.74	0.00	0.00
YPL093W	1440.87	-1.43	0.24	-6.03	0.00	0.00
YML116W-A	248.29	-1.43	0.28	-5.17	0.00	0.00
YJL167W	1238.32	-1.42	0.36	-3.96	0.00	0.00
YOL051W	116.27	-1.41	0.40	-3.52	0.00	0.01
YJR060W	190.78	-1.40	0.34	-4.09	0.00	0.00
YPL190C	136.25	-1.40	0.32	-4.34	0.00	0.00
YML051W	178.10	-1.40	0.34	-4.15	0.00	0.00
YLR190W	91.75	-1.40	0.41	-3.37	0.00	0.01
YPL075W	76.61	-1.39	0.36	-3.86	0.00	0.00
YLR023C	106.28	-1.39	0.37	-3.81	0.00	0.00
YEL024W	131.06	-1.38	0.32	-4.31	0.00	0.00
YDR524C-A	160.67	-1.38	0.38	-3.63	0.00	0.00
YAL013W	142.01	-1.37	0.40	-3.43	0.00	0.01
YKR019C	92.59	-1.37	0.38	-3.62	0.00	0.00
YIL048W	106.73	-1.36	0.33	-4.07	0.00	0.00
YDL047W	598.14	-1.36	0.31	-4.32	0.00	0.00
YNL127W	74.03	-1.35	0.39	-3.46	0.00	0.01
YMR295C	978.56	-1.35	0.32	-4.17	0.00	0.00
YGR233C	120.91	-1.33	0.32	-4.21	0.00	0.00
YPL265W	135.40	-1.32	0.35	-3.82	0.00	0.00
YFL018C	903.21	-1.32	0.26	-5.13	0.00	0.00
YNL246W	417.78	-1.31	0.28	-4.68	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
YJL078C	165.55	-1.30	0.37	-3.49	0.00	0.01
YBR241C	70.59	-1.29	0.36	-3.55	0.00	0.01
YOR127W	121.84	-1.29	0.32	-4.05	0.00	0.00
YMR109W	102.43	-1.28	0.35	-3.67	0.00	0.00
YLR109W	41567.90	-1.27	0.23	-5.45	0.00	0.00
YLR167W	38423.38	-1.27	0.38	-3.37	0.00	0.01
YEL034W	38611.40	-1.26	0.29	-4.31	0.00	0.00
YPL226W	384.19	-1.25	0.26	-4.78	0.00	0.00
YHR012W	447.26	-1.25	0.36	-3.45	0.00	0.01
YBR068C	894.51	-1.21	0.27	-4.51	0.00	0.00
YKL165C	106.27	-1.21	0.34	-3.57	0.00	0.01
YPR191W	314.96	-1.20	0.30	-4.05	0.00	0.00
YOR222W	172.95	-1.18	0.32	-3.74	0.00	0.00
YLR355C	2000.72	-1.18	0.32	-3.67	0.00	0.00
YEL017C-A	6300.82	-1.18	0.34	-3.41	0.00	0.01
YOR310C	1383.91	-1.17	0.30	-3.93	0.00	0.00
YHR089C	12542.49	-1.17	0.34	-3.43	0.00	0.01
YGR060W	2568.56	-1.16	0.28	-4.09	0.00	0.00
YDR128W	171.64	-1.16	0.32	-3.57	0.00	0.01
YIL033C	241.89	-1.16	0.30	-3.92	0.00	0.00
YGL255W	154.81	-1.15	0.33	-3.53	0.00	0.01
YLR286C	172.07	-1.14	0.34	-3.41	0.00	0.01
YDL164C	261.71	-1.13	0.33	-3.47	0.00	0.01
YLR403W	152.07	-1.13	0.33	-3.46	0.00	0.01
YGR086C	903.67	-1.11	0.30	-3.72	0.00	0.00
YBR133C	125.78	-1.11	0.33	-3.37	0.00	0.01
YDR177W	623.91	-1.11	0.26	-4.19	0.00	0.00
YDR094W	207.19	-1.09	0.28	-3.85	0.00	0.00
YJR105W	2307.61	-1.08	0.26	-4.19	0.00	0.00
YFL037W	1848.71	-1.08	0.26	-4.13	0.00	0.00
YLL050C	3581.50	-1.07	0.27	-3.90	0.00	0.00
YNL004W	245.12	-1.06	0.28	-3.81	0.00	0.00
YER151C	263.27	-1.06	0.30	-3.53	0.00	0.01
YOL098C	190.64	-1.04	0.30	-3.44	0.00	0.01
YDR046C	231.27	-1.02	0.30	-3.44	0.00	0.01
YDL130W-A	431.18	-1.02	0.27	-3.76	0.00	0.00
YOL038W	732.22	-1.02	0.29	-3.54	0.00	0.01
YGR095C	287.93	-1.01	0.28	-3.66	0.00	0.00
YFR033C	296.94	-1.00	0.29	-3.47	0.00	0.01
YDR382W	14860.28	-0.98	0.29	-3.44	0.00	0.01
YGR106C	884.73	-0.96	0.26	-3.70	0.00	0.00
YGR159C	2010.22	-0.95	0.26	-3.61	0.00	0.00
YDR037W	2486.72	-0.95	0.25	-3.82	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
YJL143W	572.64	-0.90	0.25	-3.62	0.00	0.00
YOL012C	987.96	-0.86	0.24	-3.59	0.00	0.00
YJR009C	20676.05	-0.85	0.24	-3.55	0.00	0.01
YNL055C	6876.18	-0.83	0.24	-3.48	0.00	0.01
YLR249W	16478.64	-0.80	0.22	-3.62	0.00	0.00
YOR385W	1310.82	-0.79	0.23	-3.46	0.00	0.01
YJR094W-A	10195.27	0.77	0.23	3.37	0.00	0.01
YDR064W	15211.38	0.81	0.24	3.39	0.00	0.01
YKR057W	14996.82	0.83	0.24	3.40	0.00	0.01
YGR027C	21926.10	0.89	0.24	3.64	0.00	0.00
YER019C-A	2227.32	0.92	0.25	3.69	0.00	0.00
YDL191W	4327.80	0.92	0.24	3.81	0.00	0.00
YMR230W	5931.48	0.92	0.25	3.74	0.00	0.00
YLR185W	7845.62	0.96	0.27	3.52	0.00	0.01
YGR034W	12848.12	0.98	0.24	4.12	0.00	0.00
YJL062W-A	669.71	0.98	0.29	3.37	0.00	0.01
YOR357C	462.23	0.99	0.26	3.85	0.00	0.00
YJL173C	1162.37	1.00	0.27	3.66	0.00	0.00
YGL030W	21056.45	1.01	0.28	3.60	0.00	0.00
YKR095W-A	234.12	1.01	0.29	3.50	0.00	0.01
YOR224C	1728.64	1.02	0.23	4.52	0.00	0.00
YOR367W	192.54	1.02	0.28	3.60	0.00	0.00
YNL081C	284.65	1.03	0.27	3.87	0.00	0.00
YDR533C	214.26	1.03	0.30	3.38	0.00	0.01
YDR281C	167.98	1.03	0.30	3.40	0.00	0.01
YOR326W	649.51	1.03	0.28	3.66	0.00	0.00
YDR363W-A	1519.92	1.04	0.28	3.66	0.00	0.00
YDL070W	460.42	1.04	0.28	3.73	0.00	0.00
SUT053	220.83	1.05	0.29	3.66	0.00	0.00
YGR029W	135.43	1.06	0.32	3.36	0.00	0.01
YMR130W	367.24	1.06	0.28	3.81	0.00	0.00
YCR090C	262.51	1.07	0.28	3.83	0.00	0.00
YIL156W-B	1072.75	1.08	0.24	4.48	0.00	0.00
YBR255C-A	451.91	1.08	0.26	4.19	0.00	0.00
YLR021W	307.63	1.08	0.31	3.43	0.00	0.01
YBL107C	228.25	1.08	0.30	3.66	0.00	0.00
YKL137W	177.82	1.09	0.30	3.60	0.00	0.00
YMR312W	146.95	1.09	0.30	3.66	0.00	0.00
YEL020W-A	623.61	1.09	0.32	3.45	0.00	0.01
YDL099W	625.92	1.10	0.32	3.37	0.00	0.01
YER183C	456.11	1.10	0.27	4.02	0.00	0.00
YML009C	661.08	1.10	0.29	3.78	0.00	0.00
YML081C-A	1546.65	1.12	0.25	4.42	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
YIL098C	196.37	1.12	0.32	3.54	0.00	0.01
YDR196C	155.46	1.13	0.29	3.85	0.00	0.00
YKR085C	149.63	1.13	0.32	3.55	0.00	0.01
YDR367W	162.80	1.13	0.33	3.44	0.00	0.01
YHR143W-A	401.67	1.14	0.29	3.89	0.00	0.00
YPR043W	31568.78	1.15	0.29	4.02	0.00	0.00
YDL061C	6173.72	1.16	0.28	4.14	0.00	0.00
YHR083W	177.35	1.16	0.34	3.41	0.00	0.01
YBL071W-A	358.97	1.16	0.27	4.35	0.00	0.00
YAL044W-A	308.30	1.17	0.30	3.92	0.00	0.00
YDL004W	1306.05	1.17	0.26	4.57	0.00	0.00
YLR050C	1712.56	1.17	0.35	3.39	0.00	0.01
YOR020W-A	384.69	1.17	0.29	4.04	0.00	0.00
YNL056W	113.05	1.18	0.33	3.53	0.00	0.01
YIL009C-A	105.31	1.18	0.34	3.50	0.00	0.01
YFR032C-A	12295.35	1.18	0.23	5.04	0.00	0.00
YLR112W	409.25	1.19	0.31	3.89	0.00	0.00
YLR099W-A	726.69	1.20	0.25	4.73	0.00	0.00
YER163C	284.96	1.20	0.32	3.70	0.00	0.00
YML015C	136.17	1.20	0.33	3.69	0.00	0.00
YLR429W	405.79	1.20	0.26	4.54	0.00	0.00
YPL095C	318.05	1.21	0.33	3.62	0.00	0.00
CUT465	220.59	1.21	0.30	4.02	0.00	0.00
YER074W-A	912.95	1.23	0.34	3.66	0.00	0.00
YCR020W-B	160.03	1.23	0.32	3.84	0.00	0.00
YLR325C	19944.38	1.24	0.24	5.18	0.00	0.00
YML129C	579.79	1.24	0.29	4.31	0.00	0.00
YAR002W	273.50	1.25	0.29	4.26	0.00	0.00
YBR130C	340.12	1.25	0.29	4.33	0.00	0.00
YGL224C	166.02	1.25	0.30	4.16	0.00	0.00
YLR390W	189.31	1.26	0.34	3.65	0.00	0.00
YGL226C-A	2567.62	1.26	0.30	4.21	0.00	0.00
YMR225C	186.27	1.26	0.36	3.46	0.00	0.01
YGR102C	132.39	1.27	0.36	3.52	0.00	0.01
YGL220W	2132.32	1.27	0.32	3.94	0.00	0.00
YLR104W	199.04	1.28	0.34	3.71	0.00	0.00
YCL005W-A	3735.24	1.28	0.28	4.49	0.00	0.00
YOR253W	169.36	1.28	0.33	3.89	0.00	0.00
YNR037C	477.00	1.29	0.27	4.85	0.00	0.00
YDR286C	91.04	1.30	0.38	3.45	0.00	0.01
YDR453C	75.21	1.30	0.39	3.35	0.00	0.01
YOL026C	233.95	1.30	0.36	3.59	0.00	0.00
YNL122C	140.94	1.30	0.32	4.09	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
YGR230W	72.95	1.32	0.39	3.36	0.00	0.01
YJL179W	167.18	1.33	0.36	3.70	0.00	0.00
YLR218C	332.05	1.33	0.34	3.95	0.00	0.00
YBR268W	937.05	1.34	0.30	4.45	0.00	0.00
YKL003C	259.46	1.35	0.30	4.54	0.00	0.00
XUT_10R-186	179.58	1.35	0.35	3.92	0.00	0.00
YPR145C-A	119.21	1.35	0.39	3.48	0.00	0.01
YML108W	390.87	1.35	0.29	4.68	0.00	0.00
YBR058C-A	601.08	1.36	0.26	5.29	0.00	0.00
SUT392	105.79	1.37	0.37	3.66	0.00	0.00
YER087C-B	1488.99	1.37	0.24	5.73	0.00	0.00
YBR162W-A	1977.62	1.38	0.31	4.44	0.00	0.00
YDR381C-A	269.28	1.38	0.32	4.26	0.00	0.00
YPL241C	125.36	1.38	0.32	4.38	0.00	0.00
YMR286W	294.11	1.39	0.31	4.51	0.00	0.00
YNL070W	1099.37	1.39	0.30	4.60	0.00	0.00
YDR379C-A	94.21	1.40	0.36	3.89	0.00	0.00
YFR034W-A	565.68	1.41	0.31	4.57	0.00	0.00
YKR022C	124.06	1.42	0.36	3.94	0.00	0.00
YJL152W	150.46	1.44	0.41	3.55	0.00	0.01
YOR041C	67.76	1.44	0.40	3.60	0.00	0.00
YOL077W-A	886.05	1.45	0.41	3.58	0.00	0.01
YLR283W	105.61	1.46	0.34	4.34	0.00	0.00
YKR060W	81.99	1.48	0.37	4.04	0.00	0.00
YOR238W	59.60	1.48	0.42	3.50	0.00	0.01
SUT070	63.65	1.51	0.41	3.69	0.00	0.00
YLR146C	391.68	1.51	0.36	4.18	0.00	0.00
YNL146C-A	57.85	1.51	0.43	3.51	0.00	0.01
YCR007C	69.44	1.51	0.45	3.38	0.00	0.01
YLR287C-A	11777.53	1.52	0.24	6.22	0.00	0.00
YNL138W-A	108.00	1.52	0.36	4.28	0.00	0.00
YIL014C-A	234.08	1.53	0.40	3.77	0.00	0.00
YOR045W	3773.94	1.55	0.31	4.98	0.00	0.00
YJR082C	371.91	1.55	0.39	3.97	0.00	0.00
YDL147W	1007.67	1.56	0.28	5.65	0.00	0.00
YHR134W	73.22	1.56	0.40	3.91	0.00	0.00
YMR114C	148.79	1.56	0.40	3.94	0.00	0.00
YBR233W-A	351.69	1.56	0.29	5.39	0.00	0.00
YDR469W	220.87	1.56	0.32	4.85	0.00	0.00
YML097C	138.86	1.58	0.32	5.00	0.00	0.00
YER093C-A	77.92	1.58	0.39	4.03	0.00	0.00
SUT285	78.09	1.59	0.43	3.73	0.00	0.00
YGL226W	188.81	1.59	0.42	3.82	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
YEL059C-A	66.38	1.61	0.44	3.63	0.00	0.00
YOR304C-A	152.74	1.62	0.32	5.09	0.00	0.00
YPR100W	469.83	1.64	0.30	5.50	0.00	0.00
SUT625	97.63	1.64	0.41	4.01	0.00	0.00
YDR078C	66.28	1.66	0.46	3.63	0.00	0.00
XUT_2F-141	42.24	1.66	0.47	3.57	0.00	0.01
YMR035W	271.74	1.66	0.33	5.09	0.00	0.00
YMR040W	120.57	1.67	0.35	4.85	0.00	0.00
YLR411W	42.01	1.68	0.47	3.60	0.00	0.00
YBR111W-A	75.37	1.69	0.37	4.56	0.00	0.00
YHR005C-A	1581.90	1.70	0.27	6.35	0.00	0.00
YMR074C	637.53	1.71	0.28	6.14	0.00	0.00
SUT479	35.12	1.72	0.49	3.49	0.00	0.01
YOL014W	997.19	1.72	0.30	5.81	0.00	0.00
YGR050C	65.19	1.72	0.45	3.82	0.00	0.00
YNR032C-A	304.31	1.73	0.40	4.28	0.00	0.00
YDR357C	214.54	1.73	0.33	5.24	0.00	0.00
YGL194C-A	176.36	1.73	0.31	5.65	0.00	0.00
XUT_13F-294	58.95	1.74	0.42	4.17	0.00	0.00
YHR070W	3354.93	1.75	0.34	5.21	0.00	0.00
YCR082W	1146.18	1.75	0.33	5.32	0.00	0.00
YLR388W	8572.11	1.76	0.28	6.22	0.00	0.00
XUT_11R-265	48.91	1.77	0.49	3.57	0.00	0.01
YJL127C-B	453.46	1.78	0.28	6.30	0.00	0.00
XUT_3F-71	103.04	1.78	0.36	5.01	0.00	0.00
XUT_7F-283	43.86	1.78	0.51	3.49	0.00	0.01
SUT838	63.04	1.78	0.50	3.60	0.00	0.00
SUT355	77.65	1.79	0.38	4.73	0.00	0.00
XUT_14F-190	36.30	1.81	0.53	3.45	0.00	0.01
YLL014W	380.97	1.81	0.37	4.87	0.00	0.00
YGR206W	223.55	1.84	0.38	4.87	0.00	0.00
SUT253	63.07	1.85	0.43	4.33	0.00	0.00
YMR180C	47.77	1.85	0.54	3.40	0.00	0.01
YPL071C	396.77	1.85	0.39	4.80	0.00	0.00
CUT793	152.17	1.86	0.33	5.58	0.00	0.00
YCR043C	410.66	1.87	0.27	6.80	0.00	0.00
SUT076	61.33	1.87	0.40	4.70	0.00	0.00
CUT030	61.54	1.87	0.43	4.34	0.00	0.00
YGR030C	74.89	1.88	0.48	3.87	0.00	0.00
SUT045	47.50	1.89	0.49	3.81	0.00	0.00
SUT457	97.04	1.89	0.38	5.01	0.00	0.00
YOR167C	34288.76	1.89	0.29	6.55	0.00	0.00
XUT_7F-76	39.62	1.89	0.53	3.57	0.00	0.01

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
SUT543	45.88	1.91	0.52	3.69	0.00	0.00
YHR072W-A	5737.50	1.92	0.32	5.92	0.00	0.00
CUT879	37.79	1.92	0.53	3.62	0.00	0.00
YPL096C-A	455.32	1.96	0.30	6.51	0.00	0.00
SUT199	79.58	1.99	0.39	5.04	0.00	0.00
SUT091	451.31	2.01	0.33	6.18	0.00	0.00
YPR082C	222.60	2.01	0.30	6.62	0.00	0.00
SUT737	32.98	2.02	0.56	3.61	0.00	0.00
XUT_16F-193	133.68	2.03	0.35	5.84	0.00	0.00
XUT_4F-115	47.18	2.04	0.51	4.01	0.00	0.00
SUT549	35.72	2.04	0.50	4.06	0.00	0.00
YOL159C-A	164.72	2.04	0.36	5.61	0.00	0.00
YNL012W	38.38	2.05	0.54	3.80	0.00	0.00
YPR133W-A	3349.74	2.05	0.25	8.25	0.00	0.00
YDR378C	1639.44	2.06	0.27	7.56	0.00	0.00
YCR020C-A	493.03	2.06	0.33	6.22	0.00	0.00
YER071C	221.46	2.06	0.31	6.53	0.00	0.00
YNL195C	93.02	2.06	0.39	5.28	0.00	0.00
YPL148C	66.34	2.07	0.41	5.04	0.00	0.00
XUT_12F-86	23.34	2.07	0.61	3.38	0.00	0.01
SUT141	60.98	2.08	0.46	4.52	0.00	0.00
YIL120W	88.61	2.08	0.39	5.31	0.00	0.00
YOR182C	19192.58	2.10	0.25	8.48	0.00	0.00
XUT_15R-366	25.86	2.10	0.58	3.63	0.00	0.00
SUT725	52.96	2.11	0.46	4.60	0.00	0.00
SUT706	59.21	2.11	0.43	4.91	0.00	0.00
SUT535	28.04	2.11	0.58	3.64	0.00	0.00
XUT_7R-374	47.63	2.12	0.45	4.66	0.00	0.00
YGR239C	95.44	2.12	0.37	5.75	0.00	0.00
SUT139	53.20	2.12	0.43	4.95	0.00	0.00
YBR253W	1123.06	2.13	0.39	5.45	0.00	0.00
XUT_6R-50	48.12	2.13	0.49	4.40	0.00	0.00
SUT031	43.85	2.14	0.49	4.37	0.00	0.00
YNR022C	438.25	2.14	0.27	8.04	0.00	0.00
YDR461W	13141.34	2.14	0.39	5.43	0.00	0.00
YKL160W	1471.49	2.14	0.33	6.56	0.00	0.00
YGL188C-A	7846.39	2.14	0.30	7.23	0.00	0.00
YKL217W	52.80	2.15	0.47	4.54	0.00	0.00
SUT621	45.06	2.15	0.48	4.47	0.00	0.00
XUT_7F-384	33.18	2.16	0.56	3.88	0.00	0.00
XUT_13F-350	45.50	2.17	0.46	4.72	0.00	0.00
SUT055	56.46	2.18	0.50	4.38	0.00	0.00
YBR019C	26.66	2.18	0.57	3.82	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
YPL271W	1503.72	2.23	0.31	7.17	0.00	0.00
YDR374W-A	272.68	2.24	0.32	7.02	0.00	0.00
SUT774	92.06	2.25	0.53	4.25	0.00	0.00
YLR010C	52.25	2.26	0.59	3.86	0.00	0.00
YDR079C-A	195.38	2.27	0.31	7.30	0.00	0.00
YDL232W	493.08	2.27	0.33	6.95	0.00	0.00
SUT052	32.67	2.27	0.52	4.35	0.00	0.00
XUT_10F-85	31.01	2.28	0.65	3.53	0.00	0.01
YIL002W-A	1038.16	2.31	0.25	9.11	0.00	0.00
SUT314	34.69	2.32	0.56	4.15	0.00	0.00
YKL036C	133.97	2.34	0.39	5.93	0.00	0.00
SUT442	60.29	2.34	0.56	4.15	0.00	0.00
SUT570	25.41	2.35	0.59	3.95	0.00	0.00
SUT734	19.28	2.36	0.67	3.55	0.00	0.01
SUT089	115.31	2.37	0.43	5.53	0.00	0.00
XUT_4F-238	46.90	2.40	0.49	4.85	0.00	0.00
SUT525	54.77	2.40	0.49	4.94	0.00	0.00
SUT226	43.43	2.40	0.48	5.03	0.00	0.00
SUT249	20.13	2.41	0.67	3.59	0.00	0.00
YJR112W-A	411.41	2.42	0.31	7.69	0.00	0.00
CUT479	20.41	2.42	0.67	3.60	0.00	0.00
XUT_1R-83	28.27	2.42	0.57	4.24	0.00	0.00
SUT517	42.48	2.43	0.51	4.79	0.00	0.00
SUT563	18.22	2.44	0.72	3.38	0.00	0.01
SUT530	25.71	2.44	0.70	3.48	0.00	0.01
XUT_7F-271	24.37	2.44	0.64	3.80	0.00	0.00
SUT476	44.61	2.46	0.50	4.88	0.00	0.00
XUT_7R-336	44.48	2.48	0.50	4.98	0.00	0.00
YER121W	20.02	2.49	0.75	3.35	0.00	0.01
SUT071	29.11	2.50	0.62	4.01	0.00	0.00
XUT_8R-115	33.42	2.50	0.55	4.54	0.00	0.00
YAL046C	728.79	2.50	0.28	8.93	0.00	0.00
XUT_13R-134	37.67	2.50	0.53	4.71	0.00	0.00
SUT319	32.76	2.51	0.54	4.68	0.00	0.00
SUT796	47.42	2.51	0.54	4.66	0.00	0.00
CUT118	54.99	2.53	0.47	5.36	0.00	0.00
SUT814	36.46	2.53	0.62	4.12	0.00	0.00
XUT_4F-709	15.92	2.56	0.77	3.34	0.00	0.01
YJL205C	572.25	2.56	0.31	8.20	0.00	0.00
SUT566	38.09	2.57	0.56	4.58	0.00	0.00
YDR320C-A	763.48	2.60	0.29	9.04	0.00	0.00
SUT114	124.69	2.61	0.36	7.19	0.00	0.00
SUT708	38.02	2.62	0.54	4.85	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
XUT_11R-223	48.09	2.62	0.48	5.49	0.00	0.00
SUT275	148.65	2.64	0.36	7.28	0.00	0.00
YDR139C	1110.00	2.65	0.24	11.11	0.00	0.00
YDR034W-B	148.71	2.66	0.40	6.70	0.00	0.00
XUT_13F-214	47.48	2.68	0.49	5.44	0.00	0.00
SUT699	33.11	2.69	0.55	4.91	0.00	0.00
YGR295C	98.55	2.70	0.41	6.55	0.00	0.00
XUT_9R-53	38.31	2.71	0.57	4.78	0.00	0.00
YMR046W-A	26.53	2.72	0.73	3.74	0.00	0.00
SUT335	107.80	2.73	0.42	6.44	0.00	0.00
YLR146W-A	236.42	2.73	0.45	6.11	0.00	0.00
SUT710	278.12	2.73	0.40	6.87	0.00	0.00
YJL047C-A	63.08	2.75	0.47	5.89	0.00	0.00
XUT_3R-67	34.60	2.77	0.62	4.49	0.00	0.00
SUT662	23.07	2.78	0.75	3.72	0.00	0.00
XUT_7F-478	18.37	2.81	0.72	3.90	0.00	0.00
SUT601	30.84	2.84	0.62	4.58	0.00	0.00
XUT_11F-27	23.43	2.90	0.66	4.40	0.00	0.00
XUT_14R-332	26.37	2.91	0.64	4.56	0.00	0.00
XUT_15F-129	15.99	2.91	0.83	3.52	0.00	0.01
SUT346	41.09	3.01	0.53	5.67	0.00	0.00
XUT_13R-117	37.35	3.01	0.54	5.57	0.00	0.00
SUT767	39.84	3.04	0.59	5.14	0.00	0.00
XUT_16R-278	15.45	3.07	0.80	3.85	0.00	0.00
SUT684	59.30	3.09	0.52	5.93	0.00	0.00
SUT390	24.42	3.12	0.72	4.33	0.00	0.00
YNL259C	871.83	3.14	0.31	10.21	0.00	0.00
XUT_7R-97	105.20	3.14	0.43	7.34	0.00	0.00
CUT693	13.28	3.16	0.86	3.68	0.00	0.00
YDL184C	75758.61	3.16	0.38	8.42	0.00	0.00
CUT414	54.09	3.20	0.54	5.92	0.00	0.00
XUT_13F-112	12.42	3.21	0.92	3.50	0.00	0.01
XUT_8F-2	145.58	3.26	0.39	8.26	0.00	0.00
SUT293	172.52	3.26	0.37	8.90	0.00	0.00
SUT105	32.56	3.31	0.64	5.15	0.00	0.00
YPR002C-A	16.36	3.34	0.86	3.87	0.00	0.00
SUT331	40.91	3.36	0.56	6.02	0.00	0.00
XUT_13R-293	31.10	3.41	0.68	5.01	0.00	0.00
SUT769	14.10	3.47	0.99	3.50	0.00	0.01
SUT214	36.36	3.48	0.60	5.82	0.00	0.00
YDL026W	92.04	3.50	0.51	6.89	0.00	0.00
XUT_15F-104	14.88	3.55	0.92	3.88	0.00	0.00
CUT492	16.20	3.55	0.97	3.68	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Gene	baseMean	log ₂ fold-change	lfcSE	stat	pvalue	padj
YDR536W	11.95	3.61	1.02	3.53	0.00	0.01
CUT572	20.05	3.61	0.81	4.47	0.00	0.00
YMR206W	18.50	3.64	0.81	4.51	0.00	0.00
CUT360	10.35	3.81	1.10	3.47	0.00	0.01
SUT639	10.57	3.84	1.09	3.54	0.00	0.01
YDL133C-A	16281.48	3.85	0.32	12.18	0.00	0.00
SUT556	14.65	3.87	0.94	4.11	0.00	0.00
CUT827	10.77	3.88	1.08	3.59	0.00	0.00
XUT_16F-65	25.93	3.89	0.77	5.05	0.00	0.00
CUT241	10.94	3.90	1.11	3.52	0.00	0.01
SUT783	14.98	3.95	0.97	4.05	0.00	0.00
YOL038C-A	11.42	3.96	1.10	3.59	0.00	0.00
XUT_4F-328	15.42	4.01	0.97	4.14	0.00	0.00
SUT279	48.31	4.13	0.61	6.76	0.00	0.00
XUT_13F-206	29.50	4.13	0.71	5.79	0.00	0.00
CUT040	19.27	4.34	0.91	4.75	0.00	0.00
XUT_16F-178	8.62	5.08	1.44	3.53	0.00	0.01
XUT_3F-12	10.27	5.32	1.38	3.86	0.00	0.00
CUT409	5.21	5.35	1.54	3.48	0.00	0.01
XUT_13F-116	5.75	5.50	1.51	3.63	0.00	0.00
CUT664	6.25	5.61	1.51	3.72	0.00	0.00

Table A.2: DESeq2 hits for differential association with monosomes and polysomes in *BY4741*.

Output of DESeq2 pipeline comparing uncalibrated reads from monosomal and polysomal fractions of *BY4741* sucrose gradient fractionated QuantSeq libraries. (n = 3)

Gene	log ₂ FC RO (<i>xrn1</i> Δ/ <i>BY4741</i>)	pvalue	padj
XUT_15R-249	-8.18	7.42E-08	2.61E-05
XUT_1R-16	-8.14	1.10E-05	1.10E-03
CUT409	-7.46	1.37E-07	3.97E-05
YLR279W	-7.36	2.26E-06	3.60E-04
CUT269	-6.94	5.04E-07	1.05E-04
XUT_10F-151	-6.51	2.30E-05	1.97E-03
XUT_15R-99	-6.36	2.19E-05	1.93E-03
CUT441	-6.32	6.19E-07	1.24E-04
CUT444	-5.79	2.46E-07	6.32E-05
XUT_5F-122	-5.78	2.90E-05	2.33E-03
XUT_13F-116	-5.72	3.39E-09	2.06E-06
CUT837	-5.47	1.01E-05	1.05E-03
CUT177	-5.35	1.19E-04	7.31E-03
XUT_7F-311	-5.28	5.56E-05	4.07E-03
YGR259C	-5.18	5.00E-05	3.75E-03
XUT_16F-383	-4.35	4.96E-07	1.05E-04
SUT431	-3.98	8.27E-05	5.58E-03
CUT275	-3.86	5.65E-05	4.07E-03
XUT_15R-372	-3.86	1.66E-08	9.26E-06
XUT_3F-12	-3.76	3.50E-08	1.46E-05
SUT563	-3.73	6.67E-08	2.48E-05
YHR014W	-3.54	9.44E-05	6.06E-03
XUT_4F-328	-3.38	2.74E-06	4.15E-04
XUT_10R-288	-2.96	7.05E-05	4.81E-03
SUT054	-2.82	2.53E-08	1.30E-05
XUT_13F-112	-2.82	8.75E-06	9.28E-04
XUT_16F-272	-2.75	1.32E-07	3.97E-05
YHL016C	-2.73	3.63E-06	4.93E-04
SUT083	-2.71	8.22E-10	9.16E-07
YPL068C	-2.66	2.41E-07	6.32E-05
YEL057C	-2.48	8.53E-05	5.64E-03
YPL267W	-2.46	7.79E-07	1.49E-04
YOR078W	-2.45	8.76E-07	1.63E-04
YOL038C-A	-2.42	1.62E-04	9.15E-03
YMR052W	-2.42	2.62E-11	8.75E-08
YIR010W	-2.41	6.17E-06	6.98E-04
XUT_7R-374	-2.38	4.44E-10	5.93E-07
YNL180C	-2.35	2.36E-09	1.75E-06
SUT737	-2.32	9.74E-07	1.72E-04
YDR113C	-2.27	3.39E-06	4.72E-04
YIL049W	-2.27	3.26E-09	2.06E-06
XUT_7F-478	-2.23	3.27E-06	4.65E-04

Table A.3: Xtail hits for changes in monosomal-enrichment between *xrn1*Δ & *BY4741*.

Gene	log ₂ FC RO (<i>xrn1</i> Δ/ <i>BY4741</i>)	pvalue	padj
YML053C	-2.21	1.35E-09	1.29E-06
YLR151C	-2.19	1.03E-05	1.06E-03
XUT_4F-238	-2.19	1.26E-07	3.97E-05
YBR233W	-2.12	1.98E-09	1.65E-06
XUT_14F-55	-2.11	5.82E-05	4.14E-03
XUT_6R-50	-2.10	2.13E-06	3.47E-04
YDL026W	-2.05	2.75E-05	2.24E-03
SUT476	-2.04	8.18E-06	8.81E-04
YDR532C	-2.04	1.15E-05	1.11E-03
YDR176W	-2.02	1.14E-05	1.11E-03
SUT425	-2.02	1.37E-04	8.25E-03
YLL063C	-2.02	2.73E-06	4.15E-04
YLR298C	-1.99	1.52E-04	8.93E-03
SUT684	-1.99	2.99E-06	4.39E-04
YKR058W	-1.98	6.95E-05	4.79E-03
YNL155W	-1.98	2.29E-10	3.82E-07
YIL152W	-1.89	5.26E-06	6.27E-04
XUT_16F-73	-1.87	4.86E-05	3.73E-03
YDR145W	-1.84	4.54E-07	1.01E-04
YDR073W	-1.84	2.13E-05	1.90E-03
YLR165C	-1.81	1.46E-04	8.65E-03
YEL068C	-1.73	1.16E-05	1.11E-03
YGR295C	-1.73	4.37E-07	1.01E-04
SUT479	-1.72	1.33E-05	1.25E-03
YMR195W	-1.71	5.84E-08	2.30E-05
YNL036W	-1.66	5.67E-06	6.65E-04
SUT293	-1.66	3.69E-06	4.93E-04
YIL120W	-1.66	4.99E-06	6.17E-04
YKR060W	-1.66	6.33E-07	1.24E-04
YOR342C	-1.64	3.02E-06	4.39E-04
YDL070W	-1.64	2.37E-05	2.01E-03
XUT_7F-384	-1.63	6.91E-05	4.79E-03
YBR253W	-1.62	2.50E-05	2.08E-03
YLR399C	-1.62	7.70E-06	8.43E-04
YML121W	-1.61	9.78E-07	1.72E-04
YIL045W	-1.60	4.39E-06	5.75E-04
YDR440W	-1.59	1.10E-05	1.10E-03
SUT517	-1.56	5.67E-05	4.07E-03
YOR303W	-1.55	4.71E-06	5.93E-04
YDL209C	-1.54	1.61E-04	9.15E-03
YJR084W	-1.51	1.02E-04	6.50E-03
YDR405W	-1.49	1.07E-04	6.77E-03
YGR239C	-1.48	6.00E-06	6.91E-04

Table A.3: Xtail hits for changes in monosomal-enrichment between *xrn1*Δ & *BY4741*.

Gene	log ₂ FC RO (<i>xrn1</i> Δ/ <i>BY4741</i>)	pvalue	padj
YER050C	-1.44	1.47E-05	1.33E-03
YGL096W	-1.43	1.56E-04	9.08E-03
YOR147W	-1.43	1.40E-05	1.29E-03
YNL011C	-1.41	8.90E-05	5.82E-03
YAL040C	-1.40	3.92E-05	3.05E-03
YNL050C	-1.38	9.38E-05	6.06E-03
YER020W	-1.31	1.44E-04	8.58E-03
CUT793	-1.22	1.76E-04	9.78E-03
YGL224C	-1.08	1.17E-04	7.24E-03
YAL042W	1.25	8.51E-05	5.64E-03
YHR012W	1.33	5.58E-05	4.07E-03
YPR019W	1.41	6.49E-05	4.56E-03
YGL008C	1.45	1.07E-06	1.83E-04
YGR097W	1.50	2.22E-05	1.93E-03
YJR070C	1.51	3.03E-05	2.41E-03
YDR128W	1.52	2.52E-05	2.08E-03
YBR252W	1.53	1.41E-05	1.29E-03
YDR205W	1.65	1.22E-04	7.40E-03
YMR300C	1.66	1.65E-04	9.28E-03
YLL031C	1.68	3.90E-05	3.05E-03
YNL331C	1.71	1.58E-04	9.11E-03
YLR167W	1.72	4.63E-06	5.93E-04
YIL048W	1.83	2.89E-07	7.14E-05
YJL167W	1.84	2.46E-07	6.32E-05
YKL151C	2.12	1.13E-06	1.89E-04
SUT447	2.23	1.17E-04	7.24E-03
YOR116C	2.45	1.39E-10	3.09E-07
YGL140C	2.54	2.96E-08	1.32E-05
YPL221W	2.62	2.89E-08	1.32E-05
YMR096W	2.92	7.42E-14	4.96E-10
YOR071C	3.12	6.34E-06	7.06E-04
SUT629	3.15	5.11E-06	6.20E-04
YGL176C	3.54	4.99E-05	3.75E-03
YPL137C	3.66	1.31E-07	3.97E-05
YPR115W	4.13	4.50E-07	1.01E-04

Table A.3: Xtail hits for changes in monosomal-enrichment between *xrn1*Δ & *BY4741*.

Differentially monosome-enriched genes from the Xtail pipeline (padj <0.01) comparing uncalibrated reads from monosomal and polysomal fractions between *xrn1*Δ & *BY4741* sucrose gradient fractionated QuantSeq libraries. (n = 3)

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