5.1 Inositol pyrophosphates 5-IP$_7$ and 1-IP$_7$ have a cumulative effect on protein synthesis

Yeast lacking the IP$_6$ kinase Kcs1, which have no detectable IP$_7$ and IP$_8$, show sensitivity to translation inhibitors. However, yeast that possess even one form of inositol pyrophosphate, i.e. vip1$\Delta$ yeast that have 5-IP$_7$ or kcs1$\Delta$ddp1$\Delta$ yeast that have 1-IP$_7$, display a normal phenotype. 5-IP$_7$ is the product of Kcs1 whereas 1-IP$_7$ is the product of Vip1. 5-IP$_7$ and 1-IP$_7$ can regulate protein function in two ways: 1) direct binding to proteins to modulate their function. For example, 1-IP$_7$ binds to Pho80-Pho85-Pho81 complex in yeast and regulates phosphate metabolism, but 5-IP$_7$ cannot perform this function (Lee et al., 2008), and 5-IP$_7$ binds and regulates CK2 in mammals (Rao et al., 2014); 2) by transferring their $\beta$ phosphate to pre-phosphorylated serine residues on proteins, bringing about the post-translational modification of pyrophosphorylation. For example, 5-IP$_7$ pyrophosphorylates the yeast transcription factor Gcr1 thereby regulating transcription of glycolytic enzymes (Sziggyarto et al., 2011), and pyrophosphorylation of the adaptor protein AP3$\beta$ by 5-IP$_7$ regulates vesicle trafficking in mammalian cells (Azevedo et al., 2009). Protein pyrophosphorylation can be brought about by 5-IP$_7$, 1-IP$_7$ and IP$_8$ (Bhandari et al., 2007). Therefore, modulation of protein function by pyrophosphorylation is a cumulative effect of all cellular inositol pyrophosphates, whereas functions that are specific to individual inositol pyrophosphates depend on protein binding. The mode of action of IP$_7$ can therefore vary in the regulation of different signalling pathways.

Although 1-IP$_7$ is made by Vip1 in kcs1$\Delta$ yeast, it is likely to be rapidly degraded to IP$_6$ by the enzyme Ddp1. This is because Ddp1 has been shown to preferentially hydrolyse the diphospho group on 1-IP$_7$ compared to 5-IP$_7$ (Kilari et al., 2013). This is the likely reason for very low or undetectable levels of 1-IP$_7$ in kcs1$\Delta$ cells. Therefore 1-IP$_7$ is not able to compensate for 5-IP$_7$ in these cells. In kcs1$\Delta$ddp1$\Delta$ yeast, the absence of Ddp1 leads to increased levels of 1-IP$_7$ which is able to rescue the function of 5-IP$_7$ in the ribosome biogenesis pathway and therefore restore resistance to translation inhibition. This observation that both 5-IP$_7$ and 1-IP$_7$ can support ribosome biogenesis is in agreement with our discovery that the molecular mechanism underlying this phenomenon is protein pyrophosphorylation. Therefore, in wild type yeast, it is likely that both forms of IP$_7$ and IP$_8$ have a cumulative effect on the complex
process of ribosome biogenesis. However, as 5-IP$_7$ is the predominant inositol pyrophosphate in wild type cells, and the alternative product of Kcs1, 5PP-IP$_4$ is present at negligible levels (Saiardi et al., 2002), the phenotypes observed in $kcs1\Delta$ cells have been attributed to the loss of 5-IP$_7$ and not to other inositol pyrophosphates.

5.2 Reduced ribosome levels lead to reduced protein synthesis in $kcs1\Delta$ yeast

$kcs1\Delta$ yeast show a reduced rate of protein synthesis. During normal growth conditions, protein synthesis in yeast is regulated by the availability of free ribosomes (Shah et al., 2013). During stress conditions, translation is regulated at the initiation, elongation or termination phases by different mechanisms (Shah et al., 2013). Reduced protein synthesis in $kcs1\Delta$ yeast could be due to any of the above mentioned possibilities. The availability of ribosomes in $kcs1\Delta$ yeast was measured by polysome analysis. This revealed that the active ribosomes bound to mRNA (monosomes and polysomes), that are participating in protein synthesis, are lowered in $kcs1\Delta$ yeast. This observation could be due to two reasons; 1) a defect in loading of ribosome subunits on mRNA which is the initiation step in translation, or 2) due to reduced ribosome subunit levels, i.e. 40S and 60S subunits. Analysis of individual subunits implied that reduced protein synthesis is because of reduced 40S and 60S subunit levels. Subsequent analyses indicated that reduced ribosome subunit levels are a result of compromised ribosome biogenesis. This study however did not eliminate the possibility of defects in the stages of translation initiation, elongation or termination.

The results of this study are in contrast with an earlier report where $kcs1\Delta$ yeast grown at 16°C were shown to have reduced polysome levels, whereas 80S monosome levels were higher than the wild type strain (Horigome et al., 2009). A shift from polysomes to monosomes has been observed in the process of inhibition of translation by rapamycin (Barbet et al., 1996). Loss of IP$_7$ in $kcs1\Delta$ yeast leads to pleiotropic effects on multiple cellular pathways and one of these could be inhibition of translation at low temperatures. When polysome analysis in this study was performed at the normal growth temperature of 30°C, an increase in monosomes was not observed in $kcs1\Delta$ yeast, indicating that inhibition of translation is not responsible for decreased protein synthesis.
5.3 Inositol pyrophosphates regulate rRNA synthesis although processing is unaffected

The complementation of \textit{kcs1}\textsuperscript{Δ} yeast with the catalytically active form of Kcs1 could restore protein synthesis whereas the inactive form could not, suggesting that inositol pyrophosphates, the product of Kcs1, participate in the regulation of ribosome synthesis in budding yeast. Ribosome biogenesis is a complex process consisting of three phases, rRNA transcription, co-transcriptional rRNA processing and ribosome subunit assembly. These stages were further analysed by \textit{in vivo} labelling of rRNA by \textsuperscript{14}C-uracil, and it was found that the initial stage of rRNA synthesis is compromised in the \textit{kcs1}\textsuperscript{Δ} strain.

In \textit{kcs1}\textsuperscript{Δ} yeast, there was no apparent accumulation of 27S and 20S pre-rRNA which give rise to 25S and 18S respectively. This is in turn reflected in unaltered ratios of 40S and 60S subunits in purified ribosome profiles suggesting normal rRNA processing in these cells. It has been reported that Nsr1, the \textit{S. cerevisiae} homolog of nucelolin, is a strong target of IP\textsubscript{7}-mediated pyrophosphorylation and that pyrophosphorylation of Nsr1 is reduced in \textit{kcs1}\textsuperscript{Δ} yeast cells (Saiardi et al., 2004). Nsr1 is involved in processing and maturation of 20S rRNA and its deletion was shown to result in reduced 40S subunit and increased 60S subunit levels (Kondo and Inouye, 1992; Lee et al., 1992). However, this study shows that the loss of Kcs1 does not phenocopy the loss of Nsr1, and that \textit{kcs1}\textsuperscript{Δ} yeast do not have an rRNA processing defect despite reduced Nsr1 pyrophosphorylation. This suggests that serine pyrophosphorylation does not regulate the function of Nsr1 in rRNA processing.

5.4 Defect in ribosome biogenesis in \textit{kcs1}\textsuperscript{Δ} yeast is independent of uracil uptake

Although \textit{kcs1}\textsuperscript{Δ} yeast strain display a decrease in uracil uptake, the reduction in uracil incorporation into \textit{kcs1}\textsuperscript{Δ} rRNA is substantially greater than the reduction in uptake, indicating that reduced intracellular uracil availability only partially accounts for decreased rRNA synthesis. It has been suggested that a decrease in cellular ribosome content leads to uracil accumulation, which when converted to dUTP is toxic to cells, and can be offset by down regulating uracil uptake (Seron et al., 1999). This down regulation may be brought about by endocytosis of the uracil permease Fur4 triggered by an increased amount of cytoplasmic uracil (Keener and Babst, 2013). Therefore, reduced uracil uptake in \textit{kcs1}\textsuperscript{Δ} yeast may be a consequence, rather than the cause of
reduced ribosome synthesis. To test this hypothesis a GFP tagged version of Fur4 could be expressed in wild type vs kcs1Δ yeast and its endocytosis could be monitored by fluorescence microscopy.

In addition it is also known that Fur4 protein has a PEST sequence which is a target of casein kinase 1 (Marchal et al., 2000). The phosphorylated form of Fur4 is ubiquitylated and internalized by endocytosis. It has been shown that the PEST sequence, which is an acidic serine sequence, can be a target for pyrophosphorylation by IP7 (Lolla, Unnikannan and Bhandari, unpublished). Therefore it is possible that CK1 phosphorylation of Fur4 leads to endocytosis, but pyrophosphorylation by IP7 may stabilize Fur4 to increase uracil uptake. This may present another mechanism by which IP7 supports rRNA synthesis, and would be an interesting aspect to study further.

The strains used in this study are ura3Δ (uracil auxotrophs) which cannot synthesize uracil and grow only when uracil is supplied in the medium. kcs1Δ yeast was rescued by expressing wild-type and inactive forms of Kcs1 on a plasmid that carries the URA3 gene which expresses the enzyme that supports de novo uracil synthesis. The wild-type form of Kcs1 restored the rate of protein synthesis in kcs1Δ yeast, whereas the mutant form did not, even with the support of intracellular uracil synthesis, eliminating the need for uracil uptake from the medium. This observation suggests that ribosome biogenesis is not limited by uracil uptake in kcs1Δ cells. Secondly, to eliminate the effect of uracil uptake on rRNA synthesis, a nuclear run-on assay was performed, which measures transcription by RNA polymerase that is already bound to DNA, while preventing the recruitment of new polymerase molecules to DNA. In this assay radiolabelled UTP is allowed to enter yeast cells through pores generated on the membrane by a low percentage of detergent. The nascent rRNA transcripts measured by this assay were significantly lowered in kcs1Δ yeast, suggesting that inositol pyrophosphates indeed regulate rRNA synthesis independent of uracil transport into the cell.

5.5 RNA Pol I subunits are pyrophosphorylated by IP7

At the molecular level, the inositol pyrophosphate 5-IP7 can transfer its β phosphate to phosphorylated serine residues on three RNA Pol I subunits, A190, A43 and A34.5.
The RNA Pol I subunit A135, and Uaf30, a subunit of UAF complex, possess acidic serine sequences which are predicted CK2 phosphorylation sites and yet they were not pyrophosphorylated by IP$_7$. This suggests that although pyrophosphorylation by IP$_7$ is not driven by an enzyme, it is still a substrate specific and precisely regulated post translational protein modification.

IP$_6$ has been shown to inhibit protein pyrophosphorylation by IP$_7$, possibly by binding the target site and blocking the binding of IP$_7$ (Saiardi et al., 2004). In yeast, the IP$_7$ levels vary from 1-5 µM which is 20 fold less compared to intracellular IP$_6$. Therefore it may be necessary to synthesize IP$_7$ in the vicinity of RNA Pol I to overcome the effect of IP$_6$. A study on the subcellular localization of all *S. cerevisiae* proteins demonstrated that GFP tagged Kcs1 is localised throughout the cell (Huh et al., 2003). It is therefore possible that Kcs1 present in the nucleolus synthesizes IP$_7$ responsible for Pol I pyrophosphorylation. A mass spectrometry-based global analysis of protein kinase interactions shows that both regulatory subunits and catalytic subunits of CK2 associate with RNA Pol I subunits A190 and A135 (Breitkreutz et al., 2010). Putting this together, it may be speculated that RNA Pol I may interact with both CK2 and Kcs1, and therefore be subjected to phosphorylation followed by pyrophosphorylation.

Mammalian IP6K2 has been shown to interact with the TTT complex proteins (Tel2, Tti1, Tti2) via its C-terminus (Rao et al., 2014). The CK2 catalytic subunit interacts with the TTT complex and phosphorylates a Ser residue present in the N-terminus of Tti1. IP$_7$ synthesized by IP6K2 in the vicinity of the TTT complex binds to CK2 and enhances phosphorylation of Tti1. In the same manner, it is possible that IP$_7$ synthesized by Kcs1 may enhance CK2 phosphorylation of RNA Pol I subunits, priming them for pyrophosphorylation.

In the process of localization of pyrophosphorylation sites on A43 and A34.5 subunits, it was found that Ser to Ala mutants completely eliminated or substantially reduced pyrophosphorylation whereas Ser to Asp substituents did not. RNA Pol I specific subunits A34.5 and A43 both possess multiple CK2 phosphorylation sites (Gerber et al., 2008). These sites may not be pyrophosphorylated in the native proteins but may have become the targets of IP$_7$ when the major pyrophosphorylated Ser residues were replaced with Ala. The replacement of Ser with Asp may change the net charge density on these proteins which may support pyrophosphorylation on other sites.
A34.5 has a C-terminal poly-Lys tail, which when removed, completely eliminates pyrophosphorylation on the protein. Lys being a positively charged amino acid, its presence in the C-terminal tail of A34.5 may increase the affinity of the protein towards negatively charged IP$_7$. The poly-Lys tail of A34.5 may therefore have a role in pyrophosphorylation, making this protein a strong target for IP$_7$. This is the first study to confirm the importance of Lys residues in promoting pyrophosphorylation at Ser residues surrounded by acidic amino acids.

Mass spectrometry based phosphopeptide analysis conducted by other researchers has identified Ser 1413, 1415 and 1417 in A190 but has not identified the sites Ser 322, 323 and 325 on A43, and Ser 205 and 206 on A34.5, which were therefore considered as non-phospho sites. For pyrophosphorylation to occur, phosphorylation on serine residue is prerequisite, and hence this study reveals these novel phosphorylation and pyrophosphorylation sites on A43 and A34.5.

5.6 Mutations on three RNA Pol I subunits affect RNA Pol I function

To characterize the relevance of these acidic serine sequences in the context of regulation of RNA Pol I, wild type and mutant forms of A190 1338-1448 fragment and A43 proteins were overexpressed in wild type yeast and tested for any dominant negative effects of the mutant proteins on RNA Pol I function. As A190 and A43 are part of the RNA Pol I complex, the mutant forms of these proteins are expected to interact with the enzyme complex. This could make the RNA Pol I functionally inactive and therefore retard the growth of wild type yeast. The growth of the strains overexpressing the mutant proteins was not compromised, suggesting that these overexpressed proteins were either unable to compete with the endogenous RNA Pol I complex, or that these proteins are functionally active and therefore did not reduce the activity of RNA Pol I.

To further probe the effect of the IP$_7$ target sites, mutations were generated in the yeast genome. Growth of yeast carrying mutant A190 S1413/1415/1417/A was not compromised in presence of translation inhibitors, the elongation inhibitor 6AU, or the metal ion chelating agent BPS. The three non-overlapping fragments of A190 were seen to be pyrophosphorylated by radiolabelled IP$_7$, hinting that there may be other pyrophosphorylation sites in addition to Ser 1413, 1415 and 1417. Therefore, changing only three Ser residues to Ala in A190 may not have an effect on ribosome synthesis.
Substitution of Ser with Ala in the C-terminal tail of A43, thereby eliminating both phosphorylation and pyrophosphorylation in this region, did not lead to any significant change in growth or 6AU sensitivity. However, when placed in the background of an A34.5 deletion, A43 S322/323/325A showed a substantial growth reduction in the presence of 6AU. Interestingly, there was no alteration in the rate of protein synthesis in these mutants, indicating that the significant reduction in protein synthesis observed in kcs1Δ yeast cannot be recapitulated only by deletion of the non-essential subunit A34.5 and removal of the major pyrophosphorylated regions in A190 and A43. IP7 may therefore act on other sites in RNA Pol I or on other associated elongation factors to influence transcription elongation.

5.7 Possible role of pyrophosphorylation in RNA Pol I elongation

Earlier studies show that changes in the phosphorylation status of RNA Pol I and accessory factors play a key role in the regulation of rRNA transcription. One such protein is Rrn3, which is phosphorylated at different sites that prevent (Blattner et al., 2011) its interaction with RNA Pol I, which in turn is essential for transcription initiation. This study suggests that the function of Rrn3 is not compromised in kcs1Δ yeast as chromatin immunoprecipitation analyses ruled out any defect in recruitment of RNA Pol I to the rDNA promoter.

As discussed in Section 1.2.4.2, twelve of the fourteen RNA Pol I subunits are phosphorylated at many Ser and Thr residues. The relative phosphate content on RNA Pol I determines its shift from an Rrn3 associated initiation phase to an Rrn3 free elongation phase (Fath et al., 2001), highlighting the importance of RNA Pol I phosphorylation in transcription elongation. Alkaline phosphatase can non-specifically dephosphorylate phosphoserine residues. A recent study shows that treatment of pyrophosphorylated peptides or yeast cell lysates with alkaline phosphatase for long periods of time (1-24 h) was able to remove pyrophosphorylation (Yates and Fiedler, 2015). Treatment of RNA Pol I with alkaline phosphatase for short periods of time resulted in an increase in RNA Pol I activity, whereas incubation for longer periods resulted in loss of enzyme activity (Fath et al., 2004; Fath et al., 2001). It is possible that short periods of treatment with alkaline phosphatase result in removal of phosphorylation from serine residues, whereas longer periods of incubation of Pol I
with alkaline phosphatase results in depyrophosphorylation, thereby reducing RNA Pol I elongation activity.

Although the exact pyrophosphorylated region that we have identified on A190, A34.5 and A43, all fall within mobile regions which are not visible in the recently reported RNA Pol I crystal structures (Engel et al., 2013; Fernandez-Tornero et al., 2013), they are contiguos with domains of known structure and function (Fig. 5.1). Pyrophosphorylation on A190 occurs in the ‘expander’ (Engel et al., 2013) or ‘DNA mimicking loop’ (Fernandez-Tornero et al., 2013) (Fig. 5.1), which sits in the DNA binding cleft in inactive RNA Pol I, but it is unclear how this region behaves in the elongating polymerase. A yeast strain in which most of this loop (residues 1361 -1390) is deleted shows growth retardation under thermal stress (Fernandez-Tornero et al., 2013), similar to the phenotype of kcs1 Δ yeast (Dubois et al., 2002).

**Figure 5.1** Pyrophosphorylation sites are unstructured in RNA Pol I complex. Ribbon diagram of RNA Pol I structure, created using Chimera software with the co-ordinates of the structure of an RNA Pol I monomer from PDB ID 4C2M. Colour coding is as follows: A190-grey, A135-light beige, A43-red, A14-forest green, A34.5-blue, A49-green, A12.2-yellow, A14.5-hot pink, AC40-purple, AC19-orange red, ABC27-sea green, ABC23-corn flower blue, ABC10α-cyan, and ABC10β-orange. The IP7 target sites Ser 205, 206 in A34.5 fall in the unstructured region beyond Glu 178. The IP7 target sites Ser 322, 323 and 325 in A43 fall in the unstructured beyond Ile 250. The IP7 target sites in A190 fall in the unstructured ‘expander’ region between Met 1395 and Asn 1440.
The high processivity of RNA Pol I has been attributed to its intrinsic elongation factor, the A34.5/A49 subcomplex (Kuhn et al., 2007; Schneider, 2012), which also promotes the dissociation of Rrn3 from the elongating polymerase (Albert et al., 2011; Albert et al., 2012; Beckouet et al., 2008). A34.5 interacts with Top1 (Beckouet et al., 2008), the type I topoisomerase which relaxes negative supercoiling produced in the wake of RNA Pol I (Albert et al., 2012). The mapped pyrophosphosites on A34.5, S205 and S206, extend from the positively charged C-terminal tail which anchors this subcomplex to the RNA Pol I core (Engel et al., 2013; Fernandez-Tornero et al., 2013; Jennebach et al., 2012). Pyrophosphorylation of these residues could conceivably affect the anchoring of the A34.5/A49 subcomplex onto the RNA Pol I core, thereby affecting elongation activity.

The C-terminal tail of A43 to which we have mapped three pyrophosphosites, forms a ‘connector’ which invades the cleft of the neighbouring polymerase in an inactive dimer (Engel et al., 2013; Fernandez-Tornero et al., 2013), but its role in the active elongating polymerase is unclear. Truncation of the C-terminal tail confers thermostability (Peyroche et al., 2000), and is lethal when combined with the loss of other RNA Pol I subunits A14, A49, A12.2 or A34.5, which are individually non-essential (Beckouet et al., 2011). The C-terminal region of A43 also participates in the interaction of RNA Pol I with the elongation factor Spt5 (Beckouet et al., 2011). Spt5, in a heterodimeric complex with Spt4 influences rDNA transcription, acting to close the DNA clamp, enabling the polymerase to carry out processive elongation (Albert et al., 2012; Schneider, 2012). Its interaction with RNA Pol I is mediated by binding to multiple subunits, including A190 and A34.5 (Schneider, 2012) in addition to A43. Spt5 also interacts with the yeast FACT (facilitates chromatin transcription) complex, which transiently displaces nucleosomes during transcription on chromatin templates. The phosphoprotein Nsr1, the S. cerevisiae homolog of nucleolin, is a strong target of IP₇-mediated pyrophosphorylation (Saiardi et al., 2004). Mammalian nucleolin has been shown to have FACT activity by aiding nucleosome displacement during transcription on chromatin templates, in addition to its role in pre-rRNA maturation and ribosome assembly (Rickards et al., 2007). Therefore, Nsr1, a major target of IP₇-mediated pyrophosphorylation in yeast, could contribute to the role of IP₇ in regulating transcription elongation by Pol I. Altogether, changes pyrophosphorylation on different
sites on RNA Pol I and associated factors may regulate the elongation activity of the enzyme.

Apart from elongation factors, an in silico analysis to look for acidic serine sequence motifs suggests that other proteins involved in rRNA synthesis, processing and modification possess acidic serine sequences and could be potential targets of IP\textsubscript{7}, contributing to a cumulative effect of IP\textsubscript{7} on ribosome levels (Laha and Bhandari, unpublished).

5.8 IP\textsubscript{7} signals between metabolism and ribosome biogenesis

The results in this study add to the increasing repertoire of key metabolic processes regulated by inositol pyrophosphates. It has been argued that inositol pyrophosphates are metabolic messengers or energy sensors, rather than classical second messengers (Shears, 2009; Wilson et al., 2013; Wundenberg and Mayr, 2012). Inositol pyrophosphate levels are not rapidly altered by any particular stimulus, but instead have been shown to reflect cellular ATP levels (Shears, 2009; Wundenberg and Mayr, 2012). In mammalian cells, treatment with sodium azide for 5 min caused a 40% drop in the ATP/ADP ratio, resulting in an 80% decrease in the IP\textsubscript{7}/IP\textsubscript{6} ratio (Nagel et al., 2010). Also, a reduction in ATP levels following glucose restriction has been shown to downregulate RNA Pol I activity (Hoppe et al., 2009). Ribosome biogenesis accounts for more than 75% of nuclear transcription (Moss et al., 2007) and therefore consumes most of a cell’s energy. It is conceivable that IP\textsubscript{7} acts as a conduit to transduce a decrease in intracellular ATP, signalling to reduce RNA Pol I activity and lower ribosome biogenesis to conserve energy.

5.9 Summary and future perspective

This study revealed that the inositol pyrophosphates play an essential role in maintaining normal cell proliferation in budding yeast. In the absence of these molecules, compromised cell proliferation could be attributed to a reduced rate of protein synthesis, which was in turn an effect of reduced ribosome levels. The cause of a reduction in ribosome levels was traced to compromised rRNA synthesis, which is the first and rate-limiting step in ribosome biogenesis. RNA Pol I, the enzyme responsible for the synthesis of rRNA was found to be pyrophosphorylated by 5-IP\textsubscript{7} in vitro, and in the absence of this molecule, rRNA synthesis was lowered. This
observation suggested that RNA Pol I pyrophosphorylation by 5-IP\textsubscript{7} is required for its activity. Cellular ATP levels determine not only synthesis of IP\textsubscript{7} but also the synthesis of ribosomes as this process consumes most of the cellular ATP. Thus, this study concludes that IP\textsubscript{7} acts as a mediator molecule that signals the availability of ATP to RNA Pol I to pursue rRNA synthesis.

5.9.1 Regulation of Kcs1 to synthesize IP\textsubscript{7} depending on environmental signals

IP\textsubscript{7}-mediated pyrophosphorylation of proteins is a post-translational modification and is therefore likely to be regulated. The mechanisms of regulation of IP\textsubscript{7} synthesis are still uncertain (Thomas and Potter, 2014; Wilson et al., 2013; Wundenberg and Mayr, 2012). The low cellular concentration of IP\textsubscript{7} suggests that its synthesis should occur in close proximity to the proteins that will be pyrophosphorylated. This would ensue only when Kcs1 is localized to the proteins that will be pyrophosphorylated by IP\textsubscript{7}. Kcs1 was predicted to have phosphorylation sites for many metabolic enzymes such as Gsk3

Table 5.1 RNA Pol I extrinsic elongation factors. RNA Pol I extrinsic elongation factors Spt5, Spt6 and Spt16 fragments and full length Paf1 protein are shown. Phosphorylated serines mapped by mass spectrometry (PhosphoGRID) are highlighted in blue and acidic amino acids are shown in red.

| Spt5 | 121 GSDEKRKPRE EDTKN1GDT KDEGDNKDED DDEDDDDDD DDEDDDE APT KRRQERNR F |
| Spt6 | 1 MEETGSDLKL VPRDEEIVND NDETKAPSE EEEDEVF DSS EEDDEIDE DEARKVQEGF |
| Spt16 | 961 ASDESEEVS YEASED DVS DESA SEDEE DEGE DDDISG DESEDYT GDE EEDWDEL |
| Paf1 | 1 MSKKQEIYAP IKYQNSLPVP QLPPKL LYP ESPETNAP XX QLINSLYKT NVTNLIQ D E |

Discussion
Discussion

(phosphoGRID) and yeast Protein kinase A (phosphoGRID) (Soulard et al., 2010) which are expected to be phosphorylated depending on environmental cues. Phosphorylation on Kcs1 may in turn regulate its localization and IP$_7$ synthesis. Therefore further studies should focus on the regulation of Kcs1 activity and thereby synthesis of IP$_7$, which in turn acts as an amplifier in signalling pathways by pyrophosphorylating multiple proteins.

5.9.2 Regulation of RNA Pol I mediated elongation by accessory factors

This study describes that RNA Pol I elongation is compromised in yeast lacking IP$_7$. It suggests that pyrophosphorylation of RNA Pol I specific subunits A34.5 and A43 is necessary for undisrupted elongation. This study, however, does not rule out the role of other accessory factors that are involved in RNA Pol I elongation such as Spt5, Spt6, and Spt16.

Spt5 has many phosphorylation sites identified by mass spectrometry analysis (Table 5.1) (Swaney et al., 2013) (PhosphoGRID), and interacts with CK2 (Lindstrom et al., 2003). A manual examination of Spt5 sequence suggests that these sites are likely to be the targets of IP$_7$ pyrophosphorylation. A190, A135, A34.5, A43 and A49 were found to co-purify with FLAG-tagged Spt5 upon affinity purification (Lindstrom et al., 2003). Spt4/5 may contribute to pausing of RNA Pol I early during transcription elongation to allow proper co-transcriptional processing, whereas at the 3’end of the rDNA, Spt4/5 promotes transcription elongation by cleavage of the transcripts (Anderson et al., 2011). Spt5 interacts with Spt4, Spt6 and Paf1 and this complex has been found to regulate RNA Pol I elongation (Albert et al., 2012).

Paf1 protein is part of the Paf1 complex which is required for efficient elongation during rRNA transcription (Zhang et al., 2009). Paf1 has been shown to interact with CK2 (Lindstrom et al., 2003) and is predicted to be phosphorylated at acidic serine residues (Swaney et al., 2013) (Table 5.1). Spt6 (Birch et al., 2009) and Spt16 (Albert et al., 2012; Orphanides et al.) have been shown to exhibit FACT activity (Section 5.7) thereby functioning as a chaperone to loosen chromatin, enabling RNA Pol I elongation on rDNA. Both Spt6 (Gavin et al., 2002) and Spt16 have been shown to interact with CK2 (Gavin et al., 2002; Krogan et al., 2004) and have predicted phosphorylation sites at acidic serine motifs (Swaney et al., 2013), making these proteins potential targets of pyrophosphorylation (Table 5.1).
In vivo transcription elongation studies suggested that the 3’ end of rRNA of transcript levels, i.e. 25S rRNA transcripts, were increased while early transcript levels were reduced in \( kcs1\Delta \) yeast compared to wild type yeast (Fig. 3.14), whereas RNA Pol I occupancy was not compromised (Fig. 3.13). This result is consistent with the phenotype of Spt5 mutants (Anderson et al., 2011). Therefore, it is evident that the elongation in rRNA synthesis is determined by RNA Pol I intrinsic elongation factors and recruitment of extrinsic elongation factors such as Spt5 and associated factors to the enzyme complex. This study can be extended to identify other IP\(_7\) substrates involved in elongation of rRNA transcription. These objectives can be studied by electron microscopy of rDNA chromatin in \( kcs1\Delta \) yeast, immunoprecipitations of Pol I complexes to verify the recruitment of extrinsic elongation protein complexes in wild type and \( kcs1\Delta \) yeast, and testing the IP\(_7\)-mediated pyrophosphorylation of these proteins.