Chapter 5

Conclusions and future perspectives

Luc-Alban Vuillemenot[1]

[1]Molecular Systems Biology, Groningen Biomolecular Sciences & Biotechnology Institute, University of Groningen, Netherlands

Summary of this thesis

A central goal of the work described in this thesis was to develop and characterize new readouts for the study of yeast TORC1 via single-cell microscopy. To this end, we pursued two alternative objectives: the detailed characterization of the endogenous Sfp1 protein as a TORC1 readout, and the development of new readouts based on mTORC1 substrates. As it turned out, the regulation of Sfp1 is considerably more complex than previously thought. Our findings in Chapter 2 demonstrated that Sfp1 is in fact a joint substrate of TORC1 and PKA, and that both pathways control the subcellular localization of the protein. The regulation of Sfp1 localization is partly mediated through a Nuclear Export Signal (NES) whose activity is controlled by PKA- and potentially TORC1-dependent phosphorylation of nearby residues. We further showed that TORC1 and PKA regulate Sfp1 localization independently of each other, since loss of Sfp1 phosphorylation from either pathway is sufficient to cause the translocation of Sfp1 from the nucleus to the cytoplasm. Finally, we uncovered that the C-terminal zinc finger domains of Sfp1 are involved in a parallel localization mechanism that is responsive to both TORC1 and PKA activity, providing an additional layer of regulation that needs to be further investigated.

Drawing upon our improved understanding of Sfp1 regulation, in Chapter 3 we analyzed the contributions of TORC1 and PKA to the oscillatory localization dynamics of Sfp1 during the cell cycle. By resolving the localization dynamics of Sfp1 in several different mutant strains, we showed that the two pathways control different aspects of the localization pattern of the protein throughout the cell cycle. While both PKA and TORC1 regulate the average nuclear accumulation of Sfp1 across the cell cycle, PKA appears to be the main regulator behind the peak of nuclear localization during G1. Besides TORC1 and PKA, the C-terminal zinc finger domains appear necessary for maintaining Sfp1 in the nucleus during the early S phase. On the contrary, neither TORC1 nor PKA could be associated with the peak in Sfp1 localization during G2/M. By investigating the localization dynamics of additional joint TORC1 and PKA substrates, we observed that this localization peak is shared by various substrates besides Sfp1. A mathematical model describing the nuclear accumulation of Sfp1 over the cell cycle led us to hypothesize that this peak arises from cell cycle-dependent alterations in nucleocytoplasmic translocation.

As Chapters 2 and 3 made clear, the complex (and partially unresolved) regulatory mechanisms of Sfp1 complicate its use as a TORC1-specific substrate. Therefore, in Chapter 4 we turned to mammalian TORC1 substrates and explored their potential as single-cell readouts of yeast TORC1. Our study initially showed that TORCAR, a mammalian FRET sensor based on 4E-BP1, underwent fast (de)phosphorylation *in vivo* in budding yeast in response to acute TORC1 activity changes. While TORCAR proved to be unreliable due to inconsistent FRET responses, we showed that 4E-BP1 phosphorylation was highly sensitive to TORC1 activity fluctuations. Monitoring 4E-BP1 phosphorylation dynamics in synchronized daughter cells revealed an increase in TORC1 activity during the G1 phase of the cell cycle. Furthermore, we showed that 4E-BP1 could form the basis of a translocation-based reporter of TORC1 activity compatible with single-cell microscopy. On the other hand, TFEB proved to be unsuitable as a translocation-based TORC1 reporter in yeast, as it did not accurately reflect TORC1 activity changes in our tests.

Future perspectives

Unlocking the potential of Sfp1 as a single-cell TORC1 readout

The role of zinc fingers in the regulation of Sfp1 localization

Towards the development of single-cell TORC1 readouts, in Chapter 2 we showed that the mechanism regulating Sfp1 localization is considerably more complex than previously thought. While our results have furthered our understanding of this mechanism, key details remain to be elucidated and open new questions. A critical point in this regard is the regulation of Sfp1 localization by the C-terminal part of the protein, and notably the zinc finger domains. As we found in Chapter 2, these domains mediate changes in TORC1 and PKA activity to Sfp1, providing another level of regulation besides the N-terminal NES-based mechanism. Yet, it is unclear how this C-terminal mechanism operates. A key question is whether changes in Sfp1 phosphorylation can still propagate to changes in localization when the zinc fingers are absent. To test this question, one could truncate the protein to remove the C-terminal zinc fingers, add an NLS to restore nuclear

localization, and test whether this mutant is still responsive to TORC1 or PKA activity perturbations.

The connection between the C-terminal zinc fingers and Sfp1 localization has come up before in the literature, since it was noted early on that deletion of these zinc fingers relocalizes the protein to the cytoplasm (Lempiäinen et al., 2009). It has been suggested that the zinc fingers regulate Sfp1 localization by mediating the interaction between the essential Rab-escort protein Mrs6 and Sfp1. Previous studies have highlighted the role of Mrs6 in the regulation of Sfp1 localization, proposing that Mrs6 serves as a bridge that connects the secretory system with TOR-dependent nutrient signaling and ribosome biogenesis. However, the mechanism through which Mrs6 modulates Sfp1 localization remains uncertain. Previous work has shown that the interaction of these proteins is significantly disrupted by the mutation of the Sfp1 zinc fingers. In combination with the fact that zinc finger deletion renders Sfp1 cytosolic, that work suggested that binding to Mrs6 promotes the nuclear localization of Sfp1 (Lempiäinen et al., 2009). However, other work has presented evidence suggesting that Mrs6 acts as a cytoplasmic anchor that retains Sfp1 in the cytoplasm during nutrient scarcity (Singh and Tyers, 2009). To understand the zinc finger-dependent mechanism of Sfp1 regulation, it will be critical to unravel the role of Mrs6. A way to achieve this could be to test under which conditions (replete nutrients vs starvation, TORC1/PKA inhibition) Mrs6 and Sfp1 interact, and how this interaction is altered when the zinc fingers are mutated. This could be done by immunoprecipitating epitope-tagged Sfp1 and detecting Mrs6 with mass spectrometry. Using the same setup and the phosphomutants generated in this work, one could also test whether Sfp1 phosphorylation changes its affinity for Mrs6. Finally, one should explore whether Mrs6 itself receives TORC1- or PKA-dependent inputs.

Beyond Mrs6, it cannot be excluded that Sfp1 also interacts with other proteins via its zinc fingers, and that this interaction affects its localization. A detailed characterization of Sfp1-interacting proteins using wild-type vs zinc-finger mutant Sfp1 should help answer this question.

Additional TORC1- and PKA-dependent inputs to Sfp1

Another aspect of Sfp1 that needs clarification, is the number and type of TORC1- and PKA-dependent inputs that the protein integrates. These questions are motivated by the fact that Sfp1 localization is still responsive to

acute inhibition of TORC1 or PKA in a mutant (*sfp1*^{13D}) where all known PKA- and TORC1-dependent residues - as well as recently discovered, and still unattributed residues – carry phospho-mimetic mutations. This observation could imply that Sfp1 carries additional phosphosites targeted by other kinases that have not been mapped yet. However, *sfp1*^{13D} appears completely unphosphorylated in Phos-tag immunoblots, both prior and after TORC1 or PKA inhibition. In principle, it is possible that there is additional phosphorylation that cannot be resolved by Phos-tag. While the major AGC kinase Sch9, a TORC1 substrate itself, does not phosphorylate Sfp1 *in vitro* (Lempiäinen et al., 2009), other kinases could also contribute to Sfp1 regulation. In any case, if such kinases exist, they should be downstream of TORC1 or PKA, since *sfp1*^{13D} is responsive to rapamycin and 1-NM-PP1.

Alternatively, inputs controlling Sfp1 localization could also be mediated by the C-terminal zinc fingers. For example, TORC1, PKA or other kinases could modulate the association of Mrs6 with Sfp1, thereby controlling Sfp1 localization. Two PKA consensus motifs have been identified on Mrs6 but no significant changes in Sfp1 localization was observed upon the mutation of these residues to alanine (Singh and Tyers, 2009). Still, a more extensive mapping of Mrs6 phosphorylation is still missing.

Other regulators of Sfp1 localization

Beside the early identification of Mrs6 as a major interacting protein for Sfp1, other proteins were later shown to contribute to Sfp1 activity, and these proteins might also influence the nucleocytoplasmic distribution of Sfp1. For instance, recent evidence suggests that, besides binding a set of target promoters by itself, Sfp1 also requires co-factors such as the Swi4 and Ifh1 transcription factors to bind at promoters of ribosomal protein and G1/S regulon genes respectively (Albert et al., 2019). It is not known if the interaction with these proteins affects Sfp1 translocation dynamics in response to perturbations of TORC1 or PKA activity. To get some first clues on the effects of Ifh1 and Swi4 on Sfp1 localization, one could monitor the nucleocytoplasmic distribution of Sfp1 following the rapid depletion of these proteins with auxin-inducible degron (AID) (Yesbolatova et al., 2020) or their removal from the nucleus by using the anchor-away system (Haruki et al., 2008).

Sfp1 regulation in G2/M: indications of a leaky mitosis?

Another intriguing aspect of Sfp1 regulation emerged when we observed the localization dynamics of the protein during the cell cycle. As we discussed in Chapter 3, the transient increase in the nuclear localization of Sfp1 during G1 can be explained by the transient increase of PKA activity in that phase of the cell cycle. However, we were unable to link the nuclear localization dynamics of Sfp1 during G2/M (a transient increase followed by a steep drop around karyokinesis) to corresponding changes in TORC1 or PKA activity. Moreover, several other proteins displayed a similar localization pattern during G2/M, prompting us to consider common underlying causes of this behavior. A possible explanation, revealed by our mathematical model, is that the export rate of nuclear proteins increases transiently around mitosis. However, this conjecture is at odds with the fact that budding yeast is thought to undergo closed mitosis without a breakdown of the nuclear envelope (Dey and Baum, 2021). However, recent evidence has challenged the binary classification of closed and open mitosis in eukaryotes, suggesting that the majority of eukaryotes may display a mitosis with features that are in-between open and closed mitosis (De Souza and Osmani, 2007; Dey and Baum, 2021). Some evidence for yeasts in particular, suggests that the Nuclear Pore Complex (NPC) undergoes molecular rearrangements during G2/M in budding yeast (Makhnevych et al., 2003; Rabut et al., 2004) and that the NPC disassembly causes the partial break of the nuclear envelope during closed mitosis in fission yeast (Dey et al., 2020). To test whether a leaky mitotic nucleus could drive Sfp1 nuclear export around mitosis, one could use an unregulated protein of similar size to Sfp1, such as a multimeric GFP fused to an NLS, and monitor its localization dynamics during the cell cycle. Loss of nuclear fluorescence during mitosis could indicate that the nucleus becomes partially leaky in that cell cycle phase.

Sfp1 and growth regulation during the cell cycle

A major benefit of live-cell readouts is the capability to follow the dynamic activity of signaling pathways with a high temporal resolution during cellular processes such as the cell cycle (Chapter 1). Going beyond our results from mother cell cycles presented here, it will be interesting to use Sfp1 as a readout of TORC1/PKA activity during the daughter cell cycle, and especially during G1. This is because daughters have G1 size control mechanisms that mothers do not need, as they have already reached a critical size for division

(Aldea et al., 2007; Wang et al., 2009; Ferrezuelo et al., 2012). Given the involvement of Sfp1 itself in the regulation of cell size (Jorgensen et al., 2004; Cipollina et al., 2005), it would also be interesting to elucidate the roles of Sfp1 during the G1 phase of daughters. This investigation is especially relevant since Sfp1 appears directly involved in the regulation of cell cycle progression (Xu and Norris, 1998). Moreover, it was recently shown that Sfp1 binds the promoters of several G1/S regulon genes in a glucose-dependent manner (Albert et al., 2019), potentially driving the expression of these genes together with Swi4. Importantly, PKA is also known to affect the G1/S commitment in multiple ways (Cocklin and Goebl, 2011; Mizunuma et al., 2013; Amigoni et al., 2015), but it is not known if it does so by promoting the nuclear accumulation of Sfp1.

Over the past decades, it has also become apparent that signaling pathways (e.g. TORC1 and PKA) and the cell cycle machinery are more interconnected that previously thought, and that this bidirectional communication coordinates cell growth with the cell cycle (Barbet, 1996; Mizunuma et al., 2013; Amigoni et al., 2015; Moreno-Torres et al., 2017; Talarek et al., 2017; Pérez-Hidalgo and Moreno, 2017; Odle et al., 2020; Romero-Pozuelo et al., 2020; Diehl et al., 2023). Therefore, it is not impossible that the cell cycle machinery is also involved in the regulation of Sfp1, a major effector of TORC1 and PKA. We tested this hypothesis in Chapter 2 by using an analog-sensitive mutant (Bishop et al., 2000) of the only budding yeast CDK involved in cell cycle regulation (Cdc28) (Morgan, 2007). Our tests were largely inconclusive, as the mutant strain physiology was affected by the mutation of Cdc28, and the ATP-competitive inhibitors induced large-off target effects which possibly masked the role of Cdc28 on Sfp1 dynamics. An alternative approach to test the contribution of CDK to Sfp1 activity could be the use of less disruptive cell cycle perturbations. For instance, one could employ an optogenetic system to put the expression of different cyclins under the control of a light-inducible promoter (Motta-Mena et al., 2014; Benzinger and Khammash, 2018; De Mena et al., 2018), and in this way control the activity of Cdc28 in different cell cycle phases. With such an experiment, one could perturb cell cycle progression and observe how Sfp1 localization responds when the cell cycle is slowed down or halted. A fast response of Sfp1 to these perturbations could indicate that the CDK is directly involved in its regulation.

Expanding the scope of single-cell TORC1 readouts with mTORC1 substrates

As shown by our results in Chapter 2 and 3, endogenous TORC1 substrates are difficult to use as single-cell TORC1 readouts because this approach necessitates the isolation of the different inputs that these substrates receive from other signaling pathways. As we showed in Chapter 4, the import of mTORC1 substrates into yeast offers a valuable alternative for the development of single-cell TORC1 readouts while decreasing the risk of cross-activation of these substrates by other kinases. In Chapter 4, we examined 4E-BP1 as a yeast TORC1 reporter, given its status as a direct mTORC1 substrate and its well-understood phosphorylation mechanism.

It will be interesting to compare the phosphorylation of 4E-BP1 with the phosphorylation of Sch9 (the most common TORC1 readout) under different conditions, and in particular during the cell cycle, where we observed that 4E-BP1 phosphorylation increases considerably during the G1 phase of daughter cells. A comparison with Sch9 could further support our results and provide a comparison of the temporal responsiveness of 4E-BP1 relative to endogenous TORC1 substrates.

Although we identified 4E-BP1 as a highly responsive TORC1 readout, the specific mechanism through which TORC1 phosphorylates 4E-BP1 is still to be determined. In mammalian cells, the C-terminal TOR Signaling (TOS) motif and the N-terminal RAIP motif of 4E-BP1 are involved in its recruitment by Raptor, an mTORC1 subunit (Schalm and Blenis, 2002; Tee and Proud, 2002; Böhm et al., 2021). In budding yeast, Kog1 (Raptor homolog) is also involved in substrate recognition and recruitment of TORC1 substrates (Wullschleger et al., 2006; Adami et al., 2007). However, it is unknown whether the TOS and RAIP motif recognition is conserved in yeast through Kog1. Therefore, one way to gain some insights into the mechanistic details governing 4E-BP1 phosphorylation in yeast could be to determine whether Kog1 or other TORC1 subunits bind 4E-BP1 using AlphaFold-based modelling (Jumper et al., 2021; Evans et al., 2021). These predictions could then be tested via co-immunoprecipitation experiments. Overall, determining the 4E-BP1 phosphorylation mechanism in yeast will help understand how mammalian TORC1 substrates can interact with yeast TORC1.

Despite showing changes in localization consistent with changes in TORC1 activity, our tests with a localization-based 4E-BP1/eIF4E reporter displayed

slow and moderate responses to TORC1 perturbations relative to the changes observed with endogenous TORC1 readouts (Guerra et al., 2022). This observation could be due to the high affinity of 4E-BP1 for human eIF4E, which could lead to slow localization changes. To improve the dynamic range and speed of the reporter, one could reduce this affinity by mutating eIF4E on residues mediating the binding to 4E-BP1, or by using 4E-BP1 phosphomutants with reduced affinity for eIF4E. In combination of these mutations, another reporter design could also be tested by fusing a pair of dimerization-dependent fluorescent proteins to 4E-BP1 and eIF4E (Alford et al., 2012).

As the reporter relies on the import of foreign proteins in yeast, it is important to consider whether the reporter interacts with endogenous yeast proteins. Besides altering the reporter signal, these interactions could also affect cell growth in the worst case. Specifically, it remains to be determined if the reporter interferes with the cap-dependent translation in yeast through binding of 4E-BP1 to yeast eIF4E (Cdc33) and whether the yeast functional homolog of 4E-BP1 (Caf20) binds the mammalian eIF4E. There is no direct evidence of an interaction of 4E-BP1 with Cdc33. However, an early study suggested that this could be the case since 4E-BP2 could bind Cdc33 in an NMR *in vitro* assay (Matsuo et al., 1997), albeit with an affinity that is orders of magnitude smaller than the affinity of 4E-BP1 for mammalian eIF4E. In contrast, another study observed no effect on yeast translation upon 4E-BP1 expression in yeast and suggested that 4E-BP1 does not bind yeast eIF4E, as the latter lacks some structural features required for the interaction (Hughes et al., 1999). In agreement with the latter study, we did not observe growth defects in our experiments in cells expressing both mammalian proteins either together or in isolation, despite the fact that these proteins were driven by a medium strength promoter (pSac6). Therefore, if 4E-BP1 and yeast eIF4E interact, it is likely with low affinity that is insufficient to induce a 4E-BP1-dependent repression of translation. The interaction of Caf20 with mammalian eIF4E is more elusive and remains to be determined. Caf20 does not have apparent homology with 4E-BP1 in its amino acid sequence except for a shared canonical eIF4E-binding motif which enables 4E-BP proteins to compete with eIF4G for the binding to eIF4E (Nwokoye et al., 2021; Kamada et al., 2023). While the regulation of Caf20 towards translation initiation differs from that of 4E-BP1 (Kamada et al., 2023), the presence of this shared motif could enable the binding of the yeast protein to mammalian eIF4E. AlphaFold modelling combined to co-immunoprecipitation

experiments could be used to study these interactions and improve the design of 4E-BP1/eIF4E based readouts in budding yeast.

In chapter 4, we have also tested TFEB as a localization-based readout for TORC1, but it proved to be not directly usable in the yeast context. This result suggested that the non-canonical recruitment and activation mechanism of TFEB on the vacuole (lysosome) is not conserved in budding yeast. This hypothesis could be verified by testing the interaction of TFEB with TORC1 subunits. A comprehensive analysis (via immunoblotting) of TORC1-dependent TFEB phosphorylation would also contribute to our understanding of how this substrate functions in yeast. If TFEB phosphorylation is still responsive to TORC1 but localization does respond to changes in TORC1 activity, it could also indicate that the TFEB transport mechanism is not functional in yeast, requiring further work to develop a translocation-based reporter.

Finally, other proteins of mammalian origin could be tested as TORC1 readouts. For example, the ribosomal protein S6 kinase (S6K) shares important structural similarities with its yeast ortholog Sch9 (a direct TORC1 substrate) and contains a TOS recognition motif, similarly to 4E-BP1 (Nojima et al., 2003).

TORC1 interactions with other signaling pathways

To map the full spectrum of TORC1 dynamics in single cells, a multifaceted approach will be essential. Employing substrates with diverse functions (e.g. reporters based on transcription factors or kinases) and distinct localization will provide both spatial and temporal insights into TORC1 activity. Considering its central role in cell growth regulation, TORC1 is inherently interconnected with other nutrient-sensing pathways. Therefore, unravelling the interactions between TORC1 and other major kinases at the single-cell level, will also be crucial for a comprehensive understanding of TORC1 signaling. Several single-cell PKA reporters, such as the AKAR sensors (Molin et al., 2020; Colombo et al., 2022; Botman et al., 2023), originally developed for mammalian cells have been successfully adapted for their use in budding yeast, even though the dynamic range and orthogonality of these reporters still need to be improved. In contrast, the availability of reporters for Snf1 (yeast homologue of the mammalian AMPK kinase) remains limited despite the growing evidence of the major implication of Snf1 activity in the regulation of cell growth processes. Alongside PKA, the Snf1 kinase interacts

with TORC1 signaling in multiple ways, notably by sharing the control of TORC1 and PKA pathways components such as Sch9, Pib2 and Msn2 (De Wever et al., 2005; Broach, 2012; Hughes et al., 2015; González et al., 2020; Caligaris et al., 2023). Altogether, unravelling TORC1 interactions with other signaling pathways is a long-term objective which will require the development of single-cell readouts for each pathway, and targeted dynamic perturbations of the different central kinases (e.g. TORC1/TORC2, PKA and Snf1).

Conclusions

The findings of this thesis illustrate that the development of single-cell readouts, in particular for TORC1, is a complex process that requires a good understanding of the regulatory mechanisms of the different substrates used. This understanding is often a critical limitation when selecting substrates that will form the basis of reliable readouts. In several cases, a particular substrate may turn out to be less understood than previously thought, despite initial promising results. When this occurs, one is faced with two options: discard the chosen substrate and focus on another, or delve deeper into the regulation mechanisms of that substrate. Following the second option, we took a systematic approach to decipher the regulation logic of Sfp1, and demonstrated that the protein sits at the nexus of TORC1 and PKA pathways. The new mechanistic knowledge described here lays the foundation for revisiting Sfp1 as a TORC1 readout in the future. Our systematic approach could also benefit the development of other single-cell readouts for nutrient-signaling pathways.

Moving beyond endogenous yeast TORC1 substrates, we demonstrated for the first time that the transfer of mTORC1 substrates to yeast is a viable option for the development of orthogonal TORC1 readouts, and should be further explored in the future. Altogether, the establishment of single-cell readouts for TORC1 and other major nutrient signaling pathways will allow us to gain a comprehensive overview into the temporal and spatial activities of these cell growth regulators, both in response to changing environments and during the cell cycle.

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