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Master Thesis essay:

Investigating the utility of the optogenetic toolbox for cell cycle synchronization in budding yeast

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Abstract

Cell division in eukaryotes is achieved via a conserved and tightly controlled protein network. In order to study processes that happen at specific stages during this division cycle it is important to have a culture with synchronized cells. Currently used synchronization methods often use the 'arrest-and-release' strategy, in which cells are arrested at a specific point in the cell cycle using chemicals or conditional mutants. Releasing the cells from the arresting conditions results in a synchronized re-entry to the cell cycle. However, these methods usually have severe side-effects on cell physiology and the switching between the restrictive and permissive state is slow.
To overcome these limitations, optogenetic systems may be used, as these systems can offer exact molecular control over diverse cellular processes and switching between two states can be achieved rapidly. To identify potential targets for optogenetic control an overview is given of natural existing cell cycle arresting pathways. Two exiting optogenetic systems were identified that utilize these pathways. Since these systems were not designed for cell synchronization, ways to further improve these systems for cell synchronization were discussed. Moreover, two other pathways were identified that showed high potential for cell synchronization.
Finally, two papers are discussed that developed systems for direct control of the expression or degradation of key regulators of the cell cycle. Although these systems can potentially invoke less severe side-effects, the arrest is less stringent. Overall, although most optogenetic-based systems would require more optimization to be able to compete with existing cell synchronization methods, these system have the potential to overcome the limitations of the current synchronization methods in the future.

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Chapter 1: Introduction

Key regulators of the cell division cycle:

Cell division is one of the fundamental processes of life. In eukaryotes the division cycle can be separated into four stages [Figure 1]. The two growth phases, G1 and G2, mainly serve to increase the cell mass and double the amount of proteins and organelles. In between the DNA is replicated during the S-phase and in the end the cell is divided during the M-phase (Cui, 2017). It should be noted that the budding yeast cell cycle slightly deviates from this general model, since processes characteristic to the G2 are already active during the S-phase.

The main regulator of the budding yeast cell cycle is a cyclin dependent kinase (Cdc28). As the name suggests, this kinase is dependent upon the association with a class of proteins, called cyclins, for its activation and to direct it towards specific targets. The expression and degradation of these cyclins is tightly regulated, in such a way that specific cyclins are present during each stage of the cell cycle (Cui, 2017). Together Cdc28 and the cyclins form the main regulatory unit that orchestrates the processes that are active during most of the cell cycle.

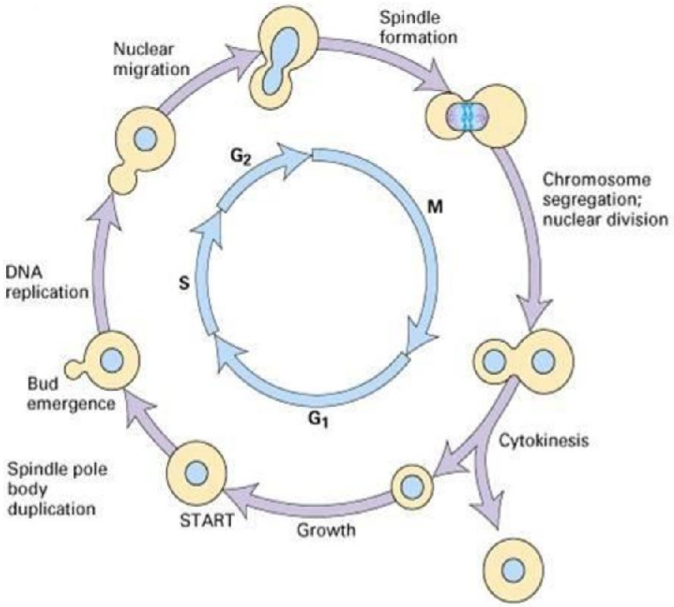

Figure 1: Overview of the yeast cell cycle
Image copied from (Cui, 2017); The middle ring shows the four stages of the cell cycle: G1, S, G2 and M. In the outer ring cartoons represent the appearance of budding yeast during each stage. Also several cell division processes that occur at characteristic points during the cell cycle are indicated.

Cell cycle synchronization:

Although the discovery of the key regulators of the cell cycle was awarded with the Nobel Prize in 2001, our knowledge about the precise regulatory mechanisms is still far from complete. One of the main challenges in studying the cell cycle is that events are usually happening in a specific stage of the cell cycle, while in an exponentially growing culture cells are present in all stages. To study the processes during a specific stage the cell cycle it can be synchronized in a cell culture. A synchronized cell culture can be obtained by separating the cells in a specific stage from those in other stages or by arresting all cells at a specific stage in the division cycle and after lifting the arrest the cells will simultaneously re-enter the cell cycle.

The separation method relies on the difference in physical properties of cells in different stages of the cell cycle, for example cells can be separated based on their size by centrifugal elutriation (Banfalvi, 2008). For the arrest and release method either chemicals or conditional mutants are used to arrest the cells. Examples of commonly used chemicals are mating pheromone, hydroxyurea and nocodazole (Banfalvi, 2017) of which the mechanisms of action will be discussed later on. Many temperature cell division cycle (cdc) mutants have been discovered during the Seventies in an attempt of finding genes involved in the cell

cycle. These mutants are functional when the cells are grown above a certain permissive temperature, but lose their function when the temperature is lowered resulting in an arrest of the cell cycle. (L. H. Hartwell, Culotti, & Reid, 1970; Leland H Hartwell, Mortimer, Culotti, & Culotti, 1973). Although all of the methods described above have specific advantages and disadvantages, they usually have severe side-effects on cell physiology and the release or synchronization process is not very fast.

Optogenetic systems for cell synchronization:

To tackle these two problems in cell cycle synchronization the optogenetic toolbox will be explored in this literature review. First of all, light can be instantly removed from a cell culture by simply switching the light off, which allows for a fast release. Secondly, optogenetic systems have been described that control precise molecular interactions without interfering with the general cell physiology. So far, optogenetic systems have been described to control gene expression, protein localization, degradation and dimerization (Repina, Rosenbloom, Mukherjee, Schaffer, & Kane, 2017). Here all of these methods will be encountered during the discussion of exiting and potentially new systems for the optogenetic synchronization of the budding yeast cell cycle.

To achieve this goal two strategies can be discriminated. The first approach aims to activate the naturally existing cell cycle checkpoints. These checkpoints are signalling pathways that cause a delay or arrest of the cell cycle upon activation. Light inducible cell cycle arrest could be achieved by putting key interactions from these pathways under optogenetic control. The other strategy relies on directly controlling the expression or degradation of the key regulators of the cell cycle, such as for example the cyclins.

In the next chapter an overview of the most important checkpoints will be given of which the most promising will be selected. In chapter 3 the selected pathways will be discussed in more detail and a strategy will be proposed to put them under optogenetic control. Next, the potential of putting the key regulators under direct optogenetic control will be discussed based on two existing systems. Finally, the results will be summarized and other potential uses for optogenetic systems in cell cycle research will be shortly discussed.

Chapter 2: Cell cycle checkpoints

2.1 Start transition checkpoints:

Cell cycle initiation:
Under constant conditions, exponentially growing yeast cells will enter the cell cycle when they have reached a certain critical mass. The exact mechanisms by which cells control their size remain a topic of discussion, which is left out of the scope of this review (Jorgensen & Tyers, 2004). Eventually, cell growth leads to the accumulation of the initial cyclin, Cln3, allowing it to activate Cdc28. The activated Cdc28/Cln3 complex triggers a positive feedback loop involving Cln1,2 and Whi5, which is well understood (Skotheim, Di Talia, Siggia, & Cross, 2008). In fact a model has been developed to simulate the START transition and it is able to capture the effect of perturbations of the central components quite well (Adames et al., 2015; Chen et al., 2004; Laomettachit, Chen, Baumann, & Tyson, 2016). Since triggering this transition irreversibly commits cells to a division cycle, there are several checkpoint pathways that can inhibit the activation of Cdc28.

Metabolism affects initiation timing:
To begin the availability of enough nutrients is essential for progression through the cell cycle. Nutrient depletion is mainly sensed through the PKA and Tor pathways and will lead to prevention of START. However, these pathways are also intertwined in regulation of the metabolism, which in turn modulates START as well. Furthermore, when nutritional depletion occurs after cells have committed themselves it can also lead to delays or arrests in other stages. More detailed overviews of the interplay between the cell cycle and nutritional sensing can be found in these two reviews (Alberghina et al., 2012; Ewald, 2018).
Closely linked to the nutrition signalling pathways is protein synthesis rate. The synthesis rate of proteins directly influences the cell cycle via a TOR mediated signalling pathway. Interestingly, inhibition of protein synthesis leads to a G1 arrest in asynchronized cells, indicating that it specifically interferes with the START transition (Polymenis & Aramayo, 2015). Further, a system has been developed to optogenetically control translation initiation in yeast, which could potentially be used to control the cell cycle (Lu et al., 2019). However, the system does not yet provide a very effective inhibition of synthesis and inhibiting protein synthesis in general is a radical intervention that will significantly effect cell physiology.

MAPK kinase induced delays:
Thirdly, cell cycle entry can be prevented by the presence of mating pheromones. Pheromones activate a MAPK (Mitogen Activated Protein Kinase) pathway that leads the activation of the MAPK Fus3. Fus3 in turn activates a Cdc28 inhibitor, Far1, and induces transcription of proteins involved in the mating response. Although the mating response leads to severe physiological changes such as shmoo formation, pheromone induced arrest is a popular method for cell synchronization in early G1 (Bardwell, 2005). Moreover, optogenetic control over this pathway has already been established (Strickland et al., 2012). The mechanism of this will be discussed in more detail in the next chapter. Also in mammalian cells optogenetic control over another MAPK pathway (the ERK/RAS pathway)

has been established already (Aoki et al., 2013; Toettcher, Weiner, & Lim, 2013), which shows that MAPK pathways are suitable targets for optogenetic systems.
Another MAPK pathway that can delay the START transition is the osmotic stress checkpoint. Activation of the MAPK of this cascade, Hog1, results in an adaptation program, which includes the increase of glycerol production. It also prevents the START transition by down-regulating cyclin expression and stabilization of Sic1, an inhibitor of cdc28 (Escoté, Zapater, Clotet, & Posas, 2004). Alternatively activation of Hog1 can also induce a pre-mitotic arrest by interfering with the morphogenesis checkpoint (Clotet et al., 2006), which will be discussed later in this chapter.

2.2 post-START checkpoints:

Although cells are committed to completing the cell cycle after passing through the START transition, there are still several checkpoints which can delay the cell cycle to allow damage to be repaired or developments to be finished. Nutritional depletion induced arrest has already been mentioned before, so it will not be discussed again.

DNA damage checkpoints:
DNA damage is sensed throughout the entire cell cycle via two pathways. One depends on Rad9 and the other is mediated via a complex consisting of Rad24/Rad17/Mec3/Ddc1. Eventually both systems activate two essential kinases: Mec1 and Rad53 (De la Torre-Ruiz, Green, & Lowndes, 1998; Longhese, Foiani, Muzi-Falconi, Lucchini, & Plevani, 1998). Rad53 activation can arrest the cell at multiple points during the cell cycle via different mechanisms, which are summarized in (Weinert, 1998). Next to cell cycle arrest Rad53 activation leads to the activation of DNA repair mechanisms, transcriptional changes and increased chromosomal movement (Smith, Bryant, & Rothstein, 2018).
Of particular interest for this review is the functioning of the DNA damage response during S-phase. Namely, Rad53 activation can inhibit DNA replication by stopping existing replication forks and preventing the formation of new forks. Further, stalled replication forks can also activate Rad53 via an Mrc1 dependent mechanism (Bacal et al., 2018). Hydroxyurea, a popular synchronization chemical, arrest cells by depleting the dNTP pools, which leads to stalled replication forks. Rad53 activation does not only stall DNA replication, but also stabilizes the replication forks during the arrest. (Ciardo, Goldar, & Marheineke, 2019).

Morphology checkpoint:
One of the first processes that is started after the START transition is bud formation [Figure 1]. During the S-phase, the progress of bud formation is monitored by the correct assembly of a septin ring around the bud neck (Lew, 2003). Septins are filamentous heteromeric protein complexes that interact with the cytoplasmic membrane. The bud-induced change in membrane curvature re-organizes the septin filaments in the bud neck, which enables the recruitment of Hsl7 in an Hsl1 dependent manner (Marquardt, Chen, & Bi, 2019). Hsl7 subsequently recruits Swe1, a Cdc28 inhibitor that prevents the mitotic entry. Recruitment of Swe1 to the bud neck leads to its degradation and thus allows for cell cycle progression. So, in contrast to the other checkpoint pathways this pathway has to be activated to induce cell cycle progression and manipulation of bud formation by for example latrunculin treatment causes a pre-mitotic arrest (Theesfeld, Zyla, Bardes, & Lew, 2003).

Spindle assembly checkpoint:
To ensure proper separation of the chromosomes correct attachment of the microtubili to the kinetochores is crucial. The spindle assembly checkpoint, which can be chemically activated by nocodazole, prevents progression into the anaphase and can be activated by two signals. The strongest signal arises from unattached kinetochores, which leads to the successive recruitment of a number of proteins on the kinetochores. Eventually this leads to the activation of the Mitotic Checkpoint Complex (MCC). The MCC binds Cdc20 inhibiting the activity of the Anaphase Promoting Complex (APC) (London & Biggins, 2014). Alternatively, low tension in microtubili can be sensed on the kinetochore, which can delay anaphase entry via an unknown mechanism that does not involve the MCC (Proudfoot et al., 2019).

2.3 Selection of checkpoints for optogenetic manipulation:

In this chapter the most important checkpoint pathways of the budding yeast cell cycle were discussed. There will be other cellular signals that affect the cell cycle, for example loss of MtDNA can induce an arrest in a Rad53 dependent manner (Crider et al., 2012), but these seem to arrest the cells at similar points during the cell cycle and are not as intensively studied. Furthermore, another checkpoint exists that prevents premature separation of Mother and daughter cells, but this was not considered as it does not seem to influence the central cell cycle timer (Brace, Doerfler, & Weiss, 2019).
The Initiation of the cell cycle may be the most tightly controlled transition and has therefore great potential for cell arrest. The nutrition and protein synthesis sensing pathways seem to be too integrated into the central cell metabolism to elicit a response that will only affect the cell cycle. The mating pheromone pathway will be discussed more thoroughly in the next chapter, because it has already been used for cell synchronization by both optogenetic as chemical stimulation. Since the signalling mechanisms in MAPK pathways are quite similar, the methods applied to the pheromone pathway may also be used to control other MAPK pathways, such as the Hog dependent arrest.
After Start, stalling DNA replication seems to be an active target as it induces a strong and specific arrest and cells actively stabilize the stopped replication forks. Further, preventing Swe1 degradation via the morphogenesis checkpoint seems to be a promising method for cell cycle arrest, as it doesn't affect other processes in the cell. Although the spindle assembly can induce a quite strong arrest, there are a lot of dynamic processes going on during mitosis making this stage less suitable for a stable arrest. Therefore, this checkpoint was left out for further characterization in the next chapter.

Chapter 3: Hijacking the natural checkpoints

3.1 The mating pheromone pathway

MAPK pathways are signalling pathways that are found in all Eukaryotes and the mating pheromone pathway is the best studied example in budding yeast. MAPK pathways are stimulated by an external signal which leads to a cellular response via a phosphorylation cascade. The kinases in these cascades are constitutively expressed and the same proteins can function in different pathways. Further, the cellular response generally affects multiple processes, which can include cell cycle arrest (Atay & Skotheim, 2017). Therefore, optogenetic control over the cell cycle via these pathways faces two main challenges: light activation of the correct cascade and prevention of side effects.

Optogenetic activation of the pheromone pathway:
Yeast cells sense extracellular mating pheromone via a G-protein coupled receptor. Once stimulated by pheromone, the released β/γ-subunit (consisting of Ste4 and Ste18) stays attached to the membrane and recruits two proteins, Ste20 and Ste5. Ste20 is a kinase that is localized at the cytosolic membrane as well and Ste5 is cytosolic scaffold protein, which binds all three components of the MAPK cascade (Ste7, Ste11 and Fus3). When these two proteins are brought together by the β/γ-subunit, Ste20 activates Ste11 (the MAPKKK), which in turn activates Ste7 (the MAPKK). Subsequently, Fus3 (the MAPK) is activated and subsequently transported into the nucleus. Here it activates Ste12, which regulates the transcriptional activation of mating factors, and Far1 which arrests the cell cycle by inhibiting Cdc28 (Bardwell, 2005) [Figure 2a]. Interestingly, Ste12 and Ste7 are also involved in the nutrient starvation pathway, but then activate the MAPK Kss1 instead of Fus3. This change in target specificity is caused by Ste5, which upon binding the membrane, can increase the affinity of Ste7 for Fus3 (Zalatan, Coyle, Rajan, Sidhu, & Lim, 2012).
Strickland and colleagues obtained optogenetic control over the pheromone pathway by controlling the localization of Ste5. They disrupted the natural interaction between Ste5 and the β/γ-subunit (Ste4 and Ste18) using an N-terminal truncated Ste5 variant, which made the cells insensitive to mating pheromones. Next they expressed a membrane anchored LOVpep domain and fused its interacting partner ePDZb to Ste5. Careful optimization of the dimerization kinetics by mutating both dimerization domains lead to a system that showed unrestricted growth under dark conditions (no signalling) and arrested cells in light conditions (constant signalling) (Strickland et al., 2012). These results show that cells can be effectively arrested via the pheromone pathway, but synchronization also requires a fast and effective release from the arrest.

Optimization of the release kinetics:
Pathak and colleagues used the same system to control the activity of the pheromone pathway, but they used 3 different photodimerization systems to control Ste5 localization. They found that background (dark state) activation was lower for a CRY2/CIB system than for the Lovpep/ePDZb system, while the arrest (light state) was equally effective (Pathak, Strickland, Vrana, & Tucker, 2014). This means that the CRY2/CIB system would result in a more efficient release than the Lovpep/ePDZb system used by Strickland and colleagues.

However, not only the release efficiency is important, but also the swiftness of the release after removal of the light

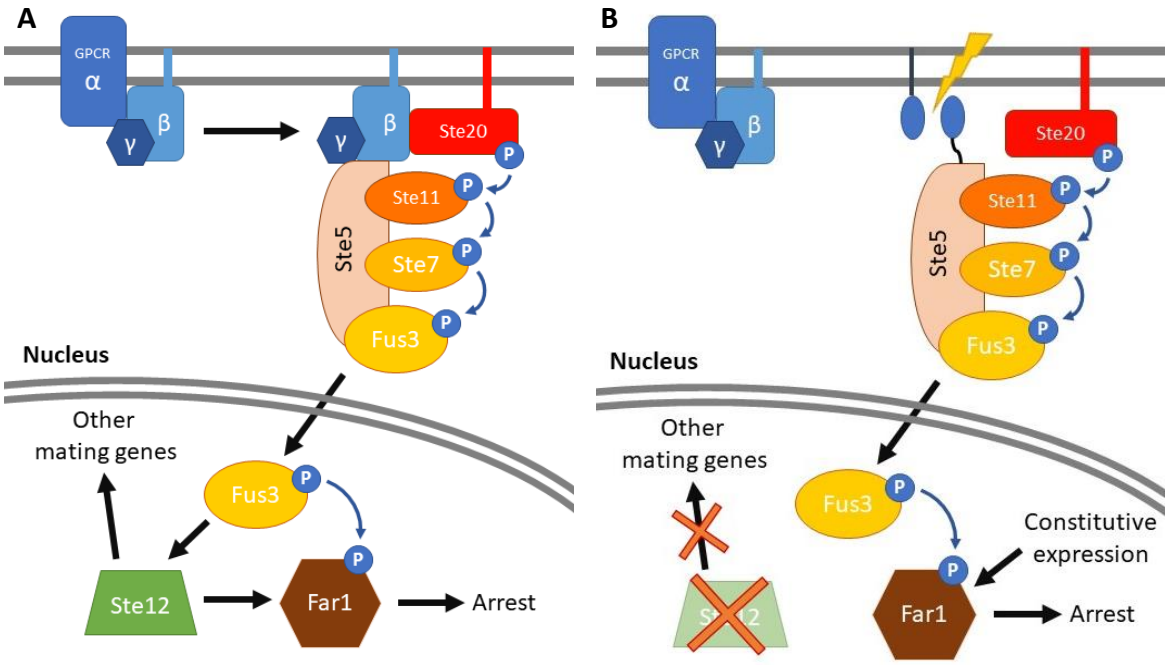

Figure 2: Schematic overview of the mating Pheromone pathway
A: natural mating pheromone pathway; In the top of the figure the cytosolic membrane is represented by the grey horizontal lines with in the top left the mating pheromone GPCR. Next (following the black arrows) the β/γ subunit is released and recruits Ste20 and Ste5. The MAPK kinase cascade is represented by the curved blue arrows. Then activated Fus3 is transported into the nucleus where it activates Ste12. Ste12 activates the transcription of Far1 and other mating genes. Finally Far1 is activated by Fus3, which leads to a G1 arrest. **B: Optogenetically manipulatable pathway;** Ste5 is recruited to the membrane by the light induced dimerization of the dimerization tags, therefore activation of the GPCR is no longer necessary. Further deletion of Ste12 prevents the transcription of mating genes. To ensure that enough Far1 is expressed it is constitutively expressed.

source. In a different study it was reported that the dark reversion half-life of a different membrane localization system was three times higher for the CRY2/CIB system than for the LOVpep/ePDZb system (164.3 vs 53.8 seconds) (Hallett, Zimmerman, Yumerefendi, Bear, & Kuhlman, 2015). It should be noted however that other variants of the tags were used and the tagged proteins differed as well, so the actual numbers are likely to differ. Nevertheless, the dark reversion half-life is an important parameter to take into account for the design of synchronization system.

The reversal kinetics of the dimerization system are important to quickly shut down the signalling activity, but the cell cycle arrest is lifted by deactivation of Far1. The deactivation mechanisms of Far1 are not exactly known. It is known that Far1 is constantly degraded in the nucleus by SCF^{Cdc4} complex (Blondel, 2000), so due to the turn-over of Far1 the fraction of activated Far1 will decrease. This process will be self-promoting as activated Far1 has an increased nuclear export and is thus more protected from the nuclear degradation. Further, this reduction of active Far1 will also lead to activation of Cdc28, which phosphorylates Far1 increasing its degradation rate even further (Tyers & Futcher, 1993). In this model the inactivation of Far1 is mainly dependent on its turnover, but there might be other

mechanisms that play a role as well. So further studies are required to investigate how cells re-enter the cell cycle after pheromone induced arrest.

Deactivation of the mating response:
To further increase the usefulness of this system triggering of the mating response should be prevented. Recently, the entire pheromone pathway was systematically deleted to construct a synthetic GPCR signalling pathway. In this research Ste12 was redirected towards a synthetic promoter sequence resulting in the loss of the entire transcriptional response upon pheromone stimulation (Shaw et al., 2019). So, removal of Ste12 seems to be promising method to remove the side-effects of pheromone stimulation.
However, Far1 expression is also affected by Ste12 (McKinney, Chang, Heintz, & Cross, 1993). Therefore, Far1 expression should be exogenously controlled by expressing it from a constitutive promoter. This has already been done before in a strain in which Cln3 was expressed from the Galactose inducible promoter. When Cln3 expression was turned off mating pheromone treatment resulted in an arrest, while cells overexpressing Cln3 did not arrest when stimulated with pheromones (McKinney et al., 1993).
The authors hypothesized that in case of Cln3 overexpression the Far1 concentration was too low to efficiently inhibit cell cycle progression. Unfortunately, it was not tested in wild type cells or with Far1 expressed from a stronger promoter. Nevertheless, this data show that the Ste12 mediated increase in Far1 expression is not necessary for G1 arrest. So, the system should at least work in a ΔCln3 strain and possibly in wildtype cells as well.
To conclude, using the optogenetically controllable pheromone pathway designed by Strickland and colleagues (Strickland et al., 2012) in a ΔSTE12 strain with a constitutively expressed Far1 seems to be an excellent way to achieve cell synchrony in yeast [Figure 2B]. The system of Strickland may be further improved by using the CRY2/CIB dimerization system, especially considering this system has recently been further optimized (Taslimi et al., 2016).

3.2 DNA replication initiation:

DNA replication is an interesting target to manipulate, since it is only required for dividing cells and the arrested state is stabilized by the cell. Therefore blocking an active replication fork using the DNA damage activated Rad53 seems like an attractive option. However, the molecular mechanism of this pathway is not exactly known, so it is difficult to identify potential targets for optogenetic control. Moreover, manipulating the very dynamic replication fork by other means seems difficult, so preventing the initiation of DNA replication will be discussed.

Phosphorylation of Sld2 is essential for DNA replication initiation:
Initiation of DNA replication is dependent on the formation of a multi-subunit complex on the origin of replication. Formation of the full complex is a multi-step process that is tightly orchestrated, for a review on this topic see (Riera et al., 2017). Essential in this process are several phosphorylation steps, one of which is the cdc28 dependent phosphorylation of Sld2 (Masumoto, Muramatsu, Kamimura, & Araki, 2002). Here a method will be discussed that allows control over the phosphorylation of Sld2 by incorporation of a non-canonical amino acid. It should be noted that this method can also be applied to other proteins of which the phosphorylation is crucial for cell cycle progression.

Masumoto and colleagues investigated the Cdc28 dependent phosphorylation of Sld2 and found 6 preferred phosphorylation sites. Interestingly they constructed a mutant in which 5 of these 6 could be mutated, which still supported growth, while deleting all 6 sites resulted in inviable cells (Masumoto et al., 2002). Thus, in this mutant, called 5A-1, controlling the phosphorylation of a single Serine residue (S100) would allow control over DNA replication. Alternatively, Tak and colleagues identified a second essential phosphorylation step of Sld2. Phosphorylation of the residues mentioned above allows the Cdc28 to phosphorylate another residue. This Threonine is located in a unusual target motif and its phosphorylation is essential for Sld2 functionality as well (Tak, Tanaka, Endo, Kamimura, & Araki, 2006).

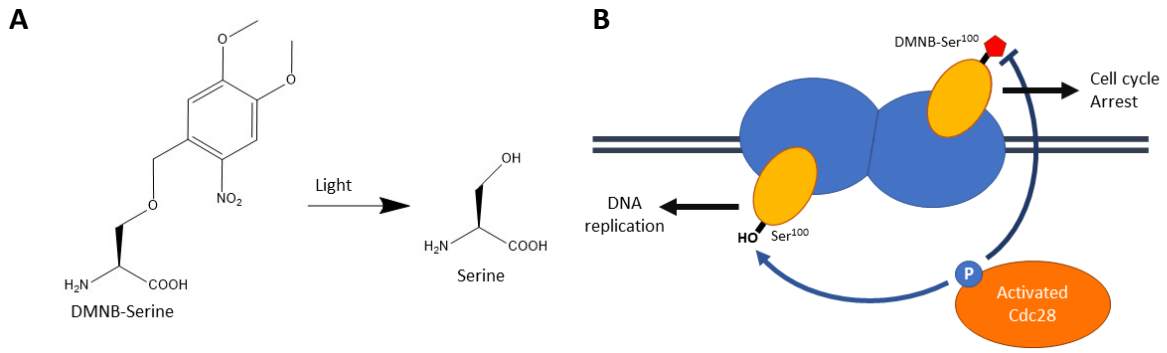

Figure 3: Blocking DNA replication with DMNB-Serine
A: Photo-activation of DMNB Serine; The reactive Oxygen of Serine is protected in DMNB-Serine, but an Auto-cleavage reaction can be activated by Blue light. The product of this reaction is a regular Serine residue of which the Hydroxyl group can be phosphorylated. **B: Schematic overview of the role of Sld2 Ser^{100} in DNA replication;** The Pre-RC complex (Blue ovals) is tightly bound to the dsDNA and has recruited un phosphorylated Sld2 (Yellow ovals). The Sld2 Ser^{100} (or photocleaved DMNB-Ser) is then phosphorylated by an activated Cdc28 complex. This leads to the subsequent recruitment of Dpb11 and other factor and eventually to initiation of DNA replication . When the DMNB-Ser^{100} (Red pentagonal) is incorporated phosphorylation by Cdc28 is not possible and Dpb11 cannot be recruited to the initiation complex.

Incorporation of a photocaged amino acid in Sld2:

Replacing one of these essential residues by a photocaged amino acid would prevent DNA replication until the protecting group is removed. An example of such a photocaged amino acid is 4,5-dimethoxy-2-nitrobenzylserine (DMNB-Ser) [Figure 3A] (Lemke, Summerer, Geierstanger, Brittain, & Schultz, 2007). To specifically incorporate this amino acid at the Amber stop codon (TAG) Lemke and colleagues engineered an orthogonal *E. coli* tRNA/aminoacyl tRNA synthetase (aaRS) pair. Recombinant expression of this tRNA/aaRS pair in budding yeast plus supplementing DMNB-Ser to the medium allowed them to incorporate the photocaged Serine into a transcription factor. Although the same protection group might be used to create a photocaged Threonine, such a system has not been described yet. Therefore it was decided to focus on the first phosphorylation step of Sld2.

Taken together, an amber suppression system is envisioned in which DMNB-Serine is incorporated at the Ser100 residue of the 5A-1 Sld2 mutant [Figure 3B]. When these cells are grown in the dark Sld2 cannot be phosphorylated, so the cells will arrest during the S-phase. Illumination with light would open free the Serine residues allowing the cells to simultaneously resume the cell cycle. However, the light would also cleave DMNB-Serine in the medium, which has not yet been incorporated. Due to the depletion of DMNB-Serine

from the medium the production of new Sld2 will be prevented, since a regular Serine cannot be incorporated on the TAG codon. Therefore the cells will probably arrest during S-phase again after a few cycles. To increase the number of cycles it may be possible to supplement the medium with additional DMNB-Serine after light activation or to specifically deprotect the DMNB-Serine in cells.
Furthermore, this also poses a problem to grow the cells without arresting them. To solve this a second copy of wild-type Sld2 may be expressed. To remove this copy during the synchronization experiment the auxin inducible degron (AID) system may be used (Papagiannakis, De Jonge, Zhang, & Heinemann, 2017). This system requires the fusion of an AID-tag to the targeted protein and recombinant expression of the TIR1 F-box protein from Oryza sativa. Addition of auxin to the medium will deplete the targeted protein in 25 minutes. Growing the cells without Auxin in the medium allows DNA replication by the wild-type copy of Sld2 independent of the photocaged A5-1 mutant.
Altogether, controlling DNA replication initiation using the Amber-suppression system described above may be achievable. Although using the Amber suppression system in yeast is well-established, combining it with the AID system is still hypothetical. Taken together these systems require the creation of a heavily engineered yeast strain that recombinantly expresses multiple proteins. Further, the system may only be used to study one or a few cycles after release from the arrest.

3.3 The morphogenesis checkpoint:

Prevention of bud initiation:
Activating the morphogenesis checkpoint can be achieved either by preventing proper bud-formation or by manipulating the Hsl1/7 mediated degradation of Swe1. A light-controlled system for bud initiation was designed by Allard and colleagues. They sequestered Bem1 to the mitochondrial membrane using an anchored PhyB/PIF dimerization system. Bem1 recruitment towards the cytosolic membrane is an essential step in the bud formation process, which is prevented by the lack of cytosolic Bem1. Light induced release of Bem1 allows it to be recruited to the membrane and progression of the cell cycle (Allard, Decker, Weiner, Toettcher, & Graziano, 2018).
The authors report ‘escape’ mutants which increase the ploidy of their DNA after several hours, which is a consequence of checkpoint failure. This likely due to the activity of the phosphatase Mih1, which removes the inhibitory phosphate that Swe1 places on Cdc28. Deletion of Hsl1 and Hsl7 in a WT background is non-lethal, whereas deletion of either gene in a ΔMih1 background is lethal and results in G2 arrested cells (McMillan et al., 1999). However, for cell synchronization purposes the escape mutants may be less problematic as they only appear after several hours.

Induction of arrest after bud initiation
Optogenetic manipulation of this checkpoint after the initiation of bud-formation has not yet been described. The Swe1 degradation pathway is initiated by remodelling of the organizational structure of septins (Marquardt et al., 2019). Manipulation of the septin organization might be a powerful tool to activate this pathway, but its complexity and the lack of knowledge of the key interactions make this too challenging for now. As a result, optogenetic control needs to be exerted on the Swe1 recruitment pathway, which consist of three steps [Figure 4a].

Fortunately, already Hsl7 mutants that are unable to bind Hsl1 have been described (Cid, Shulewitz, McDonald, & Thorner, 2001). Since this interaction is essential for the recruitment of Hsl7, these mutants arrested in G2 with elongated buds [Figure 4b]. Addition of dimerization tags to Hsl1 and the Hsl7 mutant will allow optogenetic control over this interaction and therefore over Swe1 degradation. In this system light activation will lead to the degradation of Swe1 and thus synchronized re-entry of the cell cycle. In contrast to the pheromone system in this case fast reversal kinetics of the dimerization system are less important, since there is no evidence Hsl7 localization affects cell cycle progression in other ways than via the morphology checkpoint. Therefore, the size of the dimerization tags may be a more important selection criterium as large tags may interfere with other septin interacting proteins. Recently an overview of dimerization systems has been reported, which can be used for the selection of a suitable system (Spiltoir & Tucker, 2019).
Finally, this system would support growth in the dark state, but these cells will have an elongated G2-phase and elongated buds similar to ΔHsl7 cells (Cid et al., 2001). This phenotype may be prevented by growing cells under constant light activation, which will result in constant Swe1 degradation. However, it is not known whether constant association between Hsl1 and Hsl7 interferes with other processes such as for example Hsl1 localization. Alternatively, Swe1 could be depleted using the AID-system discussed in the previous section (Papagiannakis et al., 2017). Growing these cells in the presence of auxin and under dark conditions should effectively create a ΔSwe1ΔHsl7 mutant, which does not show a growth phenotype (Cid et al., 2001).

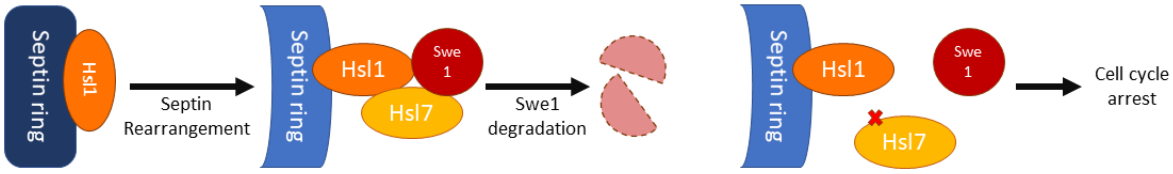

Figure 4: Schematic overview of Swe1 degradation by the Morphogenesis checkpoint
A: Normally functioning checkpoint; Hsl1 is recruited to the Septin ring that is formed around the bud neck, but in a way that is unable to interact with Hsl7. Septin rearrangement, which is induced by bud development, alters the binding conformation of Hsl1 allowing it to recruit Hsl7, which then recruits Swe1. Swe1 recruitment to the bud neck initiates its degradation allowing the cell cycle to continue. **B: Hsl7 mutants prevent its interaction with Hsl1;** The single residue Hsl7 mutants discovered by Cid and colleagues are unable to bind to Hsl1, so Swe1 cannot be recruited and degraded. Therefore Swe1 remains active and cell cycle progression by Cdc28 inhibition.

Chapter 4: Direct control over the central cell cycle regulators

Direct control over the cell cycle components is complicated due to the complex regulatory design and the existence of semi-redundant cell cycle control systems. Nevertheless, two systems have been described already in which this has been achieved. First, a system is described that allowed synchronization of the cell cycle by expression control. The second describes a system in which control is exerted over the timing of degradation.

Controlling the expression of Cln2:

Charvin and colleagues developed a system in which the cell cycle was controlled by the periodic induction of Cln2 (Charvin, Cross, & Siggia, 2009). During the natural START transition Cln3 activates the expression of Cln2 and Cln1, which in turn activate their own expression as well. This self-activating system is impaired by deletion of Cln3. However, Bck2 can still activate Cln1 and Cln2 expression in these cells, although much more slowly (Wijnen & Futcher, 1999). This fact allowed the authors to initiate the cell cycle by exogenous induction of Cln2 expression. Since the natural Cln2 is still present a short expression burst of Cln2 can activate the natural positive feedback mechanism.

A downside of this system is that it only allows cells to prematurely activate their cell cycle as they will otherwise enter it themselves. Also, if the Cln2 is expressed too early the cells are not ready to enter the cell cycle and the induction has no effect. Therefore, the authors could only synchronize either Mother or Daughter cells effectively due to the differences in the average length of the cell cycle. This may be prevented when a Cln3 Bck2 double knock-out is used, which can no longer enter the cell cycle by itself. Since the double knock-out is lethal a conditional knock-out of one of the two genes should be used. This could be achieved using the previously discussed AID system (Papagiannakis et al., 2017) or expression from for example the galacatose inducible promoter (Di Como, Chang, & Arndt, 1995).

Interestingly Charvin and colleagues used a methionine inducible transcription system to control the exogeneous Cln2 expression bursts. To quickly switch between the ON and OFF state they used a microfluidic device that allowed them to swiftly exchange the medium. They achieved switching the Cln2 expression within a few minutes, but this only works for small scale applications. Optogenetic control over gene expression would therefore be an obvious improvement on their system. Several systems exist for gene expression control, which are reviewed in (Salinas, Rojas, Delgado, & Agosin, 2017). The transcription system should have low background expression and a quick response time, but the reversal time is less important.

Degradational control over cell cycle regulators:

Besides controlling expression, degradation can also be controlled to affect the abundance of key regulatory proteins. Renicke and colleagues developed a light inducible degradation tag based on the LOV2 domain (Renicke, Schuster, Usherenko, Essen, & Taxis, 2013). As a proof of principle they showed they were able to arrest the cell cycle by preventing the degradation of two central components: Sic1 and Clb2. Both targets are not essential, so under constant illumination the cells can grow normally.

Sic1 is a Cdc28 inhibitor that is usually active in early G1 and needs to be degraded to allow entry into the S-phase (Verma et al., 1997). Sic1 consists of a cdk inhibitory domain and a

domain that directs its degradation upon phosphorylation. Removal of the N-terminal degradation domain of Sic1 results in an undegradable protein that still effectively inhibits the cell cycle (Berset et al., 2002). Replacement of this degradation domain by their light inducible degradation tag allowed Renicke and colleagues to make the degradation of Sic1 solely dependent on light. When grown under constant illumination these cells behaved like ΔSic1 cells, which do not show a growth phenotype. However, when these cells are switch to dark conditions Sic1 degradation is inhibited resulting in cell cycle arrest in G1.

Although their system efficiently blocked cell cycle progression a large fraction of the arrested cells formed multiple buds. This result is not surprising as Sic1 doesn't block the activity of Cdc28 associated with the early cyclins, which promotes bud initiation. The easiest way to prevent the formation of additional buds would be to keep the duration of the arrest as short as possible.

Clb2 is a late mitotic cyclin that is targeted for degradation by the APC/C complex. Removal of the degradation recognition box prevents the degradational control and induces a mid-mitotic arrest (Wäsch & Cross, 2002). Replacing this degradation recognition box sequence with the light inducible degradation tag allowed Renicke and colleagues to induce a metaphase arrest by preventing Clb2 degradation (Renicke et al., 2013). The efficiency of this system was not very high, after 5 hours of light stimulation only slightly more than half of the population was in metaphase. The authors do not comment on the large fraction that did not arrest, but in view of cell synchronization it is interesting to discuss this in more detail.

One possibility could be that cells can still deplete Clb2 by down-regulating its transcription. Recently, the light inducible degradation system was expanded by combining it with an optogenetic transcriptional control system (Hasenjäger et al., 2019). This system was designed in such a way that when the degradation tag was inactive (dark state) the protein was expressed and when it was degraded (light state) transcitpion was inhibited. This system was also tested by controlling Clb2 and the percentage of cells that arrested in metaphase was similar to that reported before. Therefore, it is unlikely that the cells can escape the Clb2 induced arrest by depleting Cbl2 by other means.

So, it seems that cells can eventually continue the cell cycle in the presence of Cbl2. Unfortunately, the set up of the experiment does not tell how long it takes before cells can escape the arrest. The system may still be usefull if the metaphase of the cells can be delayed long enough for cell synchronization. Further the escape from the arrest might be prevented by deleting or down-regulating backup mechanisms that allow the cells to progress past this arrest. However, this would require further research into the mechanisms and wheter they are essential.

Chapter 5: Discussion

Controlling the natural checkpoint pathways:

In the beginning of this review an overview of the important natural checkpoint pathways was given. These checkpoints can be directly or indirectly activated using chemicals. Next the optogenetic activation of the pheromone pathway was discussed, which efficiently activates the pathway. Activation of this pathway also induces a mating response, which may be prevented by deletion of the transcription factor Ste12. Although most components of this system have already been experimentally validated, it is not clear if Far1 is deactivated efficiently after release of the arrest.

Conversely, the proposed method for controlling DNA replication initiation is still mostly hypothetical. Creating the complex strain required for this system may prove quite challenging. Nevertheless, controlling the phosphorylation by incorporation of photocaged residues could become a powerful technique for cell cycle synchronization, since it can be applied to many targets. A downside is that it is not reversible like the other systems, so it can only be used to arrest and release the cells once. Further it should be expanded towards other residues that are phosphorylation targets, such as Threonine and Tyrosine. The incorporation of photocaged Tyrosine residues has already been described in both bacteria and mammalian cells (Arbely, Torres-Kolbus, Deiters, & Chin, 2012; Deiters, Groff, Ryu, Xie, & Schultz, 2006; Luo, Torres-Kolbus, Liu, & Deiters, 2017), but no evidence was found for a similar system in yeast.

The two systems that allow control over the morphogenesis checkpoint can complement each other in studying the size control mechanisms. The system described by Allard and colleagues that controlled bud initiation was used to study the size control mechanisms in Mother cells (Allard et al., 2018). While preventing cell cycle progression after bud initiation is likely to result in elongated buds and could therefore be used to study the size control mechanisms in the Daughter cells. The latter has an added advantage for cell synchronization, because decreasing the size difference between Mother and Daughter cells should also decrease the difference in G1 duration.

Direct control over the key regulators:

The systems that directly controlled abundance of the cyclins Cln2 and Clb5 could only exert a moderate level of control over the cell cycle. The initiation by exogenous Cln2 expression only worked in a short time frame and blocking Clb5 degradation for 5 hours only resulted in a 2 fold increase in cells present in the metaphase. Nevertheless, subtle control over the cell cycle can be enough to synchronize the cell cycle when it is exerted periodically as was shown by Charvin and colleagues (Charvin et al., 2009). Moreover, it could be argued that the transitions are more natural, because no inhibitors are activated. On the other hand the deletion strains that are required to increase the efficiency in these systems also affect the cell division cycle.

Finally, controlling the degradation of the CKI Sic1 proved to be a more efficient method to synchronize the cell cycles. Interestingly, controlling the abundance and activity of Cdc28 inhibitors also is the main mechanism of action of the natural checkpoint pathways. Sic1 however may not be the ideal inhibitor for cell synchronization purposes as a large fraction of the arrested cells developed multiple buds.

Conclusions:

In conclusion, optogenetic control over the cell cycle is a promising synchronization method. Already several systems have been constructed that could arrest the cell cycle. However, these systems have to be further optimized to achieve synchronization more efficiently and with less side-effects. Potential systems that can arrest during different stages of the cell cycle have been discussed as well, but these still have to be constructed and experimentally validated.

It is important to have multiple methods that can synchronize the cells at different points in the cell cycle. This allow to select the best system to study a specific process in the division cycle. Although the optogenetic systems described in this review offer a good coverage of arresting points in the interphase of the cell cycle [Figure 1], methods to arrest cells during Mitosis were left undiscussed.

The main reason to leave mitosis outside the scope of this review is that there is a fundamental switch in control during Mitosis. The key regulator of the stages discussed in this review is Cdc28, while Mitosis is mainly controlled by the Anaphase-Promoting Complex/Cyclosome (APC/C) (Chen et al., 2004). Where manipulating Cdc28 activity can be achieved by manipulating cyclins or CKI, the activity of the APC is controlled by other factors, reviewed in (Yamano, 2019). Control over this stage of the cell cycle may be achieved via the spindle assemble checkpoint discussed in this review or via the FEAR and MEN pathways which are discussed in more detail in (Queralt & Uhlmann, 2008).

Chapter 6: References

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