Intrinsic, periodic and tunable metabolic dynamics: a scaffold for cellular coherence

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Conclusions and outlook

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The cell cycle is a periodic process, a rhythmic switch between biomass (e.g. DNA and proteins) formation and segregation, in synchrony with cycles of chromatin activation, and mitochondrial energization. The CDK-centric model proposes the existence of a master oscillator, the cyclin/CDK machinery, as the driver of all periodic functions, from the cell cycle transcription to chromosome structure and dynamics. Another possibility is the existence of multiple biological oscillators, which synchronize to give rise to a higher order function, the cell division cycle. This model of coupled oscillators is supported by the late advent of the cyclin/CDK machinery (Krylov et al., 2003) in the evolution of eukaryotes, the previously reported global transcription (Orlando et al., 2008) and APC-activity oscillations in cell cycle arrested cells (Lu and Cross, 2010), or the metabolic oscillations previously reported in synchronized yeast populations (Lloyd and Murray, 2005; Slavov and Botstein, 2011; Tu et al., 2005).

The dynamic single cell metabolite (Chapter 2, Figures 1A-B, S2A-B & S3A-F, Movie S1) and cell cycle measurements (Chapter 2, Figures S5, 1C & S7), in the absence of synchronization or intercellular communication (Chapter 2, Figure S1C), performed in this thesis, show that metabolism is an autonomous oscillator, which orbits in synchrony (Chapter 2, Figures 1C, 4A-G but also without cell cycle, in cell cycle arrested (Chapter 2, Figures 2C & S6) or quiescent cells (Chapter 2, Figures 2A, 3B-C & S4C-D, Movie S2). The occurrence of metabolic oscillations across nutrient conditions and metabolic modes (respiration, fermentation and gluconeogenesis) (Chapter 2, Figures 1C & S3A-F) suggests that the metabolic oscillations are not due to the temporal activity of specific metabolic pathways but rather due to rhythmic behavior in the synthesis of biomass constituents, such as fatty acids, amino acids or nucleotide.

Consistently, the persistent metabolic oscillations in synchrony with waves of cell volume increase and DNA endo-replication in Cdc14-AID depleted cells (Chapter 2, Figure 6A-I, Movie S6), where the late cell cycle was inhibited, suggest a strong relation between the oscillating metabolism and biomass synthesis. With the protein and DNA synthesis being strongly dependent on ATP availability (Buttgereit and Brand, 1995; Wieser and Krumschnabel, 2001), and with the fatty acid synthesis requiring large amounts of redox equivalents (NADPH) (Halperin and Robinson, 1970), the metabolic

oscillations are well represented by the intracellular levels of the two metabolites, which we found to oscillate in single yeast cells oppositely in phase. However, up-to-date, the biochemical pathways which are responsible for the periodic metabolite dynamics remain unknown. Metabolic perturbation studies may help identify the functional constituents of the autonomous metabolic oscillator. Considering the strong connection between the metabolic oscillations and biomass synthesis, the deletion of any core component will possibly prove lethal. This signifies the use of novel methods for the conditional depletion (such as those used in Chapter 3) or ectopic expression of targeted proteins.

In our single cell NAD(P)H and ATP measurements we found metabolism to be a flexible oscillator, achieving periods between 1 and 24 hours depending on the available nutrients and the growth conditions (Chapter 2, Figures 1C & S4D). During fermentative growth on high glucose (10 gL^{-1}) yeast cells would exhibit metabolic oscillations with an average frequency of 0.6 h^{-1}, which corresponds to the maximum doubling time of yeast (approx. 100 min) (Chapter 2, Figure 1C). When we cultured yeast cells on low glucose (0.01 gL^{-1}) in the microfluidic device, we observed phenotypic bistability (Chapter 2, Figure 3B), and a clear metabolic threshold separating dividing from non-dividing cell fractions. Cells with metabolic frequencies above 0.15 h^{-1} would enter the cell division program, whereas single yeast cells with slower metabolism (frequency below 0.15 h^{-1}) would enter the circadian domain and quiescence. This metabolic threshold is reminiscent of the Warburg effect, and the distinct metabolic phenotype between cancer cells, fermenting even in the presence of oxygen, and non-dividing respiring cells. Consistent with the fact that the metabolic frequency sets a cell cycle threshold, it has previously been shown that somatic non-dividing cells exhibit metabolic oscillations in the circadian domain, whereas cancer (Sahar and Sassone-Corsi, 2009) or stem cells (Bessho et al., 2001, 2003; Isomura and Kageyama, 2014) exhibited ultradian oscillations with a frequency of 0.5 h^{-1}.

Next to the frequency of the metabolic oscillator, its amplitude also constitutes an accurate cell cycle predictor (Chapter 4, Figure 4A-B). Dividing cells exhibited enhanced amplitudes (NAD(P)H SD > 0.04), in contrast to the dampened metabolic oscillations (NAD(P)H SD < 0.04), which we measured in cell cycle arrested or quiescent cells. Consistently, the NADH concentration of cancer cells was found to be 1.8 times higher than normal cells (Yu and Heikal, 2009).

The autonomous metabolic oscillator may operate as a primitive timekeeper, setting the pace of global gene expression and cell division prior to the appearance of the cyclin/CDK machinery. Similarly to yeast (Causton et al., 2015), redox oscillations have been reported in different organisms across all domains of life, from archaea and cyanobacteria, to plants, insects and mammalian cells (Edgar et al., 2012). The conserved role of NAD^+ dependent deacetylases in epigenetic silencing (Vaquero, 2009), together with the A TP-dependent mechanisms of chromatin and DNA remodeling

(Gangaraju and Bartholomew, 2007), provide a link between energy (ATP and NADH) oscillations and global gene expression, which could function as a primitive cell cycle machinery. Additional cell cycle regulation might have been engrafted onto such primitive cell cycle regulation, to ensure the robust separation between cell cycle phases.

The cAMP/PKA pathway is an example of such added control. Using conditional (method presented and characterized in Chapter 3) or constitutive genetic perturbations we have exposed the redundancy of the cAMP/PKA pathway. The metabolic oscillations and their evident coordination with the cell cycle are not the result of periodic cAMP-signaling as previously conjectured (Ewald et al., 2016; Futcher, 2006; Zhao et al., 2016). Instead, the nutrient sensing pathway establishes a positive feedback loop on the metabolic dynamics (Chapter 4, Figure 5B), receiving input (Peters, 2013) from oscillating signaling metabolites (e.g. F1,6BP (Sasidharan et al., 2012)) and strengthening the amplitude of the metabolic oscillations above the minimal threshold (NAD(P)H SD > 0.04) for cell cycle initiation. Thus, the cAMP/PKA pathway ensures robust cell cycle initiation, under eventually nutritionally less optimal conditions. While the cAMP/PKA pathway is there to strengthen the metabolic oscillations, the cyclin/CDK machinery might have the function to eliminate noise in gene expression, via controlling the transcription of the early G1/S cluster (Swi6 dependent), and late G2/M cluster (Mcm1 dependent) (Haase and Wittenberg, 2014). Consistently, the existence of an autonomously oscillating transcription factor network entrained by the cyclin/CDK machinery was recently suggested (Hillenbrand et al., 2016).

In order to confirm the role of metabolism as a cell cycle regulator, it likely will be necessary to "reverse evolution" by identifying and removing the redundancy in the cell cycle control network, reproducing the primitive cell cycle engine. A cell lacking such redundant interactions would still be able to divide, but would be prone to the metabolic noise, as well as the noise in gene expression. This primitive cell would have to wait for the "right" metabolic oscillation (frequency above 0.15 h^{-1} and amplitude over 0.04 NAD(P)H SD – Chapter 4, Figure 4B) to trigger cell division. Stochasticity in gene expression would dominate the transitions between the cell cycle phases, eventually leading to DNA endo-replication and aneuploidy. Consistently, polyploidy is commonly observed in prokaryotes, and was possibly also present in amitotic proto-eukaryotes (Markov and Kaznacheev, 2016).

Next to the relevance of an autonomous metabolic oscillator in cell cycle control, a metabolic time-keeper might also be responsible for other rhythmic processes in the ultradian domain, such as the previously described periodic switch between the REM (rapid eye movement) and non-REM sleep, known as the rest-activity cycle (Kleitman, 1982; Lloyd and Murray, 2005), or the periodic secretion of growth hormone (Tannebaum and Martin, 1976). The flexible metabolic oscillator, which we have

shown to approach 24 h period during respiration on low glucose (0.01 gL^{-1} – Chapter 2, Figure 4C-D), may also constitute the basis for day-night rhythms. In fact, non- transcriptional redox circadian rhythms have been discovered in red blood cells, or in algae where RNA and protein synthesis were arrested (O'Neill et al., 2011), leading to the conclusion that metabolic circadian rhytms pre-existed the core clock components, namely transcriptional activators or inhibitors with interlocking feedback loops, traditionally thought to generate and regulate circadian rhythmicity (Takahashi, 2004).

Periodicity and synchrony is observed at all levels of biological organization, from genes, to pathways, organelles, cells, tissues, all the way up to multicellular organisms, ecosystems and human communities. Life is cycles within cycles: a higher order function emerging from the collective synchrony of coupled and mutually entrained oscillators.

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