From Department of Medicine, Solna
Karolinska Institutet, Stockholm, Sweden

TRACING THE INTERPLAY BETWEEN CELLULAR METABOLISM AND CELL CYCLE

Irena Roci

Karolinska Institutet

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Front cover image: 3D image of cells in different cell cycle phases. Credit: Ella Maru Studio
Back cover image: Overview of research strategy and main findings. Credit: Irena Roci

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Tracing the interplay between cellular metabolism and cell cycle

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Irena Roci

Principal Supervisor:
Dr Roland Nilsson
Karolinska Institutet
Department of Department of Medicine, Solna
Division of Cardiovascular Medicine

Co-supervisors:
Dr Arne Lindqvist
Karolinska Institutet
Department of Department of Cell and Molecular Biology (CMB)

Prof Yudi Pawitan
Karolinska Institutet
Department of Department of Medical Epidemiology and Biostatistics (MEB)

Opponent:
Dr Christian Frezza
University of Cambridge
MRC Cancer Unit

Examination Board:
Prof Sonia Lain
Karolinska Institutet
Department of Department of Microbiology, Tumor and Cell Biology (MTC)

Dr Erik Norberg
Karolinska Institutet
Department of Physiology and Pharmacology (FYFA)

Prof Tuulia Hyötyläinen
Örebro University
School of Science and Technology

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To people who make a good impact on others' lives and
make this world a better place.

ABSTRACT

Cancer cells are more vulnerable to targeting during S-G_2-M cell cycle phases due to common deregulations in cell cycle checkpoint mechanisms. Most common cancer drugs target processes in the S-G_2-M phases, often causing negative effects on proliferating normal cells. On the other hand, several metabolic processes are deregulated in cancer, generating metabolic dependencies and exposing vulnerabilities specific to cancer cells. Therefore, deregulated metabolic processes in the S-G_2-M phases potentially offer effective targeting strategy without harming normal cells. There are some studies reporting cyclic metabolic processes in synchronized cells, but a more systematic study of metabolism across cell cycle is lacking.

The focus of this thesis is to trace metabolic events in G_1 and S-G_2-M phases, and identify metabolic enzymes that affect cancer cell proliferation using an approach that does not interfere with cell metabolism. Initially we developed a method for performing metabolomics in sorted cells, then applied this method on G_1 and S-G_2-M sorted normal and transformed cells. We mapped hundreds of metabolites and metabolic activities in G_1 and S-G_2-M phases, and identified arginase 2 (ARG2) enzyme as a potential target which reduced cancer cell proliferation. This study can be used as a resource of metabolism across the cell cycle. This project presents a large scale investigation of metabolism in G_1 and S-G_2-M phases, contributing to the understanding of the biology of metabolism across the cell cycle and reveals metabolic activities in the S-G_2-M phases of cancer cells. This thesis is comprised of the following papers.

In **Paper I** (Roci et al, 2016) we developed a method for performing metabolomics in sorted subpopulations combining Fluorescence Activated Cell Sorting (FACS) and Liquid Chromatography Mass Spectrometry (LC-MS). We evaluated relative metabolite abundance using peak areas and metabolic activities using isotope tracing. From benchmarking of sorted sampled with extraction from culture dish we found that isotope tracing is more robust and reflects the metabolism of cells in culture dish.

In **Paper II** (manuscript) and **Paper III** (Roci et al, 2019) we mapped metabolism in G_1 and S-G_2-M phases and presented relative metabolite abundances and metabolic activities in these phases. Most metabolite abundances and ^{13}C enrichment were constant showing that they are required throughout cell cycle. Some metabolic processes like ornithine synthesis, arginine uptake showed higher activities in the S-G_2-M, while synthesis of some phosphorylated sugar metabolites was upregulated in G_1 phase.

In a follow-up study of the ornithine synthesizing enzyme, ARG2 presented in (**Paper III)**, we found that silencing of ARG2 reduced cancer cell proliferation. Cancer cells synthesized ornithine only via ARG2, but normal cells used both ARG2 and the alternative pathway via Ornithine transferase (OAT). High ARG2 expression was correlated with ER negative and Basal breast cancer subtypes, and poor survival in these cancer types.

In **Paper IV** (manuscript) we present a survey of choline metabolism by culturing several normal and transformed cell lines in ^{13}C3-choline. Betaine was synthesized only in cancer cells and was not a methyl donor in any of the cell lines. The choline dehydrogenase (CHDH) enzyme, which synthesizes the precursor of betaine, was also expressed only in cancer cell lines, and caused an increase of G_2-M phase cells when silenced. Besides, from untargeted analysis we found 122 peaks that were labeled from choline, including phosphatidylcholine synthesized in all cell lines, and other previously unknown peaks.

LIST OF SCIENTIFIC PAPERS

THESIS PUBLICATIONS

I. **Roci, Irena**, Gallart-Ayala, Hector, Schmidt, Angelika, Watrous, Jeramie, et al. (2016) **Metabolite Profiling and Stable Isotope Tracing in Sorted Subpopulations of Mammalian Cells**.
Analytical Chemistry, *88*(5), 2707–2713.

II. **Roci, Irena,** Watrous, Jeramie D., Lagerborg, Kim A., Jain, Mohit, Nilsson, Roland. (2019) **Exploring cell cycle associated metabolites in human cells**.
Manuscript/Submitted

III. **Roci, Irena**, Watrous, Jeramie D., Lagerborg, Kim A., Lafranchi, Lorenzo, Lindqvist, Arne, Jain, Mohit, Nilsson, Roland. (2019) **Mapping Metabolic Events in the Cancer Cell Cycle Reveals Arginine Catabolism in the Committed SG2M Phase.**
Cell Reports, *26*(7), 1691–1700.e5.

IV. **Roci, Irena**, Watrous, Jeramie D., Lagerborg, Kim A., Jain, Mohit, Nilsson, Roland. (2019) **Choline metabolism in cancer and normal cells**.
Manuscript

OTHER PUBLICATIONS

V. **Roci, Irena**, Gallart-Ayala, Hector, Watrous, Jeramie, Jain, Mohit, Nilsson, Roland. (2017) **A Method for Measuring Metabolism in Sorted Subpopulations of Complex Cell Communities Using Stable Isotope Tracing**.
Journal of Visualized Experiments, (120), e55011–e55011.

VI. Nilsson, Roland, **Roci, Irena**, Watrous, Jeramie and Jain, Mohit (2017) **Estimation of flux ratios without uptake or release data: Application to serine and methionine metabolism**.
Metabolic Engineering, *43*(Pt B), 137–146.

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LIST OF ABBREVIATIONS

ADC	Arginine Decarboxylase
AdoMetDC	Adenosylmethionine decarboxylase
ADP	Adenosine Diphosphate
Akt	Protein Kinase B
ARG2	Arginase 2
ASL	Arginine Succinate Lyase
ASS1	Arginine Succinate Synthase 1
ATP	Adenosine Triphosphate
Cdk	Cyclin Dependent Kinase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CHDH	Choline Dehydrogenase
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic acid
DTB	Double Thymidine Block
EGFR	Epidermal growth factor receptor
ER	Estrogen Receptor
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FH	Fumarate Hydratase
FUCCI	Fluorescence Ubiquitination Cell Cycle Indicator
GC	Gas Chromatography
GEO	Gene Expression Omnibus
GLS1	Glutaminase 1
GLUT1	Glucose Transporter 1
HBSS	Hanks Balanced Salt Solution
HER2	Human Epidermal Growth Factor Receptor 2
HMEC	Human Mammary Epithelial Cells
HMGR	HMG-Coa Reductase
HPLC	High Pressure Liquid Chromatography

LC	Liquid Chromatography
MCDB 170	Mammary Epithelial Basal Medium 170
MEGS	Mammary Epithelial Growth Supplement
MID	Mass Isotopomer Distribution
MS	Mass Spectrometry
m/z	Mass-to-charge ratio
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear Magnetic Resonance
OAT	Ornithine Amino Transferase
ODC	Ornithine Decarboxylase
ODC-AZ	Ornithine Decarboxylase Antizyme
OTC	Ornithine Transcarbamylase
PCNA	Proliferating Cell Nuclear Antigen
P-DME	Dimethylethanolamine Phosphate
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PI3K	Phosphoinositide 3-kinase
PKM2	Pyruvate kinase 2
P-MME	Monomethylethanolamine Phosphate
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute - 1640
RT	Retention Time
SAM	S-Adenosyl Methionine
SDH	Succinate Dehydrogenase
shRNA	Short hairpin RNA
siRNA	Small Interfering RNA
SSAT	Spermidine/spermine-N(1)-acetyltransferase
TCA cycle	Tricarboxylic Acid Cycle
TK	Thymidine Kinase
TMPK	Thymidylate Kinase
TS	Thymidylate Synthase

BACKGROUND

1 INTRODUCTION

Metabolism is the collection of biochemical processes that transform small molecules to form macromolecules and biomass in a cell. The life cycle of a mammalian cell, also called the cell cycle, is characterized by an increase in biomass (DNA and protein content), membrane surface and cell volume, all of which duplicate at the end of this process to give two daughter cells (Schmoller & Skotheim, 2015). These processes occur in a controlled manner. Some processes, like DNA replication, expression of some proteins, etc. are tightly controlled by cell cycle regulation at different phases. However, not much is known about how metabolism is coordinated with the cell cycle.

Being an important player in main processes in the cell, when deregulated, metabolism also contributes to cancer progression and can even cause cancer transformation (King, Selak, & Gottlieb, 2006). These deregulated metabolic processes do not occur in normal proliferating cells (Fendt, 2017). After transformation, some enzymes or metabolites can gain a special importance for cancer cells, which can be called “addictions”. Targeting these metabolic addictions of cancer offers an attractive opportunity for killing cancer cells specifically. If these addictions happen in the vulnerable cell cycle phases between DNA synthesis and cell division, their targeting might be more effective.

Metabolism is mostly measured in whole cell cultures or tissues, and not much is known about metabolism in subpopulations of culture, including cell cycle phases. In this regard there is a need to study metabolism across normal and cancer cell cycle; and identify metabolic activities upregulated in different cell cycle phases. Metabolites are high in number, very diverse in structure and chemical properties, making it technically challenging to measure cell metabolism. However, recent advances in LC-MS now offer possibilities to measure thousands of metabolites.

A more detailed information about cancer metabolism, cell cycle and study methods is provided in the sections below. The background information is intended to provide a better understanding of why we want to understand the interplay between cancer metabolism and cell cycle, what is already known, and what is the best approach to measure these phenomena.

2 BIOLOGY

2.1 Cellular metabolism and rewiring in cancer

Metabolic pathways of central metabolism involve transformation and production of small metabolites such as amino acids, carbohydrates, fatty acids, keto acids, organic acids, and other organic compounds. The main functions of metabolic pathways in a cell are to synthesize macromolecules (proteins, lipids, carbohydrates) and maintain energy homeostasis. Based on the type of end products and energetics, reactions of central metabolism can be separated into two main groups: anabolic reactions that use nutrients and energy to synthesize complex macromolecules, like in gluconeogenesis, and catabolic reactions that involve degradation of energy-source molecules and release energy, like in glycolysis. Some other type of reactions have waste disposal functions and export metabolic products not needed by the cell, preventing their accumulation inside the cells (DeBerardinis & Thompson, 2012). Lactate and non-canonical nucleotides are some examples.

The balance between anabolic and catabolic reactions is determined by the needs of cells for synthesizing or degrading macromolecules, releasing or using energy. When cells are stimulated by growth factors to grow and divide (Saltiel & Kahn, 2001), they upregulate nutrient uptake and anabolic reactions to meet the needs for macromolecules and synthesize DNA. Proliferating cells have upregulated pentose phosphate which synthesizes nucleotide precursors (ribose) and NADPH, increase glutamine uptake and fuel the TCA cycle, increase amino acid and fatty acid synthesis (Vander Heiden, Cantley, & Thompson, 2009). On the other hand quiescent cells, engage mostly in catabolic reactions to minimize macromolecule synthesis and maximize energy production in the absence of growth factor signals. While differentiated cells use the energetically efficient reactions of oxidative phosphorylation under aerobic conditions, proliferating cells have activated uptake of glucose and glycolysis in the presence or absence of oxygen (Brand & Hermfisse, 1997).

Proliferating cancer cells are very similar to normal proliferating cells regarding metabolic requirements (DeBerardinis, Lum, Hatzivassiliou, & Thompson, 2008). However, cancer cells are cell sufficient for growth factors, undergo uncontrolled proliferation, and deregulate their metabolism to gain advantage for survival and growth. Even in the absence of growth factor signals, cancer cells are able to upregulate metabolism due to activation of oncogenes, inactivation of tumor suppressors or signals from the tumor microenvironment (Hsu & Sabatini, 2008). Oncogenic signaling directs cancer cells towards increased nutrient uptake

and anabolic pathways (**Figure 1**) while avoiding apoptosis (Plati, Bucur, & Khosravi-Far, 2008).

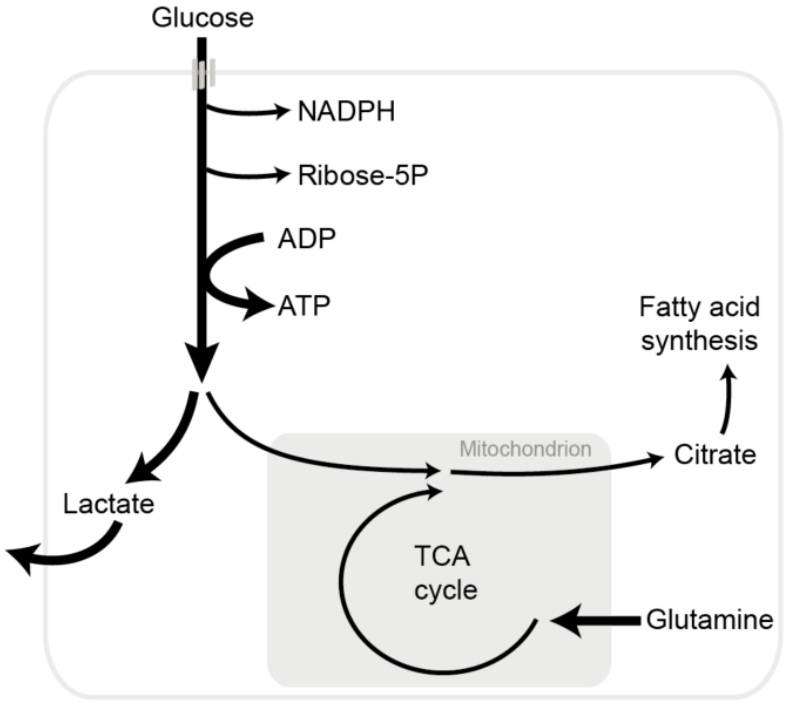

Figure 1. Upregulation of some metabolic pathways in proliferating cells.

Deregulation of metabolic pathways in cancer cells often creates metabolic dependencies that make cancer cells different from normal proliferating cells and opens a window for effective targeting (Bi, Wu, Zhang, & Mischel, 2018). Activation of oncogenic signaling and deactivation of tumor suppressors which are responsible for cancer transformation, are the main cause for deregulation of cancer metabolism (**Figure 2**). Upregulation of oncogenes like PI3k, Akt, Her2/neu, myc is found to increase activity of glycolysis and anabolic pathways, and affect expression of metabolic enzymes toward favorable pathways (Tanner et al., 2018). Overexpression of Glucose Transporter 1 (GLUT1) is an example of deregulated metabolism that increases glucose uptake as directed by oncogenes mentioned above (Benjamin, Cravatt, & Nomura, 2012; Cantor & Sabatini, 2012). Tumor environment pH and low oxygen can also cause deregulations, but some features like excessive lactate production happen even before that stage (Vander Heiden et al., 2009).

There are other scenarios of metabolic deregulation in cancer. One case is when mutated metabolic enzymes themselves cause cancer, acting as a tumor oncogene or suppressor. The TCA cycle enzymes, succinate dehydrogenase (SDH) and fumarate hydratase (FH), behave as tumor suppressors because they cause cancer when mutated (King et al., 2006). Other

cases include enzymes that have non-metabolic functions that provide advantages to cancer cells, like in the case of PKM2 which was found to translocate to nucleus and gain other roles that promote tumorigenesis (Ward & Thompson, 2012). Due to metabolic rewiring, cancer cells are known to develop dependencies on certain pathways which makes them auxotrophic for one or more amino acids, or activate other members of a metabolic enzyme family, which is different from normal cells (Cantor & Sabatini, 2012).

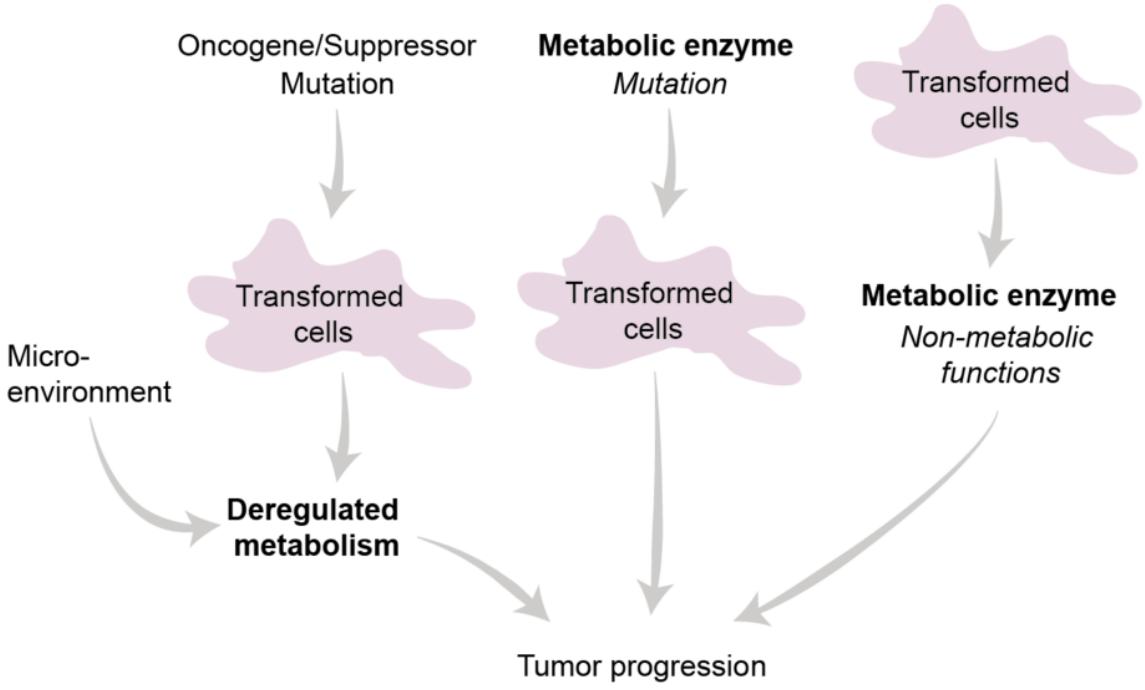

Figure 2. Deregulation of metabolism in cancer.

2.2 Cell cycle

The cell cycle is a series of events where a cell grows, duplicates DNA and other components, until it divides into two daughter cells. The first records in the literature for the terms 'cell cycle' and 'cell cycle phase' are from 1951 by Howard and Pelc where they report a study of DNA synthesis in bean cells. The phase before DNA synthesis was called Gap 1, and the phase after DNA synthesis until division was called Gap 2 (Thoday, 1954). No other events except growth were known to happen during these phases. These cell cycle phases are now called G_1 (Gap 1 or post-mitotic growth), S (Synthesis), G_2 (Gap 2 or pre-mitotic growth) and M (Mitosis).

Physical changes during cell cycle

To ensure that each daughter cell receives the same amount of material, the cell doubles all bulk properties and DNA until the end of division. Cell volume, dry mass and density (Mitchison, 2003), and even cell components like cytoplasmic organelles, plasma membrane, structural proteins and RNA (Morgan, 2007) continuously increase with cell cycle. Duplication of DNA is a major event that occurs once during the S phase of the cell cycle. Some proteins, including the cell cycle-regulating ones, have higher expression or activity, or change subcellular localization, in one cell cycle phase to ensure cell cycle progression.

While there is no consensus on how to normalize measurement of proteins, metabolites etc., well-known bulk properties, like cell volume and total protein content that increase with cell cycle can be used. Due to increase in volume, an entity has to also increase in amount in order not to be diluted. Using cell numbers for normalization might not address this physical change of cells.

Cell cycle regulation

All cell cycle-related processes have to work perfectly and without any mistake to give two cells with identical genetic material and equally distributed bulk material. Therefore, the cell cycle is a tightly regulated process. The main mechanisms that drive cell cycle progression are controlled by oscillating expression or activity of regulatory proteins which ensure that events occur in the correct order. Activity of regulatory proteins is tightly controlled in time and localization. Cyclins that are expressed periodically during the cell cycle bind and activate the cyclin dependent kinases (Cdk), which in turn activate a cascade of events (Vermeulen,K.,Van Bockstaele,D.R.,Berneman, Vermeulen, Van Bockstaele, & Berneman, 2003).

Besides, normal cells have quality control checkpoints in every phase to monitor the main processes. Checkpoints ensure that events happen in a sequential order of events, and check that certain processes are completed and are error free before initiation of other events. The first point of control is the restriction point, which occurs 3-4 hours after mitosis (Ekholm, Zickert, Reed, & Zetterberg, 2001). The restriction point checks for availability of growth signals. This is a decision point whether to enter G_0, the quiescent or resting state, until growth factors are available, or to commit to the cell cycle. Growth factors commonly initiate Ras signaling which activates a cascade of events, including Cyclin D which is a cell cycle regulating protein (Aktas, Cai, & Cooper, 1997). After the cell has decided to commit to the cell cycle, another checkpoint in late G_1 (G_1/S) checks the availability of amino acids and

energy status (Saqcena et al., 2013). This checkpoint is sensed by mTORC which is upstream of Akt and Cyclin E which is the second activated cyclin after Cyclin D. Once all checks prove successful, the cell proceeds through S phase and begins DNA synthesis. By the end of S phase, there is a checkpoint for DNA replication and DNA damage. DNA damage triggers another checkpoint active in G_2-M phase (Vermeulen,K.,Van Bockstaele,D.R.,Berneman et al., 2003), responsible for sensing the DNA damages and stopping the cell cycle until damage is repaired. In G_2 phase the cell has a last chance to check cell size, DNA replication and repair DNA damage (Chao et al., 2017). In mitosis, before cytokinesis, there is a last checkpoint which senses errors related to alignment of mitotic spindles with the chromosomes (Vermeulen,K.,Van Bockstaele,D.R.,Berneman et al., 2003). Possible misalignments lead to arrest in metaphase until repair.

Cell cycle alterations in cancer

When duplication of cell material, like DNA replication, is not done correctly and contains errors, but the cell fails to stop proliferation, these errors accumulate, and can result in further oncogenic mutations (Preston, Albertson, & Herr, 2010). Cancer cells often lose the tight control and regulatory mechanisms of cell cycle (Cantor & Sabatini, 2012), resulting in advantages like uncontrolled growth. Activation of oncogenes due to mutations provides continuous ligand-independent signal for activating pro-oncogenic processes and causing continuous uncontrolled proliferation. This happens when oncogenes like EGFR, Ras or Akt are mutated and inactivate the restriction point (Knauf et al., 2006). Some oncogenes cause overexpression of cyclins, Cdks and suppression of Cdk inhibitors in most cancer cells (Vermeulen,K.,Van Bockstaele,D.R.,Berneman et al., 2003). Having an inactivated G_1 restriction checkpoint (Diaz-Moralli, Tarrado-Castellarnau, Miranda, & Cascante, 2013), most cancer cells are forced to rely on the other checkpoints to control and repair DNA in case of mutations, incomplete replication, or other errors.

Inactive checkpoints fail to promote fidelity and completion. When tumor suppressors, the cell cycle brakes, are mutated, the cell does not stop to repair the errors but continues uncontrolled proliferation. Activity of tumor suppressor p53 is frequently lost in cancers, providing an advantage to cancer cells by making them insensitive to stress conditions like accumulation of mutations or nutrient and oxygen deficiency. In normal cells, p53 mediates arrest at the G_1 and G_2-M checkpoints, but cancer cells that have lost p53 arrest at the spindle checkpoint when stress is applied in S phase and arrest in mitosis (Benada & Macurek, 2015).

A good understanding of these checkpoints and deregulations is crucial for targeting. Combining checkpoint inactivation due to oncogenic mutations with targeting of components of other checkpoints makes cancer cells more vulnerable during the committed phase. Normal cells are more resistant to these treatments since they have functional checkpoints. Accumulation of mutations and targeting of molecules related to S-G_2 or spindle checkpoints results in defective cells in mitosis which eventually die (Gabrielli, Brooks, & Pavey, 2012).

2.3 Oscillating metabolism

Physical changes during cell growth suggest metabolic changes. In the literature, there is evidence of some metabolic events that oscillate across the cell cycle (**Table 1**). However, it still remains an open question to what extent metabolism is cell cycle-regulated in human cells. Most studies have been performed at the expression or protein level including several high throughput studies where they synchronize cells and perform microarray analysis (Bar-Joseph et al., 2008; Cho et al., 2001; Stumpf, Moreno, Olshen, Taylor, & Ruggero, 2013; Whitfield et al., 2002). Other studies have reported enzyme activity and measurement of metabolite levels. Synchronization with double thymidine block (DTB), serum starvation and/or nocodazole are the most commonly used approaches for cell cycle phase separation (Section 3.1).

DNA synthesis is the first cycling event known and the most studied. Enzymes used for nucleotide synthesis are also upregulated during S phase. Independent studies report that transcripts of Thymidine Kinase (TK) and Dihydrofolate reductase (DHFR) in fibroblasts (Denhardt, Edwards, & Parfett, 1986), and DHFR activity in Cho cells (Mariani, Slate, & Schimke, 1981) increase during S phase. A study in sorted lymphocytes reports that enzyme activity of TK, Thymidylate Synthase (TS) and DHFR reaches maximum in S-G_2-M (Pelka-Fleischer, Fleischer, Wilmanns, Sauer, & Schalhorn, 1989).

Description	Phase	Cell type	Measure	Pathway
TS	early S	3T6	mRNA	Nucleotide synth
TMPK	early S	3T6	mRNA	Nucleotide synth
DHFR	early S	3T6	mRNA	Nucleotide synth
PFKFB3	G_1-S	HeLa	Protein	Glycolysis
GLS1	G_1-S	HeLa	Protein	Glutaminolysis
ODC	S	Fibroblast	Protein	Polyamine synth
ODC-AZ	S-G_2-M	Fibroblast	Protein	Polyamine synth
AdoMetDC	S	Fibroblast	Protein	Polyamine synth
SSAT	G_1	Fibroblast	Protein	Polyamine degrad
ODC	S	Cho	Activity	Polyamine synth
Adomet-DC	M	Cho	Activity	Polyamine degrad
HMGR	G_1	HepG2	Activity	Cholesterol synth

Table 1. Oscillating metabolic enzymes reported in the literature.

Description	Phase	Cell type	Measure	Pathway
NAD+/NADH	S	HeLa	Metabolite	Redox status
Glutamine utilization	S-G_2	HeLa	Metabolite	Glutaminolysis
Lactate production	G_1	HeLa	Metabolite	Glycolysis
Putr/Spmd/Spm	G_1	Fibroblast	Metabolite	Polyamine synth
Putr/Spmd/Spm	late S	Cho	Metabolite	Polyamine synth
Isoprenyl incorporation	G_1-S	HepG2	Metabolite	Cholesterol synth
Cholesterol	G_1	HepG2	Metabolite	Cholesterol synth

Table 2. Oscillating metabolites reported in the literature.

Polyamines are a group of positively charged metabolites that have been long studied for their interaction with DNA, stabilization, and potential roles in DNA protection and replication. It is therefore not very surprising that polyamines and related enzymes are found to oscillate during the cell cycle. Putrescine, spermidine and spermine are the three types of polyamines, not containing the modified forms (acetyl- or carbamoyl- polyamines). All three contain ornithine minus a carbon dioxide in their backbone. Spermidine and spermine have three and six carbons respectively that derive from methionine via decarboxylated S-adenosylmethionine (dcAdoMet). Independent studies in fibroblasts and Cho cells found that polyamine levels are lowest in G1 and increase gradually until they reach twice the amount in S phase. Ornithine decarboxylase (ODC) and Adenosylmethionine decarboxylase (AdoMetDC) activities which are part of the polyamine biosynthetic pathway, also increase towards the S phase while being lowest in G_1 phase. (Bettuzzi et al., 1999; Fredlund, Johansson, Dahlberg, & Oredsson, 1995)

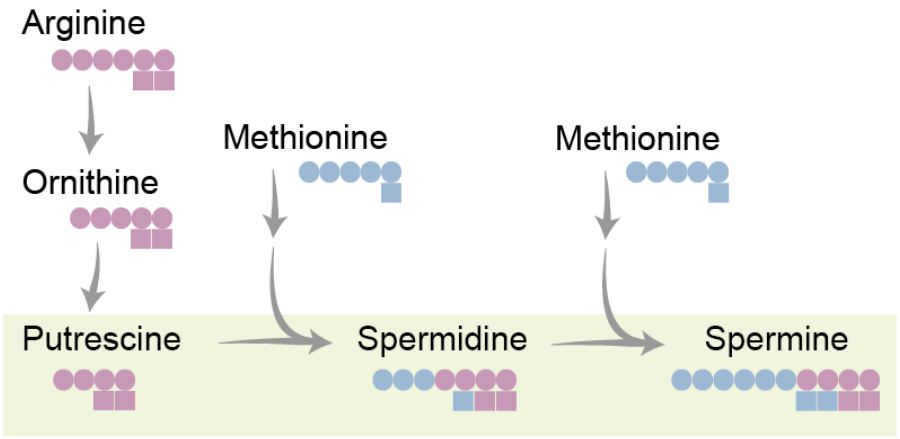

Figure 3. Polyamine synthesis from arginine and ornithine. **C**ircles represent carbon atoms and squares represent nitrogen atoms.

Also, glutamine utilization was found to be higher in the S-G_2-M phase of HeLa cells synchronized with Nocodazole and DTB, but the mRNA of glutaminase enzyme GLS1, which converts glutamine to glutamate, is highly expressed in G_1/S. Deprivation of glutamine or silencing of GLS1 caused cells to accumulate in G_1 in nocodazole treated cells and in S phase for cells treated with DTB. (Colombo et al., 2011)

As cells grow, so do cell membranes and metabolites that are required for membrane synthesis. Some studies report high levels of total fatty acids (Scaglia, Tyekucheva, Zadra, Photopoulos, & Loda, 2014) and cholesterol (Singh, Saxena, Srinivas, Pande, & Chattopadhyay, 2013) in S phase, while total phospholipid content increases throughout the cell cycle. The levels of lysophospholipids (metabolites in membrane phospholipid synthesis (Rivera & Chun, 2006)) increased steadily from G_1 to S-G_2-M (Scaglia et al., 2014). In another study, where HepG2 cells were synchronized by serum starvation and DTB, cholesterol synthesis increased in late G_1 to mid-S phase (Sepp-Lorenzino, Rao, & Coleman, 1991). HMG-Coa reductase (HMGR) activity which is the first enzyme in the mevalonate pathway that leads to cholesterol synthesis was highest in early-mid G_1 phase.

Depletion of metabolites causes cell cycle arrest

Metabolic dependencies might create vulnerabilities for cancer cells. Some findings from recent studies suggest that depriving cancer cells of certain metabolites can cause cell cycle arrest and cell death (Isono, Chano, Kitamura, & Yuasa, 2014; Patel et al., 2016; Scott, Lamb, Smith, & Wheatley, 2000). Other studies, including high throughput studies where cells were treated with siRNAs to silence different metabolic enzymes, report cell cycle arrest or

proliferation problems (Björklund et al., 2006; Grant et al., 2013; Kittler et al., 2007; Mukherji et al., 2006).

Some cancers develop amino acid dependencies, and when deprived of amino acids undergo cell cycle arrest and cell death. Asparagine depletion arrested Cho cells in G_0/G_1 phase (Fomina-Yadlin et al., 2014). Treatment with arginine deiminase (ADI), that deprives cells of arginine, was found to arrest cultured lymphatic leukemia cell line in G_1 and/or S phase bringing them to apoptosis later (Colombo et al., 2011; Gong, Zölzer, von Recklinghausen, Havers, & Schweigerer, 2000; Patil, Bhaumik, Babykutty, Banerjee, & Fukumura, 2016). Leucine deprivation was seen to induce arrest at G_1 phase of 3T3 and Cho cells (Wynford-Thomas, LaMontagne, Marin, & Prescott, 1985). Blocking FASN by enzyme inhibitors induces cell cycle arrest at S and/or G_2-M (Veigel et al., 2015).

Polyamine analogues that cannot be metabolized by cells were introduced to MCF7 and other breast cancer cell lines, and an accumulation of cells in G_1 was observed (Fredlund et al., 1995; Holst, Frydman, Marton, & Oredsson, 2006; Johansson, Oredsson, & Alm, 2008). Cho cells were depleted of polyamines by using an inhibitor that blocks ODC, which caused a delay in S phase. Addition of putrescine restored the phenotype, confirming that polyamines are required for cell cycle progression and are sensed by a checkpoint close to S phase (Harada & Morris, 1981). Other enzymes in the arginine catabolism pathway, upstream of the polyamine biosynthesis pathway, perturb growth and cell cycle regulation when inhibited.

3 METHODOLOGY

In this section, I describe available methods that are used to measure metabolites and separate cell cycle phases. Some of the methods described below were used in the research work presented in Thesis section.

3.1 Cell cycle separation

Obtaining pure fractions of cell cycle phases is crucial for generating reliable data when studying cell cycle related processes. Cell sorting based on fluorescent markers and synchronization with chemicals that block cell cycle progression are the two main approaches that have been used for separation of cells into cell cycle phases.

3.1.1 Fluorescence Activated Cell Sorting

FACS (Fluorescence Activated Cell Sorting) is by far the most versatile and widely used tool for separating cell populations. Individual cells are passed through a capillary and separated into tubes based on fluorescent markers detected by laser. A fluorescent molecule is either conjugated to antibodies binding to cycling proteins in the cell, or a gene coding a fluorescent protein is co-expressed with a cell cycle related protein in engineered cell lines (Howard M. Shapiro, 2005). Several examples of engineered cell lines are reported in the literature: PCNA (Kisielewska, Lu, & Whitaker, 2005), helicase B, DNA ligase (Easwaran, Leonhardt, & Cardoso, 2005) have been used as S phase markers; cyclin B1 as a G_2-M phase marker (Schnerch, Follo, Felthaus, Engelhardt, & Wäsch, 2013); and ubiquitin ligase based geminin (FUCCI) as a marker for S-G_2-M (Sakaue-Sawano et al., 2008) are some reported examples.

Cells can be separated based on their DNA content, since G_1 cells have one copy of DNA and S-G_2-M cells have two copies of DNA. Some small molecules bind or intercalate the DNA, providing a reading for DNA content. Hoechst-34580 is permeable to live cells, allowing for live cell staining and analysis, while other dyes like DAPI and PI are used in fixed cells.

3.1.2 Synchronization

Synchronization methods are based on cell cycle arrest due to introduction of chemicals that interfere with cell cycle processes of DNA replication, microtubule formation and cholesterol synthesis (Banfalvi, 2011a; Ma & Poon, 2011). This approach was used in early years of cell cycle research when more advanced separating tools were not developed, and it is still widely used in cell cycle studies. In the double thymidine block technique (DTB), applying two

treatment intervals with thymidine halts DNA replication due to an imbalance of nucleotides, and blocks cells in S phase (Engstrom & Kmiec, 2008). Nocodazole blocks microtubule formation which forces cells to stop in M phase, while lovastatin which blocks the mevalonate pathway and downstream cholesterol synthesis, and stops cells in G_1 phase (Keyomarsi, 1996). Serum deprivation is another approach used to study cells in G_1 phase, but it is thought to bring cells to G_0 (quiescent, non-proliferating state) due to lack of growth factors (Cooper, 1998). Physical separation of cells in different phases by centrifugal elutriation based on cell size (Banfalvi, 2011b; Diamond, 1991) and mitotic shake-off (Terasima & Tolmach, 1963) are other approaches for synchronizing cells. Synchronization is the method of choice, especially in high throughput studies, where it is practically difficult to obtain hundreds of sorted populations.

After synchronization, cells are released from the block and allowed to grow in normal medium for different periods of time and progress in cell cycle, to allow collection of cells in different phases. To assess the cell cycle progression after synchronization, methods like cell counting, incorporation of nucleoside analogues, DNA stains, cell cycle reporters, etc. are used (Henderson, Bortone, Lim, & Zambon, 2013).

3.1.3 Comparing methods

Synchronization does not require any special equipment, it is not complicated and large amount of cells can be obtained with synchronization. However, the procedure has to be optimized for each cell line and phase of synchronization, because some cells might not respond to standard synchronization procedures. Different cell lines require different concentrations and incubation times. While allowing dissection of cell cycle phases in shorter intervals due to release at different time points, cells start to lose synchrony after release. The block is not perfect, so that a high percentage of cells are synchronized in one phase while a small percentage of cells is in other phases. Moreover, treating cells with chemicals to block some processes in the cell can be “invasive”, in that their action is based on halting metabolic processes to force arrest in a particular phase. This can nonspecifically distort physiology and metabolism, sometimes making the downstream experiments more difficult to interpret.

Cell sorting, on the other hand, provides a cleaner separation of the cell cycle phases, does not disrupt metabolic reactions by drugs, and can be applied to any cell type. The disadvantage of sorting is the long sample processing time, need for specialized equipment and requirement for good fluorescent staining.

3.2 Metabolite measurements

Metabolite measurement are important when comparing different conditions like cell transformation and drug treatment. In early biochemistry studies, enzymatic assays were used to measure levels of metabolites. As technology advanced, methods like high-performance liquid chromatography (HPLC), later nuclear magnetic resonance (NMR) and mass spectrometry (MS) were used to separate and measure metabolites in samples. The popularity of these methods has changed with time, and nowadays various MS methods are used more widely, due to development of methods and technology (**Figure 4**). Today these methods continue to be used and sometimes in combination in order to increase coverage of measured metabolites. The wide range of metabolites and the variety of their chemical structure is a challenge for using one method. Often, methods that measure a specific class of metabolites with certain properties (e.g. polar metabolites) are developed.

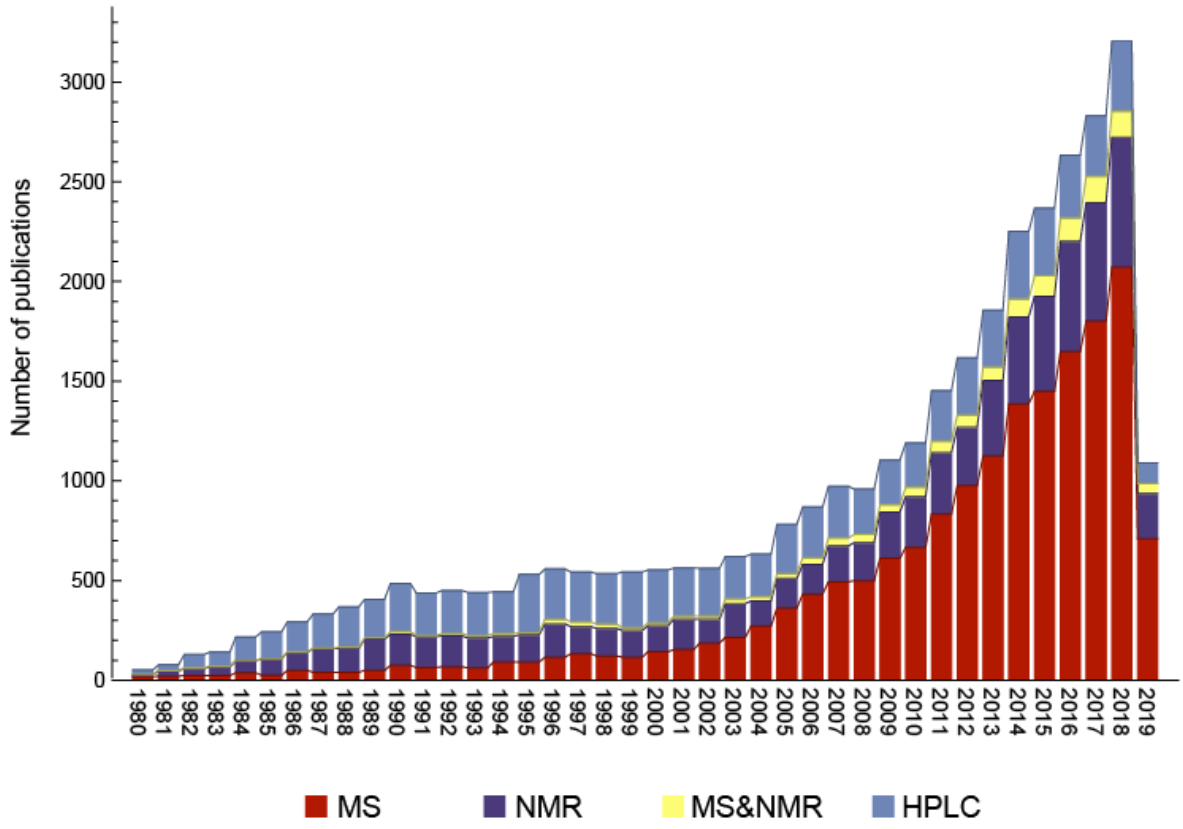

Figure 4. Publications that include one of the terms (MS, NMR, MS&NMR, HPLC) in title/abstract. Numbers are reported per year. Data are obtained from Pubmed.

3.2.1 Mass Spectrometry (MS)

Mass spectrometry (MS) and NMR are the main techniques in metabolomics, the measurement of metabolites. MS is advantageous because of the higher resolution and sensitivity, which makes it suitable to analyze low abundance molecules or small samples. MS is often coupled to GC (gas chromatography) or LC (liquid chromatography). LC is based on liquid phase separation of compounds and allows separation of most metabolites, whether they are polar, non-polar, acidic or basic; while GC works best for separation of non-volatile or highly polar compounds (Ramautar & de Jong, 2014)·(Milne, Mathews, Myers, Ivanova, & Brown, 2013). Using either of these methods, metabolites can be analyzed for their relative abundance. Quantification of metabolites in unlabeled samples is done by calculating the area under the LC peak (**Figure 5**). Peaks areas can be considered as the relative abundance of the metabolite, although it might be difficult to compare between very different types of samples (**Figure 5**).

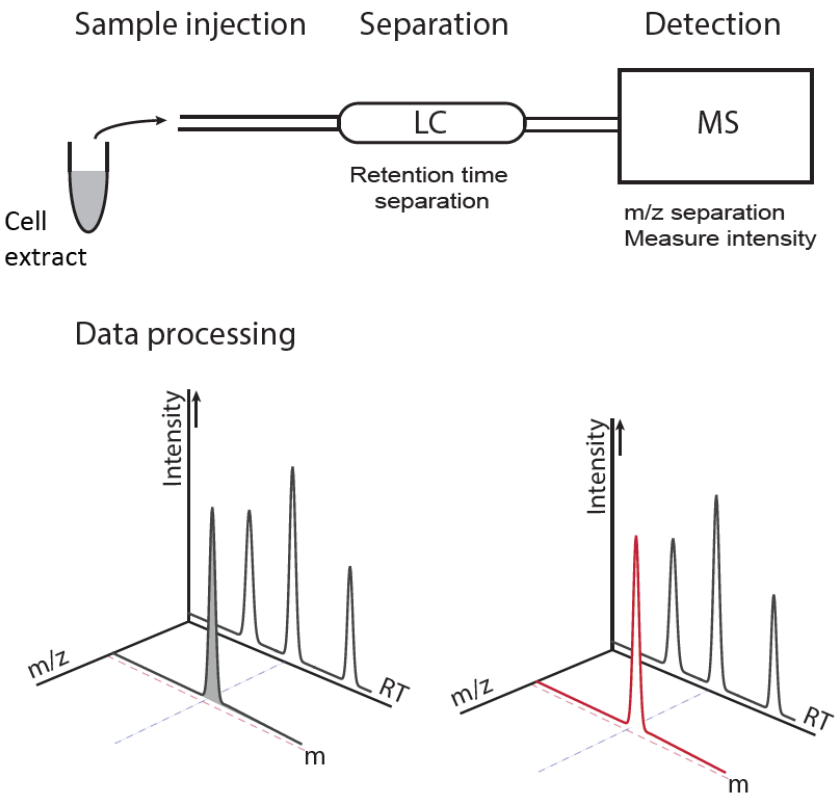

Figure 5. LCMS sample analysis and data processing.

3.2.2 Nuclear Magnetic Resonance (NMR)

NMR is based on the principle that the nucleus of some atoms can behave like a magnet, due to having a charge and spin. These nuclei, like ^{1}H, ^{13}C, ^{19}F, ^{31}P, ^{2}H, that have a charge and

spin are called “NMR active”, while others, like ^{12}C and ^{16}O are “NMR inactive” meaning that they do not produce NMR signal (Brevard & Granger, 1982). Based on these properties, in NMR, a magnetic field is applied to nuclei and the amount of energy to put nuclei in resonance in measured. The produced NMR spectrum is composed of signals reflecting the required energy to bring the NMR active nuclei into resonance. It is possible to separate molecules from each other based on the fact that different nuclei require different amount of energy depending on position in a molecule (surrounding atoms/shielding effect – affected by electronegativity of surrounding atoms). (Komoroski, Pappas, & Hough, 1991; Mlynárik, 2017)

NMR can be used to analyze both liquid and solid samples, including tissues, sample can be reused and stored for further analysis. Obtained data provides information about chemical structure, because produced signals reflect the chemical bonds between atoms (Emwas, 2015). NMR is fast and produces very reproducible data, but also requires high amount of sample and specialized staff. The instrument takes a lot of space and is expensive to operate.

3.2.3 Other methods

Other methods used for metabolite measurement and quantification are High Performance Liquid Chromatography HPLC and enzymatic assays.

HPLC is an advanced analytical technique compared to liquid chromatography developed in the mid-1960s. The separated sample is in liquid form, and it is quite fast. With HPLC it is possible to separate chemicals with high selectivity by optimizing the dissolving solution, and is widely used in pharmaceutical research to separate chemical products. (Snyder, 2000)

Enzymatic assays for metabolite measurements are nowadays mostly used as commercially available kits. These assays are based on a chain of conversion reactions. An enzyme converts the substrate (metabolite) into a product, which is coupled to oxidation or reduction of pyridine nucleotides (Newsholme & Taylor, 1968), or to a chain of reactions that involves conversion of a marker molecule. In the case of pyridine nucleotides, the light absorption changes due to oxidation or reduction and the difference in light absorption is measured. A similar principle is valid for marker molecules. In enzymatic kits that are widely used nowadays, Amplex red is converted resofurin, in a reaction that is mediated by HRP. This reaction is coupled to conversion of H_2O_2 to water. H_2O_2 is usually the byproduct in a reaction connected to the metabolite of interest (Summers, Zhao, Ganini, & Mason, 2013). Enzymatic assays can be highly specific, but the marker molecules are usually very sensitive to light and

produce high background signal. The kits are quite expensive, and measure only one metabolite at a time, but can be useful for quick measurements. When ATP/ADP, ATP/AMP (measures of energy status) and NADPH/NADP+ measurements were used to assess the methods above, MS measurements were found more accurate (Lu et al., 2017).

3.3 Assessing enzyme activity - isotope tracing

To characterize and compare cancer and normal cells, it is important to assess enzyme activities. Enzyme activity can be measured directly with enzymatic assays, which are a precise measurement of activity. However, these enzymatic assays measure activities in lysates and not in live cells. Another disadvantage is that it is very difficult to perform enzymatic assays for hundreds of metabolic enzymes in a high throughput study. Metabolite abundance, which are calculated by integrating peak areas, might be used to infer enzymatic activity, but it is difficult to give conclusions because one metabolite can be synthesized from different enzymes. Besides, metabolites abundance is a result of both synthesis and consumption of a metabolite. A better approach, which we adopted in this thesis, is the isotope tracing approach, where we infer enzyme activities from label incorporation into metabolites in living cells.

In isotope tracing, cells are cultured in labeled nutrients which are used for synthesis of other intracellular metabolites that acquire label. Isotope tracing can be performed with stable isotopes or radioisotopes. Radioisotopes were widely used in the past, but are difficult to handle. Heavy isotopes, on the other hand, are more practical because they are not toxic and can be used for tracing in animals and even patients.

Depending on the question, isotope tracing can be performed for a long time (steady state) or short time (pulse labeling). Choosing one approach or the other affects data interpretation. Pulse labeling experiments are performed to study timed processes like cell cycle phases or fast reactions. In pulse labeling experiments, isotope labeling can be analyzed by comparing peak areas of the relevant heavy isotopomer. In the case of steady state labeling, it is possible to calculate mass isotope distribution (MIDs), which sum up to one. In both cases it is possible to calculate the enrichment of the heavy isotope atoms for each metabolite, by using the formula: $\sum_{x=0}^{n} x * MIx/n$ where, n is the total number of carbons in the metabolite and MIx is the MI fraction of x. A mass isotopomer (MI) is a metabolite with higher mass due to incorporation of heavy isotope (Hellerstein & Neese, 1999). The MI fraction is defined as a fraction of abundance (concentration, pool size), calculated by dividing peak area by the total peak areas for all MIs of a metabolite. The set of all MI fractions is often termed mass

isotopomer distribution (MID) (**Figure 6**). Both peak areas and MIDs provide valuable and different information.

The isotope distribution of any given metabolite reflects the cell's metabolic activity before extraction of metabolites. Also, the MID is calculated by rescaling all MIs 0-1, so each MI fraction is considered as a ratio. This makes the data more robust (less affected by experimental condition), and gives information on the metabolic pathways (Bennett, Yuan, Kimball, & Rabinowitz, 2008). The challenge here is that it is an indirect measurement of the enzyme activity. Sometimes one metabolite is synthesized by more than one enzyme. This can be difficult to evaluate.

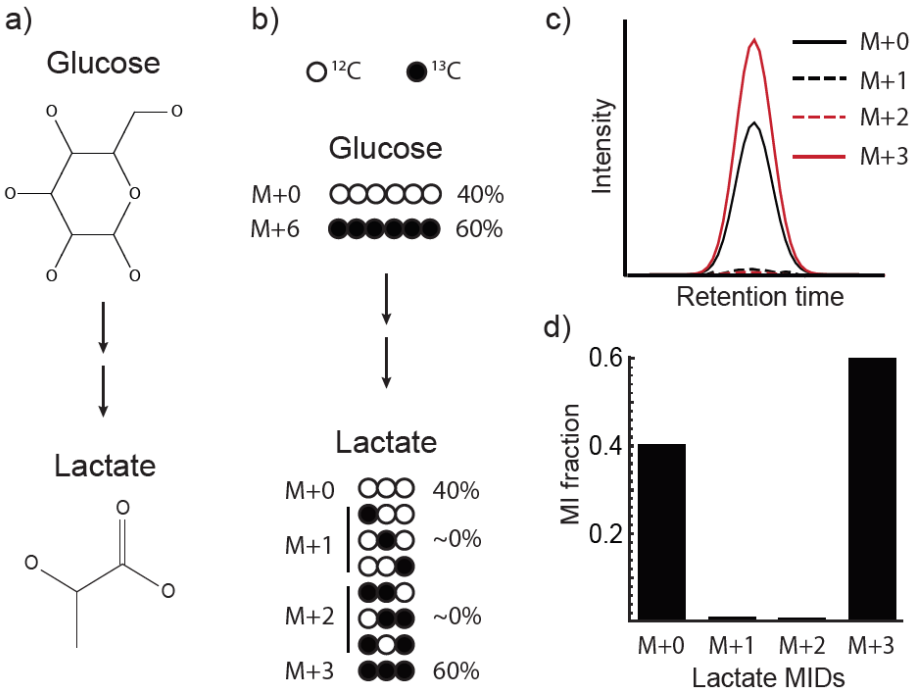

Figure 6. Illustration of peak areas and mass isotopomers. a) Chemical structures of glucose and lactate. b) Mass Isotopomer (MI) distribution of lactate when cells are cultured in medium containing 60% U-13C-glucose. Each circle represents one carbon in the molecule. M+1 is the mass isotopomer containing 1 13C in the structure. c) Peak areas of lactate MI when cultured in 60% U-^{13}C-glucose d) Lactate MIDs.

THESIS

In this thesis, I presented a study of metabolism in G_1 and S-G_2-M cell cycle phases to identify metabolic enzymes that are important for cancer cell proliferation. There is evidence in the literature that some metabolic events are cycling, and in this thesis I aim to answer related questions which are not addressed in the literature. What does the big picture of metabolism across cell cycle look like? What fraction of metabolites is oscillating? While many proteins (~15%) are oscillating at the mRNA level, is this valid for the enzyme activities as well? Are certain metabolites required for cell cycle progression? Our hypothesis is that some metabolites might be important for cell cycle progression and we want to identify those. To test this hypothesis, I investigated metabolites that are more abundant in the S-G_2-M phases and affect cell cycle progression (**Figure 7**a). I did not follow housekeeping metabolites required and synthesized throughout cell cycle (**Figure 7**b). The work presented here includes method development, metabolomics, data analysis and follow-up experiments that resulted in 4 papers, which are discussed in the following sections.

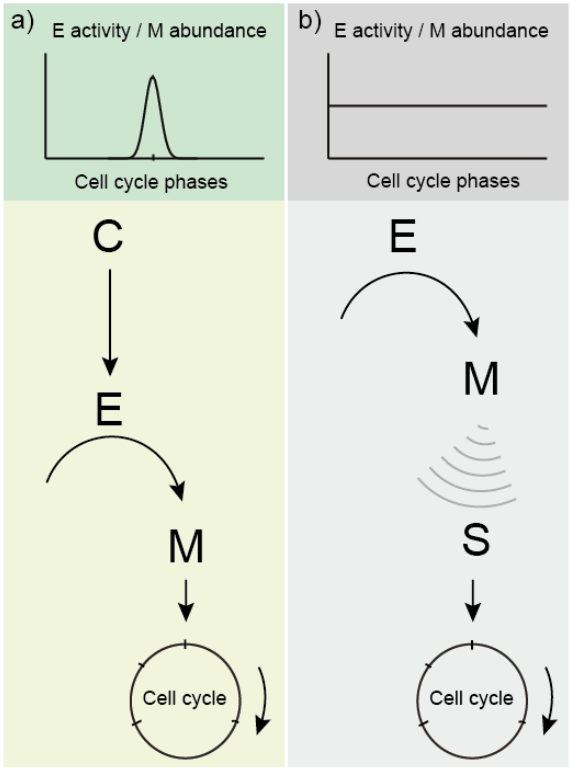

Figure 7. Models illustrating situation where metabolites affect cell cycle progression. a) A cell cycle regulator protein (C) activates E at one of the cell cycle phases, and higher M abundance in that phase is required for cell cycle progression. b) Enzyme (E) activity and metabolite (M) levels are constant across cell cycle, but presence of metabolite is sensed at a particular cell cycle checkpoint and is important for cell cycle progression.

1 AIMS

The overarching aim in this thesis is to identify metabolic enzymes that are important for proliferation of cancer cells, and make cancer cells vulnerable to targeting. To achieve this, we separated the project into sub-studies and set aims for each.

Aim 1. Develop a method for studying metabolomics of subpopulations

- Find and optimize a method for separating cells into subpopulations.
- Develop a metabolite extraction method that will minimize metabolic disturbances.
- Try different approaches for metabolic measurements, including relative metabolite abundance and isotope tracing.

Aim 2. Perform metabolomics of cells in G_1 and S-G_2-M cell cycle phases

- Optimize a cell cycle marker to separate live cells into G_1 and S-G_2-M cell cycle phases and minimize disturbances to cell metabolism.
- Optimize pulse labeling experiments with isotope tracing to study metabolic activities in G_1 and S-G_2-M phases.
- Perform metabolic experiments in cell cycle sorted normal and cancer cell lines.

Aim 3. Integrate available cell cycle data with metabolomics data generated in this project to identify interesting enzymes that show high activity and expression in the S-G2-M phases.

- Make use of available datasets on protein expression and proteomics in different cell cycle phases, as well RNAi screenings.

Aim 4. Perform follow-up experiments to verify the role of identified enzymes for cell proliferation, and compare enzyme activity in normal and cancer cells.

2 RESEARCH DESIGN

In order to address the aims in this thesis, I implemented a strategy where I combined Method development (aim 1), hypothesis-generating (aim 2, 3 and 4) and hypothesis testing (aim 5) approaches, as illustrated in **Figure 8**. A method was developed to study metabolic activities in sorted subpopulations. Application of this method to study metabolomics of G_1 and S-G_2-M phase cells generated hundreds of metabolites captured with an LC-MS method, of which a fraction with higher abundance in one phase. Mapping these results from metabolomics against other datasets on cycling expression and hits from previously published RNAi screening, generated a shortlist of "hit" enzymes with potential effect in cell proliferation. I then and performed follow-up experiments to validate these hit enzymes.

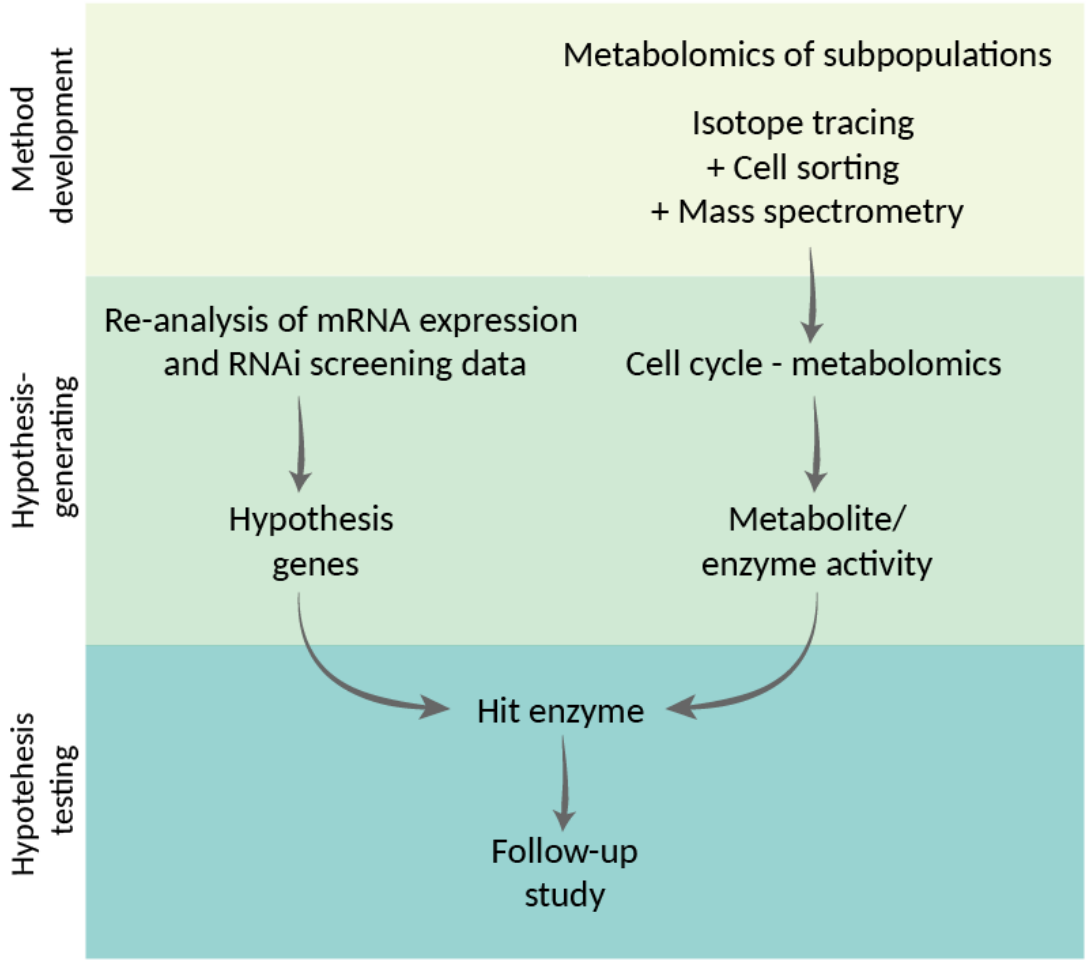

Figure 8. Research design including method development, hypothesis generating and testing.

3 RESULTS AND DISCUSSION

In this section I will summarize the findings from Papers I-IV, and discuss results and challenges.

3.1 Method development

Many biological samples, including cells in different phases of the cell cycle, are a mix of subpopulations. We developed a method that, in principle, enables metabolomics studies in any subpopulation of cells for which there are available markers. As described in Paper I, we chose the approach of combining fluorescence activated cell sorting (FACS) and liquid chromatography - mass spectrometry (LC-MS).

Measurements of cell metabolite content are prone to artifacts due to experimental procedures following cell detachment from culture dish. Therefore, we sought a solution that would allow to obtain subpopulations using FACS sorting while minimizing the metabolic distortion. We proposed a direct extraction method to shorten the sample processing time from sorting to metabolite extraction, by sorting cells into tubes containing the extracting solution, 100% methanol. When these samples were analyzed on LC-MS, metabolic signal seemed to be distorted and suppressed. When cells are sorted, they are surrounded by sorting solution which has a high salt content, similar to Hank's Balanced Salt Solution (HBSS). When cells are processed for MS analysis, they are evaporated and concentrated under vacuum using a Speed-Vac. This essential step, increases the concentration of salts several folds. Although the direct extraction provided an immediate extraction of metabolites, salt concentration was a limitation, and this method was abandoned.

We instead chose a method where we sorted cells into tubes, removed sorting solution by centrifuging and extracted metabolites from pellets. To minimize any metabolite leakage from cells due to osmosis and avoid cell lysis upon collection, cells were sorted into tubes containing HBSS and 5% dialyzed Fetal Bovine Serum (FBS). Cell sorting rate (1000events/sec) and nozzle size (100µm) were also optimized to minimize metabolic disruption during sorting.

To assess the method and for future reference (as used in Paper II, III and IV), we aimed to measure both metabolite abundance and metabolic activities and compare their integrity. We cultured cells both in unlabeled medium (all nutrients ^{12}C), and in labeled medium containing labeled U-^{13}C-glucose and U-^{13}C,^{15}N-glutamine. Glucose and glutamine feed into a large part of cellular metabolism (Palm & Thompson, 2017), giving the opportunity to evaluate several

metabolites. We extracted cells from FACS-sorted fractions, directly from culture dish, and from a pellet of cells, which is an intermediate step between the first two. We found that peak areas reflecting the relative metabolite abundance were quite different. One possible cause is metabolite leakage during sorting. However, nucleotide peaks had a higher signal in sorted samples, suggesting that the differences we observe could be due to the difference in chemical matrix. It also has to be considered that comparisons in future studies will be between sorted fractions of the same samples which should be affected in the same way by sorting procedure.

On the other hand, data obtained from isotope tracing should be more robust and less affected by sorting procedures. Isotopic labeling is a readout of the metabolic activities when cells are in culture, and should reflect what happened in culture before sorting. Label incorporation to metabolite was evaluated by calculating mass isotopomer distribution (MID), which means fraction of MI abundances (See Section 3.32.3). Since there should not be biased selection of MIs, even in the case of metabolite leakage, MIDs should be less affected by sorting. However, we found that some glycolysis metabolites like lactate, had different MIDs in sorted cells as compared to cells directly extracted from the dish. One reason could be the presence of FBS in sorting solution. Glycolysis is a fast reaction that happens within minutes, and MID differences could be due to unlabeled glucose in FBS. Due to these difficulties, control experiments are recommended to be performed when starting experiments with a new cell type. Such comparisons between extractions in culture dish and sorted cells were performed for studies reported in Paper I and II.

3.2 Mapping metabolism across the cell cycle

We used the method developed in Paper I to study metabolism in different cell cycle phases as presented in Paper II and III, which allowed mapping of metabolite abundances and metabolic activities in G_1 and S-G_2-M phases. To identify metabolic enzymes active in cell cycle phases and that may have a role in cell proliferation, we combined data from our metabolomics study with cell cycle mRNA expression data and cell cycle siRNA screening from published datasets, as illustrated in **Figure 9**.

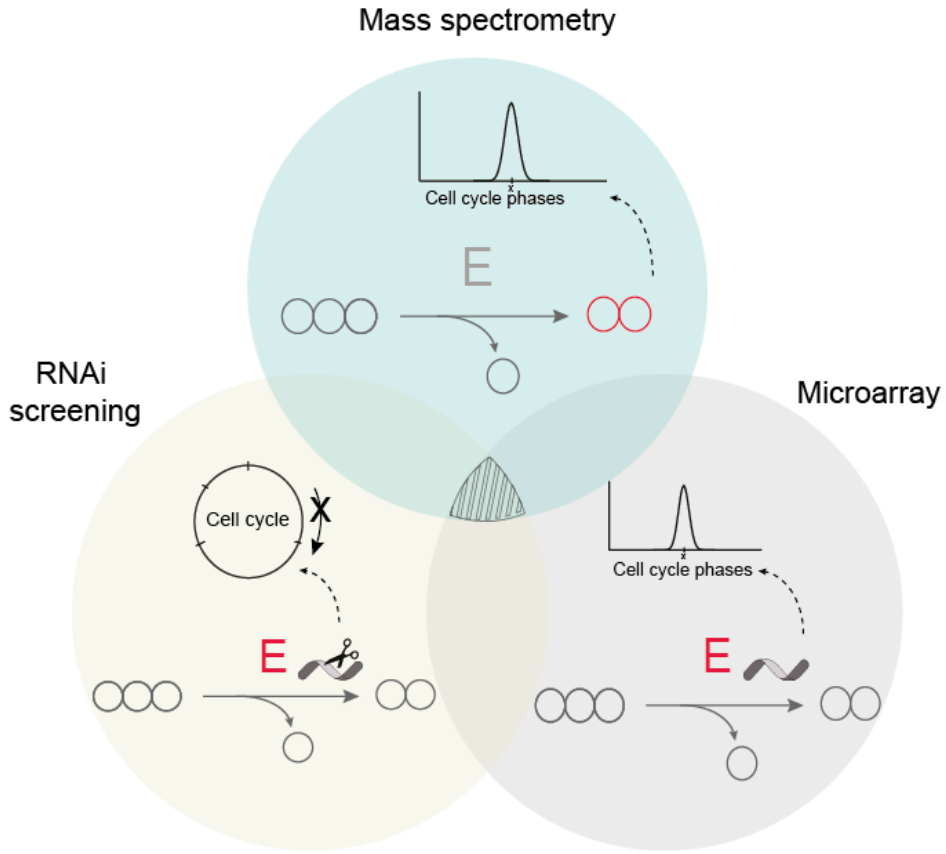

Figure 9. Integration of data from mass spectrometry, RNAi screening and microarray to identify cycling metabolic events from different levels.

3.2.1 Charting metabolism in G_1 and S-G_2-M phases

The key to sorting cell subpopulations is to have a specific marker. Fortunately, there are many cell cycle markers developed, including DNA staining and cyclin probes. Initially we sorted cells based on an internal fluorescent (Fucci) probe, Geminin, which fluoresces in S-G_2-M phases (Paper I). Cell lines with internal probes are quite easy to handle, but the experiment is limited to engineered cell lines, which require additional efforts to produce. We sought alternative methods for separating cells into cell cycle phases by staining DNA in

live cells. DNA is an established marker for cell cycle that makes possible separation into G_1 and S-G_2-M phases, which we are interested in. At the time, a new commercial dye, sir-DNA (Lukinavičius et al., 2015), promised live DNA staining without causing toxicity to cells when cultured for 24 hours. However, in our hands, micromolar concentration of sir-DNA and verapamil, which blocks Ca^{++} channels to avoid dye efflux, did not produce good intensity for sorting experiment. Next we explored live cell staining with Hoechst-34580, which can be detected with a 405nm laser which is commonly found in sorting devices (Darzynkiewicz, Juan, & Srour, 2004; H M Shapiro & Perlmutter, 2001). Different Hoechst staining concentrations and staining protocols were first tested in cell pellets, then optimized for staining of cells in the culture dish, minimizing the processing time before sorting and possible metabolite leakage that might happen when cells are detached in solution.

For these experiments we selected the tumor-derived cell lines HeLa (ovarian cancer) and MDA MB 231 (breast cancer); a telomerase-immortalized human mammary epithelial cell line (HMEC-Tert) and a derived cell line that was transformed with the SV40 early region proteins followed by a mutant Ras protein (HMEC-RAS, tumorigenic in vivo) (Elenbaas et al., 2001). Cells were cultured for 48 hours, stained with Hoechst and sorted into G_1 and S-G_2-M, extracted, and then analyzed on LC-MS. All cell lines were cultured both in unlabeled medium and in medium containing ^{13}C labeled amino acids and glucose ("fully" labeled medium). We observed a larger number of peaks and higher peak intensity in the HeLa cells dataset. Therefore this dataset was used for peak annotation and initial data analysis. Data obtained from other cells was used to compare interesting finding from the HeLa cells datasets.

Using an in-house developed peak detection software, we obtained 3426 peaks after removing unspecific peaks from blanks, and annotated 921 peaks by mapping the m/z in our list against the HMDB database. Metabolite abundances were calculated by integrating area of metabolite peaks as reported in Paper II. Considering the increase in cell volume across the cell cycle, a reasonable normalization factor is required to evaluate metabolite abundance in G_1 and S-G_2-M phases. One option is to normalize peak areas to cell size, measured indirectly by forward scatter in FACS instrument. The calculated ratio S-G_2-M / G_1 was 1.16. Thinking that a cell doubles in size over the cell cycle, a ratio of 1.16 might sound too low. However, here we take the average of cells in S-G_2-M and G_1, respectively. In fact the theoretical biomass ratio between the G_1 and S-G_2-M fractions was calculated as 1.39. On the other hand, the forward scatter calculations might have not been exactly the same as the sorted cells, because FACS instrument allows export of raw data without gating details

applied for during sorting. For this reason, we argued that the sum of peak areas for the 921 metabolites (T_{921}) cound be a good measure. It was reassuring to find that T_{921} was strongly correlated with the number of cells (a measure for biomass) analyzed in MS. The calculated T_{921} between S-G_2-M and G_1 was 1.19.

Using this normalization factor, we normalized all peak areas and found that 82% of metabolites did not change between phases. The rest was at least two fold more abundant in one of the phases. Most metabolites, including most proteinogenic amino acids and phosphatidylcholines that take part in macromolecular and membrane synthesis (Hosios et al., 2016), follow a similar trend as cell size, volume and protein content, showing a continuous need for metabolites as cells proceed to division. The fraction of cycling metabolites was 15%, and corresponded to 15-18% of mRNA transcripts that are known to be cyclic in HeLa cells (Grant et al., 2013).

To measure metabolic activities in G_1 and S-G_2-M phase cells, we applied pulse labeling with fully labeled medium as described in Paper III. Pulse labeling is suitable for cell cycle studies, because cell cycle phases occur during short windows of time, ~7 hours. In order to avoid labeling of metabolites in one phase and sorting in another, we selected an isotope tracing window of 3 hours.

DNA synthesis was used as a positive control in our experiments. Both abundance of NTPs and activity of related enzymes was higher in S-G_2-M phases. Phosphorylated pentose sugars were more labeled and in higher abundance in G_1 phase. Other cycling metabolites are discussed in Paper II and III. These experiments represent a large-scale investigation of metabolite abundances and metabolic activities in G_1 and S-G_2-M phases, and curated a large dataset of 3426 peaks, one third of which is currently annotated. Therefore, there is great opportunity for discovering other metabolites that are hitherto not known to be cycling.

3.2.2 Data integration

We selected and retrieved several datasets of mRNA gene expression data across the cell cycle, and RNAi screens for cell cycle phenotypes. We extracted data for metabolic enzymes and prepared a shortlist of metabolic enzymes that are cycling and/or cause a cell cycle phenotype when knocked down. This list was mapped against our metabolomics dataset, to identify those pathways that were more active in one cell cycle phase in at least two types of measurement. Arginase 2 (ARG2), that mediates ornithine synthesis from arginine, and

Choline dehydrogenase (CHDH), that catabolizes choline to synthesize betaine aldehyde, were in the shortlist, and are further describe below.

3.2.3 Arginase 2 is important for proliferation in S-G_2-M phases

While most amino acids acquired similar ^{13}C labeling in both sorted fractions, arginine and lysine were more labeled in S-G_2-M phases. Similarly, ornithine, but not other downstream metabolites of arginine were more labeled in S-G_2-M phases. Ornithine is synthesized from arginine through the mitochondrial arginase (ARG2), and is the precursor of polyamines that are required for DNA maintenance and cell proliferation. Interestingly, ornithine acquired more label in S-G_2-M phases of HeLa and HMEC Ras, but did not acquire any label in HMEC Tert immortalized cells during 3 hours pulse labeling with fully labeled medium. We proposed that HMEC Tert might be using another, slower reaction for ornithine synthesis. Ornithine transferase (OAT) is known to mediate an alternative reaction of ornithine synthesis from glutamine. To assess the sources of ornithine, we performed U-^{13}C-arginine and U-^{13}C-glutamine steady-state tracing in cancer and normal cells. Interestingly, cancer cells synthesized ornithine only from arginine, but normal cells used both arginine and glutamine as sources for ornithine. These results strengthened the hypothesis that ARG2 could have a role in cancer cell proliferation. To verify this, we silenced ARG2 with 7 independent hairpins from 2 different vendors, and 4 of them that showed good downregulation, reduced cancer cell proliferation by half. It is certainly important to investigate how normal cells respond to such treatments. We tried transient knockdown of HMEC Tert cells with special reagents for this cell type, but the knockdown was not successful. We also tried 3 different arginase inhibitors, which were used at high micro molar range. However, a recent study found that such inhibitors decrease cell proliferation independently of arginase enzyme (Ng et al., 2018). Indeed we found a decrease in cell number in both normal and cancer cells.

To assess the relevance of these findings to human tumors, we investigated ARG2 expression in patient samples. We searched for most available tumor normal datasets in GEO (Edgar, 2002) and Oncomine (Rhodes et al., 2004), but no overall significant difference was found between normal tissues and cancers. However, when we looked closer tumor subtypes, we found a correlation of ARG2 overexpression with estrogen receptor (ER) negative and basal (based on PAM50 classification) breast cancers. In addition ARG2 overexpression also correlated with bad survival in ER negative and basal tumor subtypes. Thus, ARG2 may be relevant in these particular tumor types.

3.3 Survey of choline metabolism

CHDH, the enzyme that converts choline to betaine aldehyde, was one of the "hit" enzymes in the data integration short list (See section 3.2.2 Data integration). Motivated by the fact that a survey of choline products in proliferating normal and cancer cells was missing, we performed a detailed characterization of choline in several cell lines and reported the data in Paper IV. We cultured HMEC Tert, HMEC SV40 (transformed with the SV40 early region proteins, (Elenbaas et al., 2001)), HMEC Ras, MCF7 (breast carcinoma), HeLa and HCT116 (colon carcinoma) cells in medium that contained 50% ^{13}C3-choline, labeled in 3 methyl groups.

Interestingly, betaine was labeled in cells with cancer origin but not in normal cells, suggesting absence of CHDH activity in HMEC cells, which was confirmed by western blot analysis of CHDH expression. Knockdown of CHDH resulted in good downregulation of the enzyme and also decreased betaine labeling in MS data. CHDH knockdown did not affect cell proliferation in our culture conditions (in normoxia). However, 2 out of 4 siRNAs caused an increase in G_2-M phases when CHDH was knocked down.

Betaine was likely not used for methylation or synthesis of other products, since sarcosine, methionine and S-Adenosyl methionine (SAM) were not labeled from ^{13}C3-choline. The question is: what is the role of betaine in cancer cells? Some previous studies suggest that betaine can act as an osmolyte. To test this, we measured betaine concentration in cell extracts and spent medium. Betaine concentration was in the low micro molar range both in cell extracts and spent media of cancer cells, indicating that betaine cannot be an osmolyte in these cells. It is possible that betaine synthesis was required for proliferation of tumor cells in vivo, but has lost this function since we are growing cells in dish. Follow-up experiments in tumor tissues will help to address this.

Finally, choline tracing produced thousands of peaks detected by LC-MS. From untargeted analysis we obtained 4908 peaks in positive and negative ion modes after subtracting unspecific peaks in blanks. 122 peaks were labeled from choline, of which 96 peaks were labeled in 3 carbons, suggesting that the head group of choline is donated. Metabolites labeled in 3 carbons include phosphocholine, CDP-choline and glycerol-phosphocholine from phospholipid metabolism, showing active phospholipid metabolism in all selected cells. 26 peaks were labeled either in 1 or 2 carbons. Since these cells do not use choline for methylation, the 1 or 2 carbon labeled peaks are likely catabolites of choline. While most of the 26 peaks are unknown, we mapped m/z with HMDB database (Wishart et al., 2013) and

found two peaks corresponding to m/z of methylethanolamine phosphate (P-MME) and dimethylethanolamine phosphate (P-DME) labeled in one and two carbons, respectively. These two metabolites are known as part of PC synthesis from PE methylation from SAM, or as part of Phosphatidyl methylethanolamine (Ptd-MME) and Phosphatidyl methylethanolamine (Ptd-DME) synthesis. However these reactions are not described in human cells before, and may be interesting for follow up studies.

4 CONCLUSIONS

4.1 Summary

In this thesis I presented a comprehensive study of metabolism in normal and cancer cells sorted into G_1 and S-G_2-M phases of the cell cycle. This was achieved through a method we developed by combining cell sorting, isotope tracing and high resolution liquid chromatography mass spectrometry (HRLC-MS), which permitted measuring and identifying hundreds of metabolite peaks. Isotope tracing was used to measure metabolic activities in sorted cell cycle phases. Culturing of cells in medium with ^{13}C nutrients in a pulse labeling experiment ensured good coverage of most metabolites synthesized internally by the cells. Most metabolic activities and metabolite abundances did not oscillate, suggesting the need for metabolites to support cell growth and building of biomass throughout the cell cycle. A few hundred metabolites acquired more label and/or had higher abundance in G_1 or S-G_2-M phases. To select hit enzyme candidates for follow up validation experiments, we integrated metabolomics data from this study, cyclic gene expression and siRNA screening. We chose to follow up on arginase 2 (ARG2) and choline dehydrogenase (CHDH) enzymes. A large part of cyclic peaks that were not annotated or were annotated but not known before to be cyclic, remain to be explored in further studies.

Ornithine was one of the metabolites that acquired more label in S-G_2-M phases of cancer cells, but was not labeled in normal cells within 3 hours pulse labeling experiment. Other steady state tracing experiments with single labeled amino acids verified that arginine, via ARG2, was responsible for higher ornithine labeling in cancer cells in pulse labeling experiment. Cancer cells used only arginine as a source for ornithine synthesis, while non-cancer cells used both arginine and glutamine via ornithine aminotransferase (OAT). ARG2 was later found to reduce the cell number by half when ARG2 was silenced for a short period of around 24 hours. The OAT enzyme, on the other hand, did not compensate for ornithine synthesis during ARG2 knockdown. In a large-scale survey of patient tissue samples, we found that ARG2 overexpression correlated with ER negative and basal breast cancer subtypes, and with poor survival in these cancers.

CHDH knockdown caused cell cycle arrest in a previous phenotypic screening, but not much was known about CHDH and choline metabolism in cancer cells. Therefore we designed a detailed experiment where we cultured normal and cancer cells in ^{13}C choline. We found that CHDH expression and activity, as shown by betaine labeling from choline, was present in cancer cells but not in the non-cancer HMEC cell lines. However, CHDH silencing did not affect cell proliferation despite the fact that there was a decrease in betaine labeling, but some

hairpins caused cell cycle arrest. The role of betaine in the studied cancer cells is still in question, but one possibility is that betaine is advantageous for cells in tumor tissues but has lost its function in cell lines. Besides, our choline tracing experiment created opportunities for exploring other reactions of choline metabolism. We provide a list of 122 metabolite peaks labeled from choline, including several unknown metabolites which suggest previously undescribed pathways in human cells.

4.2 Discussion, reflections and perspectives

The two main points of focus in this thesis are to describe and understand the biology of metabolism across cell cycle phases, and to identify potential candidates that create vulnerabilities for cancer cells. Here I present the first large survey of hundreds of metabolite abundances and activities in G_1 and S-G_2-M phases. We present a new method for tracing metabolic activities in sorted subpopulations, which in combination with HRLC-MS make possible detection and measurement of hundreds of metabolites. This approach is closer to single cell metabolomics, but at present is limited by number of cells (around 50 000) required to inject in MS in order to obtain good signal from metabolite peaks. Detected peaks were carefully curated and checked for false peaks or metabolite fragments, which is extremely important for untargeted analysis and aim identifying unknown metabolites. In several cases we encountered arginine, choline, and phosphocholine fragments, and many instances of phosphocholine polymerization products which were carefully identified and annotated. Isotope tracing is an important tool that helps identify fragments by comparing labeling patterns between metabolite and fragment. Full scan LC-MS produces a large amount of data which requires specialized skills to extract and analyze peaks, becoming a challenge to reproduce others' analysis and store data in a systematic way.

Isotope tracing is a powerful technique that reflects metabolic activities before separation of cells in the dish, tumor or other mixed population. In this way measurement of labeled metabolites is affected to a lesser extent by experimental procedures. For the cell cycle studies we cultured cells in medium where most nutrients were ^{13}C labeled in order to cover most metabolites that are synthesized by cells. Labeled metabolites reflected activities of the respective enzymes in G_1 and S-G_2-M phases.

Metabolism is very dynamic and easily disturbed by experimental handling that must ensure good to minimize leakage of metabolites. Thus, the fluorescent probe or dye that binds to the cell sorting marker should either be co-expressed with a cyclic protein in engineered cells, be easily incorporated into live cells or bind to a marker that is located at cell surface. A good

marker for cell cycle phase separation determines the resolution of the study. DNA staining is a good approach for separating G_1 and S-G_2-M phases because it can be easily applied to almost all cell lines since DNA duplicates in S phase, and there are cell permeable dyes like Hoechst-34580 which we used in our studies. Antibodies that bind to internal cyclic proteins are problematic because staining procedures require cell permeabilization. In this regard, identification of cyclic cell surface markers in future studies might allow better separation of cell cycle phases. DNA staining combined with another cell cycle marker would increase the resolution for cell cycle separation.

Despite challenges for finding a good cell cycle marker, combination of cell sorting with isotope tracing and LC-MS provides a less invasive approach as compared to other current methods like synchronization with chemicals that block metabolic reactions. Besides, sorting allows clean separation while avoiding mixing of cell cycle phases, as opposed to enrichment of a cells in a cell cycle phase during synchronization.

Selecting a good candidate metabolite to invest in follow up experiments is a challenge. To increase chances of selecting the right metabolite that is important for cancer cell proliferation, we combined metabolomics data, expression level data and phenotypic screening. Difference in ornithine labeling between phases, and the fact that ARG2 caused a cell cycle defect in the siRNA screening was encouraging for further exploration of this pathway. The knockdown experiments gave promising results during short treatment with siRNA, but was difficult to apply to specific cell types like normal cells. Validation of these experiments in normal cells with induced stable knockouts (CRISPR or shRNA) will be critical. Future ARG2 studies might cover two aspects. One aspect is to elucidate the mechanisms that ARG2 silencing decrease cancer cell proliferation. It is unclear whether the phenotype observed is due to polyamine deprivation. The other aspect is to block ARG2 for sensitizing cancer cells in tumors. In a large-scale survey of patient tissue samples, initially we did not find any correlation of ARG2 overexpression with any tumor type. However, more careful investigation revealed that ER negative and basal subtypes of breast cancers have a higher expression of ARG2. This is an important lead for further experiments, which should be directed in these subtypes of breast cancer. Targeting ARG2 could be attractive, since ER negative breast cancers are difficult to target. Arginine tracing and quantification of ornithine and polyamines in normal and tumor tissues can provide useful details about ARG2 as a potential target.

Findings from this thesis should be encouraging to perform additional studies using tissues from specific tumor types. For example, use breast tumors, one might separate normal and cancer cells and study their metabolism in G_1 and S-G_2-M phases. Besides the cell cycle –

metabolism scope, in the future will be interesting to study metabolites that cause cancer in a more systematic way (as described in Figure 2).

Overall, this thesis provides a better understanding of metabolism across cell cycle, elucidating metabolic activities and relative abundances. The work presented in this thesis is a resource for metabolism across the cell cycle data, which can be used as a reference for interesting findings, and encourage further exploration. The method of isotope tracing, sorting and LC-MS is also a valuable tool that can be used to study metabolomics in different subpopulations. ARG2 is a promising target for ER negative and basal subtype of breast cancer. The study of choline tracing provides a new avenue for exploration of choline metabolism in normal and cancer cells.

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