D8.3

Metabolic models

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Abstract	Oncogene-driven metabolic rewiring in cancer is key to allow proliferation of tumour cells in low nutrient and oxygen conditions. To study such phenomena, reconstructing context-specific metabolic models through omics data integration is crucial. Here we report the original pipeline to construct context-specific metabolic models from scRNA-seq data and we applied it to scRNA-seq data from Ewing Sarcoma.
Keywords	Metabolism modelling, context-specific genome scale models, single cell, differential flux balance analysis.

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Executive Summary

D8.3 report describes the techniques developed to construct context-specific metabolic models from single cell gene expression data and the methodology to analyse them. The goal of deliverable D8.3 is to generate and analyse in silico context-specific metabolic models to identify the metabolic pathways and metabolites involved in tumorigenesis and metabolic dependencies that can enable small-molecule therapies.

D8.3 provides a computational tool for generating context-specific constraint-based metabolic models based on an integrative analysis of scRNA-seq data, assembled in a computational pipeline written in R and Matlab.

D8.3 provides a computational data resource containing the context-specific constraint-based metabolic models constructed from scRNA-seq Ewing Sarcoma patient derived xenografts and induced cell lines to study metabolic rewiring and characterize the Warburg effect.

D8.3 describes Differential Flux Balance Analysis, a general computational tool for analyzing context-specific constraint-based models to rank metabolic pathways according to maximization/minimization of a set of metabolic goals.

The computational methodology for analysing context-specific metabolic models provided in D8.3 will be applied to the generated patient-specific metabolic models in the future.

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Chapter 1 Introduction

Systems-level understanding of metabolism is pivotal to comprehending phenotypic differences. It consists of all metabolic reactions necessary to sustain life in a cell. Cell metabolism lies downstream of other omics summarising the effects from upstream layers of epigenome, genome, and transcriptome, but also of extracellular effects, and is reflected by differences in morphology, physiology and pathology (Evers et al. 2019). This highlights the necessity to study the biochemical and physiological characteristics of individual cells and their environment. However, conventional technologies often use bulk population-level measurements, ignoring the unique behaviour resulting from cell-to-cell variations, including cellular metabolism, growth, and proliferation.

Recent development of single-cell omics technologies facilitates the investigation of intra tumoral heterogeneity at the single-cell level, enabling the exploration of cell-to-cell variations (Kashima et al. 2020; Zhu, Preissl, and Ren 2020). These approaches can help characterise the origins of genetic and non-genetic heterogeneity, which can modulate cell response to exogenous and endogenous factors such as the activation of cancer driver genes (Almendro, Marusyk, and Polyak 2013). However, single-cell metabolomics techniques are still limited: metabolic measurements are expensive, skill intensive, and need additional improvements in terms of measurement sensitivity and throughput of samples and metabolites (Kumar et al. 2020; Alghamdi et al. 2021). In addition, there is a lack of established techniques for simultaneous multi-omics measurement comprehensive of the metabolome. Because omics layers are strongly intertwined, the knowledge about omics layers that can be more easily measured in single cells, such as mRNA, together with prior knowledge about metabolism, enable the prediction of cellular metabolism.

In the field of systems biology, genome-scale metabolic models (GEMs) integrate available omics data with genome sequences to provide an improved mechanistic understanding of the intra cellular metabolism of an organism. The mathematical framework of GEMs allows to predict internal cellular fluxes from a priori knowledge of thermodynamic constraints on individual enzymatic reactions, steady state hypothesis and the genome-scale stoichiometry matrix of all metabolic reactions. The idea is to explore the system's properties at a steady state during which internal metabolites stay at a constant concentration while exchange fluxes are constant and correspond to a constant import/export rate.

In addition, GEMs provide a mechanistic cellular context that can be coupled with omics data to quantify and predict the activity of diverse metabolic pathways and cellular functions. Several algorithms exist that build context-specific GEMs from omics data integration to recapitulate the metabolism of specific cell types under specific conditions. However, they may result in considerable differences in size, functionality, accuracy, and ultimate biological interpretation (Opdam et al. 2017). A better consensus between existing algorithms is provided by integrating biological knowledge inferred from omics data (Richelle et al. 2019).

To extract a unique distribution of fluxes the standard mathematical procedure is to perform Flux Balance Analysis (FBA) (Fell and Small 1986; Watson 1984). FBA is a standard mathematical procedure to semi-quantitatively estimate the metabolic flux of each reaction in a genome-scale model at steady state when satisfying a given metabolic objective (objective function optimization), such as the production or consumption of given metabolites.

Here, we develop an original pipeline to construct context-specific models from scRNA-seq data that couples scRNA-seq analysis, functional annotation of clusters of cells, and the GIMME algorithm to integrate scRNA-seq data into GEMs. The workflow is then applied to a set of scRNA-seq data from Ewing Sarcoma (EwS) and FBA is performed to obtain a unique distribution of fluxes for each context-specific model setting as the objective the biomass pseudo flux.

We plan in the future to apply Differential Flux Balance Analysis (DFA) to rank metabolic pathways and metabolites according to a biological signature. DFA is a method developed at UNINA (Pagliarini

and di Bernardo 2013) to identify those metabolites and reactions predicted to change the most across two GEMs.

This report describes the main results achieved during the work on this deliverable:

- Building a pipeline for creating context-specific metabolic models based on integrative data analysis and the COBRA toolbox (chapter 2).
- Creating a corpus of context-specific metabolic models from scRNA-seq data from Ewing Sarcoma patient derived xenografts (PDX) (chapter 2).
- The methodology DFA and its application to the HepatoNet1 model, a metabolic reconstruction of the human hepatocyte for the analysis of liver physiology (chapter 3).

We summarize the achieved results and lay out a plan definition for the exploitation of the generated context-specific metabolic models in the last chapter.

Chapter 2 Construction of context-specific GEMs from scRNA-seq data

In this chapter, we describe the pipeline we developed to construct context-specific metabolic models using the Gene Inactivity Moderated by Metabolism and Expression (GIMME) method (Becker and Palsson 2008) through scRNA-seq integration. Since, scRNA-seq data is typically sparse, GIMME method would turn off a multitude of metabolic reactions (see Section GIMME method). To deal with it, we propose to perform scRNA-seq analysis, dimensionality reduction and clustering; and construct a unique gene expression profile per cluster. The cluster gene expression profile is then used as a proxy for the construction of the cluster context-specific genome scale model. The pipeline is built such that it can be applied to any set of scRNA-seq data. Further, we show its application to EwS PDX and cell line scRNA-seq data from Aynaud et al. 2020.

2.1 Pipeline for the construction of context-specific GEMs from PDX scRNA-seq data

To construct context-specific patient-derived xenografts from scRNA-seq PDX data, we propose an original pipeline which consists of three main steps. A first pre-processing step where dimensionality reduction, clustering and cluster annotation is performed on the scRNA-seq and a unique gene expression profile per cluster is retrieved. A second step where gene expression is integrated into a human genome-scale model by means of the GIMME algorithm. The third step consists in tuning the parameters from the previous steps with the rationale that clusters with the same annotation across multiple datasets should show a similar distribution of metabolic fluxes.

Figure 1 represents a diagrammatic representation of the pipeline. The workflow uses different tools: seurat package in R for the initial pre-processing of the data (Satija et al. 2015); gene set signatures for cluster annotation; and the COBRA toolbox (Vlassis, Pacheco, and Sauter 2014; Heirendt et al. 2019) in Matlab together with an initial constraint-based metabolic model to integrate expression data into GEMs.

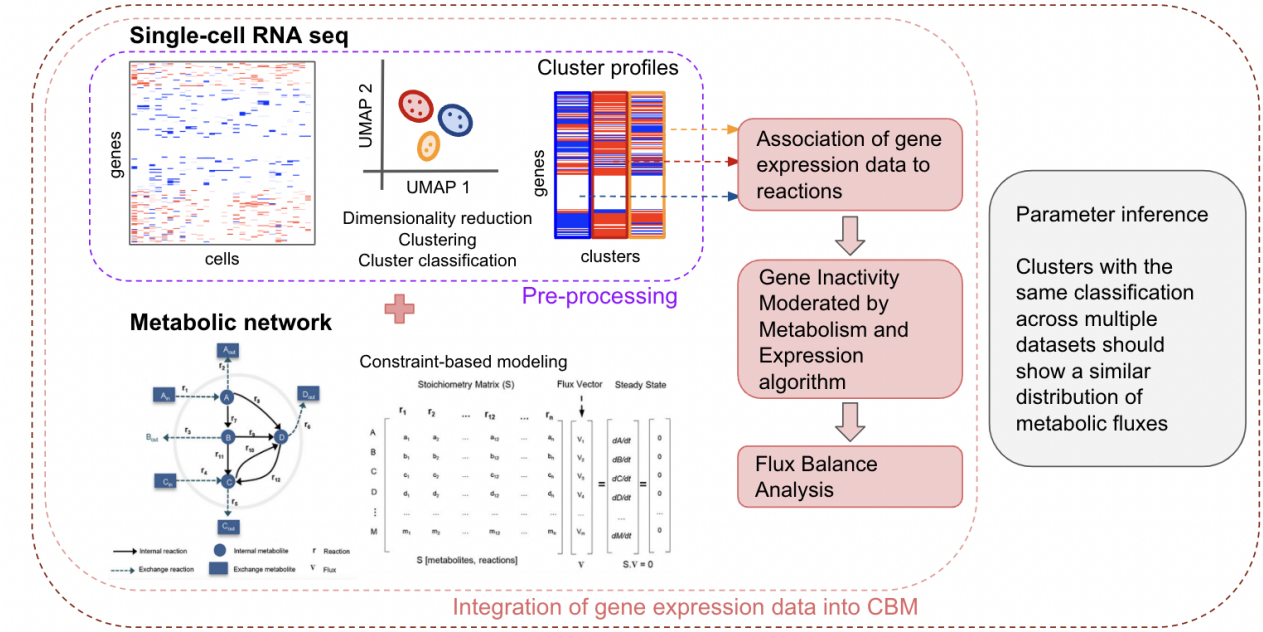

Figure 1: Schema of the pipeline for constructing context-specific metabolic models from patient-derived scRNA-seq data. Abbreviations: CBM, Constraint-Based Models; UMAP, Uniform Manifold Approximation and Projection.

2.1.1 Pre-processing and annotation of scRNA-seq data

At the first step of the pipeline, scRNA-seq analysis and cluster annotation is performed on each dataset. Clustering and cluster annotation is performed for different values of the clustering resolution. Aggregation of a unique scRNA-seq profile per cluster is then performed using the preferred aggregation method (e.g., considering the mean expression value per gene).

The pipeline is implemented such that clusters are annotated using gene set signatures which in principle are associated with one biological function. Genes associated to multiple gene signature are kept for the calculation of signature scores. The set of gene signatures to consider must be uploaded as a set of different files (one per gene signature), in '.txt' format, that contain a list of genes. Scores are then calculated using the preferred method (e.g., considering the mean expression value of genes). Cluster scores are centred and standardised independently for each dataset first. Next, clusters are jointly classified: if a score is greater than one standard deviation it is annotated as associated to the biological function of the signature.

2.1.2 Creation of context-specific GEMs

The second step of the pipeline consists in constructing for each cluster scRNA-seq profile a context-specific GEM and to infer a FBA solution of fluxes. To this end one must consider an initial GEM and the objective function to be maximize/minimize.

2.1.2.1 GIMME method

The pipeline uses the GIMME algorithm (Becker and Palsson 2008) implemented in MATLAB, to extract a context-specific model from mapped gene expression data to reactions. GIMME minimises usage of low-expression reactions while keeping the objective (e.g., biomass) above a certain threshold. In practice, cluster scRNA-seq expression is mapped to reactions in the GEM through gene-protein-reaction rules; low-expression reactions are turned off and in case the objective is not above the imposed threshold, the algorithm finds the minimum number of low-expression reactions that must be turned on to achieve it. The workflow allows to set the thresholds for the GIMME algorithm and to choose the objective function to optimize.

2.1.2.2 Association of gene expression data to GEM

Gene expression profiles were mapped to the GEMs following the conventions implemented by genome-scale metabolic models, where reactions are associated with genes and proteins via gene-protein-reaction (GPR) associations (Thiele and Palsson 2010). In general, there can be a many-to-many mapping of genes to reactions; for example, one reaction can be linked to genes (G1 and G2) or G3. The first Boolean AND relationship means that the reaction is catalysed by a complex of two gene products. Since the maximum of the complex is given by the minimum of its components, the weighting of the complex is defined as:

$$G_1 \ AND \ G_2 = min(G_1, G_2)$$

The OR relationship allows for alternative catalysts of the reaction. Thus, total capacity can be set either to the sum or to the maximum of its components. We set it to be the sum:

$$(G_1 \ AND \ G_2) \ OR \ G_3 \ = \ min(G_1 \ , G_2) \ + \ G_3$$

2.1.3 Tuning the parameters

The third step consists in tuning the parameters from the previous steps with the rationale that clusters with the same annotation across multiple datasets should show a similar distribution of

metabolic fluxes. To do so, we consider cluster annotations and a FBA solution from the associated context-specific model.

2.1.3.1 Kernel supervised PCA

Kernel supervised PCA is used to identify reactions that best discriminate the group of clusters with the same annotation. The method maximises the between group variance while minimising intra group variance. Given the $v \times n$ matrix X of v FBA fluxes and n clusters, and Y the $1 \times n$ vector of label annotations, the problem can be solved in closed form (Barshan et al. 2011).

2.1.3.2 Silhouette score

The silhouette score is then calculated in the kernel supervised PCA projection. The pipeline considers the number of kernels supervised principal components that explain 95% of the total variation. This score is calculated for all combinations of the parameters. Finally, the set of parameters for which the silhouette score was the maximum is selected.

Note that calculating the silhouette score for a group of clusters with only one element is not allowed. We propose to attribute to that cluster the same annotation of the nearest cluster in the signature score space.

2.2 Pipeline for the construction of context-specific GEMs from induced cell lines scRNA-seq data

In the context of scRNA-seq data from cell lines, we propose a similar jet simpler pipeline. It consists in grouping scRNA-seq profiles by considering cells with a similar gene signature score. A unique gene expression profile is then computed for each group of cells. Construction of a context-specific GEMs is then performed using the GIMME method as described above.

2.3 Code organization

Pipeline's scripts can be found on github: https://github.com/iPC-project-H2020/wp8-metabolic-modeling. The folder is organised as follows:

- Rcodes and MATLABcodes folders contain the R and MATLAB codes for the construction of context-specific metabolic models from scRNA-seq data.
- scRNA-seq_data folder contains the scRNA-seq datasets.
- GEM folder contains the initial GEM model.
- IC_genes folder contains the gene set signatures used for clustering annotation.

The first two steps of the pipeline are implemented in such a way that the scripts can be called from the terminal by running 'Rscript.exe /Rcodes/work_flow.R' after setting the job_profile.xlsx file in the parent folder. This file must include a:

- 'file.name' column with the scRNA-seq file names.
- 'is.cell_line' column with logicals indicating if the scRNA-seq data is from induced cell lines or not;
- 'model_name' column with the initial GEM file name;
- 'ic_score' column with the method to calculate gene signature scores (e.g. median);
- 'aggregation_by' column with the aggregation method to construct a unique gene expression profile per cluster;
- 'clustering_resolution column with the clustering resolution parameter value.

The algorithms will generate 5 folders organized in sub-folders by cell line or pdx, the initial GEM model name, clustering resolution, gene signature scoring method and clustering aggregation method. The folders are organized as follows:

- 'cluster_profiles' which contains the gene expression profiles.
- 'cluster_info' which contains the gene signature scores.
- 'ValToReact' which contains the mapped cluster gene expression values to reactions.
- 'FBA_solution' which contains the FBA solutions.
- 'csGEM' which contains the context-specific GEMs.

2.4 Context-specific metabolic models for EwS scRNA-seq data

2.4.1 Ewing Sarcoma dataset

Willing to study the metabolic switch in EwS, we considered the dataset from Aynaud et al. 2020. It comprised:

- temporal scRNA-seq data from the EwS A673 cell line upon EWSR1-FLI1 silencing through doxycycline (DOX) controlled short hairpin RNA. Following a downmodulation of EWSR1-FLI1 by 7 days of doxycycline (DOX) treatment, the dataset was issue of a time course experiment after the removal of DOX from the medium, leading to EWSR1-FLI1 re-expression.
- scRNA-seq data from A673 xenografts in a severe combined immunodeficiency (SCID) mouse without DOX (DOX-) and after 7 days of DOX treatment (DOX+).
- 8 scRNA-seq data from EwS PDXs.

In addition, we consider an independent corpus of 7 additional, yet unpublished, scRNA-seq data from EwS PDX established in CURIE.

Temporal scRNA-seq data from A673 cell lines and PDXs under DOX+/DOX- treatment were exploited to study the effect of EWSR1-FLI1 on the metabolism by clustering cells based on gene signature associated to EwS activity. With respect to the PDXs, we applied the pipeline described in chapter 2.1.

2.4.2 Context-specific metabolic models for EwS PDXs scRNA-seq data

For each PDX, we consider a range of resolution values from 0.25 to 0.8 (standard resolution value) by 0.05, and for each cluster, a unique gene expression profile per cluster is obtained by considering the mean and the median gene expression of cells in the cluster.

Clusters were annotated using the gene signatures identified in Aynaud et al. 2020 associated with EWSR1-FLI1 activity, cell cycle (G1/S and G2/M phases), oxidative phosphorylation and glucose catabolism.

We constructed a context-specific model for each cluster starting from the thermodynamically curated GEMs provided in Masid, Ataman, and Hatzimanikatis 2020. They included the thermodynamically curated version of:

- Recon2 consisting of 7440 reactions and 5063 metabolites;
- Recon3 consisting of 10602 reactions and 5825 metabolites;
- a reduction of Recon2, redHuman2, consisting of 1374 reactions and 469 metabolites;
- a reduction of Recon3, redHuman3, consisting of 1632 reactions and 591 metabolites.

Next, we infer a FBA solution for each context-specific model from each cluster, through maximisation of the biomass pseudo flux. We set the objective to be the biomass pseudo flux and we impose it to be greater than the 90% of the biomass pseudo flux from the initial GEM. We define low-expression reactions in a data-driven manner as those reactions with a mapped gene expression value below the first quantile of the distribution of mapped expression.

Therefore, for each cluster we obtain:

- a gene expression profile;
- scores associated to EwS activity, oxidative phosphorylation, proliferation and glucose catabolism;
- the mapped gene expression profile to reactions;
- a FBA profile of fluxes obtained through maximisation of the biomass pseudo flux;
- a cluster context-specific GEM.

A preliminary analysis of the generated models can be found on github: https://github.com/iPC-project-H2020/wp8-metabolic-modeling. We benchmark the fact that reactions associated with the 'Ews_high' state involve the glycine, serine, tryptophan but also the glutathione and glutamine metabolism. Analysis of such models is ongoing, and results will be the objective of a publication.

Chapter 3 Differential Flux Balance Analysis

DFA (Pagliarini and di Bernardo 2013) is based on flux balance analysis (FBA), a standard mathematical procedure to semi-quantitatively estimate the metabolic flux of each reaction in a genome-scale model at steady state when satisfying a given metabolic objective (objective function optimization), such as the production or consumption of given metabolites (Fell and Small 1986; Watson 1984). For each metabolic objective, DFA computes the difference between the metabolic fluxes predicted by FBA in the WT genome-scale model versus the perturbed model (LoF or GoF of a single enzyme or of a set of them). The LoF model lacks the enzyme under investigation, whereas the GoF model is constrained so that the reaction(s) carried out by the enzyme of interest is forced to be more active than in the WT. The result is a ranked list of metabolic fluxes of all the reactions in the model, sorted by their difference in the LoF (or GoF) model versus the WT model. This difference is computed as the average across the metabolic objectives. Hence, the reactions that are most affected by the LoF (or GoF) will be found at the top of the ranked list.

In our DFA implementation, we analyze the average change in metabolic fluxes when optimizing each of 442 metabolic objective functions included in Hepatonet1 (Gille et al. 2010) in standard physiological conditions versus perturbed non-physiological conditions. Non-physiological conditions are simulated by imposing additional constraints on metabolic reactions, metabolic objectives, or both. Finally, the change in metabolic fluxes between the standard and perturbed model are computed to yield differential fluxes. Then, the differential fluxes involving the same metabolite are aggregated to rank metabolites. In the resulting ranked list, metabolites on top will be the ones involved in the metabolic fluxes that change the most.

3.1 Application of DFA to HepatoNet1

UNINA applied an algorithm termed differential flux-balance analys (DFA) to the genome-scale metabolic network model HepatoNet1 (Gille et al. 2010). Briefly, this model includes 785 metabolites and 2,589 reactions across 8 cellular compartments, resulting in a total of 1445 compartment-specific metabolites. DFA is described in detail in Pagliarini et al. 2016 where it was used to understand metabolic changes occurring in inborn errors of liver metabolism. Here UNINA asked whether the same model could be used to understand the metabolic changes occurring in cancer.

UNINA thus used the model to understand whether it could be used to elucidate metabolic changes occuring in response to nutrient availability and cell proliferation. To this end, DFA was applied to understand the commonalities between the metabolism of heptocytes challenged with palmitate utilization (as a surrogate of increased fat availability, as fat usage is related to hepatocellular carcinoma) or proliferation (a metabolic surrogate for cellular transformation). Palmitate utilization was simulated by imposing palmitate reduction in the cytosol as an additional term (-palmitate(c)) in all the 442 metabolic objective functions. To simulate the metabolic demands of cell proliferation, we asked the model to maximise the production of all amino acids and nucleotides, by adding these as additional terms to each objective function (+ Alanine(c) + Arginine(c) ... + dATP(c) + dCTP(c)...). We then compared (absolute difference) the fluxes obtained when modelling palmitate utilization to those obtained when modelling cell proliferation across the 442 objective functions. We finally averaged such differential fluxes values over all the objective functions, and from these we ranked the metabolites to obtain the final estimates. The metabolites most altered were specifically enriched in glycolysis, serine metabolism and mitochondrial metabolism. These alterations indeed occur in hepatocellular carcinoma (Broadfield et al. 2021), thus demonstrating the suitability of DFA to elucidate metabolic changes in cancer.

Chapter 4 Conclusion and future work

The work on this deliverable produced the required methodology for the construction of context-specific models from scRNA-seq data. Its application to EwS data led to the construction of context-specific models for clusters of single cell PDX which highlighted differences in the fluxomic space between clusters annotated to be associated to different biological functions. This provides the first step toward the characterization of the metabolic rewiring in EwS and open new avenues for studying metabolic rewiring in other cancer types.

In the future, we plan to use scRNA-seq data from EwS primary tumors to validate our models and further analyze them using the DFA methodology to compare the 'altered' metabolic state of context-specific models to the 'normal' metabolic state of the initial GEM. This last analysis can provide two main results: we would be able to compare the ranking of the reactions across context-specific models that are associated with the same function but also between context-specific models associated with different biological functions.

Chapter 5 List of Abbreviations

Abbreviation	Translation
EwS	Ewing Sarcoma
GEM	Genome scale model
PDX	Patient-derived xenograft
CBM	Constraint-based model
DFA	Differential Flux balance Analysis

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