Title

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Permalink

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Journal

Cell Systems, 12(9)

Authors

Seif, Yara
Palsson, Bernhard

Publication Date

2021-09-22

DOI

10.1016/j.cels.2021.06.005

Published in final edited form as:

Cell Syst. 2021 September 22; 12(9): 842–859. doi:10.1016/j.cels.2021.06.005.

Path to improving the life-cycle and quality of genome-scale models of metabolism

Yara Seif[1,2], **Bernhard Ørn Palsson**[1,†]

Department of Bioengineering, UCSD

[1]Department of Bioengineering, University of California, San Diego, La Jolla, CA, 92093, USA

[2]Merck & Co., Inc., South San Francisco, CA 94080, USA

Summary:

Genome-scale models of metabolism (GEMs) are key computational tools for the systems-level study of metabolic networks. Here, we describe the “GEM life cycle” which we subdivide into four stages: inception, maturation, specialization, and amalgamation. We show how different types of GEM reconstruction workflows fit in each stage and proceed to highlight two fundamental bottlenecks for GEM quality improvement: GEM maturation and content removal. We identify common characteristics contributing to increasing quality of maturing GEMs drawing from past independent GEM maturation efforts. We then shed some much-needed light on the latent and unrecognized but pervasive issue of content removal, demonstrating the substantial effects of model pruning on its solution space. Finally, we propose a novel framework for content removal and associated confidence level assignment which will help guide future GEM development efforts, reduce duplication of effort across groups, potentially aid automated reconstruction platforms, and boost the reproducibility of model development.

Graphical Abstract

[†]**Corresponding author and lead contact:** To whom correspondence should be addressed: Bernhard Palsson, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, +18585345668 (tel.), +18588223120 (fax), palsson@ucsd.edu.
Author contributions:
Y.S. designed the study, conducted computational analysis and and wrote the Perspective, B.O.P. critically reviewed the content.

Declaration of interests:
The authors declare no competing interests.

Material Availability
This study did not generate new unique reagents.

Data and Code Availability
All models were downloaded from reference databases including BiGG(Norsigian *et al*., 2020), VMH(Noronha *et al*., 2019), BioModels(Chelliah *et al*., 2015), yeast.sf.net (Aung, Henry and Walker, 2013), BioModels(Chelliah *et al*., 2015), Zenodo (Peters *et al*., 2017) and Silicolife(*Home - SilicoLife*, no date), except for the starting draft AGORA models which were obtained courtesy of prof. Ines Thiele. Simulations were run using COBRApy.

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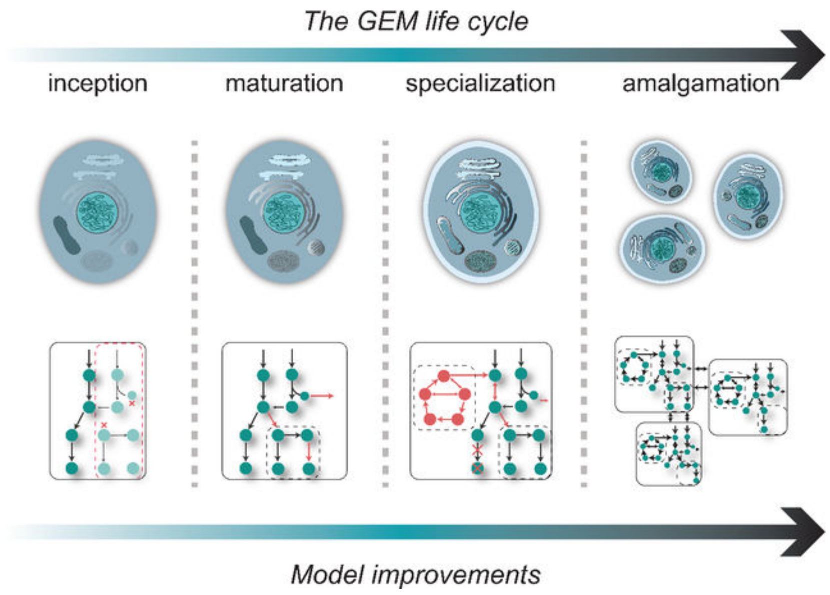

Introduction

Metabolic network reconstructions are knowledge-bases of bottom-up curated gene-linked biochemical pathways. They can be converted into a mathematical format to formulate stoichiometric genome-scale metabolic models (GEMs) capable of computing various cellular functions. The development of GEMs started shortly after the publication of the first complete genome of *Haemophilus influenzae* in 1995 with its GEM appearing in 1999 (Fleischmann *et al.*, 1995; Edwards and Palsson, 1999). As more model organisms were sequenced, more GEMs were built, covering an ever increasing breadth of phylogenetic backgrounds. The availability of sequences for an increasingly large diversity of organisms drove the need to make metabolic modeling accessible to a broader scientific community. As a result, a protocol to generate GEMs was established and computational pipelines for metabolic modeling were distributed (Becker *et al.*, 2007; Thiele and Palsson, 2010a; Wang *et al.*, 2018). Over the years, DNA sequencing cost has plummeted, and the number of available genomic sequences has increased exponentially. Thus, the challenge to build GEMs fast arose. Various semi-automated reconstruction workflows were developed, either to assemble GEMs *de novo* or to refine existing ones (Mendoza *et al.*, 2019; Norsigian *et al.*, 2019).

With the advent of new ‘omics’ technologies, it became evident that GEMs could be refined further to represent cellular states, tissue types, disease and cell lines (Uhlén *et al.*, 2015). Multiple modeling methods for ‘omics’ data integration to extract context-specific models were developed, with various levels of success (Opdam *et al.*, 2017; Richelle *et al.*, 2019). The emergence of metagenomic sequencing subsequently drove the need to build models of microbial communities in order to infer the metabolic repertoire of a microbiome sample (Magnúsdóttir *et al.*, 2017). However, questions regarding the quality of the GEMs resulting from the miscellaneous workflows have been raised (Monk, Nogales and Palsson, 2014), highlighting the need to generate robust quality metrics and reproducible and comparable reconstruction workflows (Lieven *et al.*, 2020). These shortcomings gave a new impetus to crowd-sourcing GEM development efforts, pushing teams of experts to form in order to tackle the interdisciplinary problem of systems-level metabolic modeling (Wang *et al.*, 2018; Lu *et al.*, 2019). As such, GEM development efforts are moving away from local computers and towards online platforms (Aurich, Fleming and Thiele, 2016). Monk et. al recently outlined five key challenges that face their continued development (Monk, Nogales and Palsson, 2014), including: 1) insufficiency of model curation, 2) gaps in biological knowledge, 3) narrow phylogenetic scope, 4) inconsistent adherence to modeling standards, and 5) modest biological scope. Points 3–5 are being tackled with novel metabolic modeling methods and with the emergence of standardized model test suites such as MEMOTE (Lieven *et al.*, 2020). Here, we discuss how points 1 and 2 can be addressed, drawing inspiration from past independent, but parallel efforts. We advocate for two approaches to improving the life-cycle of GEMs. First, we start by defining the GEM life-cycle. We then delve into GEM maturation, which we identify as bottleneck number 1 for GEM quality improvement because it has historically and consistently played a crucial role in improving the quality of GEM sequels. We outline how GEMs mature by drawing examples from a multitude of literature sources so as to serve as a single reference for future GEM maturation endeavors. Finally, we identify a second bottleneck in GEM quality improvement and highlight how content removal is an underappreciated feature transcending all phases of the GEM life cycle. We explicitly provide examples of content removal which can also serve as a guide for future reconstruction efforts, and outline a mathematical framework to save removed content.

Results

The four stages of the GEM life cycle: inception, maturation, specialization, and amalgamation

Multiple workflows are available to guide GEM construction (Thiele and Palsson, 2010a; Opdam *et al.*, 2017; Norsigian *et al.*, 2019). A primary distinguishing feature among workflows is the starting point, which varies depending on how well studied the organism of interest is. We illustrate this and other distinctions by outlining the "GEM life cycle" which we subdivide into four stages: 1) GEM inception, 2) GEM maturation, 3) GEM specialization, and 4) GEM amalgamation (Figure 1).

GEM inception: starting at the beginning—When no prior work has been done to characterize the metabolic capabilities of a target organism at the system level, the initial challenge is to assemble pertinent knowledge to form a first functional GEM. This first stage can be described as the "*GEM inception*" stage of the GEM life cycle, and has traditionally been divided into four steps: i) automated reconstruction, ii) manual curation, iii) conversion to a mathematical format, and; iv) and validation against experimental data (Feist *et al.*, 2009; O'Brien, Monk and Palsson, 2015). At this stage, an organism-specific bibliome is established, explicitly representing the current limits of biological knowledge available at the time of reconstruction. Curation of bibliomes (Broddrick *et al.*, 2016; Hefzi *et al.*, 2016; Levering *et al.*, 2016) takes much manual labor but is a necessary step to maximize knowledge capture in a reconstruction. Such a process is especially useful for lesser known organisms, because it offers important insights at the systems-level, and drives molecular discovery (Broddrick *et al.*, 2016; Hefzi *et al.*, 2016; Levering *et al.*, 2016).

The GEM inception can be extremely time-consuming because it is curation-driven. Advances for this step have occurred on multiple fronts including: 1) the expansion of reference pathway databases such as MetaCyc(Caspi *et al.*, 2019), KEGG(Kanehisa *et al.*, 2017), and Uniprot(UniProt Consortium, 2019) with curated content across an ever increasing number of organisms; 2) the development of automated reconstruction tools which pull content from reference network databases (Mendoza *et al.*, 2019), such as ModelSEED(Henry *et al.*, 2010), Pathway Tools(Karp *et al.*, 2016), RAVEN(Wang *et al.*, 2018), Merlin(Dias *et al.*, 2015), Kbase(Arkin *et al.*, 2018); and 3) the assembly and expansion of repositories hosting easily retrievable, interoperable, and standardized curated GEMs, such as BiGG(Norsigian *et al.*, 2020), VMH(Noronha *et al.*, 2019), BioModels(Chelliah *et al.*, 2015), yeast.sf.net (Aung, Henry and Walker, 2013), BioModels(Chelliah *et al.*, 2015), Zenodo (Peters *et al.*, 2017) and Silicolife(*Home - SilicoLife*, no date).

The quality of the starting draft reconstruction heavily affects the amount of time subsequently spent on curating the network, with a strong tradeoff between model size, model quality, and human hours spent on manual curation. Automated GEM assembly is based on identifying database genes annotated with a metabolic function sharing “sufficiently” high sequence homology(Simeonidis and Price, 2015). When validated GEMs for closely related strains exist, a faster path to a better draft starting reconstruction is to map the content of the existing GEMs by means of sequence homology. This approach was used, for instance, to build GEMs of seven closely related species of *Saccharomyces cerevisiae* (Lopes and Rocha, 2017). Oftentimes, researchers attempt to pool together the annotation results from multiple platforms as well as pre-existing GEMs, and face the laborious task of converting object identifiers. A newer and more elaborate strategy incorporates sequence homology as well as pathway homology and genomic context. It was recently applied to build 773 human gut bacterial models(Magnúsdóttir *et al.*, 2017).

Despite these advances, curating draft metabolic reconstructions is still heavily manual. In addition, in order to convert the starting draft into a high quality model, *i.e.* a model that is capable of generating meaningful predictions, each object included in the model (*i.e.* genes, reactions, metabolites, and gene-reaction mappings) should be checked against literature evidence and assigned confidence scores (Thiele and Palsson, 2010a). A number of platforms such as COBRApy (python)(Ebrahim *et al.*, 2013), COBRA toolbox (Matlab) (Heirendt *et al.*, 2019), DistributedFBA.jl (Julia)(Heirendt, Thiele and Fleming, 2017), and Sybil (R)(Gelius-Dietrich *et al.*, 2013) enable easy manual modifications.

The assembled draft reconstruction is then converted into a computational model by introducing constraints and modeling assumptions so as to formulate the network as a constraint-based optimization problem (Feist and Palsson, 2010; Palsson, 2015; Stalidzans *et al.*, 2018). At this point, the network is further refined, and the refinements make use of insights that can only be gleaned from systems level properties. Functionalities for this step are variably available across automated annotation platforms such as gap filling (which adds linkages to disconnected network edges in order to achieve biomass precursor production)(Devoid *et al.*, 2013), protein and metabolite subcellular localization predictions (for model compartmentalization)(Dias *et al.*, 2018), and Gibbs free energy calculation (for the estimation of the upper and lower bounds of a reaction)(Machado *et al.*, 2018). Emerging algorithms are now attempting to suggest which modifications to make based on gene essentiality profiles, experimentally derived nutrient utilization profiles (Hartleb, Jarre and Lercher, 2016; Machado *et al.*, 2018), or by flagging unbalanced reactions(Wang *et al.*, 2018). Despite the manual aspect of building the initial reconstruction, the corresponding workflow is decidedly the most established workflow in the GEM life cycle.

GEM maturation: the devil is in the details—In the second stage of the GEM life cycle, the starting point is an existing curated GEM which goes through a series of iterations resulting in “GEM sequels” or subsequent versions of the first GEM for the target organism. A good metabolic model should be the best representation of biological reality that we obtain given the current state of knowledge, and it therefore needs to be continually improved as new findings are published. Indeed, this on-going process takes years, or even decades, and occurs through multiple iterations yielding, hopefully, an ever increasing quality of the metabolic reconstruction that a GEM is based upon. Hence, researchers involved in such efforts ask what was previously missed, what can be improved, whether previous unknowns have become known, and whether there are new known unknowns to be discovered.

The maturation phase produces GEMs that are increasingly more organism-specific, have larger metabolic coverage, contain increasingly comprehensive manually curated pathways, and improved predictive power (Monk, Nogales and Palsson, 2014). GEM sequels are usually limited to organisms of high interest. For example, the metabolic reconstruction of *E. coli* has been updated at least five times (McCloskey, Palsson and Feist, 2013; Monk *et al.*, 2017) (iJE660, iJR904, iAF1260, iJO1366, and iML1515), the human metabolic network has been updated over four times (recon 2, recon 2.04, recon 2.2, and recon 3D) (Vieira *et al.*, 2018; Robinson *et al.*, 2020), the *S. aureus* GEM was updated 4 times (Seif, Monk, Mih, *et al.*, 2019), and the *S. cerevisiae* network has been updated over 17 times (Lopes and Rocha, 2017). Oftentimes, GEM sequel efforts occur contemporaneously across multiple groups, calling for the organization of reconstruction jamborees to produce a consensus model (Herrgård *et al.*, 2008; Thiele and Palsson, 2010b). Because this stage of the GEM life cycle is purely aimed at improving a GEM’s quality, in a subsequent section we will expand upon the characteristics and types of modifications that have been adopted historically. This will serve as a set of pointers that can guide future GEM maturation efforts.

GEM specialization: a GEM-specific evolutionary strategy—These highly curated and mature GEMs then enter the third stage of the GEM life cycle: GEM specialization. Reconstruction efforts at this stage are data-driven and either partially or fully automated, and begin to account for subtle variations across cell genotypes and gene expression levels. The general strategy is to use a mature GEM as a reference and use one or a combination of data types to guide content removal or to modify upper and lower bounds on allowable reaction rates. Multiple data types can serve to build specialized GEMs including whole genome sequences (Seif *et al.*, 2018), bulk RNA expression levels (Uhlén *et al.*, 2015), protein concentrations (Großeholz *et al.*, 2016), metabolite abundance (Campos and Zampieri, 2019; Yang *et al.*, 2019), and metabolite concentrations (Seif, Monk, Mih, *et al.*, 2019). A recent piece used instead a manual approach in which subsystems were selected based on literature evidence of alteration in a cell type (Masid, Ataman and Hatzimanikatis, 2020). The resulting specialized GEMs are described as “context-specific”, “condition-specific”, “tissue-specific”, “disease-specific”, or “strain-specific”. Here, researchers ask a large breadth of questions, and usually get their answers by comparing the specialized models against each other. To guide their conclusions, they take note of the content that was variably removed and how it changed the network properties.

Whole genome sequences are mostly used to tailor models of prokaryotes, because the variation in coding DNA sequences is significant between strains of the same species. Mature GEMs have been used as baseline reconstructions in *E. coli*, *Salmonella, S. aureus,* and *S. cerevisiae* (Fang *et al.*, 2018; Seif *et al.*, 2018; Lu *et al.*, 2019; Seif, Monk, Machado, *et al.*, 2019) to build strain-specific networks that are tailored according to each strain’s genetic background (Fang *et al.*, 2018; Seif *et al.*, 2018; Lu *et al.*, 2019; Seif, Monk, Machado, *et al.*, 2019). In some cases, the reference network is initially expanded to annotate the accessory genome and account for species-level metabolism (*i.e.*, genes and reactions which are present in some, but not all, strains) (Seif *et al.*, 2018; Seif, Monk, Machado, *et al.*, 2019). Next, the panreconstruction is tailored to each strain using the presence and absence of metabolic genes as a basis for content removal (Norsigian *et al.*, 2019). While the reductive step is easily automated through the integration of comparative genomics with metabolic modeling (*e.g.*, CarveMe) (Machado *et al.*, 2018; Norsigian *et al.*, 2019), the initial constructive step is not, and employs similar methodologies to those used for the assembly of *de novo* reconstructions.

While ‘omic’ data integration is straightforward in the case of building “strain-specific” models from whole genome sequences, or “condition-specific” models from time-course quantitative exo-metabolomics data, it remains an active area of research for the integration of all other “omic” data types (Ramon, Gollub and Stelling, 2018). There is a wide variety of approaches currently available. For example, there are four different characteristics used for transcriptomic data integration, which is especially popular for the generation of tissue-specific and disease-specific human GEMs (Rosario *et al.*, 2018; Ben Guebila and Thiele, 2019; Gatto, Ferreira and Nielsen, 2020): 1) expression level thresholding (thresholds define which genes or active/inactive), 2) the assumptions made to translate active/inactive genes to active/inactive reactions *via* the gene-protein-reaction rule, 3) the modeling extraction method, and 4) the chosen cellular objective (biomass production, ATP production, etc.) (Kim and Lun, 2014; Töpfer, Kleessen and Nikoloski, 2015; Richelle *et al.*, 2019). An emerging strategy to pinpoint the best workflow is the validation of reduced models against condition-specific gene essentiality profiles generated using CRISPR technologies (Opdam *et al.*, 2017). This highly anticipated validation strategy holds great promise for the rapid acceleration of validated ‘omic’ integration tools.

GEM amalgamation: one alone is not enough—Building multicellular GEMs is the latest development in the GEM life cycle. Here, researchers are interested in drawing a more holistic view of cellular behavior by explicitly modeling cell-cell interactions. Tailored GEMs for different cells operating in a single system are merged together into a single model to capture multicellular metabolic interactions. The modeled systems include multi-tissue organisms (Gomes de Oliveira Dal'Molin *et al.*, 2015), synthetic microbial communities (Mee *et al.*, 2014; Zelezniak *et al.*, 2015; Machado *et al.*, 2018; Pacheco, Moel and Segrè, 2019), cell populations in a single tissue (Damiani *et al.*, 2019), microbiomes (Thiele, Heinken and Fleming, 2013), whole body metabolic models (Thiele *et al.*, 2020) and host-microbe interactions (Bordbar *et al.*, 2010). Multicellular GEMs have been used to capture how metabolic diversity defines the space of allowable cell-cell interactions (Zengler and Zaramela, 2018), as well as to predict the nature of those interactions (*e.g.* synergism, antagonism) (Heinken and Thiele, 2015a). Technological advances in single cell transcriptomics, metagenomics and metatranscriptomics have accelerated the pace of community GEM development. However, each data type comes with its own challenges, complicating data-driven GEM tailoring and calling for the development of novel metabolic modeling workflows. This phase of the GEM life cycle is still in its infancy, and it holds great promise for the near future. Published protocols and guidelines have been established for stage 1 and stage 3 of a GEM life cycle (Thiele and Palsson, 2010a; Kim and Lun, 2014; Norsigian *et al.*, 2019), while details of efforts that have gone into stage 4 are reviewed by Magnúsdóttir et. al (Noronha *et al.*, 2019). Below, we discuss characteristic features shared across GEM maturation endeavors (stage 2).

Bottleneck 1: GEM maturation

GEMs mature when they are updated over the years. Here we discuss common characteristics of GEM maturation efforts which we observed across independently evolved GEM sequels contributing to increased GEM quality.

Capturing increasing cellular complexity—Early-stage GEMs tend to account for a reduced number of cellular compartments (Figure 2A). A predominant set of improvements are driven by the examination of the network structure and its reorganization. For example, the initial *E. coli* networks did not account for the periplasmic compartment which was later added in iAF1260 (Edwards and Palsson, 2000; Feist *et al.*, 2007). Similarly, the number of compartments modeled in *S. cerevisiae* increased from 3 to 16 over the course of multiple iterations (Duarte, Herrgård and Palsson, 2004; Mo, Palsson and Herrgård, 2009; Aung, Henry and Walker, 2013). The development of fully compartmentalized GEMs included the re-assignment of reactions and metabolites to new compartments and the addition of intercompartmental transport reactions to represent the metabolic exchanges between compartments (Duarte, Herrgård and Palsson, 2004; Feist *et al.*, 2007; Mo, Palsson and Herrgård, 2009; Swainston *et al.*, 2016). As a result, fewer reactions contribute to the metabolite pools in each compartment, and certain metabolites become compartment-specific. These structural modifications indirectly add constraints to the model because the exchange of metabolites between compartments is only possible through the added transport reactions (Savojardo *et al.*, 2018; Broddrick *et al.*, 2019).

Advances in knowledge driving a net expansion of scope—The accumulation of new legacy knowledge in the literature drives the need to update GEMs (Figure

2B). As knowledge specific to the organism increases, new pathways and subsystems are added, cofactor specificity is refined, additional content is pooled from expanded reference network databases, Gene-Protein-Reaction rules (GPRs, which link genes to the metabolic transformation that they participate in) are updated, new genome annotations are incorporated (along with a mapping to previous annotations), existing pathways are completed, reaction reversibility is revised, and erroneous content is removed. Frequently, added pathways are organism-specific (such as vitamin and fatty acid biosynthesis) highlighting the metabolic particularities of the cell and contributing to an increased breadth of modeled metabolic networks. Advances in knowledge also enable an expansion of scope. For example, reactive oxygen species production (Brynildsen *et al.*, 2013), host-microbiome interactions (Heinken and Thiele, 2015b), mammalian secretory pathways (Uhlén *et al.*, 2019; Gutierrez *et al.*, 2020), and strain-specific metabolic variations such as O-antigen biosynthesis in Gram-negatives (Seif, Monk, Machado, *et al.*, 2019) are more recent add-ons to metabolic networks.

Increasing organism-specificity—It is important to be aware that GEMs at early stages of development tend to have nonorganism specific information (even after a first round of curation). Just like the discovery of novel molecular mechanisms is not *ex nihilo* and relies on the available pool of knowledge in other species, the generation of GEMs relies on previously reconstructed networks which were originally tailored for different organisms. For instance, while there may be sufficient evidence supporting the addition of a metabolic transformation, details of said transformation may only be known in another (sometimes unrelated) organism. It is likely that there are variations in these processes across species, but such variations may simply remain unknown at initial stages (Dobson *et al.*, 2010; Aung, Henry and Walker, 2013; Seif, Monk, Mih, *et al.*, 2019). Therefore, GEM maturation endeavors involve the modification or removal of content as new evidence comes to light that ultimately increases organism-specificity (Figure 2B). For example, bacteriaspecific reactions participating in maltose metabolism were only detected and removed from the latest *C. elegans* GEM (Witting *et al.*, 2018), mammalian-originating free fatty acids were only removed from the latest *S. cerevisiae* model (Aung, Henry and Walker, 2013), and the glyoxylate shunt, ubiquinone biosynthesis, and vitamin K1 biosynthesis was only removed from the latest *S. aureus* model (Seif, Monk, Mih, *et al.*, 2019). Increasing organism-specificity is clearly shown in a multiple correspondence analysis plot of the content of curated GEMs by Monk et. al (Monk, Nogales and Palsson, 2014), in which more recently updated *E. coli* GEMs are located further away from the center of the plot.

Network-structure driven improvements—Flux variability analysis of a network can enable the identification of blocked reactions (*i.e.,* reactions which cannot carry flux under any condition). The incapability of a reaction to carry flux can be caused by any one of two structural features: 1) a participating substrate or upstream precursor has no producing reactions (root no-production or orphan metabolite), or 2) a participating or downstream product has no consuming reaction to allow it to leave the system (root no-consumption or dead-end metabolite) (Orth *et al.*, 2011). These instances highlight gaps in the network that can be manually subdivided into knowledge gaps and scope gaps. Knowledge gaps occur due to limitations in our knowledge of the cellular metabolic capabilities and can lead to model-driven hypothesis generation. Scope gaps feature limitations in the scope of the GEM. Their identification has guided model modifications and improved network connectivity (Dobson *et al.*, 2010; Orth *et al.*, 2011). Key advances in gap filling algorithms leveraging various types of "omics" data, including gene essentiality and transcriptomics profiles, can accelerate this process (Kumar, Dasika and Maranas, 2007; Hosseini and Marashi, 2017; Joshi *et al.*, 2020).

Increasingly informed modeling assumptions—Multiple modeling assumptions are made *de facto* across all GEMs. In order to quickly identify areas of improvement, it is important to get acquainted with where they occur. Any modification to the network made in order to achieve an objective *in silico* constitutes a modeling assumption. One commonly chosen objective is maximizing yield, *i.e.* the rate of production of biomass precursors defined *in silico* as the flux through the "biomass objective function". It is designed by the modeler and contains a set of chosen biomass precursors in specific proportions. With this addition, modelers are assuming that a cell's objective is to proliferate, which may work well in some cases (cancer cells, bacterial cells) but less so in others (terminally differentiated neurons). Modeling assumptions thus include the biomass objective function, gap filling reactions, demand and sink reactions (often added due to network structure impeding biomass production), and ATP maintenance reactions (which take into account the consumption of ATP as part of non-metabolic processes). Biomass objective functions (BOFs) can have three levels of modeling detail: 1) basic, 2) intermediate, and 3) advanced (Feist and Palsson, 2010). Initial GEMs tend to have basic level BOFs in which only major macromolecular categories (RNA, DNA, proteins, and lipids) are represented (Lachance *et al.*, 2019). They are often organism-unspecific and are later refined and expanded to include metabolites which are more specific to the organism of interest (vitamins, cofactors, inorganic ions, and membrane components), as well as energy drainage formulations to account for protein, DNA, and RNA polymerization (Figure 2C) (Duarte, Herrgård and Palsson, 2004; Dobson *et al.*, 2010; Seif, Monk, Mih, *et al.*, 2019; Széliová *et al.*, 2020). Sometimes, multiple BOFs are added to represent different cellular states, or to model the effect of stressors such as drugs and antibiotics (Heavner *et al.*, 2012; Sahoo *et al.*, 2015; Seif, Monk, Mih, *et al.*, 2019). BOFs are increasingly being replaced with "metabolic tasks," especially across GEM specialization efforts (Agren *et al.*, 2014; Pandey and Hatzimanikatis, 2019; Richelle *et al.*, 2019). "Metabolic tasks" are defined by Thiele et. al as "non-zero flux[es] through a reaction or through a pathway leading to the production of a metabolite B from a metabolite A" (Thiele *et al.*, 2013). Reactions and genes with a confidence score of 0 can also be considered as modeling assumptions and classified as prime candidates for review until experimental validation emerges. Genes and associated reactions are given a low confidence score when their inclusion is based purely on genome annotations, when they are not evaluated, or for modeling purposes (Thiele and Palsson, 2010a).

Novel technology driven improvements: genome-wide knockout mutant screens—The advent of new technologies has enabled new types of observations to be used for network refinement. When the first set of GEMs were published, gene essentiality datasets were scarce. New datasets progressively emerged or were generated for the specific purpose of validating genome-scale metabolic models (Feist *et al.*, 2007). The most pristine datasets constitute screens of complete gene knock-out mutant fitness profiles grown in a multitude of defined (preferably minimal) media (Monk *et al.*, 2017). Consequently, gene essentiality, nonessentiality, and conditional essentiality can be measured and compared directly with modeling simulations. Observed inconsistencies have brought about model corrections, highlighted knowledge gaps, and later guided the discovery of underground metabolic functions (Guzmán *et al.*, 2015, 2018, 2019). Gene essentiality datasets for complex organisms only started appearing as technologies for gene silencing and gene editing progressed. For instance, the Mouse Knockout project was announced in 2004 and launched in 2006 (Austin *et al.*, 2004) and the results were later incorporated to guide *Mus musculus* GEM updates in 2010 (Selvarasu *et al.*, 2010). Similarly, the advent of CRISPR-cas9 technologies enabled the systematic screening of tens of thousands of loss-of-function lesions affecting gene copies across multiple human cell lines, including tumor cell lines (Wang *et al.*, 2014, 2015; Blomen *et al.*, 2015; Bartha *et al.*, 2018). Initial efforts have integrated CRISPR screen results for the purpose of validating a reconstruction (Chandrasekaran *et al.*, 2017; Richelle *et al.*, 2019). However, we believe that these datasets have yet to reach their full potential in the context of human GEM reconstruction, representing an important opportunity to advance our tools and knowledge of human and cancer metabolism through the maturation of specialized GEMs.

Novel technology driven improvements: ‘omics’ data—Technological advances in profiling extracellular and endo-metabolomes have enabled the quantification of GEM metabolome coverage. Combined with manual curation and machine learning, the comparison of *in silico* modeled metabolites against compendia of metabolomics data enables: 1) the discovery of missing pathways (Costello and Martin, 2018; Toubiana *et al.*, 2019); 2) model corrections; and 3) the identification of potentially misidentified metabolites (Brunk *et al.*, 2018; Witting *et al.*, 2018). For example, extracellular metabolomics data guided the addition of secretion pathways in *S. cerevisiae* (Mo, Palsson and Herrgård, 2009) and served as a validation tool for context-specific metabolic networks (Choudhary *et al.*, 2016). Similarly, proteome-wide subcellular localization analysis is now possible using massspectrometry, and is increasingly applied to GEMs across the tree of life (Sun *et al.*, 2009; Seaver *et al.*, 2014; Jadot *et al.*, 2017; Orre *et al.*, 2019). Recent reconstruction efforts make use of this added information to identify which compartments to model and the reactome content of each compartment (Seaver *et al.*, 2014). Transcriptomics datasets have served to confirm the activity (or lack thereof) of a pathway and offer insights into the validity of network gaps (Hadadi *et al.*, 2019). Crystallized protein structures have guided the distinction between true and false isozymes in the context of gene essentiality(Broddrick *et al.*, 2016), and when integrated with GEMs, have pinpointed essential metal cofactors to be added to the biomass objective function(Seif, Monk, Mih, *et al.*, 2019). In addition, information gleaned from the primary, secondary, and tertiary protein structures is increasingly utilized to predict subcellular localization (Savojardo *et al.*, 2018). Finally, an increasing synergy between machine learning and metabolic modeling (Cuperlovic-Culf, 2018; Zampieri *et al.*, 2019) is enabling the rapid advances that have pushed the field of machine learning forward to spill over into the field of metabolic modeling.

Enriched object associated information—With the expansion and increasing specialization of knowledge bases it has become crucial and more common to enrich all objects included in GEMs with publicly available database-linked cross-references and identifiers. An especially time-consuming step in both GEM inception and maturation is the conversion of various identifier namespaces into a single consistent namespace. Adding metabolite, reaction, and gene identifiers simultaneously facilitates downstream reconstruction efforts as well as high-throughput 'omics' data integration. Crossreference databases such as MetaNetX increasingly serve as a tool to facilitate identifier mapping (Moretti *et al.*, 2016) by pulling content from over 20 databases. Protein structures are a recent addition to GEMs, constituting a fourth modeled instance (in addition to genes, reactions, and metabolites). They have enabled the examination of systems-level effects of single nucleotide polymorphisms (Brunk *et al.*, 2018). A recent package enabling automated enrichment of GEMs with protein structures (Mih *et al.*, 2018) has also made it possible to systematically identify the most recent literature relevant to the model organism because structures are predominantly linked to publications (Berman *et al.*, 2000).

Increasingly structured collaborations—As the field of GEM reconstruction matures, there is a growing realization that collaborative reconstruction efforts yield GEMs of higher quality. Owing to the multidisciplinary characteristic of GEM development, the involvement of experts specialized across various fields of study is needed. To encourage participation and facilitate cross-disciplinary collaborations, software and web servers have been recently developed (including RAVEN 2 and MetExplore) and used as tools during reconstruction jamborees. The range of functionalities provided includes collaborative development through online platforms, version control (supporting easy tracking of modifications), and the integration of 'omics' datasets with the model. MetExplore is designed to stimulate the involvement of end-users with limited computational skills by offering an all-in-one online workspace (Cottret *et al.*, 2018). In addition, their platform introduces the innovative idea of a voting system, allowing annotators to voice their opinion regarding the accuracy of each element of a GEM through an approval/disapproval rating system. In turn, votes can be easily queried by any collaborator. For the more experienced computational biologist, the use of GitHub repositories and live notebooks is increasingly accepted as the standard of practice for the development of so-called community models (Lieven *et al.*, no date; Wang *et al.*, 2018; Lu *et al.*, 2019; Robinson *et al.*, 2020). With the reproducibility of modeling and reconstruction efforts becoming a concern, GitHub offers easy tracking of modifications, a feature which is now increasingly attractive for GEM developers. In line with this trend, GEMs have made their appearance in open source repositories, such as BioModels and Zenodo, in which version control and cross-referencing to GitHub repositories and publications are supported. Going forward, communities focused on specific target organisms may develop 'crowd-sourcing' online forums to systematically harvest new knowledge and incorporate it into a consensus reconstruction. Crowd-sourcing is further enhanced by the recent establishment of standardized and consistent quality metrics and model validation tests (memote) (Lieven *et al.*, 2020).

Bottleneck 2: content removal

One overlooked and underappreciated but highly time-consuming step in the GEM life cycle is the manual removal of content (Seif, Monk, Mih, *et al.*, 2019). Both content addition and removal constitute important forms of knowledge for GEM reconstruction. Additions occur first, and algorithms designed to assemble metabolic networks attempt to annotate as many genes and reactions as possible. By lowering the threshold for sequence homology, comparing protein domains and sequence signatures (often mapping to multiple functions), and mapping content from distantly related organisms, the number of annotated metabolic genes can be artificially increased. All of these parameters can be fine-tuned but lean towards inclusion.

Reference databases: Content removal occurs naturally at every stage of the GEM life cycle. Before annotations are retrieved for automated draft reconstruction assembly, the reference network databases are themselves constantly undergoing updates. These updates include expansion to new pathways and to new species. When novel species are annotated, existing pathways that are annotated for other species are imported necessitating subsequent pruning of overly generous imports. In addition, as more pathways are added, older pathways are reviewed and improved, motivating the removal of redundant entries and lower confidence content. This process is explicitly mentioned for the updates of Metacyc (Bairoch and Apweiler, 2000; Karp *et al.*, 2002) and Uniprot (O'Donovan *et al.*, 1999; Wieser, Kretschmann and Apweiler, 2004; UniProt Consortium, 2019). For example, a total of 595 pathways have been removed from Metacyc since 2003 (Karp *et al.*, 2002; Krieger, 2004; Caspi *et al.*, 2008, 2015, 2018), with pathway removals reaching as much as 42% of additions in 2009 (Figure 3a). In total, almost as many (82%) pathways were removed from the Metacyc database as of 2017 as the total number of pathways that was present in 2003 (with a concurrent 524% increase in size).

De novo draft reconstruction: The process of *de novo* draft reconstruction assembly is also heavy in content pruning. For example, there was a significant number of manual removals in the assembly of 773 reconstructions of gut microbes (AGORA) (Figure 3b) (Magnúsdóttir *et al.*, 2017). The AGORA reconstructions were built by first automatically generating draft reconstructions using ModelSEED, which pulls the annotations from a library of manually curated subsystems and protein families. Manual and semi-automated curations were subsequently made to improve the quality of the reconstructions. In this process, an average of 3.7% of the content was removed from each reconstruction (with a concurrent 12.7% increase in model content).

GEM maturation: More superfluous content is added when converting the metabolic reconstruction to a computable format (a process which we break down in the subsequent section). As models are updated, the superfluous content is pruned out. GEM maturation efforts for *E. coli (Reed et al., 2003; Feist et al., 2007; Orth et al., 2011; Monk et al., 2017)*, *H. sapiens* (Duarte *et al.*, 2007; Thiele *et al.*, 2013; Swainston *et al.*, 2016; Brunk *et al.*, 2018) and *S. cerevisiae (Duarte, Herrgård and Palsson, 2004; Mo, Palsson and Herrgård, 2009; Heavner et al., 2012, 2013; Aung, Henry and Walker, 2013; Lu et al., 2019)* reflect the content fluctuation observed in the Metacyc database (Figure 3c). For example, the total number of metabolites removed by the third *H. sapiens* update amounts to a total of 60% of the number of metabolites originally included in the Recon 1. The active community of *S. cerevisiae* modelers update the yeast network more frequently, with multiple updates involving namespace switches (*i.e.*, the convention used to uniquely identify model instances). In Figure 3c, we follow one of the yeast sequels going through five updates, from iND750 to yeast 7. In total, 40% of the genes included in iND750 are removed.

GEM specialization: Finally, data-driven reductive workflows such as the construction of hundreds of strain-specific models of *Salmonella* led to the removal of between 2.3% and 13.2% of the total number of modeled instances (Figure 3d)(Seif *et al.*, 2018). Similarly, 53.5% and 71.6% of the starting model is removed in subsequent tissue-specific and disease-specific human metabolic models generated through the mCADRE workflow.

A framework for the removal of content

When curating GEMs, a significant amount of time is dedicated to identifying what content should be removed. While content addition is expedited by increasingly larger reference network databases and automated workflows, content removal is still a heavily manual task, performed silently on the back end of all reconstruction efforts. As a result, some of these efforts are duplicated across groups. For example, following its undocumented removal at iteration *i*, a reaction may be re-introduced at iteration *i+1*, because it still fits the thresholds set by automated reconstruction algorithms. The range of evidence that supports the absence of a capability is highly diverse in type and confidence level. Unfortunately, the removal of content is not usually saved in any format, constituting a loss of information that could be used to inform other model building efforts and boost reproducibility. The emergence of version control is likely to minimize this loss. However, such documentation is not sufficiently structured.

We thus propose a framework to enhance structured continuity in content removal as well as a confidence level scoring scheme for deleted content. When a reaction is added to a metabolic reconstruction, it is saved in a stoichiometric matrix (S) which mathematically encodes the participating substrates and products and the corresponding stoichiometries, and is assigned a confidence score. We propose that when a reaction is removed from a network, it should be likewise saved in an analogous matrix, which we henceforth call the recall-Reaction matrix (rR matrix). The rR matrix is structured similarly to the stoichiometric matrix, with rows representing each metabolite’s mass balance, and columns representing each reaction’s stoichiometry. The removal of a gene from a gene-reaction rule is also highly informative. Such removals can be saved in the recall-Gene matrix (aG matrix), with the rows representing the genes, the columns representing the reactions, and binary entries (with ‘1’ representing exclusion, and ‘0’ signifying no action). Each entry of the rR and rG matrices should be accompanied by a confidence score, a reference, and ideally a note, justifying the reason for exclusion.

We therefore outline a confidence scoring scheme which inversely mirrors the framework established by Thiele et al (Table 1), thereby offering an expanded continuity in confidence levels associated with modeled objects (Thiele and Palsson, 2010a). In our scheme, entries with the lowest confidence scores are more likely to be transferred between the two matrices. Conversely, entries scoring higher confidence values have a lower probability of being transferred at each iteration. It is more challenging to experimentally confirm the absence as opposed to the presence of a metabolic capability. However, such knowledge exists, and can be converted into a set of notes, recommendations, and warnings encoded in the rR and rG matrices (Table 1). Mathematically structured content removal has the advantage of being computationally tractable and easily accessible by the broader non-computational community. Such matrices can be easily converted into both human readable spreadsheets as well as modeling objects. They can be expanded in subsequent GEM maturation efforts and can also easily be checked against novel low confidence additions for redundancies, thereby avoiding duplication of efforts in content removal. Because they are expanded at each iteration, rR and rG matrices contain a blueprint of model modifications allowing easier reproduction of model updates. Finally, the rR and rG matrices can serve as an additional input to automated reconstruction tools when modeling novel species. For example, such tools could pool knowledge obtained from the rG and rR matrices with computed phylogenetic relatedness, pathway homology, and genome architecture similarities to extract higher confidence entries.

The recall-Reaction matrix: There are multiple conceptual shortcuts and general rules that can be applied to identify candidates for the rR matrix (Figure 4). A prime example is that of duplicate entries (confidence score of 4). Duplicate reactions and pathways are commonly found under different identifiers or with slight variations to the reaction content (Figure 4a). Reactions that are identical except for their thermodynamic reversibility (one being reversible and the other irreversible) are duplicate reactions. Such cases may arise due to uncertainty with regard to reaction directionality and tend to induce infeasible flux cycles. If reaction directionality uncertainty persists, the duplicate reactions should be replaced with a single reversible reaction (Thiele and Palsson, 2010a). Similarly, reactions with identical content, save for stoichiometric coefficients, are duplicate reactions and should be replaced with the entry with the highest confidence score. With increasing compartmentalization complexity, reactions can inadvertently be added to multiple compartments when they should only be contained in one. Duplicate metabolite entries also contribute to reaction redundancy and can be resolved once duplicate metabolites are identified. Oftentimes, duplicate reactions have identical GPRs, and this feature could serve to identify possible candidates. These issues affect both GEMs and reference network databases as suggested by Altman et. al (Altman *et al.*, 2013).

A second category of rR reactions constitutes metabolic capabilities which simply fall outside of the metabolic capabilities of the organism of interest (Figure 4b). The identification of reactions in this category usually results from literature review. With higher taxonomic ranks, pathways that should be excluded are more easily filtered out by current annotation platforms. For example, chlorophyll biosynthesis is predominant in the Plantae reactome but rare in the Animalia reactome. Knowledge of differences at lower taxonomic ranks (e.g., species-level) exists but can be more challenging to identify. Despite these known differences, as a result of running automated reconstruction workflows, pathways from unrelated organisms can get added to draft GEMs. For example, unlike *Bacillus licheniformis, Bacillus subtilis* lacks a glyoxylate shunt as evidenced by its inability to grow on C-2 carbon sources (confidence level 3, indirect biochemical evidence) (Kabisch *et al.*, 2013). Subtle variations in cofactor usage across organisms are only reflected in models when sufficient manual curations have been performed. They have been shown to affect model-predicted thermodynamic favorability and energy cost (Du *et al.*, 2018). For example, in *S. aureus*, two haem biosynthesis pathways were originally modeled; one using oxygen and the other S- adenosyl- L- methionine as a cofactor. However, recent evidence showed that *S. aureus* encodes an oxygen-independent transitional pathway, prompting the removal and replacement of both pathways (confidence score of 4, direct biochemical evidence) (Lobo *et al.*, 2015; Seif, Monk, Mih, *et al.*, 2019).

Promiscuity of gene associations is a hallmark of weak curation coverage (Figure 4c). When a single gene is associated with multiple reactions, reactions can be automatically flagged for review. Promiscuity of GPRs can arise due to annotation uncertainties, but they can also have a biological basis (e.g., substrate transporters or fatty acid biosynthesis). As more literature evidence comes to light, a subset of these reactions tends to be removed (confidence score of 4, direct biochemical evidence). The network structure can also serve to inform content removal efforts. For example, reactions with promiscuous gene assignments tend to contain multiple dead-end metabolites. GPR promiscuity can result from relaxed constraints on homology searches, or from searches encompassing a large breadth of distantly related organisms.

Reactions originating from modeling assumptions constitute a fourth category of rR reactions (Figure 4c). They technically are part of the model but not the reconstruction, and include sink, demand, and gap-filling reactions. Their addition usually fulfills a modeling purpose. However, when sufficient advances in knowledge enable their removal, they should be added to the rR matrix. For instance, the first *S. aureus* biomass reaction was adopted from the *B. subtilis* GEM which was itself partially imported from the *E. coli* GEM (Oh *et al.*, 2007). Because spermine was a precursor of the biomass objective function, a gap filling spermine biosynthesis pathway was added, with a subset of reactions being assigned erroneous GPRs. Current literature indicates that compared to *B. subtilis*, *S. aureus* lacks a polyamine biosynthesis capability and produces no spermidine or any of its precursors (confidence score of 3). In addition, alternative molecular functions of the associated genes were characterized, further supporting the removal of the pathway (confidence score of 4) (Townsend *et al.*, 1996; Joshi *et al.*, 2011).

The recall-Gene matrix: One of the hallmarks of draft reconstructions is the occurrence of GPRs with a large number of locus tags linked by the OR rule marking enzymes with redundant functions (with extreme cases containing as many as 10 “isozymes”) (Figure 4d). Extended OR rules skew transcriptomics data-integration methods which rely on the boolean encoding in the GPR to tailor the starting GEM. When a reaction is catalyzed by multiple isozymes, flux bounds are only changed when similar expression changes are observed across all genes. It should be noted that some cases with extended GPRs have a biological basis. For example, when considering underground metabolism, substrate promiscuity contributes to an extended GPR (Guzmán *et al.*, 2015, 2019).

Uncertainties in functional annotations occur across all genome annotation platforms and can be propagated through metabolic reconstruction platforms (Figure 4d). If left untouched, downstream analyses can be heavily skewed, and errors further propagated to specialized GEMs. For example, a non-metabolic gene erroneously annotated as a nutrient transporter may be upregulated under a specific condition. Following established transcriptomics data integration workflows, modelers may choose to artificially up-regulate the associated reactions based on the given data, which will incorrectly lead the model to predict that substrate uptake is increased. Indeed, this observation emphasizes the importance of using comprehensive annotation databases with both known metabolic and known non-metabolic genes as a basis for automated reconstruction workflows in order to avoid mis-assigning non-metabolic genes to a metabolic function. In addition, we suggest that homology searches be adjusted according to the availability of curated GEMs, with stricter thresholds when closely related well-annotated organisms are available (*e.g.*, 80% is used for strains of the same species (Seif *et al.*, 2018; Norsigian *et al.*, 2019)).

A second category of rG genes includes down-regulated genes in GEM specialization efforts. While a subset of computational approaches result in modified reaction constraints (e.g., eFlux (Kim *et al.*, 2016), Prom (Chandrasekaran and Price, 2010), Gimme (Blazier and Papin, 2012)), other approaches push for the removal of downregulated genes and associated reactions (e.g., mCADRE). In this case, removed content can be transferred to the rG and rR matrices and assigned a confidence level of 2 (indirect evidence from single data type). A third category of rG genes includes any genes affected by loss of function mutations (Figure 4e). A range of genetic mechanisms can indicate loss of function, including SNPs leading to early in-frame stop codons or frameshifts, large in-frame deletions/insertions, and mutation of the start codon. A strain may encode all genes participating in a linear pathway for the synthesis of a biomass precursor, but still lack the capability to produce that precursor, or produce a modified precursor due to loss of function events. For instance, *Salmonella* strains of serogroup O:2 carry the same O antigen biosynthetic gene island as O:9 strains, except for a frameshift mutation in *tyv* which abrogates the CDP-paratose 2-epimerase metabolic step, and results in a modified downstream metabolic pathway and a modified O-antigen structure (confidence score of 4, direct biochemical evidence)(Seif, Monk, Machado, *et al.*, 2019). Similarly, despite carrying the full *cap* locus, strains of USA300 and USA500 clonal lineages of *S. aureus* are incapable of producing a capsular polysaccharide due to a single nucleotide indel causing a frameshift mutation in *capD (Boyle-Vavra et al., 2015)*. Combining multiple fields of study with metabolic modeling can reveal candidate rG genes. For example, comparative genomics and evolutionary genetics together can reveal gene deletion events. Combining mutagenesis results with comparative genomics can point out single nucleotide polymorphisms causing loss of function. Analysis of structural modifications can identify possible deleterious mutations.

Content removal transforms the constraints on the solution space

There are multiple ways in which content removal or lack thereof affects the quality of a GEM. Adding complete pathways to an existing network creates additional solution space. Conversely, removing content (reactions and metabolites) removes dimensions and degrees of freedom and results in a reduction of the set of feasible steady-state flux solutions.

In the dummy pathway shown in Figure 5a consisting of five reactions and three metabolites, deleting reaction 4 results in the modification of two stoichiometric constraints (*i.e.*, the upper and lower bounds of the reaction) or, equivalently, the addition of the null constraint for the flux through reaction 4. Thus, only one pathway (p2) can carry flux. If the removal of reaction 4 is supported by literature evidence, then one of two possibilities hold: there is either a gap in the network or a new gap in knowledge for a mechanism enabling the consumption of metabolite 3.

While reaction 3 is blocked, reaction 2 becomes essential. The number of essential reactions is an indicator of biological redundancy encoded in the organism's genome. A lower number of essential reactions indicates that the organism has evolved alternative metabolic routes as a survival strategy. Superfluous content drives the number of essential reactions down by creating artificial redundancies. Despite having a larger total number of modelled reactions, updated models tend to have a larger number of essential reactions than the precursor GEM. Recent updates for *M. tuberculosis*, *P. putida,* and *S. aureus* all show this trend (Figure 5b). As a result of removing alternative pathways, the connectivity of the network is altered, forcing non-zero flux through a subset of reactions. This property is leveraged to build context-specific models in which reactions are either removed or constraints modified in accordance with an "omics" data set (Opdam *et al.*, 2017; Zampieri *et al.*, 2019).

One of the hallmarks of insufficiently curated GEMs is the presence of thermodynamically infeasible energy generating cycles (also known as futile cycles). Such cycles carry net flux at steady-state (Schellenberger, Lewis and Palsson, 2011), allow free charging of energy carriers in the absence of nutrient uptake (Fritzemeier *et al.*, 2017), yield increased biomass production, and negatively skew the predicted flux distribution following transcriptomics data integration (Wang, Eddy and Price, 2012; Machado and Herrgård, 2014). Futile cycles occur due to lack of constraints for irreversibility or the presence of erroneous reactions or cofactors. A recently published update to the *S. aureus* GEM yielded the removal of 26.5% of the starting model and the elimination of futile cycles (Seif, Monk, Mih, *et al.*, 2019). Upon reintroduction of subsets of different sizes of the removed reactions into the updated GEM, flux loops are also reintroduced (Figure 5c). Naturally, as more erroneous reactions are reintroduced, the probability of the network being capable of freely charging various energy carriers increases. For a subset of energy carriers, as few as ten added reactions are sufficient to yield their free production.

Discussion

The field of metabolic reconstruction and modeling is progressing at a steady pace. As challenges are identified, solutions banking on both theoretical and technological advances have been applied, coupled with the development of novel computational platforms and tools to overcome them. Here, we showed that GEM development can be delineated by four chronological phases: 1) inception, 2) maturation, 3) specialization, and 4) amalgamation. GEM inception is well established, GEM maturation is becoming increasingly defined, and GEM specialization and amalgamation remain as two active fields of research focused on modeling methods development. As a GEM progresses through each stage, it increases in quality and complexity, accounting for an expanded reactome and scope and a higher level of organism-specific detail. In reality, a GEM cycles through each stage multiple times, with some GEMs initially skipping one or more stages altogether depending on the whims of scientific interest. Nevertheless, when embarking on a GEM reconstruction effort, situating it within a GEM life cycle will invariable offer context and texture to both modelers and users.

We proceeded to highlight two bottlenecks for any given GEM life cycle towards reaching higher quality: GEM maturation and content removal. Interestingly, GEM maturation efforts have occurred independently across organisms but have converged towards a common set of approaches. We extracted eight characteristics of past endeavors which can serve as a starting point for future GEM maturation efforts. However, all approaches still lacked a solid framework for modification tracking, especially with respect to content removal. We thus proposed a mathematical framework in which removed content is transferred to a backup matrix which we call the recall matrices, as a strategy to circumvent duplication of effort and to mitigate the challenge of reproducing and propagating model updates. As the standard of practice is moving towards online open-source platforms for community-driven GEM development, we foresee increasing reliance on such frameworks, and in turn an increasing quality of models.

Acknowledgements:

We thank Almut Heinken and Ines Thiele for providing us with the starting draft AGORA models and Marc Abrams for reviewing this manuscript. This work was supported by NIH Grants U01 AI124316 and R01GM057089 and Novo Nordisk Foundation Grant NNF10CC1016517.

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Highlights

- The GEM life cycle delineates four steps in the evolution of metabolic models
- GEM maturation is the first rate limiting step in quality accrual
- Lack of adequate content removal hinders GEM progression

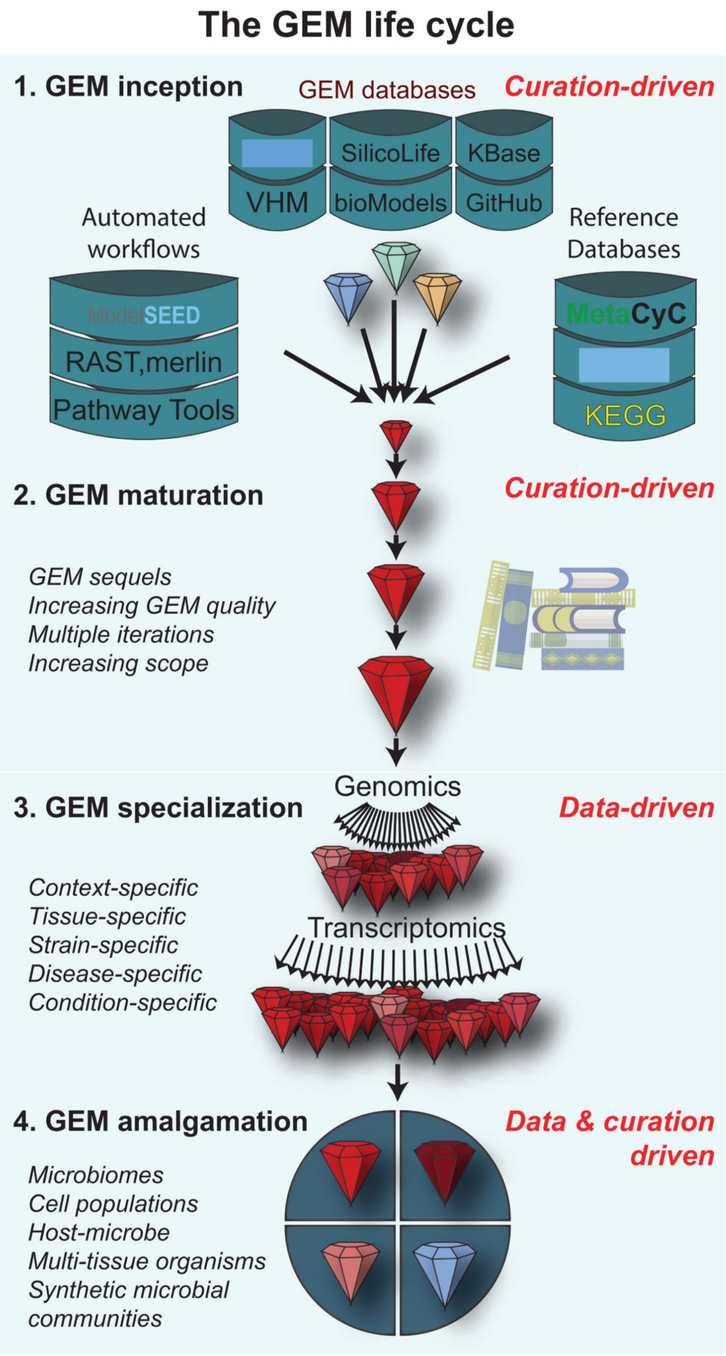

Figure 1: The GEM life cycle can be subdivided into 4 phases:

1) GEM inception; metabolic models are built for the first time by drawing from existing reference database and models and iterating through several curation cycles, **2)** GEM maturation; an existing manually curated GEM is continually updated over the years after its inception as new knowledge comes to light, **3)** GEM specialization; an existing high quality GEM is tailored to a specific strain, cell line or diseased state using an ‘omic’ data set, and **4)** GEM amalgamation; high quality GEMs are joined together to form a multi-cellular model.

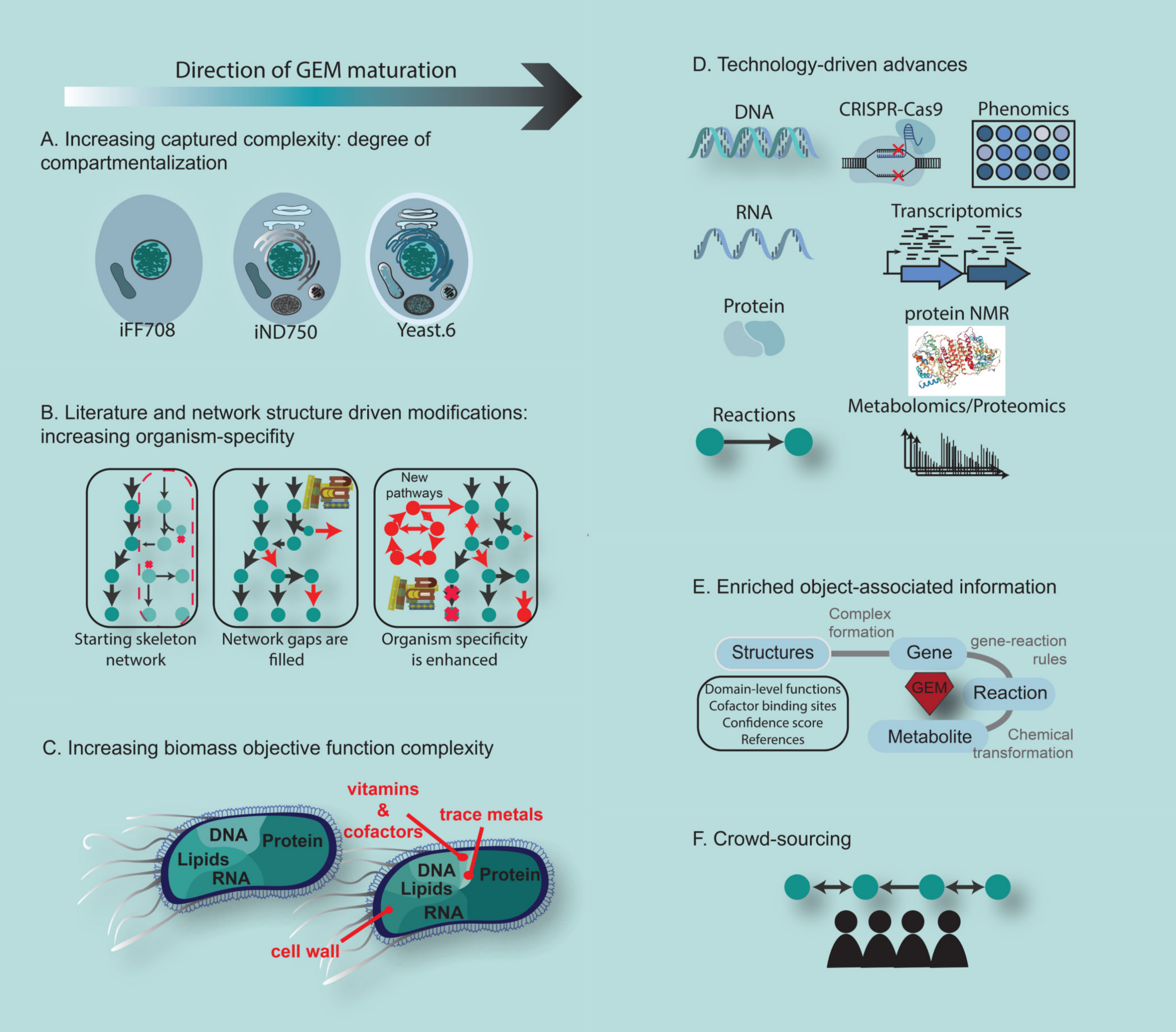

Figure 2: The eight GEM maturation characteristics:

A) increasing degree of compartmentalization; an increasing number of cellular compartments are modeled accounting for sectionalization of metabolism, **B)** advances in knowledge; as novel pathways and increased details in known pathways are uncovered, a maturing GEM reflects increasing organism specificity with network structure features driving improvements and discovery, **C)** increasingly informed modeling assumptions; as more data covering a specific organisms is generated, the biomass function of a maturing GEM increases in complexity, **D)** technology driven advances; with the emergence of ‘omics’ data, gene knockout screens, and an increasing number of Biolog phenotype microarray data sets available, the diversity of approaches to validate a model increases **E)** enriched object-associated information; with the emergence and expansion of diverse reference databases, objects in GEMs are increasingly associated with crossreferences, **F)**

crowd-sourcing; a higher diversity of expertise is used through direct (GitHub repositories, Jamborees) and indirect crowd-sourcing (sequential maturing of GEMs).

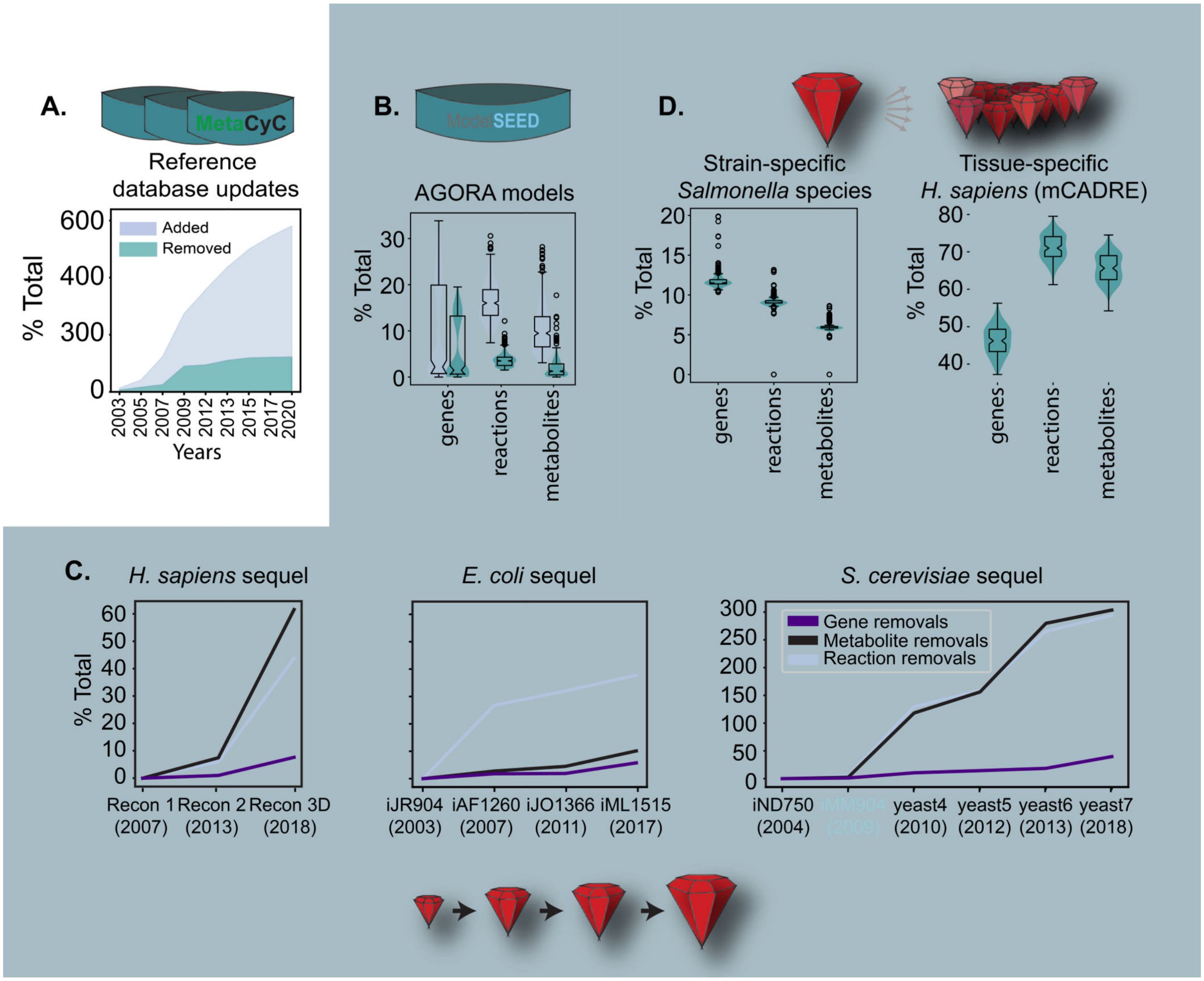

Figure 3: Content removal forms part of every stage in the GEM life cycle.

The number of removed or added instances is normalized across the plots by the total number of instances in the oldest database or model included in the analysis. **A) Reference database updates.** Fluctuations in both removed and added pathways are shown for each update going from 2003 to 2017. Numbers were obtained from the reports in the corresponding publications (Karp *et al.*, 2002; Krieger, 2004; Caspi *et al.*, 2008, 2015, 2018), **B) GEM inception.** Addition and removal of genes, reactions, and metabolites are plotted for the 773 reconstructions of gut microbes. ModelSEED draft reconstructions were obtained courtesy of Thiele et. al, and we compared their modeled content with the corresponding curated reconstructions. Counts are normalized with respect to the number of instances in the ModelSEED reconstructions. **C) GEM maturation.** Content removal occurs at every update, with genes being affected the least. Models were downloaded for *E. coli (Reed et al., 2003; Feist et al., 2007; Orth et al., 2011; Monk et al., 2017; Fang, Lloyd and Palsson, 2020)*, *H. sapiens* (Duarte *et al.*, 2007; Thiele *et al.*, 2013; Swainston *et al.*, 2016; Brunk *et al.*, 2018) and *S. cerevisiae (Duarte, Herrgård and Palsson, 2004; Mo,* *Palsson and Herrgård, 2009; Heavner et al., 2012, 2013; Aung, Henry and Walker, 2013; Lu et al., 2019)* from the BiGG database(Norsigian *et al.*, 2020), VMH(Noronha *et al.*, 2019), BioModels(Chelliah *et al.*, 2015), yeast.sf.net (Aung, Henry and Walker, 2013), and Silicolife(*Home - SilicoLife*, no date). iMM904 is shaded to mark a change of namespace which caused the sudden increase in the percent total of metabolite and reaction removals. **D) GEM specialization**. The 410 strain-specific models of *Salmonella* (Seif *et al.*, 2018; Seif, Monk, Machado, *et al.*, 2019) were obtained from the BIGG database and the 126 tissue-specific models were downloaded from Wang et. al (Wang, Eddy and Price, 2012) and we compared the content of each model with the content of the starting reference models STM.v1.0 and Human Recon 1, respectively.

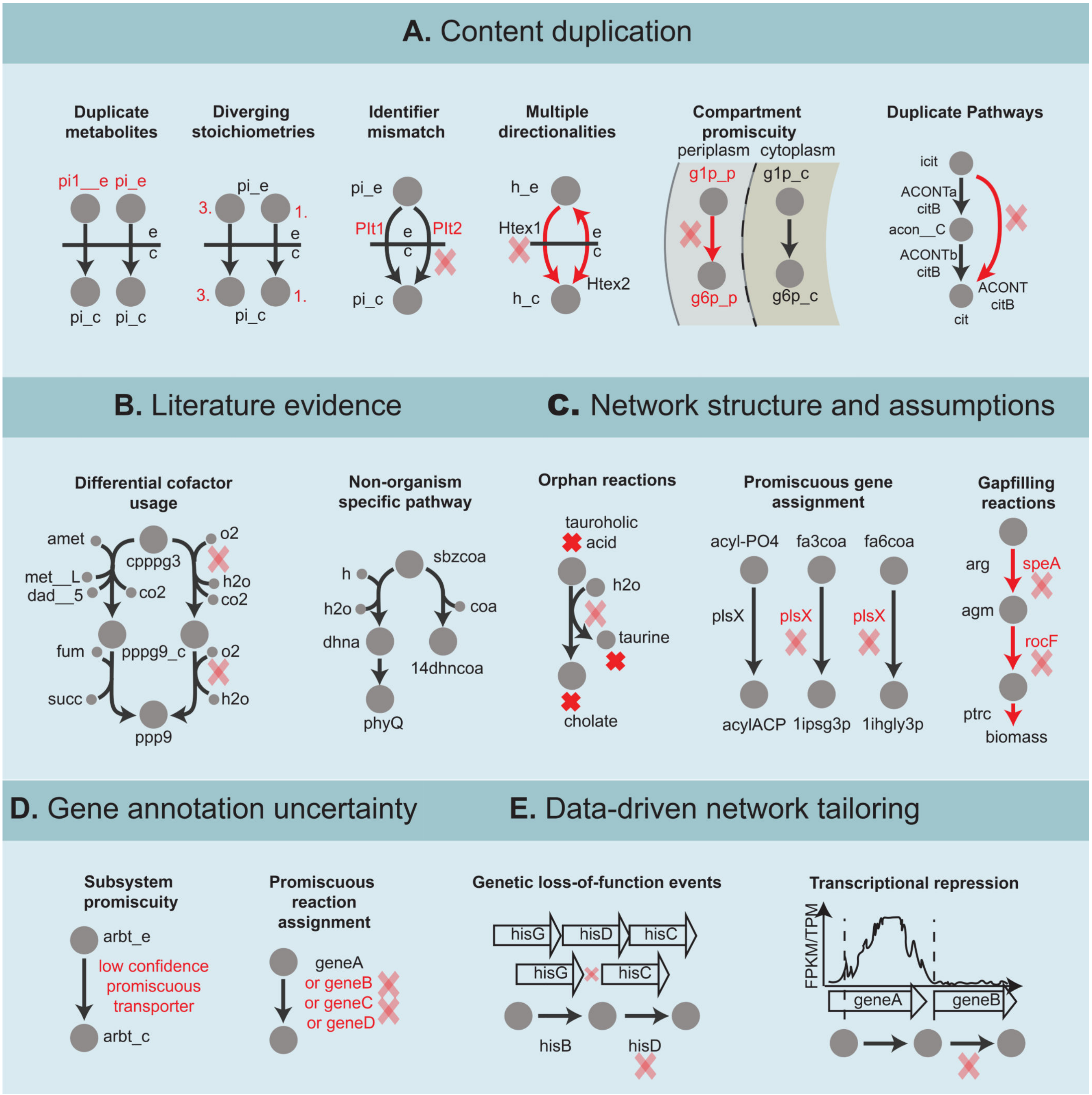

Figure 4:

Types of content removal can be subdivided into: A) content duplication. From left to right duplicate reaction caused by; inconsistent metabolite identifier (pi_e and pi__e identify extracellular phosphate), diverging stoichiometries (three phosphate molecules transported to the cytoplasm versus one phosphate molecule transported), reaction identifier mismatch (Plt1 and Plt2 are both the same transport reaction), multiple directionalities (bi-directional transport versus import of hydrogen across the outer membrane), compartment promiscuity (erroneous assignment of a phosphoglucomutase to the periplasmic compartment converting

D-glucose1phosphate to D-glucose-6-phosphate), and duplicate pathways (conversion of isocitrate to citrate *via* a two-step pathway and *via* a lumped reaction). *N.B.*, these cases serve as examples of possible content duplication but should be checked against the literature. For example, an organism could encode two phosphate transporters both of which catalyze a transport process but with different stoichiometry. B) Organism nonspecific pathways; left: example of a heme pathway initially added to the *S. aureus* GEM with incorrect differential cofactor usage due to lack of knowledge and subsequently corrected as a result of recent discoveries (Lobo *et al.*, 2015; Seif, Monk, Mih, *et al.*, 2019), right: example of a plant pathway for vitamin K1 (phylloquinone) biosynthesis which was added to the GEM of a gram-positive bacterium likely due to insufficient curation. C) Low confidence modeling assumptions. From left to right; orphan taurocholate amidohydrolase reaction annotated as being catalyzed by a C59 family penicillin amidase, promiscuous assignment of *plsX* as a phosphate acyltransferase, a isohexadecanoylglycerol-3-phosphate O-acyltransferase and an isopentadecanoyl-glycerol-3-phosphate O-acyltransferase (among others), gap filled putrescine biosynthesis pathway with erroneous gene assignment. D) Low confidence gene annotation; transport reaction assigned to a low confidence transporter, generic reaction with long “OR” based gene reaction rule, E) Datadriven evidence. Reaction catalyzed by a gene carrying a loss-of-function mutation, and reaction catalyzed by a gene that is not expressed in a cell. Abbreviations: X_c = cytoplasmic, X_e = extracellular, X_p = periplasmic, h = hydrogen, pi = phosphate, icit = isocitrate, acon__C = cis-aconitate, cit = citrate, cpppg3 = coproporphyrinogen III, amet = S-adenosyl-L-methionine, met__L = L-methionine, fum = fumarate, succ = succinate, pppg9 = protoporphyrinogen IX, o2 = oxygen, h2o = water, co2 = carbon dioxide, ppp9 = protoporphyrin, sbzcoa = O-succinylbenzoylcoA, dhna = 1,4-dihydroxy-2-naphthoate, phyQ = phylloquinone, 14dhncoa = 1,4-dihydroxy-2napthoyl-coA, fa3coa = fatty acid (Iso-C15:0)-coA, fa3coa = fatty acid (Iso-C16:0)-coA, 1ipsg3p = 1-isopentadecanoyl-sn-glycerol 3-phosphate, 1ihgly3p = 1-isohexadecanoyl-sn-glycerol_3phosphate, arg = L-arginine, agm = agmatine, ptrc = putrescine, arbt = arbutin

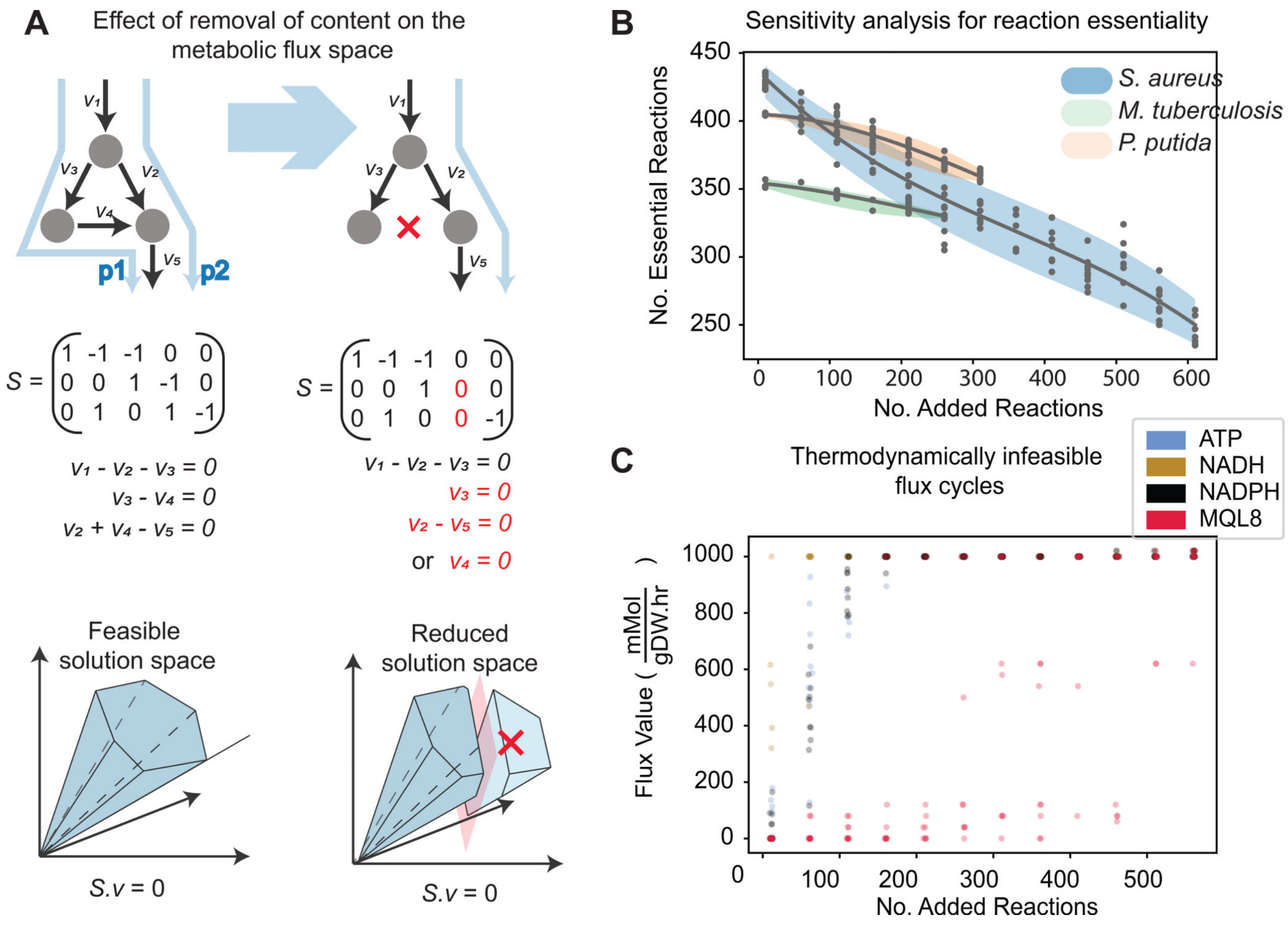

Figure 5:

Effect of removal of content on A) The solution space: We illustrate here the effect of deleting a reaction in a dummy model containing two pathways (p1 and p2). We show how the removal of reaction 4 translates to two added zeros in the stoichiometric matrix (S) and two modified constraints, **B) Reaction essentiality:** We extracted two iterations from three GEM sequels for each of *S. aureus* (Bosi *et al.*, 2016; Seif, Monk, Mih, *et al.*, 2019), *M. tuberculosis (Jamshidi and Palsson, 2007; Kavvas et al., 2018),* and *P. putida (Nogales, Palsson and Thiele, 2008; Nogales et al., 2020).* For each species, we found the set of removed reactions between the latest model and its previous version. We proceeded to re-introduce randomly sampled subsets of the deleted reactions into the latest model. We observe that as deleted content is added back to the reconstruction, fewer reactions are essential**, C) Thermodynamic consistency:** Here, we limited our analysis to the *S. aureus* GEM sequel. Similar to B, we iteratively add randomly picked subset sizes of the deleted reactions. However, as reactions are reintroduced, we simulate for the model's ability to freely produce cofactors with all nutrient exchanges blocked (Fritzemeier *et al.*, 2017). As deleted content is added back to the reconstruction, more cofactors can be freely generated in the model. Abbreviations: v1 = flux through reaction 1, p1 = pathway 1, S = stoichiometric matrix, ATP = adenosine triphosphate, NADH = reduced nicotinamide adenine dinucleotide, NADPH = reduced nicotinamide adenine dinucleotide phosphate, MQL8 = menaquinol 8.

Table 1:

Framework for confidence level scoring as part of content removal efforts in metabolic reconstructions.

Evidence type	**Confidence score**	**Examples**
Direct biochemical evidence and dereplication	4	Newly discovered biochemical reaction overriding an entry with a low confidence score (*e.g.*, through biochemical assays, resolved protein structures, etc.) (Seif, Monk, Mih, *et al.*, 2019). Removal of sinks and demands following network structure modifications brought on by new biochemical evidence. Removal of reactions associated with a single gene previously predicted to be promiscuous, when novel biochemical evidence indicates lack of promiscuity. Removal of imbalanced reactions due to lack of explicit inclusion of metabolites' formula, as new formulae are characterized and retrieved (Moreira *et al.*, 2019). Removal of duplicated content: reactions and metabolites (Zhu *et al.*, 2018)
Indirect evidence from combination of various data types	3	Removal supported by more than one 'omic' data type, for example: Discrepancies between model predictions and gene essentiality screens coupled with structural analysis, genomic neighborhood analysis, and transcriptomic data to identify false isozymes (Broddrick *et al.*, 2016). Discrepancies with gene essentiality screens coupled with metabolic modeling methods such as GLOBALFIT and updated genome annotations, or data derived genetic interaction networks (Szappanos *et al.*, 2011; Hartleb, Jarre and Lercher, 2016).
Indirect evidence from single data type	2	Indirect evidence supporting the lack of a capability, *e.g.* inability to utilize a range of nutrient sources, serotyping results (Seif, Monk, Machado, *et al.*, 2019). Updated genome annotation indicating alternative function. Transcriptomics data indicating lack of expression in a cell line (Wang, Eddy and Price, 2012).
Modeling purpose	1	Reaction with low confidence score causing significant flux alteration at the systems level and creating thermodynamically infeasible flux cycles (De Martino *et al.*, 2013).