Title: Lessons from a Minimal Genome: What Are the Essential Organizing Principles of a Cell Built from Scratch?

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A primary challenge in synthetic biology is reconstituting self-organizing systems that can undergo autonomous chromosome compaction, segregation, and cell division. Here, we discuss how the syn3.0 minimal genome sheds light on the core self-organizing principles of living cells and how these self-organizing processes can be built from the bottom up.

Biographical Sketch of the Authors:

Anthony G. Vecchiarelli grew up in Toronto, Canada and holds H.B.Sc. and Ph.D. degrees in Molecular Genetics and Microbiology from the University of Toronto. His graduate research focused on the mechanisms underlying bacterial DNA segregation. As a postdoc in Dr. Kiyoshi Mizuuchi's lab at the National Institutes of Health, Anthony developed a cell-free approach to reconstitute and visualize the bacterial systems involved in DNA segregation and cell division positioning. Anthony started his own lab in the Department of Molecular, Cellular, and Developmental Biology at the University of Michigan in January 2017. The Vecchiarelli lab uses interdisciplinary approaches to understand the mechanisms underlying subcellular organization in bacteria, with an emphasis on cell-free reconstitution and imaging of self-organizing systems. Outside the lab, you'll find him playing with his sons Ashton and Boden.

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One of the primary challenges facing synthetic biology is reconstituting a living system from its component parts. A particularly difficult landmark is reconstituting a self-organizing system that can undergo autonomous chromosome compaction, segregation, and cell division. Here, we discuss how the syn3.0 minimal genome can inform us of the core self-organizing principles of a living cell and how these self-organizing processes can be built from the bottom up. The review underscores the importance of fundamental biology in rebuilding life from its molecular constituents.

1. Introduction

Rebuilding life from its molecular constituents has been one of the greatest challenges facing biology. Reconstructing a cell can shed light on basic biological questions surrounding cellular life, such as the fundamental principles underlying cellular function and how life first emerged. Achieving this awe-inspiring feat of basic biology will also yield a number of useful biotechnological applications. In this review, we discuss the progress made on reconstituting living cells in a laboratory setting.

The diversity of life present on modern Earth and the extended variation that may be introduced with engineered cells has made it difficult to define what is meant by a "living cell." Here, we define living cells as entities that can autonomously replicate both their information-carrying molecules and the container in which these molecules reside, and that can undergo Darwinian evolution.[1] For the purposes of this discussion, we refer to any reconstituted system that satisfies this working definition of living as a synthetic cell, or SynCell.

Due to the complexity of cellular life, the first SynCells will likely resemble a stripped-down version of a modern-day prokaryotic organism (Figure 1). SynCells require a triad of features to

sustain life: metabolism, information, and self-organization (Figure 2). This review does not cover metabolism, as synthetic metabolisms will vary widely depending on the environment in which the SynCell is cultured; synthetic metabolisms have also been reviewed elsewhere.[1–3] The information component allows a cell to replicate, transcribe, and translate genetic material that can be faithfully passed down to future generations. Reconstituting the central dogma is a burgeoning area of research [4], which has also been reviewed elsewhere.[5,6] Self-organization refers to the ability of cells to sequester themselves and their genomes from the environment and coordinate efficient reactions. We restrict our discussion to self-organization and the genes that inform this feature of unicellular life.

Top-down approaches to synthetic biology can unveil minimal mechanisms of cellular growth, and as such can inform the bottom-up assembly of cells from their constituent components (Figure 1). Several groups have undertaken top down approaches to minimizing the genomes of extant bacteria, using models such as *Bacillus subtilis* and *Escherichia coli.*[7] The most successful attempt at generating a minimal genome is the J. Craig Venter Institute's syn3.0, a synthetic organism with a massively stripped-down *Mycoplasma mycoides* genome.[8] With just 473 genes, syn3.0 is the current benchmark for the minimum genetic requirements needed for cellular viability. Here, we discuss how the top-down design of the minimal genome of syn3.0 can inform the bottom-up assembly of a SynCell, wherein cellular life is built from its component parts, with an emphasis on the self-organization component of the triad of life (Figure 2). In particular, we focus on the essential systems required for the compaction of a minimal genome, its segregation after replication, and a robust cell division process that allows for reproduction. We also highlight the self-organizing systems that have already been

successfully reconstituted from purified components or show promise in a cell-free setup — a major prerequisite for using such systems in constructing a cell from the bottom up.

2. Blueprints

A hallmark of life is the possession of heritable instructions in the form of a DNA genome. A SynCell should express characteristics of the organism(s) from which its genome originated. In the interest of an engineering approach, this genome should also be minimal, where all components are essential, well-understood, and accounted for. Progress in this field has accelerated due to recent technological advances, including fast and inexpensive DNA synthesis, the ability to "boot-up," or successfully express, synthetic DNA in a host cell, and high-throughput genome engineering techniques.[9] Here, we consider a minimal genome to be one that only includes genes absolutely necessary to satisfy the requirements for a cell to be considered living.

2.1. Core, accessory, and quasi-essential genes

The genes of a truly minimal genome can be divided into two subsets: core and accessory. The core is a gene set that encodes the housekeeping functions undertaken by all life. Mushegian and Koonin used a comparative genomic method to search for conserved sequences in distantly related bacteria and found that 262 genes were shared.[10] This gene set shrinks as the scope of species widens, with 60 core genes found in common between a collection of prokaryotes and eukaryotes.[11] However, a minimal set of core genes on its own is not sufficient for life. Accessory genes are also required for viability. These accessory genes are found only in a subset of organisms, as the biological functions they encode for can be achieved in diverse ways and are

typically optimized for specific environments.[9] A prime example are metabolic genes essential for growth on a specific media.

When designing a minimal genome that supports life, an arbitrary time limit for cell division is often set to render the cell-line experimentally practical.[8,12] This is a necessary caveat as doubling time varies widely among extant bacteria.[13] Therefore, in addition to core and accessory genes, minimal genomes also contain quasi-essential genes (i.e., genes required for an organism to meet an arbitrarily set maximum doubling time, but may not be necessary if that parameter were removed). An essential step in building a streamlined genome that supports unicellular life is the identification of the core, accessory, and quasi-essential genes, and their associated functions.

2.2. The minimal genome of syn3.0

The Venter Institute has taken a top-down approach to creating a minimal genome by stripping genes from *Mycoplasma mycoides.* Mycoplasmas are ideal chassis organisms for the design of minimal genomes. Mycoplasmas typically enjoy an intracellular lifestyle and rely on their host for many functions, which has allowed them to undergo significant genome erosion (reviewed in McCutcheon and Moran[14]). The naturally minimized genomes of Mycoplasmas eliminate much of the guesswork required to pare down a genome. It is important to note that other host-associated bacteria, such as *Stammera*, have even more reduced genomes than mycoplasmas.[15] However, mycoplasmas are the smallest organisms that can be cultured in host-free media, rendering them easier to grow in the lab than many other taxa that have undergone genome erosion. Minimizing these genomes further necessitates growing these cells in highly specialized

and enriched media, which can become costly and potentially limit biotechnological applications.

In 2010, a synthetic reproduction of a modified *M. mycoides* genome was produced and transplanted into a *Mycoplasma capricolum* cell, resulting in JCVI-syn1.0, a self-replicating cell with an entirely synthetic genome. It phenotypically resembled *M. mycoides*, but contained additional watermark sequences as well as gene deletions, polymorphisms, and mutations.[16] Consecutive cycles of sequence design and genome construction were then performed on syn1.0.[8] The most recent end product is JCVI-syn3.0 where 428 genes were removed from the original *M. mycoides* genome, leaving only 473 genes remaining.

Syn3.0 has the smallest known genome of any free-living organism.[8] Among its characterized genes, nearly half are involved in gene expression (41%) and preservation (7%). Genes encoding the structure and function of the cell membrane make up 18% of characterized genes, while 17% are associated with metabolism. The remaining one-third of essential genes in the syn3.0 genome (149 genes) are of unknown function. Such a massive gap in knowledge underscores how little we understand the basic principles of life. Elucidating the functions of these essential genes is one of the main bottlenecks in minimal genome research as well as in the bottom-up construction of a SynCell.

Although syn3.0 has been called a 'minimal cell,' its 473-gene set is much larger than the 262-gene core set suggested by comparative genomics.[10] Current *in silico* research suggests many more genes could be deleted from syn3.0 while retaining viability. For example, a recent computational approach produced minimal genomes smaller than syn3.0 and identified 11 redundant essential genes.[12] Such *in silico* approaches will be key to overcoming the main

challenges associated with minimal genome design: quasi-essential genes, genetic redundancy, and the significant fraction of essential genes annotated as unknown function.[17]

2.3. The minimal gene set of self-organization

Dynamic spatial organization is crucial for faithful and efficient cellular replication. The chromosome of a SynCell will require compaction and segregation prior to division (Figure 2). The division machinery itself must also be spatiotemporally organized so that septation occurs after the partitioning of essential components and at a location that maintains this distribution. Here, we discuss minimal mechanisms for: 1) genome compaction, 2) active segregation of replicated chromosomes, and 3) spatial organization of cell division. Top-down synthesis of the minimal genome of syn3.0 informs us of what genes may be essential for the spatial organization of a SynCell built from the bottom up. Specifically, it sheds light on what genes are required for the self-organizing principles associated with chromosome compaction, chromosome segregation, and cell division. Combined, only nine genes remain in these subcategories of self-organization (Table 1).[8] This remarkably small gene set implies that bottom-up engineering of the self-organizing principles required for unicellular life is possible. Here, we discuss this gene set and how it informs the self-organization of a SynCell.

3. Compaction of a Minimal Genome

The contour length of a typical bacterial chromosome, such as that of *E. coli* at 4.6 Mb, is ~1.5 mm — three orders of magnitude longer than the cell itself (Figure 3).[18] Several physical and biochemical processes are required to compact a chromosome so that it fits into its cellular confines. To what degree is chromosome compaction required for a SynCell with a minimized,

and therefore physically smaller, genome? The syn3.0 chromosome is 531 kbp, which equates to a contour length of 180 microns. Given that the cellular diameter of syn3.0 is ~0.4 microns,[8,16] its chromosome requires at least a 450-fold compaction. Here, we discuss the physical and biochemical processes that could be used for the organized compaction of a minimal chromosome in a SynCell.

3.1. Physical mechanisms of chromosome compaction

The minimal chromosome of a SynCell needs to be compacted into a spatially ordered but pliable structure, and many physical factors can contribute to this compaction. First, polymer dynamics predicts that even in the absence of cellular confines, the chromosome will mold into a globule, providing an estimated 100-fold compaction (Figure 3).[19] Compaction is further aided by cellular confinement, macromolecular crowding of the cytoplasm, and the resulting excluded volume effects.[20–22] Finally, the DNA is supercoiled, which is essential for maintaining a compact yet accessible genome. Consistent with this supercoiling requirement, only five genes were found to be essential in the functional category of DNA topology in the minimal genome of syn3.0. These genes encode three different topoisomerases: DNA gyrase, topoisomerase I (Topo I), and topoisomerase IV (Topo IV).[8] (Figure 3 and Table 1). Topo IV relaxes positive supercoils [23] but also decatenates the circular chromosome copies following replication,[24] which likely serves an essential function in syn3.0.

3.2. Biochemical mechanisms of chromosome compaction

Aside from topoisomerases introducing DNA compaction via supercoiling, nucleoid-associated proteins (NAPs) and structural maintenance of chromosome (SMC) complexes are key

biochemical factors that play an important role in compacting and organizing bacterial genomes. NAPs are functionally analogous to eukaryotic histones in that their binding to DNA can generate kinks (i.e., IHF and HU) or bridges (i.e., H-NS) that ultimately result in chromosome compaction.[25–27] NAP homologs were not identified as essential in the genome of syn3.0.[8] However, NAPs could be hidden in the one-third of genes whose function is designated as unknown. Consistently, a more detailed bioinformatics analysis of the essential genes of unknown function in syn3.0 identified the NAP homolog HU.[17] In conjunction with these bioinformatics approaches, a survey of DNA binding proteins in syn3.0 would also serve as an excellent starting point in identifying other NAPs essential for chromosome compaction in a SynCell.

The minimal genome of syn3.0 suggests the SMC complex is of critical importance. Only three genes were found to be required in the chromosome segregation category, and their gene products (ScpA, ScpB, and SMC) make up the tripartite SMC complex (Figure 3 and Table 1).[8] Eukaryotic cells rely on a number of SMC proteins for proper gene expression, chromosome organization, genome duplication, and segregation.[28–31] All of these functions are likely the result of the co-entrapment of DNA loops within the circumference of the SMC ring.[32] In prokaryotic cells, such as *Bacillus subtilis* as well as syn3.0, a single SMC-ScpAB complex is essential for growth and survival.[8] The SMC-ScpAB complex has been shown to load onto the chromosome in a manner that compacts, organizes, and aligns the two chromosome arms.[28] Bacterial SMC complexes have also been shown to play a role in DNA replication.[33]

The underlying molecular mechanism describing how SMC complexes compact a chromosome remains elusive. Recently, however, the Dekker group provided direct visualization of an SMC complex (*Saccharomyces cerevisiae* condensin) forming and processively extruding

DNA loops in a cell-free setup using purified components.[34] This work is a significant step forward in the bottom-up reconstitution of chromosome compaction. An attractive next step would be to combine circular DNA molecules with SMC complexes and topoisomerases within liposomes. Such experiments are necessary to determine if physical processes (polymer dynamics, confinement, crowding) combined with supercoiling and SMC-based looping are indeed necessary and sufficient in the formation of a minimal nucleoid (Figure 3).

4. Minimal Systems for Chromosome Segregation

All living cells must segregate and position replicated DNA to ensure faithful genetic inheritance. A hallmark feature of a SynCell would therefore be the segregation of its replicated chromosomes to opposite sides of the cell prior to division. The small size of a bacterial cell allows passive diffusion to equally segregate high copy components such as proteins. But if an essential cellular component, such as the chromosome, is of a low copy number, active segregation and positioning are likely required. Therefore, some form of active chromosome segregation will be useful and possibly essential for a SynCell.

In prokaryotes, most chromosomes and almost all naturally-occurring low-copy plasmids encode for an active segregation, or partition (Par) system (Figure 4). While only some bacterial chromosomes may be actively partitioned by these specialized systems, low copy plasmids require active segregation machinery. Par systems are useful tools in the bottom-up assembly of a SynCell because they are self-organizing and minimalistic, encoding only three components: a cis-acting partition site on the DNA target and two trans-acting proteins (reviewed in Baxter and Funnell [35]). The partition site is functionally analogous to a eukaryotic centromere and is specifically bound by one of the proteins to form a partition complex. The second protein is an

NTPase that uses ATP or GTP hydrolysis to drive chromosome segregation. Par systems have been categorized according to whether the NTPase contains a Walker ATP-binding motif (ParA), or resembles eukaryotic actin (usually called ParM) or tubulin (TubZ) (reviewed in Gerdes et al.[36]). Other modes of segregation are emerging (reviewed in Hürtgen et al.[37]). Here, we focus on the systems that have been successfully reconstituted using purified components in a cell-free setup, as this is a critical bottleneck in the creation of a minimal DNA segregation system in a SynCell.

The ParM system has been found only on a subset of plasmids, but its polymer-based DNA segregation mechanism is well understood because of cell-free reconstitution [38] that correlates with in vivo fluorescence microscopy.[39] In vivo, actin-like polymers of ParM push plasmids to opposite cell poles through a mechanism of insertional polymerization (Figure 4A). In a cell-free setup, purified ParM polymerized and pushed apart beads that were coated with the partition complex.[39] TubZ systems have also been reconstituted in vitro[40] and have been shown to use a polymer-based mechanism that can undergo dynamic instability[41] and treadmilling.[42] Simple polymer-based mechanisms similar to those provided by ParM or TubZ are attractive options for building a minimal DNA-segregating machine for a SynCell.

ParA-based segregation systems are widespread, encoded by most bacterial chromosomes and low copy plasmids (reviewed in Baxter and Funnell[35]). Studies of ParA-based plasmid partitioning have been particularly useful in elucidating the general mechanism. In vivo, the ParA ATPase coats the nucleoid, while its partner protein, ParB, forms the partition complex on the plasmid (Figure 4B). The partition complex stimulates the release of ParA proteins from the nucleoid in the vicinity of the plasmid, resulting in a concentration gradient of ParA. Following plasmid replication, the sister copies bidirectionally segregate as they chase high concentrations

of ParA in opposite directions. This gradient-based mechanism ensures that a copy of the plasmid is inherited by both daughter cells after division. A similar gradient-based mechanism for the segregation of bacterial chromosomes has also been proposed.[43]

This gradient-based method of transport has been reconstituted in a DNA-carpeted flow cell, which served as a biomimetic of the nucleoid.[44–46] ParA was mixed with beads coated with the partition complex. As found in vivo, ParA coated the DNA carpet and its concentration was depleted in the vicinity of the beads.[45] The beads then utilized the ParA gradient on the DNA carpet for directed movement. Despite ParA systems being widespread in bacteria, the requirement of the nucleoid as a matrix for segregation is an additional layer of complexity that must be considered when choosing a DNA segregating machine for a SynCell, compared to polymer systems that are truly autonomous.

Par systems are not found in all bacterial genomes. Therefore, it can be debated as to whether a Par system would be needed for segregating the chromosome of a SynCell. In fact, syn3.0 does not have a Par system.[8] How then is its chromosome segregated? *E. coli* also lacks a Par system, but encodes for an SMC complex called MukBEF (reviewed in Rybenkov et al.[47]). It has been proposed that the extrusion of DNA from replication forks may help push the sister chromosome copies toward opposite sides of the cell (Figure 5). In combination with this extrusion, chromosome segregation may result from, or be assisted by, entropic forces.[48,49] SMC complexes could bind and condense DNA as it is being spooled out of the replisome, thereby facilitating the entropic demixing of sister chromosomes during their replication and ensuring the two nucleoids are separated to opposite sides of the cell prior to division (Figure 5). It remains to be tested whether SMC-based compaction coupled to entropic-demixing would be a sufficient mechanism for faithful chromosome segregation in a SynCell.

5. Positioning the Cell Division Machinery

Positioning copies of the replicated genome to opposite sides of a SynCell implies that division itself must be positioned at mid-cell. There are a number of positive and negative regulators that position the division machinery in bacteria (reviewed in Monahan et al.[50]). However, only one has been successfully reconstituted in a number of cell-free setups: the *E. coli* Min/FtsZ system (reviewed in Loose et al.[51] and in Mizuuchi and Vecchiarelli[52]). The Min system acts on a tubulin-like GTPase highly conserved in the microbial world called FtsZ. FtsZ polymerizes into a structure called the Z ring, which acts as a dynamic scaffold for the recruitment of downstream cell division proteins required for septation. The Min system is composed of three proteins (MinC, MinD, and MinE) that self-organize into a cell pole-to-cell pole oscillator on the inner membrane that spatially aligns FtsZ polymerization into a Z ring at mid-cell.[53] MinD associates with the inner membrane when bound to ATP.[54,55] MinE associates with MinD on the membrane[52,56] and stimulates its release.[57] The dynamic interplay between MinD and MinE on the inner membrane results in a pole-to-pole oscillation.[58] The final protein, MinC, is not required for oscillation, but associates with MinD on the membrane and inhibits FtsZ polymerization into a Z ring (reviewed in Lutkenhaus[59]). The pole-to-pole oscillation of MinD (and MinC) therefore prevents Z ring formation near the poles, promoting symmetric cell division at mid-cell.

The Schwille group has been successful in reconstituting the pole-to-pole oscillation of the entire MinCDE system in lipid-lined microcompartments that were rod shaped to mimic the geometry of an *E. coli* cell.[60] Also introduced into the cell-free setup was an FtsZ-YFP fusion protein that also encoded for a membrane targeting sequence (FtsZ-YFP-mts). The mts was required to recruit FtsZ to the membrane, bypassing the need for proteins FtsA and ZipA

(otherwise essential in recruiting FtsZ to the membrane). Strikingly, the pole-to-pole oscillation of the MinCDE system restricted the polymerization activity of FtsZ-YFP-mts to the center of the rod-shaped compartments. These findings suggest that if an FtsZ-based division mechanism were chosen for a SynCell, the MinCDE system may be a viable approach to positioning cell division at mid-cell.

6. Minimal Cell Division Systems

For a SynCell to be considered living, it must undergo cell division. There are several hurdles to building division machinery from the bottom up. Across the biotic world, cells divide in various ways, including eukaryotic mitosis, binary fission, budding, and other exotic mechanisms.[61] Regardless of mechanism, division machineries are highly complex. Eukaryotic cells typically have over 130 genes involved in division,[62] whereas prokaryotes have around 20–30 genes.[63] As expected for such an important process, there is plenty of redundancy and plasticity in division mechanisms. The variability makes it difficult to pinpoint an ideal minimal division system. However, a corollary to the plasticity of cell division mechanisms is that there are many possible ways to divide a SynCell. A full understanding of protein-based division machineries, particularly those that exist in simpler prokaryotic systems, will help us determine what is truly necessary and sufficient for dividing a SynCell. Here, we highlight the cell division genes remaining in the minimal genome of syn3.0 and discuss recent bottom-up reconstitution research that has provided promising protein-based machines for SynCell division.

6.1. FtsZ as a division scaffold for a SynCell

The most extensively studied protein involved in the division of a prokaryotic cell is FtsZ.[64] As mentioned above, FtsZ has multiple spatial regulators that allow it to coalesce in the form of a Z ring on the inner membrane at mid-cell (reviewed in Monahan et al.[50]). The Z ring: 1) acts as a scaffold for the recruitment and assembly of several additional divisome components, 2) contributes to the invagination force, and 3) organizes cell wall remodeling during septation (reviewed in den Blaauwen and Luirink[65]). FtsZ cannot bind to the membrane by itself but depends on adaptor proteins such as FtsA and ZipA that recruit FtsZ to the membrane.[66,67] FtsZ polymerization and treadmilling dynamics have been successfully reconstituted on supported lipid bilayers using its native membrane anchors FtsA and ZipA purified from *E. coli*.[68,69] When combined in liposomes, FtsZ and FtsA have been shown to form continuous helical co-polymers that provide enough of a mechanical force to constrict liposomes and generate narrow necks, but complete division events were not supported.[70]

Given the ubiquity of FtsZ in the microbial world and its extensive study, the Z ring can potentially be a useful division scaffold in a SynCell. But how to faithfully generate robust division events via an FtsZ-based mechanism in a liposome is still unclear. An important next step in reconstituting an FtsZ-based division system that is spatially regulated is to combine MinCDE with FtsZ and its native anchors FtsA and ZipA into a liposome. But FtsZ, even with its native membrane anchors, has not been shown to create consistent abscission between daughter liposomes.[70] Additional downstream factors known to associate with the Z ring are likely required for reconstituting an FtsZ-based division machine from scratch. Identifying the necessary and sufficient set of divisome components is a critical goal towards using FtsZ as a divisome scaffold in a SynCell.

6.2. Cell division genes in syn1.0 vs. syn3.0

Compared to *M. mycoides* cells (or syn1.0), syn3.0 cells exhibit a 3-fold slower doubling time (3 hours vs. 1 hour) and are polymorphic in appearance, presenting a spectrum of cell morphologies reminiscent of L-forms (Figure 6).[8] L-forms are bacterial cells that lack a cell wall and have an irregular mode of division.[71] The loss of a cell wall has been shown to induce overproduction of membrane material, causing a surface area to volume imbalance that can result in cell division.[72] Therefore, L-form division can occur independent of protein-based division machinery via irregular and erratic protrusions and budding events.[73]

The minimal gene set of syn3.0 serves as a useful benchmark in determining what is necessary and sufficient for its irregular division, and potentially, what proteins could be used for the division of a SynCell. Syn1.0 has only four genes in the cell division category, encoding four proteins: Fic, SepF, FtsA, and FtsZ (Figure 6 and Table 1).[8] Fic functions in a metabolic pathway to regulate cell division.[74] SepF has been shown to have redundant functions to FtsA in cell division, polymerizing into rings that bind and recruit FtsZ to the membrane.[75] Of these four cell division genes, only FtsA remained in syn3.0. Early iterations of the Venter Institute's minimized genome had SepF as essential and FtsA as dispensable. This essentiality flipped to FtsA in later iterations of genome design and was maintained in syn3.0, consistent with FtsA and SepF having redundant functions.[8] Remarkably, even FtsZ was no longer essential in syn3.0. This is surprising given FtsA's function in recruiting FtsZ to the membrane.[76] Whether FtsA is necessary and sufficient in generating the constriction forces needed for the division process in syn3.0 is a question that remains to be addressed.

A recent bioinformatics analysis of the essential genes of unknown function in syn3.0 unveiled a homolog of DivIVA.[17] DivIVA is involved in the later stages of division, as its

recruitment depends on the negative membrane curvature that occurs during invagination of the dividing septum.[77–79] DivIVA homologs are present in a number of bacterial phyla, including some mycoplasmas that neither produce a cell wall nor require FtsZ for viability.[80] However, mycoplasma DivIVA homologs remain uncharacterized. It is attractive to speculate that FtsA and DivIVA work together in the division of syn3.0 via a currently unknown mechanism.

It is important to reiterate that syn3.0 devolves into an L-form-like mode of division (Figure 6). The removal of FtsZ may contribute to the polymorphic appearance of syn3.0 cells, due to an irregular division process. When FtsZ was removed from *M. mycoides* in a previous study, this strain was still able to divide, but the division forces were attributed to gliding motility genes[80] which are also absent in syn3.0. Together, the findings show that there are likely multiple modes of FtsZ-less division, reminiscent of L-forms, that the first SynCells could rely on for reproduction.

6.3. Eukaryotic approaches to SynCell division

Actin is a possible candidate for SynCell division due to its dynamic polymerization, which generates strong forces within the cell, especially when coupled with myosin.[81,82] It has been shown that actin can form contractile rings when confined.[83] This experiment was performed in droplets, but it would be interesting to observe this process within a liposome. Reconstitution of a minimal actin cortex has been demonstrated on supported lipid bilayers,[84] and dynamic stretching of giant unilaminar vesicles with actin has also been observed.[85] The spatiotemporal control of actin polymerization is extremely complex, and though these advancements are fascinating, utilizing actin filaments or microtubules for the controlled and faithful division of a SynCell will be difficult. However, there is one particular system in eukaryotes that has provided

insight into how a minimal cell division system might be achieved — the endosomal sorting complex required for transport (ESCRT) system.

6.4. The eukaryotic ESCRT system and the archaeal CdvABC system

The ESCRT system is required for a variety of budding processes, including cellular abscission (reviewed in Christ et al.[86]). ESCRT is composed of five complexes (ESCRT-0, -I, -II, -III, and Vps4), along with an assortment of associated proteins. Though the eukaryotic ESCRT system is currently too complex to be used as the division machine for a SynCell, the detailed study of ESCRT has provided valuable information about an analogous but simpler system that is a promising candidate for SynCell division — the archaeal CdvABC system.

In *Sulfolobales*, an archaeal model organism, division is mediated through Cdv proteins, which are homologs of eukaryotic ESCRT proteins.[87,88] Cdv proteins are organized into two groups. The first group is encoded by *cdvA*, *cdvB*, and *cdvC* genes organized on one chromosomal locus, and the second group is encoded by three *cdvB* paralogs organized at different locations along the chromosome.[89] Current research suggests that the four CdvB genes are homologs to the eukaryotic ESCRT-III class,[89] and CdvC is a homolog of Vps4.[90] CdvA can bind to the membrane, so it is often modeled as the recruiter of CdvB to the membrane (Figure 7).[91] CdvB is proposed to be important for early stage division, and the paralogs CdvB1 and CdvB2 are suggested to have roles in cellular abscission.[92] CdvC interacts with CdvB and is essential for abscission.[93] CdvB3 is thought to be important but not essential for cell division and CdvA localization.[94] CdvB proteins form coils, and it is thought that the reduction in diameter of CdvB coils creates the constriction force for division.[95] Understanding the role of

CdvA and building a comprehensive model of this relatively simple archaeal division system is an area of active research.

The CdvABC system is a promising candidate for dividing SynCells due to its simplicity. The system is comprised only of three core proteins and three accessory proteins.[89] Though not much is currently known about this particular system, there is a significant body of work on the eukaryotic ESCRT system that helps inform our understanding of the CdvABC system. Reconstituting the CdvABC system in a liposome to induce a successful division event would mark a significant step forward in creating a robust but simple division system for a SynCell.

7. Summary and Future Directions

The design and fruition of a minimal divisome will represent a major milestone in the development of a living cell from the bottom up. Unfortunately, we still have large gaps in our understanding of the protein-based division systems that are likely to be the most amenable to bottom-up reconstitution. Syn3.0 will be a useful tool for exploring the effect of re-introducing FtsZ and other division-related proteins to observe changes in this organism's L-form division phenotype.

Liposomes will likely be the confinement material of choice for the first SynCells given their biocompatibility; however, other materials are also under study (reviewed in Spoelstra et al.[96]). While a variety of protein-based mechanisms for liposome division are in their infancy, several physical and chemical methods have already proven successful in dividing a liposome.[97] For example, microfluidics provide a reliable method for producing liposomes of homogenous size and shape.[98] Flowing these liposomes in a microfluidic device towards the sharp edge of a wedged-shaped splitter forces division into two separate liposomes.[99] But an important tenet for

life as we have defined it here is that the cell must be self-sufficient. Therefore, it can be argued that a dependence on physical or chemical mechanisms for division precludes meeting this requirement. We have, however, outlined several promising protein-based division machineries, including the bacterial FtsZ system, actin and microtubule systems, the eukaryotic ESCRT system, and its homolog in archaea, the CdvABC system.

The archaeal CdvABC system requires further study, but has enormous potential as a simple division machinery that could be introduced into liposomes. Reconstitution of robust protein-based division of a liposome will be a biological feat. Accomplishing this task will bring us much closer to creating the first SynCell. In the context of reconstituting all the essential self-organization principles of a living cell, successful integration will be a milestone achievement. Ideally, this reconstitution will involve coupling the segregation of a sufficiently compacted minimal genome to the spatially-regulated division of a liposome.

We have shown how top-down design of the syn3.0 genome can inform the bottom-up assembly of a SynCell by specifically focusing on the self-organization component of the triad of life (Figure 2). Several sobering findings are revealed in this comparison: 1) we do not know the function of one-third of the genes in the syn3.0 minimal genome, 2) many of the self-organizing systems of syn3.0 have not been reconstituted in vitro, and 3) many of the self-organizing systems that have been reconstituted in a cell-free setup are not present in syn3.0. Top-down and bottom-up strategies towards the development of a minimal form of life are powerful in their own right, but these approaches should not be siloed as the data can be complementary and informative. Progress towards building the first SynCell will accelerate a great deal when these strategies are combined and integrated. Above all, we need to elucidate the functions of the essential genes whose functions remain unknown (one-third of the syn3.0 genome). As stated by

the late Nobel Prize-winning biologist Sydney Brenner (1927-2019), "To understand how all of this works we will need something more than merely lists of components…the great difference between the telephone directory and a Shakespeare play is that, while both have a grand cast of characters, only the play has a plot."

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Conflict of Interest

The authors declare no conflict of interest.

Keywords:

Minimal Genome, Synthetic Cell, Self-organization, Synthetic Biology, Bottom-up Biology

Figure Legends:

Figure 1. Top-down versus Bottom-up approaches towards the goal of a SynCell. The top-down approach strips non-essential genes from extant living organisms. The bottom-up approach aims to build a "living" cell from purified and reconstituted functional modules.

Figure 2. The triad of life: Metabolism, Information, and Self-organization. This review focuses on self-organizing processes essential for chromosome compaction/topology, chromosome segregation, and cell division. The protein products of the genes remaining in Syn 3.0 that remain under the functional categories of DNA Compaction/Topology, Segregation, and Division are shown on the right.

Figure 3. **Chromosome compaction is required for a SynCell**. The contour length of the *E. coli* chromosome is compared to that of syn3.0. Several physical and biochemical processes are required to compact a chromosome so that it fits into its cellular confines.

Figure 4. **Polymer- and gradient-based mechanisms of DNA segregation.** (**A**) Polymer-based segregation. Actin-like polymers of ParM push plasmids to opposite cell poles through a mechanism of insertional polymerization. (**B**) Gradient-based segregation. ParA binds the nucleoid, and ParB-bound plasmid DNA (the partition complex) stimulates the release of ParA from the nucleoid. The resulting concentration gradient of ParA allows replicated plasmids to bidirectionally segregate as they chase high concentrations of ParA in opposing directions.

Figure 5. **Chromosome segregation via extrusion from the replisome, entropic demixing, and SMC-based compaction**. Extrusion of DNA from replication forks may push the sister chromosome copies toward opposite sides of the cell. Segregation may also be assisted by entropic forces. SMC complexes bind and condense DNA as it is spooled out of the replisome, thus ensuring the two nucleoids are separated to opposite sides of the cell prior to division. OriC is the origin of chromosome replication.

Figure 6. Electron micrographs comparing morphologies of syn1.0 (left) and syn3.0 (middle and right). Three cell division genes were removed from the syn1.0 genome (*ftsZ, sepF, and fic*), leaving only *ftsA* in syn3.0. Scale bars: 10 microns (top images) and 1 micron (bottom images). Image adapted with permission from Hutchison et al.[8]

Figure 7. A model for division via the archaeal CdvABC system. CdvA (red) binds the cell membrane and is thought to mediate the localization of the division structure by recruiting downstream CdvB proteins (orange). CdvB may be important for early stage division, and the paralogs CdvB1 and CdvB2 may have roles in abscission. CdvC (blue) interacts with CdvB proteins and is essential for abscission. CdvB3 is thought to be important but not essential for cell division and CdvA localization. CdvB proteins form coils, and it is thought that the reduction in diameter of these coils creates the constriction force for cytokinesis.

Table 1. Genes and the proteins they encode that remain (green) or are deleted (red) in the genomes of syn1.0 and syn3.0 for functional categories relating to the spatial organization of a SynCell.[8]

Functional category	Genes	Proteins	Syn1.0	Syn3.0
DNA topology (or compaction)	*gyrA, gryB*	Gyrase	✓	✓
	topoIVA, topoIVB	Topoisomerase IV	✓	✓
	topA	Topoisomerase I	✓	✓
Chromosome Segregation	*scpA, scpB, smc*	SMC complex	✓	✓
Cell division	*ftsZ*	FtsZ	✓	✗
	ftsA	FtsA	✓	✓
	sepF	SepF	✓	✗
	fic	Fic	✓	✗

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