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Title: Origins, evolution, and physiological implications of *de novo* genes in yeast

Running Title: *De novo* genes in yeast

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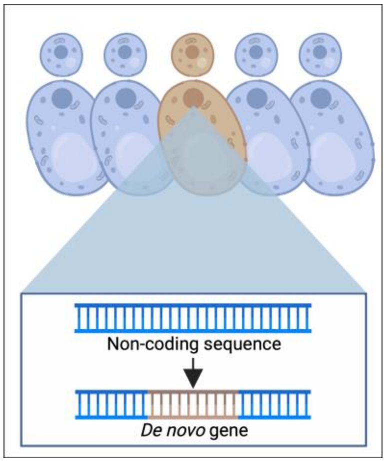
Take aways

- Several yeast genes of recent *de novo* origin play important cellular roles
- Yeasts express thousands of *de novo* sequences with unknown biology
- Yeasts are well suited to address fundamental questions about *de novo* gene birth

Abstract: *De novo* gene birth is the process by which new genes emerge in sequences that were previously non-coding. Over the past decade, researchers have taken advantage of the power of yeast as a model and a tool to study the evolutionary mechanisms and physiological implications of *de novo* gene birth. We summarize the mechanisms that have been proposed to explicate how non-coding sequences can become protein-coding genes, highlighting the discovery of pervasive translation of the yeast transcriptome and its presumed impact on evolutionary innovation. We summarize current best-practices for the identification and characterization of *de novo* genes. Crucially, we explain that the field is still in its nascency, with the physiological roles of most young yeast *de novo* genes identified thus far still utterly unknown. We hope this review inspires researchers to investigate the true contribution of *de novo* gene birth to cellular physiology and phenotypic diversity across yeast strains and species.

Graphical abstract



This paper makes a case for why yeasts are particularly well suited to address fundamental questions about *de novo* gene emergence. Several yeast genes of recent *de novo* origin are known to play important cellular roles. Yeasts also express thousands of species-specific and strain-specific de novo open reading frames with unknown biology that offer exciting avenues for discovery.

1. A brief history of *de novo* gene birth research

De novo gene birth is the process by which new genes evolve from sequences that were previously non-coding (Tautz, 2014; Tautz & Domazet-Loso, 2011; Van Oss & Carvunis, 2019). Once thought to be exceedingly rare (Jacob, 1977), *de novo* gene birth has now been observed in a wide variety of taxa (Baalsrud et al., 2018; Cai et al., 2008; Chen et al., 2010; Heinen et al., 2009; Khan et al., 2020; Li et al., 2010; Reinhardt et al., 2013; Weisman, 2022; Xie et al., 2019). Several studies have described how young *de novo* genes that exist in only a single species can play important biological roles through species-specific molecular mechanisms (Bungard et al., 2017; Cai et al., 2008; Li et al., 2010; Li et al., 2014; Xie et al., 2019; Zhuang et al., 2019). The process of *de novo* gene birth has therefore received considerable recent attention as a major potential source of genetic, structural, and phenotypic novelty (Abrusan, 2013; Bornberg-Bauer et al., 2021; Capra et al., 2010; Chen et al., 2013; Knopp et al., 2019; Lee et al., 2019; McLysaght & Guerzoni, 2015; McLysaght & Hurst, 2016; Reinhardt et al., 2013; Schlotterer, 2015).

Yeasts have played a central role in the field of *de novo* gene birth since its inception. When the *Saccharomyces cerevisiae* genome was sequenced in 1996, approximately 6,000 open reading frames (ORFs) longer than 300 nucleotides were predicted to be protein-coding genes (Goffeau et al., 1996). Of these, around 30% lacked identifiable homologs among known genes from other species—i.e., they were "orphan genes" (Dujon, 1996). The sequencing of additional genomes over the course of the subsequent decades led to the identification of homologs for many of these orphans (Brachat et al., 2003; Cliften et al., 2003; Kellis et al., 2003; Riley et al., 2016; Shen et al., 2018; Weisman et al., 2020). Nevertheless, several hundred remained homolog-free, unable to be grouped into any gene family. Since *de novo* gene birth was considered highly implausible, such lack of cross-species conservation combined with the absence of experimental evidence was thought to indicate a lack of function. The remaining orphans were therefore initially presumed to correspond to mis-annotations, unlikely to encode functional protein-coding genes, and relegated to the status of "dubious" ORFs (Fisk et al., 2006). However, a 2008 survey of dubious ORFs showed that most were in fact expressed and detected in high-throughput functional genomics assays, suggesting that they did not correspond to mere mis-annotations but may encode *bona fide* orphan genes (Li et al., 2008). The same year, Cai et al. demonstrated that the *S. cerevisiae* orphan gene *BSC4* was of *de novo* origin (Cai et al., 2008).

BSC4 was originally identified as a translated ORF exhibiting Sup35-dependent translational readthrough (Namy et al., 2003). Cai *et al*. then showed that *BSC4* evolved recently in the *S. cerevisiae* lineage via point mutations in a locus that was previously non-coding (Cai et al., 2008). This was the first demonstration that a full-length protein-coding gene can emerge *de novo* in any species. The authors showed that *BSC4* increases in expression level throughout stationary phase and, based on synthetic lethal interactions with *RPN4* and *DUN1*, proposed that Bsc4 is involved in DNA repair during stationary phase to enable the transition from nutrient-rich to nutrient-poor environments. A decade after this initial characterization, Bsc4 became the first protein encoded by a *de novo* gene to be structurally characterized in any species. Unlike typical conserved yeast proteins, it was found to exhibit a rudimentary 'molten globule' fold with high beta sheet content and a hydrophobic core (Bungard et al., 2017).

Shortly following the characterization of *BSC4*, Li et al., in 2010 and 2014, deployed an exhaustive set of experiments and analyses (**Table 1**) to generate the most complete characterization of a yeast *de novo* gene to date (Li et al., 2010; Li et al., 2014). Their studies showed that the *MDF1* ORF emerged *de novo* in *S. cerevisiae* in the previously non-coding sequence anti-sense to a conserved protein-coding gene, *ADF1* (**Figure 1A**). Interestingly, Adf1 represses transcription of *MDF1* by binding to its promoter such that sense and anti-sense expression at this locus have antagonistic physiological effects. When expressed, Mdf1 promotes fermentation and suppresses mating by physically interacting with Snf1 and Matα2 (**Figure 1B**). In other words, the young *de novo* gene *MDF1* mediates the crosstalk between reproduction and vegetative growth through a *S. cerevisiae*-specific molecular mechanism. The case of *MDF1* illustrates how, contrary to prior assumptions, young ORFs that emerged *de novo* in non-coding sequences and lack cross-species conservation can encode proteins with key cellular roles.

2. Methods for inferring *de novo* origin

The most convincing evidence that an ORF originated *de novo* is the identification of a set of one or more "enabling mutations" that arose in previously non-coding sequences within the lineage resulting in a new ORF (e.g., mutation/s creating a new start codon) (McLysaght & Hurst, 2016). This is done by aligning the locus containing the ORF of interest with syntenic orthologous DNA regions in closely related species and showing that the enabling mutations are absent in these species, *i.e.* showing that the orthologous DNA regions are truly non-coding (Vakirlis & McLysaght, 2019) (**Figure 2**). A study applying this approach confirmed the *de novo* origin for *30 Saccharomyces* ORFs (Vakirlis et al., 2018).

Such synteny analyses for *de novo* gene birth inference can be further refined using ancestral sequence reconstruction approaches. This was demonstrated for the first time in any species with the *de novo S. cerevisiae* ORF YBR196C-A (Vakirlis, Acar, et al., 2020). Ancestral reconstruction at this locus showed not only how enabling mutations conferred coding potential to an ancestrally non-coding DNA region, but also how subsequent frameshifts and substitutions have led to the rapid evolution of the initial ORF, leading to loss in some lineages and substantial changes in length and primary sequence in others. These mutational processes led to the emergence of a small species-specific transmembrane protein in *S. cerevisiae* that localizes at the endoplasmic reticulum and promotes larger colony growth when overexpressed. A subsequent study (Papadopoulos et al., 2021) used ancestral reconstruction to retrace the evolutionary history of 70 candidate *de novo* genes identified by previous studies (Carvunis et al., 2012; Lu et al., 2017; Vakirlis et al., 2018; Wu & Knudson, 2018), and reported that most *de novo* enabling mutations corresponded to frameshifts and loss-of-stop events leading to the merging of two small intergenic ORFs.

This complexity can obfuscate the identification of enabling mutations from syntenic alignment alone when one aims to use automated sequence analyses. To circumvent this challenge, a recent study (Wacholder et al., 2021) adapted a Reading Frame Conservation metric (Kellis et al., 2003) calculated from syntenic alignments to identify all ORFs in the *S. cerevisiae* genome with non-coding orthologous regions in other *Saccharomyces* species. These ORFs were then classified into candidate pseudogenes, when distant homologs could be identified through sequence similarity searches in the fungal lineage, or candidate *de novo* ORFs when the corresponding protein sequence was lineage-specific. This analysis estimated that 251 annotated S. cerevisiae ORFs (7 Verified, 96 Uncharacterized, 148 Dubious) emerged *de novo*.

While approaches based on synteny and enabling mutations are now considered standard, phylostratigraphy-based approaches have been widely used in earlier studies of gene birth. In phylostratigraphy, the origin of a new gene is inferred in the most recent common ancestor of all species with a homolog identified by sequence similarity searches (Domazet-Loso et al., 2007). Three groups have performed phylostratigraphy analyses on the *S. cerevisiae* genome, providing lists of hundreds of *S. cerevisiae* orphan genes (Carvunis et al., 2012; Lu et al., 2017; Vakirlis et al., 2018); such analyses have also been conducted on *Lachancea* yeasts (Vakirlis et al., 2016). However, these results must be interpreted with caution because orphan genes can originate via several evolutionary mechanisms other than *de novo* gene birth, including lateral transfer and duplication followed by extreme sequence divergence (Long et al., 2003; Van Oss & Carvunis, 2019). When an orphan gene identified by phylostratigraphy is present in at least two taxa, it is possible to estimate the likelihood that it has acquired a unique sequence through extreme sequence divergence by extrapolating an estimate of its evolutionary rate (Weisman et al., 2020). However, when a gene is found only in one species with no detectable homolog at all, analyses of synteny and enabling mutations are required to infer the mechanism of origin. A recent analysis (Vakirlis, Carvunis, et al., 2020) showed that sequence divergence is not the main source of orphan genes in *S. cerevisiae*, suggesting that *de novo* gene birth may play a major role in generating molecular novelty.

A fundamentally different strategy for *de novo* gene birth inference consists in comparing genome expression patterns, rather than ORF sequences, between yeast strains and species. Indeed, *de novo* gene birth can take place when a pre-existing non-coding RNA acquires a novel ORF and becomes translated (“transcription first”), or when a pre-existing ORF becomes transcribed and translated (“ORF first”) (Schlotterer, 2015). In this latter case, the “enabling mutations” would be those that lead to a novel transcription or translation event rather than those that lead to a novel ORF. These are harder to identify by DNA sequence analysis than mutations enabling the emergence of an ORF, as the genetic determinants of expression changes over evolutionary time are not as well understood. It is however well established that the yeast lineage undergoes substantial evolutionary transcriptional turnover (Li & Johnson, 2010). Lu and colleagues identified 4,340 putative *S. cerevisiae*-specific *de novo* genes that are transcribed but share no orthologues in other *Saccharomycetaceae*, most of which were inferred to have arisen from transcript isoforms of ancient genes (Lu et al., 2017). By comparing the transcriptomes of *S. cerevisiae* and ten other yeast species, Blevins et al. identified 213 *de novo* originated transcripts in *S. cerevisiae*, half of which were in the anti-sense orientation of other genes and many of which appeared to be translated (Blevins et al., 2021). At a finer evolutionary scale, Durand et al. analyzed the turnover of ribosome-associated transcripts among wild *S. paradoxus* strains and identified 447 lineage-specific translation events (Durand et al., 2019). While most were attributable to lineage-specific ORF gains and losses, several instances appeared to have been potentiated by lineage-specific increases in expression level.

The prevalence of *de novo* gene birth in yeast is supported by overwhelming comparative genomic evidence from the aforementioned studies. Yet, there is currently no definitive, community-approved list of which yeast genes have originated *de novo*. Different approaches yield different – though overlapping – results (Blevins et al., 2021; Papadopoulos et al., 2021). The issue lies, in part, in that there is no consensus operational definition of what constitutes a "gene" in the context of *de novo* gene birth, where the signatures of evolutionary conservation typically relied on to predict functionality are absent (Keeling et al., 2019). Further developments of computational methods for the detection of *de novo* originated genes are also much needed for the advancement of the field (Li et al., 2022). Such advances are more challenging to attain in the yeast lineage than in other eukaryotic lineages whose genomes tend to evolve more slowly. Yet, as a plethora of yeast genomes have now been sequenced (Kurtzman et al., 2011; Peter et al., 2018; Shen et al., 2018; Vakirlis et al., 2016), exciting opportunities for large-scale comparative and evolutionary studies of *de novo* gene emergence in yeasts are arising.

The increasing availability of intraspecies genome sequences in yeast has also revealed substantial genetic diversity, distinguishing between the 'core' and the 'accessory' genomes, the latter containing genes specific to sets of isolates or individual strains (McCarthy & Fitzpatrick, 2019). Exploring the extent to which the accessory genome is comprised of *de novo* genes will likely shed light on the mechanisms by which rapid genetic evolution mediates rapid phenotypic and ecological adaptation. Along these lines, a recent study found that only 41% of young *de novo* ORFs identified in the S288C reference annotation were fixed in the *S. cerevisiae* species, while most were still segregating (Vakirlis, Acar, et al., 2020). Future studies integrating evolutionary dynamics of sequence and expression variation at the population level will be instrumental in deriving models of ORF and transcript evolution in real time, to shed light on the full extent of *de novo* gene emergence and its impacts on the diversity of the yeast lineage.

3. The "noncanonical translatome" as a reservoir for *de novo* gene birth

The first unbiased genome-scale transcriptomic studies reported that most of the *S. cerevisiae* genome is transcribed (David et al., 2006; Nagalakshmi et al., 2008). Soon after, the first ribosome profiling studies revealed widespread translation outside of annotated *S. cerevisiae* genes (Brar et al., 2012; Carvunis et al., 2012; Ingolia et al., 2009; Wilson & Masel, 2011). Shortly following these discoveries in yeast, the phenomenon was also reported in other taxa spanning the tree of life (Ruiz-Orera & Alba, 2019). All these "non-canonical" translated elements had been missed by genome annotations because they tend to be short and rapidly evolving. The "translatome" is much larger, and much more diverse, than currently reflected in genome annotation databases.

Noncanonical translation was originally predicted by early models of *de novo* gene birth that were largely built on data from yeast (Cai et al., 2008; Carvunis et al., 2012; Masel, 2006; Wilson & Masel, 2011). These models postulated that some of the hundreds of thousands of short ORFs that appear and disappear continuously during the evolution of non-coding sequences could, if transcribed, become translated and expose new genetic variation to the action of natural selection. Those "proto-genes" (Carvunis et al., 2012) with deleterious translation products would be purged away, while those with nearly neutral or adaptive effects would constitute a reservoir for *de novo* gene emergence. Multiple studies have now uncovered that many, if not most, non-canonical translated elements in yeast are of *de novo* origin (Durand et al., 2019; Spealman et al., 2018; Wacholder et al., 2021). Most recently, Wacholder et al. combined Ribo-seq data from 42 published studies and identified strong translation evidence for almost twenty thousand noncanonical *S. cerevisiae* ORFs, including 12,129 of apparent *de novo* origin based on Reading Frame Conservation analyses (Wacholder et al., 2021).

Future empirical studies are needed to estimate the true size of the yeast translatome, considering the expanding genetic diversity of the yeast genome and pan-genome. Computational predictions are not yet possible, as the molecular signals governing which noncanonical ORFs are translated *in vivo* remain unknown. A small number of proteomic and microscopy studies have detected the protein products of some of these noncanonical translation events in yeast cells (He et al., 2018; Lu et al., 2017; Yagoub et al., 2015), but the vast majority remain undetected. The *de novo* translated ORFs include uORFs and dORFs (translated ORFs located upstream and downstream of annotated coding sequences, respectively) as well as ORFs translated from transcripts containing no annotated gene (Blevins et al., 2021; Carvunis et al., 2012; Durand et al., 2019; Li et al., 2021; Smith et al., 2014; Wacholder et al., 2021; Wilson & Masel, 2011). To what extent the noncanonical translatome generates an entirely novel proteome or yields rapidly degraded products that serve to regulate translation and transcript stability remains an open research question. Unknown too is the proportion of the noncanonical translatome that corresponds to translation “noise” and does not contribute to fitness. The fraction of *de novo* emerged noncanonical translated ORFs that become fixed into *de novo* genes maintained by selection is estimated to be low (Carvunis et al., 2012; Vakirlis et al., 2018; Wacholder et al., 2021). The fact that cells exert a considerable amount of energy to translate so many novel ORFs raises the question of whether such pervasive translation confers an adaptive fitness advantage, beyond providing the raw material for gene birth.

4. Insights into the features of *de novo* genes and mechanisms of *de novo* emergence

Yeast *de novo* ORFs, whether annotated or not, tend to share general characteristics: their primary sequences tend to be very similar to those of intergenic ORFs, and they tend to be short, rapidly evolving, and often expressed in both lineage-specific and condition-specific manners (Basile et al., 2017; Blevins et al., 2021; Carvunis et al., 2012; Durand et al., 2019; Ekman & Elofsson, 2010; Li et al., 2021; Papadopoulos et al., 2021; Vakirlis et al., 2018; Wu & Knudson, 2018). These characteristics are thought to derive directly from their *de novo* emergence and to be associated with possible physiological corollaries. For example, condition-specific expression of yeast *de novo* ORFs has been reported in the context of various stresses (Blevins et al., 2021; Carvunis et al., 2012; Doughty et al., 2020; Li et al., 2021; Wacholder et al., 2021). Could these species-specific translated elements represent a rapidly evolving part of the cell's response to stress?

Vakirlis et al. provided some evidence to this question by showing that overexpression of young *S. cerevisiae de novo* ORFs with predicted transmembrane domains can increase colony growth under nitrogen or carbon limitation (Vakirlis, Acar, et al., 2020). Transmembrane domains are overrepresented among annotated *de novo* ORFs in *S. cerevisiae* (Carvunis et al., 2012; Vakirlis, Acar, et al., 2020), but the cellular mechanisms by which increased expression of species-specific transmembrane domains would allow cells to adapt to starvation stress remain to be elucidated. Interestingly, Vakirlis et al. did elucidate the evolutionary mechanisms giving rise to the de novo origination of ORFs with transmembrane domains (Vakirlis, Acar, et al., 2020) as a direct result of codon biases in the genetic code, whereby transmembrane residues tend to be encoded by thymine-rich codons. A "transmembrane-first" model was therefore proposed whereby translation of intergenic sequences that are rich in thymine have a high propensity to generate transmembrane peptides, which in turn are more likely to be adaptive and retained by natural selection. The transmembrane-first model is, to date, the only proposed model that directly ties molecular mechanisms of *de novo* gene birth to a specific biophysical protein property associated with an adaptive fitness advantage.

Several studies, however, have identified additional properties of yeast *de novo* ORFs that are also linked to their evolutionary trajectories and possibly to their physiological roles. In particular, the specific genomic location where *de novo* emergence takes place appears to greatly influence primary sequence, transcriptional regulation, and evolutionary rate. Vakirlis et al. (Vakirlis et al., 2018) reported a strong over-representation of *de novo* ORFs at GC-rich loci across multiple yeast lineages. These loci are depleted in stop codons and often correspond to divergent gene promoters, suggesting a regulatory relationship between these *de novo* ORFs and their conserved neighbors. Blevins et al. identified many *de novo* transcripts located on the opposite strand of conserved genes and co-regulated with their overlapping counterparts in response to stress (Blevins et al., 2021). Loci opposite protein-coding genes also tend to be depleted in stop codons. An over-representation of yeast *de novo* ORFs has been reported in rapidly evolving subtelomeric regions (Carvunis et al., 2012) and recombination hot spots (Vakirlis et al., 2018). It is tempting to speculate that genomic regions that are transcriptionally active, fast-evolving, or depleted in stop codons favor not only the emergence but also the functional evolution and retention of *de novo* genes.

Collectively, these studies suggest the existence of diverse evolutionary avenues for *de novo* gene birth, each possibly associated with different biophysical protein properties and phenotypic impacts. It is unclear how young *de novo* ORFs change over time, although some evidence suggests a trend towards increasing foldability (Papadopoulos et al., 2021). It may be that distinct selective pressures favor the emergence of distinct types of proteins in different environments. For example, while intrinsic disorder is predicted to be rare among yeast *de novo* genes in general (Basile et al., 2017; Carvunis et al., 2012; Ekman & Elofsson, 2010; Vakirlis, Acar, et al., 2020; Vakirlis et al., 2018), it is observed in high excess in older *de novo* genes from the *Lachancea* lineage (Vakirlis et al., 2018). It is thought that *de novo* genes increase in length, expression level and cellular interactivity over time (Abrusan, 2013; Carvunis et al., 2012; Lu et al., 2017; Tautz, 2014), but more mechanistic research is needed to fully understand the long-term evolutionary dynamics of *de novo* gene origination and evolution. The physiological implications of *de novo* gene emergence are in dire need of further study as well. No noncanonical *de novo* translated ORFs, and very few annotated *de novo* genes, have been deeply characterized to date.

5. Proposed Evolutionary Systems Biology framework for future investigations of *de novo* gene birth in yeast

The study of *de novo* gene birth offers an unprecedented paradigm to understand the role of genetic novelty in the emergence of novel protein structures, functions, and phenotypes. By studying genetic elements that are transitioning from non-coding to protein-coding, we can unravel how novelty arises on multiple scales, from the DNA sequence to integration into cellular networks and the possible emergence of new phenotypic traits. Given that novel genes, in general, have been shown to rapidly integrate into cellular networks (Abrusan, 2013; Tsai et al., 2012), network-based approaches may turn out to be very fruitful for understanding what makes a strain or species unique from a molecular standpoint. The example of *MDF1* demonstrates how an emergent *de novo* protein can rapidly integrate into an existing cellular network and evolve a critical biological role (**Figure 1**, **Table 1**) (Li et al., 2010; Li et al., 2014). For future studies in the emerging field of *de novo gene* research, we propose a novel framework guided by an evolutionary systems biology approach to utilize yeast's potential as a model and a tool for this field of study (**Figure 3**). Guided by this framework, related levels of evidence and function (Keeling et al., 2019) can be investigated to characterize *de novo* ORFs.

For a given *de novo* candidate, some key questions would be: In what context is it transcribed and translated? When and how did it acquire the regulatory signals controlling expression? Does it participate in genetic or protein-protein interactions? Does it stably localize to a specific sub-cellular compartment? When and how did its protein sequence acquire the necessary residues or domains to specify its localization and/or interactions? Does its expression impact fitness in a particular biological context? Concomitantly researching a *de novo* candidate's characteristics along with when and how these characteristics arose is expected to yield insights into the interrelated evolutionary and physiological forces at play. A particular candidate may be required for survival in a specific context, or it may modulate traits that do not impact fitness. As more proto-genes and *de novo* genes are discovered, the wealth of resources and the repertoire of techniques available to researchers working in yeast combined with our proposed framework (**Figure 3**) offer a unique opportunity to explore this untapped font of molecular diversity.

Yeasts are established as an exceptional model for molecular genetics, cell biology, and biochemistry due to their ease of culture, simple life cycles, short generation times, a paucity of multi-intronic genes, and their relatively small genomes (~10-20Mbp.) Genome-wide deletion and over-expression libraries have been developed for multiple yeast strains, particularly in *S. cerevisiae* (Alberti et al., 2007; Brachmann et al., 1998; Douglas et al., 2012; Fasanello et al., 2020; Gelperin et al., 2005; Giaever et al., 2002; McIsaac et al., 2013; Sopko et al., 2006), enabling advanced, high-throughput approaches that can be expanded to characterize phenotypes for *de novo* candidates (Costanzo et al., 2010; Costanzo et al., 2016; Douglas et al., 2012; Parsons et al., 2006; Piotrowski et al., 2017; Vizeacoumar et al., 2010). Once a phenotype is detected with confidence, mechanisms can be inferred with many tools, e.g. with deep mutational scanning (Fowler & Fields, 2014) or network-based computational approaches (Li et al., 2021). As *de novo* ORFs often overlap with non-coding sequences that may function as regulatory elements or ncRNAs, it can be important to experimentally dissect which aspects of null mutant phenotypes are truly caused by loss of translation or loss of the protein product. This can be achieved with single nucleotide genome editing of the translation start site, for example (Wacholder et al., 2021).

Yeasts have also long been at the forefront of the ‘omics’ revolution, offering the opportunity to conduct systems-level studies that can investigate the genome, transcriptome, translatome, interactome, metabolome, and phenome (Yu & Nielsen, 2019). Yeast offers yet another advantage over other systems as not only are more and more strains being sequenced every day (Libkind et al., 2020), but such strains are also being used for “comparative-phenomics” in the laboratory setting (Robinson et al., 2021). The exploitation of natural yeast isolates and diverse experimental conditions that attempt to recreate their natural environment may shed light on why so many *de novo* translated elements exist, and why they evolve so rapidly. It will be informative to compare the tolerance of *de novo* ORF expression in wild strains and natural environments with that of commonly used laboratory strains and experimental settings.

Yeasts are also well-positioned as a model system for addressing the "holy grail" of *de novo* gene birth: the opportunity to observe the phenomenon in real-time. While this is not a trivial endeavor, yeasts are amenable to experimental evolution (Voordeckers & Verstrepen, 2015). One can imagine applying selective pressure to an experimentally evolving population and combining it with sequencing to observe the order of events that lead to the formation of *de novo* genes, and indeed, if a particular path or order is "preferred". The ability to control this phenomenon opens the possibility that applying appropriate selective pressures may lead to evolved populations with unique genes to overcome current limitations in using yeasts for agricultural, industrial, or medical purposes.

6. Conclusion

Yeasts are well suited to address some of the fundamental questions and promising opportunities in the *de novo* gene birth field. The pliability of the system allows us to ask nearly any question: How do *de novo* ORFs acquire the signals necessary for expression? How are new genetic elements integrated into the vast pre-existing *S. cerevisiae* transcriptional and protein-protein interaction networks? How can we perturb these networks to dissect the function(s) of these novel genetic elements? The rapidly evolving field of *de novo* gene birth can shed new light on our understanding of genes, proteins and how they evolve. Furthermore, it opens the door for exciting medical and industrial possibilities. Yeasts are uniquely situated to exploit these opportunities.

Author Contribution

Conceptualization, S.B.P. and A.-R.C. Writing – Original Draft, S.B.P., S.B.V.O., C.H., A.W. Writing – Review & Editing, S.B.P., C.H., S.B.V.O., A.W., A.-R.C. Supervision, A.-R.C.

Conflict of Interest

A.-R.C. is a member of the scientific advisory board for Flagship Labs 69, Inc (ProFound Therapeutics).

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Tables

Table 1. Applying the evolutionary systems biology approach to the investigation of *MDF1*.

Categories	Test for Evidence	Results
Sequence	Comparative genomics	It is under positive selection
	PSI-BLAST	There are no significantly homologous ORFs in all of the other organisms examined beyond two short, truncated ORFs in the close relatives *S. bayanus* and *S. mikatae*.
	Synteny	The intergenic region between flanking genes could not encode a protein in other species due to the presence of multiple stop codons
Expression	Strand-specific RT-PCR	*MDF1* is only expressed in *S. cerevisiae*
	Western-blot	Positive signal for the protein

Structure	Structure prediction server - PORTER	Mdf1 mimics Mata1 in having a three-helix-domain that can bind to Matα2
Localization	Fluorescent tagging	Mdf1 exists in the cytoplasm and nucleus
Interaction & Mechanism	Chromatin immunoprecipitation (ChIP)	Adf1 binds to the upstream region of *MDF1*
		Mdf1 binds haploid specific genes (*MATα1*, *STE4*, *STE5*, *FUS1*, *FUS2*, *FUS3*, *GPA1*, *SST2*, and *RME1*)
	Gel electrophoresis	*ATP1*, *PGK1*, *MDH1* & *SAM1* expression is increased in *MDF1 ADF1Δ* strains
	Microarray	Downregulation of mating pathway (MAPK)
	Semi-quantitative RT-PCR	MAPK pathway genes (*STE3*, *STE12*, *FUS1*, *FUS3*) are downregulated
	Complementation assay	Overexpression of *MATα1* gene rescues the mating ability of an *mdf1Δ* mutant
	Yeast two-hybrid assay	Mdf1 interacts with Matα2
	Pull-down assay	Mdf1 interacts with Matα2
	Electrophoretic mobility shift assays (EMSAs)	Mdf1 and Matα2 are bound to each other and function in a mutually dependent manner
Phenotype & Fitness	Competition experiment	*MDF1 ADF1Δ* strain grows more quickly than the wild-type strain
	Growth rate analyses	
	Mating assay	*MDF1 ADF1Δ* is less successful at mating. No such effect is seen in closely related species.

Figure legends

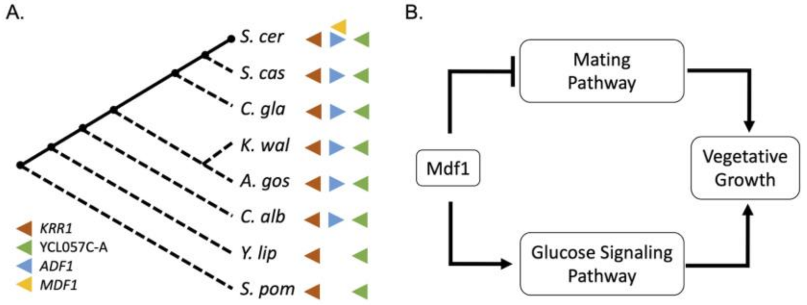

Figure 1. ***MDF1:*** **A** ***de novo*****-evolved gene that integrates into essential biological pathways. A.** Phylogeny- and synteny-based analysis of various fungi revealed that *MDF1* emerged specifically in *S. cer* subsequent to its split from *S. cas*. At the same time, *ADF1*, an antisense gene to MDF1, is conserved in all but the most distant member of the hemiascomycete subdivision of fungi. The *MDF1* syntenic block is shown to the right of the phylogenetic tree. *S. cer*: *Saccharomyces cerevisiae*; *S. cas*: *Saccharomyces castellii*; *C. gla*: *Candida glabrata*; *A. gos*: *Ashbya gossypii*; *C. alb*: *Candida albicans*; *Y. lip*: *Yarrowia lipolytica*; *S. pom*: *Schizosaccharomyces pombe* (Li et al., 2010). **B.** Mdf1 promotes vegetative growth by suppressing the mating pathway and enhancing the glucose signaling pathway (Li et al., 2014).

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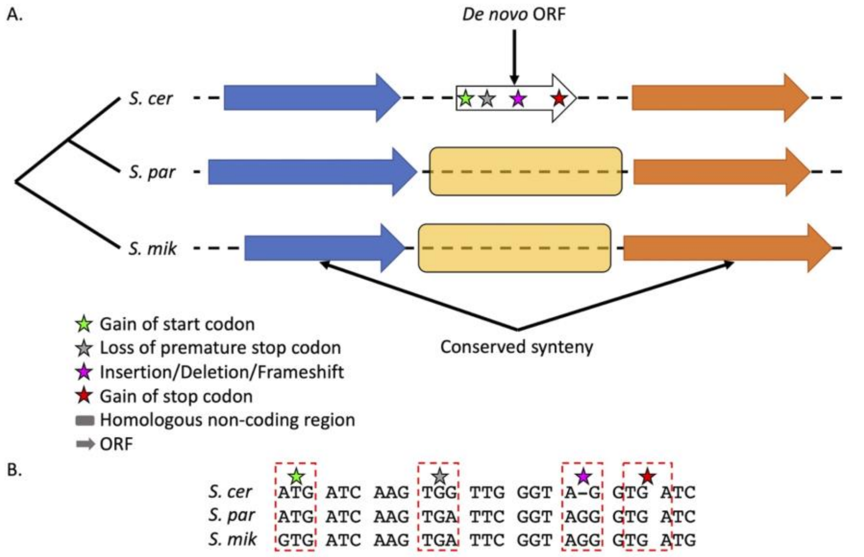
A.
De novo ORF
S. cer
S. par
S. mik
Conserved synteny
★ Gain of start codon
★ Loss of premature stop codon
★ Insertion/Deletion/Frameshift
★ Gain of stop codon
▬ Homologous non-coding region
➡ ORF
B.
S. cer ATG ATC AAG TGG TTG GGT A–G GTG ATC
S. par ATG ATC AAG TGA TTC GGT AGG GTG ATC
S. mik GTG ATC AAG TGA TTC GGT AGG GTG ATG

Figure 2. Pictographic representation of a hypothetical *de novo* ORF in *Saccharomyces Cerevisiae*. A. A combination of conserved synteny and phylostratigraphy is used to identify the homologous region of interest (highlighted in yellow) in the closely related species. This region of interest can be used to identify enabling mutations across the lineage that led to the *de novo* ORF in the focal species (*S. cerevisiae* in this case.) The enabling mutations can include but are not limited to a gain of start codon (green star), loss of premature stop codon (grey star), insertion-deletion and/or a frameshift (pink star) and a gain of stop codon (red star.) Figure inspired by Vakirlis & McLysaght, 2019 (Vakirlis & McLysaght, 2019). **B.** A hypothetical example of enabling mutations that occurred along the lineage to result in a *de novo* ORF in the focal genome. Changes highlighted within boxes are possible enablers. Identification of one or more of such mutations (example gain of start codon) are needed to provide convincing evidence of *de novo* ORF emergence. *S. cer*: *Saccharomyces cerevisiae; S. par: Saccharomyces paradoxus; S. mik: Saccharomyces Mikatae*.

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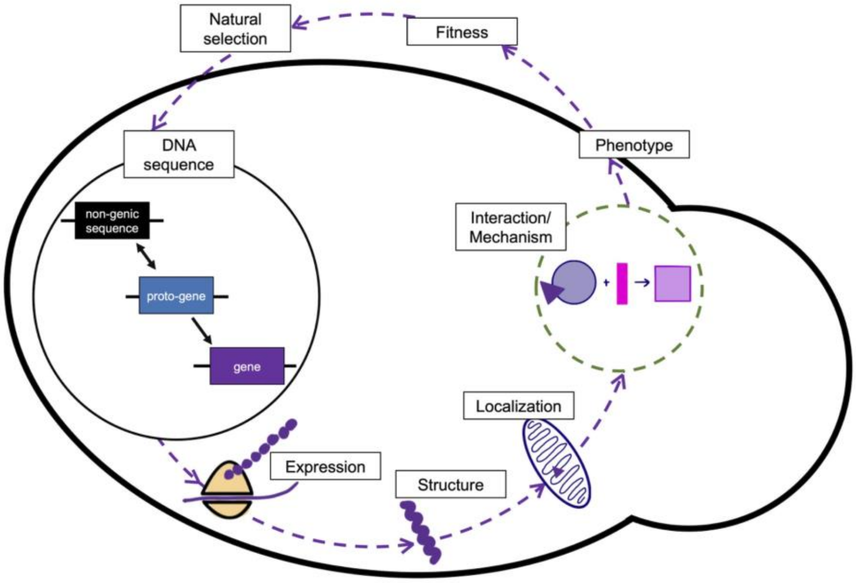

Figure 3. Evolutionary systems biology approach for characterizing the biological role of a candidate *de novo* gene. The framework proposes a combination of evolutionary and molecular approaches that may be used to identify and investigate a candidate *de novo* gene. Insights drawn from these varied approaches can then be put together to provide a holistic understanding of the ORF's biological role. Overall, this framework represents a circular continuum that is under the influence of natural selection.

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