High Throughput Screening of Functionally Replaceable Human Gene Suppressors in Yeast Reveals New Mechanistic Insights into the Proteasome Function

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Abstract

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Sarmin Sultana

Orthologous genes in diverged species tend to perform similar functions. This conjecture forms the foundation of most biomedical research and justifies the use of model organisms in research with implications in human biology. Humans and the baker's yeast *Saccharomyces cerevisiae* diverged from a common ancestor approximately one billion years ago. Yet, they share several thousand genes accounting for nearly one-third of the yeast genome. Several systematic studies have shown that many human orthologs are replaceable in yeast. However, nearly half of the tested human genes are non-replaceable in yeast. This work focuses on the non-replaceable human genes; we establish a genetic suppressor screen that allows the selection for replaceability of otherwise non-replaceable human genes in yeast. We focused on the non-replaceable human catalytic beta subunit $\beta 2$ (*PSMB7*) of the proteasome core to test this strategy.

Traditional suppressor screens are tedious and time-consuming; therefore, we developed a high-throughput strategy using a robotic setup. Using error-prone PCR, we generated a mutant pool of the non-replaceable human catalytic subunits, which were transformed in heterozygous knockout diploid yeast strains and screened for yeast colonies carrying a replaceable human mutant gene. After validation by re-transformation followed by sequencing, we identified the amino acid changes in the human genes that allow replaceability. To identify the mode of suppression, we performed structural analysis of the mutants by modelling human protein structure in the yeast proteasome. The mutants are broadly categorised to three classes. 1) The

mutations close to the interacting surfaces of the neighbouring proteins in the complex, 2) The mutations that affect the catalytic activity of the protein thus, affecting the assembly in the yeast proteasome core and lastly, 3) The mutations that lie in the C-terminal region of β2. The mutants reveal divergence of regulation and function in human and yeast proteasome core assembly. Broadly, this research will create an extended resource to study human gene function in yeast by swapping even more as yet non-replaceable human orthologs in yeast. Additionally, the genetic approach allows us to discern whether the gain of replaceability is driven by changes in protein-protein interactions (PPIs); thus, uncovering core evolutionary principles. Through characterization of the essential PPIs out of many, the approach may help decode genotype-phenotype relationships in organisms, especially in the cases where aberrant phenotypes are associated with diseases in humans.

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Dedications

I would like to dedicate this thesis to my parents and my siblings for their support easing my way throughout this project. A special dedication to my father who passed away in August 2020 and never saw me graduate. A special gratitude to my mother who has always been there for me and gave me constant support even though she had faced hardships of her own.

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Chapter 1: Background

1.1 The orthologue-function conjecture

The concept of orthology was introduced by Walter Fitch to understand homologous relationships between genes and to distinguish between the mode of descent from the common ancestor [47]. Orthologs are a set of homologous genes that evolved from an ancestral gene through speciation event. Since orthologous genes trace back to the ancestral gene of the common ancestor, it is considered a precise way of characterizing the similarities between the genes of the species compared [46, 47]. The ortholog-function conjecture posits that orthologous genes in diverged species should perform similar or identical functions [23]. This forms the foundation of most modern biological research, including annotating newly sequenced organisms, functional genomics, evolutionary studies of biological systems and phylogenomic analyses [46]. All organisms share extensive similarities in their genetic makeup, and the knowledge of processes of one organism can be deemed beneficial for studying other organisms, including humans [2]. Humans and *Saccharomyces cerevisiae* (budding yeast) diverged from a common ancestor approximately one billion years ago [24]. Yet, they share many genes that makeup about one-third of the yeast genome [48], with amino acid identity ranging from 9 to 92% and a genome-wide average of 32% [1]. Comparison between human and yeast genes showed approximately 2100 groups of orthologs represented by 2300 yeast genes and 3900 human genes [2, 49].

Budding yeast is considered an ideal model organism to study human genes not only because it shares genes with humans, but it is also inherently amenable and can be manipulated easily in both haploid and diploid forms compared to mammalian cells [50]. Even though one of the early humanization experiments was done using *Schizosaccharomyces pombe* (fission yeast) [2,76], budding yeast was the first eukaryotic organism to have its complete genome sequenced in 1996, thus consequently leading to the rapid development of several experimental

methodologies in molecular and systems biology for manipulation of cellular machinery in an inexpensive and easy manner [50, 51]. These include the development of functional genomic tools like yeast deletion mutant collections, gene over-expression libraries and GFP tagged yeast strains [53-57, 68]. It also led to the development of high throughput techniques like proteomics and metabolomics [58-60, 68] as well as protein-protein interaction studies [68-71]. It has been used as a model organism to study cellular processes like regulation of gene expression, cell cycle, metabolism, signal transduction and apoptosis [61-66, 52]. Using yeast as a model organism also helps avoid the ethical and experimental constraints that might come with the target organism that is being studied [52]. Therefore, accessibility of functional genomic tools, the ease of genetic manipulation of yeast and short generation time combined with low cost and availability of selection markers makes yeast an ideal platform for the systematic studies.

1.2 Humanization of yeast

Even if two orthologous genes perform similar functions in different organisms, it may not be possible to replace one for the other, particularly if the organisms have widely diverged such as in the case of humans and yeast. However, several systematic studies show that many yeast genes can be successfully replaced with their human equivalents [1, 2]. Humanized yeast, therefore, provide a valuable template for understanding human biology. One of the early humanization experiments was done by Lee and Nurse where they isolated and characterized the human homologue of *cdc2* gene by expressing a human cDNA library in fission yeast and selecting clones that rescued a mutation in the *cdc2* gene [76]. The data also revealed that the human *CDC2* gene, with amino acid identity of 63%, encoded for a protein of same molecular weight as the fission yeast *cdc2*, thus indicating a likely conservation of cell cycle elements between humans and fission yeast [76]. For 30 years, humanization of yeast has been achieved

in several forms [2]. Laurent *et al.* described the humanization of yeast in five degrees which range from using yeast to identify human drug targets (degree 0) to the expression of human proteins in yeast regardless of orthology (degree 1) to the humanization of specific amino acids in yeast proteins (degree 2), or entire genes (degree 3) and complete pathways and complexes (degree 4) into yeast [2].

Previously, Kachroo *et al.* tested the replaceability of orthologous human genes with 1:1 yeast-human orthology that are essential for survival in yeast. Out of 414 yeast-human orthologs, 176 human genes (43%) could replace their yeast counterparts [1]. Upon analysis of factors that play a role in replaceability, it was observed that sequence similarity of yeast and human genes was not enough to explain the humanization of yeast. Rather it was the specific properties of the gene modules that allowed replaceability [1]. As described by Wagner et al. (2007), gene modules are a set of genes or proteins that work together to perform a function semiautonomous to other gene modules [67]. The data suggested that modules in diverged organisms evolve as a whole. Upon further analysis of the relationship between gene modules and replaceability, it was observed that genes in specific modules are either near entirely replaceable, as in the case of sterol and heme biosynthesis pathways, or not. Out of 19 genes involved in the sterol biosynthesis pathway, 17 genes were replaceable and functionally rescued yeast mutants suggesting a conservation of functionality of sterol biosynthesis between yeast and humans [1]. Similarly, several modules were completely non-replaceable, for example, systems responsible for DNA replication and repair [1,2]. One such example is the origin recognition complex (ORC) where none of the four genes tested for complementation were replaceable. The complex interacting with ORC, minichromosome maintenance (MCM) complex also showed similar results with five of the tested genes being non-replaceable in yeast [1]. These examples indicate strong association between gene modularity and replaceability.

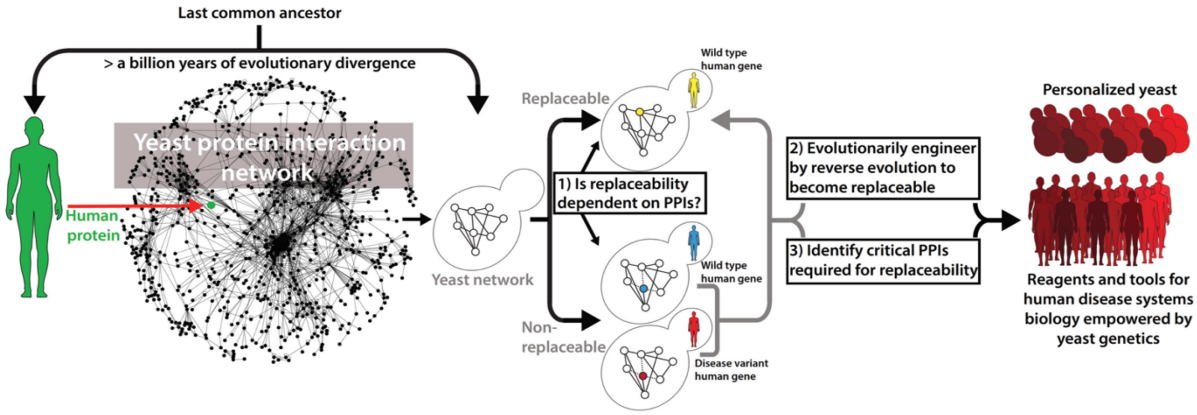

Figure 1. Humanization of yeast could shed light on divergent regulatory mechanisms, and critical genetic or protein-protein interactions

The humanization of yeast allows the accommodation of replaceable human genes into the yeast network. Non-replaceable human genes may lack the critical interactions or regulatory mechanisms required for replaceability in yeast. Identification of the missing interactions or regulatory pathways may serve as a tool for studying human disease.

As described in **Figure 1**, humanization of yeast indirectly shows that the replaced human genes have mostly assimilated with the vast genetic/PPIs network, thus performing interactions similar to their yeast counterparts. However, given that yeast and humans diverged over a billion years ago [24], some of these genetic and protein-protein interactions (PPIs) may have changed, thus leading to the non-replaceability of many human genes which are otherwise performing similar functions in either species. These changes could be at the level of regulation, expression or genetic and physical interactions. Therefore, our project focuses on a "reverse evolution" approach to humanize orthologous but non-replaceable human genes. We hypothesize that this will allow the analysis and identification of properties responsible for humanization in yeast such as critical protein-protein interactions and novel regulatory mechanisms. Characterization of these properties may help us understand the likely new biology in humans and yeast that leads to incompatibility.

Attributes of yeast like inherent tractability, manipulability and short generation time combined with availability of functional genomic tools for systematic studies in yeast make it an appropriate model to study human diseases. One of the early examples of using yeast models for studying human diseases was cystathione β-synthase (CBS) which is encoded by the *CBS* gene in humans and *CYS4* gene in yeast [73]. Mutations in the human *CBS* gene leads to a genetic disorder called Cystathione β-synthase (CBS) deficiency causing homocystinuria and mental retardation [73]. The *CBS* gene functionally rescues the *cys4* mutations in yeast [73], therefore, this system has been helpful in studying the *CBS* variants related to disease in yeast [74]. In another study, several other *CBS* disease variants, previously sequenced from patients, were also tested for function using yeast as a model [75].

However, one of the limitations of studying human disease in yeast is that not all human orthologs can functionally complement their yeast counterparts [1]. Since yeast and human diverged a long time ago [24], important interactions between genes or proteins might be missing that are required for replaceability. Therefore, by identifying the mechanisms that explain complementation of human genes in yeast, the non-replaceable human genes can also be successfully replaced in yeast. These genes and their disease variants, if any, can be studied easily in yeast models, thus possibly providing insights to understanding related human diseases.

1.3 Proteasome core structure and assembly

My project focuses on the proteasome complex involved in the highly regulated degradation of the cellular proteome [4]. In humans, localized in the nucleus and cytoplasm, it is involved in the degradation of abnormal and misfolded proteins as well as the processing of intracellular antigens presented by the immune system [4]. The proteasome complex is a highly conserved machine essential for the regular function of cellular regulatory mechanisms like

cell cycle, DNA repair, immune response, signal transduction, etc. [5, 6, 7]. A dysfunctional proteasome is known to cause cancer and neurodegenerative diseases in humans [8].

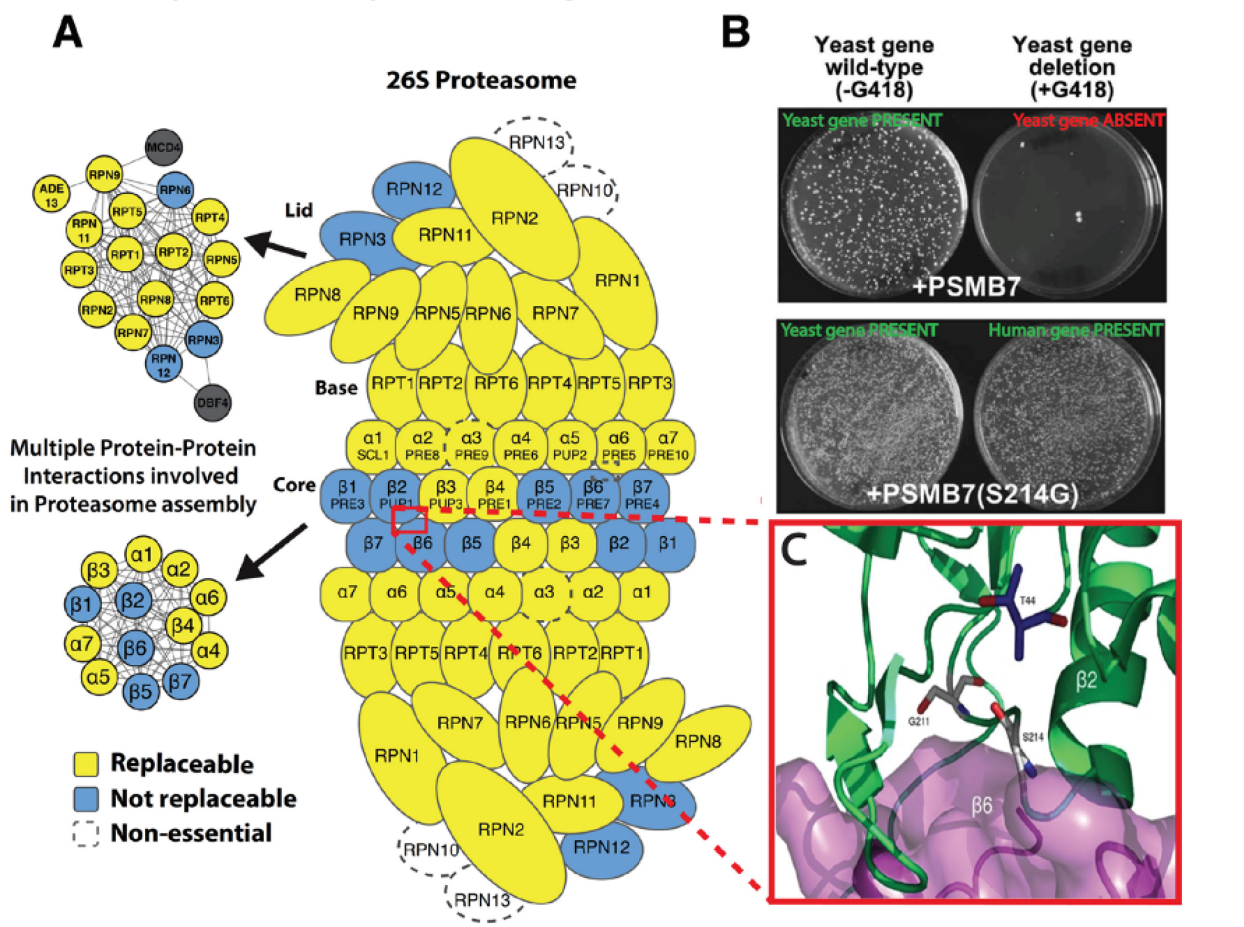

Figure 2. Yeast 26S proteasome complex genes are largely replaceable by its human equivalents

(A) Many yeast 26S proteasome complex genes are replaceable (yellow) by their corresponding human counterparts through plasmid-based complementation (one gene at a time). However, certain subunits in the *β* ring and the lid are non-replaceable (blue). **(B)** Previously, a plasmid-based complementation assay revealed a single amino acid substitution (Ser^{214} -> Gly^{214}) could lead to replaceability of non-replaceable *PSMB7* gene (human *β*2) [1]. **(C)** S214G mutation mapped onto the human *β*2 protein modelled into the yeast proteasome protein shows close proximity to the active site (T44) and the neighbouring yeast *β*6 subunit [1].

The 26S proteasome is a complex that consists of two submodules – the 19S Regulatory Particle (RP) and the 20S Core Particle (CP) (**Figure 2A**). The 19S RP covers the 20S CP on each end and is responsible for identifying the ubiquitinated substrates and unfolding them. In contrast, the core, with the help of its proteolytically active subunits, degrades the substrate into smaller peptides [6, 9, 14]. The 20S barrel-shaped proteasome core is a complex made of four heptameric rings stacked together [10, 11]. Among the four rings, the two outer rings are made of seven α subunits, and the two inner rings are made of seven β subunits each [6]. The two outer rings are responsible for the generation of channels that allow substrate entry to the proteolytic chamber. Precisely, the highly conserved N-termini of the α subunits control the passage of substrates through the gate [7, 40]. Three active protease subunits are present on each β ring, specifically β1, β2 and β5, that are responsible for substrate degradation. These subunits are threonine proteases comprising N-terminus threonine as an active site residue. These catalytic β subunits are synthesized as proenzymes, which activate upon N-terminal propeptide cleavage after CP assembly, thus, exposing the active site threonine residue [10, 12, 13]. The process is highly regulated and appropriate cleavage of the propeptides occurs only up on the optimal assembly of proteasome core. Among the other β subunits, non-catalytic β6 and β7 subunits are also synthesized with propeptides that, upon cleavage, form the mature CP. The remaining β3 and β4 subunits do not undergo cleavage and retain their primary form [10]. Assembly of the proteasome core is a combination of the self-assembly of subunits and the guidance of N-terminal and C-terminal regions of specific subunits and external factors like chaperones. During the assembly, the subunits are added sequentially while creating a series of intermediates [11, 20]. With the help of the chaperones, the α subunits are assembled into a ring which functions as a template for the addition of β subunits to form a half proteasome [7, 41]. The incorporation of β subunits to the α ring starts with the β2 subunit [7, 20]. The C-terminal of the β2, crucial for viability, wraps around β3 and interacts with β4. This is followed

by the incorporation of β5, β6, β1 and lastly β7 [20, 21, 22]. Extrinsic chaperones and the N-terminal and C-terminal regions of certain β subunits (functioning as 'intramolecular chaperones') assist in the assembly of β subunits as well [7, 20]. The β5 propeptide, in the presence of extrinsic chaperones, is responsible for propeptide autocleavage during proteasome maturation, dimerization of the half proteasomes and incorporation of β6 [12, 20, 42, 43]. The β2 propeptide is responsible for the addition of β3 [20]. Once β7 is incorporated into the β ring, its C-terminal region extends into space between β1 and β2 of the opposite ring, which results in the dimerization of half proteasomes [22, 42, 44, 45].

Humanization of the proteasome complex genes revealed that out of 28 subunits that were tested for replaceability, 21 subunits were successfully replaced by their human counterparts (one-at-a-time). Moreover, the subunits that were not replaceable (some subunits of the β ring and two interacting subunits of 19S regulatory particle) were clustered together in a sub-module rather than being present randomly throughout the complex (**Figure 2A**) [1]. In case of the proteasome core, the human catalytic βs (β1, β2 and β5) and non-catalytic β6 and β7 subunits cannot functionally replace their yeast orthologs [1]. Incidentally, only the unprocessed human β3 and β4 subunits are replaceable in yeast suggesting the improper assembly of human subunits with propeptides as a likely reason for non-replaceability. This demonstrates that genetic modularity, within a specific module, is essential for replaceability. Therefore, in this study, we use the proteasome complex to test the hypothesis.

Even though the wildtype human β2 subunit failed to replace in yeast, a single amino acid substitution (Ser^{214} -> Gly^{214}) in β2 subunit allowed replaceability [1] (**Figure 2B**). Through structural analysis of the human β2 and identification of the location of the mutation S214G, it was observed that the mutation resides close to the active site T44 and the neighbouring subunit yeast β6 (**Figure 2C**). However, the mechanism of the functional replaceability remains unknown [1]. We hypothesize that the local protein-protein interactions

are restored due to a mutation. We, therefore, speculate that finding more such mutations that allow replaceability can help provide more insights towards the likely mechanism of replaceability of human β2.

In the next few chapters, I describe the pipeline to screen for replaceable human β2 variants in yeast, characterize the variants and aided by the suppressors test new variants that allow functional replaceability to eventually guide the incorporation of wildtype human protein in the yeast.

2. CHAPTER 2: Development of a pipeline for high-throughput suppressor screening for replaceable variant of human gene in yeast

2.1 Description of the suppressor screen previously employed

The suppressor screening uses a yeast magic marker heterozygous knockout strain (HetKO). This is a collection of heterozygous diploids with one copy of the chromosome carrying the wild-type gene and the other copy containing the knockout of the gene by harbouring a *KanMX* cassette in place of a particular gene [53, 54]. These strains also have an SGA reporter with MATa-specific pMFA1-*HIS3* (also called the magic marker) cassette incorporated into the *CAN1* locus [15,17, 53, 54].

As shown in **Figure 3**, the previous screening strategy involves the transformation of the appropriate HetKO strain with the plasmid containing the human gene variant flanked by a constitutive yeast promoter (pGPD) and an auxotrophic selection (Ura+) [1]. The transformed heterozygous diploids are grown on a synthetic medium without uracil and sporulated followed by selection on a synthetic magic marker medium (-LEU -HIS -ARG -URA + Canavanine) in the presence of geneticin. This medium only selects for the knockout haploids that carry the knocked copy of the yeast gene. When the yeast gene knockout is lethal, growth is observed only when the human gene (or its variant) complements the yeast gene function. The assays were further confirmed by tetrad dissection [1].

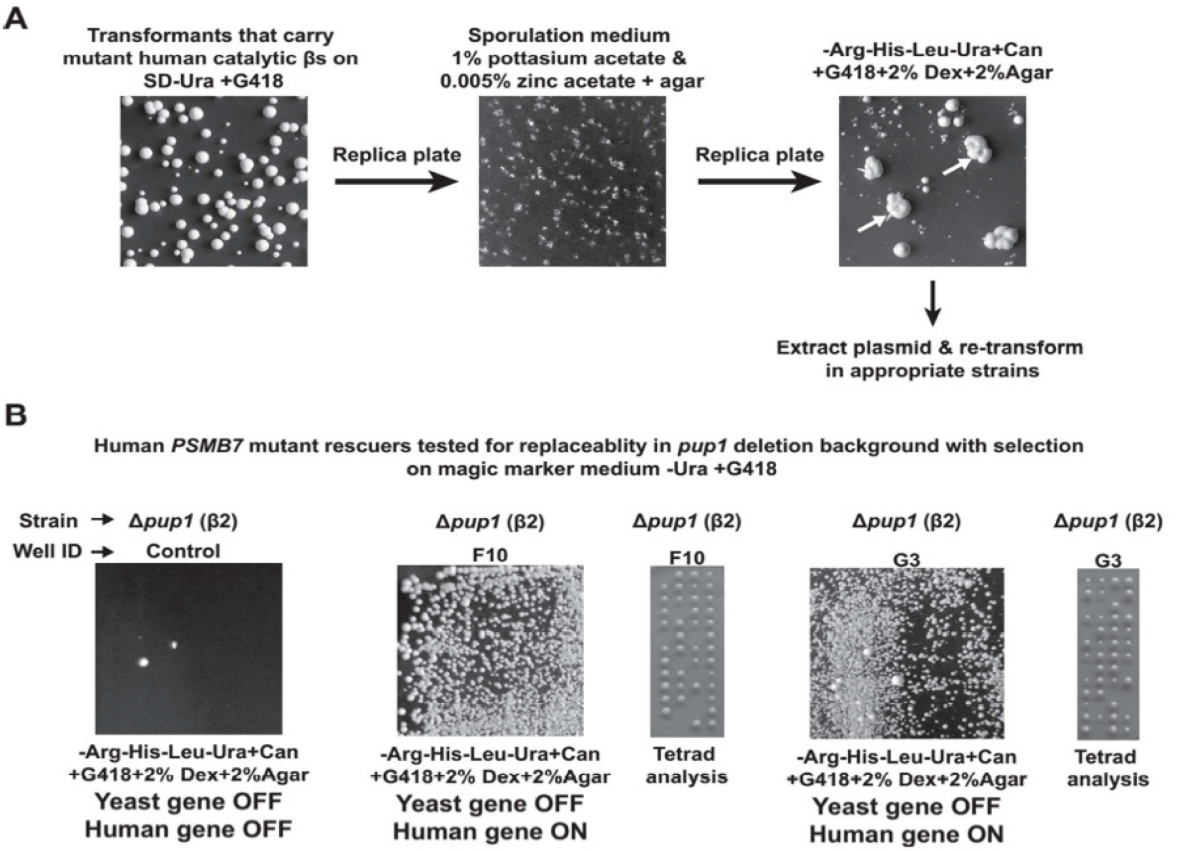

Figure 3. Conventional suppressor screening for the identification of mutant replaceable human genes in yeast

A. *PSMB7* mutant library was transformed into yeast heterozygous diploid knockout *Pup1/Δpup1*::KanMX strain. Transformants were replica plated onto sporulation medium followed by selection on magic marker medium with G418 selection. Plasmids were isolated from surviving colonies (considered as potential suppressors) for further experiments [1]. **B.** Plasmids (Mutants F10 and G3) were retransformed into the yeast *Pup1/Δpup1*::KanMX strain and selected on magic marker medium with G418 selection to confirm complementation by human gene. Functional replacement by human gene was further confirmed by tetrad dissection [1].

While the method described above successfully allowed the identification of replaceable human gene suppressors, the screening was tedious. First, the technique required manual isolation of colonies, including tetrad dissection, which could be excruciatingly time-consuming. Second, despite the arduous labour, the likelihood of finding suppressors is 1 in 100 to 1 in 1000; therefore, manual screening couldn't yield as many suppressors. Finally, the likelihood of contamination was also higher.

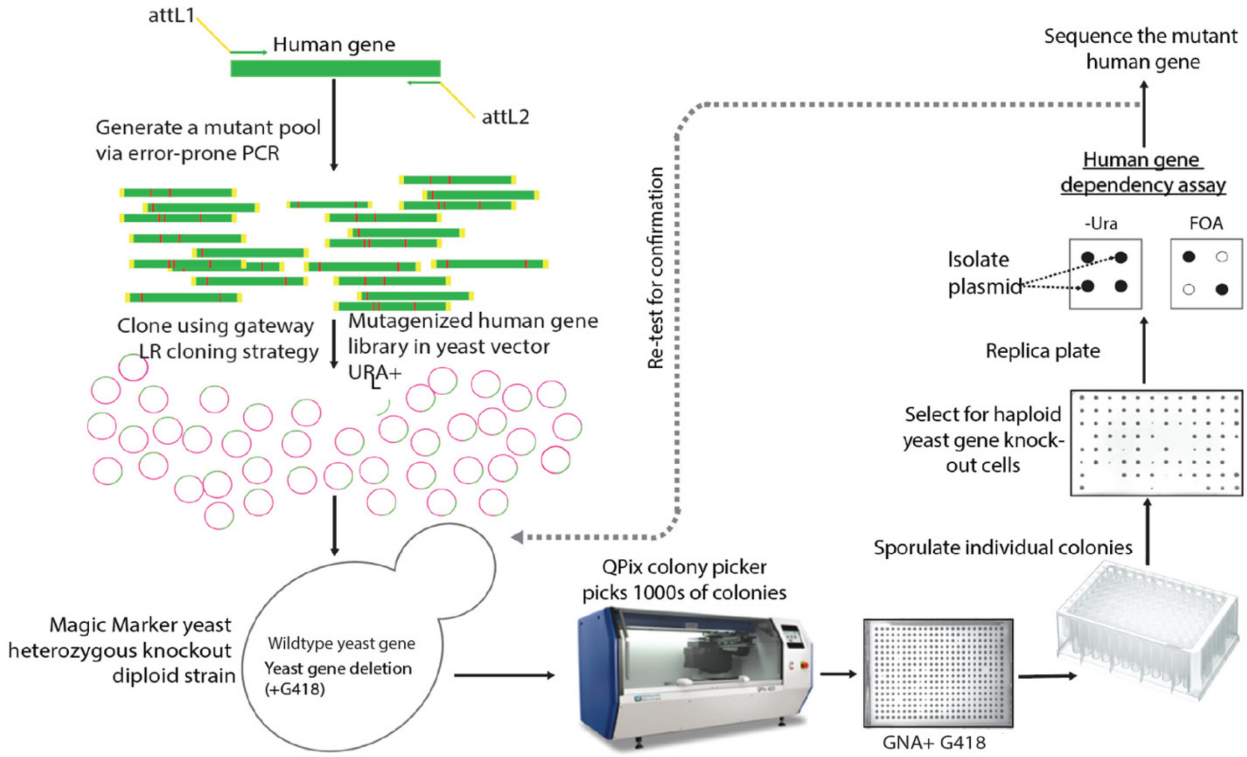

Figure 4. High-throughput pipeline developed to screen suppressors quickly and efficiently using robotics

A human gene mutant library is generated by error-prone PCR and cloned into the expression vector. This is followed by transformation into yeast heterozygous diploid knockout *Pup1/Δpup1*::KanMX strain. Single colonies are picked with the help of robotics (QPix colony picker) and selected on a pre-sporulation GNA-rich media. Individual colonies (from GNA media) are then subjected to sporulation followed by selection on magic marker medium with or without G418 selection. Colonies growing on magic marker medium + G418 are further verified by plasmid dependency assay using 5-FOA selection.

Therefore, my **first objective** was to develop a pipeline to screen for suppressors in a high-throughput manner with the help of robotics available at the Concordia Genome Foundry (**Figure 4**). We specifically focused on screening for suppressors for the non-replaceable catalytic human β2 subunit (*PSMB7*). If successful, this method would allow a faster, extensive scale screening while minimizing contamination and errors in a shorter time. Though the concept behind this pipeline remains the same, we have tweaked specific steps to carry out the experiments in a 96-well format. We have also eliminated tetrad dissection and included plasmid-dependency assay that uses 5-FOA selection to confirm the complementation of defective yeast by human gene.

2.2. Large-scale transformation and selection of functionally replaceable human genes in yeast

The wild type human *PSMB7* (human *β2*) gene cannot functionally complement the orthologous yeast gene *Pup1* (yeast *β2*) [1]. Therefore, a *PSMB7* (human *β*2) mutant library was generated previously by Dr. Aashiq Kachroo using the error-prone PCR strategy that introduced ~0-4 mutations per kbp [1]. The *PSMB7* gene mutants were cloned into the yeast expression vector pAG416GPD-ccdB (CEN) harbouring a constitutive GPD promoter and an auxotrophic selection (-Ura) using the LR Gateway cloning strategy [31] (**Figure 5A**).

To verify if a mutant *PSMB7* gene can functionally complement the loss-of-function of the yeast *Pup1*, we transformed the mutant library of *PSMB7* expression vectors in the yeast diploid HetKO strain (*Pup1*/Δ*pup1::KanMX*). We performed a large-scale transformation of the *PSMB7* gene mutant library by scaling up (upto 4X) the transformation protocol such that several thousand well separated yeast colonies were obtained, each carrying a plasmid with a unique *PSMB7* gene mutant. We plated the transformation mix on a Q-tray (instead of a small petri dish) to pick each distinct colony using robots. Nearly 1000 colonies were picked in an automated manner using the QPix 460 Colony Picking System available at the Concordia Genome Foundry (**Figure 5B**). The colonies picked by the QPix were spotted in a 96-well format on the pre-sporulation GNA medium with G418 selection (**Figure 5B**).

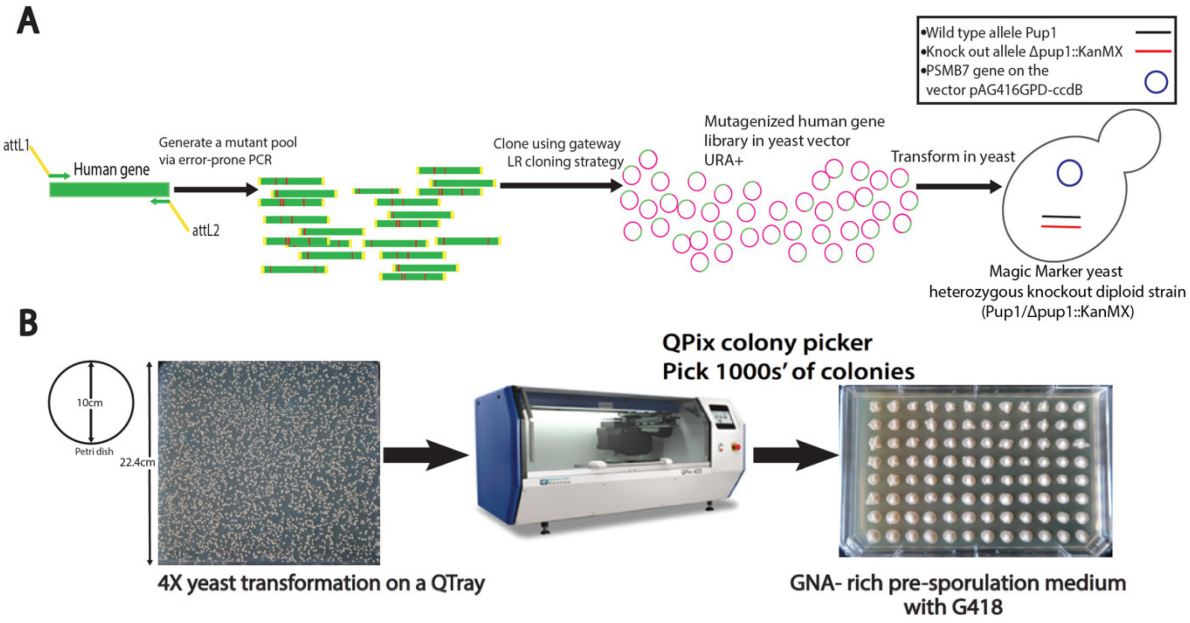

Figure 5. Large-scale transformation of *PSMB7* mutant library in *Pup1/Δpup1::KanMX* HetKO strain

A. Human gene *PSMB7* was randomly mutagenized using error-prone PCR (0-4 mutations per kbp) to create a mutant pool and cloned into the yeast expression vector pAG416GPD-ccdB (+Ura) followed by transformation into yeast HetKO strain (*Pup1*/Δ*pup1::KanMX*) **B.** Scaled up *PSMB7* mutant library transformation (4X) was performed and the mixture plated on a QTray and each colony was picked by QPix 460 Colony Picking System (1000 colonies were picked) and spotted on GNA rich pre-sporulation medium with antibiotic selection G418 in a 96-well format.

2.3. Sporulation and selection of viable haploid knockout strains that express a replaceable variant of a human gene

As previously indicated, the HetKO strains used in this study carry an SGA selection marker (also called a Magic Marker) that allows the selection of haploid strains from a mixture of unsporulated diploids and haploid spores [72] (**Figure 6A**). To obtain haploid spores, the transformed diploid HetKO yeast were subjected to starvation by transferring to a sporulation medium [29, 30, 72]. After sporulation, the mixture would contain the wild-type and the knockout haploid strains (MAT a and MAT α types), both harbouring the mutant *PSMB7* human gene on the plasmid in addition to unsporulated diploids [29, 30]. The mixture was plated on a synthetic Magic Marker medium (-Leu -Arg -His -Ura +CAN in the presence or

absence of the antibiotic G418) in a 96-well format. This selection only allows the growth of MATa type haploid yeast strains harbouring the yeast knockout allele when grown in the presence of G418 while selecting for the mutant *PSMB7* human gene (-Ura) simultaneously [1,15,17]. The *Pup1* gene is essential for viability of the yeast cell, therefore, Δ*pup1::KanMX* haploid strain is inviable. However, if the human gene (or its variant) successfully replaced the function of the gene, the strain shows growth on the Magic Marker medium with G418 [1]. As an internal control for sporulation efficiency, we also tested the growth of wild type *Pup1* haploid spores on Magic Marker medium in the absence of G418 (MM-G418) which show growth (**Figure 6B, left panel**). Out of 1000 colonies, 19 grew on MM+G418 (see representation image in **Figure 6B, right panel**), which were considered the potential strains carrying human gene suppressors that functionally replace the corresponding yeast gene. The potential haploid suppressor mutants (19 selected) were re-selected on MM-G418 and MM+G418 medium separately on petri dishes to obtain single colonies and perform further assays to confirm the mutant human gene plasmid dependency as described in **Section 2.4**.

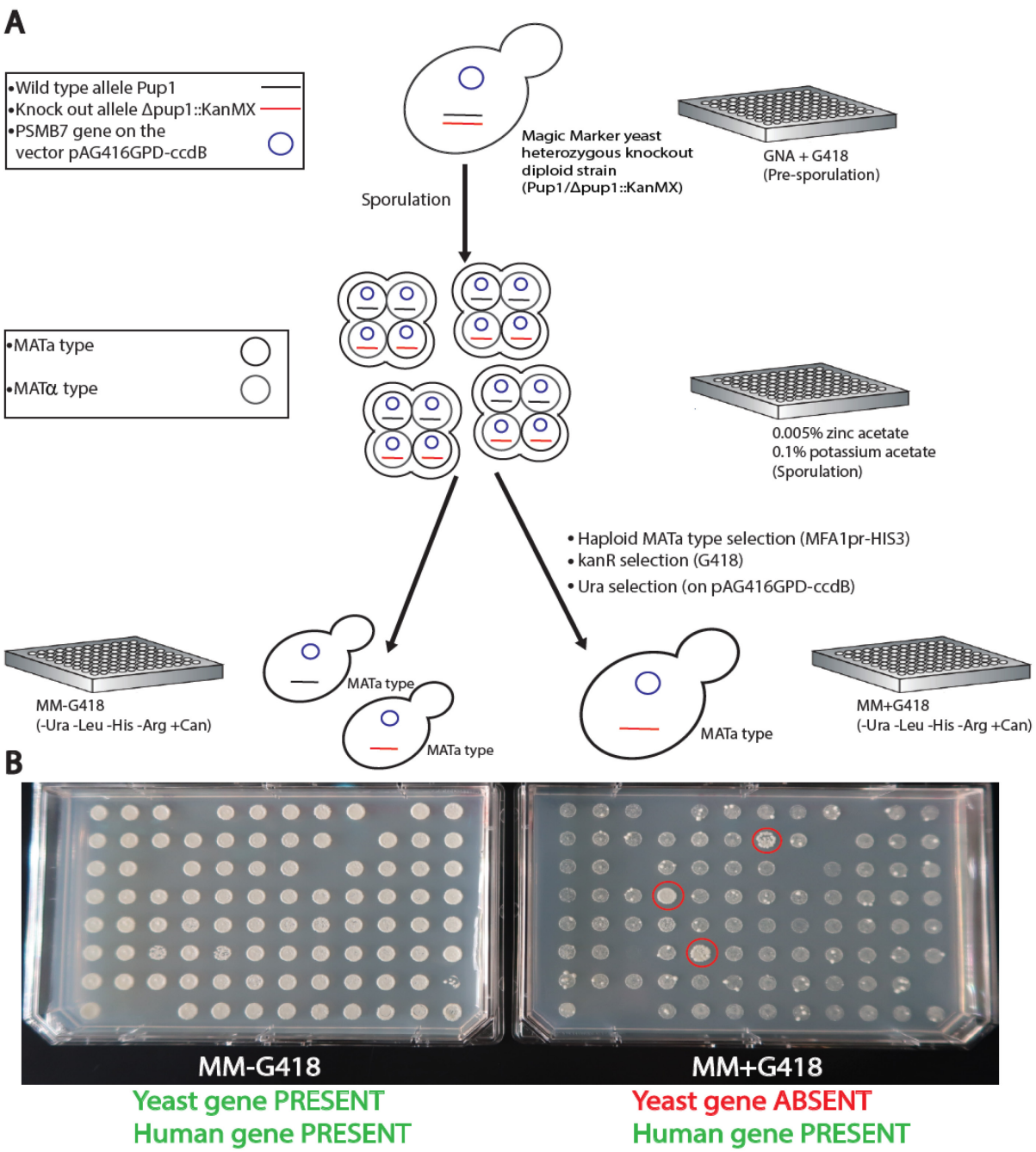

Figure 6. Selection of viable haploid knockout strains to find the replaceable human genes

A. The human gene containing plasmid transformed yeast strains are sporulated to generate wild-type and the knockout haploid strains (MAT a and MAT α types), both containing the mutant *PSMB7* human gene on the plasmid (pAG416GPD-ccdB). The haploid spores were grown on MM-G418 and MM+G418 medium to select for MATa type haploid yeast knockout strain (G418) while simultaneously selecting for human gene on the vector (+Ura) to screen for potential human gene suppressors [72]. **B.** A representative plate shows two unique mutants growing similarly on both MM-G418 and MM+G418 and are considered as potential suppressors.

2.4. Human gene plasmid dependency assay

Selection on Magic Marker medium with or without G418 is limited in revealing the true nature of suppression. The suppression may occur as a consequence of three main events such as **mis-segregation** (that allows both the homologous chromosomes carrying the wild type and the knockout copy of the yeast gene to segregate in the same cell), **intergenic suppression** (that may be due to the mutation on another yeast locus) or an extragenic suppression due to the mutant human gene on the plasmid [19]. To verify if the replaceability is due to the expression of the mutant human gene, the potential suppressors were tested for plasmid dependency assay. This is achieved by selecting the potential suppressors on a synthetic medium with a minimum amount of uracil and 5-FOA (5-Fluoroorotic Acid). If metabolized by the *URA3* gene (present on the human gene harbouring plasmid, fluoroorotic acid converts to a toxic compound 5-FU (5-fluorouracil) that is fatal to yeast (**Figure 7A**) [32]. The medium allows the selection of the yeast cells that can successfully eliminate the human gene expressing plasmid. If the yeast cell survival is dependent on the human gene, those cells cannot grow in the absence of the human gene. 7 out of 19 suppressors could not survive on the medium containing 5-FOA, suggesting that the human gene variant is essential for their viability (the representative example is shown in **Figure 7B**). Next, we isolated the human gene expressing plasmid from each of the yeast strains and purified it by transformation and plasmid isolation in *E. coli*.

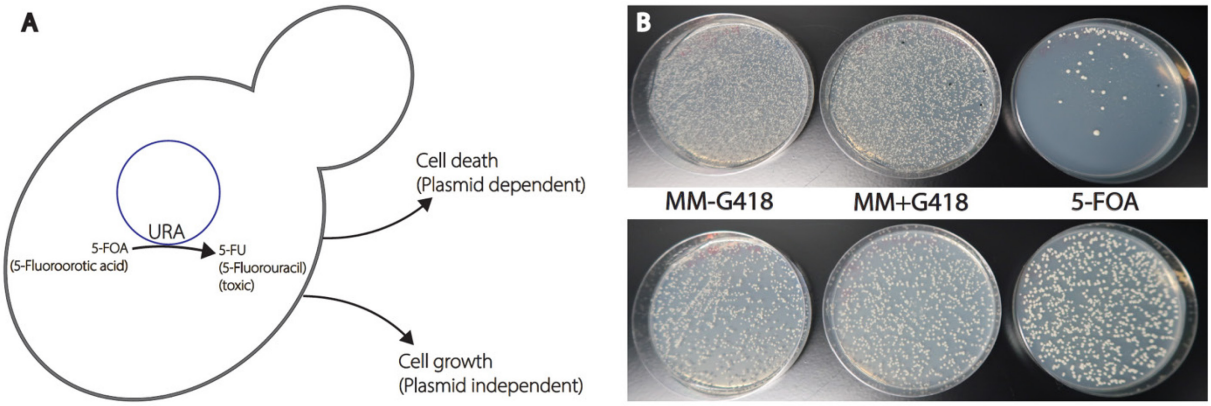

Figure 7. Human gene plasmid dependency assay to confirm if the replaceability is due to the human gene variant

A. 5-FOA in the presence of URA3 gene is metabolized into 5-FU (toxic compound fatal to yeast)**.** Therefore, 5-FOA can be used to perform plasmid dependency assay by selecting yeast cells on 5-FOA containing medium. Yeast cells that can eliminate the plasmid and are viable, grow in the presence of 5-FOA whereas the yeast cells whose survival is dependent on the human gene containing plasmid cannot grow on 5-FOA medium. (B) A representative example shows human gene dependent suppressors fail to grow on 5-FOA plates indicating their plasmid dependency **(Top panel**), whereas plasmid independent mutants showed growth on 5-FOA plates (**Bottom panel**).

2.5. Confirmation of complementation by potential human gene suppressors by retransformation in *Pup1*/Δ*pup1::KanMX* HetKO strain

To further confirm the complementation by the mutant human gene variant, we isolated the plasmid from each individual strains that passed the test of plasmid dependency. Next, we retransformed the *Pup1*/Δ*pup1::KanMX* HetKO strain, each with a purified plasmid using the same pipeline as described above in **Figure 4**. Upon re-analysis, all 7 out of the 19 suppressors, as previously identified, grew on MM+G418 medium (**Figure 8A)** and also showed plasmid dependency on 5-FOA medium. We performed quantitative growth assays to show that the expression of the variant human genes complements the deletion of the corresponding yeast ortholog similar to the yeast gene (*PupI*) when expressed similarly on the plasmid (**Figure 8B**).

2.6. Identification and characterization of replaceable human gene mutations

All the 7 complementing human gene variants were sequenced to identify the mutations responsible for functional replaceability in yeast. Sequence analysis showed that each plasmid contained a unique variant of the human gene. We also identified a previously characterized suppressor S214G (Ser^{214} -> Gly^{214}), further confirming the optimal functioning of the pipeline to discover replaceable human gene suppressors (**Figure 8C**).

Table 1 also shows the human gene *PSMB7* suppressors with the corresponding amino acid changes and their phenotype compared to the growth phenotype of wild type *Pup1* (yeast *β*2 subunit) gene when expressed on the plasmid. Some human gene variants harboured more than one mutation (K249R-R32W, S161T-T260I-E263K, T44A-E26K-M67V-N224D, T233R-D96V, T44A-L116H), while some were single-site mutants (A70V and S214G). To assess the fitness of the human gene suppressors, quantitative growth assays were performed.

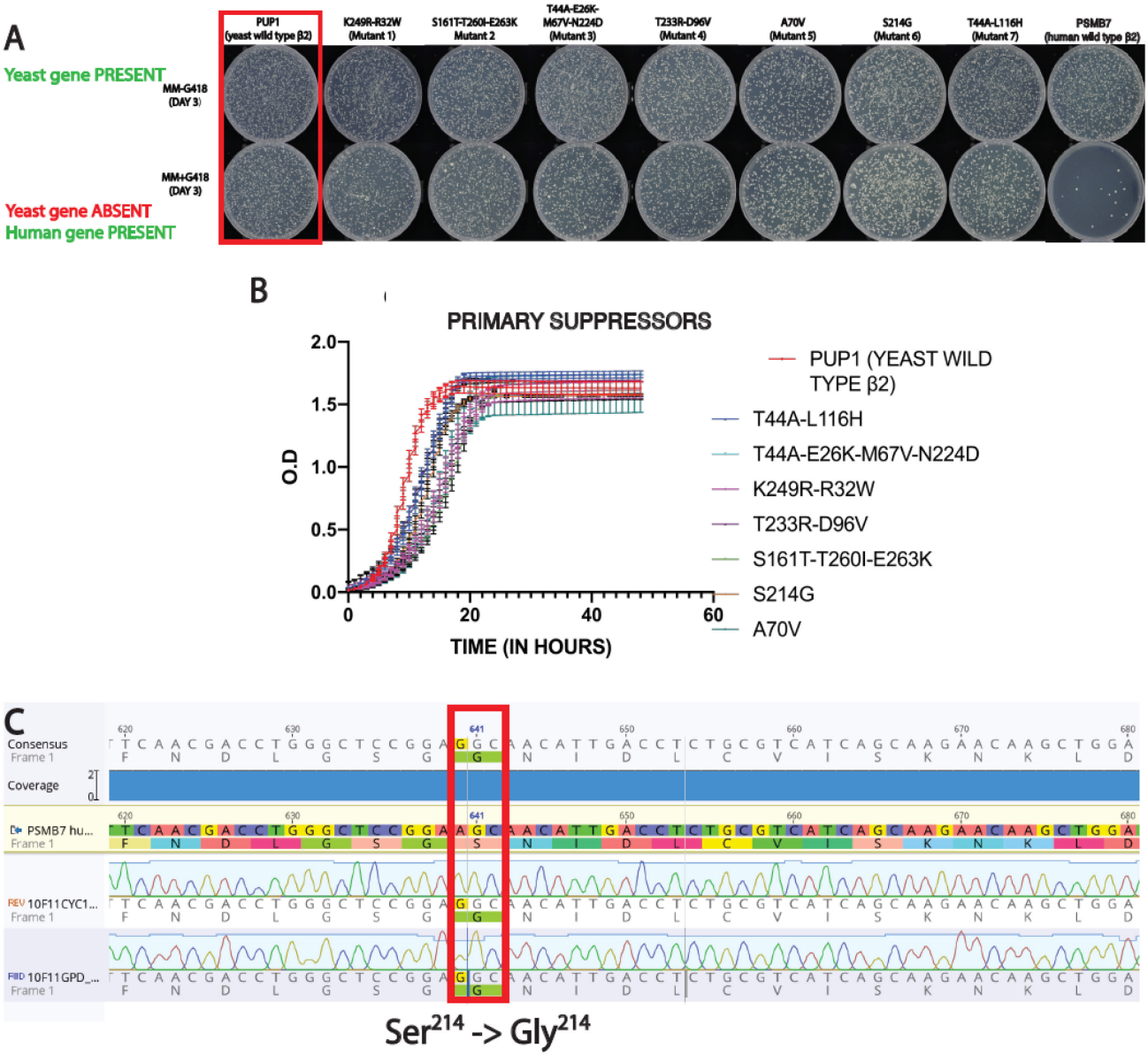

Figure 8. Conformation, identification and growth analysis of replaceable human gene suppressors

A. Selection of human gene suppressor mutants (K249R-R32W, S161T-T260I-E263K, T44A-E26K-M67V-N224D, T233R-D96V, A70V, S214G, T44A-L116H) on MM-G418 (native yeast gene PRESENT) and MM+G418 (native yeast gene ABSENT & Human gene PRESENT) showing growth similar to a positive control (i.e., in the presence of plasmid-borne yeast *Pup1*; highlighted in red) after 3 days. **B.** Quantitative growth assays of replaceable human gene suppressors show similar growth profile compared to the yeast wild type *Pup1* when expressed on plasmid (in red). **C.** Representative Sanger sequencing analysis of one of the human gene mutants revealed a previously identified suppressor *PSMB7* - S214G (Ser^{214} -> Gly^{214}) (highlighted in red). Both Forward and Reverse primer reads are aligned to the wild-type reference sequence.

Table 1: Summary of all the human gene mutations associated with each replaceable human gene variant

Primary mutants	Amino acid change	Phenotype of replaceable human gene variant in yeast knockout strain (growth compared to yeast *β*2 wild type *Pup1*)
Mutant 1 **(K249R-R32W)**	Lys -> Arg (K249R)	Normal growing
	Arg -> Trp (R32W) (Part of the propeptide)	
Mutant 2 **(S161T-T260I-E263K)**	Ser -> Thr (S161T)	Normal growing
	Thr -> Ile (T260I)	
	Glu -> Lys (E263K)	
Mutant 3 **(T44A-E26K-M67V-N224D)**	Glu -> Lys (E26K) (Part of the propeptide)	Normal growing
	Thr -> Ala (T44A) (Active site)	
	Met-> Val (M67V)	
	Asn -> Asp (N224D)	
Mutant 4 **(T233R-D96V)**	Asp -> Val (D96V)	Normal growing
	Thr -> Arg (T233R)	
Mutant 5 **(A70V)**	Ala -> Val (A70V)	Normal growing
Mutant 6 **(S214G)**	Ser -> Gly (S214G)	Normal growing
Mutant 7 **(T44A-L116H)**	Thr -> Ala (T44A) (An active site)	Normal growing
	Leu -> His (L116H)	

3. Chapter 3: Characterization of single nucleotide substitutions in human *PSMB7* contributing to the replaceability of yeast *Pup1*

3.1 Using site-directed mutagenesis to construct single-nucleotide human variants to identify mutation(s) that allow functional complementation in yeast

Several primary replaceable human gene suppressors identified through the initial screen (**Chapter 2**) were observed to harbour multiple mutations in human *PSMB7* gene. To determine which of these mutations alone or in combination contributed to the functional replacement of yeast *Pup1*, we performed site-directed mutagenesis to engineer each of the mutations into the wild-type human *PSMB7* (**Figure 9A**). Each expression vector was transformed into the *Pup1*/Δ*pup1::KanMX* HetKO strain to test for functional complementation as previously described in **Chapter 2.** The approach should identify which single nucleotide change is sufficient to complement the lethal growth defect or if a combination of mutations was required to achieve complementation (**Figure 9C & 9D**). As shown in **Table 2**, we observed 8 out of the 14 single mutants, namely R32W, T260I, E263K, E26K, M67V, N224D, D96V and L116H, failed to complement the *pup1* deletion independently. Thus, the experiment showed which mutations are not required for replaceability and narrowed down the most critical suppressor mutations necessary for human gene replaceability in yeast (**Figure 9B**). 6 of 14 mutants resulting in a single amino acid change (K249R, S161T, T44A, T233R, A70V and S214G) complemented the lethal yeast growth defect. 3 of 6 single mutants (A70V, T44A and S214G) show growth comparable to the yeast *Pup1* (**Figure 9C**). In contrast, the 3 remaining point mutations (S161T, T233R, K249R) show delayed growth after 5 days compared to wild-type *Pup1* (**Figure 9D**).

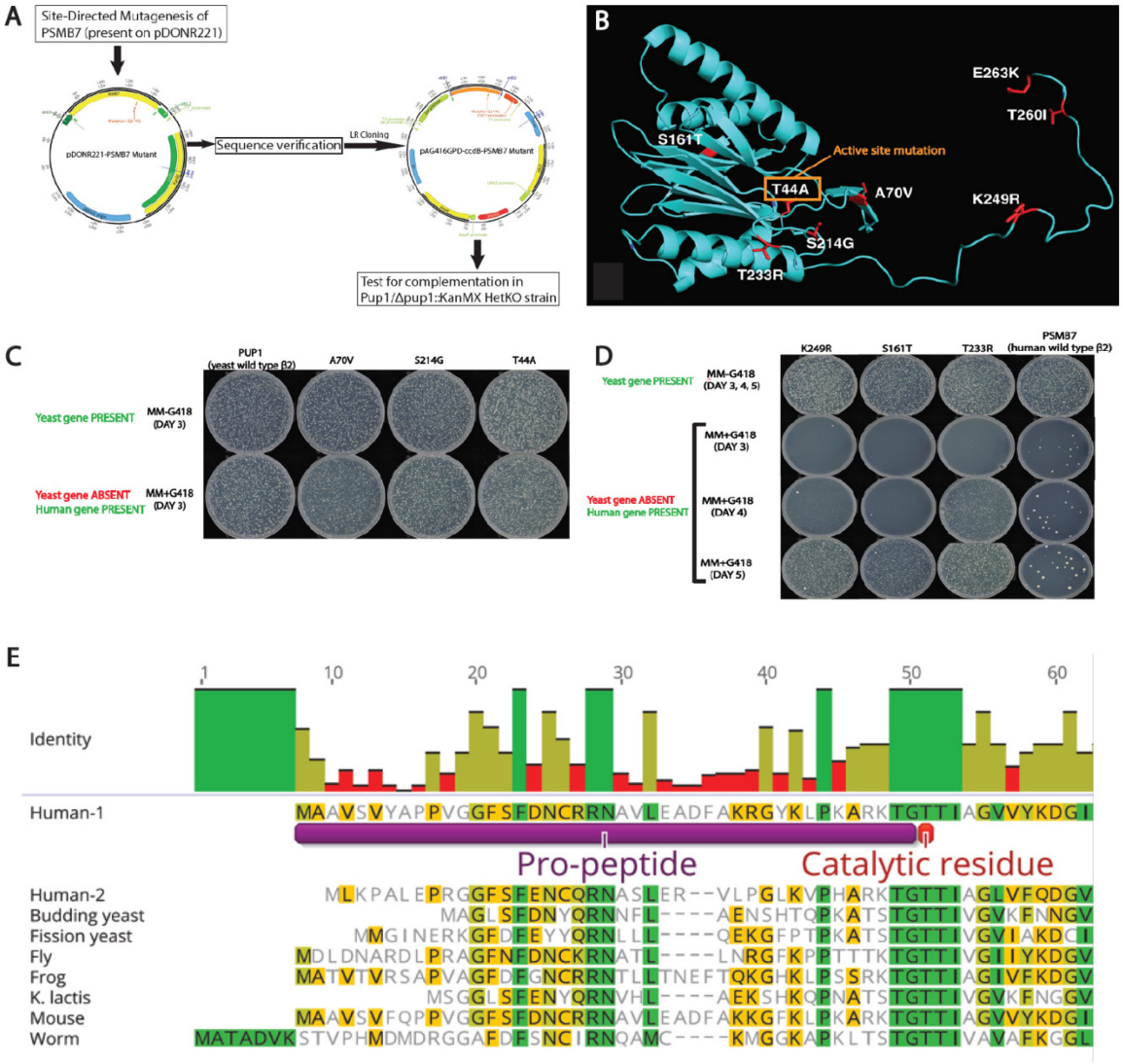

Figure 9. Construction and characterization of single-nucleotide human *PSMB7* variants to identify specific mutation(s) sufficient for functional replaceability in yeast

A. Site-directed mutagenesis was performed to create single-site *PSMB7* mutants (in an entry clone pDONR221). After sequence verification, the single-site *PSMB7* mutants were cloned into the yeast expression vector pAG416GPD-ccdB and tested for complementation in *Pup1*/*Δpup1::KanMX* HetKO strain. **B.** Single amino acid substitutions in human *PSMB7* are shown in a crystal structure of *PSMB7* (PDB 1IRU) that allow the functional rescue of the corresponding yeast ortholog (highlighted in red). The structure reveals critical amino acids and domains the might be necessary to allow interaction and assembly in the yeast proteasome core. Surprisingly, T44A mutation (highlighted in orange) is a catalytically inactive *PSMB7* that was able to rescue the *pup1* deletion. **C.** Selection of single-site mutants on MM-G418 (native yeast gene PRESENT) and MM+G418 (native yeast gene ABSENT). The yeast *Pup1* and human wild type gene *PSMB7* on plasmids serve as positive and negative controls respectively. Single mutants showed growth (after 3 days) similar to the positive control *Pup1*. **D.** Some single-site mutants showed delayed growth (after 5 days) unlike *Pup1*. **E.** Alignment of *β*2 gene sequences shows conservation of the Threonine (Thr) residue at the active site (highlighted in red).

To further quantify the efficiency of functional replaceability, we performed quantitative growth assays. In the cases where more than one mutation contributed to the functional replaceability, we expect the single mutants to grow slowly or show no growth at all when compared to the primary mutant. Thus, the quantitative growth assays should also test the requirement of more than one mutation for optimal functional complementation. For example, in the case of **mutant 1** (K249R-R32W), **mutant 2** (S161T-T260I-E263K) and **mutant 4** (T233R-D96V) (**Table 2**) (**Figure 9C & 9D**), while the single amino acid change is enough to confer functional replaceability, the accessory mutations tend to allow more efficient functional complementation. The quantitative growth assays are described in detail in the next section.

Table 2: Phenotype of the single mutants created from the previously identified primary suppressors through site-directed mutagenesis

Primary mutants	Single mutants	Phenotype of single mutants in yeast knockout strain (growth compared to yeast *β*2 wild type *Pup1*)
Mutant 1 (K249R-R32W)	K249R	Slow growing
	R32W (part of propeptide)	No growth
Mutant 2 (S161T-T260I-E263K)	S161T	Slow growing
	T260I	No growth
	E263K	No growth
Mutant 3 (T44A-E26K-M67V-N224D)	T44A (Active site)	Normal growth
	M67V	No growth
	E26K (part of propeptide)	No growth
	N224D	No growth
Mutant 4 (T233R-D96V)	D96V	No growth
	T233R	Slow growing
Mutant 5 (A70V)	A70V	Normal growth
Mutant 6 (S214G)	S214G	Normal growth
Mutant 7 (T44A-L116H)	T44A	Normal growth
	L116H	No growth

3.2 Quantitative growth assays reveal which single amino acid changes in *PSMB7* are sufficient to confer functional replaceability in yeast

Based on the data shown in **Figure. 9C & 9D**, some single mutants (S161T, K249R and T233R) appeared to show delayed growth compared to their corresponding primary suppressors with multiple mutations (i.e., S161T-T260I-E263K, K249R-R32W and T233R-D96V, respectively). To further verify that these suppressors needed more than one mutation to rescue the loss of yeast gene function optimally, we tested this assumption by performing growth assays for single-site mutants and comparing the assay with the primary *PSMB7* suppressors (**Figure 10**).

We show that the single-site suppressors, A70V and S214G (also the primary suppressors), showed a similar growth profile to the yeast β2 wild-type version *Pup1* (**Figure 10B & 10C**). Interestingly, in the case of *PSMB7*-T44A active site mutant, we also observed comparable growth to the wild type yeast, which in turn was similar to the primary suppressors T44A-L116H and T44A-E26K-M67V-N244D (**Fig. 10D**). Thus, in the case of these suppressors, the other mutations (L116H, E26K, M67V and N224D) are just carriers due to random mutagenesis and are not required for replaceability. Threonine residue serves as an active site for the catalytic proteasome β subunits and are highly conserved [12,14,37,38] (**Figure 9E**). Previously, it was shown that an active site mutation T30A in the *Pup1* gene (yeast β2) revealed growth defect [21,38]. In contrast, we do not observe any obvious growth defect in case of the *PSMB7* T44A mutant. The β2 subunit is the first to be assembled during the β-ring formation and its N-terminal pro-peptide acts as a chaperon during this process [20]. Since a catalytically dead human *PSMB7* mutant (T44A) was able to complement the corresponding yeast ortholog, it suggests a possible previously uncharacterized role of an active site in proper proteasome assembly.

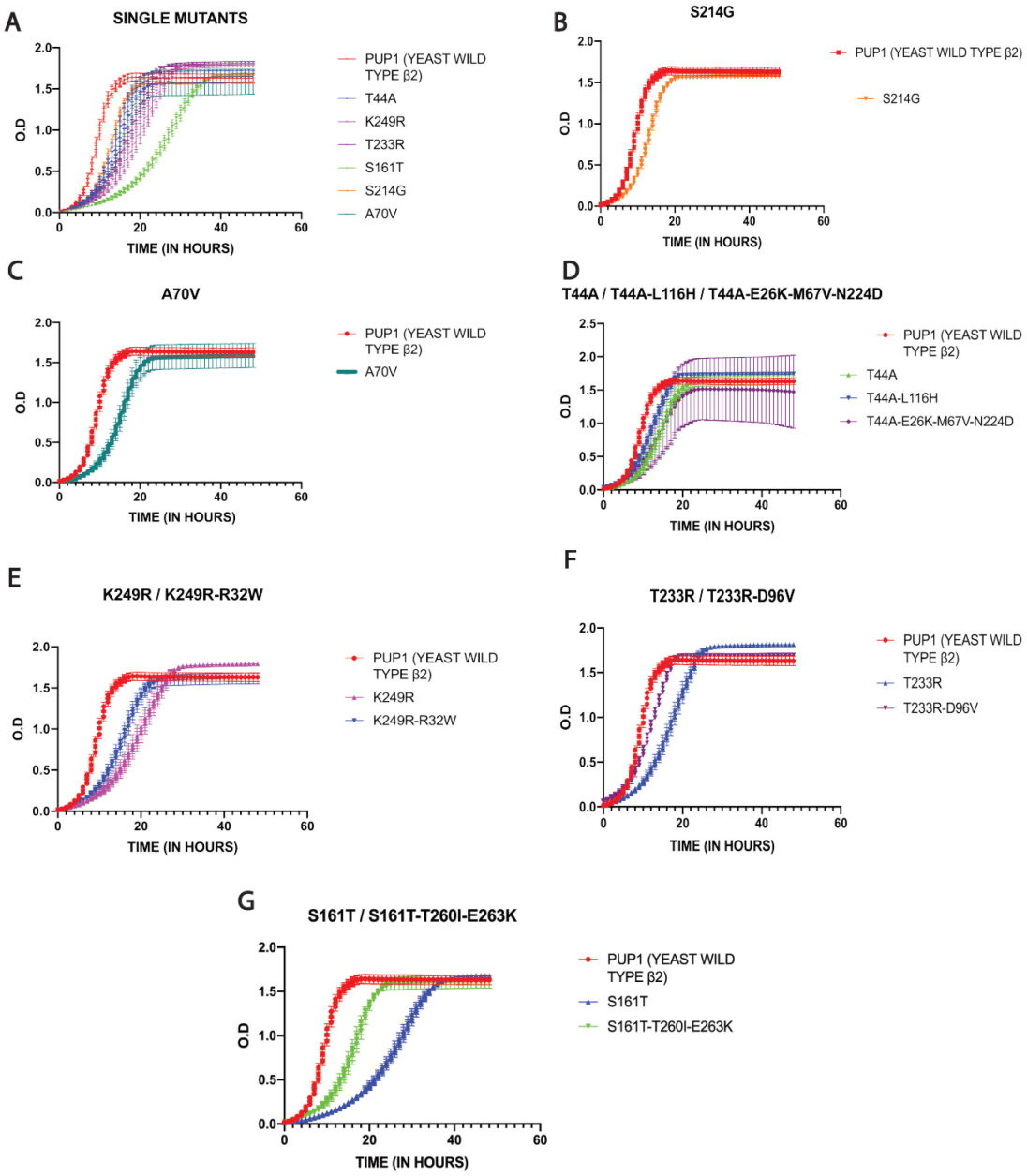

Figure 10. Quantitative growth assays to compare replaceability of single-site mutants to their primary suppressors

A. Representation of the growth profile of all the single-site *PSMB7* mutants compared to the growth profile of the positive control *Pup1*(yeast wild type *β*2 in red). **B.** *PSMB7* (S214G) mutant compared to yeast *Pup1* **C.** *PSMB7* (A70V) mutant compared to yeast *Pup1* **D.** *PSMB7* (T44A) mutant compared to the primary suppressors *PSMB7* (T44A-L116H), *PSMB7* (T44A-E26K-M67V-N224D) mutant and the positive control yeast *Pup1* **E.** *PSMB7* (K249R) mutant compared to the primary suppressor *PSMB7* (K249R-R32W) and the positive control yeast *Pup1*. **F.** *PSMB7* (T233R) mutant compared to the primary suppressor *PSMB7* (T233R-D96V) and the positive control yeast *Pup1* **G.** *PSMB7* (S161T) mutant compared to the primary suppressor *PSMB7* (S161T-T260I-E263K) and the positive control yeast *Pup1*.

Similarly, we observed a comparable growth for single-site mutants K249R and T233R compared to their primary counterparts and the wild type yeast (**Figure 10E** & **10F**), therefore, eliminating the role of mutations R32W and D96V in suppression. Finally, the single mutant S161T showed a significant delay in the start of their exponential phase thus, indicating a growth defect when compared to both the wild type yeast and its primary mutant S161T-T260I-E263K (**Figure 10G**). This suggests a possible contribution of the C-terminal tail mutations, T260I or E263K, in complementation of the loss of function of the yeast ortholog.

3.3 Structural characterization of functionally human gene variants identifies residues and domains responsible for functional replaceability of human *PSMB7* in yeast

The ultimate aim of large-scale suppressor screen was to identify several mutations that confer functional replaceability of human *PSMB7* gene in yeast. Together, the screen should allow the identification of incompatible amino acids or domains in human-yeast proteins respectively that do not allow functional replaceability. We had previously modelled the human *PSMB7* structure in the yeast proteasome core structure [1]. We highlighted the amino acids required for functional complementation in the human *PSMB7* and classified the mutations based on their proximity to the neighbouring yeast proteasome subunits (**Figure 11**).

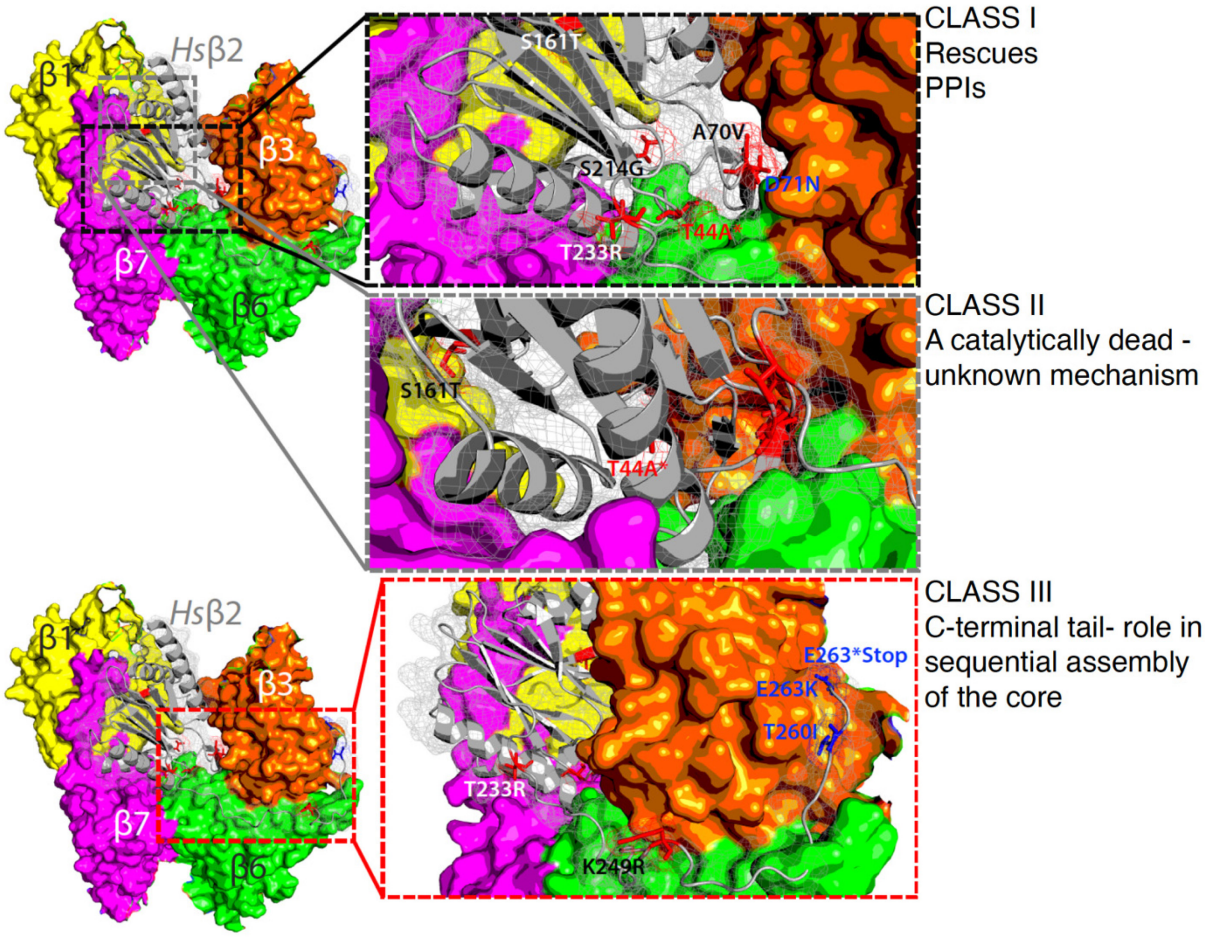

Figure 11. Modelling of human *PSMB7* in the yeast proteasome core reveals the nature of replaceable human gene suppressors

Human *PSMB7* (grey ribbon; PDB 1IRU) was modelled in the yeast proteasome core structure (indicated as coloured subunits in yellow- β1, orange- β3, pink- β7 and green- β6; PDB 1RYP). Amino acids changes that contribute to functional replaceability are highlighted (single amino acid substitutions alone in red & as accessory substitutions as blue).

We broadly classify the mutations in three classes. **Class I** mutations likely rescue the PPIs with neighbouring yeast proteasome subunits (such as with yeast β1, β3 and β6). **Class II** are those that affect the activity of the proteasome subunit. While T44A mutant falls in this class, S214G mutations lies close to the active site and may also fall in this category. We are in the process of testing if the T44A mutation in *PSMB7* gene leads to a catalytically dead β2. In other words, we are testing if the catalytic activity is linked to the assembly of the β core in yeast. In turn, the assays may reveal novel and diverged mechanism of core assembly in human

proteasome compared to the yeast. **Class III** mutations reside on the C-terminal tail of the *PSMB7*. C-terminal tails of *PSMB7* interacts with the neighbouring β3 and α subunits [20, 21, 22]. The tail plays an important role in proper sequential assembly of the β proteasome core [7, 20]. In the next chapter, we test the role of C-terminal tail in the assembly of the human *PSMB7* in the yeast proteasome core.

4. Chapter 4: C-terminal tail swaps from yeast to human β2 show possible role of the tail in proteasome assembly

4.1 Swapping human C-terminal domain with yeast C-terminal domain

The structural analysis of the human β2 subunit revealed that 4 out of 14 mutations reside on the C-terminal tail of human *PSMB7* (**Figure 11**). The substitutions such as E263-Premature STOP (previously identified) [1], T233R, K249R, T260I and E263K lie in the C-terminal region (**Figure 11)**. The β2 subunit is the first to be assembled during the formation of the β-ring in the proteasome core which then recruits β3 subunit [20]. The C-terminal region of the β2 subunit wraps around the β3 subunit of the proteasome while also interacting with the β4 and α subunits [20, 21, 22]. The C-terminal of the β2 subunit also acts as 'intramolecular chaperones' during the proteasome assembly [20]. Moreover, sequence alignment of β2 subunit across diverse genera also show that the C-terminal region has diverged compared to the rest of the protein (**Figure 12A**). Therefore, we hypothesize that the interactions involving these domains may have changed between humans and yeast, thus, leading to incompatibilities and failure in proteasome assembly in the humanized yeast. Therefore, to test this hypothesis, we asked if swapping the yeast C-terminal tail on the human *PSMB7* would be enough to allow functional replaceability of the human gene in yeast. We engineered three human-yeast hybrid protein with different lengths of C-terminal tail swaps (**Figure 12B**), whereby a part of the C-terminal region of the β2 subunit is of yeast origin and the rest of it is of human origin. The lengths of the yeast C-terminal domain were chosen based on the mutations on the human C-terminal region (T233R, K249R, T260I and E263K) and the structure of the tail. These hybrids human-yeast genes were cloned into the expression vector pAG416GPD-ccdB and transformed into the *Pup1*/Δ*pup1::KanMX* HetKO diploid yeast to test functional replaceability.

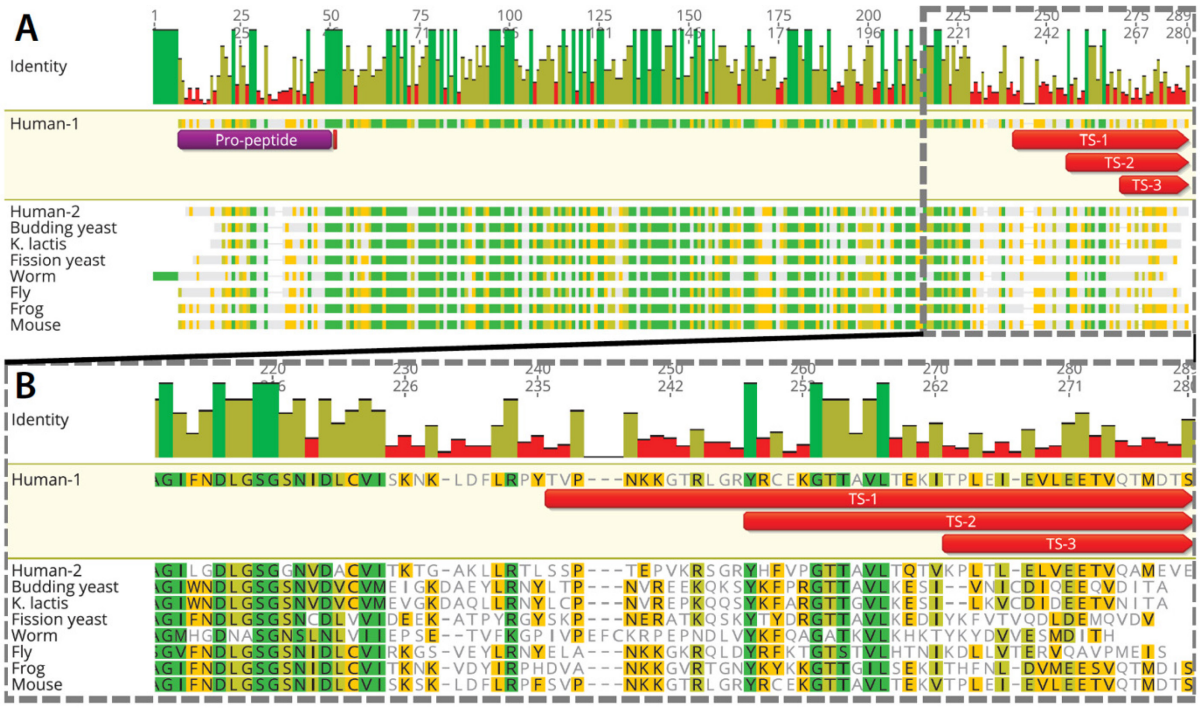

Figure 12. Pro-peptide and C-terminal domains of *β*2 subunits have diverged compared to the rest of the protein

A. Alignment of *β*2 subunits show that the C-terminal domain has diverged among diverse organisms. C-terminal tails are critical for subunit interaction and sequential proteasome assembly. The identity score is represented as bars with 100% identity in green and medium in light green and low in red. **B.** The region of tail swaps performed from yeast to human protein are shown as red arrows. Tail Swap-1 (TS-1) swaps the entire yeast *β*2 tail in humans *PSMB7*, Tail Swap-2 (TS-2) & Tail Swap-3 (TS-3) carry C-terminal tail swap of progressively smaller region respectively.

We named these domain swaps as Tail-Swap-1 (TS-1), Tail-Swap 2 (TS-2) and Tail Swap-3 each with progressively smaller regions of yeast protein sequence respectively with TS-1 harbouring an entire C-terminal tail from yeast *β*2 subunit (**Figure 13A**). The functional complementation test revealed that an entire tail swap i.e., TS-1 showed functional complementation similar to the wildtype yeast *β*2 gene (**Figure 13B**) whereas TS-2 and TS-3 failed to do so. The human *PSMB7*-TS-1 showed growth profile similar to the positive control *Pup1* (yeast wildtype *β*2) as confirmed by quantitative growth assays (**Figure 13C**). The C-terminal swap in human-yeast **TS-1** hybrid harbours the longest yeast C-terminal compared to

the other domain swap hybrids (**Figure 13A**) by swapping the human C-terminal with the 42 amino acid length yeast C-terminal.

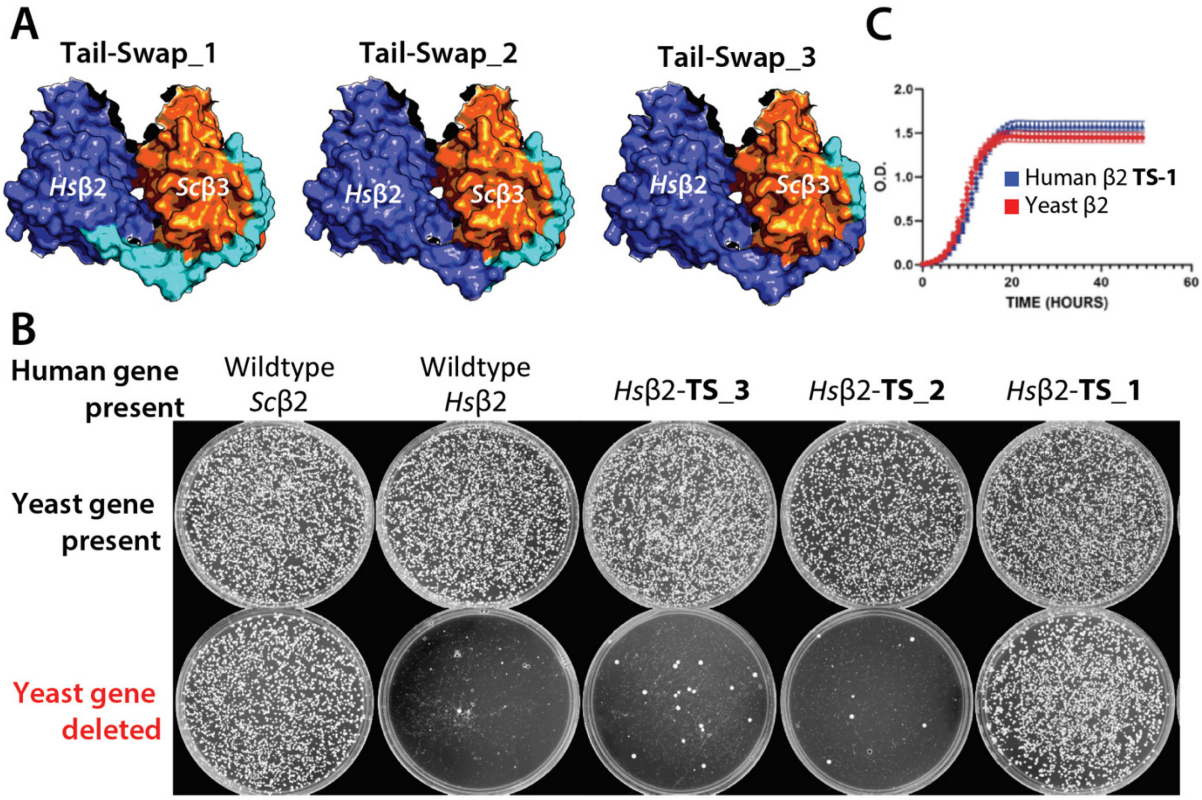

Figure 13. The C-terminal tail swaps from yeast to human *β*2 shows the role of the tail in proper proteasome assembly

A. Structure of *β*2 (blue) (PDB 1IRU) and *β*3 (orange) (PDB 1RYP) in the proteasome core shows the C-terminal tail of *β*2 wrapping around the *β*3 subunit. The C-terminal tail swapped from human to yeast is shown in cyan. Tail Swap-1 (TS-1) represents the entire yeast C-terminal tail swapped in the human *β*2. The progressively smaller C-terminal tails of yeast are shown in the case of Tail Swap-2 & 3 respectively. **B.** The functional replaceability test performed on MM-G418 (native yeast gene present) and MM+G418 (native yeast gene deleted) shows the yeast *Pup1* and human wild type gene *PSMB7* on plasmids serving as positive and negative controls respectively. Yeast *Pup1* successfully complements the deletion of the native yeast gene copy whereas the wildtype human *β*2 does not. The full-length C-terminal tail swap containing human *β*2 (TS-1) showed growth similar to the positive control *Pup1* after 3 days incubation at 30°C. **C.** Representation of the growth profile of TS-1 mutant compared to the growth profile of the positive control *Pup1* (yeast wild type *β*2). The mean of 3 independent growth curves is plotted (N=3).

Previously, the deletion of the yeast β2 C-terminal region caused lethality in yeast [22]. The experiment further supports the critical role of C-terminal tail in core assembly. The deletion of the yeast β2 C-terminal also led to the accumulation of intermediates that only contained β2, but did not contain β3, suggesting that the β2 subunit was unable to recruit β3 subunit thus, halting the proteasome assembly [20]. In our study, since we were able to make yeast-human hybrids replaceable by a mere swap of C-terminal regions (from human to yeast), it proves the importance of yeast β2 C-terminal in assembly. It also suggests that wildtype human β2 could become replaceable in a humanized β3 context; the hypothesis that we are testing in the lab. We are yet to test if the C-terminal tail swap affects the protease activity of the human β2.

5. Chapter 5: Conclusion

My Master's thesis project focused on evolutionarily conserved human genes that are not swappable in yeast. While the orthology conjecture postulates the broad equivalence of functions between a diverse range of organisms, the functional replaceability tests the extreme version of the hypothesis by swapping orthologous genes from humans into yeast. Yeast and humans share more than 4000 genes [24, 48]. Previous systematic tests have already revealed many human genes that are functionally swappable in yeast, however, many human genes fail to complement the corresponding yeast equivalents [1, 2]. This study was aimed to characterize the incompatibilities in human genes that lead to their non-replaceability in yeast to eventually characterize key features of the orthologous genes that have uniquely evolved between two distinct species. The genetic assays represented in this thesis reveal subtle variations at the amino acid sequence level (mostly single-site changes) needed to efficiently replace previously non-replaceable human genes in yeast.

To identify the amino acid changes that allow replaceability of non-replaceable human genes, we first established a high throughput screen to identify suppressors. In this project, we screened for suppressors of the non-swappable *PSMB7* gene (human *β*2 subunit of the proteasome core). **Chapter 2** describes a pipeline to screen for human *PSMB7* gene suppressors in a robotic setup. The previously generated *PSMB7* mutant library was used to transform into yeast HetKO strain (*Pup1*/Δ*pup1::KanMX*). Several thousand colonies were individually picked and screened for functional replaceability. We narrowed down the potential suppressors through the selection of viable haploid knockout strains to find the replaceable human genes. We also confirmed the human gene suppressors using plasmid dependency assays by 5-FOA selection. Through this screening process, not only were we able to rediscover a previously identified suppressor (S214G), but we also discovered several new suppressors which had a similar fitness profile compared to the wild-type yeast *β*2.

The primary suppressors harboured more than one mutation per gene. To identify the critical changes in amino acid that contribute to the replaceability of *the PSMB7* gene in yeast, we created mutants by introducing a single amino acid change into the *PSMB7* gene (**Chapter 3**). These single-site mutants were tested for functional complementation using the pipeline from **Chapter 2**. Next, the fitness of these mutants was assessed through quantitative growth assays comparing the functional rescue of these *PSMB7* mutants with the wild-type yeast *β*2 as well as the corresponding primary suppressors. We were able to further narrow down the specific mutations that contribute to replaceability. Surprisingly, we discovered that an active site mutation (T44A) contributes to replaceability in yeast, suggesting there might be a possible role of an active site in proteasome assembly (Class II). The mutations need to be further characterized biochemically to establish the rationale for the role of active site in proteasome assembly.

We modelled human *β*2 structure in the yeast proteasome to map the identified mutations. We observed that several of these mutations were close to the interacting domains (Class I). These suppressors likely rescue the PPIs required for proper assembly of the human *PSMB7* in yeast *β* core. Several suppressors lie on the C-terminal region of the human *β*2 subunit. During the proteasome assembly, the C-terminal region of the *β*2 subunit cloaks around the *β*3 subunit [20, 21, 22]. The N and C-terminals also act as chaperones during proteasome assembly [20]. The sequence alignment of the *β*2 subunits from diverse organisms showed a significantly diverged C-terminal tail regions of *β*2 compared to the rest of the protein suggesting the region has evolved independently, leading to non-replaceability. Therefore, we created different human-yeast hybrids of the *β*2 subunit by swapping the human C-terminal tail with the yeast C-terminal of various lengths. Upon testing for functional complementation, we discovered that the complete yeast C-terminal region was necessary to allow replaceability (**Chapter 4**). In contrast, hybrids with shorter yeast C-terminal failed to complement the

defective yeast, thus confirming our speculations about the missing interactions in the β2 tail region necessary for assembly.

To identify the mode of suppression and the critical interactions between neighbouring subunits, we are in the process of quantifying protein-protein interactions followed by biochemical tests for the activity of the proteasome core.

Systematic replaceability of orthologous genes helps identify the contributions of each amino acid's individual properties to the conservation of function. This feature will become particularly useful in characterizing the mutations that lead to fatal diseases in humans. We aim to utilize the high-throughput strategy that we have developed to screen for suppressors in other non-replaceable human genes, thus expanding the number of replaceable human genes in yeast. Furthermore, by analyzing the amino acid residue changes that allow complementation, we can identify vital protein-protein interactions (PPI) in the context of protein complexes, such as the proteasome, that are functionally critical for efficient assembly and activity. Protein complexes such as proteasome, involve many PPIs and distinguishing which PPIs are critical for proper assembly of the complex is challenging. Therefore, genetically characterizing essential PPIs is critical to understand the genotype-phenotype relationships and their role in human disease.

6. Chapter 6: Materials and Methods:

Media:

The following media components used were from Sigma Aldrich: YPD agar, YPD Broth, yeast nitrogen base without amino acids or ammonium sulfate, yeast nitrogen base without amino acids, yeast synthetic drop-out medium supplements without uracil, dextrose, agar, L-glutamic acid monosodium salt hydrate, nutrient broth, yeast extract and canavanine. G418 (Geneticin) (50 mg/mL) for antibiotic selection was used from Gibco and SC -Ura -Leu -Arg -His (dropout media) from Sunrise. *E. coli* cells were always grown on LB medium (Sigma Aldrich).

The following media were used in this project:

- YPD: yeast extract, peptone, dextrose
- GNA: dextrose, nutrient broth, yeast extract, agar
- SD-URA: yeast nitrogen base without amino acids or ammonium sulfate, L-glutamic acid monosodium salt hydrate, yeast synthetic drop-out medium supplements without uracil, agar, dextrose
- Sporulation media: potassium acetate 10%, zinc acetate 0.5%, autoclaved MilliQ water
- Magic Marker media: yeast nitrogen base without amino acids and ammonium sulphate, L-glutamic acid monosodium salt hydrate, SC -Ura -Leu -Arg -His (dropout media), agar, dextrose
- 5-FOA: yeast nitrogen base without amino acids, yeast synthetic drop-out medium supplements without uracil, uracil, dextrose, 5-FOA, agar

Constructing *PSMB7* mutant library into yeast expression vector:

The *PSMB7* gene mutant library was created by Dr. Aashiq Kachroo by error-prone PCR (GeneMorph II Random Mutagenesis Kit from Agilent) to introduce mutations and add attL1 and attL2 sites at the 5’ and 3’ ends of the gene. The mutant library was then cloned into the

expression vector pAG416GPD-ccdB (+Ura; CEN) under the constitutive promoter GPD via the LR cloning strategy using the Gateway® LR Clonase® II enzyme mix kit from Invitrogen [31]. pAG416GPD-ccdB was used as the expression vector due to the presence of attR1 and attR2 sites, thus making the vector Gateway cloning compatible [25]. It is a shuttle vector that can propagate in both *E.coli* for Gateway cloning and yeast to test for expression [26, 27, 28]. It is also a centromeric plasmid that includes two key features: autonomously replicating sequences (ARS) and centromeric (CEN) sequences. ARS sequences allow the plasmid to replicate only once during the S-phase of every cell cycle [33, 34, 35]. CEN sequences are responsible for chromosome segregation along the mitotic spindle apparatus thus, allowing the plasmids to replicate as independent chromosomes while maintaining its stability without integration [36].

Transformation of *PSMB7* mutant clones into Yeast Knockout strain:

For transformation, yeast HetKO cells (*Pup1*/Δ*pup1::KanMX*) (obtained from ATCC) were made competent using the Frozen-EZ Yeast Transformation II Kit from Zymo Research. The *PSMB7* mutant clones were transformed into the yeast HetKO competent cells at a large scale using the same kit mentioned above and plated on Q-trays containing synthetic medium without Ura in the presence of G418 (200 µg/ml) (SD-URA+G418). The trays were incubated at 30°C for 2-3 days. Single colonies were then picked using the QPix 460 colony picker. This automated system picks around 1000 colonies in 20 minutes, making the picking quicker while producing consistent and dependable results in a sterile environment. The single colonies were spotted on pre-sporulation GNA medium with G418 selection (200 µg/ml) in a 96-well format and incubated at 30°C for 1-2 days to allow the colonies to grow abundantly.

Selection of haploid yeast strains with *PSMB7* mutant clones:

To select for the viable haploid yeast knockout strains depending on the mutant *PSMB7* gene, colonies from pre-sporulation GNA medium were inoculated in 700 mL of liquid sporulation medium (0.1% potassium acetate (Sigma P1190), 0.005% zinc acetate (Sigma Z0625) and MilliQ water to make up the total volume) in 96-well deep well tubes. The mutant clones are incubated at room temperature for 3-5 days while shaking at 230 rpm or by using a rotator, forcing the diploid yeast to undergo meiosis and sporulating to form tetrads to survive the harsh conditions.. After the confirming spore formation by brightfield microscope, the spores were plated uniformly in a 96-well format on plates containing synthetic Magic Marker (MM) medium (-His -Arg -Leu -Ura +Can (60 µg/mL)) with or without G418 (200 µg/ml) and incubated at 30°C for 2-3 days. For the *PSMB7* mutants that grew equally on MM-G418 and MM+G418, their corresponding spores were diluted (1:20 dilution), plated again on MM-G418 and MM+G418 petri plates, and incubated at 30°C for 2-3 days to obtain single colonies.

Human gene dependency assay:

To test for human gene dependency, the haploid spores that grew on MM (+G418) medium were replica plated on synthetic medium containing 5-FoA (1g/1L) from Thermo Fisher and uracil (50mg/L) from Sigma Aldrich and incubated at 30°C for 1-2 days.

Plasmid preparation from yeast:

Once the suppressors were confirmed to be dependent on the human gene, plasmids containing the mutant *PSMB7* gene were extracted from yeast colonies. Yeast colonies of the confirmed suppressors (grown on GNA medium) were inoculated in YPD medium and incubated overnight at 30°C. The plasmids were extracted from yeast the following day using the QIAprep® Spin Miniprep kit. Since the plasmid yield from yeast was low, the plasmids were

re-transformed into *E.coli* and extracted again using the QIAprep® Spin Miniprep kit to obtain a higher yield.

E.coli transformation:

The desired plasmid (50ng) was added to 50 µL of *E.coli* competent cells and kept on ice for 30 minutes. The cells were then incubated at 42°C for 45 seconds for heat shock treatment and kept on ice again for 5 mins. 950 µL of LB media was added to the transformation mix and incubated at 37°C for 1 hour to allow the cells to divide and grow. After 1 hour, the cells were centrifuged at 4000rpm for 5 minutes, resuspended in 100 µL of LB media and plated on LB agar with appropriate selection. The plates were incubated overnight at 37°C.

Construction of _PSMB7_ single mutant clones:

PSMB7 single mutants were created with the use of the Q5® Site-Directed Mutagenesis kit from New England BioLabs. The primers for this experiment were designed using the software Geneious. We first obtained the wildtype human *PSMB7* entry clone in pDONR221 using BP Gateway strategy followed by sequence-verification [31]. Using the Q5® Site-Directed Mutagenesis kit, the primers were used to introduce a specific single nucleotide change in the wild type *PSMB7* gene (present on the gateway compatible donor vector pDONR221 containing kanamycin selection) as well as amplify it. The linear vectors were then treated with three enzymes from the kit: Kinase, Ligase, and DpnI to obtain circular human b2 single mutant clones. These circularized vectors were transformed into *E.coli*, plated on LB medium with kanamycin selection, and incubated overnight at 37°C. The *PSMB7* single mutant clones were digest verified using restriction enzymes EcoRV and HindIII from New England BioLabs and then sent for sequence verification. Once the clones were sequence-verified, they were cloned into the expression vector pAG416GPD-ccdB (+Ura; CEN) using Gateway® LR Clonase® II

enzyme mix kit from Invitrogen [31]. All the clones were transformed into competent *E.coli* cells and then isolated from *E.coli* using the QIAprep® Spin Miniprep kit.

Growth assays:

For growth assays, haploid single and primary mutants were inoculated in SD-URA+G418 and grown overnight after confirming their plasmid dependency. 1 µL of mutants were then inoculated in 149 µL of SD-URA+G418 at the initial OD_{600} of 0.01. The growth assay was performed with Synergy H1 plate reader for 48 hours while continuously shaking at 282 cpm while measuring the OD_{600} at 20-minute intervals. The OD_{600} measurements were then plotted to obtain growth curves for comparison using the Graph Prism software.

Construction of Yeast-Human hybrid clones into yeast expression vector:

Three primers, each containing a part of the *Pup1* C-terminal and a part of the *PSMB7* gene, were designed and used to create four different Yeast-Human hybrids by PCR (*AccuPrime Pfx* DNA polymerase from Invitrogen) while simultaneously adding attB1 and attB2 sites.

The reverse primers used for the construction of the yeast-human hybrids are mentioned in **Table 3**.

Table 3: List of reverse primers used for the construction of yeast-human hybrids

Primer name	Primer sequence
PSMB7-Tail Swap 1 - Rp-TS-1	5'-GATCACCACTTTGTACAAGAAAGCTGGGTC AAGCCGTTATATCGACTTGTTCTTCTTGTATGT CACAAATATTGACAATACTCTCCTTCAGCACA GCAGTTGTACCCCTGGGGAATTTGTAGCTTTTC TGCTTTTCTTCTCTAACATTTGGAGTCAAGTAT GGGCGGAGAAAATCCAGC-3'
PSMB7 -Tail Swap 2 - Rp-TS-2	5'-GATCACCACTTTGTACAAGAAAGCTGGG TCAAGCCGTTATATCGACTTGTTCTTCTTGT ATGTCACAAATATTGACAATACTCTCCTTCA GCACAGCAGTTGTACCCCTCTCACACCTGTA CCGGCCAA-3'
PSMB7 -Tail Swap 3 - Rp-TS-3	5'-GATCACCACTTTGTACAAGAAAGCTGGGTCAA GCCGTTATATCGACTTGTTCTTCTTGTATGTCACA AATATTGACGATTTTCTCAGTGAGGACTGCAGT-3'

The following protocol was used for the PCR reaction: initial denaturation step at 95°C for 2minutes followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 62-64°C for 30 seconds and extension at 68°C for 1 minute and 30 seconds. The 30 cycles were followed by a final extension step for the hybrids at 68°C for 10 minutes. The hybrid PCR products were purified by gel extraction (QIAquick® Gel extraction kit) and then cloned into the gateway compatible donor vector pDONR221 (+Kan) via the BP cloning strategy using

Gateway® BP Clonase® II enzyme mix kit from Invitrogen [31]. The hybrid clones were confirmed by restriction digestion using EcoRV and HindIII (from New England BioLabs) and sequence verification. The verified hybrid clones were finally cloned into the yeast expression vector pAG416GPD-ccdB (+Ura; CEN) by LR cloning (Gateway® LR Clonase® II enzyme mix kit from Invitrogen) [31]. All the clones were transformed into competent *E.coli* cells and then isolated from *E.coli* using the QIAprep® Spin Miniprep kit.

7.Chapter 7 - Bibliography

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