Membrane localization of paralogous leucine permeases Bap2 and Bap3 is regulated by Bul1 (Long title)

Bul1 regulates leucine permeases Bap2 and Bap3 (short title)

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Abstract

Timeliness in expression and degradation of the nutrient permeases is vital for any organism. In *Saccharomyces cerevisiae* while transcriptional regulation of permeases has been studied in great detail, post translational events such as trafficking, sorting and turnover are poorly understood. We observed that loss of *BAP2* and but no other leucine permeases lead to growth retardation in a routine used SCGlu/Gal but not in a SCGlylac medium. Leucine prototrophy suppressed the growth retardation, showing that *BAP2* was synthetically lethal with *LEU2*. We revealed that loss of *BUL1* but not *BUL2* an arrestin involved in trafficking of other diverse permeases suppressed this lethality. The suppression was dependent on another leucine permease *BAP3*. Further experiments revealed that in *bul1Δ* cells, both *BAP2* and *BAP3* accumulated in plasma membrane and their turnover is reduced. Based on our results and what is known, we propose that *BUL1* regulates TORC1 activity by controlling the leucine uptake.

Introduction

Leucine occupies a unique status among the proteinogenic amino acids. It has the highest frequency of occurrence in polypeptides across genomes and also functions as a signal (Crozier et al., 2005; Stipanuk, 2007; Suryawan et al., 2011; Ananieva et al., 2016). For instance, Target of Rapamycin (TOR) the king pin of cell growth and proliferation in eukaryotes is activated by leucine (Bonfils et al., 2012; Han et al., 2012; Gonzalez and Hall, 2017). Leucine consumption has implications in several diseases. Sarcopenia, the loss of muscle mass and strength in old age is treated by leucine supplementation, which promotes protein synthesis and inhibits protein breakdown (Komar et al., 2015; Martinez et al., 2019). Leucine deprivation results in stalling of breast cancer growth and induces apoptosis (Xioa et al., 2016). Leucine and its breakdown products reduce the glucose uptake, in adult cardiomyocytes even after insulin treatment by affecting translocation of GLUT4, a glucose transporter, establishing a possible link between diabetes and leucine consumption (Renguet et al., 2017).

S.cerevisiae has several permeases to import leucine and other amino acids from the external milieu (Ljungdahl and Daigan-Fornier, 2012). It constantly remodels the plasma membrane landscape to adapt to the changing environment, by removal of existing and insertion of new permeases into the cell membrane (Forsberg et al., 2001). Permeases destined for removal are ubiquitylated, endocytosed and degraded in the vacuole (Rotin et al., 2000; Hettema et al., 2004; Erpapazoglou et al., 2008). Factors such as availability of the substrate, change of carbon and nitrogen sources, stresses like pressure, temperature, pH, heavy metal ions, fold of the permease and drugs like cycloheximide and rapamycin stimulate the turnover of plasma membrane permeases (Abe and Iida, 2003; Kim et al., 2007; Lin et al., 2008; Nikko et al., 2008; Nikko and Pelham, 2009; Hatakeyama et al., 2010; O'Donnell et al., 2010; Becuwe et al., 2012a; Zhao et al., 2013; Suzuki et al., 2013; Ghaddar et al., 2014; Crapeau et al., 2014; Talaia et al., 2017; Hovsepian et al., 2017; Hovsepian et al., 2018; Savocco et al., 2019).

Permease ubiquitylation is achieved by the combined action of Rsp5 ubiquitin ligase and yeast arrestins (Belgareh-Touze et al., 2008; Leon and Tsapis., 2009). Yeast arrestins, commonly known as Arrestin Related Trafficking adaptors (ARTs), are ancestral orthologs of mammalian β and visual arrestins (Lin et al., 2008; Alvarez, 2008). ARTs provide the physical link between the Rsp5 ligase and permease cargo, by acting as a scaffold. This is because many of the yeast permeases lack PY motif necessary to bind Rsp5 ligase (Belgareh-Touze et al., 2008; Leon and Tsapis., 2009; Lauwers et al., 2009). For diverse signals, a permease may hire different ARTs for its ubiquitylation and vice versa (O'Donnell and Schmidt, 2019). Also a fair amount of redundancy exists where for a given signal a cargo is targeted by more than one ART to facilitate ubiquitylation (O'Donnell and Schmidt, 2019). Because of this redundancy and extensive network of interactions, it is difficult to establish the signal-ART-cargo relationship.

Here, we show that the growth of *S.cerevisiae* is critically dependent on *BAP2*, a high affinity leucine permease, either in a synthetic medium containing several other amino acids or in a synthetic medium with low concentration of leucine. This observation comes as a surprise considering the ability of several other permeases to transport leucine. We observed that loss of *BUL1*, an ART, overcame the dependency on *BAP2*, by stabilizing *BAP3* in the plasma membrane. We show that the turnover of leucine permeases Bap2 and Bap3 is accomplished by Bul1. Finally, we propose that by regulating the abundance of these permeases, Bul1 modulates the activity of TORC1, which in turn regulates Bul1, thus setting up a feedback loop. Hence, this study for the first time provides a possible mechanism as how leucine regulates its own uptake.

Results

Discovery of a nutrient dependent growth phenotype for yeast lacking an arrestin

Our lab has been investigating the role of *MRG19* (aka *CSR2*, *ART8*) in carbon and nitrogen metabolism (Kabir et al., 2000; Khanday et al., 2002; Das & Bhat, 2005). A *mrg19*Δ strain grew better than its WT (wild type) colleague, when branched chain amino acids were used as nitrogen sources (Das., 2005). We continued the study with strains of BY4741 background. We studied the growth of WT and strains lacking *CSR2 (ART8)* or/and *ECM21 (ART2)* the paralogue of *CSR2* in SMAaGlu medium (Synthetic Minimal Amino acid Glucose) (Fig.1&2).

All the strains grew equally well in every media except *ecm21*Δ and *mrg19Δecm21*Δ strains which grew poorly in SMTGlu medium, which had 2mM of Tyrosine as Nitrogen source (Fig.2A). Decreasing the Tyrosine concentration to 0.5mM restored the growth and increasing the concentration to 4mM or 6mM halted even the growth of WT (Fig.2B). We speculated that high conc. of Tyrosine inhibits growth.

Even in the routinely used SCGlu medium, *ecm21*Δ and *mrg19Δecm21*Δ strains grew poorly (Fig.3). The poor growth phenotype disappeared in STDGlu medium (SCGlu medium lacking Tyrosine) (Fig.3). We inferred that Tyrosine was growth inhibitory to WT and the strain lacking *ECM21*. Loss of *ECM21* only made cells more sensitive to Tyrosine. We pursued further investigations related to *ECM21* and not *MRG19*.

Isolation of second site suppressor of ecm21Δ growth defect

To understand the mechanistic basis of the poor growth or growth defect, we resorted to mutant hunt. We isolated two independent spontaneous suppressor mutants, which grew equally well as WT in both SCGlu and SMTGlu media (Fig.4). Segregation analysis and study of heterozygous diploids (of suppressor mutants) implicated that the suppression was effected from a single locus and dominant in nature (data not shown). Genome sequencing and analysis of the suppressor mutants enabled us to identify $BUL1^{H402N}$ as the suppressor. Like *ECM21*, *BUL1* is a yeast arrestin responsible for the endocytosis of several transporters like Gap1, Tat2, Tat1, Put4, Ptr2, Ctr1, Dal5 and Jen1 (O'Donnell and Schmidt, 2019).

As it was dominant in nature, the suppression could due to a dominant negative function or gain of function or haploinsufficiency. Plasmid expressing $BUL1^{H402N}$ allele did not suppress the growth defect of *ecm21*Δ strain (Fig.5), indicating $BUL1^{H402N}$ was neither a dominant negative nor a gain-of-function allele.

On the other hand *ecm21*Δ homodiploid strain (*ecm21*Δ/*ecm21*Δ *BUL1/BUL1*) exhibited the growth defect, but not the *ecm21*Δ heterodiploid strain (*ecm21*Δ/*ecm21*Δ *BUL1/bul1Δ*)

(Fig.6). As the deletion of one copy of *BUL1* (haploinsufficiency of *BUL1*), suppressed the growth defect, we expected $BUL1^{H402N}$ to be a loss of function allele.

A *bul1Δbul2Δ* strain but not a *bul1Δ* or *bul2Δ* strain suffers a mild growth defect at 37ºC in YPD (Yashiroda et al., 1998). A *bul1Δ* strain of BY4741 background is naturally a *bul1Δbul2Δ* strain due to possession of the loss of function allele of *BUL2* ($BUL2^{F883L}$) (Kwan et al., 2011). To avoid confusion we denote *bul1Δ* strain of BY4741 background not as *bul1Δbul2Δ*.

A plasmid carrying *BUL1* but not $BUL1^{H402N}$ restored growth of the *bul1Δ* strain, at 37ºC in YPD (Fig.7). The experiment was repeated in SMTGlu medium where the growth defect of the *bul1Δ* was distinct (Fig.7). These results proved that $BUL1^{H402N}$ was a loss of function allele. Finally we demonstrated that deletion of *BUL1* suppresses the growth defect of *ecm21*Δ strain (Fig.8).

Isolation of LEU2 as a suppressor of the growth defect

Parallel to the mutant hunt, a yeast multicopy library was screened and *LEU2* was isolated as a suppressor of the growth defect (Fig.9). It is to be noted that strains of BY4741 background are leucine auxotrophs (*leu2Δ0*).

Many studies have isolated *LEU2* as a suppressor, in different genetic screens (Garrett, 1989; Cohen and Engelberg, 2007; Journo et al., 2009; Tardiff et al., 2013). It was reasoned that *LEU2* was a suppressor by virtue of its ability to confer leucine prototrophy, as the strains were *leu2Δ* and experienced impaired leucine uptake. We surmised whether the growth defect of *ecm21*Δ strain also presented similar case and an elevated concentration of leucine in the medium might alleviate the growth defect.

As anticipated when leucine was supplied 5 times the normal concentration, growth defect of *ecm21*Δ strain disappeared (Fig.9). We concluded that growth defect *ecm21*Δ strain was caused by impaired leucine uptake and was overcome by leucine prototrophy or increased leucine supplementation or loss of *BUL1*.

Growth defect is due to an unknown mutation and not linked to ECM21 locus

ECM21 was not picked up in the multicopy library screen. When transformed e*cm21*Δ strain with a plasmid carrying *ECM21*, the growth defect did not recede (Fig.10A). Also a freshly created *ecm21*Δ strain from WT did not exhibit any growth defect in SMTGlu and SCGlu (Fig.10B). We were surprised and surmised that the original *ecm21*Δ strain had acquired a cryptic mutation, causing the growth defect. Attempts to identify the cryptic mutation from the genome sequence data were unsuccessful.

BAP2 and LEU2 are synthetically lethal

Commonly used WT strains of BY4741, W303 and ∑1278b genetic background suffer growth defect in a modified SCGlu medium (Cohen and Engelberg, 2007). The growth defect is due to impaired leucine uptake and is suppressed by multicopy plasmids either bearing *LEU2*, *BAP2* or *TAT1*.

It is intriguing as to why WT strains are unable to import leucine, despite enormous leucine present in the medium (580µM), beside the ability of the cells to express several leucine permeases like Bap2, Bap3, Tat1, Tat2, Agp1 and Gnp1. We surmised that the insufficient leucine uptake was a consequence due to a fierce competition between leucine and other competing amino acids for their cellular entry through a common transporter(s).

If the prediction was true, a drop in the abundance of the competing amino acids should favor leucine import and growth. As anticipated WT grew well in the routinely used SCGlu, which has only 12 amino acids in lower concentrations (Fig.3), as compared to all 20 amino acids in high concentrations in the modified SCGlu.

We sought to establish a leucine import deficient growth defect even in the routine SCGlu medium. For this we deleted the major leucine transporters in the WT and studied the growth (Fig.11A). We observed none of the transporter mutants but *bap2Δ* and *tat1Δ* strains showed severe and moderate growth defect respectively. The growth defect of *bap2*Δ strain was overcome by *LEU2* complementation (Fig.11B). To the best of our knowledge, this is the first report of a synthetic lethal relationship between *BAP2* and *LEU2*. We continued further investigations pertaining to *BAP2* and not *TAT1*.

Synthetic lethality of BAP2 and LEU2 is carbon source dependent

Leucine uptake in *S. cerevisiae*, increases by eight to tenfold when galactose or glycerol replace glucose as carbon source (Peter et al., 2006). Interestingly, *bap2*Δ strain exhibited the growth defect when the carbon source was switched to galactose but not when glycerol plus lactate was used (Fig.12).

Growth defect of bap2Δ strain is suppressed by loss of BUL1 which requires BAP3

We wondered if loss of *BUL1*, would suppress the growth defect of the *bap2Δ* strain, similar to the *ECM21* case (with the cryptic mutation). As expected, loss of *BUL1* had suppressed the growth defect of *bap2*Δ strain in SCGlu medium (Fig.13A). We speculated that loss of *BUL1* would lead to accumulation of a transporter(s) in the plasma membrane to import sufficient leucine and restores the growth. We found that the suppression was dependent on *BAP3* and but not on others like *TAT2*, *AGP1* and *GNP1* (Fig.13A,B).

We confirmed this finding by studying the growth of other control strains (Fig.13C). We found this suppression to operate also in SCGal (Fig.13C).

Loss of BUL1 leads to accumulation of Bap3 in PM

We believed that in a *bap2*Δ strain loss of *BUL1*, accumulates Bap3 in the plasma membrane which imports enough leucine to restore the growth. As expected Bap3 accumulation was enriched in the plasma membrane of a *bap2Δbul1Δ* strain than the *bap2Δ* strain (Fig.14A). Opposing the above, the vacuolar Bap3 distribution was enriched in a *bap2Δ* strain than the *bap2Δbul1Δ* strain (Fig.14A).

We tried to know how loss of *BUL1*, affected Bap3 localization in WT. Bap3 was enriched in plasma membrane of the *bul1Δ* strain than the WT and the vacuolar distribution was reversed (Fig.14B). Combining these results we made a generalized inference that irrespective of the genetic background (WT or *bap2*Δ) loss of *BUL1*, leads to increased plasma membrane accumulation and decreased vacuolar accumulation of Bap3. Upholding our inference, immunoblotting results revealed that Bap3 degradation was remarkably reduced in the *bul1Δ* strain than the WT (Fig.14C). Combining these results we inferred that *BUL1* was responsible for the endocytosis of *BAP3*.

Unlike SCGlu medium, loss of *BUL1* resulted only in minimal difference in plasma membrane and vacuolar Bap3 distribution in SCGal medium (Fig.14D,E). We did not perform immunoblotting of Bap3-EGFP in SCGal medium.

Loss of BUL1 empowers WT to grow in an extremely low leucine environment

We extended our study to SD medium. WT and *bap2Δ* strains grew equally well in SD75 medium (75µM leucine), possibly because of the uninterrupted leucine import due to lack of heavy load of amino acids as in SCGlu medium (Fig.15). As the leucine concentration dropped, in SD25 medium (25µM leucine) (Fig.15), *bap2Δ* strain ceased to grow. Growth was restored through loss of *BUL1*, requiring *BAP3*.

A Surprising observation came when WT, failed to grow in SD5 medium (5µM leucine) (Fig.15). Again, loss of *BUL1* restored its growth which required *BAP2* not *BAP3*. We inferred the following: (1) Loss of *BUL1* leads to accumulation of *BAP2* and *BAP3* in the plasma membrane. (2) *BAP2* and *LEU2* are synthetically lethal at low but not at high leucine concentration. The lethality is overcome through accumulation of *BAP3* in plasma membrane. (3) Loss of *LEU2* is lethal in an extremely low leucine environment. The lethality is overcome through accumulation of *BAP2* but not *BAP3* in plasma membrane.

As inferred, the plasma membrane localization of Bap3-EGFP was marginally increased in the *bul1Δ* strain than the WT (Fig.16A). We also observed a remarkable enrichment of Bap2-yeGFP in the periphery of *bul1Δ* strain than the WT (Fig.16B). Also the vacuolar distribution of Bap2-yeGFP was dramatically reduced in *bul1Δ* strain than the WT. Substantiating the above, degradation of Bap2-yeGFP was much reduced in the *bul1Δ* strain than WT (Fig.16C). From these results we concluded that loss of Bul1 lead to accumulation of Bap2 and Bap3 in the

plasma membrane, and Bul1 was responsible for the endocytosis of Bap3 and Bap2 in SD medium.

We were curious to know whether loss of *BUL1*, leads to PM accumulation of Bap2 in SCGlu medium (Fig.17). We did not observe any discernible difference in Bap2 localization between WT and *bul1*Δ strain.

Bul1 could be responsible for the endocytosis of a third permease

We were curious to know the behavior of the *bap2*Δ strain in SMTGlu medium where *ecm21*Δ strain displayed growth defect (Fig.2A). Here too, the *bap2*Δ strain suffered growth defect and was suppressed by loss of *BUL1* (Fig.18A). Despite deleting several permeases, we were not able to identify the one that favors growth of *bap2*Δ*bul1*Δ strain (Fig.18A-C). We assumed that the suppression was not reliant on a single transporter, instead more than one. Hence the strategy of deleting the transporters one at a time was not right to identify them.

Loss of BUL2 does not suppress the growth defect of bap2Δ strain

BUL2 and *BUL1* are paralogues and share many cargoes for endocytosis (Yashiroda et al., 1998; O'Donnell and Schmidt, 2019). As mentioned before, WT is naturally *bul2*Δ as it harbors a loss of function allele of *BUL2* (*BUL2*^{F883L}) (Kwan et al., 2011). So, it is possible that suppression of the *bap2*Δ growth defect is due to the combined loss of both *BUL1* and *BUL2*.

To check this idea, *bap2*Δ*bul1*Δ strain (originally *bap2*Δ*bul1*Δ*bul2*Δ) was complemented with functional *BUL2* allele from W303-1A strain background (Fig.19). Though the functional *BUL2* did not confer the growth defect back in the *bap2*Δ*bul1*Δ strain, *BUL1* did. This confirmed that the suppression was due to the loss of *BUL1* but not *BUL2*. The functionality of the *BUL2* of W303-1A strain was verified as it successfully rescued the growth of the *bul1*Δ strain (originally *bul1*Δ*bul2*Δ) which suffers a severe growth defect at 37˚C in SMTGlu (Fig.20).

BAP2 is the major leucine transporter in SCGlu medium

After studying their effect on growth we tried to know how genetic manipulations such as loss of *BAP2*, *BUL1* affect leucine uptake, in SCGlu medium (Fig.21). All the strains were made leucine prototrophs to support growth of *bap2Δ*, *bap2Δbap3Δ* and *bap2Δbap3Δbul1Δ* strains which otherwise do not grow in SCGlu medium.

Considering uptake capacity of WT as 100%, *bap3*Δ, *bap2*Δ and *bap2*Δ*bap3*Δ strains respectively displayed 10%, 40% and 40% drop in uptake. This revealed *BAP2* as the major and *BAP3* as a minor transporters of leucine. Loss of *BUL1*, in *bap2*Δ strain doubled its uptake which is 10% more than the WT. This was due to PM accumulation of *BAP3*, as loss of *BAP3* eliminated this effect. Similarly, loss of *BUL1* in *bap3*Δ strain increased the uptake 50% more than the WT. This was due to apparent PM accumulation of *BAP2*, as loss of *BAP2* not only

eliminated this effect but also reduced its uptake equivalent to the *bap2*Δ strain. Loss of *BUL1*, in WT increased the uptake by 30%.

We found the uptake data for loss of *BAP2*, *BAP3* is in close agreement with the previous reports (Grauslund et al., 1995; Didion et al., 1998).

Discussion

Our initial aim was to identify the role of arrestins, *ART2/ECM21* and *ART8/MRG19* in the utilization of different nitrogen sources. We observed that a strain lacking *ECM21* to exhibit a growth defect, due to impaired leucine uptake. Further analysis indicated that the above growth defect was not due to the loss of *ART8 per se*, but because of a cryptic mutation. Point mutations in *ecm21Δ* strain in combination with other founder mutations confer growth advantage (Kryazhimskiy et al., 2014). It is possible that the cryptic mutation which probably occurred by chance have provided a growth advantage during propagation. Nevertheless, the growth defect conferred by the cryptic mutation opens up an unchartered domain in leucine uptake.

BAP2 and *LEU2* are synthetically lethal and to the best of our knowledge, this is the first report of this genetic interaction. This led us to suspect whether the cryptic mutation resides in *BAP2*. Analysis of the *BAP2* ORF of *ecm21Δ* strain did not identify any aberration. When observed for its localization in WT, much of Bap2 remained at the plasma membrane. But in the *ecm21Δ* strain, Bap2 was present both in plasma membrane and vacuole (Fig.22). Perhaps the cryptic mutation reduces the abundance of Bap2 in the plasma membrane and causes a moderate if not exactly a *bap2Δ* like growth defect.

SPS (Ssy1-Ptr3-Ssy5) amino acid sensor complex triggers transcription of permeases *AGP1*, *GNP1*, *BAP2*, *BAP3*, *TAT1* and *TAT2* in response to extracellular amino acids (Forsberg et al., 2001). Loss of SPS components *SSY1* or *PTR3* and *LEU2*, causes synthetic lethality in SCGlu, suppressed by loss of *BUL1* (Forsberg et al., 2001). In our case loss of *LEU2* and *BAP2* alone is sufficient to establish such lethality not only in SCGlu and other media too. Loss of no other permease could establish such lethal phenotype, as *BAP2* is the major leucine importer in SCGlu, with *TAT1* coming second (Didion et al., 1998). Leucine import by *BAP3* is negligible unless it accumulates at plasma membrane. We believe that PM accumulation of other leucine permeases through loss of cognate ARTs can also restore growth in *bap2Δ* strain.

An exciting observation was the carbon source dependency of the *bap2Δ* growth defect. Though galactose and glycerol favor increased leucine uptake by ~10 fold than glucose (Peter et al., 2006), growth defect persists in SCGal not in SCGlylac. We think that the leucine permeases expressed in SCGal are different from SCGlylac. Perhaps the uptake hike in SCGal is due to upregulation of *BAP2*, while it is due to upregulation of other permeases like *AGP1* or *GNP1* in SCGlylac. Leucine uptake mechanisms seem more intricate than what is imagined.

Leucine activates TORC1 (Target of Rapamycin Complex1), the apex regulator of cell growth (Gonzalez and Hall, 2017). Leucine binds Cdc60, a leucyl tRNA synthetase cum intracellular leucine sensor to activate TORC1, via EGO complex bound Rag GTPases Gtr1 and Gtr2 (Bonfils et al., 2012). Active TORC1 in turn activates Bul1 by inhibiting Npr1 kinase (Merhi and Andre, 2012). Active Bul1 promotes Bap2 and Bap3 endocytosis causing drop of intracellular leucine levels, ultimately reducing TORC1 activity. We propose that this signaling cascade represents a TORC1-Leucine feedback loop that functions autonomously (Fig.23).

The TORC1-Leucine feedback loop has implications in the lifespan of *S.cerevisiae*. Loss of *BUL2*, reduces chronological lifespan (CLS) and prolongs replicative lifespan (RLS) concurrently (Kwan et al., 2011). The change in the lifespans is due to the suspected increased TORC1 activity, as a result of increased intracellular amino acid pool due to loss of *BUL2*. If that is true, *BUL1* may exert greater control over lifespan than *BUL2* in view of its role in regulating TORC1. Also, CLS of a *leu2Δ* strain grown in SD medium is extended by leucine prototrophy or increasing leucine concentration in the medium (Alvers et al., 2009). In such case, loss of *BUL1* may also extend CLS.

We wonder why strains experiencing 40% drop in leucine uptake exhibit severe growth defect in SCGlu (Fig.24)? Possibly, the intracellular leucine concentration drops below the threshold required for Cdc60 (1) to activate TORC1. Growth ceases when TORC1 the mastermind of translation is not active or (2) to charge a leucyl tRNA for translation. It is proposed that leucine being one of the most sought amino acids for translation, a slight fall in its availability sternly affects growth (Alvers et al., 2009).

BUL1 alleles with loss of function mutation ($BUL1^{Q146K}$, $BUL1^{Q146P}$) have been isolated previously (Tardiff et al., 2013; Sunshine et al., 2016). They bear mutations at the same residue near the PY motif (PPSY, 157-160), essential for binding Rsp5 ligase. It is speculated that these mutations abolish Bul1 binding to the Rsp5 ligase. Trying to understand the loss of function of $BUL1^{H402N}$, we identified a PF motif (PPSF, 406-409) near residue 402 and another PF motif (LPSF, 131-134). Proteins having PY or PF motif can physically bind Rsp5 through its WW domains (Hesselberth et al., 2006; Lauwers et al., 2010; Crapeau et al., 2014). We believe that H402N mutation close to the PF motif (PPSF, 406-409) abolishes Bul1 binding to Rsp5 ligase. Astonishingly, loss of an Art-Rsp5 ligase interaction due to mutation in PF motif or its adjoining regions has not been reported yet in *S.cerevisiae*. We have identified PF motifs in other ARTs (Fig.25A).

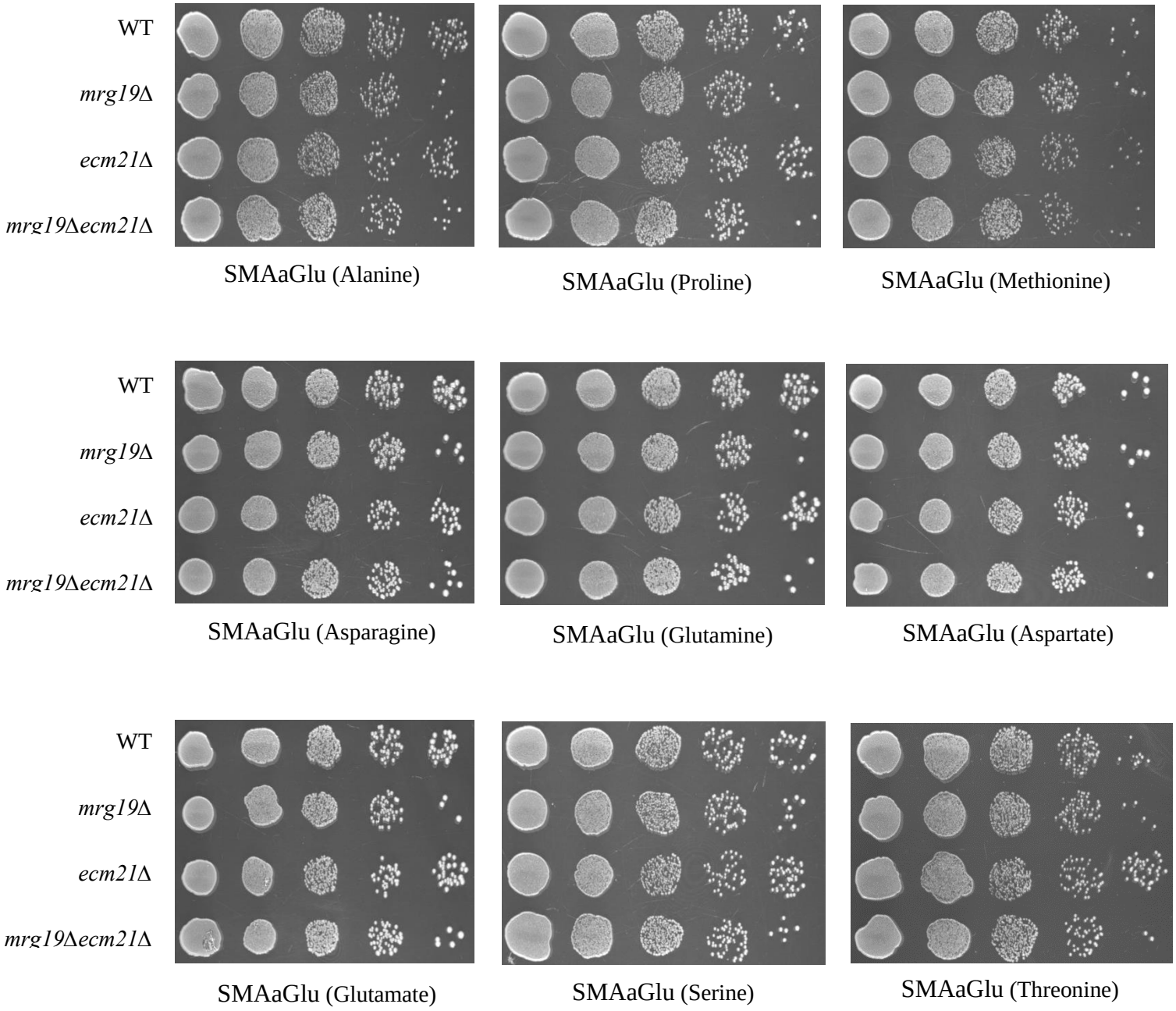
With the identification of PF motifs in Bul1, we think Q146K/P mutation abolishes Rsp5 ligase binding not only through PY motif (157-160) but also PF motif (131-134) (Fig.25B). The case of loss of function allele of *BUL2* ($BUL2^{F883L}$) is very interesting, as there are no PY/F motifs near the residue 883. This suggests possible occurrence of other unidentified motifs that function like PY/F motifs (Fig.25B).

A major challenge is to understand how a handful of ARTs in unison with just one ubiquitin ligase regulate a multitude of transporters for diverse physiological signals. For instance, how for a specified signal Bul1 would precisely downregulate Bap2 without affecting Gap1, Tat2, Jen1 which are also its cognate cargos? We hypothesize that each PY/F motif is responsible for downregulating a subset of cargoes regulated by Bul1. Rsp5 Binding of PY motif (157-160) may favor downregulation of Gap1, binding of PF motif (406-409) may favor downregulation of Bap2/3 and PF motif (131-134) may favor downregulation of another cargo. Each motif having a specific set of interacting partners is not new. The three WW domains of Rsp5 ligase have their own subsets of interacting proteins with PY/F motifs (Hesselberth et al., 2006).

If our idea is right, we could setup screens using ART alleles with selectively mutant PY/F motifs to identify the corresponding permeases. Currently we are exploring this possibility.

Figures

Fig.1.



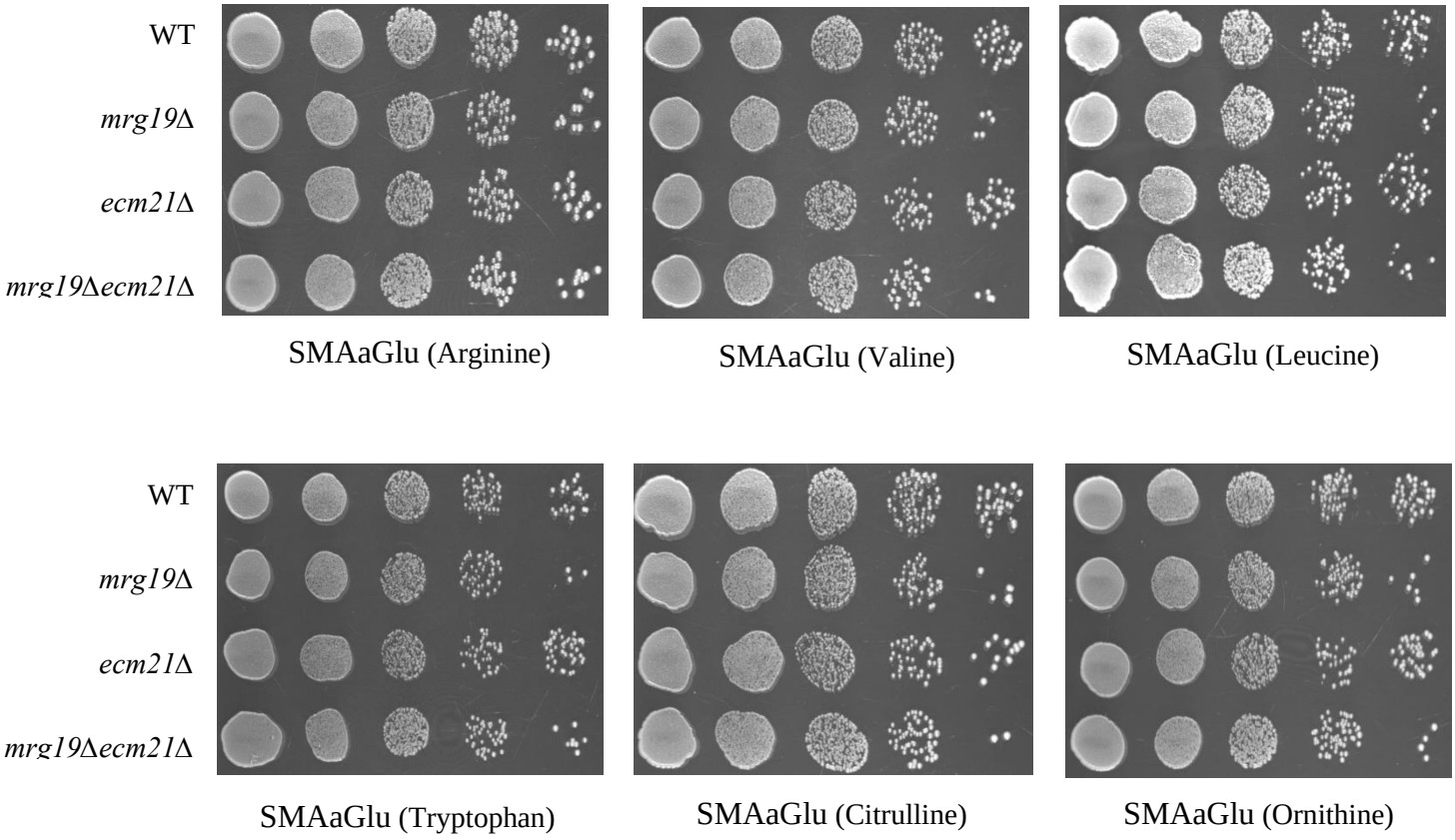
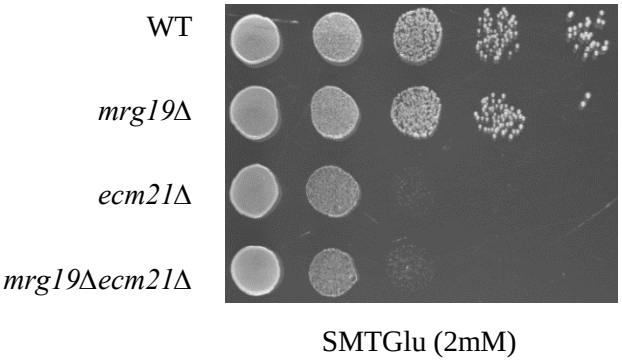


Figure 1. Different amino acids serve as nitrogen source. WT, *mrg19Δ*, *ecm21*Δ and *mrg19Δecm21*Δ strains grown in SCGlylac broth were spotted for growth in SMAaGlu (Amino acid) media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 30˚C.

Fig.2.

A



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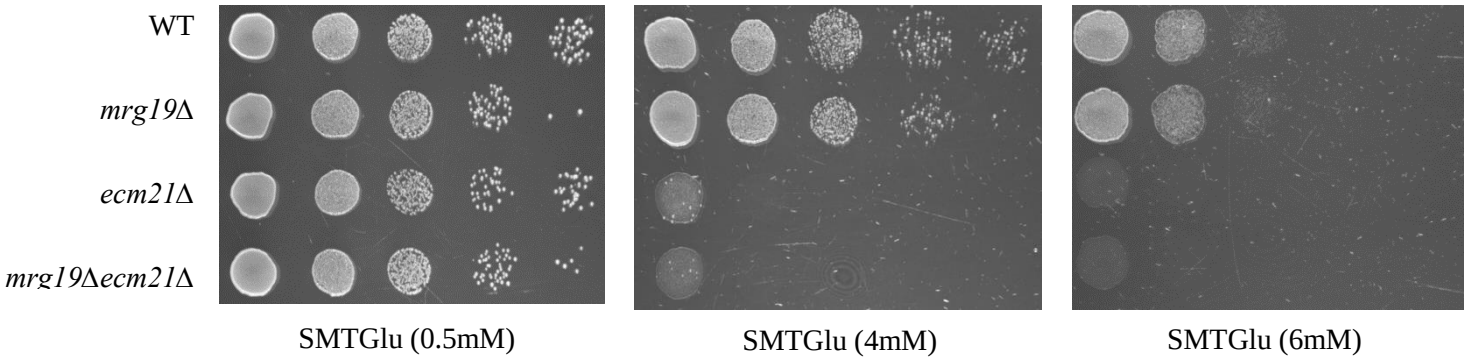


Figure 2. High concentration of Tyrosine causes growth inhibition. WT, *mrg19Δ*, *ecm21*Δ and *mrg19Δecm21*Δ strains grown in SCGlylac broth were spotted for growth in (A) SMTGlu (2mM Tyrosine) and (B) SMTGlu (0.5/4/6mM Tyrosine) media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 30˚C.

Fig.3.

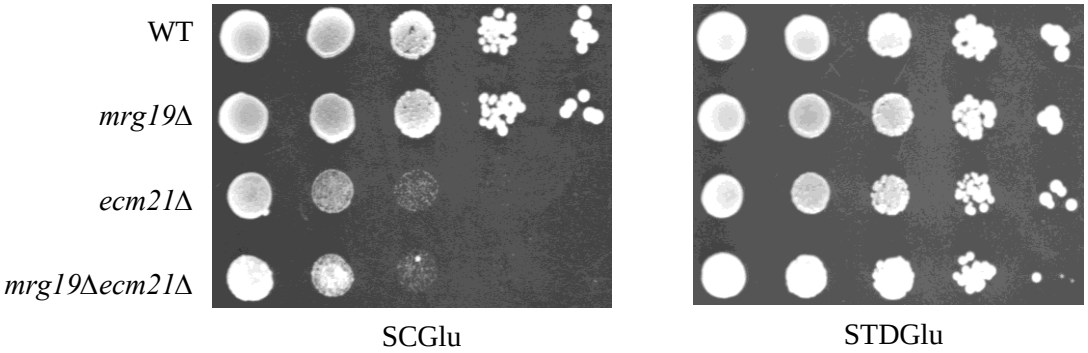


Figure 3. Withdrawal of Tyrosine restores growth in *ecm21*Δ strain. WT, *mrg19*Δ, *ecm21*Δ and *mrg19*Δ*ecm21*Δ strains grown in SCGlylac broth were spotted for growth on SCGlu and STDGlu media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 30˚C.

Fig.4.

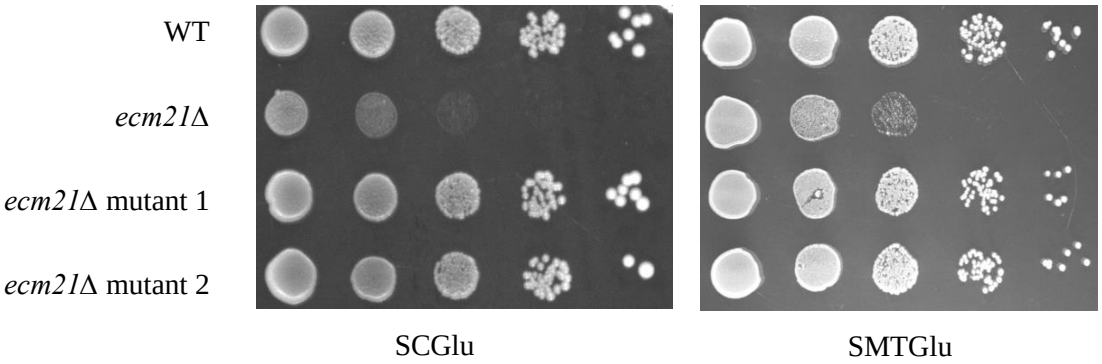


Figure 4. Spontaneous mutants overcome the growth defect. WT, *ecm21*Δ and *ecm21*Δ spontaneous mutant strains grown in SCGlylac broth were spotted for growth on SCGlu and SMTGlu media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 30˚C.

Fig.5.

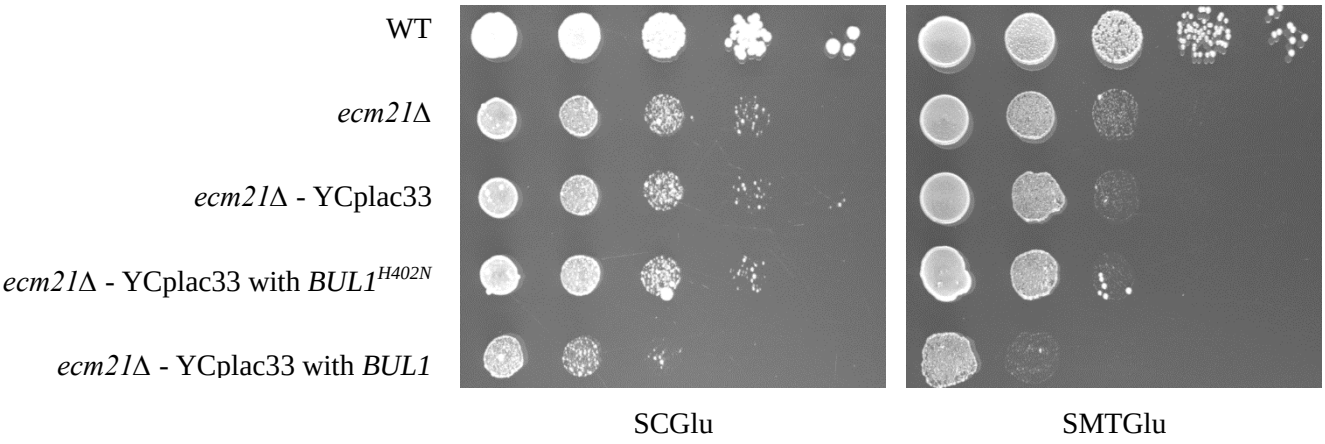


Figure 5. *BUL1^{H402N}* does not exhibit dominant negative effect or is a gain of function allele. WT, *ecm21*Δ strains and *ecm21*Δ transformants carrying YCplac33 or YCPlac33 with *BUL1^{H402N}* or *BUL1* grown in SCGlylac or SUra^{-}Glylac broth were spotted for growth on SCGlu and SMTGlu media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 30˚C.

Fig.6.

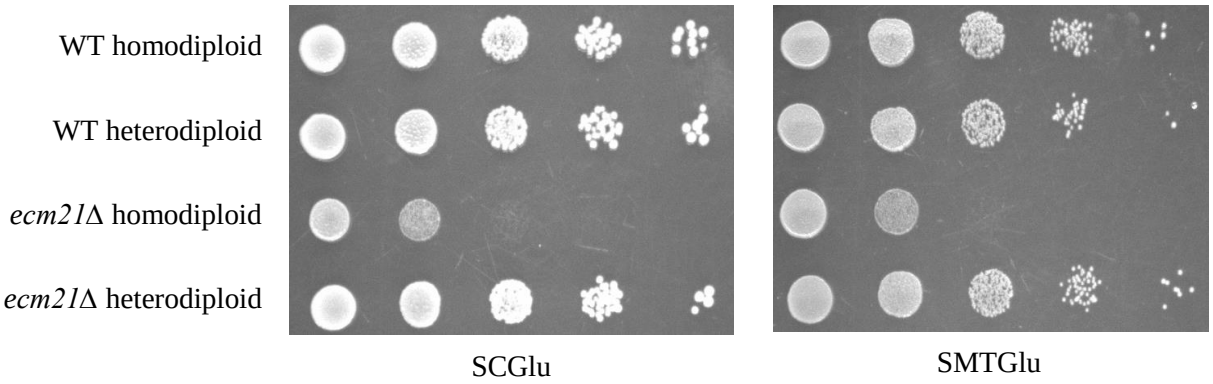


Figure 6. Haploinsufficiency of *BUL1* suppresses the growth defect. WT homodiploid (*ECM21*/*ECM21 BUL1*/*BUL1*), WT heterodiploid (*ECM21*/*ECM21 BUL1*/*bul1*Δ), *ecm21*Δ homodiploid (*ecm21*Δ/*ecm21*Δ *BUL1*/*BUL1*) and *ecm21*Δ heterodiploid (*ecm21*Δ/*ecm21*Δ *BUL1*/*bul1*Δ) strains grown in SCGlylac broth were spotted for growth on SCGlu and SMTGlu media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 30˚C.

Fig.7.

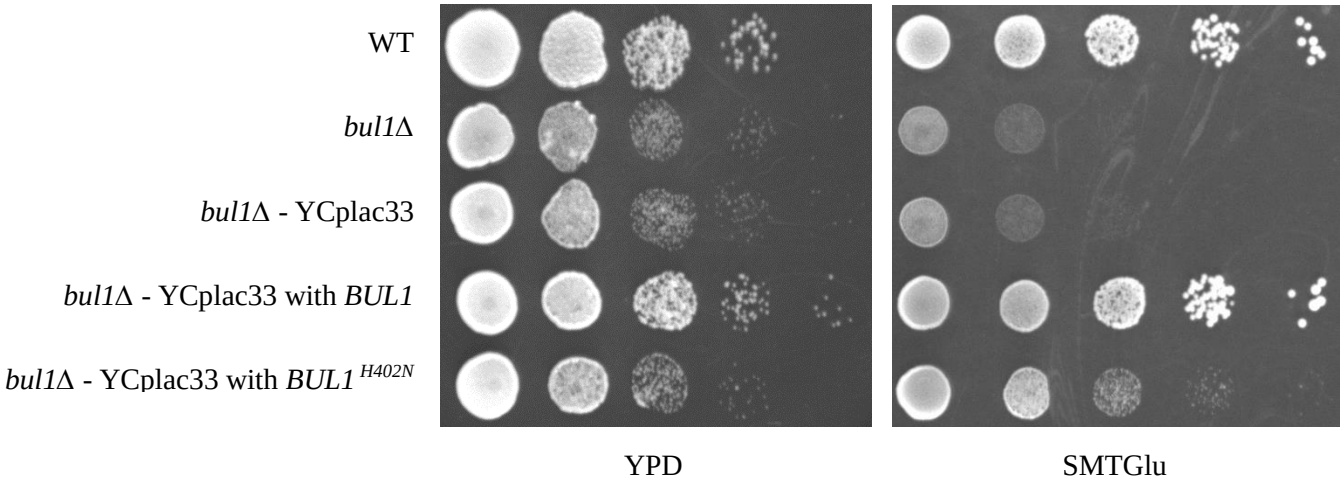


Figure 7. *BUL1^{H402N}* is a loss of function allele. WT, *bul1*Δ strains and *bul1*Δ transformants carrying YCplac33 or YCPlac33 with *BUL1* or *BUL1^{H402N}* grown in SCGlylac or SUra^{-}Glylac broth were spotted for growth on YPD and SMTGlu media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 37˚c.

Fig.8.

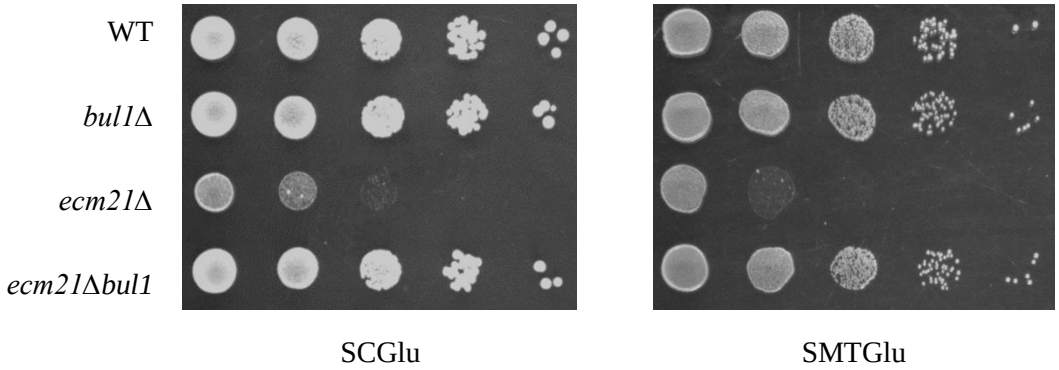


Figure 8. Loss of *BUL1* suppresses the growth defect of *ecm21*Δ strain. WT, *bul1*Δ, *ecm21*Δ and *ecm21*Δ*bul1*Δ strains grown in SCGlylac broth were spotted on SCGlu and SMTGlu media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 30˚C.

Fig.9.

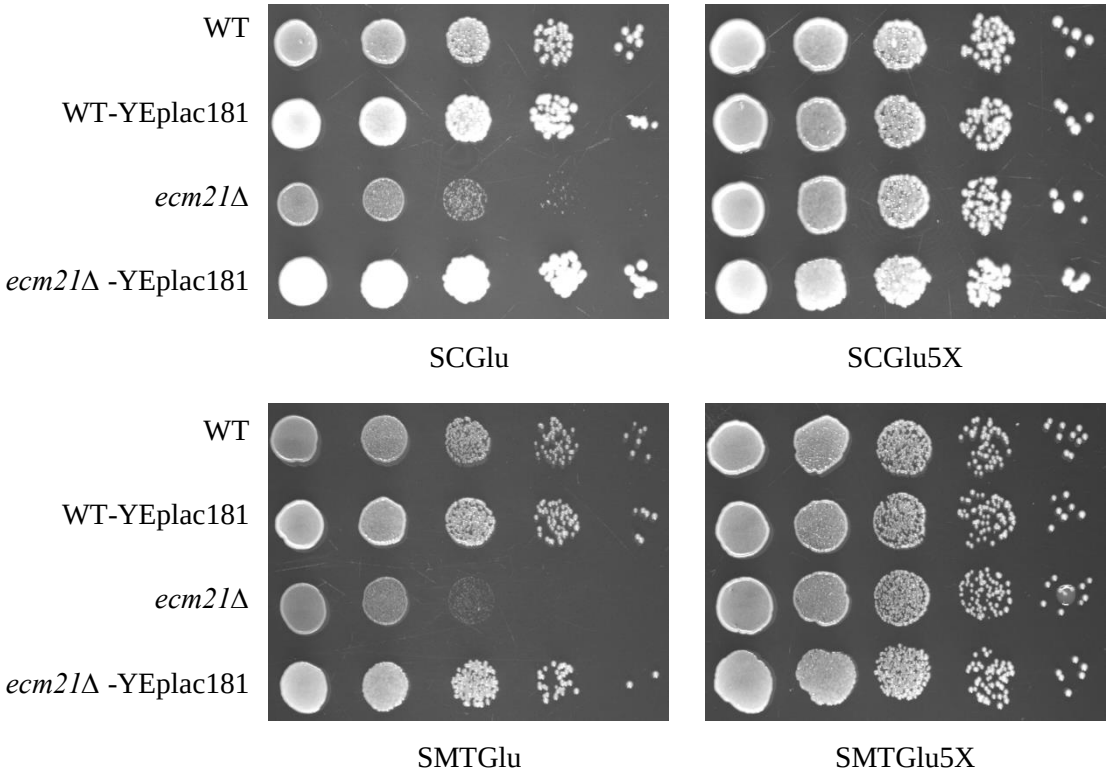
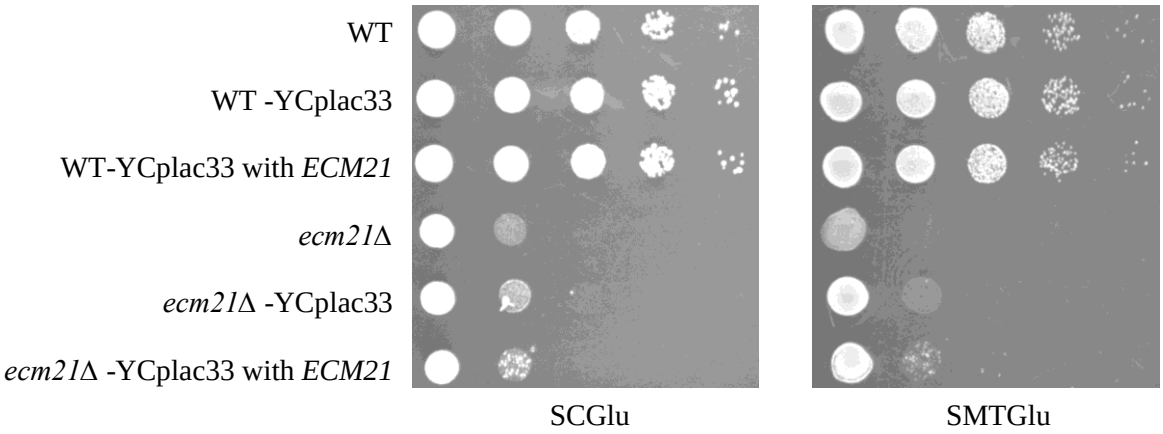


Figure 9. Leucine prototrophy or excess leucine availability suppresses the growth defect. WT, *ecm21*Δ strains and their transformants carrying YEplac181, a *LEU2* plasmid grown in SCGlylac or SLeu⁻Glylac broth were spotted on SCGlu (188µM leucine), SCGlu5X (1mM leucine), SMTGlu (75µM leucine) and SMTGlu5X (375µM leucine) media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 30˚C.

Fig.10.

A



B

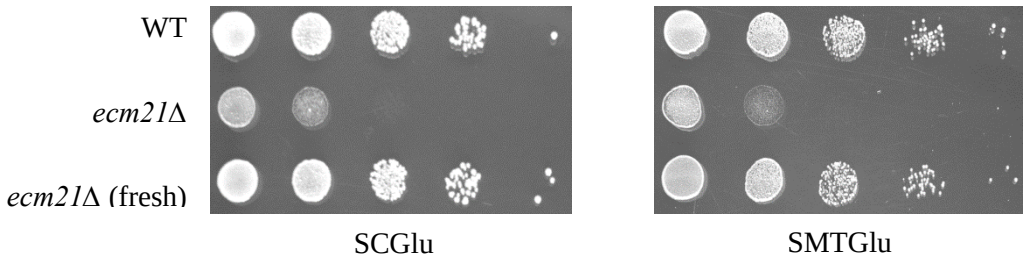


Figure 10. The growth defect of *ecm21*Δ strain is originally due to a cryptic mutation. (A) WT, *ecm21*Δ strains and their transformants carrying YCplac33 or YCplac33 with *ECM21* were grown in SCGlylac or SUra$^-$Glylac broth were spotted on SCGlu and SMTGlu media. (B) WT, *ecm21*Δ and *ecm21*Δ strains (freshly constructed) grown in SCGlylac broth were spotted on SCGlu and SMTGlu media. Images were captured and analyzed after incubating the Petri plates for 2-3 days at 30˚C.

Fig.11.

A **B**

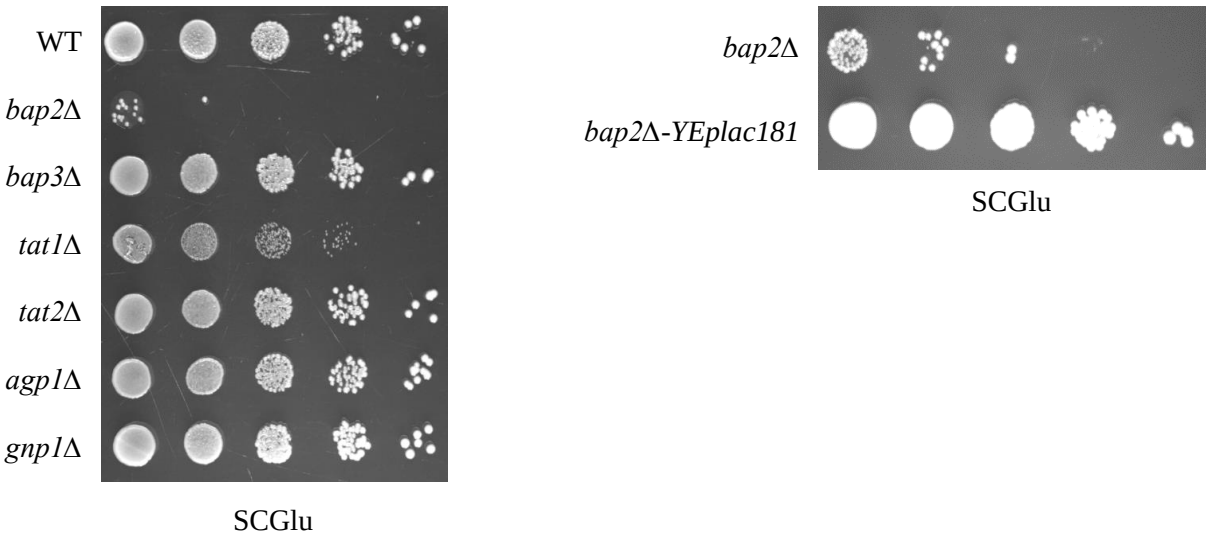


Figure 11. *BAP2* and *LEU2* are synthetically lethal. (A) WT, *bap2*Δ, *bap3*Δ, *tat1*Δ, *tat2*Δ, *agp1*Δ and *gnp1*Δ strains grown in SCGlylac broth were spotted on SCGlu medium. (B) *bap2*Δ strain and its transformant carrying YEplac181 grown in SCGlylac or SLeu$^-$Glylac broth were spotted on SCGlu media. Images were captured and analyzed after incubating the petri plates for 2-3 days at 30˚C.

Fig.12.

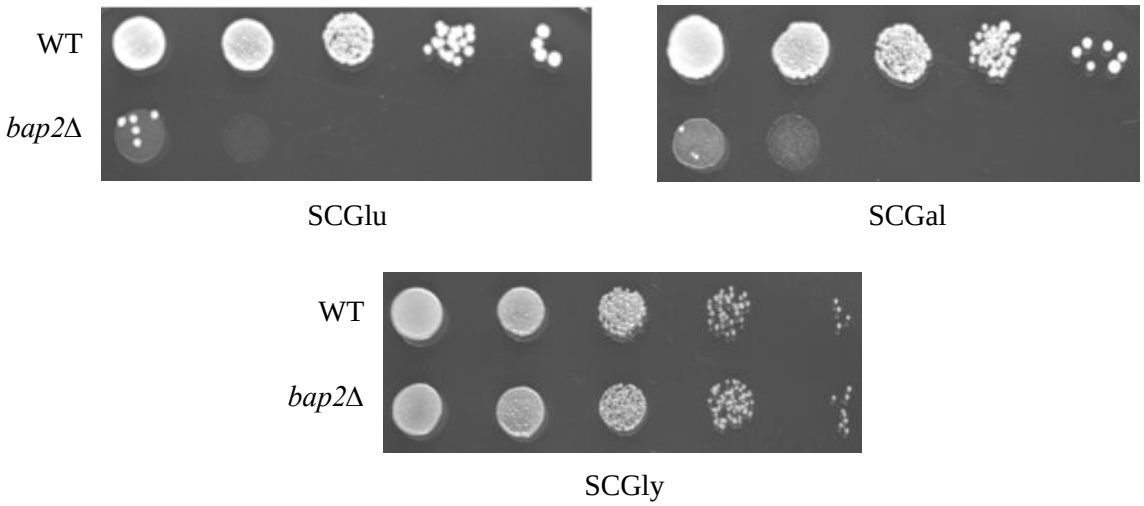
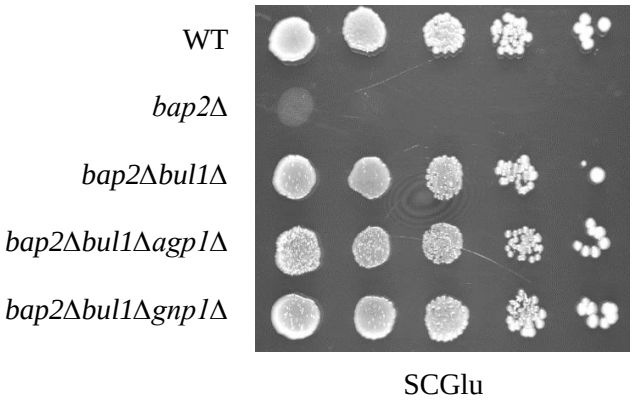


Figure 12. Synthetic lethality of *BAP2* and *LEU2* is not observed in presence of glycerol plus lactate. WT and *bap2*Δ strains grown in SCGlylac broth were spotted on SCGlu, SCGal and SCGlylac media. Images were captured and analyzed after incubating the petri plates for 2-3 days at 30˚C.

Fig.13.

A



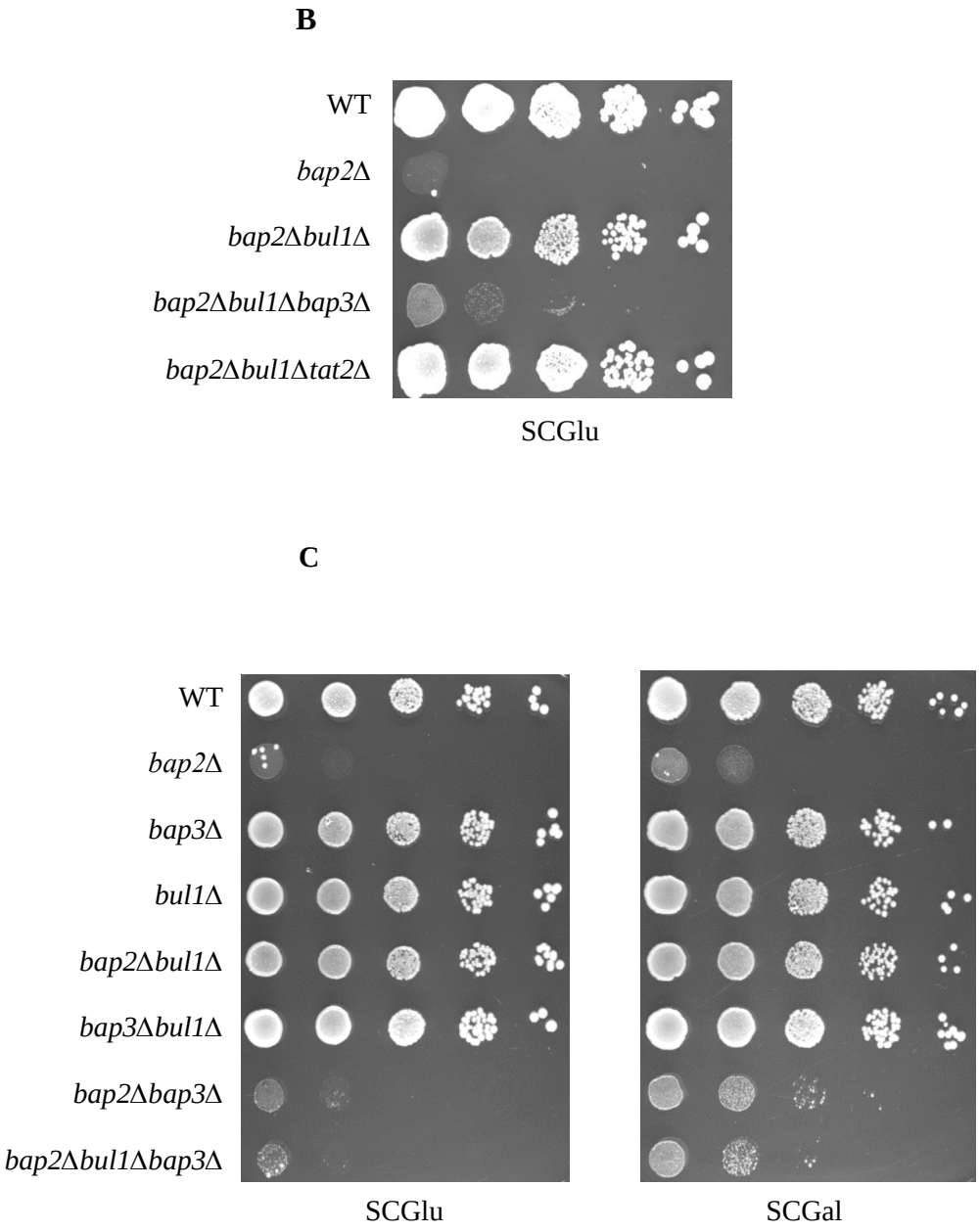
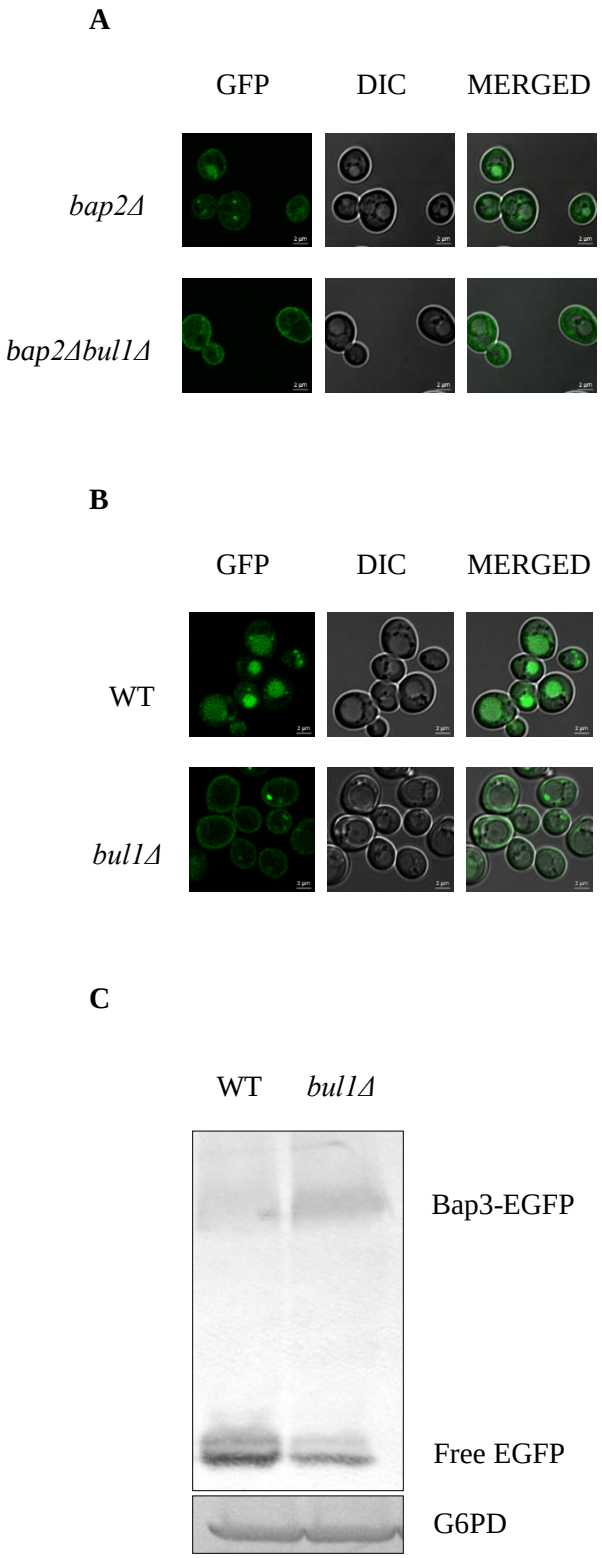


Figure 13. Loss of *BUL1* suppresses the *bap2*Δ growth defect, in a *BAP3* dependent manner. (A) WT, *bap2*Δ, *bap2*Δ*bul1*Δ, *bap2*Δ*bul1*Δ*agp1*Δ and *bap2*Δ*bul1*Δ*gnp1*Δ strains grown in SCGlylac broth were spotted on SCGlu medium. (B) WT, *bap2*Δ, *bap2*Δ*bul1*Δ, *bap2*Δ*bul1*Δ*bap3*Δ and *bap2*Δ*bul1*Δ*tat2*Δ strains grown in SCGlylac broth were spotted on SCGlu medium. (C) WT, *bap2*Δ, *bap3*Δ, *bul1*Δ, *bap2*Δ*bul1*Δ, *bap3*Δ*bul1*Δ, *bap2*Δ*bap3*Δ and *bap2*Δ*bul1*Δ*bap3*Δ strains grown in SCGlylac broth were spotted on SCGlu and SCGal media. Images were captured and analyzed after incubating the petri plates for 2-3 days at 30˚C.

Fig.14.


A
GFP
DIC
MERGED
bap2Δ
bap2Δbul1Δ
B
GFP
DIC
MERGED
WT
bul1Δ
C
WT
bul1Δ
Bap3-EGFP
Free EGFP
G6PD

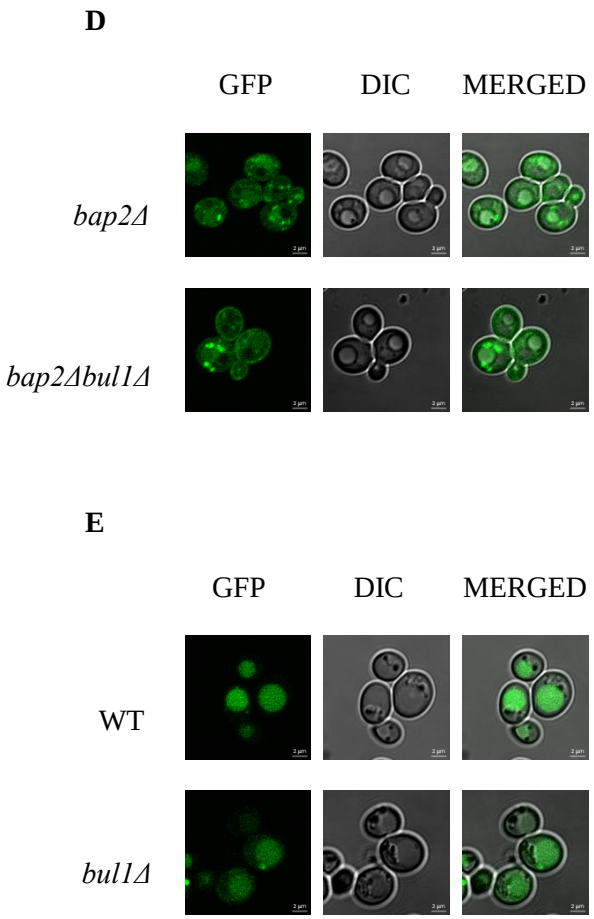


Figure 14. Bul1 is responsible for the endocytosis of Bap3. (A) Localization of endogenously EGFP tagged *BAP3* in *bap2*Δ and *bap2*Δ*bul1*Δ strains grown in SCGlylac broth and subcultured in SCGlu broth upto mid logarithmic phase. Scale-2µM. (B) Localization of endogenously EGFP tagged *BAP3* in WT and *bul1*Δ strains grown in SCGlylac broth and subcultured in SCGlu broth upto mid logarithmic phase. Scale-2µM. (C) Immunoblotting of total cell lysates of endogenously EGFP tagged *BAP3* in WT and *bul1*Δ strains, grown as mentioned in B. Anti-GFP and anti-G6PD antibodies (for loading control) were used for immunoblotting. Free EGFP emanates from vacuolar degradation of Bap3-EGFP chimera. (D) Localization of endogenously EGFP tagged *BAP3* in *bap2*Δ and *bap2*Δ*bul1*Δ strains grown in SCGlylac broth and subcultured in SCGal broth upto mid logarithmic phase. Scale-2µM. (E) Localization of endogenously EGFP tagged *BAP3* in WT and *bul1*Δ strains grown in SCGlylac broth and subcultured in SCGal broth upto mid logarithmic phase. Scale-2µM.

Fig.15.

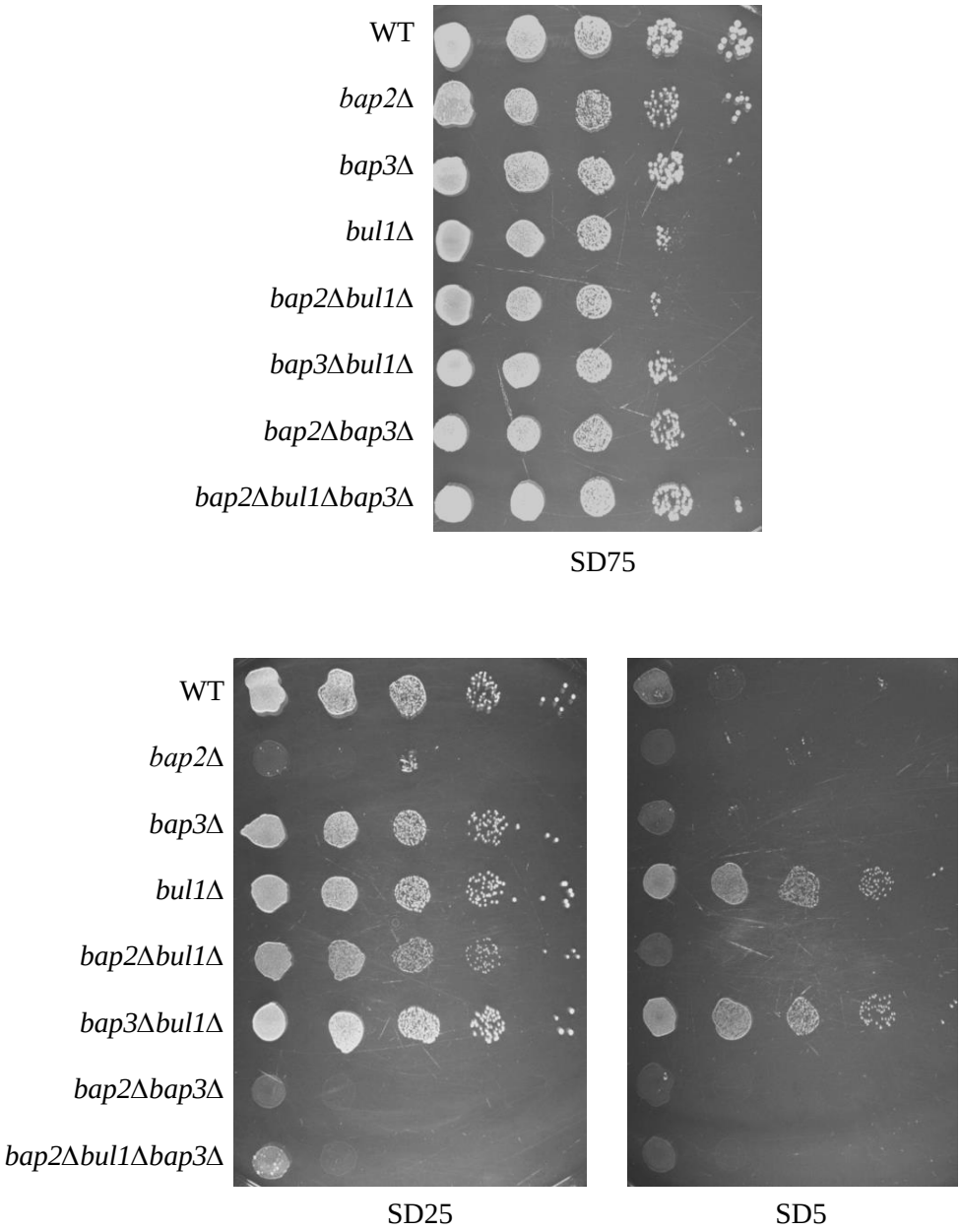


Figure 15. Loss of *BUL1* suppresses the growth defect of WT and *bap2*Δ strains in medium containing low leucine. WT, *bap2*Δ, *bap3*Δ, *bul1*Δ, *bap2*Δ*bul1*Δ, *bap3*Δ*bul1*Δ, *bap2*Δ*bap3*Δ and *bap2*Δ*bul1*Δ*bap3*Δ strains grown in SCGlylac broth were spotted on SD75, SD25 and SD5 media. Images were captured and analyzed after incubating the petri plates for 2-3 days at 30˚C.

Fig.16.

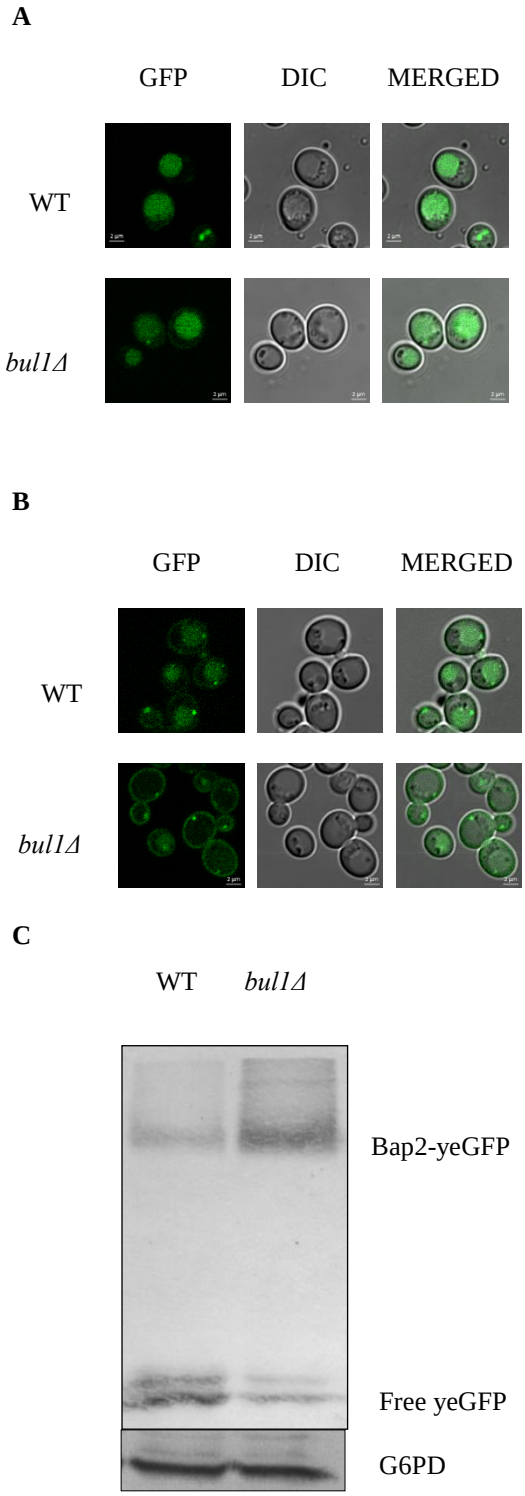

A
GFP
DIC
MERGED
WT
bul1Δ
B
GFP
DIC
MERGED
WT
bul1Δ
C
WT
bul1Δ
Bap2-yeGFP
Free yeGFP
G6PD

Figure 16. Bul1 is responsible for the endocytosis of Bap3 and Bap2. (A) Localization of endogenously EGFP tagged *BAP3* in WT and *bul1*Δ strains grown in SCGlylac broth and subcultured in SD25 broth upto mid logarithmic phase. Scale-2µM. (B) Localization of endogenously yeGFP tagged *BAP2* in WT and *bul1*Δ strains grown in SCGlylac broth and subcultured in SD25 broth upto mid logarithmic phase. Scale-2µM. (C) Immunoblotting of total cell lysates of endogenously yeGFP tagged *BAP2* in WT and *bul1*Δ strains, grown as mentioned in B. Anti-GFP and anti-G6PD antibodies (for loading control) were used for immunoblotting. Free EGFP emanates from vacuolar degradation of Bap2-yeGFP chimera.

Fig.17.

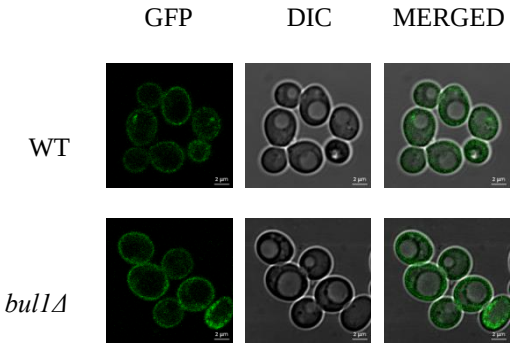
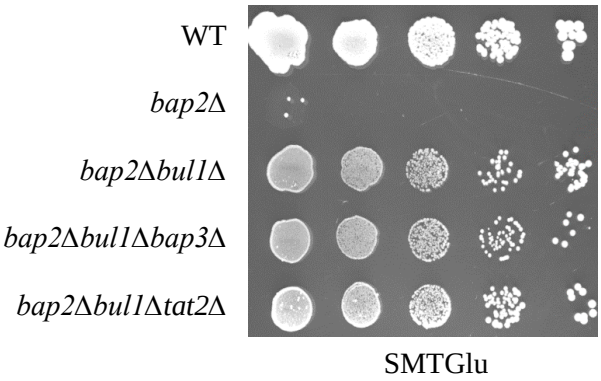


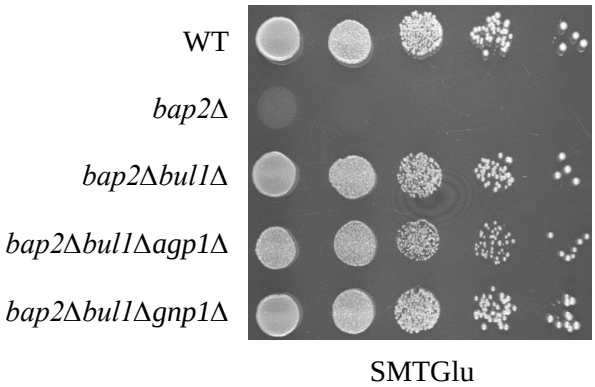
Figure 17. Loss of Bul1 does not affect Bap2 localization in SCGlu medium. Localization of endogenously yeGFP tagged *BAP2* in WT and *bul1*Δ strains grown in SCGlylac broth and subcultured in SCGlu broth upto mid logarithmic phase. Scale-2µM.

Fig.18.

A


WT
bap2Δ
bap2Δbul1Δ
bap2Δbul1Δbap3Δ
bap2Δbul1Δtat2Δ
SMTGlu

B


WT
bap2Δ
bap2Δbul1Δ
bap2Δbul1Δagp1Δ
bap2Δbul1Δgnp1Δ
SMTGlu

C

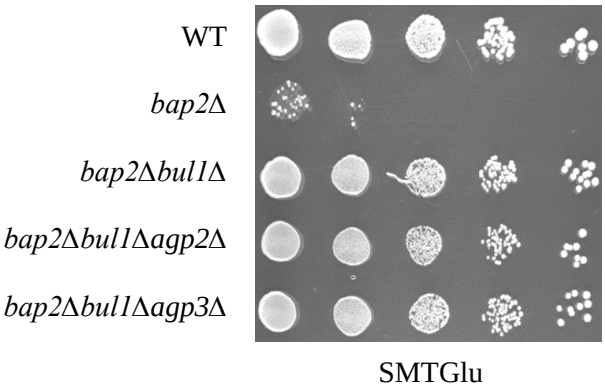

WT
bap2Δ
bap2Δbul1Δ
bap2Δbul1Δagp2Δ
bap2Δbul1Δagp3Δ
SMTGlu

Figure 18. Loss of *BUL1* suppresses the growth defect of *bap2*Δ strain in SMTGlu medium. WT, *bap2*Δ, *bap2*Δ*bul1*Δ and (A) *bap2*Δ*bul1*Δ*bap3*Δ, *bap2*Δ*bul1*Δ*tat2*Δ (B) *bap2*Δ*bul1*Δ*agp1*Δ, *bap2*Δ*bul1*Δ*gnp1*Δ (C) *bap2*Δ*bul1*Δ*agp2*Δ, *bap2*Δ*bul1*Δ*agp3*Δ strains grown in SCGlylac broth were spotted on SMTGlu medium. Images were captured and analyzed after incubating the petri plates for 2-3 days at 30˚C.

Fig.19.

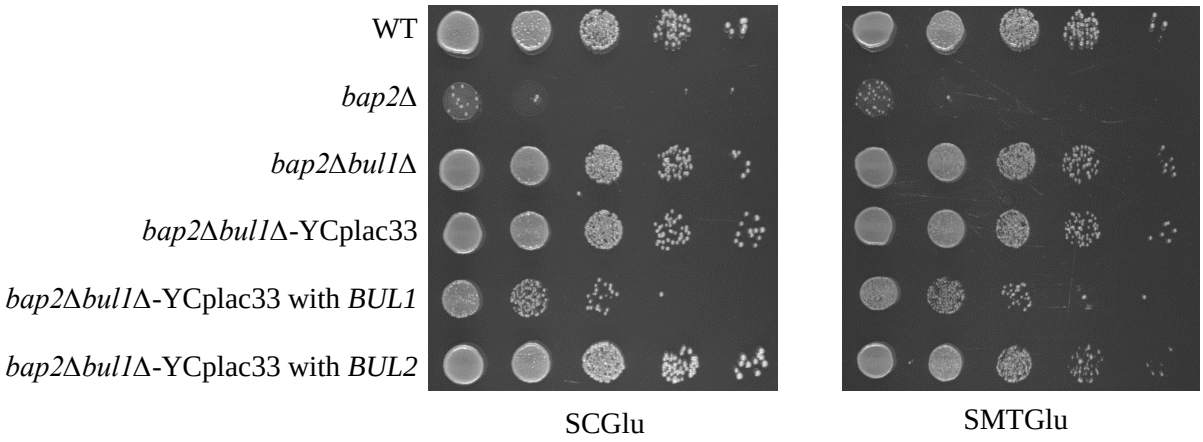


Figure 19. Complementation by *BUL1* not *BUL2* confers the growth defect back in the *bap2*Δ*bul1*Δ strain. WT, *bap2*Δ, *bap2*Δ*bul1*Δ strains and *bap2*Δ*bul1*Δ transformants carrying YCplac33 or YCplac33 with *BUL1* or *BUL2* grown in SCGlylac or SUra$^-$Glylac broth were spotted for growth on SCGlu and SMTGlu media. Images were captured and analyzed after incubating the petri plates for 2-3 days at 30˚C.

Fig.20.

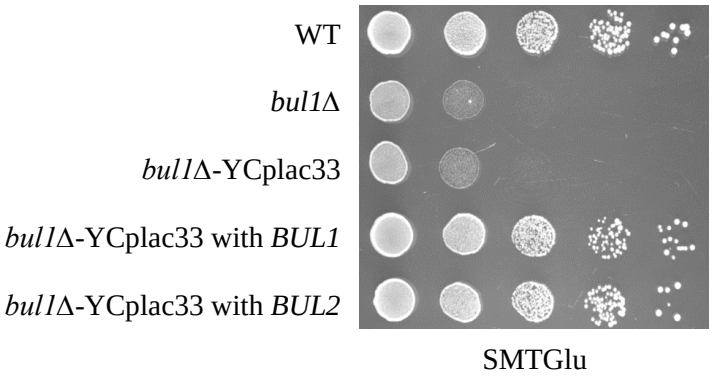


Figure 20. *BUL2* of W303-1A strain is functional. WT, *bul1*Δ strains and *bul1*Δ transformants carrying YCplac33 or YCplac33 with *BUL1*/*BUL2* grown in SCGlylac or SUra⁻Glylac broth were spotted for growth on SMTGlu medium. Images were captured and analyzed after incubating the petri plates for 2-3 days at 37˚C.

Fig.21.

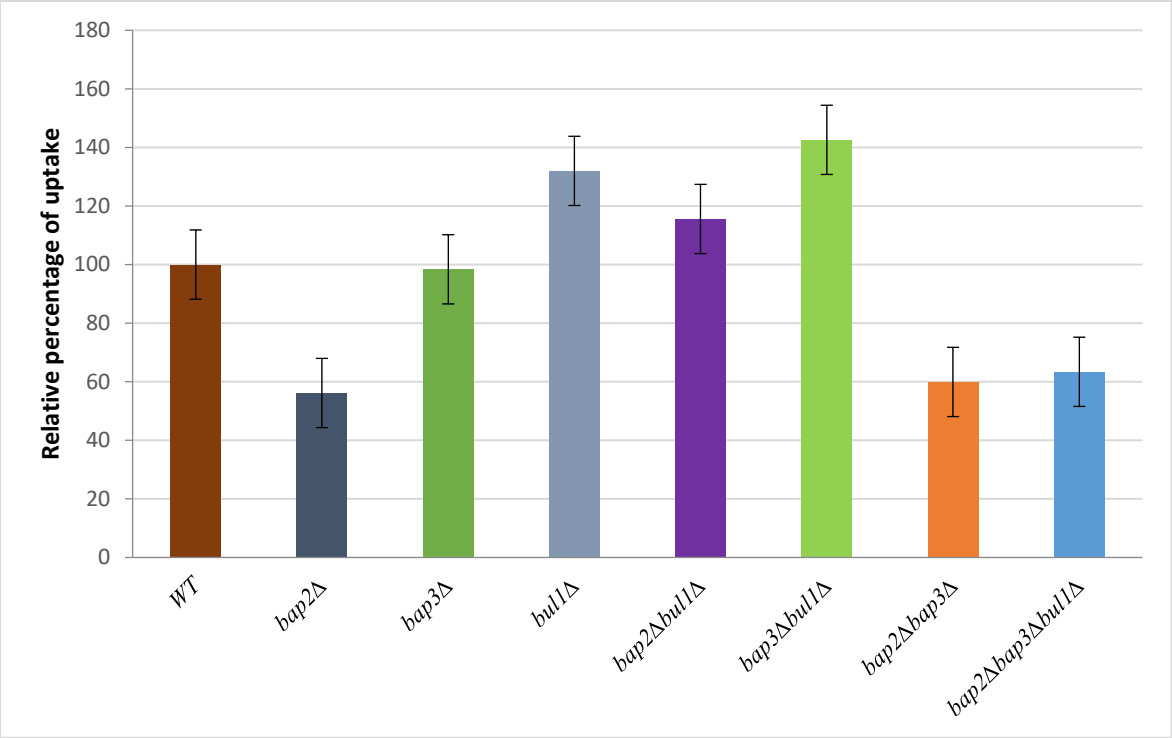


Figure 21. Uptake of C14-L-Leucine. WT, *bap2*Δ, *bap3*Δ, *bul1*Δ, *bap2*Δ*bul1*Δ, *bap3*Δ*bul1*Δ, *bap2*Δ*bap3*Δ and *bap2*Δ*bul1*Δ*bap3*Δ transformants carrying YEplac181, a *LEU2* plasmid were assayed for the uptake of C14 labelled L-Leucine. Data was from three independent experiments involving three biological and technical replicates. Uptake was determined as picomoles/0.8x10^6cells/min and expressed as mean relative percentage ± SE, considering uptake of WT as 100%.

Fig.22.

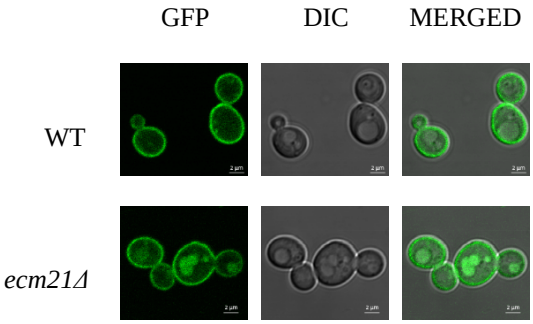


Figure 22. A cryptic mutation in *ecm21*Δ strain affects localization of Bap2. Localization of endogenously yeGFP tagged *BAP2* in WT and *ecm21*Δ strains grown in SCGlylac broth and subcultured in SCGlu broth upto mid logarithmic phase. Scale-2µM.

Fig.23.

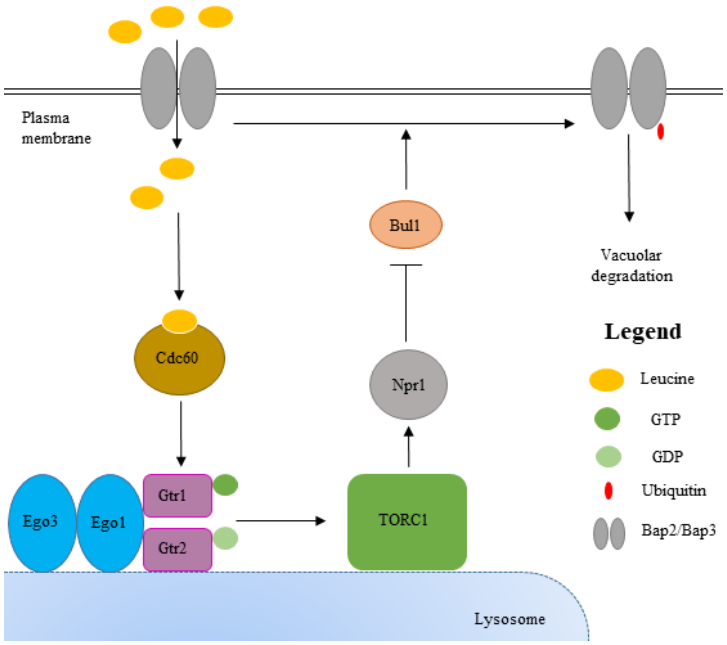


Figure 23. The proposed TORC1-Leucine feedback loop. Schematic representation of the TORC1-Leucine feedback loop representing how leucine regulates TORC1 activity.

Fig.24.

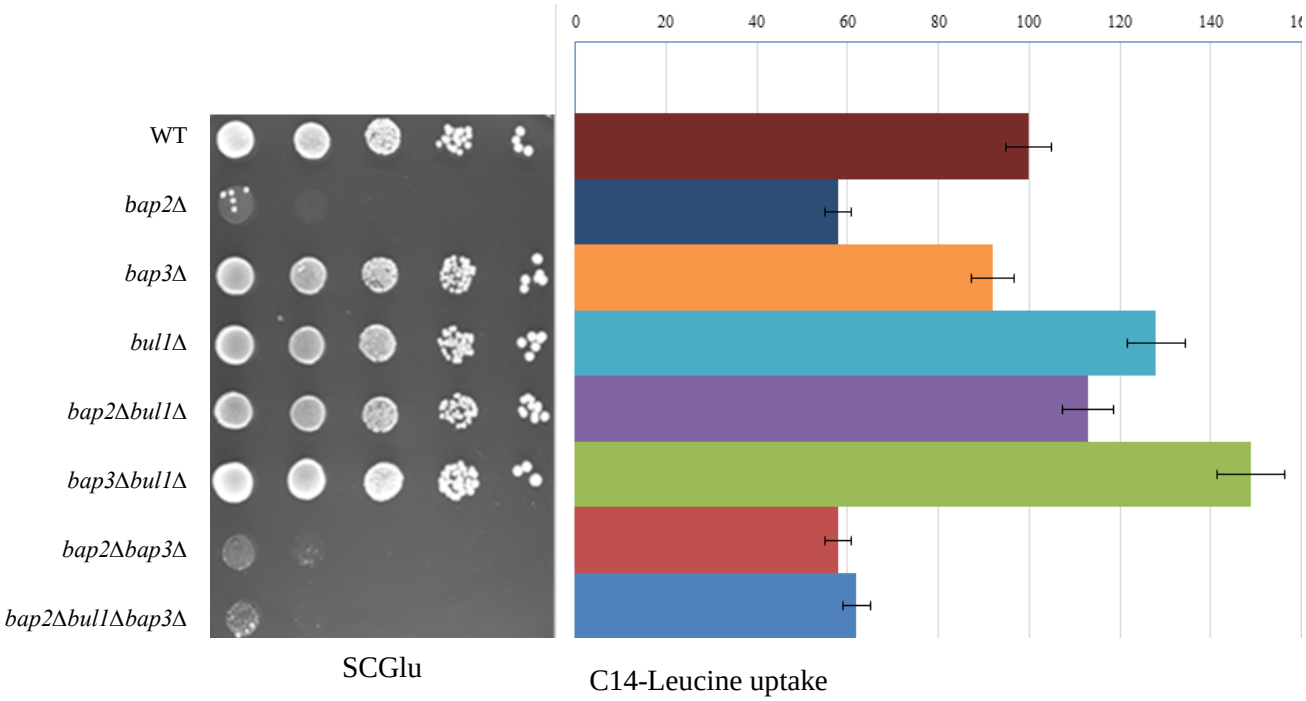
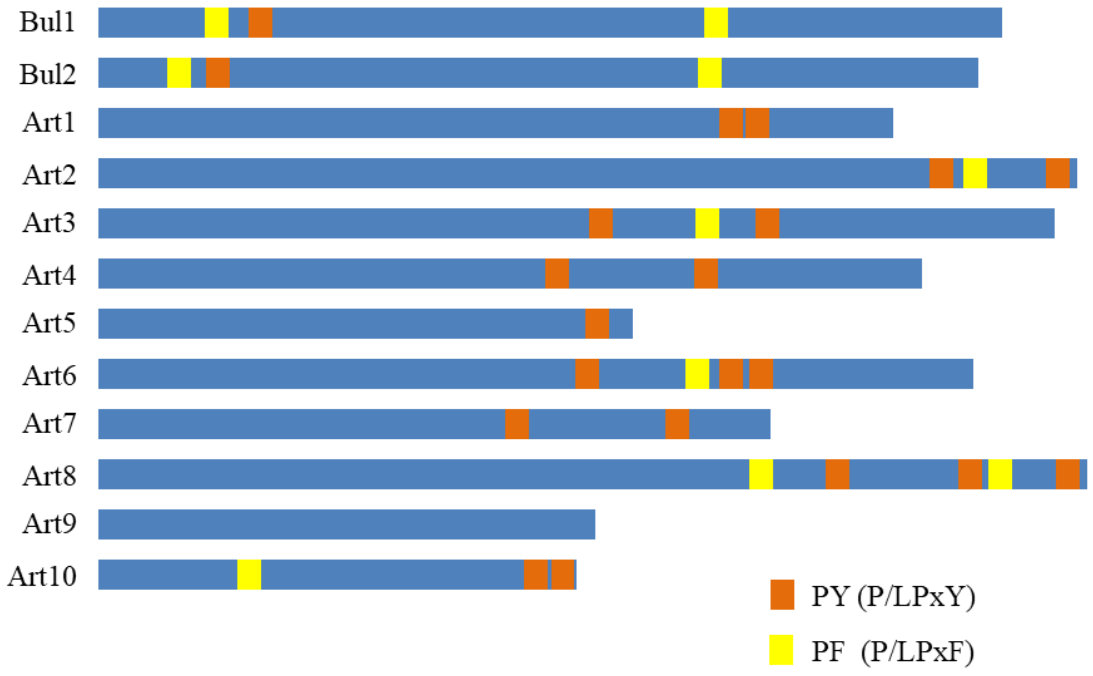


Figure 24. Growth and relative leucine uptake. Correlation of growth phenotype of WT, *bap2*Δ, *bap3*Δ, *bul1*Δ, *bap2*Δ*bul1*Δ, *bap3*Δ*bul1*Δ, *bap2*Δ*bap3*Δ and *bap2*Δ*bul1*Δ*bap3*Δ strains grown on SCGlu medium as shown in Fig.13C and their relative leucine uptake as shown in Fig.21.

Fig.25.

A



B

Figure 25. PY and PF motifs of ARTs. (A) Schematic representation of the ARTs and their PY (P/LPxY) and PF (P/LPxF) motifs. (B) Bul1 and Bul2 with their loss of function mutations highlighted.

Materials and methods

Yeast strains and plasmids

All yeast strains are of BY4741 background (ST.1). They were either purchased from Euroscarf or constructed. Yeast transformation was performed by LiAc-PEG method. Yeast genomic DNA was isolated by glass bead method. Genetic manipulations like disruption and tagging were performed by PCR based homologous recombination method. These manipulations were again confirmed by PCR. Primers used for the above procedures are listed (ST.3).

E.coli strain XL1 or DH5α were used for plasmid cloning and propagation. Plasmid isolation was done by alkaline lysis method and *E.Coli* transformation was done by PEG method. Plasmids used in the study are listed (ST2). Plasmid carrying *ECM21*, was constructed by cloning of a 4590 bp BamHI-SalI fragment of *ECM21* gene (1000 bp upstream of start codon, 3354 bp CDS and 236 bp downsream of stop codon) amplified from the genomic DNA of WT BY4741, into the SalI-BamHI site of YCplac33. Plasmid carrying either *BUL1* or $BUL1^{H402N}$ was constructed by cloning of 4020 bp SalI-EcoRI fragment of *BUL1* or $BUL1^{H402N}$ gene (1000 bp upstream of start codon, 2931 bp CDS and 89 bp downsream of stop codon), into SalI-EcoRI site of YCplac33. *BUL1* or $BUL1^{H402N}$ were amplified from the genomic DNA of *ecm21Δ* BY4741 and *ecm21Δ* dominant mutant BY4741 respectively. Plasmid carrying *BUL2*, was constructed by cloning of a 4019 bp SalI-SacI fragment of *BUL2* (1000 bp upstream of start codon, 2763 bp CDS, 250 bp downsream of stop codon, 6 bp of primer region), amplified from genomic DNA of WT W303-1A strain, into the SalI-SacI site of YCplac33.

Make – Primers (Sigma, BLOT solutions), Restriction enzymes, polymerases and ligases (Thermo scientific, NEB), PCR/Gel purification kits (Qiagen), Agarose (Sigma, Himedia), Incubator and shakers (Trishul, Pooja), PCR machine (Applied biosystems), Centrifuge (Eppendorf, Hettich), Spectrophotometer (Hitachi), Autoclave (Equitron), Laminar air flow chamber (Klenzaids, Biolinx).

Media recipe

Synthetic Minimal Amino acid Glucose (SMAaGlu) – 0.17% Yeast nitrogen base, 2% glucose, 0.01% of auxotrophic supplements and 2mM of corresponding amino acid. **Synthetic Minimal Tyrosine Glucose (SMTGlu)** – 0.17% Yeast nitrogen base, 2% glucose, 0.01% of auxotrophic supplements and 2mM Tyrosine. **Important note:** The usual amino acid concentration of the above described media is 2mM, but varied to 0.5mM, 4mM or 6mM whenever needed.

YPD – 0.5% Yeast extract, 1% bacto peptone and 2% glucose. **Synthetic Complete Glucose** or **Synthetic Complete Galactose (SCGlu/SCGal)** – 0.17% Yeast nitrogen base, 0.5% ammonium sulfate, 0.05% amino acid mixture (ST4) and 2% glucose or galactose. **Synthetic Complete Glycerol plus lactate (SCGlylac)** – 0.17% Yeast nitrogen base, 0.5% ammonium sulfate, 0.05% amino acid mixture (ST4) and 3% (V/V) glycerol and 2% (V/V) of 40% lactate pH 5.7. **Synthetic Tyrosine Dropout Glucose (STDGlu) -** 0.17% Yeast nitrogen base, 0.5% ammonium sulfate, 0.05% amino acid mixture (ST4) minus Tyrosine and 2% glucose. **Synthetic Minimal Dextrose (SD)** – 0.17% Yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose and 0.01% of auxotrophic supplements Uracil (89µM), Histidine (64µM), Methionine (67µM) and Leucine (76µM). SD75, SD25 and SD5 refers to SD with 75µM or 25µM or 5µM leucine. For solid media 2%agar was used. $SUra^{-}Glylac$ and $SLeu^{-}Glylac$ refers to SCGlylac lacking Uracil or Leucine respectively. All the media were sterilized by autoclaving at 121ºC at 15 lbs for minutes before use.

Make – Glucose (Sisco research laboratories), galactose (Sigma), glycerol (Merck), lactate (Merck), amino acids & uracil & adenine (Sigma), yeast nitrogen base (Difco), ammonium sulfate (Sisco research laboratories), yeast extract (Himedia), bacto peptone (Difco), agar (Difco), Pipettes (Gibson), Glass ware (Borosil, Asco, Merck, Actira), Plastic ware (Tarson).

Spotting assay (Growth assay)

Cells were grown in SCGlylac broth (SCGlylac broth lacking uracil or leucine for transformants) to mid log phase, usually for 48 hours, harvested by centrifugation at 13000 rpm and washed with sterile distilled water and cell density was normalized to OD_{600} 1.0. The normalized cell suspension was serially diluted to tenfold dilutions upto 10^{-4} dilution. 5µl from each dilution was

spotted onto appropriate medium, air dried and incubated at 30ºC or 37ºC for 2-3 days. Images of the petriplates were captured by UVITEC, CAMBRIDGE gel documentation system of GeNei.

Protein extraction and immunoblotting

Protein was extracted by alkaline lysis method. Cells grown in suitable medium to mid log phase were harvested at 4ºC and lysed with 0.5ml of lysis solution (0.2M NaOH, 0.2% mercaptoethanol) for 10 min on ice. Trichloroacetic acid was added to a final concentration of 5%, and incubated for another 10 min on ice. Samples were centrifuged for 5 min at 13000 x g for 5 min. Pellets were resuspended in 35 µl of dissociation buffer (4% SDS, 0.1M Tris-HCl pH 6.8, 4mM EDTA, 20% glycerol, 2% 2-mercaptoethanol, 0.02% Bromophenol blue) and mixed with 15 µl of 1M Tris base, and heated at 37ºC for 10 min.

Total cell free extracts were resolved by SDS-PAGE and transferred to 0.45 micron nitrocellulose membrane. Mouse bi-clonal anti-GFP antibodies were used to probe GFP tagged proteins and chased by secondary anti-mouse goat antibody conjugated with alkaline phosphatase. Rabbit anti-G6PD antibody was used to probe G6PD, and chased down by secondary anti-rabbit mouse antibody conjugated with alkaline phosphatase. Blots were developed and images were captured by UVITEC, CAMBRIDGE gel documentation system of GeNei and analyzed by UVITEC1D software.

Make – Mouse bi-clonal anti-GFP antibody (Roche Diagnostics, Mix of 13.1 and 7.1 monoclonal antibodies), Secondary anti-mouse goat antibody (), Rabbit anti-G6PD antibody (Santacruz Biotechnology) and Secondary anti-rabbit mouse antibody (Santacruz Biotechnology). Nitrocellulose membrane (Amersham Biosciences). SDS-PAGE and blotting transfer apparatus (GeNei).

Fluorescence microscopy

Cells were grown in appropriate medium to mid log phase were harvested, washed in sterile distilled water and resuspended in the same (To avoid auto fluorescence due to nutrient broth). Cells were readily analyzed for GFP fluorescence at room temperature under Zeiss Axio-Observer Z1 inverted motorized and computer-controlled laser scanning fluorescence microscope (Carl Zeiss, LSM 780) equipped with iPlan-Apochromat 100X oil immersion

objective 1.4 NA and PMT detector camera. Argon+ laser was used to excite the fluorophore with excitation wavelength of 488nm and emission wavelength was 526nm. Images were captured and later processed by Zen 2012 blue acquisition software from Zeiss.

Leucine uptake assay

Yeast cells were grown in SCGlylac broth lacking leucine; subcultured in SCGlu broth and grown upto mid log phase (OD_{600} 1-1.5). Cells were harvested, washed with SCGlu broth twice and resuspended in the same to $0.4x10^7$cells/ml. To 200µl of this suspension added was 2.8KBq (500 picomoles) of C14-labelled leucine. The suspension contained 188µM of cold leucine and 2.5µM of C14-Leucine. The cell suspensions were incubated in 30ºC incubator cum shaker for 0 min, 60 min and 120 min time points. The C14-labelled leucine uptake was stopped by adding cold leucine of 100µl to a final leucine concentration of 12.6mM. The cells from the suspension were collected on a 0.45 micron nylon membrane (Amersham Biosciences) by suction. Cells were washed with ice cold water for 3 times and the membrane was dried for 10 minutes. The membrane with cells was counted for radioactive pulses for 1min/sample in Perkin Elmer Liquid scintillation analyzer. For each repetition biological replicates were taken and technical triplicates were taken for each strain for each time point. C14-Leucine with specific activity of 5.5GBq/mmole (150mci/mmole) was purchased from BRIT, India.

Genome sequencing and analysis

The genomes of *ecm21*Δ and *ecm21*Δ dominant mutants 1 and 2 were sequenced by Medgenome labs, Bangalore, through Illumina Hiseq4000 platform. Using Burrows Wheeler Alignment, (BWA-MEM) raw reads were aligned to individual fasta files of chromosomes. The fasta files of chromosomes were obtained from Saccharomyces Genome Database. A pipeline consisting of samtools and bcf tools (Li., 2011) was used to create variant files. Using these variant files, annotation of variants were done.

ST1. Yeast strains used in this study.

Strain	Genotype	Source
WT	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
mrg19Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mrg19Δ::KanMX*	Euroscarf
ecm21Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ecm21Δ::KanMX*	Euroscarf
mrg19Δecm21Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mrg19Δ::KanMX ecm21Δ::hphMX*	Lab stock
ecm21Δ dominant mutant 1	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ecm21Δ::KanMX*	This study
ecm21Δ dominant mutant 2	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ecm21Δ::KanMX*	This study
WT homodiploid	MAT a/ MAT α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0*	This study
WT heterodiploid	MAT a/ MAT α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0 BUL1/bul1Δ::hphMX*	This study
ecm21Δ homodiploid	MAT a/ MAT α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0 ecm21Δ::KanMX/ecm21Δ::KanMX*	This study
ecm21Δ heterodiploid	MAT a/ MAT α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0 ecm21Δ::KanMX/ecm21Δ::KanMX BUL1/bul1Δ::hphMX*	This study
bul1Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bul1Δ::hphMX*	This study
bul2Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bul2Δ::hphMX*	This study
ecm21Δbul1Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ecm21Δ::KanMX bul1Δ::hphMX*	This study
bap2Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX*	This study
bap3Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap3Δ::KanMX*	This study
tat1Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 tat1Δ::KanMX*	This study
tat2Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 tat2Δ::KanMX*	This study
agp1Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 agp1Δ::KanMX*	This study
gnp1Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gnp1Δ::KanMX*	This study
bap2Δbul1Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bul1Δ::hphMX*	This study

bap2Δbul2Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bul2Δ::hphMX*	This study
bap3Δbul1Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap3Δ::Sphis5 bul1Δ::hphMX*	This study
bap2Δbap3Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bap3Δ::Sphis5*	This study
bap2Δbul1Δbap3Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bul1Δ::hphMX bap3Δ::Sphis5*	This study
bap2Δbul1Δtat2	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bul1Δ::hphMX tat2Δ::KlURA3*	This study
bap2Δbul1Δagp1Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bul1Δ::hphMX agp1Δ::KlURA3*	This study
bap2Δbul1Δagp2Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bul1Δ::hphMX agp2Δ::KlURA3*	This study
bap2Δbul1Δagp3Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bul1Δ::hphMX agp3Δ::KlURA3*	This study
bap2Δbul1Δgnp1Δ	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bul1Δ::hphMX gnp1Δ::KlURA3*	This study
bap2ΔBAP3-EGFP	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX BAP3::EGFP-Sphis5*	This study
bap2Δbul1ΔBAP3-EGFP	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bap2Δ::KanMX bul1Δ::hphMX BAP3::EGFP-Sphis5*	This study
BAP3-EGFP	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BAP3::EGFP-Sphis5*	This study
bul1ΔBAP3-EGFP	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bul1Δ::hphMX BAP3::EGFP-Sphis5*	This study
BAP2-yeGFP	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BAP2::yeGFP-hphMX*	This study
bul1ΔBAP2-yeGFP	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bul1Δ::kanMX BAP2::yeGFP-hphMX*	This study
ecm21ΔBAP2-yeGFP	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ecm21Δ::KanMX BAP2::yeGFP-hphMX*	This study
W303-1A	MAT a *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Lab stock

ST2. Plasmids used in this study.

Plasmid	Description	Source
YCplac33	Yeast centromeric shuttle vector with *AMP*^{R} and *URA3* markers	Lab stock
YCplac33-*ECM21*	Yeast centromeric shuttle vector with *AMP*^{R} and *URA3* markers carrying *ECM21*	This study
YCplac33-*BUL1*^{H402N}	Yeast centromeric shuttle vector with *AMP*^{R} and *URA3* markers carrying *BUL1*^{H402N}	This study
YCplac33-*BUL1*	Yeast centromeric shuttle vector with *AMP*^{R} and *URA3* markers carrying *BUL1*	This study
YCplac33-*BUL2*	Yeast centromeric shuttle vector with *AMP*^{R} and *URA3* marker carrying *BUL2*	This study
Yeast genomic DNA library	Yep24 based yeast genomic library	Lab stock
YEplac181	Yeast episomal shuttle vector with *AMP*^{R} and *LEU2* markers	Lab stock

ST3. Primers used in this study.

Description	**Sequence**
Fwd Pr for disruption of *MRG19*	5’TGGCTCTCCAAGGGTAATCAGTCAGACTAGTAAGCAAAGTCAATCAGCTTGCCTTGTCCCCGCCGG3’
Rev Pr for disruption of *MRG19*	5’TATATGTGTGTGTGAGGTTTATAAAAAAAGTGAAGATATTAATATTCGACACTGGATGGCGGCGTT3’
Fwd Pr for confirmation of disruption of *MRG19*	5’AGTACACCGTGAGTACATATTG3’
Fwd Pr for disruption of *ECM21*	5’ATGCCGTTTATAACATCAAGACCGGTGGCGAAAAATTCATCCCACTCTTAAGCCAGCTGAAGCTTCGTACG3’
Rev Pr for disruption of *ECM21*	5’ATCGTAGAATAATTCAAAAAAAAATAAAAAACGAAAAAATTCAGGCCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *ECM21*	5’GAACGACTCATTATTATTGCTCGGAAGGTC3’
Fwd Pr for disruption of *BUL1*	5’ACTGTTCGTGTGTGTCAACAGGTATATCGTACGCTAAATGGCCAGCTGAAGCTTCGTACG3’
Rev Pr for	5’TATAAGAAAAGTAACGAGAATTTTTTCTAATGTTTTTTTAGG

disruption of *BUL1*	CCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *BUL1*	5’AGCGGGCCGTGACCCGCTCTCAGAGGCTAT3’
Fwd Pr for cloning of *BUL1*	5’TCTAGAGTCGACGTGTTCGTGTTGGAGCCTTCCTCTGGAGCATCGCCACCAG3’
Rev Pr for cloning of *BUL1*	5’GAGCTCGAATTCAGGACGCGATAAATACCTTCATCTATAAGC3’
Fwd Pr for cloning of *BUL2*	5’ATTACTGCAGGTCGACTACATATATTAGTACCATGACGTTATATATATATCTGTGAAGAG3’
Rev Pr for cloning of *BUL2*	5’TAATGAGCTCGTCTCTCTTCTTCTTTAGTAATACCTGTGCCATGTGATAGCC3’
Fwd Pr for cloning of *ECM21*	5’CTGCAGGGATCCGTCAAGCGGCGCATCAAGTGTACGCTT3’
Rev Pr for cloning of *ECM21*	5’AAGCTTGTCGACGGTGTCAATTCGGCAAGAGGTGTTCTGCGC3’
Fwd Pr for disruption of *BAP2*	5’CGATACTATCAATTTATTTAGCTTCAATAAAACTCAAGTGATTTTAGAAATGGCCAGCTGAAGCTTCGTACG3’
Rev Pr for disruption of *BAP2*	5’ATCAACCGGCTCTAATGGGAAGTGTCCAGACCTGAGTGGTGTAGTTAAGTTTAGGCCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *BAP2*	5’GATGAGCACATCTCCTCGTATATATACGTA’
Fwd Pr for disruption of *BAP3*	5’CCGGGCATTAAAAACTACGTTGTAATTAGCGTGGTTACTATTGATGGATTGGCGAAGTAAGCCAGCTGAAGCTTCGTACG3’
Rev Pr for disruption of *BAP3*	5’TAGACAACGTTGAGTAAGGTGGTTCGGTCCGAACAACAGTTTCATGCTTGTGTTACTTCGGGCCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *BAP3*	5’CAGTGGCAGTATGTAGTTTGTTCTTGCGTTTCCTTGTGGT3’
Fwd Pr for disruption of *TAT1*	5’GTAGCTACCTAATATAGTTTCTCGATAAAAAGGCGTAAAAATGGCCAGCTGAAGCTTCGTACG3’
Rev Pr for disruption of *TAT1*	5’AAGCCCGATGAAGCCAAGCGGAAAATGAATGGAATTGCTGTTAGGCCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *TAT1*	5’GTTGGAGTAAAAAAGTATATAAGATGCTGT3’
Fwd Pr for disruption of *TAT2*	5’ACTGACTTTCTCAACACGTCGAGCACGACATTTCCCAAAAATAGCTGAAGAACATAAGCTAAGCAAATTACGCAACACACTCTCATATGGCCAGCTGAAGCTTCGTACG3’
Rev Pr for disruption of *TAT2*	5’TGGAATAATTAATTATGAAGGATGAGTACTAGAGAAGAGAAAAAAAAATATTCTACAAAAATAAATTGAACTTGTTTCTTCGGT

	ATTAGGCCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *TAT2*	5’GAGATTTCCCACCATTCACTCCAG3’
Fwd Pr for disruption of *AGP1*	5’TATTATGTAATCTTTATAGAAGAAGCACGCTAATATAGACAAAGATAGCTTCGCACAATGGCCAGCTGAAGCTTCGTACG3’
Rev Pr for disruption of *AGP1*	5’ACTACAAAACAGAAGTACAAAAATGAATAAATATAAAAGAAGTAAATGCTTTTTTTTTAGGCCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *AGP1*	5’CCCGGGGGATTCCTGAAAGAGCGCCTTTACCTCAACCTAC3’
Fwd Pr for disruption of *AGP2*	5’TCAGCCTTGTCCCCCCTAAGCTGCACTTACATTTTGCTTCCATAACTTTTGCCAAGCATGGCCAGCTGAAGCTTCGTACG3’
Rev Pr for disruption of *AGP2*	5’TGACAATAAATTTGGAGGCAGTCAATGTAAATTTGTGAATATAACGACATAATTGCATTAGGCCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *AGP2*	5’GTAAGGGTAGTGTTAGTTCACCATACTTCG3’
Fwd Pr for disruption of *AGP3*	5’TTGTCAGTTCTCCCCTCATTGCTCTGAAAATATACAAGATTTCAGCAATCCTCGCTAATGGCCAGCTGAAGCTTCGTACG3’
Rev Pr for disruption of *AGP3*	5’GGGGGCGGCTGTAAAAAGGTAAAATAATTCTCAAATAGCTTATATTCACAATAATGGTCAGGCCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *AGP3*	5’GGAGATCATGTTTCCGGAGTAACTCCGCTG3’
Fwd Pr for disruption of *GNP1*	5’AGAAAAAAAAACTATCGCTTTCTCAAGTAGCTTATATAATATCAAATATTGCACATTATGGCCAGCTGAAGCTTCGTACG3’
Rev Pr for disruption of *GNP1*	5’GCTAATTGAATTGTTTCTTCAAGTTTTTTTTTTTTTTTTTGAATCGTGATTTCTGCTTTAGGCCACTAGTGGATCTG3’
Fwd Pr for confirmation of disruption of *GNP1*	5’GGGGATCCCTTGACCTCTGGCTTAGCTTCTTG3’
Fwd Pr for C terminal GFP	5’GAATATGTCTTTGATGAGAAAAGCTTATCATTTCTGGTGTGGAGCAGGTGCTGGTGCTGGTGCTG3’

tagging of *BAP2*	
Rev Pr for C terminal GFP tagging of *BAP2*	5’AATGGGAAGTGTCCAGACCTGAGTGGTGTAGTTAAGTTTATAGTCCTGTCGGGTTTCGCCACC3’
Fwd Pr for confirmation of C terminal GFP tagging of *BAP2*	5’ATGATCCAGAGCTAATGAGACAAGAGGACG3’
Fwd Pr for C terminal GFP tagging of *BAP3*	5’AGAAAGGTTAAAGAACTCTTCAATATTCGTCAGAGTCTACAAATTTTGGTGTGGAGCAGGTGCTGGTGCTGGTGCTG3’
Rev Pr for C terminal GFP tagging of *BAP3*	5’GTTGAGTAAGGTGGTTCGGTCCGAACAACAGTTTCATGCTTGTGTTACTTCGTAGTCCTGTCGGGTTTCGCCACC3’
Fwd Pr for confirmation of C terminal GFP tagging of *BAP3*	5’CGAGAGTTATCTGGCTGCCCCGCTATGGAT3’
Primer for confirmation of disruption by KanMX	5’ CTCTGGCGCATCGGGCTTCCC3’
Primer for confirmation of disruption by hphMX	5’CCCCGAACATCGCCTCGCTCC3’
Primer for confirmation of disruption by *KlURA3*	5’ATGTCCACAAAATCATATACCAGTAGAGCTGAGACT3’
Primer for confirmation of disruption by *ScURA3*	5’ CTAGGTTCCTTTGTTACTTCTTCCGCCGCCTGCTTC3’
Primer for confirmation of disruption by *SpHIS5*	5’CCAAGAATCCAATTCCCGTGTCTACTTGGA3’
Primer for confirmation of tagging by yeGFP	5’ TTATTTGTACAATTCATCCATACCATGGGTAATACC3’
Primer for confirmation of tagging by EGFP	5’CCGCCGAATCCTTACTTGTACAGCTCGTCCATGCCG3’

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