Biomedical Journal 47 (2024) 100694

Contents lists available at ScienceDirect

Biomedical Journal

journal homepage: www.elsevier.com/locate/bj

Inactivation of Ymr1, Sjl2/3 phosphatases promotes stress resistance and longevity in wild type and Ras2G19V yeast

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ARTICLE INFO

Keywords:
Aging
Ras activity modulators
Phosphoinositides
Ras Pathway
Transposon mutagenesis
Ras suppressors

ABSTRACT

In *Saccharomyces cerevisiae*, Ras (RAt Sarcoma) activity plays a central role in mediating the effect of glucose in decreasing stress resistance and longevity, with constitutive Ras activation mutations promoting cell growth and oncogenesis. Here, we used transposon mutagenesis in yeast to identify suppressors of the constitutively active Ras2G19V, orthologue of the KRASG12C mammalian oncogene. We identified mutations in *YMR1* (Yeast Myotubularin Related), *SJL2* (SynaptoJanin-Like) and *SJL3* phosphatases, which target phosphatidylinositol phosphates, as the most potent suppressors of constitutive active Ras, able to reverse its effect on stress sensitization and sufficient to extend longevity. In *sjl2* mutants, the staining of Ras-GTP switched from membrane-associated to a diffuse cytoplasmic staining, suggesting that it may block Ras activity by preventing its localization. Whereas expression of the Sjl2 PI 3,4,5 phosphatase mediated stress sensitization in both the Ras2G19V and wild type backgrounds, overexpression of the phosphatidylinositol 3 kinase VPS34 (Vacuolar Protein Sorting), promoted heat shock sensitization only in the Ras2G19V background, suggesting a complex relationship between different phosphatidylinositol and stress resistance. These results provide potential targets to inhibit the growth of cancer cells with constitutive Ras activity and link the glucose-dependent yeast pro-aging Ras signaling pathway to the well-established pro-aging PI3K (PhosphoInositide 3-Kinase) pathway in worms and other species raising the possibility that the conserved longevity effect of mutations in the PI3K-AKT (AK strain Transforming) pathway may involve inhibition of Ras signaling.

1. Introduction

At least 25 % of all human tumors are driven by RAS mutations [1] but for some cancer types mutations in this gene affect a larger portion reaching 95 % for pancreatic cancer (KRAS), 45 % for colorectal cancer (KRAS), and 35 % for lung cancer (KRAS) (Data from National Cancer Institute Ras initiative). The impairment of its GTPase activity, blocking RAS in the GTP-bound form, is the biochemical defect shared by the different mutated proteins found in human tumors. Even though the early onset of *RAS* mutations in cell transformation has been assessed, persistent expression of mutated *RAS* is necessary for tumor maintenance [2] rendering RAS a central target also in metastatic cancers. On the other hand, genome manipulation in a wide array of model systems ranging from yeast to mammals has identified the RAS pathway as a major pro-aging pathway and certain *HRAS* alleles alone or in combination with *APOE* (APOlipoprotein E) alleles have been associated with longevity in humans [3,4]. Mammalian cell studies also reveal that while immortalized cells are transformed by active RAS proteins, primary mammalian cells become instead senescent with high expression of p53 and p16 proteins and phenotypic similarities with senescent cells that have reached the Hayflick limit [5]. This phenomenon, which is called oncogene-induced senescence, is believed to be a protective mechanism against neoplastic transformation [6–8]. These observations link aging, cancer and senescence but indicate that the relationship between oncogene activity and cancer is complex since constitutive Ras activity can accelerate aging and promote cancer growth but can also promote cell senescence to prevent cancer.

Unfortunately, the lack of hydrophobic pocket in the RAS protein

Peer review under responsibility of Chang Gung University.
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https://doi.org/10.1016/j.bj.2023.100694
Received 5 November 2023; Received in revised form 18 December 2023; Accepted 19 December 2023
Available online 27 December 2023

Table 1
List of Ras2G19V suppressors identified by transposon mutagenesis.

gene ID	clone no	Function	Mammalian orthologue
RPL1A	3	Ribosomal 60S subunit L1Ap	L10 large ribosomal pt subunit
FMP45	4	sphingolipid metabolism	N/A
SJL2	2	PI metabolism	1 and 2
SEC4	1	Secretory pathway	RAB33B
YDL085C-A	3	unknown	SERF1a
PHO23 v	1	Rpd3L HDAC complex	p33-ING1/ING3 partial homology
ECM8	1*	unknown	N/A
MSN1	1	transcription factor	N/A
RGT1	1	glu-responsive transcription factor	N/A

Table 2
Yeast strains and plasmids used in this study.

Strain	Genotype	Source	Figures
DBY746	*MATα leu 2–3, 112 his3Δ1 trp1-289 ura 3*–52 GAL$^+$	Gralla, 1991, J Bacteriol	1, 2, 3, 4B, 4C, 5
msn1Δ	DBY746 *msn1::HIS3*	This study	2B-D
sjl3Δ	DBY746 *sjl3::HIS3*	This study	5A, B
sjl2Δ	DBY746 *sjl2::HIS3*	This study	2B, 5A,B
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	YKO	3B,C, 4A
fmp45	BY4741 *fmp45::URA3*	YKO	3D, 2F
sjl2Δ	BY4741 *sjl2::URA3*	YKO	3D
sjl1Δ	DBY746 *sjl1::HIS3*	This study	3D
sjl2-3Δ	DBY746 *sjl2::HIS3 sjl3::LEU2*	This study	5A
sjl2Δras2Δ	DBY746 *sjl2::HIS3 ras2::LEU2*	This study	5A
sjl3Δymr1	DBY746 *sjl3::HIS3 ymr1::LEU2*	This study	5B

Plasmid	Description	Source	Figures
pVPS34	*URA3VPS34*	Gift from Scott Emr	4C
pRS416R2G19V	*URA3 Ras2G19V*	Gift from Kevin A Morano	1
sjl2C446	*Sjl2C446 URA3*	Gift from Scott Emr	4A
sjl2D850	*Sjl2D850 URA3*	Gift from Scott Emr	4A
Sjl2	*Sjl2 URA3*	Gift from Scott Emr	3 B, C
pMSS4	*MSS4 LEU2*	*Gift from Scott Emr*	4B
Yep51 Ras2G19V	*Ras2G19V LEU2*	*This study*	1, 2B, C, 4 A, C, 5B,
YEpeGFP-RBD3	*GFP-RBD3 LEU2*	Gift from Sonia Colombo	6

structure, has challenged the identification of small molecules capable to inhibit RAS function. RAS has thus been classified as undruggable for decades [9]. More recently, inhibitors targeting the mutated form of RAS have been developed and many clinical trials started (e.g. for non-small cell lung cancer: NCT04303780 and NCT04185883, NCT03785249, NCT04613596, NCT04685135, NCT05472623, NCT05375994) but toxicities and off-target effects have limited their usage so far [10]. Recently, specific inhibitors of KRASG12C mutant RAS protein have been developed, but, after initial responses, acquired resistance have limited the overall survival [11].

The activated GTP-bound form of RAS transmits the signal downstream thanks to its affinity for at least 11 different RAS effectors [12]. Interaction with RAS increases their membrane concentration enhancing their catalytic activity [12]. It is worth noting that some of these effectors are mutated in cancer and have stimulated the search for inhibitors of these effectors or of their RAS binding activity. It is interesting to note that some of these effectors such as MAPK (Mitogen Activated Protein Kinase), TOR (Target Of Rapamycin), and AKT were also identified through genetic screens aimed at identifying longevity genes in several model systems.

Other researchers focused on the requirement for active RAS proteins to be bound to the inner plasma membrane through a posttranslational process involving the addition of a farnesyl isoprenoid moiety to its C-terminal CAAX [13]. This posttranslational modification of the RAS proteins has been successfully targeted with selective inhibitors [14]. However, even though preclinical studies have been successful in blocking HRAS-dependent tumors in mice with no significant side-effect, clinical trials with those inhibitors were disappointing. It was later discovered that inhibition of isoprenylation resulted in geranyl geranylation of the C-terminal of the RAS proteins, another post-translational modification which is also effective in linking RAS to the plasma membrane [15–18] thus overriding the efficacy of isoprenylation inhibitors.

It must also be noted that RAS posttranslational modification, even though necessary for membrane localization, is not sufficient by itself but needs to be coupled with electrostatic membrane association. In fact, the preferential plasma membrane localization is mainly due to the higher plasma membrane negative charge compared to other cellular membranes [19]. Physiological modification of membrane lipid composition has been demonstrated during phagocytosis, a phenomenon associated with the rapid release of K-RAS, RAC (RAs-related C3 botulinum toxin substrate 1) and cSRC (cellular SaRComa) from the membrane. This observation led to the idea that dissociation and reassociation cycles of membrane proteins, in response to change in membrane lipid composition, can be another layer of signaling regulation

Table 3
List of oligonucleotides used to generate gene deletions.

Oligo used to obtain the indicated gene disruption:	
msn1fw	atccttggaagagtattcagcaaatccacctaacaatgatg AGA TTG TAC TGA GAG TGC AC
msn1 rev	gaagagttgctgcttatgtacagtccttgactttggccac CTG TGC GGT ATT TCA CAC CG
sjl1fw	cgaatccccttgttaaacatttgtaaaccggaaagttggaac AGA TTG TAC TGA GAG TGC AC
sjl1rev	caaaaagttccagccaaaaatagagagtacgctcacaga CTG TGC GGT ATT TCA CAC CG
sjl2fw	ctttgcctggaaataatggtaagtcctactttcaacctat AGA TTG TAC TGA GAG TGC AC
sjl2rev	gtaatccgagggtttgctgaaacaatttttagctatatta CTG TGC GGT ATT TCA CAC CG
sjl3fw	acagtcaatatccaatatcatattaagaaagctagcagtaccAGA TTG TAC TGA GAG TGC AC
sjl3rev	ttgtttctcttcgttcatttaaagggatacaaacggaacaac CTG TGC GGT ATT TCA CAC CG
pho23fw	ggacgaacccggaaggagaaaagtttgtagtcttgcagt AGA TTG TAC TGA GAG TGC AC
pho23rev	attcgcccgagagctatttcacagttttttttgcagtcg CTG TGC GGT ATT TCA CAC CG
rgt1fw	cggtggtcccaagctattaaacatttccatccaaggttcta AGA TTG TAC TGA GAG TGC AC
rgt1rev	caccattttgagcccaaatcattagaaaaaaatctttctg CTG TGC GGT ATT TCA CAC CG
fmp45 fw	atagtggaccaaatggcgttcacgagcatcaagaagaccg AGA TTG TAC TGA GAG TGC AC
fmp45rev	gcaaggctgggacccaaaaactttgcgttcatctggactt CTG TGC GGT ATT TCA CAC CG
ymr1fw	gggtcacttggtgagattaggtcgtcgttattatttttattttg AGA TTG TAC TGA GAG TGC AC
ymr1rev	gaaatgcttgaacttttctatacttctatcattgtatgcaaaatac CTG TGC GGT ATT TCA CAC CG

lowercase the gene-specific sequence; uppercase the sequence of the pRS vector series used to amplify the selectable marker.

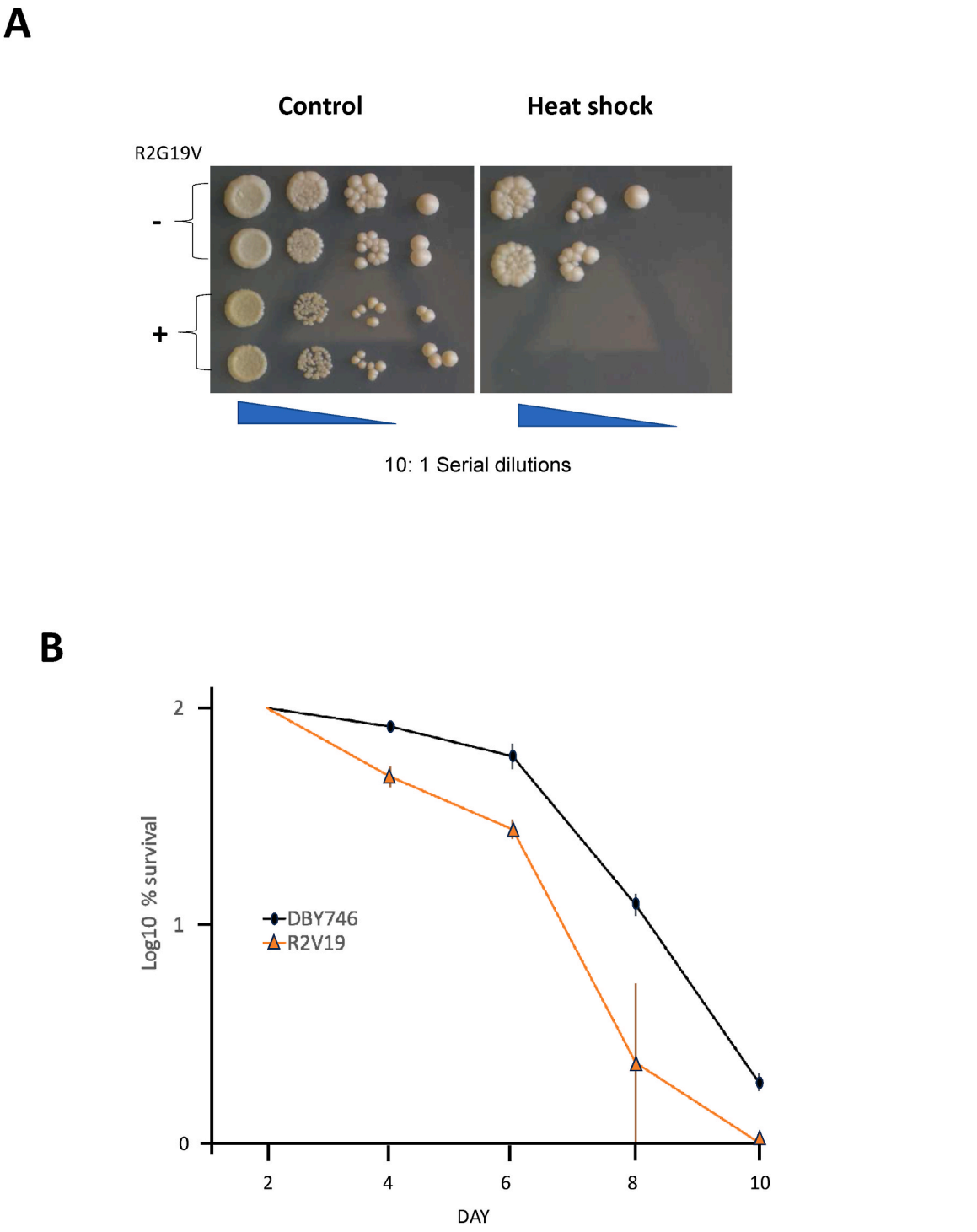

Fig. 1. Ras2G19V increases stress sensitivity and shortens lifespan. A) wild type yeast strain transformed with an expression vector for Ras2G19Vp (R2G19V) or with the corresponding empty vector. Aliquots of cells after 2 days of growth were serially diluted and heat shocked. B) Chronological lifespan (average of three expp.) of the same strains.

[19–21].

In the present study we performed a genome-wide screen, in the model system *Saccharomyces cerevisiae*, aimed at finding genes and pathways whose impairment can suppress the deleterious effects of constitutively active Ras2p, thus providing potentially druggable targets for drugs against aging and cancer.

2. Material and methods

2.1. Mutagenesis

Transposon mutagenesis and allele rescue were realized as previously described [22].

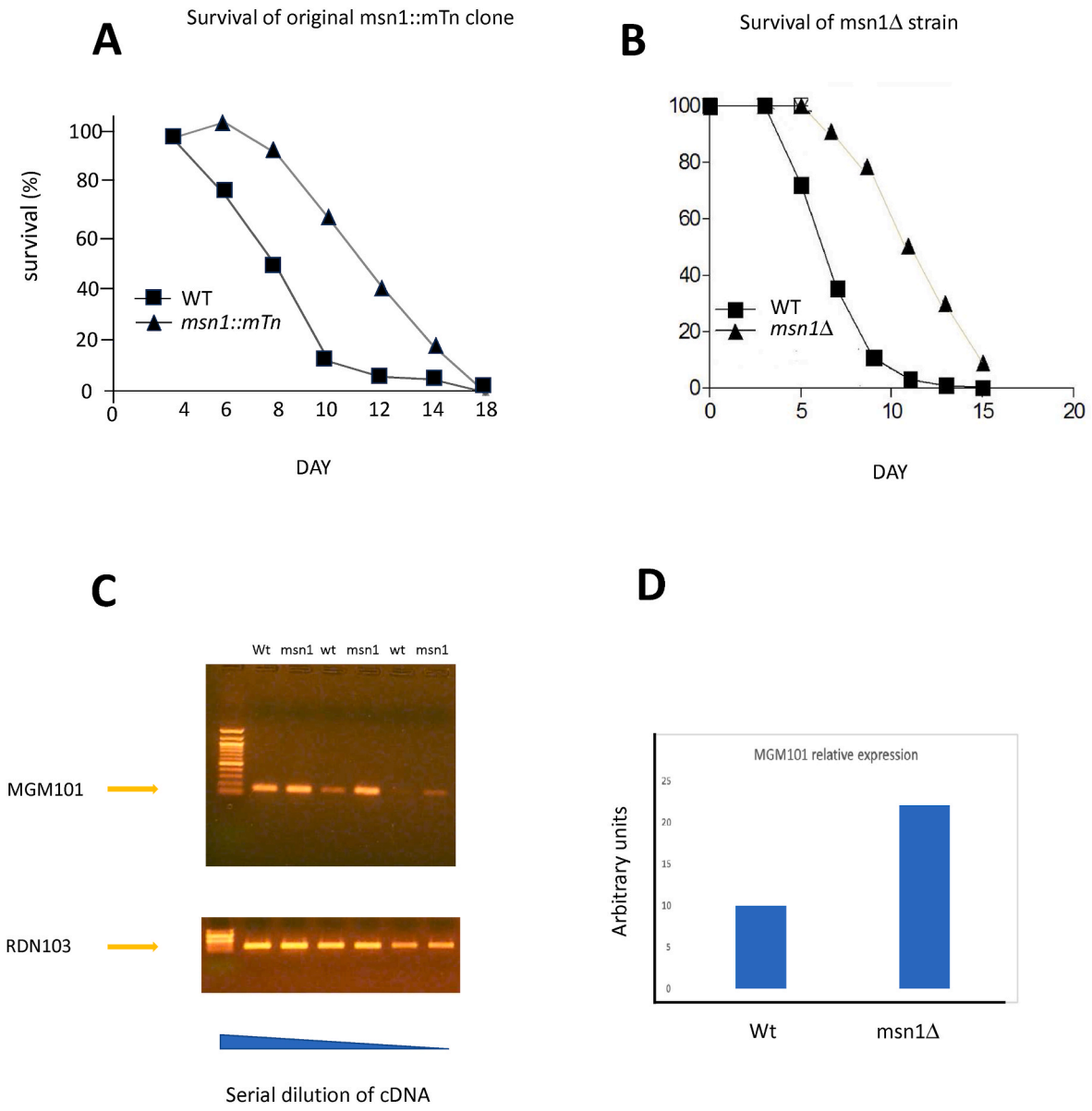

Fig. 2. Characterization of the Msn1p mutant. A) Chronological lifespan of the original mutant with transposon inserted within the *MSN1* gene. B) Chronological lifespan of the strain with the deletion of the gene *MSN1*. C) PCR of the cDNA of the *MGM101* gene (serial dilutions). D) Results confirmed by real time PCR.

2.2. Plasmid and strains

Plasmids and Strains used in the present study are listed in Table 2 Gene Knockouts were generated by one-step gene disruption [23] the list of the oligonucleotides used for gene disruption is reported in Table 3.

2.3. Growth condition

Cells were grown in rich medium YPD (1 % Yeast extract, 2 % Peptone, 2 % Dextrose) or minimal medium SDC (Synthetic Dextrose Complete: 0.17 % yeast nitrogen base, 0.5 % ammonium sulfate, 0.08 % amino acids, pH 6). To maintain selection of plasmids, minimal media were prepared in the absence of specific amino acid or nucleotide (see table S2 for a complete list) containing 2 % glucose as carbon source. Cells were grown at 30 °C.

2.4. Chronological life span assay

The assays were made as previously described [24]. Briefly, small starting cultures were grown onto SDC or onto the appropriate selective media for an overnight and then diluted (1:10) with fresh medium. Glass flasks, covered with aluminum foil caps, were prepared assuring a flask to culture ratio of 5:1. After dilution, flasks were kept at 30 °C with vigorous shaking (200 rpm) to ensure proper aeration. This starting point was considered day 0. Every 48 h, small aliquots from each culture were plated onto YPD plates after proper dilution. After incubation at 30 °C for 2–3 days the YPD plates were recovered, and the Colony Forming Unit (CFUs) counted. Viability was assessed attributing the 100 % survival at the CFUs obtained at day 2.

2.5. Stress resistance assays

Assays performed as previously described [23]. Briefly, cells grown in standard conditions on complete or selective liquid media were serially diluted at the appropriate time points and spotted on rich (YPD), minimal (SDC) or minimal selective plates. Heat shock resistance was assessed incubating at either 55 °C (heat-shocked) or 30 °C (control) for a variable time ranging from 30 min to 120 min. After the heat-shock,

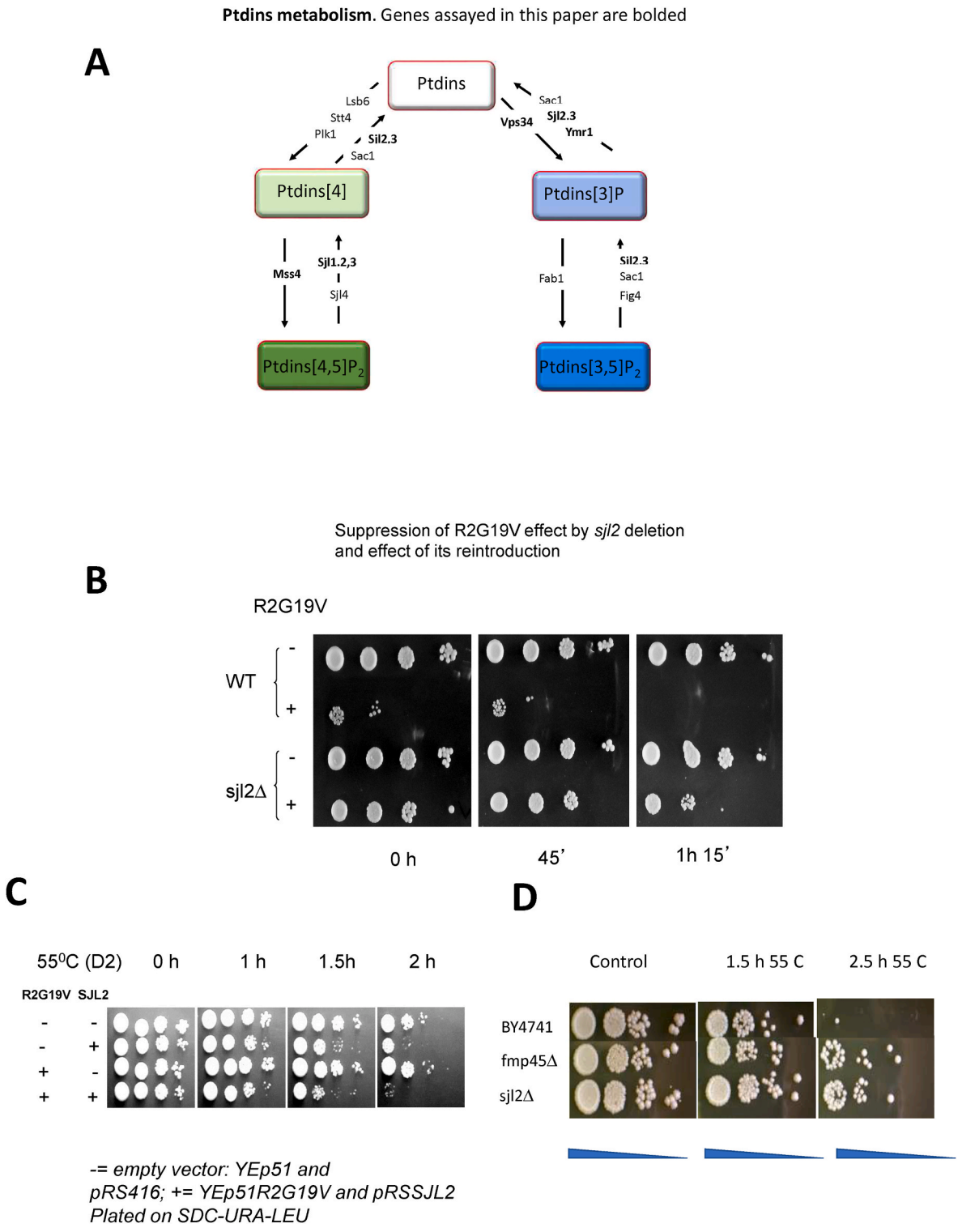

Fig. 3. Sjl2p and Ras2p inhibition. A) A scheme of the PIs metabolism, in bold are indicated the genes assayed in this paper. B) Effect of the introduction of the constitutively active *Ras2G19V* allele (R2G19V) on wild type and on *sjl2Δ* strain after heat shock. Duration of the heat shock is reported. C) Reintroduction of *SJL2* alone or in combination with *RAS2G19V* allele.Effect on heat shock resistance. D) Deletion on a different genetic background of SJL2 and of FMP45 genes. Effect on heat resistance.

plates were incubated at 30 °C for 2–3 days to count the colony forming units.

Oxidative stress resistance was determined as follow. Cells, grown in standard condition, were washed-diluted in K-phosphate buffer 0.1 M, pH 6 at the appropriate time points, and then treated with of hydrogen peroxide (different concentrations) for 30 min at room temperature. Aliquots of untreated and treated cells were then spotted onto rich or selective plates and incubated at 30 °C for 2–3 days.

2.6. Ras2-GTP subcellular localization

Wild type yeast cell or sjl2Δ cells were transformed with the

YEpeGFP-RBD3 plasmid (a kind gift from Sonia Colombo) the encoded eGFP-RBD3 (Green Fluorescent Protein- Ras Binding Domain) probe is specific for Ras-GTP. Ras2-GTP subcellular localization was monitored after 2 and 4 days of growth under standard condition. Cells were viewed with an Olympus BX50 fluorescence microscope using the filter U-MSWB [24].

2.7. RNA extraction and quantitative PCR assay

Total RNA was isolated according to the kit's instructions (RiboPure-Yeast kit Ambion). RNA was treated with RNase-free DNase I (Promega) to remove contamination of genomic DNA. 0.5 μg of total RNA was reverse transcribed into cDNA using ImProm-II Reverse Transcriptase (Promega) with sequence specific primers (ACT1, GAATCCAAAACAA-TACCAGTAG; MGM101, gttggttatgccggcttcgttga). Quantitative PCR experiments were performed on StepOne Real-Time PCR instrument (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems) and the gene specific primers: ACT1, fw-TCGTGCT GTCTTCCCATCTATC and rev-GTAGAAGGTATGATGCCAGATC; MGM101, fw-AGCCCGTGCAGTGAGGAAAAGTT and rev-CGGTGCTGCCGGTTTCCAAA. Thermo-cycling conditions were as follows: 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Relative gene expression was calculated using the $2-\Delta\Delta CT$ method and normalized to ACT1 mRNA levels [24].

3. Results and discussion

To identify genes and pathways capable of attenuating the effects of Ras2G19V protein, we transposon-mutagenized wild type yeast cells carrying the episomal *RAS2G19V* allele. The latter, orthologue of the mammalian oncogenic G12V allele, blocks Ras protein into the constitutively active GTP-bound form. The resulting constitutively active signaling cascade significantly decreases both resistance to multiple stresses and longevity [25] [Fig. 1 A, B]. We thus took advantage of this detectable Ras2G19Vp phenotype to identify mutants capable of surviving 1 h under heat stress at 55 °C.

300 individual thermotolerant mutants were selected and from these, 16 clones were confirmed to be resistant after further phenotype analysis. Transposon insertion on 9 different genes was confirmed to rescue the Ras2G19Vp-dependent detrimental phenotype. Gene identity was determined by DNA sequencing as reported in [Table 1]. The list also reports the number of times each gene has been found during the screening as judged by restriction enzyme analysis of the recovered fragments (data not shown). We then performed Gene ontology analysis to identify role, mechanisms and pathways that could be involved in the phenotype observed.

3.1. Protein synthesis

RPL1a (Ribosomal Protein of the Large Subunit), orthologue of the human gene coding the L10 ribosomal protein, is a constituent of the large ribosomal subunit in eukaryotes. Manipulation of protein synthesis rate has been reported to increase the lifespan in organisms ranging from yeast to mammals [26,27] and genome-wide screen identified the ability of ribosomal protein coding genes to increase the replicative lifespan in yeast when deleted [28]. Interestingly, it was also demonstrated that deletion of ribosomal proteins increases the expression of the *GCN4* (General Control Nonderepressible) transcription factor, and that this overexpression is required for the longevity extension observed. *GCN4* is also upregulated when the concentration of uncharged tRNAs increases and inhibition of cytosolic tRNA synthetase extends yeast lifespan. The same observation has been made in *C. elegans* with a mechanism involving the *GCN4* orthologue atf-4 [28]. Therefore, it is not surprising that interruption of a component of the ribosomal large subunit suppresses the pro-aging effect of *RAS2G19V* allele possibly by involving Gcn4p. It must also be noted that *RPL1* gene duplicated during yeast evolution giving rise to *RPL1a* and b paralogs. Deletion of both genes has detrimental effect [29] but even though they code for identical proteins, ribosomes with the paralog *RPL1b* have a specialized function translating genes involved in cellular respiration at a higher rate than ribosomes carrying the a paralog [30].

3.2. Mitochondrial DNA stability

MSN1 (Multicopy Suppressor of SNF1 mutation) was originally identified as a multicopy suppressor of *SNF1* (Sucrose Non-Fermenting) deficient strains [31]. It is classified as a transcriptional activator of genes involved in nutrient utilization and was also involved in osmotic stress response [32]. The *msn1::mTn*, identified by our screening as capable to suppressing the effect of *RASG19V* on heat shock resistance, also resulted in chronological lifespan extension [Fig. 2a]. A complete deletion of the *MSN1* coding region confirmed the longer survival observed in the original mutant [Fig. 2b]. Regarding the possible mechanism of this suppression, Msn1p is predicted to bind the *FLO11* (FLOcculation) promoter, which in turn is involved in pseudohyphal differentiation in glucose-limiting condition. However, there is a strong similarity between *FLO11* promoter and *MGM101* (Mitochondrial Genome Maintenance) promoter. *MGM101* is instead involved in mitochondrial DNA stability and is required to repair oxidative damaged mitochondrial genome. We thus evaluated gene expression of *MGM101* in wild type and *msn1::mTn* mutant yeast by real time PCR. The results shown in [Fig. 2C and D] indicate increased expression of *MGM101* in mutant cells (msn1) respective to wild type suggesting a role for increased protection of mitochondrial genome in constitutively active Ras mutants.

3.3. Autophagy

PHO23 (PHOsphate metabolism) has been described as a suppressor of autophagosomes with a molecular mechanism involving various *ATG* (AuTophaGy-related) genes but mainly *ATG9*. Cells lacking *PHO23* have an increased number of autophagosomes, as judged by transmission electron microscopy and increased expression of *ATG9* [33]. This result is strengthened by the observation that increased expression of *ATG9*, obtained by other means, results in increased autophagy [30]. It is therefore interesting to speculate that increased autophagy may be involved in the reversal of Ras2G19Vp phenotype. This speculation is supported by the observation that substances like erianin and alisertib inhibit invasion and epithelial-mesenchymal transition of colorectal cancer cells carrying KRASG13D by a mechanism involving increased autophagy [34,35].

3.4. Phosphoinositides signaling

The involvement of the phosphoinositides (PI) signaling pathway [Fig. 3 A] in Ras2G19Vp suppression was suggested by the observation that *sjl2::mTn*, whose wild type allele encodes a component of the PIs metabolism, was identified through our screening for suppressors of Ras2G19Vp stress resistance sensitization and confirmed by a similar phenotype in deletion mutants lacking the complete coding sequence [Fig. 3B]. Reintroduction of *SJL2* on a centromeric plasmid re-established Ras2G19Vp sensitivity further validating the previous observation [Fig. 3C]. We also confirmed the increased stress resistance of *sjl2* deletion mutants in the BY4741 genetic background [Fig. 3D] excluding the possibility of a genetic background-dependent effect. It must be noted that the phosphatase Sjl2 protein is constituted by two different domains each with a specific biochemical function: Sac1-like (Suppressor of ACtin, shared by Sjl3p) and inositol 5 PPase (shared by Sjl1p and Sjl3p). The Sac1-like domain possesses polyphosphoinositide activity which *in vitro* dephosphorylates phosphatidylinositol 4-phosphate (PtdIns(4)P), PtdIns(3)P and phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2) to PtdIns [Fig. 3A] [36]. The 5-PPase domain

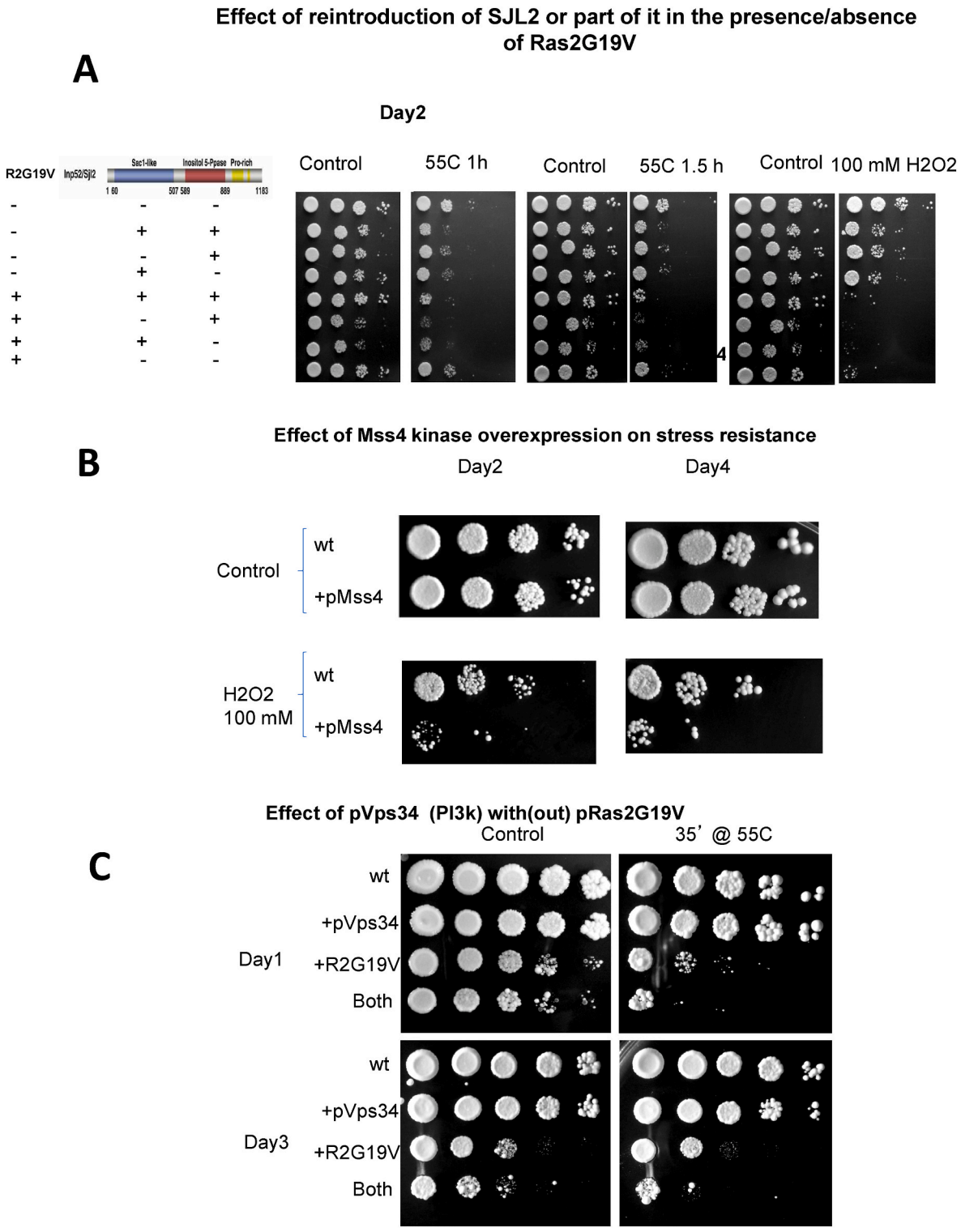

Fig. 4. Effect of PIs manipulation on Ras2p sensitivity. A) Reintroduction of single domain of Sjl2 protein in the presence/absence of *RAS2G19V*. B) Effect of the overexpression of the PI (4)P 5 kinase Mss4p on oxidative stress resistance. C) Effect of the overexpression of the PI kinase Vps34p on heat resistance in the presence/absence of Ras2G19Vp.

hydrolyzes PtdIns(4,5)P2 to form PtdIns(4)P.

To determine if specific PIs were responsible of Ras sensitization, we used different *SJL2* episomal vectors expressing either only the Sac1like domain because the point mutation D850S inactivated the 5Pase domain or only the 5-PPase domain because the C446S mutation inactivated the Sac-1 like domain [37]. The vectors were then introduced in a *sjl2Δ* strain in the presence/absence of the *RAS2G19V* allele [Fig. 4A]. The results showed that reintroduction of the entire Sjl2 protein or of any single domain equally affected yeast cells to Ras2G19Vp-dependent sensitization to stresses [Fig. 4A]. In addition, both the expression of the kinase Mss4p (Multicopy Suppressor of the temperature-sensitive Stt4-1 mutation, [Fig. 4B] which results in increased production of Ptdins 4,5 P2 (see [Fig. 3A] for a scheme) or the overexpression of the kinase Vps34p which increased the production of Pteridins 3P resulted

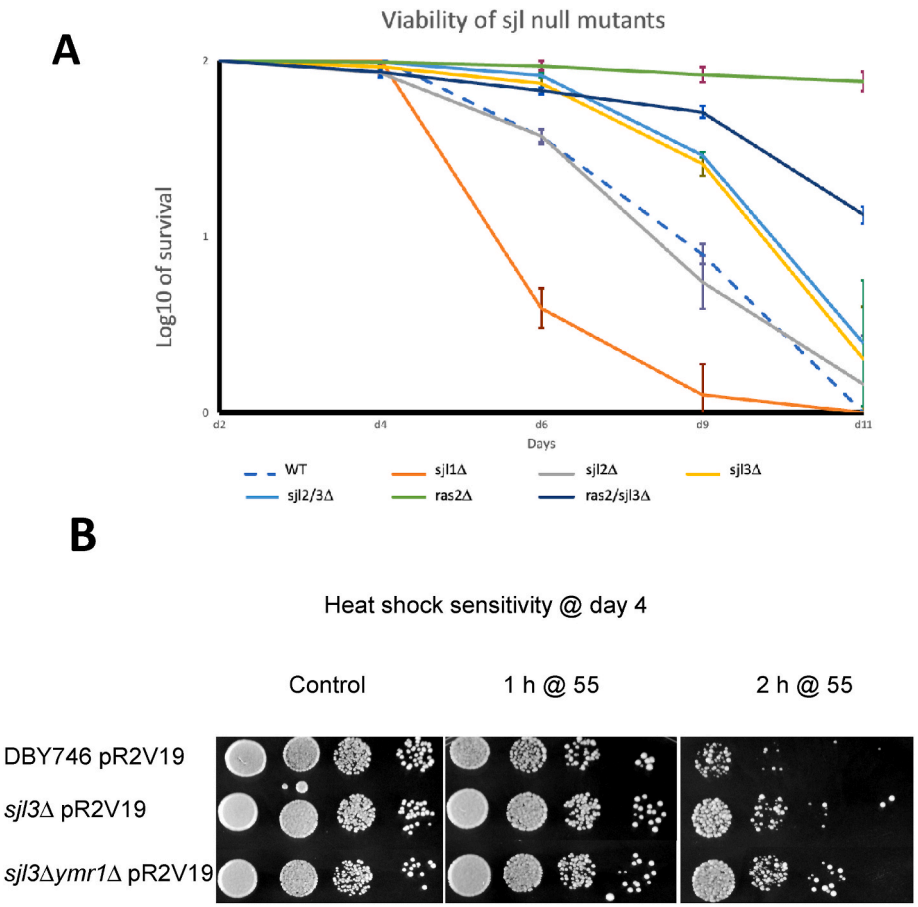

Fig. 5. PIs phosphatases affect longevity and Ras2-GTP sensitivity. A) Chronological lifespan of isogenic strains with deletion on different PI phosphatases coding genes. B) Sensitivity of phosphatase mutants to constitutively active Ras2-GTP.

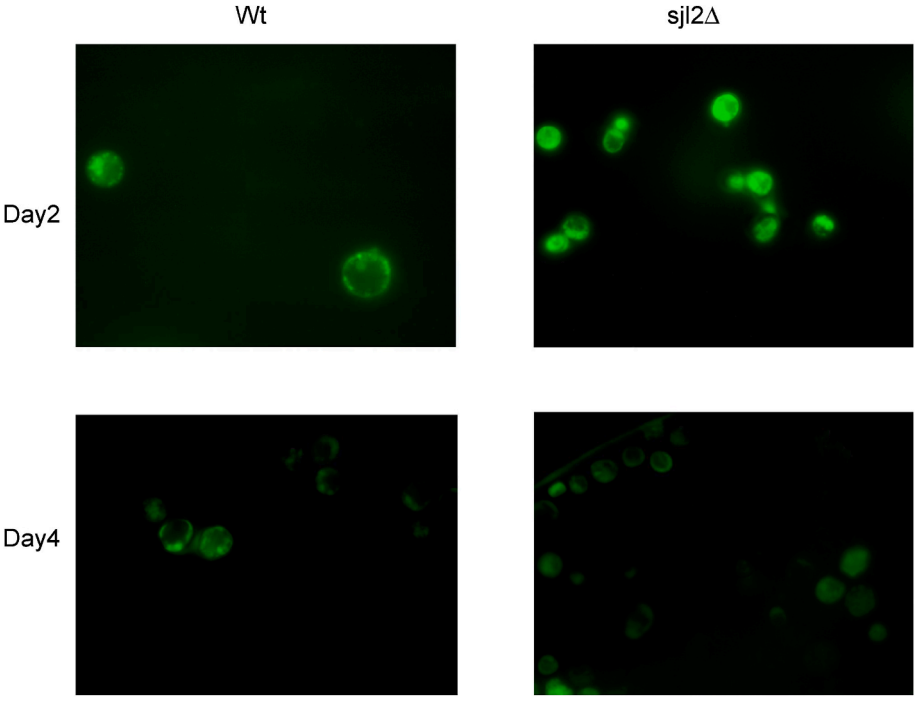

Fig. 6. staining of Ras2-GTP in wild type and sjl2Δ strain.

in increased stress sensitivity [Fig. 4C] suggesting that increased production of phosphorylated products of both branches of PIPs metabolism enhances Ras2G19Vp-dependent sensitivity. Interestingly Vps34p overexpression showed increased stress sensitivity only in the presence of the constitutively active Ras2 protein suggesting a direct interaction between these two pathways [Fig. 4C].

These results are particularly important, because they may link the role of the Ras pathway in accelerating aging in yeast to that of the Daf-2 -PI3K (DAuer Formation) pathway in accelerating aging in worms [38, 39]. On the contrary, single and double deletion of each phosphatase, even though all phosphatases contribute to many steps of PIPs dephosphorylation, differentially affects longevity [Fig. 5A] with *sjl3* deletant reaching maximal longevity. It must also be noted that Sjl proteins have specific localization to the plasma membrane [40], therefore the different phenotype of each *SJL* deletant could be explained by their cellular localization rather than by their biochemical activity. This speculation is reinforced by the observation that the contemporary deletion of the phosphatases *YMR1* and *SJL3*, which is known to affect the asymmetric subcellular localization of PIP3 [41], fully rescued the Ras2G19V-dependent phenotype [Fig. 5B]. It is interesting to note that *fmp45*(Found in Mitochondrial Proteome), and sec*4* (SECretory), isolated in our screening (see [Table 1] and [Fig. 3C]), are predicted to share the same localization of Sjl2p into the endocytic actin patches [42, 43].

We thus examined the possibility that PIs manipulation was affecting the membrane localization of Ras2G19V protein resulting in modulation of Ras-dependent phenotype.

We then performed Ras-GTP imaging taking advantage of a probe made by the fusion of GFP with a trimeric Ras binding domain of Raf (eGFP-RBD3). This fusion protein binds Ras-GTP with higher affinity than Ras-GDP [44,45] allowing subcellular localization of the active Ras protein. Wild type or *sjl2Δ* cells were transformed with eGFP-RBD3 expressing vector and fluorescence was monitored after 2 or 4 days of growth in standard condition. The result in [Fig. 6] shows that at both time points the localization of active Ras to the membrane is blocked by the absence of the Sjl2 protein, suggesting that the suppression of the stress sensitization and lifespan decrease caused by constitutive active Ras by mutations in Sjl2p, may be achieved by preventing Ras membrane localization [Fig. 6].

One of the most common suppressor mutations identified in the transposon mutagenesis screening is in the unknown gene YDL085A, which we named *RAR1*, for Ras Activity Regulator 1.

4. Conclusion

This study confirmed the previously identified role of ribosomal proteins, mitochondria integrity and autophagy in stress resistance and longevity but also proposes that these pathways can mediate RAS activity. In fact, even though further studies are needed to confirm our observations, we found that several proteins of these pathways and especially the enzymes involved in PIs phosphorylation and dephosphorylation can play important roles in aging but also in the activity of oncoproteins, thus serving as potential longevity and cancer drug targets.

These observations are of particular interest since they suggest an interconnection between the yeast Ras pro-aging pathway and the worm and mammalian PI3K pro-aging pathway and raise the possibility that the well-established role of PI3K in aging in multiple organisms may be associated, at least in part, with its effect on Ras activity. Since the deletion and overexpression of PIs phosphatases and kinases are predicted to increase the concentration of different PI species in both branches of PIs metabolism, the suppression of the Ras2G19V phenotype observed may be the result of altered phospholipid metabolism and not of an increased/decreased concentration of a specific phospholipid moiety.

Acknowledgements

This study was funded in part by Associazione Italiana per la RIcerca sul Cancro (AIRC IG# 17605 and 21920 to VDL).

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