Lithium Chloride Sensitivity Connects the Activity of *PEX11* and *RIM20* to *PGM2* translation

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Abstract

Lithium chloride (LiCl) is a widely used and extensively researched drug for the treatment of bipolar disorder (BD). As a result, LiCl has been the subject of research studying its toxicity, mode of action, and downstream cellular responses. LiCl has been shown to influence cell signalling and signalling transduction pathways through protein kinase C and glycogen synthase kinase-3 in mammalian cells. LiCl's significant downstream effects on the translational pathway necessitate further investigation. In yeast, LiCl is found to lower the activity and alter the expression of *PGM*2, a gene encoding a sugar-metabolism phosphoglucomutase. When phosphoglucomutase activity is reduced in the presence of galactose, intermediates of galactose metabolism aggregate, causing cell sensitivity to LiCl. In this study, we identified that deleting the genes *PEX11* and *RIM20* increases yeast LiCl sensitivity. We further show that *PEX11* and *RIM20* regulate the expression of *PGM2* mRNA at the translation level. The observed alteration of translation seems to target the structured 5'-untranslated region (5'-UTR) of the *PGM2* mRNA.

1. Introduction:

Bipolar disorder (BD) has been linked to signaling transduction dysregulation [1]. Over the past several decades, owing to demonstrated therapeutic potential, treatment with lithium chloride (LiCl) has proven to be a successful treatment for BD patients [2]. LiCl has since been used to combat other psychoses, and its potential effectiveness against neurodegenerative diseases [3, 4] is notable. Signal transduction mechanisms of glycogen synthase kinase-3 and protein kinase C, which are implicated in neuronal plasticity and progression, were shown to be influenced by LiCl [5, 6]. BD patients who received lithium therapy were reported to have altered expression of protein kinase C and phosphoinositide enzymes [7, 8]. Furthermore, LiCl exhibits anti-inflammatory, protein homeostasis, cytoprotective, and synaptic repair properties, culminating in LiCl as a potential multi-directed treatment, applicable in relieving a wide range of conditions, including Alzheimer's [2, 3]. Multiple downstream targets, such as brain-derived neurotrophic factor and β-catenin, have been shown to interact with lithium [9, 10]. Although LiCl has been a standard treatment for decades, further research is needed to reveal its possible effect on established and unknown gene pathways in the human body.

Previous research has established the budding yeast, *Saccharomyces cerevisiae* cell sensitivity to LiCl, primarily when galactose is used as the primary carbon source. LiCl sensitivity in yeast was observed to be induced by a difference in *PGM2* expression and activity. *PGM2*, a phosphoglucomutase, facilitates the entry of galactose into the glycolysis pathway. *PGM2* enzyme catalyzes the transfer of glucose-1-phosphate to glucose 6-phosphate, which, if impaired, triggers intermediate metabolite aggregation and toxicity in yeast cells [11]. Consequently, when yeast cells are grown on galactose media containing LiCl, cell growth is severely hampered due to metabolite aggregation and glycolysis impairment. Subsequently, uridine diphosphate glucose levels are significantly hindered in yeast cells during LiCl treatment, further inhibiting glucose-related pathways (Masuda et al., 2001). Prior research indicates that LiCl can inhibit enzymes involved in the processing of rRNA, ribosomal protein precursors, and the amount of mature mRNA in the cytoplasm, implying inhibition at the translational level.

Eukaryotic initiation factors (eIFs) are heavily involved in the regulation of translation initiation [13]. The initiation factor *eIF4A* (*TIF2*) is a prototypical DEAD-box RNA helicase that functions together with *eIF4B, eIF4H* and *eIF4F* to untangle and prepare the ribosome binding mRNA template by unwinding the secondary structure proximal to 5' untranslated region (UTR). It is reported that *eIF4A* is involved in LiCl stress in yeast [14]. Overexpression of eIF4A is shown to recover sensitivity of the yeast cells to LiCl in galactose media. Similar observation has also been reported for other genes linked to the translation of structured mRNAs in yeast [15, 16]. Deletion mutants for these genes showed increased sensitivity to LiCl.

In the current study, we report that deletion of *PEX11 and RIM20* increase cell sensitivity to LiCl when the gene mutant strains are grown in galactose. The primary reported function of *PEX11* is to mediate fatty acid oxidation and induce peroxisome proliferation[17]. *RIM20* is shown to be actively involved in pH response and multivesicular body formation[18, 19]. Our subsequent genetic studies indicate that these genes are involved in regulating the translation of *PGM2* mRNA.

2. Materials And Methods:

2.1 Strains and Plasmids

The MAT “α” mating strain, Y7092 can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu21Δ0 ura3Δ0 met15Δ0 and MAT “a” mating strain, Y7092 Y4741 orfΔ::KanMAX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0are utilized in this study. To construct double deletion mutant strains, non-essential gene deletion mutant strains were collected from the Yeast knockout library [20] and used for Synthetic Genetic Analysis (SGA). SGA gene knockouts in MATα mating strains were accomplished using PCR and homologous recombination after the Lithium acetate process, as followed by previous PCR studies [21, 22]. The yeast overexpression plasmid library was used to generate overexpression plasmids for the gene candidates [23]. Thermofisher® Yeast gene-GFP fusion library is purchased and used to generate *PGM2-GFP* fusion strain for qRT-PCR and western blot analysis as shown before [24–26]. Chemical sensitivity and PCR analyses are used to validate the overexpression plasmid. A modified SGA approach coupled with random spore analysis is used to establish deleted genes reinserted into their respective deletion mutant strains [27, 28]. Crossing MATα with nourseothricin sulphate (clonNAT) and MATa with a G418 resistance gene in lieu of the gene deletion of interest results in modified SGA. For MATa haploid progeny selection, spores germinated on selective media containing thialysine and canavanine after sporulation. G418 sensitivity was examined using replication plating after the cells were transferred to clonNAT media. PCR analysis was conducted to validate the target gene inside the chromosome in colonies immune to clonNAT but sensitive to G418. The p416 plasmid carried a non-complex construct, used as the control plasmid and carried a LacZ expression cassette under gal promoter control. HIV1 (TAR), RTN4IP1 (RTN), BCL-2 (Bcell) and 2-hair hairpin constructs were designed and cloned into the p416 expression vector upstream of LacZ mRNA using XbaI restriction site between the *β-galactosidase* reporter gene (LacZ expression cassette) and gal promoter.

Expression constructs for pTAR construct contains the 5’ UTR of HIV1-TAR gene (5’ GGTTCTCTGGTTAGCCAGATCTGAGCCCGGGAGCTCTCTGGCTAGCTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTGTGTGCC 3’) and pRTN contains 5’ UTR of FOAP-11 gene(5’GGGATTTTTACATCGTCTTGGTAAAGGCGTGTGACCCATAGGTTTTTTAGATCAAACACGTCTTTACAAAGGTGATCTAAGTATCTC3’), pBCell contains 5'UTR of BCL-2 gene (5' GGGGGCCGUGGGGUGGGAGCUGGGGGGGCCGUGGGGU GGGAGCUGGG 3') and 2-hair has 5’ UTR of construct (5' CTTGGTAAAGGGGGUGGTCTGAGCCCGGGAGCTCTCTGCTGCTTAAGCCTCGGATTTT 3’). Plasmids included an ampicillin resistance gene for selection in DH5α (in E.Coli) and a URA3 gene selection in yeast.

2.2 Media Inoculation

Yeast cultures were grown with either YP media (1% Yeast extract and 2% Peptone) or Synthetic Complete media with selective amino acids (0.67% Yeast nitrogen base w/o amino acids and 0.2% amino acid dropout mixture). The carbon source in yeast media was either 2% glucose or 2% galactose. Agar at 2% was used to make solid media. LB media (Lysogeny Broth) was used for E.Coli cultures. GeneJET plasmid miniprep kit (Thermofisher®, Ottawa, ON, Canada and Bio-Basics®, Toronto, ON, Canada) was used for E. coli plasmid extraction, and yeast plasmid miniprep kit (Omega Biotek®, Norcross, GA, USA) was used for yeast plasmid extraction; all kits were used as directed by the manufacturer.

2.3 LiCl sensitivity analysis

Specific colonies of yeast are cultivated for two days at 30^{o}C in liquid YPgal (YP + 2% galactose) media before growth saturation. Liquid cultures are standardized to an OD 0.1 at 600nm after incubation, serially diluted (10^{-1} to 10^{-4}) and spot platted (10ul per spot) onto agar YPgal media with 10 mM LiCl treatment. Sensitivity to LiCl could be assessed by comparing gene deletion strains with wild type (WT). Overexpression constructs p*PEX11* and p*RIM20* are transformed into our gene deletion strains to validate observed sensitivities due to the deletion of our candidate genes. For quantification analysis, colony counting was performed using 100 µL of diluted 10^{-4} concentration cell cultures then spread onto YPgal plates with and without 10 mM LiCl. The colonies were counted after a 48h incubation at 30°C. Each experiment was conducted in triplicates. One way ANOVA analysis (P-value ≤ 0.05) was used to determine significant statistical differences.

2.4 mRNA quantification analysis

PGM2-GFP yeast strain grown in liquid YPgal media with 10 mM LiCl treatment was used to determine *PGM2* mRNA concentrations in different deletion strains. Max RNA is collected using the Qiagen® RNeasy Mini Pack. iScript The Bio-Rad® cDNA Synthesis Kit (Mississauga, ON, Canada) was used to create complementary DNA and Bio-Rad® iQ SYBR Green Supermix along with CFX connect real-time system (Bio-Rad®) are used to perform qRT-PCR. The housekeeping gene *PGK1* is used as an internal control. Experimentation and data processing is carried out in compliance with MIQE guidelines [29]. Experiments are replicated with three technical and biological replicates. One way ANOVA analysis (P-value ≤ 0.05) was used to determine significant statistical differences. Primers *PGK1* Forward: ATGTCTTTATCTTCAAAGTT; Reverse: TTATTTCTTTTCGGATAAGA; *PGM2* Forward: GGTGACT CCGTCGCAATTAT; R: CGTCGAACAAAGCACAGAAA

2.5 PGM2 Protein content measurement

Quantitative western blotting is used to determine the protein content of the PGM2p-GFP fusion protein. Mutant strains were cultured in liquid YPgal media with 10 mM LiCl. The protein extraction process is carried out as mentioned previously [30]. Bradford Protein Assay (BSA) was used to determine the protein concentration. 50ug of total extracted protein were loaded onto a 10% SDS-PAGE gel and ran through the Mini-PROTEAN Tetra cell electrophoresis apparatus method (Bio-Rad®). Trans-Blot Semi-Dry Transfer (Bio-Rad®) was used to transfer protein through a 0.45 µm nitrocellulose membrane. PGM2p-GFP protein levels are detected using a mouse monoclonal anti-GFP antibody (Santa Cruz®), and *PGK1* protein levels are measured using a mouse monoclonal anti-PGK1 antibody (internal control). Immunoblot visualization and densitometry research are carried out using chemiluminescent substrates (Bio-Rad®), the Vilber Lourmat gel doc Fusion FX5-XT (Vilber®), and the FUSION FX app (Vilber®). Experiments are replicated with three technical and biological replicates. One way ANOVA analysis (P-value ≤ 0.05) was used to determine significant statistical differences.

2.6 Quantitative β-galactosidase Assay

Translation initiation activity of our gene deletion strains is investigated using, LacZ reporter constructs (as previously described). LacZ activity was assessed using the assay as a quantitative tool to quantify translational activity in mutant yeast strains. The assay was carried out using O-nitrophenyl—d-galactopyranoside [31, 32]. Experiments are repeated three times with technological and biological replicates. Meaningful variations are evaluated using a one-way ANOVA study (P-value 0.05).

2.7 Genetic Interaction Analysis

Genetic interactions analysis for our candidate genes (GI) were investigated using a 384 formatted SGA. The yeast double deletion mutant library is generated as previously shown [24, 25, 28]. A query strain (MATα, Y7092) carrying a gene deletion of interest and a clonNAT marker is mated with our three non-essential gene deletion sets, comprising ~1000 MATa mutants. Following rounds of selection, haploid double gene deletion mutants are selected. Successful gene knockout transformations were identified using PCR analysis with pAG25 plasmid as the template. The clonNAT resistance gene marker was amplified, with NAT and Kanamycin used as selection markers. Conditional SGA is carried out by growing the generated double mutants in a chemical stress condition [33] on sub-inhibitory, 3 mM LiCl agar media. This facilitates the investigation of genes that only express under LiCl stress conditions [25, 34]. Phenotypic Suppression Array (PSA) is performed by mating an empty plasmid, MATa single deletion library, and an overexpression plasmid of a query gene carried by MATα yeast strain, as mentioned previously for the SGA mating protocol. Strains with and without the overexpression plasmid are compared and grown in media with LiCl treatment. Overexpression of query genes was used to examine whether there is some phenotypic shift with inhibitory LiCl treatment. A proposed functional relation between these two genes could then be established [22, 33].

2.8 Genetic Interaction data Analysis

The size of the colony determines cell fitness [28, 35]. Colony size comparisons between the control (WT) and single deletion mutants are performed to determine if any phenotypic changes (synthetic illness or synthetic lethality) exist. Normalized colony sizes were then compared. The fitness threshold for yeast cells is set to a minimum of 30% size reduction in contrast to the control. SGA Software was used to measure colony sizes and similarities [36]. Only size reductions of 50–95% are taken into consideration. This experiment was repeated three times, each time with a separate technical replication. Only hits of at least two replicates are taken into consideration. Finally, identified hits are then classified according to their biological processes and molecular function and then enriched with Gene ontology terminology using tools including the Genemania database. http://genemania.org (accessed on January 18, 2021) and complex functionality profiling http://webclu.bio.wzw.tum.de/profcom/start.php (Referenced on January 23, 2021).

3. Results And Discussion:

3.1 Yeast tolerance to Lithium Chloride is eroded by gene deletions of PEX11 and RIM20

Chemical-genetic approaches have the ability to allow the detailed annotation of the functional properties of various chemicals and bioactive compounds. They can shed light on a compound's primary mode of action as well as its secondary sites of interactions within a cell [24]. The sensitivity of mutant strains to a target compound is a powerful approach for determining the target pathways and the impact a compound has on a cell at the molecular level. In the current study, while investigating yeast gene deletion mutants that are sensitive to LiCl, we discovered two deletion mutants for *PEX11 and RIM20*, that exhibited increased sensitivity to LiCl (Figure 1A, B) compared to a control strain (Figure 1A). Using a spot test analysis, we demonstrate that when cells are grown in media containing galactose as the primary carbon source, deletion of *PEX11 and RIM20* resulted in a substantial reduction in growth in the presence of 10 mM LiCl, indicating increased sensitivity. Deletion of *TIF2* (*eIF4A*) was used as a positive control. Further we demonstrated that incorporating the deleted genes back into the corresponding gene deletion mutants reversed the observed growth reduction establishing a correlation between the observed phenotypes and the deleted gene. Colony count measurement analysis further validated these results by offering a quantitative viewpoint (Figure 1B). The formation of colonies observed in the presence of LiCl is compared and normalized to the number of colonies observed in the control strain. Lower number of colonies formed for gene deletion mutants indicates increased sensitivity to LiCl compared to the control strain. As shown in Figure 1B, deletion of *TIF2, PEX11* and *RIM20* under the influence of LiCl results in a significant reduction in colony formation. As before reintroduction of the deleted genes back into the corresponding mutant cells increased the number of colonies formed to levels comparable to that of the control strain. Additionally, we examined yeast strains' exposure to LiCl when glucose was used as the carbon source. As expected, no increased sensitivity for the mutant strains was observed when glucose was used (S1. Figure).

Previously, it was stated that overexpression of *TIF2* reversed the toxicity of susceptible strains to LiCl. We inserted overexpression plasmids for our candidate genes into corresponding deletion strains spotted on media containing 10 mM LiCl to see whether they would similarly restore LiCl sensitivity. When the plasmids were integrated into the mutant strains, fitness was restored, suggesting that they may function similarly to *eIF4A* in the cell.

When galactose is used as the carbon source, LiCl inhibits *PGM2* expression, resulting in the accumulation of galactose intermediate metabolites that are toxic to yeast cells [11]. *GAL1* is the galactokinase in yeast that phosphorylates -D-galactose to -D-galactose-1-phosphate in the initial stages of galactose metabolism. By converting glucose-1-phosphate to glucose-6-phosphate, *PGM2* promotes galactose entry into glycolysis. We investigated the impact of *PEX11 and RIM20* on LiCl toxicity when galactose metabolism was disabled. For this purpose, we developed double gene deletions for *PEX11* and *RIM20* with the *GAL1* gene. Not surprisingly, we observed *GAL1* double mutant cells were no longer hypersensitive to LiCl treatment. *PEX11* and *RIM20* have not been previously linked to LiCl sensitivity or the underlying molecular mechanisms associated with it, rendering them intriguing gene candidates to investigate.

3.2 PGM2 Expression is Controlled at the Translational Level by PEX11 and RIM20.

PGM2 plays a key role in the sensitivity of yeast cells to LiCl[12], we investigated whether *PEX11 and RIM20* will influence *PGM2* expression. For this PGM2p was GFP-tagged, and western blot analysis was performed using anti-GFP antibodies to quantify protein content (Figure 2A). The deletion of *PEX11 and RIM20* had no substantial effect on PGM2p protein levels in the absence of LiCl treatment. Interestingly, when cells were treated with LiCl, *PEX11* and *RIM20* gene deletion resulted in a significant reduction of PGM2p protein levels compared to the WT. To analyze the effect of *PEX11* and *RIM20* gene deletion on *PGM2* transcription, the mRNA content of *PGM2* was measured using qRT-PCR. Figure 2B indicates that deletion of *PEX11 and RIM20* had similar levels of *PGM2* mRNA levels as the WT in control media. However, *PGM2* mRNA content increased in cells treated with LiCl, but there was no statistically relevant difference between mutant strains and WT. Thus, the deletion of *PEX11* and *RIM20* seems to have little influence on *PGM2* mRNA content. As a result, it appears that *PEX11 and RIM20* influence PGM2p at the level of protein synthesis. These findings are similar to the observations by Hajikarimlou et al., where in the presence of LiCl, deletion of *YTA6 and YPR096C* inhibited *PGM2* expression at the translational level [16].

3.3 Deletion of PEX11 and RIM20 Influences Translation of β-Galactosidase Reporter mRNAs with a Hairpin Structure, but not Those Without

Several genes that affected *PGM2* expression at the translation level, used the 5′-UTR of *PGM2* mRNA to exert their activities. The 5′-UTR of *PGM2* mRNA is predicted to have a structured region (S2. Figure) and in the absence of TIF2, a protein that unwinds mRNA structures during translation, *PGM2* expression is reduced [14]. Next we intended to investigate whether *PEX11* and *RIM20* affect translation using the structured 5′-UTR of *PGM2* mRNA. For this experiment, we utilized pPGM2 plasmid where the *PGM2* 5′-UTR is placed in front of a *LacZ* expression cassette in the p416 expression plasmid. The pPGM2 plasmid, as well as the parental p416 plasmid lacking a structural region in front of the *LacZ* gene were transformed into the deletion mutant strains for our candidate genes as well as the WT strain. *β -galactosidase* activity was quantified as a measure of translation (Figure 3A). No significant difference was observed for *β*

-galactosidase activity derived from mRNAs lacking a 5'-UTR structure (p416) in different strains. *β -galactosidase* activity was significantly reduced however in *pex11*Δ and *rim20*Δ when the mRNA carried *PGM2* 5'-UTR suggesting a connection between the activity of *PEX11* and *RIM20*, and the translation of structured mRNAs.

Next, we examined the effect of *PEX11* and *RIM20* on additional structured mRNAs. For this we utilized four additional constructs each carrying different structures at their 5'-UTR. pTAR carries a structure derived from HIV1 mRNA containing a ΔG= -57.9kcal/mol and pRTN carries the structured 5'-UTR of RTN4IP1 mRNA with ΔG= -29.8kcal/mol. Similarly, pBcell carries a structure derived from BCL-2 mRNA and a ΔG= -20kcal/mol. The last construct, p2hair contains a synthetic structure designed to have a high degree of complexity ΔG= -33kcal/mol. In our analysis. we observed that deletion of *PEX11* and *RIM20* significantly reduced the expression of all four highly structured mRNAs, comparing the WT suggesting a general role for these two genes in the translation of structured mRNAs (Figure 4).

3.4 Genetic Interaction Analysis Further Connects the Activity of PEX11 and RIM20 to Protein Biosynthesis

Genetic interaction (GI) analysis is based on the principle that parallel pathways allow for flexibility and tolerance to random harmful mutations, preserving cells viability and maintaining cell homeostasis [16]. A gene in one pathway might compensate for a gene in another, allowing the cell to survive. Consequently, when two genes in parallel pathways are deleted, cell fitness can be unexpectedly reduced (sickness) or even the cell dies (lethality). Therefore, when deletion of two genes result in a phenotype that is unexpected from the phenotypes of individual gene deletions, it is commonly said that the two genes are forming a genetic interaction. Due to the reduced fitness of double mutant this type of interaction is called negative genetic interaction (nGI). Many studies use nGIs to investigate gene function and pathway interactions [15, 16, 29, 37].

The high throughput analysis of GIs in yeast is performed by mating the two yeast mating types: α-mating type (Mat α) and a-mating type (Mat a). Mat "α" carries the target gene deletion and is crossed with an array of single gene deletions of Mat "a," mating type, to produce double gene deletions[28]. Colony size is used to measure the fitness of the strains[35, 38]. We utilised this method to investigate genetic connections between our query genes, *PEX11* and *RIM20*, with approximately 1000 additional genes that includes approximately 700 genes associated with gene expression pathway and a random collection of 300 genes used as a control. (S1. Table).

In our analysis, we discovered numerous intriguing nGIs and several common gene hits between *PEX11* and *RIM20.* Functional enrichment analysis of the hits revealed that a significant number of them are connected to relevant biological processes that includes translation regulation, ribosome biology,, mRNA catabolic processes, and protein synthesis and protein synthesis associated factors (Figure 5). For *PEX11*, we identified nGIs including *DPH5, PCI8, EAP1*, and *TIF1* among others. The methyltransferase, *DPH5* is engaged in the diphthamide biosynthesis pathway, responsible for assembly of translation elongation factor 2 (Eft1p or Eft2p[39]., *PCI8* forms the subunit of translation initiation factor, eIF3b, contributing to the formation of 43S pre-initiation complex during translation initiation. It is a key factor in cellular signalling at all stages of protein synthesis, including elongation, termination, and ribosome recycling[40]. *EAP1*, is also involved in translation initiation process. It is an associated protein of translation initiation factor eIF4E, which forms a part of eIF4F complex. It could potentially enhance/inhibit the overall translation rate by facilitating mRNA degradation and promoting mRNA decapping[41].

Similarly, *RIM20* interacted with several translation machinery associated genes including *RPS23A, TOR1*, and *TMA19* (Figure 5). *RPS23A* codes for the ribosomal protein 28 (rp28) and forms a component of the 40S small ribosomal subunit[42]. Translation regulator protein *TOR1* is a member of the TOR complex, which is known to regulate global translation rate through signal transduction. Under stress environments, the TOR-related proteins *TOR1* and *TOR2* control translation initiation and promote early G1 progression in yeast[43]. *TMA19* codes for Translation Machinery Associated protein 19 that interacts with ribosomes during translation initiation. [44].

We also observed several common interactors between *PEX11* and *RIM20* including *SLH1*, and *CAF20. SLH1* is a putative RNA helicase that is directly implicated in the translation inhibition of non-poly(A) mRNAs. [45]. *CAF20* is a translational control phosphoprotein found in the mRNA cap-binding complex. It acts as a regulator of cap-dependent translational initiation [46].

Conditional nGIs are those interactions that are formed under a specific condition that includes the presence of a sub-inhibitory concentration of a bioactive compound, cold shock, heat shock, minimum media etc. They represent gene functional associations that are formed in response to a certain environment[30, 47]. For example, in the presence of DNA damage, the functions of certain genes may be altered, and it is the altered functions that are functionally related and hence form the basis of GIs in the presence of DNA damage[48]. We investigated nGIs for *PEX11* and *RIM20* using a mild sub-inhibitory concentration of LiCl (3 mM). The observed GIs in this case are those that are formed as a response to the presence of LiCl. As seen in Figure 6, we found new nGIs for our candidate genes *PEX11* and *RIM20*. *PEX11* interacted with several translation regulating genes including *PBP1*, and *EBS1* among others. *PBP1* is a component of glucose-deprived induced stress granulates that influences global translation rate through the TORC1 signalling pathway and autophagy. It is also known to have an active role in controlling mRNA polyadenylation by interacting with Pab1P, which influences initiation of translation during protein biosynthesis[49]. *EBS1* codes for a protein involved in translation inhibition by interacting with cap binding proteins *CDC33* and *NAM7* upon glucose starvation. It also regulates translation initiation factor eIF4E, another cap binding protein that binds to 5' end of the mRNA during the formation of 48S initiation complex in translation initiation step[50]. Interestingly, eIF4E is a component of the eIF4F complex, that comprises of eIF4A, eIF4G and eIF4E[51]. eIF4G is a scaffolding protein and eIF4A is a DEAD-box helicase protein that is actively involved in unwinding structured mRNAs during translation initiation. PEX11 interacting with EBS1 may indirectly influence helicase activity of eIF4A and potentially affect other helicases influencing global translation rate.

Similarly, *RIM20* interacted with several news genes involved in translation control and regulation of translation. *ETT1* is a nuclear protein that regulates overall translation rate and enhances/inhibits protein synthesis by influencing initiation and termination step during translation[52]. Under extreme stress conditions, *PTP2* modulates phosphorylation in the MAPK signalling pathway, affecting mRNA decapping and mRNA stability influencing overall translation rate[53]. *RPS19B* is a protein component of the 40s ribosomal subunit that participates in the assembly of 43s pre-initiation complex by binding matured mRNA to small ribosomal subunit with translation initiation factors eIF1A and eIF3 during protein synthesis[54].

Notably, we also observed several common interactors between *PEX11* and *RIM20* including *DHH1, TIF2*, and *SHE3* that are involved in translation control. *DHH1* is a cytoplasmic DEAD-box helicase that regulates translation process influencing mRNA stability, mRNA degradation and polyadenylation at 5' end of mRNA[55]. As explained above, translation initiation factor, *TIF2* is another DEAD box RNA helicase that facilitates the binding and unwinding of mRNA during translation initiation[51]. *SHE3* is a RNA binding protein that constitutes a part of the mRNA localization machinery and plays an active role in recruiting Myo4p-She3 complex that is involved in mRNA export / localization into the cytoplasm, influencing global translation rate[56].

Phenotypic suppression array (PSA) analysis focuses on another kind of interaction in which overexpression of one gene compensates for the absence of another [24, 33, 57, 58]. This is an important type of interaction as it can highlight certain functional overlaps between two genes. To this end we exposed the gene expression mutant arrays to 10 mM LiCl. Some of the investigated strains showed increased sensitivity. Then, by introducing *PEX11* and *RIM20* overexpression plasmids, we attempted to compensate for the observed sensitive phenotypes. Interestingly, the overexpression of either *PEX11* or *RIM20* compensated for the sensitivity of two gene deletions for *RPS30A* and *SLF1* (Figure 6A). *RPS30A* encodes a cytosolic small ribosomal subunit protein that can also bind to mRNAs and contributes to the selection of the correct tRNA molecules[59]. *SLF1* is an RNA binding protein that regulates mRNA translation, especially under stress conditions. [60].

The recovery of the sick phenotype in gene deletions for *SLF1, RPS30A* by introducing *RIM20* and *PEX11* overexpression plasmid was confirmed using spot test (Figure 6B). This was further verified by cell quantification analysis using colony count (Figure 6C). Using spot test and colony count analysis we verified that the overexpression of *RIM20* and *PEX11* compensated the sensitivity to LiCl that was seen for deletion strain for *RPS30A* and *SLF1*. Both *SLF1* and *RPS30A* have reported genetic interactions with *TIF2*. Consequently, we also included the deletion strain for *TIF2* in the spot test for analysing LiCl sensitivity compensation. Interestingly, when *PEX11* and *RIM20* are overexpressed in *TIF2* deletion, LiCl sensitivity caused by *TIF2* deletion was also recovered further connecting the activity of these genes.

4. Concluding Remarks:

When used as a therapeutic molecule, LiCl has a broad impact on the expression of various genes and pathways among other activities. Its precise mechanism of action however remains unclear. Here, we examined yeast gene deletion mutant strains for LiCl sensitivity and discovered that when two genes, *PEX11* and *RIM20*, were deleted, the corresponding deletion mutant strains were more sensitive to LiCl. *TIF2* overexpression, which codes for eIF4A, the helicase enzyme involved in translation initiation, has been shown to restore LiCl sensitivity in yeast cells cultured on galactose media[14]. We determined that overexpression of our candidate genes causes a phenotypic recovery similar to *TIF2*, suggesting that these genes may have a similar/related function. In the literature the molecular activities for *PEX11* and *RIM20* have not been linked to LiCl sensitivity making these genes intriguing target for further study. LiCl toxicity in yeast is thought to be caused by the accumulation of galactose intermediate metabolites, particularly galactose-1-p[14, 15]. It has been shown that LiCl inhibits the conversion of glucose-1-phosphate to glucose-6-phosphate via modifying the expression of the phosphoglucomutase enzyme *PGM2*[11]. The current study shows that *PEX11* and *RIM20* influence *PGM2* expression. We demonstrate that *PEX11* and *RIM20* regulate the translation of *PGM2* mRNA via its 5'-UTR region. Similar observations were made using other mRNAs with various 5'-UTRs that carry different structures, but not for the ones without. We recently reported on the finding of several additional genes involved in the translation of structured mRNAs, [15, 16]. The identification of new gene functions that influences the translation of structured mRNAs suggests that translation of mRNAs with structures seems to be more complex than what was originally thought. Furthermore, it shows that the structured mRNAs might be subjected to a more complicated regulatory network during translation than previously hypothesized.

The impact of *PEX11* and *RIM20* on structured mRNA translation may be explained in a variety of ways. The simplest explanation is that one or more of these components may have mRNA helicase activity, which may directly contribute to the unwinding of the structured mRNAs at 5'UTR. In the absence of recognizable helicase motifs, the possibility of a helicase activity for these proteins is unlikely. It is also possible that these factors might affect the activity of other helicases and consequently, mediate the translation of structured mRNAs. Modifying the activity of ribosome can be another way that that *PEX11* and *RIM20* might influence the translation of structured mRNAs. There are no reported physical interactions between these factors and ribosomes making this a less likely scenario. However, in the current study we uncovered several interactions for *RIM20* with several ribosomal proteins suggesting a potential link between *RIM20* and ribosomes. An alternative explanation is that these factors may exert their activity on the biology of the mRNAs. In this way *PEX11* and *RIM20* might affect mRNA biogenesis or affect factors that contribute to mRNA biogenesis.

The findings of the current study further connect the biological activity of LiCl to the translation of structured mRNA in yeast. This knowledge may contribute to our understanding of the biology of BD. LiCl is widely considered to be a neuroprotective drug that prevents neuronal cell death. According to the observations made in the current study, LiCl's mode of action may include altering translation of certain structured mRNAs linked to the process of cell death and apoptosis. In future, it would be interesting to study the effect of LiCl on the translation of such mRNAs. Inhibiting the expression of these mRNAs may be considered for therapeutic purposes.

Declarations

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Author Contributions

Professor Golshani is responsible for conceptual development of this study, co-executor of all experiments, and coordinators of the grants that supported this work. S. Jagadeesan, a PhD candidate, contributed to translation experiments as well as data analysis. M. Algafari, a PhD candidate, contributed to the experimental approaches and data analysis. M. Hajikarimlou, contributed to the experimental analysis. All authors contributed to the writing of the manuscript.

Data Availability

All data generated and/or analyzed during this study are included in this research article and/or its Supplementary Material Files).

Competing Interests

The authors declare no competing interests.

Compliance with Ethical Standards:

The authors declare that they have no known conflicting interests / financial interests or personal relationships that could have appeared to influence the work reported in this paper

The authors declare that there are no research activities involved with human participants or animals during this study

The authors declare that the research was carried out with full awareness and informed consents from all researcher groups involved in the study, meeting standard ethical guidelines.

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Figures

Figure 1

Spot test and colony count analysis reveal increased LiCl sensitivity for *tif2Δ, pex11Δ and rim20Δ,* in comparison to WT, with respective overexpressions and *GAL1* overexpression causing similar tolerance compared to WT. A) Yeast cells are serially diluted (10^{-1} to 10^{-4}) and spotted onto Ypgal media with 10 mM LiCl. Each experiment was repeated at least three times with similar outcomes as shown. WT is used as a control. **B)** Mutant strains *tif2Δ, pex11Δ and rim20Δ* formed significantly reduced number of colonies under LiCl treatment compared to the WT. Re-introduction of the target genes results in a reversal of LiCl sensitivity in gene deletion mutants, with *GAL1* overexpression of target genes resulting in similar reversal of sensitivity. Bars represent mean values (± S.D., n ≥ 3), error bars represent standard deviation, and different letters indicate statistically significant differences (One-way ANOVA with Post Hoc Tukey Test, a = 0.05).

Figure 2

Protein and mRNA content analysis for *PGM2-GFP* with and without 10 mM LiCl treatment via western blot and qRT-PCR analysis. A) PGM2p-GFP protein content is reduced in deletion strains *pex11*Δ and *rim20*Δ when cells are treated with LiCl. PGK1p is used as the housekeeping gene/control with obtained values normalized to it. **B)** No difference was observed for the mRNA content for the deletion strains. Please note that there was an approximately 40% increase in the absolute values for the mRNA content level between LiCl treated and non-treated cells. Each experiment is repeated at least three times. Bars represent mean values (± S.D., n ≥ 3), error bars represent standard deviation and '*' represents statistically significant results compared to the WT (One-way ANOVA with Post Hoc Tukey Test, a = 0.05). Data are normalized to those for the WT strain under the same condition (with or without LiCl treatment)

Figure 3

***β-galactosidase* activity analysis of simplistic construct p416, with and without *PGM2* hairpin. A)** Each strain carries a p416 mRNA construct with no complex structure upstream at the 5' UTR of the *LacZ* reporter. No significant difference in *β-galactosidase* activity between yeast strains. **B)** Each strain carries a complex *PGM2* mRNA construct at the 5'UTR, upstream from the *LacZ* reporter. *β-galactosidase* activity is reduced in *tif2*Δ, *pex11*Δ and *rim20*Δ strains in comparison to WT. Bars represent mean values (± S.D., n ≥ 3) and '*' represents statistically significant results compared to WT, (One-way ANOVA with Post Hoc Tukey Test, a = 0.05).

Figure 4

***β-galactosidase* activity analysis in yeast strains containing varying complex secondary structures. A)** Each strain carries a complex *p2-hair* mRNA construct at the 5'UTR, upstream from the *LacZ* reporter. *β-galactosidase* activity is reduced in *tif2Δ, pex11Δ and rim20Δ* strains in comparison to WT. **B)** Each strain carries a complex pRTN mRNA construct at the 5'UTR, upstream from the *LacZ* reporter. *β-galactosidase* activity is reduced in *tif2Δ, pex11Δ and rim20Δ* strains in comparison to WT**. C)** Each strain carries a complex pTAR mRNA construct at the 5'UTR, upstream from the *LacZ* reporter. *β-galactosidase* activity is reduced in *tif2Δ, pex11Δ and rim20Δ* strains in comparison to WT. **D)** Each strain carries a complex pBcell mRNA construct at the 5'UTR, upstream from the *LacZ* reporter. *β-galactosidase* activity is reduced in *tif2Δ, pex11Δ and rim20Δ* strains in comparison to WT. Each analysis was normalized to each hairpin structure's respective WT. Bars represent mean values (± S.D., n ≥ 3) and '*' represents statistically significant results compared to WT, (One-way ANOVA with Post Hoc Tukey Test, a = 0.05).

Figure 5

Negative genetic interactions (nGIs) for *PEX11* and *RIM20*. A cluster of interactors fall in the category of protein synthesis and protein synthesis associated factors*PEX11* (p = 4.49E-06) and *RIM20* (p = 6.32E-07). *SLH1*, *SGN1*, *DPH2* and *CAF20*, are mutual hits shared between two target genes. Genes are represented via circles (nodes) and lines/dotted lines (edges) represent nGIs identified. The red circle within the inset represents an example of a typical negative genetic interaction. Intensity of interactions is shown through color gradients, darker coloration indicating reduced fitness. Existing genetic interactions refer to those GIs that have been previously reported in the literature.

Figure 6

Conditional nGIs for *PEX11* and *RIM20* in the presence of 3mM LiCl. In the presence of LiCl, a new set of genetic interactors are revealed that are involved in the regulation of translation for both *PEX11* (p=7.83E-06) and *RIM20* (p= 5.33E-6). *CPA1*, *SHE3*, *DHH1* and *TIF*2 are mutual interactors shared between

PEX11 and *RIM20*. Genes are represented via circles (nodes) and lines/dotted lines (edges) represent nGIs identified. The red circle within the inset represents an example of a typical negative genetic interaction. Intensity of interactions is shown through color gradients, darker coloration indicating reduced colony growth. Existing genetic interactions refer to those GIs that have been previously reported in the literature.

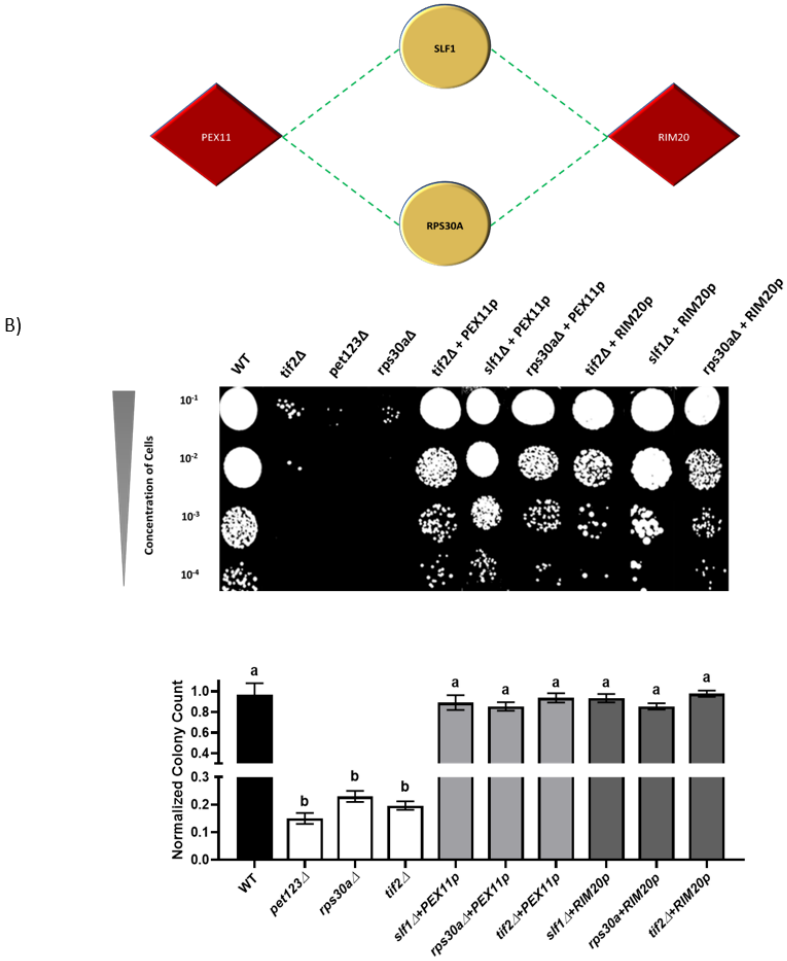

Figure 7

Overexpression of *PEX11* and *RIM20* compensate for the sensitivity of *SLF1*Δ, and *RPS30A*Δ to 10mM LiCl A) *RPS30A* and *SLF1* are both involved in translation control. Overexpression of *PEX11* and *RIM20* recovers the phenotype caused by LiCl sensitivity for the deletion strains for *RPS30A* and *SLF1*. **B)** Spot test analysis further showed that *PEX11* or *RIM20* overexpression increased tolerance to 10 mM LiCl in the deletion strains for *SLF1*, *RPS30A* and *TIF2*. For this experiment, yeast cells are serially diluted from 10^{-1} to 10^{-4} and spotted onto Ypgal media with 10 mM LiCl. Each experiment was repeated at least three times with similar outcomes as shown. WT is used as a control. **C)** Colony count analysis further verified that *PEX11* and *RIM20* overexpression compensated for the LiCl sensitivity in *SLF1*, *RPS30A* and *TIF2* gene deletion strains. Bars represent mean values (± S.D., n ≥ 3), and different letters indicate significant differences (One-way ANOVA with Post hoc Tukey Test, α = 0.05).

Supplementary Files

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