学　位　論　文

Dysregulation of DPYSL2 expression by mTOR signaling in schizophrenia: Multi-level study of postmortem brain

（統合失調症における mTOR シグナルを介した DPYSL2 発現の調節異常: 死後脳マルチスケール研究）

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論　文　内　容　要　旨

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学位論文題名	Dysregulation of DPYSL2 expression by mTOR signaling in schizophrenia: Multi-level study of postmortem brain（統合失調症における mTOR シグナルを介した DPYSL2 発現の調節異常: 死後脳マルチスケール研究）

近年の統合失調症研究において、mTOR（mechanistic target of rapamycin）シグナル伝達系と DPYSL2（dihydropyrimidinase-like 2）は、それぞれ統合失調症の生物学的メカニズムへの関与が注目されており、また、新たな治療標的候補分子として検討がなされている。一方、in vitro 研究において、DPYSL2 は、mTOR シグナル伝達系による Cap 依存的翻訳調節を受ける分子であることが知られていた。これまで、統合失調症におけるこれらの分子群の発現量変化は個々には測定された既報があったが、mTOR シグナル伝達系と DPYSL2 の相互関係にどのような変化が生じているかは検討されていなかった。

そのため、我々は、統合失調症患者 24 名と対照者 32 名の剖検脳組織の前頭前野（PFC）と上側頭回（STG）における mTOR シグナルを構成する分子(mTOR, ribosomal protein S6 kinase 1: S6K, phospho-p70 S6 kinase: pS6K, ribosomal protein S6: S6, phospho-S6: pS6)と DPYSL2 のタンパク質発現を ELISA 法を用いて定量し、個々の分子の疾患-非疾患間の分子発現量比較を行うと共に、mTOR シグナル伝達系と DPYSL2 の相関解析を実施した。また、これらの分子群のタンパク質発現量と SNPs との関連解析、生前の臨床プロファイル(diagnostic instrument for brain studies: DIBS)との関連解析も行なった。

その結果、PFC と STG の S6 のタンパク質発現量の平均値は、統合失調症患者で低かった（$p < 0.01$）。DPYSL2 のタンパク質発現は、mTOR 翻訳制御のエフェクターである pS6 タンパク質発現量と有意な正の相関を示した。また、これらの mTOR シグナル伝達と DPYSL2 の変化と遺伝子多型との関連解析において、pS6 と DPYSL2 発現の相関の傾きは、遺伝子多型、疾患-非疾患によって異なっており、5'TOP を含む領域に特定のハプロタイプをもつ DPYSL2 では、pS6K や pS6 などの mTOR 翻訳制御のエフェクター分子の高い発現を必要とする傾向を認めた。

以上のことから、統合失調症における S6 発現量の低下が確認され、mTOR シグナル伝達と DPYSL2 が 5'TOP Cap 依存的な翻訳を介して関係していることが支持された。また、5'TOP を含む領域に位置する DPYSL2 の特定のハプロタイプは、mTOR 翻訳制御の効率性に影響することが示唆された。本研究で得られたこれらの結果により、mTOR シグナル伝達と DPYSL2 が関わる主要な統合失調症治療戦略に関する知見がもたらされた。

※日本語で記載すること。１２００字以内にまとめること。

Title: Dysregulation of DPYSL2 expression by mTOR signaling in schizophrenia: Multi-level study of postmortem brain

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Abstract

The mammalian target of rapamycin (mTOR)-signaling and dihydropyrimidinase-like 2 (DPYSL2), both of which have received increasing interest as potential therapeutic targets for schizophrenia, are connected via Cap-dependent translation of the 5'TOP motif. We quantified the expression of molecules constituting the mTOR-signaling and DPYSL2 in the prefrontal cortex (PFC) and superior temporal gyrus (STG) of postmortem brain tissue samples from 24 patients with schizophrenia and 32 control individuals and conducted association analysis to examine abnormal regulation of DPYSL2 expression by the mTOR-signaling in schizophrenia. The average S6 expression levels in the PFC and STG were lower in patients with schizophrenia ($p < 0.01$). DPYSL2 expression showed a significant positive correlation with pS6 expression levels, which were effectors of mTOR translational regulation. Association analyses of these mTOR-signaling and DPYSL2 alterations with genetic polymorphisms and the clinical profile showed that the slope of the correlation between pS6 and DPYSL2 expression differed depending on disease and genetic polymorphisms, suggested that certain genetic variants of DPYSL2 require high mTOR-signaling activity. Thus, the findings confirmed decreased S6 expression levels in schizophrenia and supported the relationship between the mTOR-signaling and

DPYSL2 via 5'TOP Cap-dependent translation, thus providing insights connecting the two major schizophrenia treatment strategies associated with the mTOR-signaling and DPYSL2.

Keywords: schizophrenia, postmortem-brain, mTOR, RPS6, DPYSL2

Abbreviations:

ANCOVA	Analysis of covariance
BSA	Bovine serum albumin
DIBS	Diagnostic Instrument for Brain Studies
DLPFC	Dorsolateral prefrontal cortex
DNR	Dinucleotide repeat
DOI	Duration of illness
ELISA	Enzyme-linked immunosorbent assay
GWAS	Genome-wide association study
mTOR	mammalian target of rapamycin
PFC	Prefrontal cortex
PMI	Postmortem interval
SNP	Single-nucleotide polymorphism
STG	Superior temporal gyrus
UTR	Untranslated region

1. Introduction

Dysfunction or hypofunction of the mammalian target of rapamycin (mTOR) signaling has been recently hypothesized to be involved in the pathology of neuropsychiatric disorders, including schizophrenia. In fact, rapamycin, an inhibitor of mTOR functional complex 1 (mTORC1), is known to be a potential pharmacological agent for autism spectrum disorder (Gururajan and van den Buuse, 2014; Berry et al., 2016). In the central nervous system, the mTOR-signaling participates in the intracellular regulation of protein synthesis, specifically for proteins involved in controlling neuronal morphology and facilitating synaptic plasticity (Berry et al., 2016). Several extracellular and environmental factors known to be associated with schizophrenia, such as glutamate, reelin, BDNF, and serotonin, functionally converge on the mTOR-signaling in vivo (Gururajan and van den Buuse, 2014). In addition, AKT family members, which lie closely upstream of the mTOR-signaling, are widely known to be schizophrenia-associated molecules, since multiple studies, including ours, reported elevated protein expression of phosphorylated Akt1 in schizophrenia (Emamian et al., 2004; Hino et al., 2016; Howell et al., 2017).

In animal studies, methamphetamine administration caused expression changes in molecules known to be associated with the mTOR-signaling, including those encoded

by major susceptibility genes for schizophrenia, such as DISC1, NRG1/ErbB4, and DPYSL2. Additionally, rapamycin exposure was shown to induce altered behaviors with cognitive decline similar to human neuropsychiatric disorders in mice (Ryskalin et al., 2018). Consistent with these findings, two recent postmortem brain studies reported altered expression of mTOR signaling in schizophrenia to confirm the effects of these pathways on the pathophysiology of schizophrenia (Ibarra-Lecue et al., 2020; Chadha and Meador-Woodruff, 2020).

Dihydropyrimidinase-like 2 (DPYSL2; CRMP2) is one of the molecules that has been attracting attention for its association with the pathophysiology of schizophrenia. DPYSL2 regulates neuronal polarity for cytoskeletal dynamics, vesicle trafficking, and synapse elongation in the process of brain development (Hensley et al., 2011; Niwa et al., 2017). *DPYSL2* has been identified as one of the schizophrenia susceptibility loci in genome-wide linkage studies (Schizophrenia Working Group of the Psychiatric Genomics Consortium., 2014), and various genetic association studies have revealed relationships between the following single-nucleotide polymorphism (SNP) variants of *DPYSL2* and schizophrenia susceptibility: rs17666 (Nakata et al., 2003), rs12155555 (Fallin et al., 2005), rs17088251 (Shifman et al., 2008), rs73229635 and rs3837184 (Liu et al., 2014), rs367948 and rs445678 (Pham et al., 2016). Moreover,

DPYSL2 is also associated with the carbonyl stress hypothesis of schizophrenia (Toyoshima et al., 2019). A very recent report suggested that carbonyl stress caused abnormal multimerization of DPYSL2, and carbonylated DPYSL2 impaired neurodevelopmental function. Interventions from this point of view can also be potential targets for the treatment of schizophrenia (Yoshihara et al., 2021).

DPYSL2 is also one of the candidate molecules that connects the mTOR-signaling with the pathophysiology of schizophrenia (Liu et al., 2014; Pham et al., 2016). One of the functions of the mTOR-signaling is to regulate Cap-dependent translation according to the nutritional and oxygen status in the local tissue via downstream effectors of the mTOR-signaling, such as the ribosomal protein S6 kinase 1 (S6K), the ribosomal protein S6 (S6), and the eukaryotic translation initiation factor 4B (eIF4B) (Morita and Sobue, 2009). S6K activation and the resulting S6 phosphorylation induce translational activation of mRNAs with a 5′TOP motif in the 5′ untranslated region (UTR), and the S6 gene itself is known to have a 5′TOP motif (Levy et al., 1991; Fumagalli et al., 2009). In addition, DPYSL2 has been reported to be encoded in 5′TOP RNA, and the function of the mTOR-signaling, which regulates neuronal viability and proliferation, was mediated by local translational regulation of DPYSL2 mRNA (Morita and Sobue, 2009; Na et al., 2017). Furthermore, a genetic variant of *DPYSL2* has been

shown to be vulnerable in schizophrenia in an mTOR translational regulation in vitro assay, linking these dysregulations to the pathophysiology of schizophrenia (Liu et al., 2014; Na et al., 2017).

In previous postmortem brain studies, the mTOR-signaling and DPYSL2 were independently examined (Toyoshima et al., 2019; Ibarra-Lecue et al., 2020; Chadha and Meador-Woodruff, 2020). However, the mutual relationship of these molecules has not been reported. As previously stated, both the mTOR-signaling and DPYSL2 are candidate targets for pharmacological treatment of schizophrenia. Clarification of the relationships between these molecules is required to advance the treatment strategy for schizophrenia. Thus, the purpose of the current study is to examine evidence of abnormal regulation of DPYSL2 expression by the mTOR-signaling in schizophrenia by using accumulated data from the postmortem brain.

2. Materials and Methods

2.1. Human postmortem brain tissue collection

Postmortem brain tissue samples from 24 patients with schizophrenia (cases) and 32 control participants were obtained from Fukushima Brain Bank at the Department of Neuropsychiatry, Fukushima Medical University, and Brain Research

Institute at Niigata University, as described previously (Hino et al., 2016). Use of postmortem human brain tissues was approved by the Ethics Committee of Fukushima Medical University and Niigata University, and the study complied with the Declaration of Helsinki and its later amendments. Each patient with schizophrenia fulfilled the diagnostic criteria established by the American Psychiatric Association (Diagnostic and Statistical Manual of Mental Disorders: DSM-5). Control participants, on the other hand, were individuals who had never been diagnosed with any psychiatric disorders according to DSM-5 during their lifetime. The Diagnostic Instrument for Brain Studies (DIBS; Hill et al., Victoria, Australia; Mental Health Research Institute, 2005) was used for rating the symptoms of each patient. Detailed demographic information of each group is summarized in Table 1a, b.

2.2. Tissue characterization

Pieces (about 100 mg) of gray matter from the PFC (Brodmann area: BA10) and STG (Brodmann Area: BA22) were obtained from the frozen brain. The tissues were suspended in N-PER™ Neuronal Protein Extraction Reagent (Thermo Fisher Scientific, USA) and sonicated using a sonicator (W385; Heatsystems, USA). The samples were diluted twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7

mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4). The samples were centrifuged at 12,000 × *g* for 5 min and filtered (Ultrafree-MC-GV, 0.22 μm; Merck Milipore, Bedford, MA, USA). The protein concentration was determined by the Bradford method (Bradford protein assay kit, Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as the standard.

2.3. Protein expression analysis by enzyme-linked immunosorbent assay (ELISA)

The protein expression levels of mTOR, S6K, pS6K, S6, pS6, and DPYSL2 in each brain sample were determined by ELISA. The kits used in this study were as follows: mTOR ELISA Kit (ab206311; Abcam), p70S6K ELISA Kit (ab176652; Abcam), p70S6K (pT389 + Total) ELISA Kit (ab225582; Abcam), Mouse/Human/Rat Phospho-RPS6 /Ribosomal Protein S6 (Ser235, Ser236) ELISA kit (LS-F1743; LifeSpan BioSciences), and Human DPYSL2/CRMP2 Sandwich ELISA Kit (LS-F18366; LifeSpan BioSciences).

2.4. SNP genotyping and SNP selection for association analysis

SNP genotyping was performed on the basis of SNP data obtained in our previous studies (Hino et al., 2016; Kunii et al., 2019). In brief, genomic DNA was

extracted from the frozen cerebellum or occipital cortex. Genotyping was performed using the HumanCoreExome -24 v1.0 Beadchip (Illumina, Tokyo, Japan) on an iScan system (Illumina). In the present study, we examined 5 SNPs in the *MTOR* gene, 3 SNPs in the *RPS6KB1* gene (rs1292032, rs1051424, and rs180515), and 14 SNPs in the *DPYSL2* gene. For analyses of the associations between SNPs and the expression of each protein, we excluded SNPs with call rates < 99%, minor allele frequencies < 5%, and Hardy-Weinberg equilibrium test p-values < 0.05.

In order to connect the results of SNP analysis with the pathophysiology of schizophrenia, linkage disequilibrium analysis was performed to clarify the haplotype in the gene region. The analysis was performed on Japanese population controls in the database of the 1000 Genomes Project (Siva and *1000 Genomes project*, 2008) by using Haploview v.4.2 software (https://www.broadinstitute.org/haploview).

2.6. Statistical analysis

Two-group comparisons (control vs schizophrenia) were performed by unpaired Student's t-test. Linear regression analysis was used to assess the contribution of age and postmortem interval (PMI) to the protein immunoreactivity values. When significant differences were observed between groups, subsequent analyses of

covariance (ANCOVA) were performed to discard the effect of these potential confounding variables on the observed differences. Pearson's correlation analysis after linear regression analysis was used to evaluate correlations between each protein expression level and the clinical symptom score of DIBS. A p-value of <0.05 was considered statistically significant. SPSS ver. 25.0 (SPSS, Chicago, IL, USA) was used for the analysis.

3. Results

3.1. Protein expression levels of DPYSL2 and mTOR-S6K-S6 pathway

The results of analysis of the protein expression levels in the PFC and STG are shown in Figure 1. The average mTOR protein expression levels in the PFC were significantly higher in patients with schizophrenia than in the controls (control: 1.69 [SD 0.89] vs. schizophrenia: 2.83 [SD 1.66], $p = 0.01$), while the two groups showed no significant differences in mTOR expression in the STG (Fig. 1a). The average S6 level was significantly lower in both PFC and STG in patients with schizophrenia than in controls (BA10: control, 0.10 [SD 0.05] vs. schizophrenia, 0.07 [SD 0.03], $p < 0.01$; BA22: control, 0.11 [SD 0.07] vs. schizophrenia, 0.07 [SD 0.03], $p < 0.01$) (Fig. 1e). In addition, the S6 phosphorylation rate in schizophrenia (pS6/S6) showed a significant

increase in the STG (control, 34.4 [SD 22.9] vs. schizophrenia, 60.3 [SD 35.6], $p < 0.01$) (Fig. 1g). On the other hand, no significant difference between cases and controls was observed for protein expression of DPYSL2 (Fig. 1h).

Then, in order to investigate the relationship between downregulation of S6 and mTOR translational regulation in schizophrenia, we also performed correlation analysis of the protein expression levels of S6 and DPYSL2, encoded by 5′TOP RNA, and pS6K and pS6 in the PFC, both of which were effectors of mTOR translational regulation. The schematic diagram of the relationship between these molecules is presented in Fig. 2a. In the correlation analysis, Figure 2b shows a significant correlation between pS6K and pS6 in both control and schizophrenia groups (control: $r = 0.58$; $p < 0.01$; schizophrenia: $r = 0.79$; $p < 0.01$). The correlations of pS6 expression with both S6 (Fig. 2c) and DPYSL2 (Fig. 2d) were significantly positive in both the case and control groups (Fig. 2c: control group, $r = 0.44$, $p = 0.01$; schizophrenia, $r = 0.48$, $p = 0.03$; Fig. 2d: control group, $r = 0.56$, $p < 0.01$, schizophrenia: $r = 0.50$, $p < 0.01$). Furthermore, the slope of the correlation between pS6 and DPYSL2 protein expression levels was parallel in the cases and controls (Fig. 2d). However, the slopes of the correlations between pS6K and pS6 and between pS6 and S6 protein expression levels differed in the cases and controls (Fig. 2b, c).

3.2. Effects of SNP variants on protein expression of the molecules constituting the mTOR-signaling and DPYSL2

On the basis of the linkage disequilibrium analysis performed on Japanese population controls in the database of the 1000 Genomes Project (https://www.internationalgenome.org/), the *MTOR* gene region was divided into 9 haploblocks, *RPS6KB1* was divided into 10 haploblocks, and *DPYSL2* was divided into 14 haploblocks. *RPS6* did not show SNP variants in the Japanese control database, and the SNP analysis performed in this study also did not show SNP variants. Of these haploblocks, we examined 3 *MTOR*, 2 *RPS6KB1*, and 3 *DPYSL2* haploblocks that contained the SNP investigated in this study (Fig. 3a, Fig. 4a).

The results of χ^2 tests for each haplotype, and the case and control groups were negative for *MTOR*, *RPS6KB1*, and *DPYSL2*. Therefore, we analyzed the effect of haplotype variants on the expression of each protein constituting the mTOR-signaling and DPYSL2 in the PFC. In the *MTOR* haploblock3, minor haplotype showed significantly lower pS6K and pS6 protein expression levels than in the major haplotype (pS6K: major haplotype: 3.19 [SD 1.59] vs. minor haplotype: 2.05 [SD 0.87], $p = 0.02$, pS6: major haplotype: 10.8 [SD 5.08] vs. minor haplotype: 7.39 [SD 2.70], $p = 0.03$)

(Fig. 3b). On the other hand, no significant differences were detected in protein expression between *RPS6KB1* haplotypes.

In addition, *DPYSL2* haploblock 10 showed a minor haplotype with elevated protein expression levels of pS6K and pS6 compared to those of the major haplotype (pS6K: major haplotype: 2.37 [SD 1.28] vs. minor haplotype: 3.53 [SD 1.55], $p = 0.02$; pS6: major haplotype: 8.39 [SD 3.43] vs. minor haplotype: 11.74 [SD 5.86], $p = 0.04$), and haploblock 14 had a minor haplotype showing depressed protein expression levels of mTOR compared to that of the major haplotype (mTOR: major haplotype: 2.44 [SD 1.08] vs. minor haplotype: 1.58 [SD 0.74], $p = 0.02$) (Fig. 4b).

Figure 4d shows the results of additional analysis of the effect of haploblock 10 of DPYSL2, which is known as a schizophrenia susceptibility gene region, on 5′TOP RNA-mediated expression regulation in the case and control groups. The correlation between the expression levels of pS6 and DPYSL2 indicated the efficiency of DPYSL2 translational regulation via the mTOR-signaling. As for the effect of haplotype, the slope of the correlation between the expression levels of pS6 and DPYSL2 in the control group was smaller for the minor haplotype than for the major haplotype. In addition, with respect to the difference between the case and control groups, the slope of

the correlation between the expression levels of pS6 and DPYSL2 in schizophrenia patients was lower than that in the controls.

3.3. Association analysis between protein expression and the antemortem clinical profile

The results of correlation analysis for the association of the antemortem clinical profile (DOI: duration of illness, CPZeq: chlorpromazine equivalent dose, and clinical score of DIBS) and protein expression levels of the molecules in this study are shown in Table 2. S6K protein expression in BA10 was negatively associated with the DOI of schizophrenia ($r = -0.44$ [$p = 0.04$]) (Table 2a). DPYSL2 expression in BA22 was positively associated with positive symptom scores ($r = 0.55$ [$p < 0.01$]) and total scores of DIBS ($r = 0.50$ [$p = 0.02$]) (Table 2b). On the other hand, no significant correlation was seen with CPZeq, which represented the dose of antipsychotic treatment. Moreover, S6 expression did not significantly correlate with any particular clinical profile.

4. Discussion

In this study, we quantified the levels of DPYSL2 and several proteins related to the mTOR-signaling in the PFC and STG of the postmortem brain. Subsequently, we

conducted an association analysis of the intermolecular interaction of molecules related to the mTOR-signaling and DPYSL2 by utilizing multi-layered data based on SNP information and clinical profiles. This study provided several unique results and insights that reinforced past hypotheses of alterations in the relationship between mTOR translational regulation and DPYSL2 in schizophrenia.

Protein-expression analysis showed a significant increase in the expression of mTOR in the PFC in schizophrenia, and a significant decrease in the expression of S6 in both the PFC and STG in schizophrenia. Radhika Chadha et al. conducted detailed measurements of mTOR, including phosphorylated mTOR and two functionally distinct complexes—mTOR complex I (mTORC1) and mTOR complex II (mTORC2)—in the dorsolateral prefrontal cortex (DLPFC) of the postmortem brain of schizophrenia patients. They reported reduced expression of phospho-AKT S473 and phospho-mTOR S2448Ser 473 (S473), and increased mTOR complex formation binding to Ricter and Rapter. However, the total mTOR expression was not altered significantly in schizophrenia (Chadha and Meador-Woodruff, 2020). Although our results showed that the total mTOR expression in the STG did not significantly change between cases and controls, a previous animal study confirmed that mTOR signaling could have potentially both increased and decreased in distinct brain regions (Gururajan and van den Buuse,

2014). Thus, our findings showing increased mTOR protein expression in the PFC were reasonable.

Ibarra Lecue et al. reported decreased S6 protein expression levels in the PFC in schizophrenia (Ibarra-Lecue et al., 2020), consistent with our findings showing downregulation of S6 protein expression in the PFC and STG. On the other hand, Ibarra Lecue et al. also reported a decrease in the phosphorylation ratio of S6 (pS6/S6) in schizophrenia, which contradicted the corresponding finding in this study. However, several previous animal studies have shown that administration of antipsychotics may alter the S6 phosphorylation rate (Bonito-Oliva et al., 2013, Ibarra-Lecue et al., 2020), so these findings could be attributed to the effects of antipsychotics on pS6 protein expression levels. Nevertheless, in this study, pS6 expression and S6 phosphorylation levels (pS6/S6) were not significantly correlated with CPZeq, indicating the lack of a relationship between the dose of antipsychotics and S6 phosphorylation levels in the postmortem brain. Accordingly, the differences in the results for S6 phosphorylation among these studies require further evaluation, and investigations of the neuro-molecular mechanisms underlying the downregulation of total S6 expression consistently observed in schizophrenia should take into account factors other than the effects of antipsychotics.

Next, we investigated the relationship between several proteins related to the mTOR-signaling and DPYSL2 in schizophrenia. Although several comprehensive proteome analyses using postmortem brains have previously reported quantitative changes in DPYSL2 expression in schizophrenia, their results have been inconsistent (Toyoshima et al., 2019). In the present study, no significant difference was observed in the protein expression of DPYSL2 between cases and controls.

On the other hand, in the correlation analysis, the expression of pS6, which is the effector of mTOR translational regulation, showed a significant positive correlation with DPYSL2 and S6, which are encoded by 5′TOP RNA. Thus, the expression levels of the molecules encoded by 5'TOP RNAs might also be regulated by mTOR translational regulation in the human brain. Furthermore, the slope of the correlation between pS6 and S6 differed between the schizophrenia and control groups. This result indicated a decrease in the efficiency of mTOR translational regulation in schizophrenia and suggested that this dysfunction may explain the reduced protein expression of S6 in schizophrenia via reduced mRNA translation.

The expression level of DPYSL2 was maintained despite a possible decrease in the efficiency of mTOR translational regulation. This may be explained by the presence of a compensatory mechanism. S6 depletion has been reported to be compensated by

the induction of p53, thereby preserving the translation of 5'TOP RNA (Volarevic et al., 2000; Fumagalli et al., 2009). Thus, when S6 levels are reduced, the correlation of pS6, which is effector of mTOR translational regulation, with the molecules encoded by 5'TOP RNA is expected to be disturbed (Catts and Catts, 2000; Zhuo et al., 2019). The gene encoding p53 is known to be susceptible to schizophrenia (Allen et al., 2008), and further studies of abnormalities in Cap-dependent translation of 5'TOP RNA, including the compensatory pathway mediated by p53 in schizophrenia, are needed.

Furthermore, we analyzed whether downregulation of S6 translation was associated with genetic variation of *MTOR*, *RPS6KB1, RPS6*, and *DPYSL2*. Haploinsufficiency of the *RPS6* gene was shown to be associated with decreased gene expression of S6 and activation of the compensatory pathways mediated by p53 in an animal study (Panić et al., 2006), but the *RPS6* gene did not recognize haplotype variants in both the Japanese control database and the SNP analysis performed in this study.

On the other hand, the minor haplotype in haploblock 3 of *MTOR*, which included rs2076655 and rs7525957, was associated with increased pS6K and pS6 expression. To the best of our knowledge, there are no reports describing the association between genetic variants at the 3′ end of *MTOR* and risk of schizophrenia. Although the

direction of expression changes of the mTOR-signaling in schizophrenia was inconsistent across previous studies, our results provide a new insight showing that genetic variants of *MTOR* may affect the expression of mTOR signaling via TORC1 function, which is involved in phosphorylating downstream effectors.

In addition, the *DPYSL2* haplotype in haploblock 10, which consists of rs2585456, rs3808564, rs388047, rs415524, and rs12678418, showed a significant increase in the protein expression of pS6K and pS6, and the haplotype in haploblock 14, which included rs708621, exhibited a significant decrease in the protein expression of mTOR. This retrograde effect of *DPYSL2* on the mTOR-signaling suggests that maintenance of protein expression of DPYSL2 with a particular haplotype requires large levels of phosphorylating activity of S6 and S6K, which are effectors of the mTOR-signaling.

Previous reports have supported not only the hypothesis that specific genetic variants in DPYSL2 require high mTOR-signaling activity but also its relevance to the pathogenesis of schizophrenia. Several GWAS studies have reported that SNP at the 3′ end of DPYSL2 [rs17666 (Nakata et al., 2003), rs12155555 (Fallin et al., 2005), rs17088251 (Shifman et al., 2008)] are schizophrenia-susceptible genes. Liu et al. (2014) and Pham et al. (2016) reported that the polymorphic dinucleotide repeat (DNR)

variant in the 5′UTR of one of the DPYSL2 transcripts, which was located at the 5′ end of NM_001386.6 (Fig. 4a), was associated with schizophrenia by affecting the translation efficiency of DPYSL2. They reported reduced binding to the effecters of mTOR translational regulation in DPYSL2 with the schizophrenia-associated DNR variant, and showed that these DNR variants diminished the neuronal development-related functions of DPYSL2 in response to mTOR signaling. In addition, the results in a small Chinese sample set reported that polymorphisms in rs367948 and rs445678, which were linked to the 5′ TOP region of DPYSL2B, were susceptibility genetic variations for schizophrenia (Liu et al., 2014; Pham et al., 2016).

In the current study, haploblock 14, which contains the 3′ end of DPYSL2, and haploblock 10, which contains the 5′ end of NM_001386.6 and rs367948 and rs445678, showed differences in the expression of mTOR-signaling proteins upstream of DPYSL2. Furthermore, analysis of DPYSL2's haplotype effect on its expression via 5′TOP RNA for the case and control groups showed that the slope of the correlation between pS6 and DPYSL2 was lower in schizophrenia with both haplotypes than in controls with the major haplotype. Altogether, the minor haplotype of DPYSL2, located in the region containing 5′TOP, may require higher expression of effectors such as pS6K and pS6, which exacerbates the inefficiency of mTOR translational control.

Furthermore, it was suggested that the effect of schizophrenia pathology may secondarily exacerbate the inefficiency of mTOR translational regulation, although further studies are needed to confirm this hypothesis.

The analysis related to antemortem clinical symptoms showed that the expression level of DPYSL2 could be the endophenotype of the clinical positive symptoms score and total DIBS score for schizophrenia. On the other hand, the protein levels of S6, S6K, pS6, and pS6K were not directly related to the symptoms of schizophrenia. These findings were consistent with the proposition suggesting that the effects of disruption of the mTOR pathway in schizophrenia were mediated by the regulatory function of DPYSL2 neurogenesis (Pham et al., 2016). The results of this study support the assumption that interventions to the mTOR-signaling, including DPYSL2, can serve as a potential therapeutic target for schizophrenia. These treatment strategies may be especially promising in the schizophrenia subtype with a specific haplotype variant in the 5′TOP motif of *DPYSL2*.

This study had some limitations. First, our study population was relatively small. Thus, our findings must be confirmed via postmortem examination in a larger brain cohort and a future meta-analysis. Moreover, with respect to sample quality, the findings for some proteins were affected by confounding factors such as pH and PMI,

although we made statistical corrections to account for the effects of confounding factors. Second, to confirm the disruption of 5′TOP translational regulation in schizophrenia, integration with gene expression analysis and proteome analysis of more 5′TOP-encoded proteins is needed. We plan to perform further research using gene expression data and other resources to address this limitation. Thirdly, due to technical issues, we cannot exclude the effect of heterogeneity in cell composition of brain tissue samples on protein expression. These will need to be confirmed by single-cell RNA-seq and other methods in the future to determine the alterations in each cell type.

5. Conclusion

In this study, mTOR expression was increased in the PFC, while S6 expression was decreased in the PFC and STG in schizophrenia. These findings suggested that these expressions may be downregulated by the inefficiency of mTOR translational regulation in schizophrenia. In addition, a minor haplotype of *DPYSL2*, which was located in the region including the 5′TOP that is known to be a schizophrenia susceptibility gene region, required higher expression of mTOR translational regulation effectors such as pS6K and pS6. These inefficiencies regarding the mTOR-signaling and

DPYSL2 via mTOR translational regulation may be associated with the pathology of schizophrenia.

Since the findings of this study show an association of the clinical score with the expression level of DPYSL2, especially for positive symptoms, the results reinforce the findings of previous reports showing that the effects of mTOR pathway disruption in schizophrenia are mediated by the regulatory function of DPYSL2. Further research is needed to establish a consensus on decreased expression of S6 and abnormal regulation of DPYSL2 expression via the mTOR-signaling in schizophrenia.

DECLARATIONS OF INTEREST

None.

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Figure legends

FIGURE 1

Quantification of proteins in the prefrontal cortex (PFC; Brodmann area, BA10) and the superior temporal gyrus (STG; Brodmann area, BA22) of patients with schizophrenia and controls. a, b: Concentration (pg) of mammalian target of rapamycin (mTOR), p70S6 kinase 1 (S6K) per mg of total protein. c, e, f, h: The expression levels of phospho-p70 S6 kinase (pS6K), S6 ribosomal protein (S6), phospho-S6 ribosomal protein Ser235/236 (pS6), and dihydropyrimidinase-like 2 (DPYSL2) are presented in relation to their expression in HeLa cells. d,g: The scale of pS6K/S6K, pS6/S6 was the standardized value. (Cont: control, Sz: schizophrenia, a.u.: arbitrary unit, *: $p < 0.05$)

FIGURE 2

Correlation analysis of the protein expression levels of DPYSL2 encoded by 5′TOP RNA and S6 and pS6 in the PFC, both of which were effectors of mTOR translational

regulation. a: A schematic diagram of the relationship between the mammalian target of rapamycin (mTOR)-signaling and dihydropyrimidinase-like 2 (DPYSL2) via 5'TOP Cap-dependent translation. b, c, d: Pearson's correlation analysis following linear regression analysis of protein expression in the prefrontal cortex (PFC) of patients with schizophrenia and controls. The expression levels of phospho-p70 S6 kinase (pS6K), S6 ribosomal protein (S6), phospho-S6 ribosomal Protein Ser235/236 (pS6), and dihydropyrimidinase-like 2 (DPYSL2) are expressed as normalized values; b: pS6K vs pS6, c: pS6 vs S6, d: pS6 vs DPYSL2. (*: $p < 0.05$, **: Approximate line with a suppressed intercept)

FIGURE 3

Effects of genetic variants of *MTOR* on protein expression of the molecules constituting the mTOR-signaling and DPYSL2. a: The haplotypes of *MTOR* by linkage disequilibrium analysis using a healthy Japanese gene database (https://www.internationalgenome.org/). The figure shows the haplotypes that were present above 10%. b: The haploblock containing the single-nucleotide polymorphisms (SNPs) selected in this study was used for association analysis of the expression of proteins of the mammalian target of rapamycin (mTOR) pathway and

dihydropyrimidinase-like 2 (DPYSL2). c: The allele frequencies of each SNP of *MTOR*. (M: major haplotype, m: minor haplotype, *: $p < 0.05$)

FIGURE 4

Effects of genetic variants of *DPYSL2* on protein expression of the molecules constituting the mTOR-signaling and DPYSL2. a: The haplotype of *DPYSL2* by linkage disequilibrium analysis using a healthy Japanese gene database. The figure shows the haplotypes that were present above 10%. b: The haploblock containing the single-nucleotide polymorphisms (SNPs) selected in this study was used for association analysis of the expression of proteins of the mammalian target of rapamycin (mTOR) pathway and dihydropyrimidinase-like 2 (DPYSL2) . c: The allele frequencies of each SNP of *DPYSL2*. d: The DPYSL2's haplotype effect on its expression via 5 TOP RNA for case-control group. The correlation of phospho-S6 ribosomal protein Ser235/236 (pS6) and DPYSL2 expression was compared between haplotypes of haploblock 10 in cases and controls. Linear regression analysis was performed to evaluate the equality of slopes for the correlations. (M: major haplotype, m: minor haplotype, SD: standard deviation, *: $p < 0.05$)

Table legends

TABLE 1

Subject demographic and clinical characteristics. a: Demographic data are reported as Mean ± SD: standard deviation. Demographic variables (sex, age, pH and PMI; postmortem interval) were compared between groups using a chi squaretest and Student's t-test, for categorical and continuous variables, respectively. b: Clinical status (DOI: duration of illness, CPZeq: chlorpromazine equivalent dose, score of DIBS: The Diagnostic Instrument for Brain Studies) are reported as Mean ± SD: standard deviation. (* $p<0.05$)

TABLE 2

Association analysis between protein expression and antemortem clinical profile. a: Pearson's test was used for correlation analysis between the proteins expression of the mammalian target of rapamycin (mTOR) signaling and dihydropyrimidinase-like 2 (DPYSL2), and clinical profile including DOI: duration of illness, CPZeq: chlorpromazine equivalents. b: The same correlation analysis as above was performed for clinical symptom score of DIBS: The Diagnostic Instrument for Brain Studies. (a.u: arbitrary unit, * $p<0.05$)

a)

Variables	Controls	Schizophrenia	P value
Number of samples	32	24	
Gender			
Female	12	9	1.00 [a]
Male	20	15	
Age at death (years)	62.1 (SD 15.0)	68.4 (SD 11.0)	0.09 [b]
PMI (hour)	8.1 (SD 10.6)	16.5 (SD 11.8)	0.01* [b]
pH	6.2 (SD 0.4)	6.3 (SD 0.4)	0.59 [b]

b)

Clinical status	average (SD)
DOI (years)	42.3 (SD 10.2)
CPZeq (mg/day)	545.9 (SD 568.9)
DIBS	
Total scale	21.0 (SD 13.7)
Positive symptom score	14.5 (SD 10.1)
Negative symptom score	2.7 (SD 2.4)
General psychopathology score	3.8 (SD 3.1)

a)

	DOI		CPZeq	
	PFC	STG	PFC	STG
mTOR (pg/mg)	r=-0.15 (p=0.49)	r=-0.33 (p=0.12)	r=-0.16 (p=0.48)	r=0.02 (p=0.92)
S6K (pg/mg)	r=-0.44 (p=0.04)*	r=-0.37 (p=0.09)	r=0.13 (p=0.57)	r=0.09 (p=0.68)
pS6K (a.u.)	r=-0.07 (p=0.76)	r<-0.001 (p=0.99)	r=-0.08 (p=0.71)	r=0.05 (p=0.84)
pS6K/S6K	r=0.40 (p=0.05)	r=0.21 (p=0.33)	r=-0.26 (p=0.22)	r=-0.01 (p=0.96)
S6 (a.u.)	r=-0.27 (p=0.21)	r=-0.13 (p=0.55)	r=-0.10 (p=0.65)	r=-0.07 (p=0.75)
pS6 (a.u.)	r=-0.20 (p=0.36)	r=-0.19 (p=0.38)	r=-0.09 (p=0.68)	r=-0.12 (p=0.61)
pS6/S6	r=-0.03 (p=0.89)	r=0.41 (p=0.05)*	r=-0.09 (p=0.68)	r=-0.18 (p=0.39)
DPYSL2 (a.u.)	r=-0.04 (p=0.85)	r=-0.14 (p=0.52)	r=0.08 (p=0.74)	r=0.19 (p=0.40)

b)

	Positive score		Negative score		General psychopathology scale		DIBS total scale	
	PFC	STG	PFC	STG	PFC	STG	PFC	STG
mTOR (pg/mg)	r=0.39 (p=0.07)	r=-0.06 (p=0.80)	r=0.19 (p=0.39)	r=0.02 (p=0.93)	r=0.01 (p=0.95)	r=-0.28 (p=0.19)	r=0.32 (p=0.13)	r=-0.10 (p=0.65)
S6K (pg/mg)	r=-0.05 (p=0.81)	r=-0.12 (p=0.60)	r=-0.27 (p=0.22)	r=-0.15 (p=0.51)	r=-0.19 (p=0.40)	r=-0.22 (p=0.31)	r=-0.13 (p=0.57)	r=-0.16 (p=0.46)
pS6K (a.u.)	r=-0.10 (p=0.66)	r=-0.09 (p=0.70)	r=0.05 (p=0.84)	r=-0.25 (p=0.26)	r=-0.24 (p=0.28)	r=-0.21 (p=0.34)	r=-0.12 (p=0.50)	r=-0.15 (p=0.49)
pS6K/S6K	r=-0.13 (p=0.55)	r=-0.05 (p=0.80)	r=0.17 (p=0.43)	r=0.20 (p=0.35)	r=0.15 (p=0.48)	r=-0.28 (p=0.19)	r=-0.10 (p=0.65)	r=-0.11 (p=0.60)
S6 (a.u.)	r=0.05 (p=0.83)	r=0.23 (p=0.29)	r=0.11 (p=0.63)	r=0.22 (p=0.32)	r=0.06 (p=0.79)	r=-0.02 (p=0.92)	r=0.07 (p=0.76)	r=0.20 (p=0.36)
pS6 (a.u.)	r=-0.19 (p=0.39)	r=-0.21 (p=0.34)	r=-0.02 (p=0.94)	r=-0.01 (p=0.98)	r=-0.26 (p=0.23)	r=-0.27 (p=0.22)	r=-0.20 (p=0.36)	r=-0.21 (p=0.33)
pS6/S6	r=0.39 (p=0.07)	r=-0.06 (p=0.80)	r=-0.28 (p=0.18)	r=0.02 (p=0.93)	r=0.34 (p=0.10)	r=-0.27 (p=0.20)	r=-0.28 (p=0.18)	r=-0.39 (p=0.06)
DPYSL2 (a.u.)	r=-0.23 (p=0.29)	r=0.55 (p<0.01)*	r=-0.17 (p=0.44)	r=0.32 (p=0.14)	r=-0.27 (p=0.22)	r=0.18 (p=0.42)	r=-0.26 (p=0.23)	r=0.50 (p=0.02)*

Figure. 1

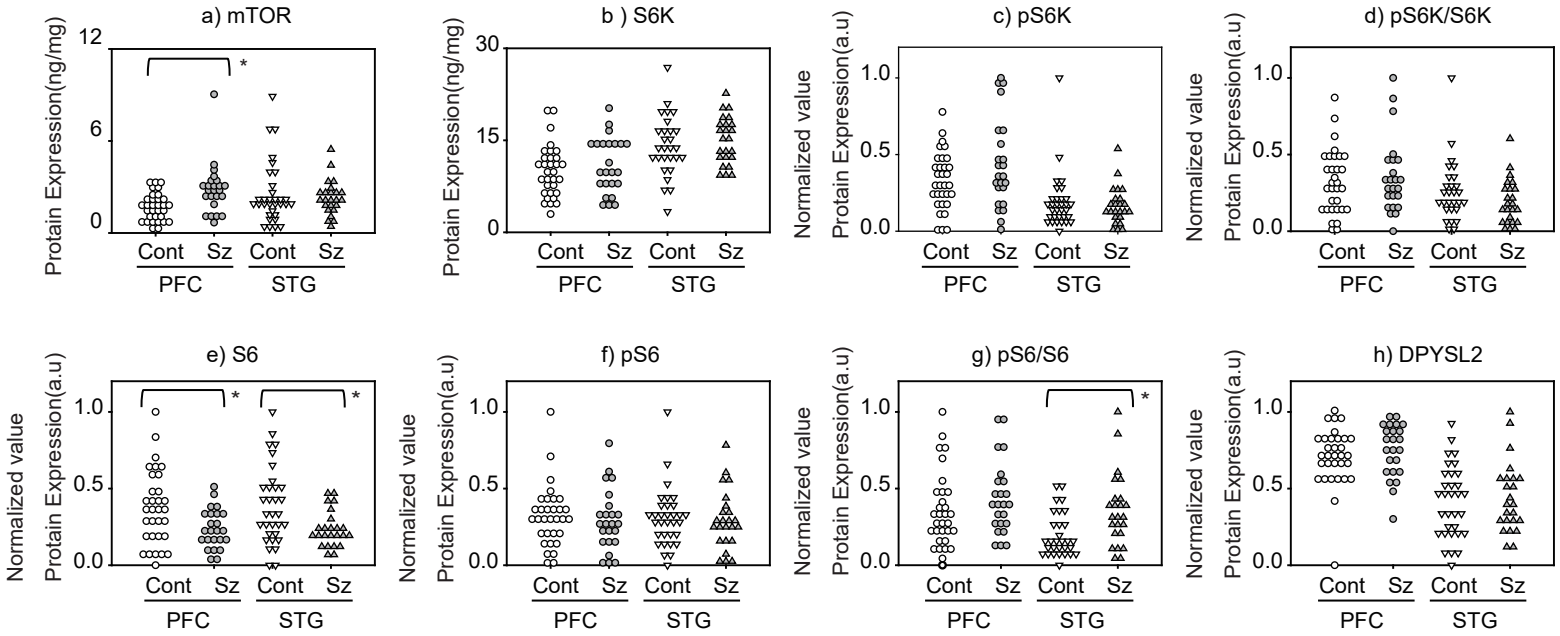

a) mTOR
Protain Expression(ng/mg)
12
6
0
*
Cont Sz Cont Sz
PFC STG
b) S6K
Protain Expression(ng/mg)
30
15
0
Cont Sz Cont Sz
PFC STG
c) pS6K
Normalized value
Protain Expression(a.u)
1.0
0.5
0.0
Cont Sz Cont Sz
PFC STG
d) pS6K/S6K
Normalized value
Protain Expression(a.u)
1.0
0.5
0.0
Cont Sz Cont Sz
PFC STG
e) S6
Normalized value
Protain Expression(a.u)
1.0
0.5
0.0
*
*
Cont Sz Cont Sz
PFC STG
f) pS6
Normalized value
Protain Expression(a.u)
1.0
0.5
0.0
Cont Sz Cont Sz
PFC STG
g) pS6/S6
Normalized value
Protain Expression(a.u)
1.0
0.5
0.0
*
Cont Sz Cont Sz
PFC STG
h) DPYSL2
Normalized value
Protain Expression(a.u)
1.0
0.5
0.0
Cont Sz Cont Sz
PFC STG

Figure. 2

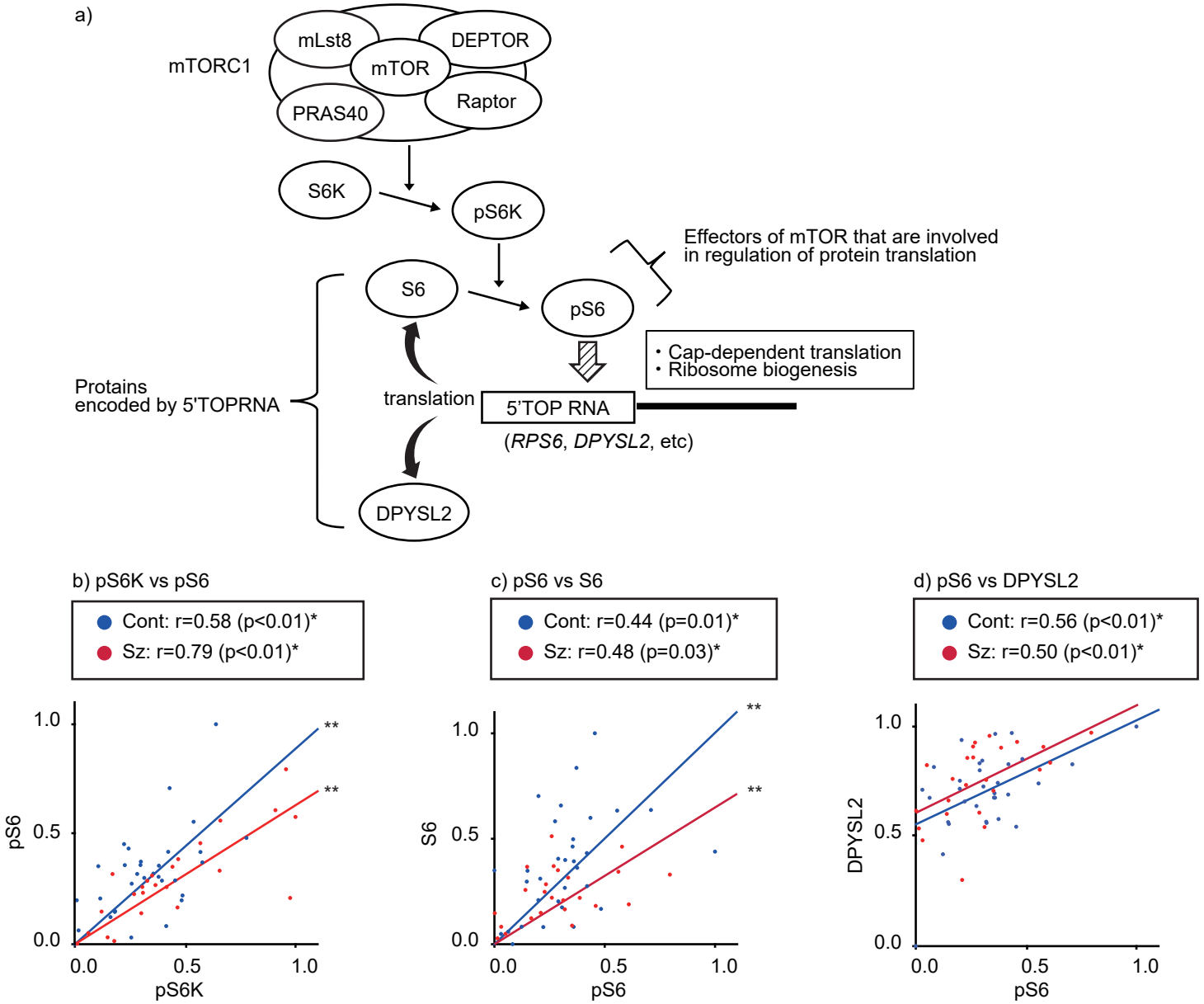
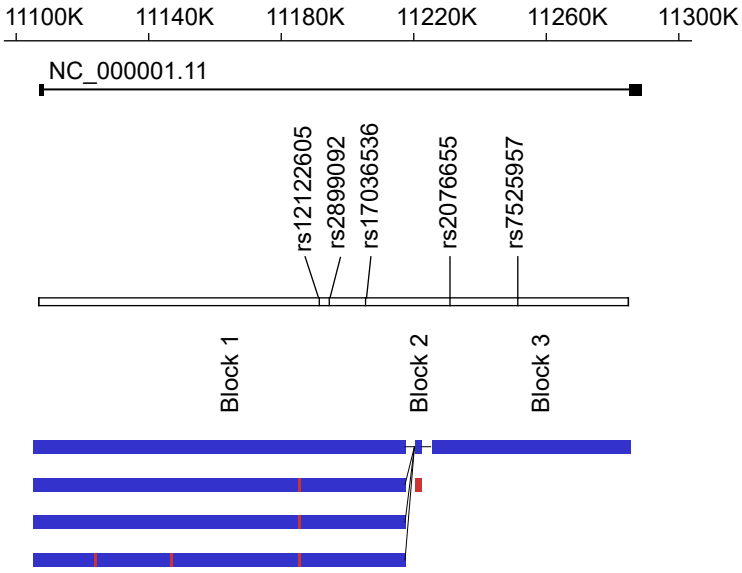
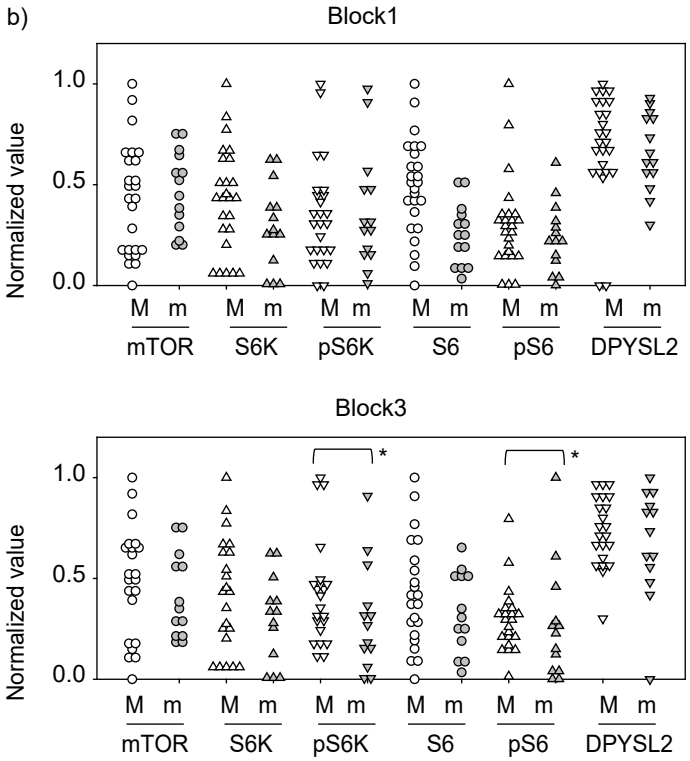

a)
mTORC1
mLst8
DEPTOR
mTOR
Raptor
PRAS40
S6K
pS6K
Effectors of mTOR that are involved in regulation of protein translation
S6
pS6
• Cap-dependent translation
• Ribosome biogenesis
Protains encoded by 5'TOPRNA
translation
5'TOP RNA
(RPS6, DPYSL2, etc)
DPYSL2
b) pS6K vs pS6
Cont: r=0.58 (p<0.01)*
Sz: r=0.79 (p<0.01)*
pS6
pS6K
c) pS6 vs S6
Cont: r=0.44 (p=0.01)*
Sz: r=0.48 (p=0.03)*
S6
pS6
d) pS6 vs DPYSL2
Cont: r=0.56 (p<0.01)*
Sz: r=0.50 (p<0.01)*
DPYSL2
pS6
0.0
0.5
1.0
**

Figure. 3

a) *MTOR*(Chr1:11106535-11273497)



b)

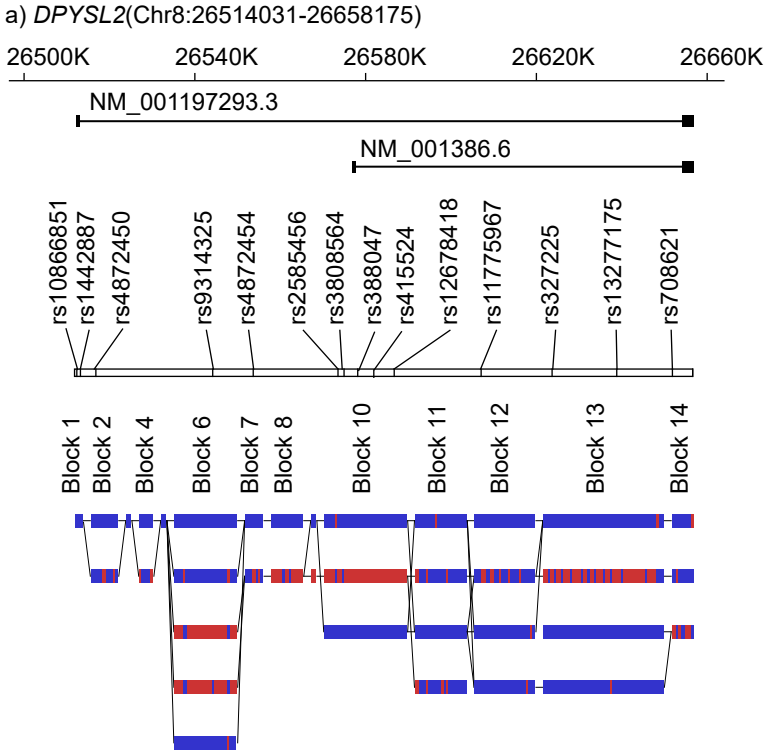


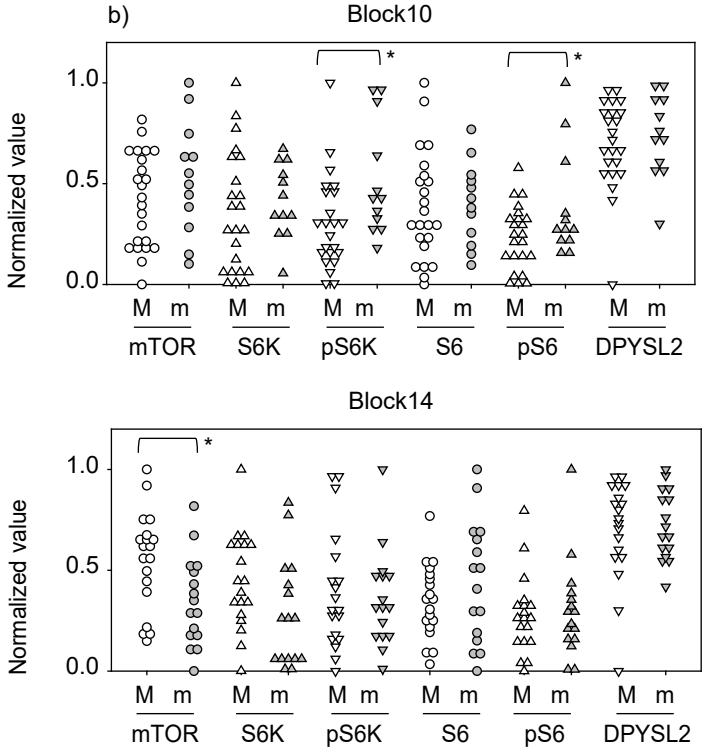
c)

Block1	rs12122605	rs2899092	rs17036535	Frequency
Major	CC	GG	CC	63.2%
	TC	GG	CC	18.4%
Minor	TC	CG	CG	7.9%
		Other		10.6%

Block3	rs2076655	rs7525957	Frequency
Major	AA	TT	55.3%
	AG	TC	15.8%
Minor	AG	TT	13.3%
	AG	TT	7.9%

Figure. 4





c)

Block10	rs2585456	rs3808564	rs388047	rs415524	rs12678418	Frequency
Major	GG	GG	CC	TT	TT	42.9%
Minor	AG	AG	TC	TC	TC	29.4%
	GG	AG	TC	TC	TC	14.7%
			Other			14.3%

Block14	rs708621	Frequency
Major	TT	54.3%
Minor	TC	28.6%
	CC	8.6%

d)