# MAP2 is Hyperphosphorylated in Schizophrenia and Alters its Function

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**Abstract**

Schizophrenia (Sz) is highly polygenic disorder, with common, rare, and structural variants each contributing only a small fraction of overall disease risk. Thus, there is a need to identify downstream points of convergence that can be targeted with therapeutics. Profound reduction of Microtubule-associated Protein 2 (MAP2) immunoreactivity (MAP2-IR) is present in ~60% of individuals with Sz, despite no change in MAP2 protein levels. MAP2 is phosphorylated downstream of multiple receptors and kinases identified as Sz risk genes, altering its immunoreactivity and function. Using an unbiased phosphoproteomics approach we quantified 18 MAP2 phosphopeptides, 10 of which were significantly altered in Sz subjects with low MAP2-IR. Network analysis grouped MAP2 phosphorylations into 3 modules, each with a distinct relationship to dendritic spine loss, synaptic protein levels, and clinical function in Sz subjects. We then investigated the most hyperphosphorylated site in Sz, serine1782 (pS1782). Computational modeling predicted phosphorylation of S1782 reduces binding of MAP2 to microtubules, which was confirmed experimentally. Because only a limited number of MAP2 interacting proteins have been previously identified, we combined co-immunoprecipitation with mass spectrometry to characterize the MAP2 interactome in mouse brain. The MAP2 interactome was enriched for proteins comprising the ribosomal complex, including FMRP. This association was shown to be functional as overexpression of wildtype and phosphomimetic MAP2 reduced protein synthesis *in vitro*. These findings provide a new conceptual framework for Sz in which a large proportion of affected individuals may have a “MAP2opathy” – in which, hyperphosphorylation of MAP2 may mediate impairments of neuronal structure and function.

## Introduction

Schizophrenia (Sz) is highly polygenic, with common, rare, and structural variants each contributing only a small fraction of overall disease risk[1]. Thus, there is a need to identify points of convergence, i.e. pathologies shared by large proportions of individuals with Sz, that can be therapeutically targeted to halt or reverse the synaptic[2] and functional deterioration[3,4] that occurs in affected individuals. Reduced Microtubule-Associated Protein 2 (MAP2) immunoreactivity (IR) has been reported in multiple cortical regions in Sz[5-10], leading one recent author to characterize it as a hallmark pathology in Sz[11]. Reduced MAP2-IR was not a consequence of common technical and clinical confounds of postmortem studies, suggesting it reflects an underlying pathogenic process [10,12].

In ~60% of individuals with Sz, the reduction in MAP2-IR is profound [10,12,13]. Even in these cases in which MAP2-IR is markedly reduced, it is not due to loss of MAP2 protein itself[10], and thus must reflect reduced detectability of MAP2 by antibody. Phosphorylation of MAP2 is known to alter its structure, function, and immunoreactivity (IR)[14], and to be regulated downstream of multiple genes now established by unbiased methods as Sz risk genes[15-23]. As the predominant regulator of the dendritic microtubule cytoskeleton, as well as a noted coordinator between microtubule and actin dynamics[24], alterations to MAP2 function could explain a variety of abnormalities in dendritic structure which have been observed across multiple cortical regions in Sz (reviewed in [25]). For instance, microtubule organization is disrupted in olfactory neuronal precursors derived from individuals with Sz[26]. Thus, we hypothesized that MAP2 phosphorylation would be altered in Sz and alter MAP2 functions, providing a potential hub for pathogenesis.

## Methods

### Preparation of expression constructs

IRES-MAP2c-EGFP plasmids containing cDNAs encoding human MAP2c (NM_031845) was purchased from GeneCopoeiaTM (Cat#: CS-E2438-M61-01). Although MAP2c is a shorter isoform, we use the amino acid numbering designated by full-length MAP2 throughout unless specifically referring to an experiment using the MAP2c plasmid (i.e. S426 in MAP2c corresponds to S1782 using numbering based on MAP2b). MAP2c phosphomimetic mutant S426E was generated using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies) and mutagenized primers (Table 1). Plasmids were purified using the EndoFree Plasmid Maxi Kit (Qiagen). Presence of the mutation at the desired site without additional off-target effects was evaluated by Sanger sequencing (Table 1).

### Cell culture and transfection

HEK cells were grown in RPMI-1640 containing 5% fetal bovine serum (Cellgro, Manassas, VA) without antibiotics. They were grown in a humidified chamber at 37 degrees Celsius with 5% $CO_2$. They were passaged appropriately during linear growth phase to maintain sub-confluent culture. For transfections they were seeded at $1.5x10^5$ cells/well into 12-well culture-treated plates. They were serum starved for 16h overnight, then replenished with complete media at the time of transfection the following morning.

<u>Transfection</u>

All transfections were performed using Lipofectamine LTX with Plus reagent (ThermoFisher Scientific, Grand Island, NY) in the absence of antibiotics. Media was changed 16h after transfection.

### Western Blotting

Cell-free protein lysates were obtained from cells using radioimmune precipitation assay buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS), and total protein levels were quantified using the Lowry protein assay (Bio-Rad). 15 µg of total

protein was loaded per well. Samples were electrophoresed on a NuPage Bis-Tris 4-12% gradient polyacrylamide gel (ThermoFisher Scientific) and transferred to a PVDF membrane (Millipore, Danvers, MA) at 100V for 1h. Membranes were blocked in Licor Blocking buffer (Odyssey, Lincoln, NE) for 1h at room temperature and primary antibody was added and incubated overnight at 4 degrees Celsius. The following primary antibodies were used in this study: mouse anti-MAP2 (HM-2, Abcam, 1:1000), rabbit anti-β tubulin (Abcam, 1:10,000), and mouse anti-puromycin (12D10, Millipore Sigma, 1: 25,000). Membranes were washed 5x5min in TBS containing 0.1% Tween and incubated with fluorescent-conjugated secondary antibodies (1:10,000) for 1h at room temperature. Five more washes were performed and membranes were imaged using the Odyssey Licor System. Densitometric analyses were performed using MCID Core Analysis software (UK).

**Table 1. Primer sequences**

| TARGET | FORWARD (5'-3') | REVERSE (5'-3') |
|---|---|---|
| S1782E mutagenesis | CCACGCTGGATCTGCCTGGTTCCTGT GTAATGATCTCAGCC | N/A |
| MAP2c sanger sequencing | Primer 1: GCGGTAGGCGTGTACGGT<br>Primer 2: TTCACGCACACCAGGCACT<br>Primer3: TAGACCTAAGCCATGTGACAT | Primer 1: AGCAGTCCCCAAGTCAGT<br>Primer 2: AGTGCCTGGTGTGCGTGAA<br>Primer3: GAGAAGGAGGCAGATTAGCTG |

### Co-immunoprecipitation

For co-immunoprecipitation (Co-IP) from 4-week old mouse brain tissue, mice were sacrificed by lethal CO2 followed by rapid decapitation. Whole mouse brains were then extracted, snap frozen and stored at -80 °C until use. Frozen brain tissue was manually homogenized in ice-cold 0.5% NP-40 IP lysis buffer with RNAse (100uL per 1mL of lysis buffer) by micro-pestle and passing through 23G needle multiple times. The lysate was then incubated on ice for 10 min followed by centrifugation at 14,000g for 20 min at 4 °C to remove the insoluble fraction. The supernatant was centrifuged again at 12,000g for 10 min at 4 °C. The supernatant was transferred to a new tube, 50µl of which was saved as input, and the rest of the supernatant was precleared by incubation with 1mg 1xPBS pre-washed Dynabeads (Thermo Fisher Scientific) on a rotator at 4°C for 1h. To prepare the antibody-coupled beads, HM-2 antibody (Abcam, Cambridge, MA, USA) was incubated together with washed Dynabeads (8µg antibody per mg beads used) in 1x PBS buffer with 0.1M citrate (pH 3.1) on a rotator at 4°C overnight. The precleared supernatant was incubated with antibody-coupled beads on a rotator at 4°C overnight. Controls were generated using washed beads not coupled to antibody. Following overnight incubation, the supernatant was collected and saved in a new tube. The Co-IP beads were washed three times in ice-cold 1xPBS buffer by gentle pipetting and directly boiled in 60 µl 1x SDS sample buffer (30 mM Tris-HCl, pH 7.4, 1% SDS, 12.5% glycerol, 2.5% β-mercaptoethanol, 0.05% Orange G) at 95 °C for 5 min to elute protein complexes from the beads.

### In-gel trypsin digestion

Immunoprecipitated samples were shortly separated (1cm) by SDS-PAGE and stained with SimplyBlue™ SafeStain (Thermo Fisher Scientific). The stained gel regions were excised and in-gel trypsin digested as previously described [27]. In brief, the excised gel bands were washed with HPLC water and destained with 50% acetonitrile (ACN)/25mM ammonium bicarbonate until no visible staining. Gel pieces were dehydrated with 100% ACN, reduced with 10mM dithiothreitol (DTT) at 56°C for 1 hour, followed by alkylation with 55mM iodoacetamide (IAA) at room temperature for 45min in the dark. Gel pieces were then again dehydrated with 100% ACN to remove excess DTT and IAA, and rehydrated with 20ng/µl trypsin/25mM ammonium bicarbonate

and digested overnight at 37°C. The resulting tryptic peptides were desalted with Pierce C18 Spin Columns, speed-vac dried and resuspended in 18µL 0.1% formic acid. A pooled instrument control (PIC) was made by taking 3µL from each of the samples.

## Human Subjects and Animals

Tissue was obtained from postmortem brains recovered during autopsies conducted at the Allegheny County Medical Examiner's Office, Pittsburgh, PA, following informed consent from the next of kin. All procedures were approved by the University of Pittsburgh Committee for the Oversight of Research and Clinical Trials Involving the Dead and the Institutional Review Board for Biomedical Research as previously described[28,29]. An independent committee of experienced clinicians made consensus Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition diagnoses, or absence thereof, for each subject using medical records, structured interviews with surviving relatives, and, when available, toxicology reports. A cohort comprising 16 individuals with a consensus diagnosis of schizophrenia (Sz) and 16 matched non-psychiatric control (NPC) subjects was generated (Table S1). The Sz subjects were further divided by MAP2 immunoreactivity (MAP2-IR) as either low (MAP2-$IR_{LOW}$) or normal (MAP2-$IR_{NL}$)[30]. Tissue slabs containing the superior temporal gyrus with Heschl's Gyrus (HG) located medial to the planum temporal were identified, and the superior temporal gyrus (STG) removed as single block. The samples were then distributed in a block design for preparation and analysis to evenly distribute SZ and control samples and blind the experimenters during sample preparation, analysis, and peak integration. Gray matter was collected from HG by taking 40 µm sections and frozen at -80 °C[31].

In order to account for the effects of chronic antipsychotic drug administration, a cohort of 18 male rhesus monkeys, n = 6 per group, were administered therapeutically relevant doses of haloperidol, olanzapine, or vehicle. The tissue utilized here has been extensively described elsewhere[32,33]. Briefly, tissue slabs containing the superior temporal gyrus were removed in a single block and 20 mg of gray matter was harvested as described above. A mouse model of postmortem interval (PMI) was generated using wildtype C57/Bl6J mice purchased from Jackson Laboratories (Bar Harbor, ME, USA). Adult mice, n= 2 per timepoint (0, 6, 12, 18, 24 hrs), were sacrificed by $CO_2$ asphyxiation followed by cervical dislocation. The carcasses were incubated at room temperature for 2/3 the PMI duration and then placed at 4°C for the final 1/3. The brain was then removed from the skull, the cerebellum discarded, and the hemispheres separated (giving n = 4 hemispheres/time point), flash frozen in isopentane on dry ice, and stored at -80°C until use.

## Mass Spectrometry

### Sample Preparation – Phosphopeptide enrichment

Gray matter from primary auditory cortex was harvested as previously described[34]. Protein quantification was determined by using a micro bicinchoninic acid assay. The samples were processed across multiple runs, using a balanced block design to evenly distribute schizophrenia and control subjects. Each run was processed identically. To account for biological and experimental variation, pooled controls were included in the designed and interspersed throughout. 500µg of protein from each gray matter homogenate sample were in-solution digested overnight at 37C with trypsin at a ratio of 1:50 (ug trypsin: ug total protein). Samples were then desalted using Oasis cartridges. Phosphorylated peptides were enriched using Titanium Dioxide Phosphoenrichment. To perform the titanium dioxide phosphoenrichment, ToptTips $TiO_2$ + $ZrO_2$ (Glygen Corporation, Columbia, MD, USA)) were washed three times with loading buffer (19% lactic acid, 80% ACN, 1% formic acid) and centrifuged 30 sec x 0.5g. Beads were suspended to 50% slurry with the loading buffer and 20µL of bead slurry was added to each sample. The sample + bead mixtures were rotated for 1 hour. Samples were spun 30sec x 0.5g and supernatant was removed. 1 mL of the loading buffer was added to each sample, and rotated for 5 minutes. Samples were centrifuged and supernatant removed. Sample was

suspended in 200µL washing buffer (80% ACN, 5%TFA) and centrifuged through a C18 Stage tip. C18 stage tip was subsequently washed x4 with washing buffer. Phosphopeptides were eluted with 50µL elution buffer (450µL Ammonium Hydroxide (28%) in 4.5 mL DI $H_20$ pH 11) 2 times. Elution buffer was evaporated and samples re-suspended in 20µL 98% buffer A (0.1% formic acid/DI $H_20$) 2% buffer B (0.1% formic acid/ACN). Samples were then analyzed by LC-MS/MS.

Liquid Chromatography Tandem Mass Spectrometry: Phosphopeptides

Phosphopeptide enrichments were analyzed by reversed phase nanoflow liquid chromatography tandem mass spectrometry (nLC-MS/MS) using a nanoACQUITY (Waters Corporation, Milford, MA, USA) coupled to an Orbitrap XL hybrid ion trap mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). For each nLC-MS/MS analysis, 1 µL of peptides was injected onto a C18 column (PicoChip™ 25 cm column packed with Reprosil C18 1.9 µm 120Å chromatography media with a 75 µm ID column and 15 µm tip; New Objective, Inc., Woburn, MA, USA) and eluted into the mass spectrometer with a 60 min linear gradient of 5-35% ACN/0.1% FA at a 300 nL/min. A data-dependent top 4 method was used with full scan resolution set at 60,000. Ions were isolated for MS/MS analysis with a 2.0 Da window. Dynamic exclusion was enabled to minimize the redundant selection of peptides previously selected for MS/MS. Ion chromatograms of 10 selected peptides were extracted using Skyline software[35] and used to monitor during the instrumental analysis to ensure robust instrument performance.

LC--MS/MS – MAP2 co-IP

Extracted tryptic peptides were analyzed by nano reversed-phase liquid chromatography tandem mass spectrometry (nLC-MS/MS) using a nanoACQUITY (Waters Corporation, Milford, MA, USA) online coupled with an Orbitrap Velos Pro hybrid ion trap mass spectrometer (Thermo Fisher Scientific). For each LC-MS/MS analysis, 1 µL of peptides was injected onto a C18 column (PicoChip™ 25 cm column packed with Reprosil C18 3 µm 120Å chromatography media with a 75 µm ID column and 15 µm tip; New Objective, Inc., Woburn, MA, USA) and then eluted off into the mass spectrometer with a 66 min linear gradient of 2-35% ACN/0.1% FA at a 300 nL/min. A data-dependent top 13 method was used with full scan resolution set at 60,000. Ions were isolated for MS/MS analysis with a 2.0 Da window. Dynamic exclusion and chromatograms from 20 selected peptides were utilized as described above.

Data analysis

MS/MS spectra from both the phosphopeptide and MAP2 co-IP experiments were searched using MASCOT search engine (Version 2.4.0, Matrix Science Ltd, Boston, MA, USA) against the UniProt human and mouse proteome databases. The following modifications were used: static modification of cysteine (carboxyamidomethylation, +57.05 Da), variable modification of methionine (oxidation, +15.99Da). The mass tolerance was set at 20ppm for the precursor ions and 0.8 Da for the fragment ions. Peptide identifications were filtered using PeptideProphet™ and ProteinProphet® algorithms with a protein threshold cutoff of 99% and peptide threshold cutoff of 90% implemented in Scaffold™ (v4.7.5; Proteome Software, Portland, Oregon, USA). Skyline MS1 filtering[36] was used for batched integration of ion chromatograms of all identified peptides. MS2 spectra for MAP2 were manual sequenced to confirm the phosphopeptide-spectra match and confidence of phospho-site assignment.

**Microtubule-binding (MTB) assay**

Transfected HEK293 cells were harvested 48 h post transfection with ice-cold microtubule-binding buffer (80 mM Pipes, 5 mM MgCl2, 0.5 mM EGTA) supplemented with 1% Triton X-100 and protease/phosphatase inhibitors (Millipore, Burlington, MA, USA; and Sigma, St. Louis, MO, USA, respectively) and lysed by vortex and passing through a 26G needle multiple times. Protein lysates were first centrifuged at 17,000g for 10 min at 4 °C to remove insoluble cell debris, after which the supernatant was collected and further pre-cleared by

ultracentrifugation at 100,000 g for 40 min at 4°C. *In vitro* microtubule-binding assay was performed using the microtubule-binding protein spin down assay kit (Cytoskeleton, Denver, CO, USA) according to the manufacturer's instructions. Briefly, microtubules were freshly polymerized and kept at room temperature prior to use. For each microtubule binding reaction, 20µl freshly prepared microtubules were incubated with a series of volumes (10 µl /20 µl /30µl) of pre-cleared cell lysate for 30 min at room temperature. Pre-cleared cell lysate alone in incubation reaction was included as negative control. Moreover, MAP fraction (contains 60% MAP2 at 280 kDa and 40% tau proteins at 40-70 kDa) and BSA, each incubated with the polymerized microtubules, served as a technical positive and negative control, respectively. Reaction solution was then placed on top of 100 µl taxol supplemented Cushion buffer (80mM PIPES pH 7.0, 1 mM MgCl2, 1 mM EGTA, 60% Glycerol) and centrifuged at 100,000 g for 40 min at room temperature. The pellets containing microtubules and their binding proteins were resuspended in 1x SDS sample buffer as described above. Supernatant and pellet samples were analyzed by SDS-PAGE and Western blot.

**MD simulation**

The molecular dynamic (MD) systems were built using cubic TIP3P water box with 12 Å buffer around the solute following the general procedure of protein system building in CHARMM-GUI. Cl- and Na+ ions were added to the solvent to neutralize the charge of the systems. All MD simulations were performed using NAMD and CHARMM C36m FF (optimized for disordered proteins). Secondary structure analysis of MD simulation trajectories were carried out using Timeline Plugin available within VMDv1.9.2. Initially all systems were minimized twice and then equilibrated them before beginning production runs. In the first minimization solute, atoms were held fixed through 500 steps of steepest descent and 500 steps of conjugate gradient minimization. In the second minimization, only the solute backbone atoms were restrained through 2000 steps of steepest descent and 3000 steps of conjugate gradient. After minimization, system temperature was raised to 300 K over the course of a 100 ps constant volume simulation during which the solute is fixed with weak (10.0 kcal/mol) restraints. For the production run, the 500 ns simulations was held at constant temperature 300 K and under constant pressure of 1 bar without any restraints used during equilibration step. For this, Langevin dynamics was used to maintain constant temperature with a Langevin coupling coefficient of 1 ps–1, and a Nosé-Hoover Langevin piston was used to maintain constant pressure with a piston period of 50 fs and a piston decay time of 25 fs while keeping barostating isotropic. A 2-fs timestep was used for integration together with the SHAKE algorithm where all covalent bonds between hydrogen and the heavy atom are constrained. The van der Waals interactions were smoothly switched off at 10 – 12 Å by a force-switching function while the long-range electrostatic interactions were calculated using the particle-mesh Ewald method.

**Surface sensing of translation (SUnSET) Assay**

Surface sensing of translation (SUnSET) assay was performed as previously described[37] with minor modifications: HEK293 cells were incubated with 10 µg/ml of puromycin in cell culture media for 1 h before harvest. As a negative control, cells were pretreated with 10 µL of cycloheximide (CHX) to inhibit protein synthesis for 1h before puromycin treatment. No puromycin incorporation was detected under these conditions (data not shown). Levels of newly synthesized proteins incorporating puromycin were measured using western blots as described above.

**Statistical analysis**

Student's t-test on log2 transformed peptide peak area was used for the statistical inference to select Map2 interacting partners. A peptide was considered differentially ID'd if the q-value was <.1 and a positive Map2 CoIP:Control ratio value.

Statistical analyses for the MTB and puromycin incorporation assays were performed in SPSS software with One-way ANOVA and Bonferroni post-hoc test to determine statistically significant differences for all the comparisons. All statistical tests were two-sided with $\alpha = 0.05$. Data are displayed ± standard error of mean (SEM).

**Differential expression analysis on phosphorylation**

ANCOVA was performed to detect the difference across Sz with MAP2-$IR_{LOW}$, Sz with MAP2-$IR_{NL}$, and NPC samples in 18 MAP2 phosphopetides. PMI and assay run were adjusted as covariates. Fold changes, p-values, and q-values between each Sz group and control samples are presented (Table 2).

We also analyzed the association between MAP2 phosphopetides and each demographical or clinical variable using a similar ANCOVA model to detect potential confounders, where diagnosis, PMI and assay run were adjusted. P-values and q-values for each variable are presented (Table S2).

**Network Analysis:** Weighted Gene Co-expression Network Analysis (WGCNA)[38] was used to investigate the pattern of co-regulated phosphopeptide expression in all samples. Topological overlap matrix will be used to identify modules with clustered phosphopetides. The three identified networks were visualized using Cytoscape. To quantitate the representation of expression levels in each module, we calculated the first principal component of modules given by WGCNA, referred to as the module-specific eigenpeptide. Linear regression models were fitted to model the correlation between each module-specific eigenpeptide and dendritic spine density, synaptic protein levels, and socioeconomic status (SES), with diagnosis (Sz or control) adjusted.

**Results**

***MAP2 is hyperphosphorylated in auditory cortex in Sz***

We conducted an unbiased proteomic survey of MAP2 phosphorylation in primary auditory cortex (A1) obtained from Sz subjects with either profoundly reduced MAP2-IR in A1 (MAP2-$IR_{LOW}$, N=11)[10] or normal MAP2-IR (MAP2-$IR_{NL}$, N=5), and from non-psychiatric control (NPC, N=16) subjects (see Table S1 for subject characteristics). We identified 18 unique MAP2 phosphopeptides in our samples, confirmed by manual sequencing of MS2 psectra, 10 of which were significantly altered ($q<0.1$) in MAP2-$IR_{LOW}$ Sz subjects relative to NPC subjects. The most altered phosphopeptide, VDHGAEIITQS[+80]PGR, containing a single phosphorylation at serine 1782 (pS1782, numbering per the canonical MAP2b isoform, Uniprot identifier: P11137-1), was increased up to nearly 9-fold relative to NPC subjects (Fig 1B upper panel, Table 2). In MAP2-$IR_{NL}$ Sz subjects, phosphopeptide levels were unchanged or substantially attenuated (Table 2). Elevated levels of MAP2 phosphopeptides were largely unassociated with potential tissue and clinical confounds (Table S2) with two notable exceptions. First, levels of VDHGAEIITQS[+80]PGR were significantly lower in Sz subjects taking antipsychotics at time of death than in those subjects off antipsychotics, raising the possibility that antipsychotic treatment could act, in part, through effects on phosphorylation of S1782. However, this effects was not recapitulated by a monkey model of long-term antipsychotic exposure, nor did this model induce elevations of any phosphopeptides found to be elevated in MAP2-$IR_{LOW}$ Sz (Fig 1A, lower panel). Second, phosphopeptide abundance may be subject to substantial alterations from postmortem interval (PMI). Thus, we further evaluated this potential confound in 2 ways. We first tested the effect of PMI on phosphopeptide abundance in our subjects, finding no significant associations (all $q>0.1$, Table S2). Second, we surveyed MAP2 phosphopeptide abundance in a mouse model of increasing PMI. We detected 9 MAP2 phosphopeptides in this model. Fig 1B shows the detected MAP2 phosphopeptides, 8 of which demonstrate stable or linear changes over time (and thus readily accounted for by PMI matching in our subjects). Of note, one peptide (RL*S[+80]*NVS*S[+80]*SG*S[+80]*INLLESPQLATLAEDVTAALAK) had a nonlinear decay, so although included in network analysis (see below) it was not considered a candidate for further study.

We next applied Weighted Gene Co-Expression Network Analysis (WGCNA)[38] to the MAP2 phosphopeptide

data from the Sz and NPC subjects. MAP2 phosphopeptides clustered into three modules (Fig 2A-C). We then used these three modules to compute eigenpeptide values for all subjects and tested these values for their associations with known pathological features of disease, namely decreased dendritic spine density and decreased synaptic protein levels, both of which had been previously measured in these subjects[29,39]. Furthermore, socioeconomic status (SES) was included as a proxy measure for functional impairment in Sz[40], thus we also tested the eigenpeptide values for their association with subject SES (Table 3). Levels of phosphorylation of blue module sites, which include pS1782, are associated with lower spine density, reduced synaptic protein levels, and poorer outcomes (lower SES). Figure 2D shows the negative correlation between dendritic spine density and pS1782 levels. In contrast, increasing phosphorylation of brown module sites, which are only present in the MAP2a and MAP2b isoforms, are correlated with better phenotypic outcomes. Turquoise module phosphopeptides show little relationship to the measured phenotypes.

***Phosphorylation of MAP2 at S1782 alters its predicted structure and reduces microtubule binding***

The phosphopeptide with the most elevated levels in MAP2-$IR_{LOW}$ Sz was VDHGAEIITQS[P]PGR, phosphorylated at S1782. This peptide belongs to the blue module, which includes negative correlations with dendritic spine density and synaptic protein levels. Furthermore, S1782 is conserved in the MAP2 homolog microtubule-associated protein tau (MAPT), as S396 (numbering by common convention as per Isoform Tau-F, Uniprot identifier: P10636-8), which when phosphorylated confers synaptic pathology[41-44]. We used all atom Molecular Dynamics simulations (MDS) to predict potential effects of pS1782 on MAP2 structure. Like its homolog MAPT, structure of the intrinsically disordered MAP2 is difficult to characterize. Because MAP2 and MAPT share high sequence homology, to validate our model we first checked for consistency with available information in both MAP2[45,46] and MAPT[47,48]. For this validation we relied on the known effect of phosphorylation at T231 in MAPT (homologous to T1649 in MAP2). For example, our model of pT1649 MAP2 revealed a salt bridge interaction between pT1649 and R1648 and helical propensity for the region $^{299}$ATPKQLRLR$^{307}$, both of which predictions recapitulated prior observations for the corresponding residues in MAPTau (data not shown)[49-52]. We then examined phosphorylation at S1782 by simulating a region of MAP2 containing residues 1718 to 1827 (covering the C-terminal domain). Our models indicated two structural changes upon phosphorylation of S1782: (a) an antiparallel β hairpin structure observed in wild-type (WT) MAP2 opened up to create a solvent-exposed extended β structural conformation between A1776 and S1782; and (b) pS1782 induced a solvent-exposed extended β structural conformation between residues V1755 and A1770, whereas helical structural propensity was present in WT MAP2 (Fig. 3). The homologous residues K369 to A384 in Tau are part of the C-terminal (residues K369 to S400) that flank the MT binding region and have been proposed for positioning Tau on the MT surface allowing MT binding[53-55]. More recently, NMR showed that pS396 MAPT had weaker tubulin affinity than the wild type Tau peptide with signal attenuation centered around residues H374, F378, H388 and Y394[56]. Based on these data, we predicted that MAP2 pS1782 decreases MT binding.

To empirically evaluate these predictions, HEK cells, which lack endogenous MAP2, were transfected with plasmid containing either wildtype MAP2c (Uniprot identifier: P11137-2) or MAP2c with the phosphomimetic mutation S426E, homologous to S1782 in full length MAP2b (see Methods for plasmid construct information), and a microtubule (MT) binding assay was performed. The results of the MT binding assay are shown in Figure 3B&C and confirm the computationally-predicted reduction of MT binding by S426E MAP2c.

***MAP2 interacts with ribosomal complexes and inhibits protein synthesis***

MAP2 binding to MTs can sequester MAP2 and its binding partners[57]. Conversely, reduced MAP2 binding to MTs increases the availability of MAP2 to associate with alternate interaction partners[58], creating what in essence is a gain of function of these latter effects. We therefore sought to use an unbiased proteomic screen of the MAP2 interactome to identify additional potential interaction partners. Co-IP of MAP2 from RNAse-treated whole brain homogenate from C57Bl6J mice (N=3 mice, 6 hemispheres total as biological samples) resulted in identification of 590 proteins that were potential interactors of MAP2 ($q<.05$) for MAP2 IP compared to no antibody control (Fig 4A-C ,Table S3). Our unbiased approach highlighted an interaction previously observed in neurons, although underappreciated, between MAP2 and ribosomal complexes[59]. Functional annotation analysis[60] of these proteins revealed enrichment for terms related to translation (e.g. GO:0006412~translation, corrected $p=1.4E^{-30}$, N=71 proteins, 44 of which are ribosomal subunits). Table S4

shows the 3 highest scored GO categories within the top 8 functional annotation clusters.

Based on the above association of MAP2 with ribosomal complexes, we next hypothesized that MAP2 may play a role in translational regulation. To test this, we measured incorporation of puromycin, an indicator of new protein synthesis[37]. Expression of both WT or S426E MAP2c in HEK293 cells suppressed new protein synthesis compared to empty vector control, though the magnitude of this effect was slightly greater in S426E (Fig 4D&E).

## Discussion

MAP2 is the predominant dendritic MAP in the brain, where its modification by phosphorylation downstream of glutamate signaling[17] has been postulated to be a requisite step linking experience to structural plasticity of dendrites[61]. We implemented a phosphoproteomics approach to evaluate this post-translational modification of MAP2 in Sz subjects in comparison to NPC subjects. We identified 18 unique MAP2 phosphopeptides in our samples, 9 of which were significantly altered in Sz compared to NPC subjects. Abnormally phosphorylated peptides were located predominantly in the Proline Rich and C-terminal domains which flank the MT Binding domain and are present in all isoforms. The most altered site was pS1782, which was found to be increased nearly 9-fold in Sz subjects compared to NPC subjects. We carefully considered potential confounds (e.g. PMI, pH, tissue storage time, age, race, sex, antipsychotic treatment, substance use, tobacco use) in our human subjects, none of which accounted for the elevation in phosphopeptides in Sz. Instead, a module of co-occurring phosphopeptides including pS1782, correlated with increasing severity of structural, proteomic, and functional measures in our subjects, suggesting the possibility that some or all of these co-occurring phosphorylations may causally contribute to the pathogenesis of Sz. To evaluate potentially causality, we chose the most elevated phosphopeptide, containing pS1782, as a candidate for further study.

Prior studies have shown that phosphorylation of MAP2 within its MT binding domain and proline rich domains reduce MT binding by MAP2,[14] and thus alter their dynamics.[62] Given the proximity of S1782 to the microtubule binding domain of MAP2, we utilized computational modeling to predict the effects of this modification on structure. We now show that phosphorylation of S1782 in the c-terminal domain of MAP2 can act similarly to phosphorylation at the homologous S396 in MAPT, which (in combination with pS404) is primarily responsible for MAPT dissociating from MTs.[63] Subsequent *in vitro* studies confirmed this finding, demonstrating reduced MT-binding in an S426 (MAP2c) phosphomimetic construct.

Our computational models similarly identified a set of β-strands induced by S1782 phosphorylation between K368-V372 and A420-Q425, a more open configuration that provides more conformational combinations required for protein-protein interaction and thus the potential for an additional gain of pathologic functions. Specifically, we hypothesized that the reduced MT-binding affinity caused by (pseudo)phosphorylation of S1782 would lead to increased MT-unbound MAP2 levels. Previous work has shown binding by some proteins to only MT-unbound MAP2[64], suggesting that a careful balance between MT-bound and -unbound MAP2 may be necessary for proper cellular functioning. Hence this increase, in combination with new protein interactions enabled by the conformational changes, provides a means by which phosphorylation of MAP2 may yield additional pathological gains of function in schizophrenia. To determine the nature of these additional functions, we combined immuno-enrichment with LC-MS/MS to characterize the MAP2 interactome. These studies revealed that the MAP2 interactome was enriched for interactions with ribosome and RNA-binding proteins forming the translational machinery (Table S3). This finding is unlikely to be artifactual, as we eliminated the possibility of residual RNA binding through RNAse treatment prior to the co-IP. This finding suggests a novel role for MAP2 in regulation of protein translation. To determine the nature of this alternative function of MAP2 we used puromycin incorporation to measure protein synthesis. We found that overexpression of MAP2c suppresses protein translation, an effect which is slightly, though non-signficantly, enhanced by pseudophosphorylation at S426 (MAP2c). Taken together, these findings are the first to suggest that MAP2 may have an additional function in regulating protein translation. Although there was a non-signficant difference in this function between WT and S426E MAP2c, it warrants further consideration in future studies that phosphorylation at other sites, alone or in combination, may alter this novel function of MAP2.

Nevertheless, the association of MAP2 with the protein translation machinery, and more importantly our novel demonstration that overexpression of WT or S426E MAP2 inhibits protein synthesis, provide a potential mechanism by which MAP2 may mediate synaptic plasticity. A number of mRNAs are translated explicitly in dendrites after synaptic stimulation to provide rapid regulation of local protein levels in support of structural and functional plasticity.[65] Specific mRNAs are maintained in a translationally repressed state before being transported to dendrites.[66] This translational repression is mediated, to a large extent, by Fragile X Mental Retardation Protein (FMRP), encoded by the FMR1 gene, and related protein family members. Altered FMRP levels,[67] and genetic variants in targets of FMRP,[21] have been previously associated with schizophrenia. Our data suggest this might involve dysfunction of MAP2 which is a known target of FMRP[68] and which was associated with FMRP and related proteins in our co-IP experiments (Table S3). In fact, FMRP maps into the most enriched cluster, Intracellular ribonucleoprotein complex (GO:1990904), of these proteins (Table S4). The possibility that repression of synaptic protein synthesis by MAP2 has a role in the pathogenesis of Sz was further supported by our observation of the correlation of the blue module, containing pS1782, with reduced levels of multiple synaptic proteins in synaptosomes of subjects with Sz.

In summary, we identified a novel pathology of Sz, hyperphosphorylation of multiple peptides within MAP2. We further demonstrated that at least one of these phosphorylations, pS1782, impairs MAP2 MT binding and inhibits new protein synthesis, functions critical for dendritic plasticity. These findings provide a new conceptual framework for schizophrenia as a “MAP2opathy”. That is, hyperphosphorylation of MAP2, like its homolog MAPT in dementia pathogenesis, may mediate impairments of neuronal structure and function downstream of many different genetic (and possibly environmental) risk factors. The potential impact of this conceptualization is that it identifies a bottleneck of molecular pathology providing a narrow set of targets for prevention and treatment for a large proportion of individuals with schizophrenia, regardless of the polygenic origins of their illness. Identification of the specific binding partners of MAP2 within the protein translational machinery, e.g. via approaches such as chemical cross-linking,[69] may provide leads to small molecules or peptides that disrupt this interaction and could then be tested as novel therapies for these individuals.


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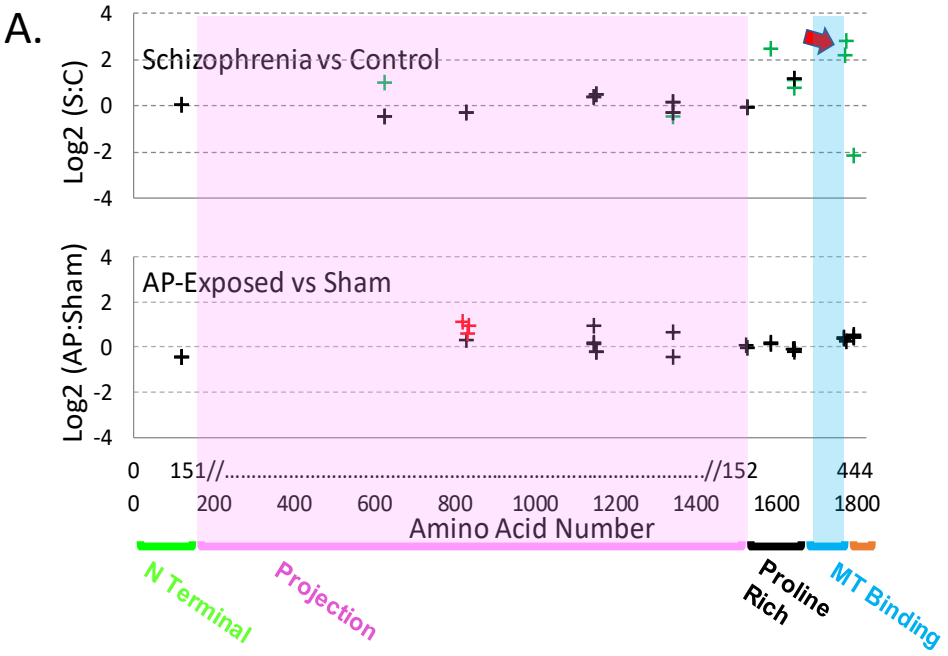
## Figure Legends

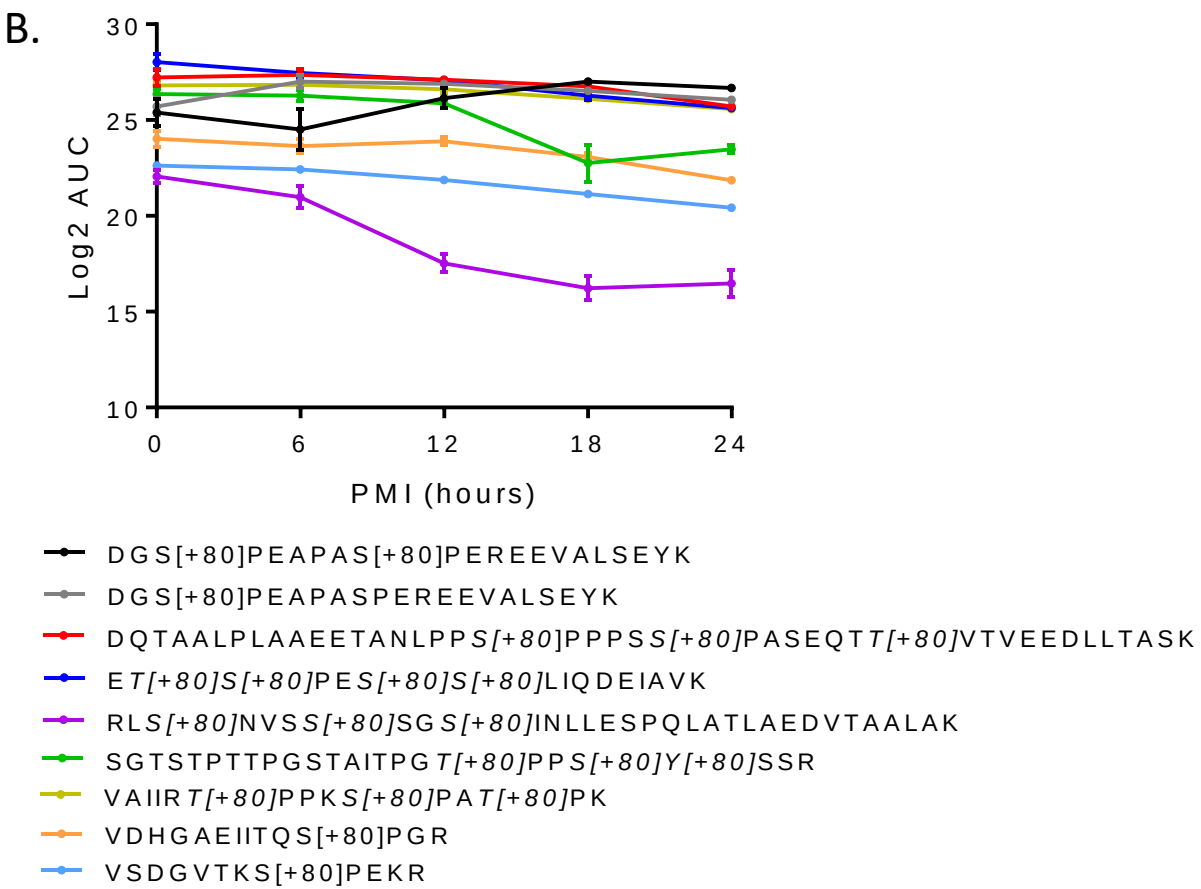
**Fig 1. MAP2 is hyperphosphorylated in Sz.** (A) Top panel shows Sz vs NPC, phosphopeptides are denoted by crosses at the position of their starting amino acid. Nine phosphopeptides had FDR significant alterations (q<0.1, green crosses). Note significantly altered phosphopeptides were present in the Projection domain (unique to high MW MAP2A/B) and surrounding the MT binding domain. Red arrow denotes pS1782. No phosphopeptides were detected in the MT binding domain. Bottom panel shows antipsychotic-exposed (AP) vs sham-exposed monkey. Three peptides were nominally significant (p<0.05, red crosses). Most peptides were identified in both species (indicated by alignment on x-axis), but peptides significantly altered in Sz did not correspond to those increased by antipsychotic exposure. X-axis shows amino acid location in MAP2C and in canonical MAP2B. MAP2 functional domains are denoted. (B) Log2 area under the curve (AUC) values for significant MAP2 phosphopeptides across increasing postmortem intervals in mouse tissue demonstrates relatively linear stability that is easily accounted for in our PMI matching of subjects. The one exception to these linear trends is RLS*[+80]*NVSS*[+80]*SGS*[+80]*INLLESPQLATLAEDVTAALAK. Individual peptides indicating phosphorylated residue(s) are listed in the legend below the graph. Data shown are ±SEM from N=4 samples per timepoint.

**Fig 2. WGCNA analysis of MAP2 phosphopeptides.** (A) Three distinct modules (blue, brown, and turquoise, were identified. (B) Network diagram of phosphopeptide modules. Arrow indicates S1782. Node size is proportional to connectivity, line thickness is proportional to correlation. (C) Modules shown on MAP2B sequence. Arrow indicates S1782. (D) pS1782 negatively correlates with dendritic spine density. NPC = nonpsychiatric control, Sz subjects grouped by MAP2 immunoreactivity as either normal (MAP2-$IR_{NL}$) or low (MAP2-$IR_{LOW}$)

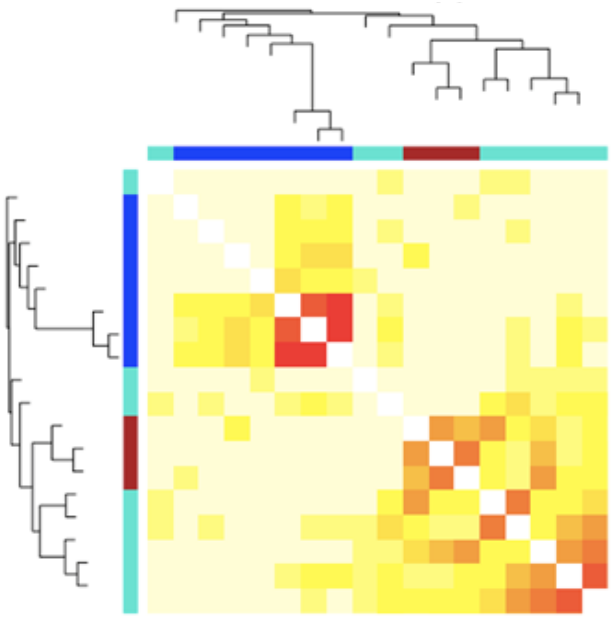
**Fig 3. Computational modeling of MAP2 pS1782 alters predicted structure and reduces microtubule binding.** (A) Snapshot of the C-terminal domain of MAP2. Amino acids A1776 to Q1781 form an extended β strand (red β strand) that is occluded by an opposing β strand. In much of the non-phosphorylated ensemble, there is found to be helical propensity in or near regions S1759 to R1765 (yellow) which is not found in the phosphorylated ensembles. In pMAP2 (pS1782) the β strand at A1776 to Q1781 is no longer occluded by another β strand; this is predicted to reduce microtubule binding and enhance interactions with other proteins. The effect of phosphorylation is recapitulated by phosphomimetic mutation S1782E (data not shown). (B) Reduced MT-binding in MAP2c with the phosphomimetic mutation S426E, homologous to S1782 in full length MAP2b relative to MAP2c-WT. 30µL of cell lysate from transiently transfected HEK cells was subjected to *in vitro* MT-binding assay and supernatant (S) and pellet (P) fractions were subjected to SDS-PAGE. (C) Densitometric analysis of gels shows decreased MT binding in the S426E mutant compared to WT. Data shown are from 3 independent experiments, ± SEM. *p < 0.05

**Figure 4. Co-IP of MAP2 from mouse brain and functional effects of S1782E on protein synthesis.** (A) Agarose gel image confirming degradation of RNA in tissue lysate prior to co-IP. (B) MAP2c was IP'd from RNAse treated mouse brain cortical homogenate (sup = supernatant, IP = immunoprecipitant). Beads without antibody coupling were used as a control. Individual channels are shown vertically (MAP2 in 800 channel, β-tubulin in 700 channel). (C) Using conservative cutoff criteria of q<.05, 6095 peptides (red) from 590 proteins were identified as potential MAP2 interactors. (D) Puromycin incorporation was detected via western blotting following a 1h treatment of transiently transfected HEK293 cells expressing MAP2c-WT or MAP2c-S426E for 48h. (E) Densitometric quantification of relative puromycin incorporation normalized to MAP2 expression levels. N=4, data shown are average across experiments ± SEM. *p < 0.1, **p<.05.

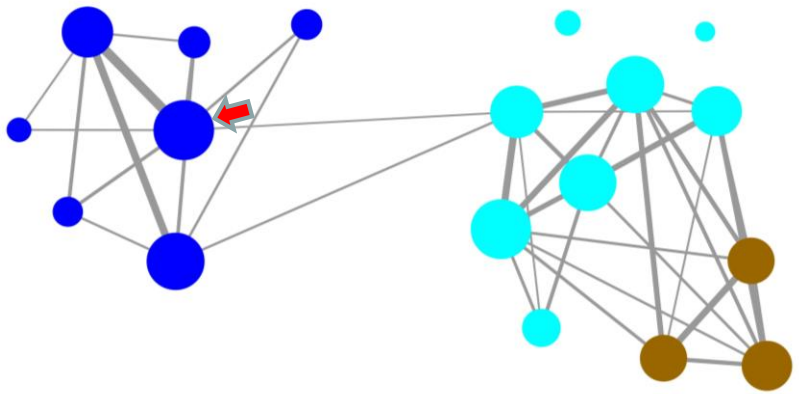

A.
Schizophrenia vs Control
Log2 (S:C)
AP-Exposed vs Sham
Log2 (AP:Sham)
4
2
0
-2
-4
0
151//
//152
444
200
400
600
800
1000
1200
1400
1600
1800
Amino Acid Number
N Terminal
Projection
Proline Rich
MT Binding


B.
Log2 AUC
30
25
20
15
10
0
6
12
18
24
PMI (hours)
DGS[+80]PEAPAS[+80]PEREEVALSEYK
DGS[+80]PEAPASPEREEVALSEYK
DQTAALPLAAEETANLPPS[+80]PPPSS[+80]PASEQTT[+80]VTVEEDLLTASK
ET[+80]S[+80]PES[+80]S[+80]LIQDEIAVK
RLS[+80]NVSS[+80]SGS[+80]INLLESPQLATLAEDVTAALAK
SGTSTPTTPGSTAITPGT[+80]PPS[+80]Y[+80]SSR
VAIIRT[+80]PPKS[+80]PAT[+80]PK
VDHGAEIITQS[+80]PGR
VSDGVTKS[+80]PEKR

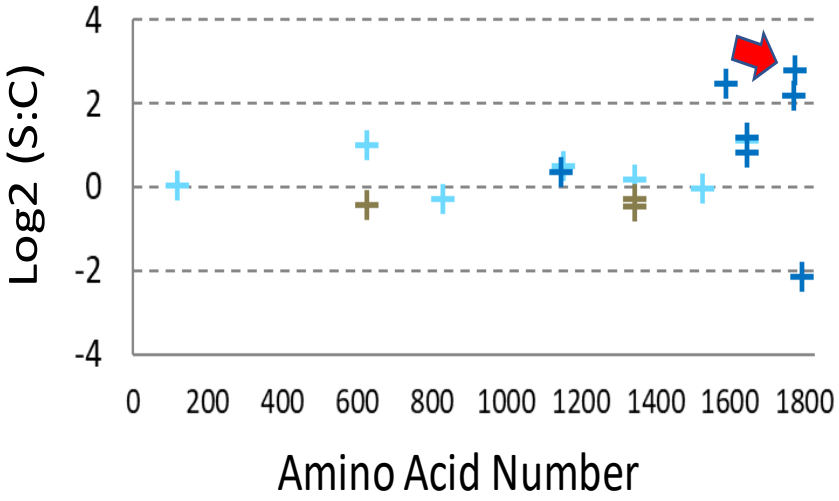
A.



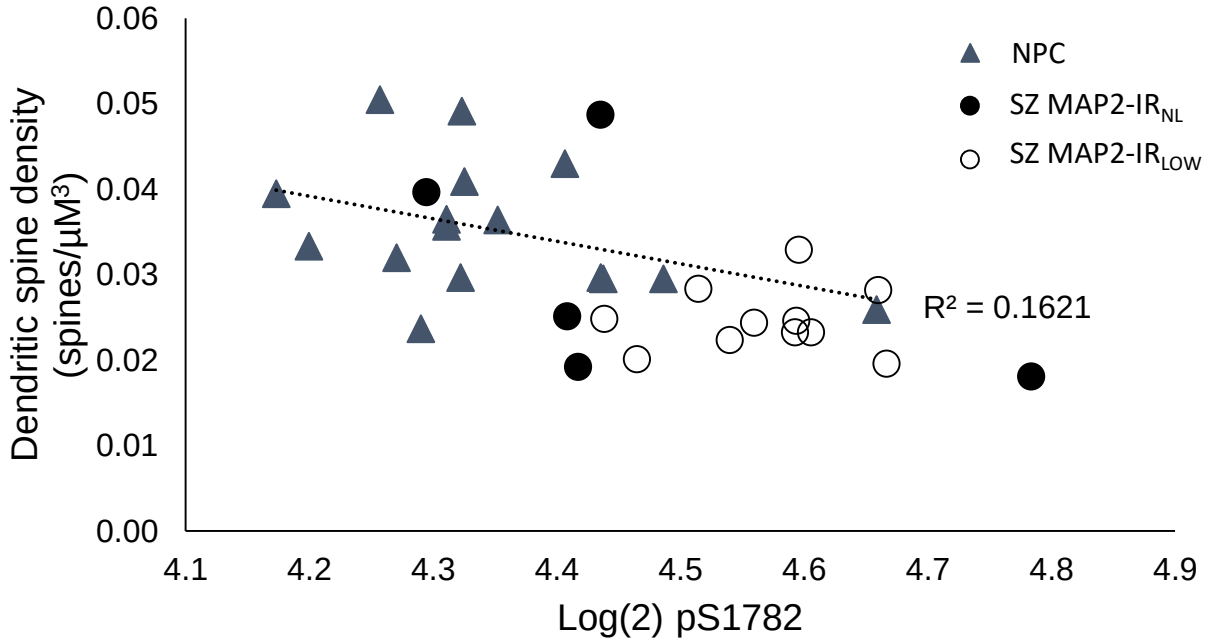
B.



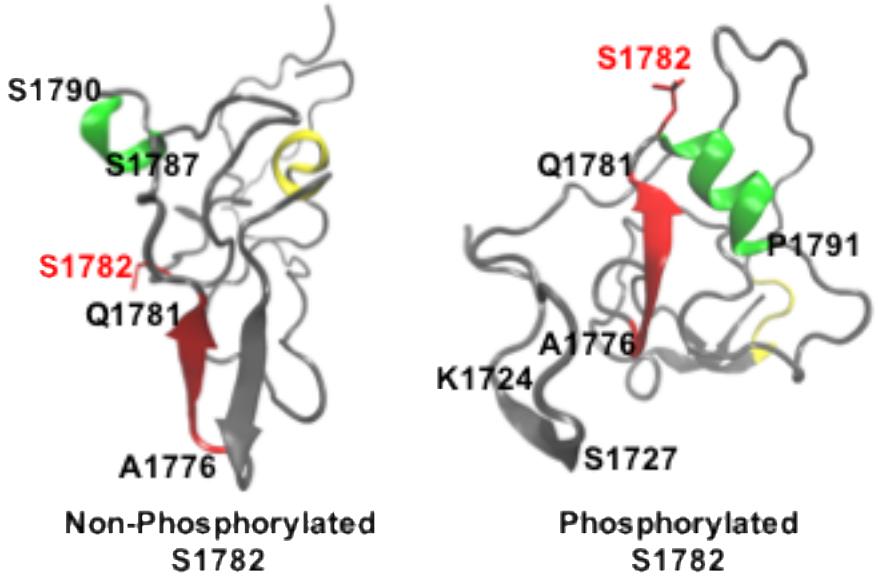
C.


Log2 (S:C)
4
2
0
-2
-4
0
200
400
600
800
1000
1200
1400
1600
1800
Amino Acid Number

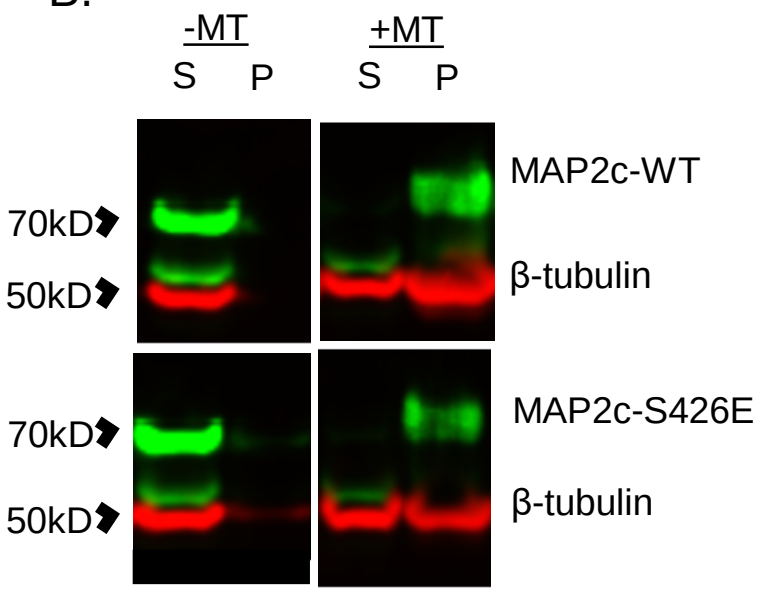
D.


Dendritic spine density (spines/μM³)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
Log(2) pS1782
NPC
SZ MAP2-IR$_{NL}$
SZ MAP2-IR$_{LOW}$
R² = 0.1621

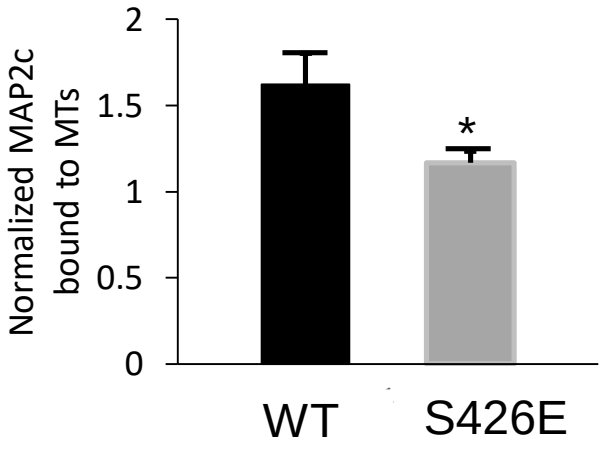
A.

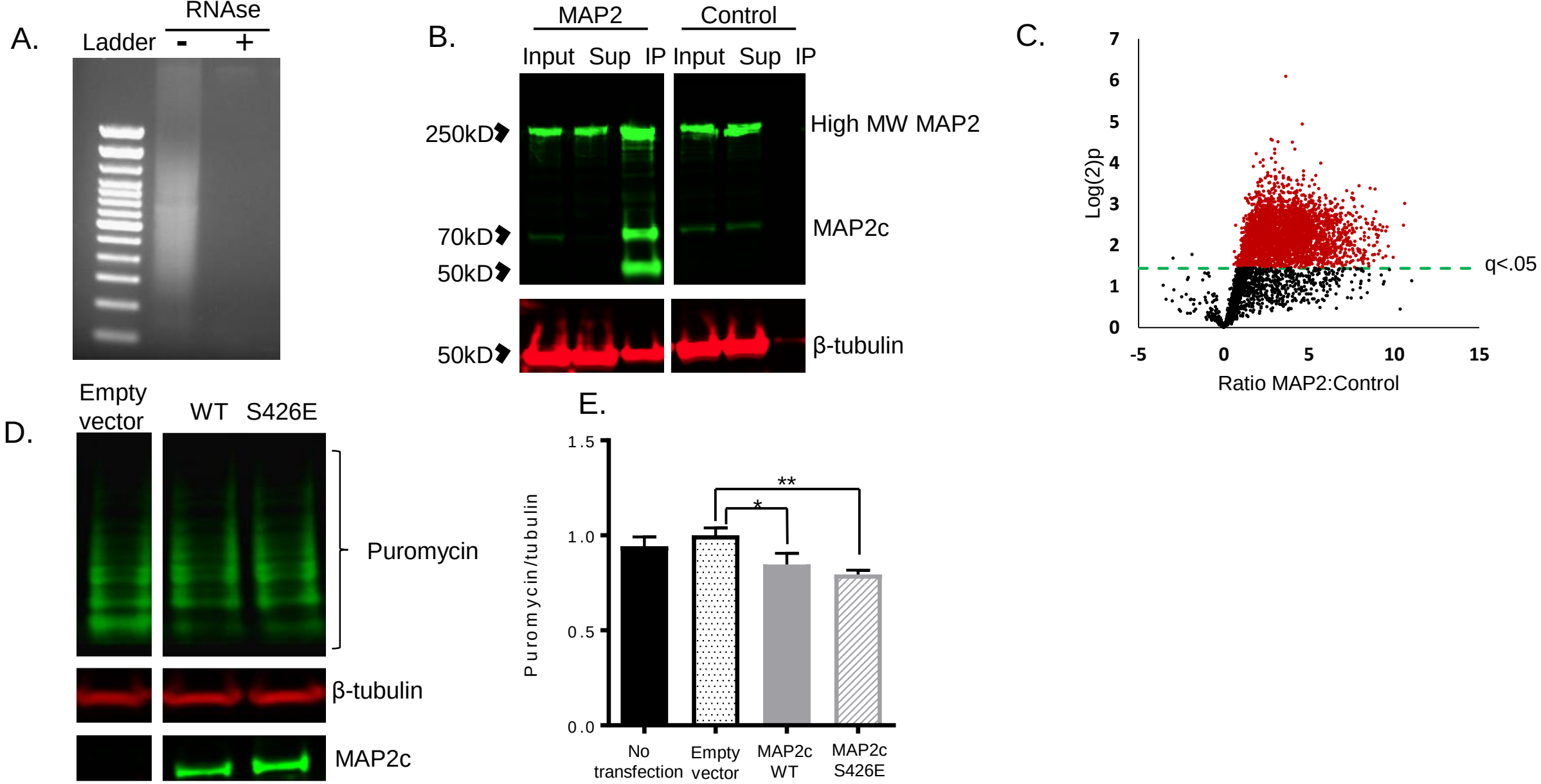

S1790
S1787
S1782
Q1781
A1776
Non-Phosphorylated
S1782
S1782
Q1781
P1791
A1776
K1724
S1727
Phosphorylated
S1782

B.


-MT
+MT
S
P
S
P
70kD
50kD
MAP2c-WT
β-tubulin
70kD
50kD
MAP2c-S426E
β-tubulin

C.


Normalized MAP2c bound to MTs
2
1.5
1
0.5
0
*
WT
S426E


A.
RNAse
Ladder
-
+
B.
MAP2
Control
Input
Sup
IP
Input
Sup
IP
250kD
70kD
50kD
50kD
High MW MAP2
MAP2c
β-tubulin
C.
Log(2)p
Ratio MAP2:Control
q<.05
D.
Empty vector
WT
S426E
Puromycin
β-tubulin
MAP2c
E.
Puromycin/tubulin
No transfection
Empty vector
MAP2c WT
MAP2c S426E

| Protein Name | Module |
|---|---|
| MTAP2_HUMAN | Blue |
| MTAP2_HUMAN | Blue |
| MTAP2_HUMAN | Blue |
| MTAP2_HUMAN | Blue |
| MTAP2_HUMAN | Blue |
| MTAP2_HUMAN | Blue |
| MTAP2_HUMAN | Blue |
| MTAP2_HUMAN | Brown |
| MTAP2_HUMAN | Brown |
| MTAP2_HUMAN | Brown |
| MTAP2_HUMAN | Turquoise |
| MTAP2_HUMAN | Turquoise |
| MTAP2_HUMAN | Turquoise |
| MTAP2_HUMAN | Turquoise |
| MTAP2_HUMAN | Turquoise |
| MTAP2_HUMAN | Turquoise |
| MTAP2_HUMAN | Turquoise |
| MTAP2_HUMAN | Turquoise |

| Peptide Modified Sequence |
| --- |
| ADEGKKE*T[+80]S[+80]* PESSLIQDEIAVK |
| SGTSTPTTPGSTAITPG*T[+80]* PP*S[+80]Y[+80]* SSR |
| VAIIR*T[+80]* PPK*S[+80]* PA*T[+80]* PK |
| VAIIRT[+80]PPKS[+80]PATPK |
| ARVDHGAEIITQS[+80]PGR |
| VDHGAEIITQS[+80]PGR |
| RL*S[+80]* NVS*S[+80]* SG*S[+80]* INLLESPQLATLAEDVTAALAK |
| ESQP*S[+80]* PPAQEAG*Y[+80]* S*T[+80]* LAQSYPSDLPEEPSSPQER |
| DGS[+80]PEAPASPEREEVALSEYK |
| DGSPEAPAS[+80]PEREEVALSEYK |
| DQTAALPLAAEETANLPP*S[+80* ]PPPS*S[+80]* PASEQT*T[+80]* VTVEEDLLTASK |
| ESQPSPPAQEAGYSTLAQSYPSDLPEEPSS[+80]PQER |
| RKS[+80]VPSETVVEDSR |
| KE*T[+80]S[+80]* PE*S[+80]S[+80]* LIQDEIAVK |
| E*T[+80]S[+80]* PE*S[+80]S[+80]* LIQDEIAVK |
| DGS[+80]PEAPAS[+80]PEREEVALSEYK |
| VSDGVTKS[+80]PEKR |
| VAIIRT[+80]PPK |

| Starting AA number | FoldChange (MAP2-$IR_{LOW}$ SZ vs Ctrl) |
|---|---|
| 1147 | 1.236 |
| 1588 | 7.293 |
| 1644 | 1.782 |
| 1644 | 1.665 |
| 1772 | 5.947 |
| 1774 | 8.791 |
| 1792 | 0.141 |
| 625 | 0.638 |
| 1344 | 0.794 |
| 1344 | 0.687 |
| 118 | 0.921 |
| 625 | 1.795 |
| 829 | 0.556 |
| 1152 | 1.350 |
| 1153 | 1.314 |
| 1344 | 0.941 |
| 1527 | 0.953 |
| 1644 | 2.455 |

| Log2 FoldChange (MAP2-$IR_{LOW}$ SZ vs Ctrl) | p value | q value |
|---|---|---|
| 0.306 | 0.22470 | 0.31113 |
| 2.867 | 0.00001 | 0.00019 |
| 0.833 | 0.01940 | 0.04364 |
| 0.735 | 0.40104 | 0.48125 |
| 2.572 | 0.00147 | 0.00663 |
| 3.136 | 0.00018 | 0.00162 |
| -2.825 | 0.00064 | 0.00385 |
| -0.649 | 0.01021 | 0.03675 |
| -0.333 | 0.12603 | 0.20623 |
| -0.541 | 0.01610 | 0.04141 |
| -0.118 | 0.66940 | 0.75308 |
| 0.844 | 0.04746 | 0.08543 |
| -0.848 | 0.32844 | 0.42228 |
| 0.433 | 0.04485 | 0.08543 |
| 0.394 | 0.15557 | 0.23336 |
| -0.088 | 0.80149 | 0.84863 |
| -0.069 | 0.90921 | 0.90921 |
| 1.296 | 0.01234 | 0.03703 |

| FoldChange (MAP-$IR_{NL}$ SZ vs Ctrl) | Log2 FoldChange (MAP-$IR_{NL}$ SZ vs Ctrl) | p value |
|---|---|---|
| 1.393 | 0.478 | 0.16049 |
| 3.064 | 1.615 | 0.03217 |
| 1.657 | 0.729 | 0.11801 |
| 4.401 | 2.138 | 0.07646 |
| 2.452 | 1.294 | 0.19721 |
| 4.060 | 2.022 | 0.04749 |
| 0.617 | -0.697 | 0.48590 |
| 1.002 | 0.003 | 0.99259 |
| 0.886 | -0.175 | 0.54275 |
| 0.803 | -0.317 | 0.27391 |
| 1.293 | 0.370 | 0.32429 |
| 2.571 | 1.362 | 0.01925 |
| 1.971 | 0.979 | 0.40090 |
| 1.527 | 0.611 | 0.03613 |
| 1.653 | 0.725 | 0.05586 |
| 1.668 | 0.738 | 0.12435 |
| 0.995 | -0.007 | 0.99310 |
| 1.624 | 0.700 | 0.29189 |

| q value |
|---|
| 0.32098 |
| 0.20111 |
| 0.27978 |
| 0.22938 |
| 0.35498 |
| 0.20111 |
| 0.58308 |
| 0.99310 |
| 0.61059 |
| 0.43783 |
| 0.44902 |
| 0.20111 |
| 0.51545 |
| 0.20111 |
| 0.20111 |
| 0.27978 |
| 0.99310 |
| 0.43783 |

| Module | Starting Amino Acid # for Included Phosphopeptides | Correlation With Dendritic Spine Density | Signficant Correlations with Synaptic Protein Levels N (% R < 0) | Correlation With Subject SES |
|---|---|---|---|---|
| **Blue** | 1147, 1588, 1644, 1644, 1769, 1774, 1792 | R = -0.56<br>P = 0.001 | 57 (88%) | R = -0.47<br>P = 0.01 |
| **Brown** | 625, 1344, 1344 | R = 0.44<br>P = 0.01 | 10 (40%) | R = 0.50<br>P = 0.007 |
| **Turquoise** | 118, 625, 831, 1152, 1153, 1344, 1527, 1644 | R = 0.10<br>P = 0.57 | 0 (N/A) | R = 0.22<br>P = 0.26 |