Mammalian Target of Rapamycin (mTOR) Tagging Promotes Dendritic Branch Variability through the Capture of Ca\(^{2+}\)/calmodulin-dependent protein kinase II α (CaMKIIα) mRNAs by the RNA-binding Protein HuD*

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*Running Title: HuD directs CaMKIIα expression in specific dendritic branches

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Background: Memory requires protein synthesis of dendritic CaMKIIα.

Results: HuD directs CaMKIIα expression in a branch-specific manner. mTOR inhibition reduces HuD binding and promotes deadenylation of CaMKIIα mRNA.

Conclusions: mTOR activity tags synapses allowing HuD to capture CaMKIIα in a branch-specific manner.

Significance: mTOR and HuD provide a molecular model for the synaptic tagging and capture hypothesis.

ABSTRACT

The fate of a memory, whether stored or forgotten, is determined by the ability of an active or tagged synapse to undergo changes in synaptic efficacy, requiring protein synthesis of plasticity related proteins. A synapse can be tagged, but without the “capture” of plasticity related proteins it will not undergo long-lasting forms of plasticity (Synaptic tagging and capture hypothesis; STC). What the “tag” is and how plasticity related proteins are captured at tagged synapses is unknown.

Ca\(^{2+}\)/calmodulin-dependent protein kinase II α (CaMKIIα) is critical in learning and memory and is synthesized locally in neuronal dendrites. The mechanistic (mammalian) target of rapamycin (mTOR) is a protein kinase that increases CaMKIIα protein expression; however, the mechanism and site of dendritic expression are unknown. Herein, we show that mTOR activity mediates the branch-specific expression of CaMKIIα, favoring one secondary, daughter branch over the other in a single neuron. mTOR inhibition decreases the dendritic levels of CaMKIIα protein and mRNA by shortening its poly(A) tail. Overexpression of the RNA-stabilizing protein HuD increases CaMKIIα protein levels and preserves its selective expression in one daughter branch over the other when mTOR is inhibited. Unexpectedly, deleting the third RNA recognition motif (RRM) of HuD, the domain that binds the poly(A) tail, eliminates the branch-specific expression of CaMKIIα when mTOR is active. These results provide a model for one molecular mechanism that may underlie the STC hypothesis where mTOR is the tag, preventing deadenylation of CaMKIIα mRNA.
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Activation of mTOR kinase is required for protein synthesis-dependent, late-phase, long-term potentiation (L-LTP) and memory consolidation (1,2). mTOR consists of two subunits, mTORC1 and mTORC2. mTORC1, a serine/threonine kinase, promotes cap-dependent translation by phosphorylating p70 S6 kinase (S6K) and eIF-4E binding protein (4E-BP) (3). One notable mRNA whose translation is regulated by mTORC1 is Ca²⁺/calmodulin-dependent protein kinase II α (CaMKIIα) (4,5). CaMKIIα is important for the induction and maintenance of LTP and memory (6). The importance of locally translated CaMKIIα mRNA in memory consolidation was demonstrated in a mouse where the dendritic targeting sequence of CaMKIIα in the genome was disrupted (7). Moreover, synapses that express protein synthesis-dependent LTP tend to occur on one dendritic daughter branch as opposed to the synapses of both daughter branches (8). Thus, further insight into the subcellular loci of CaMKIIα expression in dendrites may yield information regarding the importance of dendritic branches in memory formation.

The expression of the RNA binding protein HuD is correlated with both spatial learning and contextual fear conditioning (9-11). Furthermore, expression of several HuD target mRNAs are associated with improved cognition (9,12,13). Recently, we determined that mTORC1 kinase serves as a switch for translation of specific mRNAs such as CaMKIIα through HuD. We demonstrated that when mTORC1 is active HuD binds to its high affinity target mRNAs including CaMKIIα, stabilizing the mRNA and promoting its translation. When mTORC1 is inhibited, CaMKIIα mRNA degrades, thus releasing HuD and allowing it to bind to low affinity target mRNAs, such as the voltage-gated potassium channel Kv1.1(5). How CaMKIIα mRNA is degraded is unclear. Collectively, these data strongly support a role for mTOR activity and HuD to promote the translation of mRNAs that support learning and memory.

The synaptic tagging and capture hypothesis proposes that the synapses activated during early LTP (E-LTP) become tagged in a protein synthesis-independent manner (14). For the tagged synapse to undergo lasting changes in synaptic efficacy it must capture plasticity-related proteins or the mRNAs that code for these proteins(15). The requirement for protein synthesis comes from studies that demonstrate that the conversion of E-LTP to L-LTP is blocked with the addition of protein synthesis inhibitors such as the mTORC1 inhibitor rapamycin (16,17). While a great deal is known about global mTORC1-regulated translation, it is unknown whether mTORC1 regulates protein expression in a site-specific manner. In this study, we demonstrate an unexpected role for HuD in mTORC1-regulated branch-specific CaMKIIα protein expression. Using immunocytochemistry and in situ hybridization to map CaMKIIα protein and mRNA, we show that CaMKIIα is preferentially expressed in one daughter branch versus the other when mTORC1 is active, suggesting that mTORC1 serves as a tag for CaMKIIα expression.

We determined that HuD mediates the branch-specific expression of CaMKIIα, likely through the binding of its poly(A) tail. Furthermore, we found that HuD expression is branch-specific and that this process does not rely on mTORC1 activity. Thus, our findings provide a model where mTORC1 activity and the branch-specific targeting of HuD determines which mRNAs are available to be translated and in turn, the propensity of a dendritic branch to undergo site-specific and long-lasting forms of plasticity.

EXPERIMENTAL PROCEDURES

Transfection and Immunocytochemistry
Neurons were cultured as previously described in Sosanya et al., 2013 (5). Briefly, hippocampi from E18-19 rats were collected, dissociated, and plated. Neurons were plated at a density of 50,000 neurons/12 mm coverslip. Cultured hippocampal neurons were transfected with pcDNA+eGFP, pcHuD+eGFP, and pcHuDI+II + eGPF, at 17-20 days-in-vitro (DIV) using Lipofectamine2000 (Life Technologies), as described by the manufacturer using neurobasal media (Life Technologies). Cloning of pcHuD and pcHuDI-II is described in Anderson et al, 2000 (18). At DIV 21-24, 4 days post-transfection, neurons were treated with 200 nM Rapamycin, 100µM AP5,
DMSO (vehicle for Rapa), or H$_2$O (vehicle for AP5) for 75 min. Following treatment, neurons were fixed for 20 min at RT in 4% paraformaldehyde followed by 3 washes in 1X Phosphate Buffer Saline (PBS). Neurons were then permeabilized for 5 min with 0.25% triton in 1X PBS (Sigma) and blocked for 1 hour in blocking solution (8% Goat serum, 0.25% Triton, 1X PBS) at RT. Primary antibodies were incubated overnight (ON) at 4°C in blocking solution followed by secondary antibody incubation for 1 hour at room temperature (RT) in blocking solution. The cells were then washed (PBS), mounted (Fluormount-G; SouthernBiotech), and imaged.

**Antibodies**

Primary antibodies used: mouse anti-CaMKII$\alpha$ (1:200, LifeSpan Biosciences), chicken anti-GFP (1:200, Aves Labs), mouse anti-Kv1.1 extracellular (Neuromab), rabbit anti-myc (1:200, Sigma), rabbit anti-PS6 (1:50, Cell Signaling Technology), chicken anti-MAP2 (1:2000, Abcam). Secondary antibodies from Life Technologies used at 1:400: Alexa488 anti-chicken, Alexa555 anti-mouse, and Alexa647 anti-rabbit.

**Fluorescent in situ hybridization (FISH)**

For CaMKII$\alpha$ or HuD mRNA detection, fluorescent *in situ* hybridization was conducted using the ViewRNA ISH Cell Assay kit (Affymetrix) as described in Cajigas et al., 2012 (19). The CamkII$\alpha$ and HuD probe sets were designed commercially by Affymetrix. Briefly, primary hippocampal neurons (DIV 20–21) were fixed at room temperature for 30 minutes with a 4% paraformaldehyde solution (4% paraformaldehyde, 5.4% glucose, 0.01M sodium metaperiodate, in lysine-phosphate buffer). Proteinase K treatment was omitted and the rest of the hybridization was completed according to the manufacturer’s instructions. The cells were then washed with PBS and blocked with 4% goat serum in PBS for one hour followed by incubation in primary antibody (chicken anti-MAP2 or chicken anti-GFP) overnight at 4°C. After three washes with PBS the cells were incubated with the appropriate secondary antibody for one hour at room temperature and washed with PBS. The coverslips were then mounted with an antifading mounting medium and imaged as described above.

**Quantification of Phospho-S6 puncta and CaMKII$\alpha$ mRNA puncta**

Images were acquired using a Leica SP5 confocal microscope (63X objective lens, N.A. 1.2) with sequential scanning. Series of z-stacks were collected at 0.5 µm intervals for a total of 5.0 µm. Dendrites were chosen blindly based on MAP2 or eGFP signal. Following image acquisition, a binary mask of equally thresholded images was created using Meta Imaging Series 7.8. To measure branch variability, 10 µm long regions of interest (ROI) were drawn before and after the 1st branch point of the MAP2 or eGFP dendrite, as described by Govindarajan et al. (8). P-S6 puncta intensity in the primary and secondary branches and CaMKII$\alpha$ puncta intensity in the cell body, primary, and secondary branches was measured using the integrated morphometry image analysis. P-S6 intensity in the cell body was taken as a ratio over eGFP or MAP2, as the signal was not punctate in the cell body. Individual puncta were counted in the primary and secondary branches, similar to Cajigas et al. (19). To determine if mTOR was equally or differentially active between daughter branches the number of P-S6 puncta/10µm area after the branch point was determined for each daughter branch emerging from a single parent dendrite. The absolute value of the difference in puncta number per 10µm area between the arbitrarily assigned daughter branch A and daughter branch B was determined.

**Image Analysis, Branch Variability Index (BVI) and 3-D rendering**

Following image acquisition as described above, the ratio of CaMKII$\alpha$, Kv1.1, or myc-HuD over eGFP signal (volume control) was determined by the ImageJ plugin ROI Manager under Stacks-T-Functions. Daughter branches emerging from a single parent dendrite were arbitrarily assigned A and B (see Figure 1A). In order to avoid negative numbers, we used the absolute value of branch A minus B. BVI was calculated by the following equation: BVI= |Daughter Branch A – Daughter Branch B| / average BVI of control neurons for their individual culture. 3-D rendering
was achieved using the ImageJ plugin Interactive 3D surface plot.

**Poly(A) Tail Length (PAT) Assay**

PAT assay was carried out according to Wu et al. and similar to Udagawa et al. (20, 21). Cultured cortical neurons between DIV21-28 were treated in artificial cerebrospinal fluid (aCSF) for 10 min at time point 0 or Rapamycin for 60, 90, or 180 min. Neurons were harvested in HB buffer B (20 mM HEPES, pH 7.4, 5 mM EDTA, pH 8.0, with RNaseOut and TCEP) and homogenized. Following a low-speed spin (900rpm, 10min) total RNA was isolated using Tri-LS following the manufacturer protocol (ABI). Reverse transcription was performed with the anchor-oligo dT primer (5' GCGAGCTCCGCGGCCGCGT 3'; oligo #1) using the superscript III first strand cDNA synthesis kit (Life Technologies). Subsequent PCR was performed with 100ng of cDNA using Amplitaq Gold DNA polymerase (ABI) with specific CaMKIIα forward (5'CCGAAGCTTCTCTCTTTCTTTTTATTATGTGCTGTG 3'; oligo #1) and reverse (5' GCTCTAGACACATAATTTGTAGCTATTTATTCC 3') oligos or Kv1.1 forward (5' GCCGCCGCAGCTCCTCTACTATCAG 3'; oligo #1) and reverse (5' GCTTTTGATTGCTTGCCTGGTGCTT 3') oligos (13, 14). To detect the poly(A) tail oligo #1 for CaMKIIα or Kv1.1 was used in combination with the anchor-oligo dT primer. PCR was done with an initial denaturation step (95°C, 5min) followed by 10 cycles of (15 sec at 95°C, 15 sec at 45°C, 1min at 72°C) and then 50 cycles of (15 sec at 95°C, 15 sec at 58°C, 1min at 72°C) and finally 7 min at 72°C. The PCR products were resolved in 2% agarose gel. As a control for the PAT assay, 600 ng of total RNA was treated with RNase H and oligo dT for 20 min at 37 °C prior to RT-PCR.

**Knockdown of HuD with shRNA**

For shRNA-mediated knockdown of HuD primary hippocampal neurons were transfected on DIV 17 with pEGFP and either the pRetro-shHuD plasmid (CGCATCCTGGTGTACAGT) (22) or the pRetro control plasmid. The transfection protocol is described above. Following 72 hours the cells were fixed and prepared for either CaMKIIα immunocytochemical analysis or HuD FISH.

**Local Translation of myr-dGFP CaMKIIα UTR**

Detailed methods regarding the detection of local translation of the coding sequence for a myristoylated, destabilized GFP flanked by the 5' and 3' untranslated regions of α-CAMKII have been reported by Aakalu et al. and Sutton et al. (23, 24). In brief fluorescent recovery after bleaching - live imaging of new CaMKIIα translation using the myr-dGFP reporter was performed 36 hours post transfection with either myr-dGFP-CaMKIIα UTR +HuD or myr-dGFP-CaMKIIα UTR ΔHuD and dsRED. Neurons were live imaged in HEPES based aCSF using 20x immersion lens on Leica SP5 spinning disk confocal microscope. An initial z-stack of the full extent of the neuron was acquired. The dGFP signal was then bleached by acquiring 1 frame/second with 488 argon laser set at full power for 100 frames. Following this, a z-stack of each neuron was then acquired approximately every six minutes for thirty minutes. The average change in green fluorescence was calculated at time t=0(F₀), t=6(F₆), t=12(F₁₂), t=18 (F₁₈), and t=30 (F₃₀) for the same ROIs 10 μm before and after each branch point. The percentage change in green fluorescence (ΔF/F) was calculated as: \((F₃₀ - F₀) / F₀\) at 30 min postbleach. A similar equation was used for F₆, F₁₂, and F₁₈. The branch variability index (BVI) was calculated at t=30 min as the absolute difference between the percentage changes in fluorescence for each daughter branch: \((ΔF/Fₐ - ΔF/Fₗ)\).

**RESULTS**

**NMDAR signaling activates mTORC1 in cultured hippocampal neurons**

For synapses to be tagged they must be stimulated by synaptic activity, usually requiring N-Methyl-D- aspartate (NMDA) receptor activation. Using a simplified system, we have shown previously that NMDAR activity promotes the phosphorylation of mTORC1 and acute rapamycin treatment reduces it in cultured hippocampal and cortical neurons (21 – 28 DIV) (25). However, the subcellular localization of mTORC1 activity has not been determined. To
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In this end, we treated neurons with vehicle, (2R)-amino-5-phosphonovaleric acid (AP5), or the mTORC1 inhibitor rapamycin and stained for the downstream marker of active mTORC1, Phospho-S6 (P-S6), a key ribosomal protein. Thus, we predict that during elevated spontaneous NMDAR activity, such that occurs in dissociated neurons after several weeks in culture (14), mTORC1 will be active throughout the dendritic arbor (Figure 1A). As expected, mTORC1 was strongly activated in the cell body (Figure 1B), primary and secondary branches (Figure 1C and D) of cultured primary hippocampal neurons, as indicated by the change in signal intensity of P-S6 puncta or hotspots with rapamycin. To determine if mTORC1 activity is equally or differentially stimulated between two daughter branches that emerge from a single parent dendrite, we counted the number of P-S6 hotspots per 10 µm area directly after the branch point, similar to Govindarajan et al. (8). We arbitrarily assigned one daughter branch A and the other B (cartoon, yellow and white boxes, Figure 1A). We then took the absolute value of the difference in P-S6 hotspots between branch A and B. As predicted, there was relatively little difference between the two daughter branches, only differing in P-S6 puncta number by ~1 hotspot (branch with most puncta averaged ~3±0.24 vs. the branch with the fewest number of puncta averaging ~2±0.41). Notably, the signal intensity of the P-S6 hotspots was significantly reduced with mTORC1 inhibition; however, the remaining signal between branches was relatively the same (Figure 1E; BV1 = DMSO: 0.94±0.30, Rapa: 0.71±0.35; single t-test not significantly different from zero).

In contrast to rapamycin, blocking NMDAR signaling with AP5 had a smaller effect of ~30% versus a ~60% reduction of the P-S6 signal in the cell body (Figure 1A – B and 1F - G). Interestingly, AP5 significantly reduced the signal intensity of P-S6 in the primary, parent dendrite by ~90% (Figure 1H; Vehicle, water: 1 ± 0.27; AP5: 0.10±0.03) and the secondary branch by ~79% (Figure 1I and J; Vehicle, water: 1.00±0.17; AP5: 0.21±0.05). Similar to rapamycin, the number of detectable puncta did not change between daughter branches (Figure 1E and J). These data suggest that NMDAR activity stimulates mTORC1 throughout the dendritic arbor and can be specifically blocked with either AP5 or rapamycin treatment.

NMDAR and mTORC1 activity is required for CaMKIIα branch-specific expression

NMDAR activation stimulates the mTOR-dependent, local protein synthesis of CaMKIIα mRNA (4). However, it is unknown whether mTORC1 differentially regulates CamKIIα protein expression in one daughter branch over the other. To answer this question, we determined if CaMKIIα protein expression was branch-specific using immunofluorescence with blockers of NMDAR/mTORC1 activity. EGFP expression allowed us to clearly visualize individual neurons and normalize signal by volume. While the cell body CaMKIIα signal in neurons is highly variable, NMDAR inhibition with AP5, but not mTORC1 inhibition with rapamycin reduces CaMKIIα protein expression dramatically by ~66% (Figure 2A – B and F-G). These results suggest that CaMKIIα expression in the cell body may be independent of mTORC1 activity.

Next, we measured CaMKIIα in the dendrites by determining the average signal intensity in the primary apical dendrite prior to the first branch and normalized by eGFP as a volume control (Figure 2A, C, F, and H). Inhibition of mTORC1 by rapamycin (Figure 2A, C) or NMDARs by AP5 (Figure 2F and H) reduced the dendritic expression of CaMKIIα in the primary parent branch by ~50% and 40%, respectively (Figure 2C; DMSO: 1.00 ± 0.22, Rapa: 0.49 ± 0.05; Figure 2G: Vehicle: 1.00 ±0.15, AP5: 0.60±0.10). Next, we measured the average signal intensity of CaMKIIα in each daughter branch, 10 µm from the branch point normalized by eGFP. Again, reducing mTORC1 activity either by NMDAR antagonism (AP5) or rapamycin decreased the overall expression of CaMKIIα in the secondary branches (Figure 2A, D, F, and I). To determine if CaMKIIα was differentially expressed between daughter branches, we determined its Branch Variability Index or BVI by measuring the signal intensity of CaMKIIα normalized to eGFP for each daughter branch and then took the absolute value of paired daughter branch A minus daughter branch B. This difference was divided by the average BVI for
HuD directs CaMKIIα expression in specific dendritic branches. In this case, a value of 0 indicates that the protein is equally distributed between daughter branches (see Experimental Procedures for equation). As the BVI moves away from 0, protein expression becomes more polarized in one daughter branch over the other. Indeed, CaMKIIα protein is enriched in one daughter branch by at least ~2 fold when mTORC1 is active relative to neurons treated with AP5 or the mTORC1 inhibitor rapamycin (Figure 2E: BVI for DMSO: 1.00 ± 0.06, BVI for Rapa: 0.45 ± 0.07; Figure 2J: BVI for Vehicle: 1.00±0.27, BVI for AP5: 0.24±0.09).

Branch-specific expression of Kv1.1 does not require mTORC1 activity

To determine if branch-specific expression is generally dependent on mTORC1, we examined the expression of Kv1.1 protein, whose dendritic expression is negatively regulated by mTORC1 activity. As previously observed (5,25,26), mTORC1 inhibition increased Kv1.1 protein ~100% in the primary and ~70% in the secondary dendrites (primary dendrite: DMSO: 1.00 ± 0.17, Rapa: 2.04 ± 0.45; secondary dendrite: DMSO: 1.00 ± 0.13, Rapa: 1.72 ± 0.27; Figure 3A-D). Although we did not observe changes in BVI when mTORC1 activity is disrupted, the BVI of ~1 suggests that Kv1.1 protein expression is more abundant in one daughter branch over the other under both conditions (Figure 3E). These results altogether suggest that both CaMKIIα and Kv1.1 expression is branch-specific, favoring one daughter branch over the other in a single neuron. In contrast, mTORC1 activity regulates the drop in the branch-specific expression of CaMKIIα but not of Kv1.1.

CaMKIIα mRNA targets one daughter branch over the other in a single neuron when mTORC1 is active

An unresolved debate concerning the STC hypothesis is whether it is the mRNA or protein that is “captured” in a site specific manner. While CaMKIIα protein is branch-specific (Figure 2), it is unclear if the mRNA is as well. To answer this question we performed fluorescent in situ hybridization (FISH) against CaMKIIα mRNA when mTORC1 is active or inhibited with rapamycin. Control and rapamycin treated neurons were probed for CaMKIIα mRNA and quantified (Figure 4A). As a negative control we used a sense probe that did not detect any signal (Figure 4B). Consistent with CaMKIIα protein levels, there was no change in the number of mRNA granules in the cell body when normalized by area (Figure 4A and C). In contrast, there was a significant reduction in total CaMKIIα positive mRNA granules in the primary and secondary dendritic branches of rapamycin treated neurons (Figure 4D and E).

To determine if the mRNA targets one branch over the other, we determined if there were more CaMKIIα mRNA positive granules in one daughter branch over the other in a single neuron. As expected, we detected a range between ~4 (high branch) and ~1 (low branch), with ~3 more granules per 10 μm segment that were selectively targeted to one branch over the other (Figure 4F) when mTORC1 is active (Figure 4F; DMSO: 2.5±0.28; Rapa: 0.65±0.15). As seen with the protein, the number of mRNA positive granules was decreased with rapamycin, reducing the branch-selective expression of CaMKIIα mRNA. Of note, the signal intensity of the granules that remain present after rapamycin treatment is relatively equal to those that are present when mTORC1 is active (Figure 4G). Consistent with the branch-selective expression of CaMKIIα protein, these results suggest that when mTORC1 is active CaMKIIα mRNA localizes to one daughter branch over the other within a single neuron.

mTORC1 inhibition results in the rapid degradation of CaMKIIα mRNA by shortening of the poly(A) tail

In light of these data, two questions remain unanswered: (1) what mediates the branch-specific targeting of CaMKIIα mRNA and (2) how does inhibition of mTORC1 reduce it? By first determining the mechanism that reduces branch-specific mRNA targeting, we might glean insight into the factors that mediate the process. In yeast, inhibition of TORC1 accelerates the deadenylation-decapping pathway (27). mRNAs that decay rapidly in the presence of rapamycin have shorter poly(A) tails possibly through rapid deadenylation (27). Thus, we hypothesized that deadenylation of CaMKIIα mRNA underlies the reduced CaMKIIα mRNA when mTORC1 is
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inhibited. Using the poly(A) tail length (PAT) assay, we measured CaMKIIα mRNA poly(A) tail length when mTORC1 kinase was active or inhibited by rapamycin. The reduction in poly(A) tail length, band intensity (Figure 5A) and the sensitivity to RNase H treatment (Figure 5B) all indicate that inhibiting mTORC1 activity shortens the CaMKIIα mRNA poly(A) tail. Notably, Kv1.1 mRNA levels remain roughly the same (5), while the poly(A) tail length remains relatively the same with mTORC1 inhibition (Figure 5C). These results favor the hypothesis that the mTORC1-dependent reduction in CaMKIIα mRNA is mediated by its rapid deadenylation and subsequent mRNA degradation.

The binding of the RNA-binding protein HuD to the 3'UTR of CaMKIIα is required for its branch-selective expression

Since the branch-specific expression of Kv1.1 was not affected by mTORC1 activity, we considered the possibility that HuD, an RNA-binding protein that binds to both mRNAs, could mediate branch-specific expression of CaMKIIα. We recently demonstrated that HuD-CaMKIIα mRNA interaction mediates the mTORC1-dependent expression of CaMKIIα protein (5). Furthermore, we showed that CaMKIIα mRNA and Kv1.1 mRNA compete for HuD binding with CaMKIIα mRNA “winning” when mTORC1 is active, due to CaMKIIα’s higher affinity for HuD and abundance (5). In agreement with our data suggesting that reduced mTORC1 activity leads to the shortening of the CaMKIIα mRNA poly(A) tail (Figure 5), HuD stabilizes its target mRNAs by delaying the onset of mRNA degradation and has an ~10 fold higher affinity for mRNAs with long poly(A) tails (> 150 nt) (18,28,29). Thus, if HuD is required for the branch-selective expression of CaMKIIα, then we would predict that deletion of HuD binding sites from the 3'UTR of CaMKIIα would eliminate the polarized expression of CaMKIIα. Since CaMKIIα mRNA has 35 HuD binding sites (30) we turned to a reporter construct coding for myristoylated (myr) destabilized GFP (dGFP) fusion to the dendritic targeting sequence within the 3'UTR of CaMKIIα that contains 8 overlapping HuD binding sites (5,23). As expected, neurons that express this reporter construct show selective expression of myr-dGFP in one daughter branch over the other, having a BVI of ~1 (Figure 6A and B). In contrast, when we express this reporter construct with the HuD binding sites removed, the myr-dGFP signal seems to accumulate at the branch point, reducing the BVI by 55% (Figure 6A and B; myr-dGFP CaMKIIα UTR: 1.00±0.19, myr-dGFP CaMKIIα UTR ΔHuD: 0.45±0.13). These results suggest that HuD mediates the branch-selective expression of CaMKIIα mRNA.

Knockdown of endogenous HuD reduces the branch-selective expression of CaMKIIα

As a further test to assess the relative importance of HuD in mediating CaMKIIα’s branch-specific expression in neurons, we transfected neurons with a short hairpin RNA (shRNA) designed and characterized to reduce or knockdown (KD) HuD mRNA expression (22). To verify that the shRNA was effective at reducing HuD mRNA expression we performed fluorescent in situ hybridization using an antisense probe set specific for HuD mRNA. As expected only neurons transfected with eGFP and the HuD shRNA showed reduced HuD mRNA in the cell body (Figure 7A, right panel, white arrow = nontransfected cells versus outlined transfected cell body). Furthermore, HuD mRNA was reduced by ~66% 72 hours post-transfection in HuD KD neurons when compared to eGFP expressing neurons that were transfected with vector alone (Figure 7A-B; control: 1.00±0.21, HuD shRNA: 0.34±0.12). As a negative control we used a sense probe that did not detect any signal (Figure 7C).

To determine if HuD impacts the subcellular localization of CaMKIIα expression we immunostained control and HuD shRNA expressing neurons with an antibody against CaMKIIα. Notably, the cell body expression was highly variable with no significant overall change between groups of neurons. In contrast, the expression of CaMKIIα showed a downward trend in the primary dendrite and a significant decrease in the secondary branches in HuD shRNA expressing neurons relative to control neurons transfected with vector alone (Figure 7E-G; primary dendrite: control: 1.00±0.1, HuD shRNA: 0.68±0.13; secondary dendrite: control: 1.00±0.08, HuD shRNA: 0.87±0.18). Similar to what we observed with our reporter construct, the branch-
specific expression of CaMKIIα was dramatically reduced by ~56% in HuD KD neurons (Figure 7G). Thus, a decrease in HuD expression results in reduced CaMKIIα polarized expression, with more protein being distributed between daughter branches.

**HuD targets one daughter branch over the other in a single neuron**

We next examined the possibility that HuD itself may be selectively targeted to one daughter branch over the other and hence, mediate the branch-specific expression of CaMKIIα when mTORC1 is active. Since, the antibodies available to detect HuD are not reliable for immunofluorescence, we measured HuD protein with an anti-myc antibody directed against the overexpressed myc tagged HuD protein in hippocampal neurons. Unlike CaMKIIα, total myc-HuD levels do not change with mTORC1 activity, as indicated by the quantification of signal in both the primary and the secondary branches (Figure 8A-C). Surprisingly, the BVI of myc-HuD more than doubles upon mTORC1 inhibition (HuD, DMSO: 1.00 ± 0.16; HuD, Rapa: 2.43 ± 0.37; Figure 8D).

To ensure that HuD overexpression does not increase the diameter of one daughter branch over the other, thus favoring increased protein expression in the larger branch over the smaller branch by diffusion, we measured the diameter of both daughter branches using eGFP in control and HuD overexpressing neurons. There was no significant difference in the diameter between daughter branches within the same neuron when comparing control and HuD expressing neurons (Figure 8E). Collectively, these results suggest that HuD may direct the branch-specific expression of its target mRNAs.

**HuD rescues CaMKIIα protein expression and branch variability when mTORC1 is inhibited**

Next, we examined the possibility that overexpression of HuD would increase CaMKIIα protein and hence restore its BVI when mTORC1 is inhibited. We predicted that CaMKIIα BVI would be maintained in HuD overexpressing neurons in the presence of rapamycin due to the increased targeting of HuD to one daughter branch over the other. Similar to what was observed in Figure 2, CaMKIIα protein levels in the cell body in HuD overexpressing neurons relative to control neurons remained the same regardless of mTORC1 activity (Figure 8F). As predicted, in contrast to the cell body, HuD overexpressing neurons had significantly more CaMKIIα protein in the primary dendrite when mTORC1 was inhibited by rapamycin (Figure 8A and G). Furthermore, in rapamycin treated neurons, HuD restored CaMKIIα expression back to control levels in the secondary branches (Figure 8A and H). Consistent with the increased targeting of HuD when mTORC1 activity was reduced, HuD restored CaMKIIα BVI back to control levels (Figure 8A and I). These results suggest that HuD increases CaMKIIα protein and maintains its BVI when mTORC1 is inhibited.

**Branch-specific expression of CaMKIIα requires the poly(A) tail-binding RNA recognition motif of HuD**

If HuD protects CaMKIIα mRNA from deadenylation in a branch-specific manner, then expressing a truncated form of HuD that does not bind to the poly(A) tail of its targets (18,28,29) will not rescue CaMKIIα BVI. HuD has three RNA recognition motifs (RRM), two of which bind specific HuD binding motifs in the mRNA sequence of its targets. The third RRM binds the poly(A) tail of its mRNA targets (28,29). Since shortening of the CaMKIIα poly(A) tail leads to mRNA degradation, we examined whether the 3rd RRM and linker region of HuD are required to mediate the HuD-dependent rescue of CaMKIIα BVI when mTORC1 is inhibited. Indeed, overexpression of a HuD construct lacking the 3rd RRM (HuD I+II) alone did not block the reduction of CaMKIIα protein or BVI as had the full-length protein when mTORC1 is inhibited (Figure 8A and F-I). Interestingly, in HuD I+II expressing neurons CaMKIIα levels are high when mTORC1 is active and the expression of HuD remains polarized (myc HuD: Figure 8C and D; CaMKIIα: Figure 8G-H). Unexpectedly, HuD’s ability to mediate branch-specific expression of CaMKIIα is greatly reduced (red arrow; Figure 8A and I). These findings suggest that binding of the poly(A) tail to the 3rd RRM of HuD underlies the differential expression of CaMKIIα between daughter branches.
HuD directs CaMKIIα expression in specific dendritic branches

Since HuD mediates the polarized expression of CaMKIIα mRNA, we hypothesized that CaMKIIα mRNA local translation is also branch-specific. To test our hypothesis, we performed fluorescence recovery after photobleaching (FRAP) of our reporters—destabilized, myristoylated GFP (dGFP) that is fused to the 3' UTR of CaMKIIα, in which HuD binding sites were maintained (myr-dGFP-CaMKIIα UTR+HuD) or removed (myr-dGFP-CaMKIIα UTRΔHuD) (Figure 6) (23, 24, 31). We then measured the translation-dependent recovery from photobleaching of each reporter construct. An increase in fluorescence during recovery represents newly synthesized dGFP protein (Figure 9A; red arrows) (31). Because dGFP contains a myristoylation site, which tethers the protein to the membrane and thus limits protein movement, increased dGFP fluorescence is due to local protein synthesis and not protein diffusion (23, 24, 31). Removal of HuD binding sites did not alter the total expression of new myr-dGFP in the primary and secondary dendrites at 30 minutes of recovery (Primary: HuD = 1.00 ± 0.40; ΔHuD = 1.48 ± 0.31; Secondary: +HuD = 1.00 ± 0.23, ΔHuD = 0.83 ± 0.12; Figure 9B - C). However, dendrite A—a daughter dendrite conventionally assigned as expressing more dGFP with the HuD binding sites contained more dGFP than dendrite A without the HuD binding sites (HuD A = 1.00 ± 0.17; ΔHuD A = 0.44 ± 0.09; Fig 9D). Dendrite B—a daughter dendrite designated as expressing less myr-dGFP—exhibited similar levels of myr-dGFP regardless of the presence of HuD binding sites (+HuD B = 0.26 ± 0.08; ΔHuD B = 0.30 ± 0.08; new Fig 9C). Additionally, the levels of new myr-dGFP between HuD daughter dendrites A and B are significantly polarized compared to ΔHuD daughter dendrites (HuD A-B: p < 0.0001; ΔHuD A-B: p = 0.8389; One-way ANOVA, Tukey’s multiple comparison test). These findings suggest that between daughter branches the presence of HuD binding sites generally supports the polarized, dendritic translation of myr-dGFP-CaMKIIα mRNA.

DISCUSSION

The temporal and spatial regulation of protein expression is critical for a neuron to modify its synaptic input in an experience-dependent manner (32, 33). “Synaptic tag and capture,” in which proteins are localized in response to strong stimuli at one set of synapses are available to other nearby synapses to facilitate plasticity at both sets of synapses, is thought to underlie long-term plasticity (14). Synapses that are “bound” together and distributed on a single dendritic branch increase the probability that excitatory postsynaptic potential (EPSP) amplification will occur (34-37). Molecular mechanisms that mediate branch-specific expression of proteins that facilitate plasticity are unknown.
Reductionist Model for the Synaptic Tagging and Capture Hypothesis

Here we use a simplified model where dendritic mTORC1 is active and can be inhibited with rapamycin, to ask if phosphorylated mTORC1 can serve as a tag. We determined that mTORC1 is active in both daughter branches by NMDAR activity (Figure 1), a specified requirement to serve as a tag during late-stage plasticity (15). Interestingly, NMDAR/mTOR activity only mediates the selective expression of the plasticity related protein CaMKIIα in one branch. We have discovered that HuD, the RNA-binding protein that has been previously characterized to stabilize CaMKIIα mRNA and promote its translation (38), mediates its branch-specific expression by targeting its mRNA to one daughter branch over the other. Deletion of the HuD binding sites in the 3’UTR of CaMKIIα mRNA removes its branch-specific local expression (Figure 6 and 9).

We show that degradation of CaMKIIα mRNA occurs through deadenylation when mTORC1 activity is reduced and may explain the rapamycin-dependent reduction in CaMKIIα branch variability. Remarkably, HuD overexpression protects and rescues the rapamycin-reduction in CaMKIIα protein, confirming that HuD is limited when mTORC1 activity is reduced (5). As predicted, overexpression of HuD I+II, notably missing the 3rd RRM that binds to the poly(A) tail, fails to rescue CaMKIIα protein and BVI reduction when mTORC1 is inhibited. Although, our evidence is strong for changes in mRNA abundance reflecting changes in protein expression, we cannot discount the fact that there may be corresponding changes in CaMKIIα protein stability. It should be noted that mTORC1 inhibition also promotes autophagy (39), suggesting the mTORC1 is an important signaling pathway in protein homeostasis.

One of the most surprising results herein, is that the absence of HuD binding to the poly(A) tail results in the equal distribution of CaMKIIα protein in both daughter branches, suggesting that poly(A) binding is required to mediate CaMKIIα branch-specific expression. These results lead to the intriguing possibility that the length of the poly(A) tail of plasticity related mRNAs may serve as the bait for HuD capture and branch-selective expression.

Redondo and Morris have suggested that there may be multiple tags that can facilitate synaptic capture of plasticity related proteins (15). Consistent with this idea, previous reports have suggested that CaMKIIα itself serves as a tag. Notably, inhibiting the phosphorylation of CaMKIIα prevents L-LTP, a requirement to serve as a tag (40). Although we did not detect CaMKIIα in both branches, blocking CamKIIα activity in activated synapses where it is localized may be sufficient to block long-term changes in plasticity. Through the discovery of HuD as the RNA-binding protein that mediates CaMKIIα expression herein, future experiments may help elucidate the tag-plasticity related protein interactions in more complex systems.

The question of how specific mRNAs target activated synapses is perplexing. It has been suggested that neuronal RNPs patrol a group of synapses (41). Consistent with this idea, bidirectional movement of mRNAs in dendrites has been observed (41-45). Global mRNA “exploration” may be required for the local protein synthesis at stimulated synapses during early events that set the stage for long-term plasticity (46,47). Interestingly, HuD protein levels increase with neuronal/mTORC1 activity (5,48) and the protein is targeted in a branch-specific manner. Collectively these data suggest that HuD is a good candidate to target the mRNAs coding for proteins required to strengthen neighboring synapses to facilitate late-stage plasticity.

In summary, our previous study, demonstrating that HuD can switch target mRNAs from CaMKIIα when mTORC1 is active to Kv1.1 when mTORC1 is inhibited, combined with these findings, suggest that HuD’s branch-specific expression may be what “captures” mRNAs to specifically shuttle and stabilize them in one daughter branch based on their affinity and abundance. How HuD protein targets one daughter branch over the other in a single neuron is yet to be determined. However, what is clear is that the mRNA that it captures, be it CaMKIIα mRNA when mTORC1 is active or Kv1.1 mRNA when mTORC1 activity is reduced, will depend on the level of mTORC1 activity, serving as the tag and dictating the strength of the synapse (5,41).
References

HuD directs CaMKIIα expression in specific dendritic branches


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**Figure 1. NMDAR signaling leads to mTORC1 activity throughout the dendritic arbor.**

(A) Cartoon of neuron (above) shows where puncta signal intensity measurements were taken for quantitative analysis throughout all figures. Boxed regions (primary branch: green, daughter branch A:
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yellow and daughter branch B: white) represent 10µm segments prior to and after branch point used for analysis. Immunostaining of Phospho-S6 (P-S6) hot spots in DMSO (carrier) and Rapamycin (200nM) treated cultured hippocampal neurons. Cell body images were taken at with a lower gain relative to dendrites to avoid saturation of signal (left). MAP2 expression (not shown) was used to outline the dendrites of the representative neurons. Colored arrows refer to the corresponding blown up region of the dendrite that is outlined with the dotted line in the same color to the right. Images were pseudocolored and subject to 3-D rendering was achieved using the Interactive 3D surface plot plugin in ImageJ to demonstrate differences in signal intensity. Scale bar= 10 µm.

(B) P-S6 puncta signal intensity was measured in the cell body and normalized by area for DMSO and Rapamycin treated neurons. Note, P-S6 puncta intensity decreases with rapamycin treatment in the cell body. *<0.05 by unpaired Student’s t-test. DMSO: n=11 neurons, Rapa: n=12 neurons. (C) P-S6 puncta signal intensity was measured in a region 10 µm before the primary branch point of DMSO and Rapamycin treated neurons. DMSO: n=13 primary dendrites, Rapa: n=17 primary dendrites.

(D) P-S6 puncta signal intensity was measured in a region 10 µm after the primary branch point of DMSO and Rapamycin treated neurons. *<0.05 by unpaired Student’s t-test. DMSO: n=26 secondary branches, Rapa: n=24 secondary branches.

(E) Change in mTORC1 activity between daughter branches was determined by counting the number of P-S6 puncta in each daughter branch/ 10µm area after each branch point and using the equation: ΔP-S6 hotspots = |Daughter Branch A/area – Daughter Branch B|. DMSO: n= 16 and Rapa: n=14 daughter branch pairs.

(F-J) Cultured hippocampal neurons were treated with Vehicle (H2O) or AP5 (100µM) and immunostained for P-S6 puncta. MAP2 (not shown) expression was used to outline the dendrites of the representative neurons. Quantification was performed on non-saturated images of cell bodies. Quantification of P-S6 puncta was performed as described above for the rapamycin treatment. Images were pseudocolored and 3-D rendering was achieved using the Interactive 3D surface plot plugin in ImageJ. Scale bar=10 µm. Vehicle: n=19 cell bodies, 19 primary branches, 39 secondary branches, and 20 paired daughter branches; AP5: n=16 cell bodies, 18 primary branches, 31 secondary branches, and 17 paired daughter branches.

Figure 2. NMDAR and mTORC1 activity lead to branch-specific expression of CaMKIIα protein levels which is reduced with NMDAR/mTORC1 blockade.

(A) eGFP expressing cultured hippocampal neurons were treated with DMSO or rapamycin and immunostained for CaMKIIα. Representative neurons are shown. Colored arrows refer to the corresponding blown up region of the dendrite that is outlined with the dotted line in the same color to the right. CaMKIIα images were pseudocolored and 3-D rendering was achieved using the Interactive 3D surface plot plugin in ImageJ to demonstrate differences in signal intensity. Scale bar= 10 µm.

(B) The signal intensity of CaMKIIα in the cell body of neurons treated with DMSO or rapamycin was measured as a ratio over eGFP. Only unsaturated cell bodies were used for the quantification. DMSO: n=18, Rapa: n=13 neuronal cell bodies.

(C-D) The signal intensity of CaMKIIα in neurons treated with DMSO or rapamycin was measured as a ratio over eGFP. Note, CaMKIIα protein decreases with rapamycin treatment. *<0.05, ***<0.005 by unpaired Student’s t-test. Primary branch: DMSO: n=21; Rapa: n=24. Secondary branch: DMSO: n=72; Rapa: n=71.
HuD directs CaMKIIα expression in specific dendritic branches

(E) Branch variability index (BVI) was determined by normalizing all signals by eGFP within the same branch and using the equation: BVI= |Daughter Branch A – Daughter Branch B| / Averaged Control BVI. Note CaMKIIα BVI is reduced when mTORC1 is inhibited with rapamycin. ***<0.005 by unpaired Student’s t-test. CaMKIIα: DMSO: n=33 and Rapa: n=29 paired daughter branches.

(F) eGFP expressing cultured hippocampal neurons were treated with Vehicle or AP5 and immunostained for CaMKIIα. Representative neurons are shown. CaMKIIα images were also pseudocolored and 3-D rendering was achieved using the Interactive 3D surface plot plugin in ImageJ. Scale bar= 10 µm.

(G) The signal intensity of CaMKIIα in the cell body of neurons treated with vehicle or AP5 was measured as a ratio over eGFP. Vehicle: n=16, AP5: n=14 neuronal cell bodies.

(H) The signal intensity of CaMKIIα in neurons treated with Vehicle or AP5 was measured as a ratio over eGFP, 10 µm before the branch point. Note, CaMKIIα protein decreases with AP5 treatment. *<0.05 by unpaired Student’s t-test. Vehicle: n=14, AP5: n=12 primary branches.

(I) The signal intensity of CaMKIIα in neurons treated with Vehicle or AP5 was measured as a ratio over eGFP, 10 µm after the branch point. **<0.01 by unpaired student t-test. Vehicle: n=28 branches, AP5: n=26 branches.

(J) Branch variability index (BVI) was determined by normalizing all signals by eGFP within the same branch and using the equation: BVI= |Daughter Branch A – Daughter Branch B| / Averaged Control BVI. Note that CaMKIIα BVI is reduced when NMDAR is inhibited with AP5. *<0.05 by unpaired Student’s t-test. Vehicle: n=14, AP5: n=11 paired daughter branches.

Figure 3. Branch-specific expression of Kv1.1 does not require mTORC1 activity.

(A) Representative images of neurons treated with DMSO (control) or Rapamycin and immunostained for Kv1.1. Colored arrows refer to the corresponding blown up region of the dendrite that is outlined with the dotted line in the same color to the right. Note that the dendritic expression of Kv1.1 increases with mTORC1 inhibition. Kv1.1 images were also pseudocolored and 3-D rendering was achieved using the Interactive 3D surface plot plugin in ImageJ to demonstrate change in signal intensity. Scale bar=10 µm.

(B-D) Quantification of Kv1.1 signal intensity normalized by eGFP in the cell body (B) as well as the primary (C) and secondary dendrites (D). The signal intensity of Kv1.1 in neurons treated with DMSO or rapamycin was measured as a ratio over eGFP, 10 µm before or after the branch point. *<0.05 by unpaired student t-test. Cell body: DMSO: n=11; Rapa: n=9. Primary branch: DMSO: n=11; Rapa: n=9. Secondary branch: DMSO: n=24; Rapa: n=18.

(E) Branch variability index (BVI) was determined by normalizing all signals by eGFP within the same branch and using the equation: BVI= |Daughter Branch A – Daughter Branch B| / Averaged Control BVI. Note that Kv1.1 expression in secondary dendrites is polarized in both control and rapamycin treated neurons. Kv1.1: DMSO: n=12 and Rapa: n=9 paired daughter branches.
Figure 4. mTORC1 activity leads to the branch-specific expression of CaMKIIα mRNA which is reduced with mTORC1 inhibition.

(A) Fluorescent in situ hybridization (FISH) using the CaMKIIα mRNA specific antisense probe. eGFP staining was used to outline the dendrites of the representative neurons shown. Colored arrows refer to the corresponding blown up region of the dendrite that is outlined with the dotted line in the same color to the right.

(B) Fluorescent in situ hybridization (FISH) using a sense probe (negative control) to verify the specificity of the antisense probe used in A. For visualization purposes the images showing the mRNA puncta were dilated once.

(C) CaMKIIα mRNA puncta number was measured in the cell body of DMSO and rapamycin treated neurons and normalized by area. DMSO: n=25 neuronal cell bodies, 2000 puncta; Rapa: n=21 neuronal cell bodies, 1544 puncta.

(D) CaMKIIα mRNA puncta number was measured in the primary branch of DMSO and rapamycin treated neurons. Note that puncta number is reduced with rapamycin treatment. **<0.01 by unpaired Student’s t-test. DMSO: n=45 primary branches, 212 puncta, Rapa: n=30 primary branches, 68 puncta.

(E) CaMKIIα mRNA puncta number was measured in the secondary branch of DMSO and rapamycin treated neurons. Note that puncta number is reduced with rapamycin treatment. **<0.01 by unpaired Student’s t-test. DMSO: n= 92 secondary branches, 185 puncta, Rapa: n= 60 secondary branches, 36 puncta.

(F) Branch variability was determined by counting the number of CaMKIIα mRNA puncta in each daughter branch. Quantification of the difference in puncta number between the two branches is shown. *<0.05 by unpaired Student’s t-test. DMSO: n=45 paired daughter branches, Rapa: n=30 paired daughter branches.

(G) CaMKIIα mRNA puncta signal intensity was measured in DMSO and rapamycin treated neurons from both the primary (10 µm before the branch point) and the secondary (10 µm after the branch point) branches. DMSO: n=17 neurons, 26 dendrites; Rapa: n=16 neurons, 30 dendrites.

Figure 5: mTORC1 inhibition degrades CaMKIIα mRNA by deaddeylation of its poly(A) tail.

(A) Neurons treated with rapamycin for 0, 60, 90, and 180 min were harvested from cultured neurons and PCR was performed with specific primers. Above, schematic of CaMKIIα internal primers (a and b) and primers that recognize the poly(A) tail of CaMKIIα. Note that CaMKIIα poly(A) tail is deadenylated due to mTOR inhibition as indicated by the reduced poly(A) tail length and intensity of the PCR product, over time.

(B) As a control, PCR was performed following RNase H treatment to validate amplification of the PCR product containing the poly(A) tail.

(C) PCR was performed using Kv1.1 specific primers and primers that recognize the poly(A) tail of Kv1.1. Note that neither the poly(A) tail or the mRNA levels of Kv1.1 change in rapamycin treated neurons.
Figure 6: Deletion of the HuD binding motifs in the 3’ untranslated region of CaMKIIα mRNA prevents its branch specific expression.

(A) Representative neurons transfected with myr-dGFP-CaMKIIα UTR or myr-dGFP-CaMKIIα UTRΔHuD and stained for GFP. Colored arrows refer to the corresponding blown up region of the dendrite that is outlined with the dotted line in the same color to the right.

(B) Quantification of neurons transfected with myr-dGFP-CaMKIIα UTR or myr-dGFP-CaMKIIα UTRΔHuD and stained for GFP. Branch variability index (BVI) was determined by measuring GFP signal intensity in each daughter branch followed by using the equation: BVI= |Daughter Branch A – Daughter Branch B|/Dendritic Area. *<0.05 by unpaired Student’s t-test. myr-dGFP-CaMKIIα UTR, myr-dGFP-CaMKIIα UTRΔHuD: n=15 paired daughter branches.

Figure 7: Knockdown of endogenous HuD decreases the Branch-specific expression of CaMKIIα.

(A) Representative images of neurons co-transfected with control vector or shRNA against HuD and eGFP cDNA. 72 hours post-transfection neurons were fixed and HuD mRNA was detected using an antisense probe set against HuD mRNA. Notice strong detection of HuD mRNA containing granule structures in the cell bodies of control eGFP neuron (left, white dotted outline of cell body) compared to HuD shRNA (right, white dotted outline of cell body) eGFP neuron. Note that untransfected neurons in HuD KD panel still express HuD mRNA as indicated by red in situ signal (white arrows). For visualization purposes the images showing the mRNA puncta were dilated twice.

(B) Quantification of the fold change of reduced HuD mRNA containing puncta per area as determined by eGFP signal in the cell body of control and shRNA expressing neurons. Note the ~65% reduction in HuD mRNA detection in shRNA expressing neurons.

(C) Representative neurons showing specificity of HuD antisense probe set. Note red in situ puncta signal only with antisense probe set.

(D) Representative images of CaMKIIα expression in control and HuD shRNA expressing neurons. Colored arrows refer to the corresponding blown up region of the dendrite that is outlined with the dotted line in the same color to the right. CaMKIIα images were also pseudocolored and 3-D rendering was achieved using the Interactive 3D surface plot plugin in ImageJ.

(E) The signal intensity of CaMKIIα in the cell body of control and HuD shRNA expressing neurons measured as a ratio over eGFP.

(F) Quantification of CaMKIIα expression in the primary branch 10µm prior to branch point.

(G) Quantification of CaMKIIα expression in the secondary branch. Note, the non-significant trend in reduced CaMKIIα protein levels in the primary dendrite.

(H) Branch Variability Index (BVI) for control and HuD shRNA expressing neurons. Note the significant reduction in BVI in HuD shRNA neurons compared to control neurons. Significance determined by Student t-test, *p<0.05. Error bars represent SEM. Cell Bodies: control n=17, HuD KD n=10; Primary dendrites: control n= 26, HuD KD n=13; Branch Pairs: control n= 28, HuD KD n=13.
Figure 8. Differential branch expression of full-length myc-HuD but not its truncated form missing the domain that binds the poly(A) tail rescues CaMKIIα branch variability when mTORC1 is inhibited.

(A) Above, schematic showing HuD constructs used. Cultured hippocampal neurons were transfected with pcDNA+eGFP, pCHuD+eGFP, or pCHuD I+II+eGFP and treated with either carrier (DMSO) or rapamycin. Representative neurons immunostained for CaMKIIα and myc-HuD are shown. CaMKIIα and myc-HuD images were also pseudocolored and 3-D rendering was achieved using the Interactive 3D surface plot plugin in ImageJ. Yellow arrow indicates daughter branch with more HuD and CaMKIIα protein relative to the other daughter branch (white arrow). Scale bar= 10 µm.

(B) Primary (10 µm before branch point) dendritic myc-HuD protein was measured as a ratio over eGFP. HuD DMSO: n= 11 and HuD Rapa: 13 primary dendrites. HuD I+II DMSO: n=15 primary branches; HuD I+II Rapa: n=10 primary branches. **<0.01; ***<0.005 by One way ANOVA Newman-Keuls post-test.

(C) myc-HuD protein in secondary, daughter branches (10 µm after the branch point) was measured as a ratio over eGFP. HuD DMSO: n= 22 and HuD Rapa: n=26 secondary branches. HuD I+II DMSO: n=30 secondary branches; HuD I+II Rapa: n=20 secondary branches. ***<0.005 by One way ANOVA Newman-Keuls post-test.

(D) The difference between myc-HuD/eGFP protein was measured between daughter branches and normalized to myc-HuD DMSO and graphed as BVI. Note the increased BVI for myc-HuD between daughter branches in rapamycin treated neurons. **<0.01 by One way ANOVA Newman-Keuls post-test. HuD DMSO: n=11 and HuD Rapa: 11 paired daughter branches. HuD I+II DMSO: n=15 and HuD I+II Rapa: n=10 paired daughter branches.

(E) The difference in diameter between daughter branches of neurons transfected with eGFP+pcDNA or eGFP+myc-HuD was measured using eGFP. Note there is no significant difference in branch diameter between pcDNA and myc-HuD neurons. pcDNA: n=10 and HuD: n=20 secondary branches.

(F) CaMKIIα protein was measured in the cell body of HuD or HuD I+II transfected neurons that were then DMSO or rapamycin treated. HuD DMSO: n=27, HuD Rapa: n=24, HuD I+II DMSO: n=16, HuD I+II Rapa: n=15. *<0.05 by One way ANOVA Newman Keuls post-test.

(G) Primary dendritic CaMKIIα protein (10 µm before branch point) was measured as a ratio over eGFP. Note, HuD rescues reduced CaMKIIα levels in rapamycin treated neurons. #<0.05 and ##<0.01 significantly different from pcDNA DMSO by one sample t-test. *<0.05; ***<0.005 by One way ANOVA Newman Keuls post-test. HuD DMSO: n=26 and HuD Rapa: 25 primary dendrites. HuD I+II DMSO: n=19 and HuD I+II Rapa: n=14 primary dendrites. The dark gray bar represents the mean±S.E.M of pcDNA DMSO neurons, as determined in Figure 2. The light gray bar represents the mean ±S.E.M of pcDNA Rapa neurons, as determined in Figure 2.

(H) CaMKIIα protein in secondary, daughter branches (secondary branch; 10 µm after the branch point) was measured as a ratio over eGFP. **<0.01 and ***<0.005 by One way ANOVA Newman Keuls post-test. HuD DMSO: n=78 and HuD Rapa: 78 secondary branches. HuD I+II DMSO: n=36 and HuD I+II Rapa: n=30 secondary branches.

(I) The absolute value of the difference between CaMKIIα/eGFP protein was measured between daughter branches and normalized to pcDNA DMSO BVI and graphed as BVI. Note, HuD rescues reduced
CaMKIIα BVI when mTOR is inhibited. In addition, removing the linker region and 3rd RRM significantly reduces the branch-specific expression of CaMKIIα when mTORC1 is active, red arrow.

*<0.05 and **<0.01 by One way ANOVA Newman Keuls post-test. HuD DMSO: n = 36 and HuD Rapa: n= 44 paired daughter branches. HuD I+II DMSO: n=18 and HuD I+II Rapa: n=15 paired daughter branches.

**Figure 9: Fluorescence recovery after photobleaching of myristoylated dGFP-CaMKIIα UTR** demonstrates that HuD binding sites facilitates branch-specific local synthesis.

A) Representative images of dendrites expressing myristoylated destabilized GFP (myr-dGFP) fused to CaMKIIα-UTR, in which HuD binding sites are maintained (HuD, left) or deleted (ΔHuD, right). dGFP fluorescence of the same dendrites before (top), immediately after (0 minutes), and 30 minutes after photobleaching. Bottom panel is overlay of dGFP at 0 minutes (green) and 30 minutes (red). Yellow indicates unbleached dGFP. After 30 minutes of recovery, myr-dGFP-CaMKIIα-UTR HuD display polarized translation of dGFP while myr-dGFP-CaMKIIα-UTRΔHuD show approximately equal dGFP translation. Red arrowheads indicate GFP signals that disappear or are reduced immediately after bleaching (time 0) and reappear 30 minutes after recovery. dGFP signals are overlaid on their respective dendrites (gray). Scale bar = 10 μm.

(B) Deletion of HuD binding sites does not affect the total expression of new myr-dGFP in primary (HuD = 1.00 ± 0.40; ΔHuD = 1.48 ± 0.31; p = 0.35) and secondary (HuD = 1.00 ± 0.23; ΔHuD = 0.83 ± 0.12; p = 0.55) dendrites 30 min after bleaching.

(C) Deletion of HuD binding sites reduces polarized translation between daughter dendrites that emerge from a single primary dendrite. Dendrite A is assigned to a daughter dendrite that expresses more dGFP, and dendrite B is designated to a daughter dendrite that expresses less dGFP. Data points of designated A and B in a single neuron are connected by dotted lines (HuD A = 1.00 ± 0.17; HuD B = 0.26 ± 0.08; ΔHuD A = 0.44 ± 0.09; ΔHuD B = 0.30 ± 0.08). At 30 min after recovery, HuD daughter dendrites display more polarized expression of new dGFP than ΔHuD daughter dendrites (+HuD A-B; p < 0.0001; ΔHuD A-B: p = 0.8389; One-way ANOVA, Tukey’s multiple comparison test). Among designated dendrite As (highly expressing daughter dendrite) with HuD binding sites contain more dGFP than ΔHuD expressing cells (HuD A = 1.00 ± 0.17; ΔHuD A = 0.44 ± 0.09; p < 0.006, One-way ANOVA, Tukey’s multiple comparison test).

(E) New myr-dGFP protein synthesis is observed after 6 min of recovery in both reporter constructs (HuD = 105.9 ± 1.3%; ΔHuD = 106.2 ± 1.3%; #, p < 0.006 by Student’s t test compared to baseline (dashed line)) Baseline is fluorescence intensity immediately after photobleaching (time = 0 min, F0). Removing HuD binding sites abrogates the polarized new translation of myr-dGFP between daughter dendrites (HuD slope = 0.40 ± 0.11; ΔHuD slope = 0.01 ± 0.06). Slopes were determined by linear regression analysis; §, p < 0.003. 30 min after photobleaching, HuD daughter dendrites exhibit greater polarity in myr-dGFP expression compared to ΔHuD daughter dendrites (HuD = 114.5 ± 2.4%; ΔHuD = 106.1 ± 1.0%; *, p < 0.003)

(F) Deletion of HuD binding sites reduces dendritic BVI of new myr-dGFP protein after 30 min of recovery (HuD = 1.00 ± 1.5; ΔHuD = 0.48 ± 0.1; p < 0.007). n = 11 neurons for myr-dGFP- CaMKIIα UTR HuD. n = 10 for myr-dGFP- CaMKIIα UTR ΔHuD. Statistical analyses for B, C and E: Student’s t test.
Figure 1 (Sosanya et al)
Figure-2 (Sosanya et al.)
Figure-3 (Sosanya et al.)
Figure 4 (Sosanya et al.)
A

CaMKIIα

\[ \overset{\text{A}\cdots}{\overset{a}{\overset{b}{\overset{\sim}{175\text{bp}}}}}} \]

dT12-anchor

Rapamycin

Time (min) 0 60 90 180 ladder

CaMKIIα Poly(A) tail (a+dT12-anchor)

CaMKIIα Internal primers

Dedenylation

B

CaMKIIα poly(A) tail internal RNase H

- + - +

bp

300 250 200

C

ladder Rapamycin

Kv1.1 Poly(A) tail (a+dT12-anchor)

Kv1.1 Internal primers

Time (min) 0 60 90 180

bp

500 400 300 200

Figure-5 (Sosanya et al.)
A

![Image of fluorescence microscopy showing Myr-dGFP and Myr-dGFP-CaMKIIα UTR and Myr-dGFP-CaMKIIα UTR ΔHuD](image)

B

![Bar chart showing Myr-dGFP/Area (Branch Variability Index) for HuD and ΔHuD with BVI and asterisk](image)

Figure-6 (Sosanya et al.)
Figure 9 (Sosanya et al)
Neurobiology:
Mammalian Target of Rapamycin (mTOR) Tagging Promotes Dendritic Branch Variability through the Capture of Ca2+/calmodulin-dependent protein kinase IIα (CaMKIIα)mRNAs by the RNA-binding Protein HuD

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