The Me31B DEAD-box helicase localizes to postsynaptic foci and regulates expression of a CaMKII reporter mRNA in dendrites of Drosophila olfactory projection neurons

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INTRODUCTION

The formation of long-term memory (LTM) and the underlying persistent synaptic changes require protein synthesis at stimulated synapses (Steward and Schuman, 2001; Martin and Zukin, 2006; Sutton and Schuman, 2006; Costa-Mattioli et al., 2009; Richter and Klann, 2009). For this local translation, mRNAs have to be transported in a repressed state from soma to synapse, and then stored at sites where translational activation must occur. Several studies have implicated a role for ribonucleoprotein (RNP) particles in this process (Martin and Zukin, 2006; Sossin and DesGroseillers, 2006; Bramham and Wells, 2007; Banerjee et al., 2009; Mikl et al., 2010). RNP particles, which contain proteins involved in mRNA translation, transport, repression, and decay, can form large complexes that have been identified in neurons of different species by light microscopy (Krichevsky and Kosik, 2001; Barbee et al., 2006; Kiebler and Bassell, 2006; Cougot et al., 2008; Zeitelhofer et al., 2008; Miller et al., 2009; Tubing et al., 2010).

Staufen- and FMRP-containing RNPs in the neurites of cultured Drosophila neurons contain proteins found in P-bodies, somatic RNPs that are sites of mRNA degradation and translational control (Barbee et al., 2006; Parker and Sheth, 2007). The discovery that neuritic RNPs are related to P-bodies has suggested a hypothesis that P-body components may play an important role in the regulation of synaptic mRNAs and thereby in the regulation of synaptic plasticity (Barbee et al., 2006; Cougot et al., 2008). Consistent with this hypothesis, some components of these large neuritic mRNPs including Staufen, Pumilio, Gld-2 and FMRP are required in Drosophila for the formation of LTM (Dubnau et al., 2003; Bolduc et al., 2008; Kwak et al., 2008) and/or dendrite morphogenesis (Lee et al., 2003; Ye et al., 2004). However, for most granule proteins, their localization, traffic, and functions are poorly understood in mature nervous systems.

One such granule protein found in P-bodies, maternal RNA granules, and neuritic RNPs, is a DEAD-box helicase with conserved orthologs from S. cerevisiae (Dhh1), to mammals (RCK/DDX-6) (Navarro et al., 2001; Cougot et al., 2002; Kiebler and Bassell, 2006; Weston and Sommerville, 2006). In yeast and mammalian cells, the protein is required not only for translational repression and mRNA decay (Coller et al., 2002; Minshall and Standart, 2004), but also...
for P-body formation (Andrei et al., 2005; Coller and Parker, 2005). In contrast to these findings, which point to function as a negative regulator of mRNAs, observations in the *C. elegans* germline and in *P. falciparum*, indicates that the ortholog CGH1/DOD1 proteins may have additional roles in stabilizing subsets of mRNAs and protecting them from degradation (Mair et al., 2006; Boag et al., 2008).

The *Drosophila* homolog Me31B is associated with maternal sponge bodies where it appears to repress translation of many granule-associated mRNAs (Nakamura et al., 2001). Me31B is associated with dFMR1/FRMRP in neuritic mRNPs, regulates dendritic morphogenesis in sensory neurons, and, like its human homolog RCK, is required for optimal miRNA function (Barbee et al., 2006; Chu and Rana, 2006). Together, these observations point to a role for Me31B in the repression of dendritically localized mRNAs, their aggregation into transport mRNPs, and in control of locally translated synaptic mRNAs such as CaMKII, which regulate synaptic plasticity (Mayford et al., 1996; Aakalu et al., 2001; Ashraf et al., 2006).

However, many aspects of the above model have not been experimentally tested. It remains to be shown that mRNP aggregates exist in differentiated adult synapses in vivo. Therefore the composition and characteristics of such mRNP particles is unclear. In addition how Me31B in particular, and neuronal mRNP components in general, regulate target dendritic mRNAs also remains largely unknown in vivo.

To address these outstanding questions, we studied the neuronal expression and localization of Me31B in the adult *Drosophila* brain and the effect of Me31B mutations on the expression of synaptic CaMKII reporter protein (Mayford et al., 1996; Aakalu et al., 2001; Ashraf et al., 2006). We chose to analyze neurons and synapses in the olfactory circuit, which is one of the best-understood and genetically accessible neuronal networks in the adult brain (Davis, 2005; Vosshall and Stocker, 2007; Fiala, 2008). Not only is it well characterized in terms of its connectivity and function, but also the plasticity of synapses on projection neurons (PNs), as well as local translation of CaMKII mRNA in PNs, has been associated with olfactory memory (Ashraf et al., 2006; Keene and Waddell, 2007; Fiala, 2008).

Our data show: (i) that Me31B is expressed widely in the nervous system; (ii) that it is present on a postsynaptic particle in vivo; and (iii) that Me31B is required in PNs for the repression of CaMKII reporter mRNA. These observations represent an early step in the process of linking translational control proteins, synaptic mRNP dynamics and local mRNA translation with synaptic plasticity in a well-characterized behavioral circuit.

**MATERIALS AND METHODS**

**DROSOPHILA STOCKS**

Fly stocks were raised at 25°C on standard cornmeal and agar media. Wild-type stocks (*Oregon-R*), *UAS-Me31B* strains, *me31B*Δ2, *FRT40A* (Barbee et al., 2006), and *UAS-myrfGFpΔGAMEFUTR* were from the Ramaswami stock collection. *MZ-317-Gal4, tub-Gal80ts* 2x, and *GH146-Gal4 (on X)* were from Liuqin Luo, V. Rodrigues, and S. G. Gajendra (NCBS, Bangalore). *NP529-Gal4* was from Rachel Wilson and Kei Ito; *NP225-Gal4* from Kei Ito; *UAS-Me31B RNAi 4916R-1 (on X; Barbee et al., 2006) was from the NIG-FLY collection; *UAS-Me31B RNAi 49379* (on 2nd; Dietzl et al., 2007) was from the VDRC stock collection; *UAS-SytGFP, Or83B-Gal4, GH146-Gal4, UAS-mdCD8-GFP, hsFLP, FRT40A, FRT82B, tub-Gal80, FRT82B* and *tubGal80, FRT40a* were from the Bloomington Stock Centre.

**IMMUNOHISTOCHEMISTRY**

Preparations of *Drosophila* adult brains were performed after R. Stocker (Stocker et al., 1990). Decapitated fly heads were fixed in 4% PFA in 1× PBS + 0.2% Triton for 3 h on ice, washed with 1× PBS + 0.2% Triton and brains were dissected in blocking solution (PBS + 0.2% Triton and 5% NGS). Brains were incubated with primary antibodies overnight on 4°C diluted in blocking solution, followed by incubation with secondary antibodies for 3 h at room temperature diluted in blocking solution.

For preparations of *Drosophila* antenna whole flies were fixed in 4% PFA in PBS + 3% Triton for 1 h on ice. Antenna were cut off and fixed for an additional 4 h on ice. The antenna were washed quickly with 1× PBS + 3% Triton for 3× 15 min, followed by a 2-day wash on 4°C. Incubation with primary antibodies in 1× PBS + 0.1% triton over night at 4°C and incubation with secondary antibody as well in 1× PBS + 0.1% triton over night at 4°C.

Primary antibodies used: mouse anti-Me31B (Nakamura et al., 2001), rabbit anti-GFP (Molecular Probes), goat anti-GFP FITC conjugated and chicken anti-GFP (Abcam), rabbit anti-Pacman (S. Newbury; Barbee et al., 2006; Hillebrand et al., 2007; Zabolotskaya et al., 2008), rabbit anti-Dcp1 (J. Wilhelm; Barbee et al., 2006; Hillebrand et al., 2007; Lin et al., 2008; Zabolotskaya et al., 2008), rabbit anti-Stauken (D. St Johnston; St Johnston et al., 1991), mouse anti-NC82 (Developmental Studies Hybridoma Bank), rabbit anti-CaMKII (L. Griffith; Koh et al., 1999), and mouse anti-CaMKII (S. Ohsako; Takamatsu et al., 2003).

Secondary antibodies used: Alexa 488-, Alexa 555-, and Alexa 647-conjugated anti rabbit and anti mouse IgG (Molecular Probes). Preparations were mounted in Vectashield Mounting Medium (Vetc Labs) and imaged on a Zeiss LSM 510-meta confocal microscope.

**IMAGE ACQUISITION**

All images were taken on a Zeiss LSM 510 confocal microscope. To image mRNP particles in fixed brain tissue, we used a 63× objective (Zeiss Plan-Apochromat, 1.4 Oil Ph3) and the “digital zoom” software feature of the LSM 510 software (zoom factor between 3 and 4) that allows to scan a region of interest at a higher pixel resolution, constrained of course by the normal ~300 nm resolution limit of light microscopy. Regions of particular interest were selected in Photoshop and presented in a way to allow a better evaluation by the reader.

**MARCM**

For the induction of MARCM clones *Drosophila* crosses of the respective genotype were raised at 25°C and the flies were transferred to a new vial every 4 h for timed egg collections. A heat-shock pulse at 37°C in a water bath for 1 h was given at optimal time points to induce single-cell clones in PNs projecting to the DL-1 glomerulus. The time points (between 0 and 60 h after larval hatching) were chosen after G. Jefferis (Jefferis
et al., 2001). Heat-shocked larvae were transferred back to 25°C, raised to adulthood and the adult flies were dissected 1–4 days after eclosion.

AUTOMATED SPOT COLocalIZATION ANALYSIS

We created “Spotnik” a MatLab plug-in which allowed us to automate the quantitative analysis of spot colocalization across red and green channels of a double-stained image. The details of the signal-processing algorithms used have been described elsewhere (Pan et al., 2010). However, the main steps and logic were as follows.

Spot region extraction

Given that spots can be seen in regions of the image with highly variable levels of local background labeling, a direct image thresholding tool does not allow spots to be identified. Thus, we created a spot extraction tool that sharpened and enhanced details of the image, using a Wiener filter coupled with a gamma operation, and then modified the contrast of the image such that local features have the same contrast independent of their local background. The image was now thresholded with a user-defined threshold, to create a mask that included regions well-contrasted from the background. This mask was then used to select regions of pixels from the original sharpened image for subsequent processing.

Spot detection and analysis

Individual regions defined by the mask are first normalized to remove the background (by fitting a local plane to the background). A subsequent modeling step identified Gaussian parameters for each spot in each identified region using a Split-Merge Estimation-Maximization technique (Ueda et al., 2000) and synthesized Gaussians at their modeled positions. The center of these spots and their variances (widths) are then recorded for analysis. At each step, we ensured that manually detected spots were not lost and that computationally detected spots were not artifactual.

Colocalization analysis

Several different types of colocalization analysis are possible. However, for each spot imaged in one spectral channel, a simple MatLab tool allowed us to compute the distance of the closest spot in the other channel. Plots were then created in which the percent of spots that have corresponding closest spots closer than a given distance was plotted against distance.

RESULTS

Me31B IS EXPRESSED IN THE ADULT BRAIN

The expression of neuronal Me31B/DDX6/RCK/Dhh1/Cgh1 and the composition of associated neuronal mRNPs has been previously examined in dendrites of primary cultured neurons (Barbee et al., 2006; Cougot et al., 2008; Zeitelhofer et al., 2008; Miller et al., 2009). In order to extend these studies to an in vivo system, we used confocal microscopy and immunohistochemistry to examine Me31B expression in adult Drosophila brains, focusing primarily on the well-studied olfactory system (Figure 1A).

Me31B is ubiquitously expressed throughout the brain (Figure 1B). Closer examination of Me31B expression in the antennal lobes (AL) area showed punctate expression in cell bodies and in the neuropil (synaptic) area of the AL (Figure 1C).

Combining anatomical information with genetic markers for specific neuronal cell types, we directly confirmed Me31B expression in olfactory PNs (Figure 1D), local interneurons (LNs), and mushroom-body (MB) Kenyon cells (Figure 1E). In addition to neurons, Me31B was also strongly expressed in glial cells, which sent processes into the AL (Figure 1F). In control experiments,
we confirmed that the anti-Me31B antibodies did not label cell bodies or synapses of me31B null mutant neurons (see Figure 6; Figure S1 in Supplementary Material). Thus, Me31B is present in both somatic and synaptic particles.

We further attempted to examine the composition of these particles and their relationship to P-bodies, cytoplasmic mRNP aggregates that mediate specific forms of translational repression and mRNA decay (Eulalio et al., 2007a; Parker and Sheth, 2007).

**SOMATIC AND SYNAPTIC Me31B FOCI DIFFER IN THEIR COMPOSITION**

In cultured Drosophila neurons, Me31B-positive mRNPs in neurites are related to P-bodies based on the high frequency at which they contain various P-body markers, including the RNA decapping enzymes Dcp1 and Pcm (the Drosophila homologue of the 5′–3′ RNA exonuclease Xrn-1) (Barbee et al., 2006). We asked how these neuritic mRNPs compared with Me31B containing particles in vivo, by examining Me31B-positive foci in the adult Drosophila brain for presence of the P-body markers Dcp1 and Pcm as well as the neuronal granule/transport mRNP marker Staufen, which may also be a component of P-bodies (Anderson and Kedersha, 2006; Eulalio et al., 2007a). In neuronal cell bodies, double-immunostained to visualize Me31B and P-body markers, we observed that a very large fraction of the Me31B-positive, cytoplasmic foci also contained each of the three proteins (Figure 2). This indicates that the Me31B foci in neuronal soma are similar to P-bodies and to mRNP granules in neurites observed in primary cell culture (Barbee et al., 2006).

In contrast to particles in the soma, Me31B foci in the synaptic neuropil areas appeared to be distinct and to only rarely contain the P-body markers tested (Figure 3). Thus, although Dcp1, Pcm, or Stau-labeled foci were present in the neuropil, these showed relatively weak colocalization with Me31B.

In order to rigorously assess levels of colocalization, we developed an algorithm, tentatively named Spotnik that allowed us to efficiently identify foci in one spectral channel and to measure the distance to the closest foci in the other spectral channel. Two key features of this program were: (a) local field thresholding, which

![Image of Me31B foci in neuronal soma containing Staufen and P-body associated mRNA hydrolases.](https://example.com/image.png)
M31B foci are predominantly dendritic in OSNs and PNs.

The rigorous colocalization analysis enabled by Spotnik confirmed that particles labeled with M31B colocalized much more efficiently with Dcp1, Pcm, and Staufen immunostained foci in cell bodies than it did at synapses. For example, based on using a 200-nm cut-off for perfect colocalization, Me31B showed 58% colocalization with Dcp1 in soma, but only 7% at the synapse. Thus, the data indicate that Me31B is present in a class of synaptic particle that lacks RNA degradative enzymes, reminiscent of a class of storage mRNP described recently in the C. elegans germline (Boag et al., 2008; Gallo et al., 2008). Alternative interpretations of these data are considered in the Section “Discussion”.

**Me31B FOCI ARE PREDOMINANTLY DENDRITIC IN OSNs AND PNs**

The observations above suggest an obvious hypothesis in which synaptic Me31B foci represent sites where mRNAs are held repressed until released for stimulation-induced, local translation. To ask whether Me31B particles were enriched in either the presynaptic terminal or dendrites, we performed a series of experiments, which examined the localization of Me31B and Staufen foci in a double-stained olfactory glomerulus. Computationally analyzed levels of colocalization are presented **Figure 4**.
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DL-1 PNs contained Me31B foci (Figure 6A). Loss of these foci in MARCM-generated me31B<sup>Δ2</sup>/me31B<sup>Δ2</sup> mutant PNs confirmed the specificity of Me31B staining (Figure 6B).

Me31B foci were similarly enriched in dendrites of olfactory sensory neurons (OSNs; Figures 5G–I). OSN soma and sensory dendrites in the antenna visualized in Or83bGal4-UAS-GFP (Or83b > GFP) animals contained several Me31B particles. However, OSN presynaptic terminals imaged in the AL of Or83b > SytGFP animals, showed very little overlap with Me31B foci. Indeed, we observed many instances where presynaptic endings revealed by Syt-GFP appeared to surround Me31B labeled particles. Thus, in at least two of the major classes of AL neurons, Me31B foci are predominantly localized to dendrites.

Me31B REGULATES Dcp1 POSITIVE PARTICLES IN PN DENDRITES

In non-neuronal cells, Me31B and its non-fly orthologs are required for the normal assembly of P-body like particles which contain not only Me31B, but also RNA hydrolases such as Dcp1 and Pcm (Barbee et al., 2006; Hillebrand et al., 2007). The observation that most dendritic Me31B-positive particles are not marked by anti-Dcp1 pointed to the possibility that dendritic Dcp1 particles may assemble in an Me31B independent way.
We examined Me31B and Dcp1 in single-cell clones of wild-type or me31b\textsuperscript{25}\textsubscript{2}/me31b\textsuperscript{25}\textsubscript{2} PNs generated using the MARCM technique. CD8-GFP marked, wild-type MARCM clones showed both somatic and dendritic Me31B and Dcp1 particles within areas marked by CD8-GFP (Figures 6A,E and not shown). And as expected, me31b\textsuperscript{25}\textsubscript{2}/me31b\textsuperscript{25}\textsubscript{2} mutant cells lacked Me31b-positive particles.
foci in soma and dendrites (Figures 6B,C). In addition, these cells showed a striking reduction of Dcp1 foci (Figures 6D,F), which is quantified in Figure 6G. Thus, although synaptic Dcp1 foci appear to be largely free of Me31B (or to have particularly low levels of the protein), the integrity of these foci is dependent on Me31B.

Me31B REPRESSES A CaMKII REPORTER mRNA IN PN DENDRITES

The presence of Me31B in postsynaptic particles suggested that the protein might repress synaptic mRNA translation, similar to its role in oskar and bicD regulation. We therefore asked whether loss of Me31B would result in significant upregulation of CaMKII translation in dendrites, much as its loss causes precocious translation of Oskar and BicD in oocytes (Nakamura et al., 2001; Ashraf et al., 2006).

We knocked down Me31B in PNs by coexpressing two double-stranded RNAi constructs that have been previously used to knockdown Me31B in non-neuronal cells (Barbee et al., 2006; Hillebrand et al., 2007) and analyzed the effects of this knockdown on expression of a translational reporter for the CaMKII mRNA (Ashraf et al., 2006) (Figures 7D,E). In the CaMKII reporter, GFP coding sequences are placed under control of the 3′ UTR for CaMKII. When transcribed in PNs, the reporter mRNA is transported to PN dendrites where it is repressed by the miRNA pathway and activated by behavioral experience (Ashraf et al., 2006). Knockdown of Me31B in PNs caused a substantial increase in levels of CaMKII reporter protein in the AL (Figures 7D,E). Thus, Me31B is required in PNs for repression of a synaptically targeted, miRNA regulated mRNA. Me31B repression influences both dendritic and somatic levels of the CaMKII reporter protein; indeed, knockdown of Me31B is associated with roughly two-fold increase in levels of the reporter in PN cell bodies (not shown). This might be expected if Me31B is involved in the repression of CaMKII transcript in the soma in preparation for its dendritic targeting.

DISCUSSION

Experimental observations presented here are of interest because they: (a) provide new information on neuronal mRNPs in vivo; (b) reveal a novel function for Me31B in the regulation of dendritic mRNA expression; and (c) together highlight important aspects of mRNA regulation at synapses.

PROPERTIES AND FUNCTIONAL IMPLICATIONS OF SYNAPTIC ME31B GRANULES OBSERVED IN VIVO

The Me31B/Dhh1p/DDX6/CGH-1 class of DEAD box helicases is associated with many different kinds of mRNP aggregates, including maternal RNA storage granules, P-bodies, stress granules, as well as various granule subtypes observed during...
The depletion of Me31B by RNAi leads to a reduction of Me31B foci in PN cell bodies. Two independent RNAi lines for Me31B were coexpressed with GH146-Gal4 in PNs, which were marked by the expression of UAS-myrGFPCaMKII-3′UTR. The use of tub-Gal80ts allowed a specific temporal expression of the Me31B hairpin constructs in adult flies (crosses were kept on 18°C till late pupal stage and Gal4 expression was induced by switching the crosses to 30°C for 4 days).

(A) Me31B foci in wt PN cell bodies (magenta, arrowheads); insets show both channels for the boxed cell; Genotype for wt: +/+, GH146-Gal4, UAS-myrGFPCaMKII-3′UTR/+; 2x tub-Gal80ts/+.

(B) Reduction of Me31B foci after the expression of Me31B RNAi hairpin constructs in PNs; insets show both channels for the boxed cell; Genotype for RNAi: UAS-me31B-RNAi/+, GH146-Gal4, UAS-myrGFPCaMKII-3′UTR/UAS-me31B-RNAi; 2x tub-Gal80ts/+.

(C–E) Expression of two Me31B RNAi lines in PNs using GH146-Gal4 increases the level of CaMKII translational reporter protein expression (myrGFPCaMKII-3′UTR). (C) Representative image of myrGFPCaMKII-3′UTR expression driven in GH146 Gal4, presented as a false color image (the gray value intensity for the associated color is annotated); tub Gal80 flames which restricts reporter expression to adults; Genotype: +/+, GH146-Gal4, UAS-myrGFPCaMKII-3′UTR/+; 2x tub-Gal80ts/+.

(D) Representative image of myrGFPCaMKII-3′UTR fluorescence quantified in the DA1 glomerulus of antennal lobes of flies expressing two RNAi constructs for Me31B (intensity scale bar in C). Genotype: UAS-me31B-RNAi/+, GH146-Gal4, UAS-myrGFPCaMKII-3′UTR/me31B-RNAi; 2x tub-Gal80ts/+.

(E) myrGFPCaMKII-3′UTR fluorescence quantified in the DA1 glomerulus of antennal lobes of wt and experimental flies in which Me31B is depleted in ∼60% of all PNs. A two-fold increase of CaMKII reporter expression in the DA1 glomerulus is observed following Me31B depletion in adult flies: P < 0.001 (Student’s t-test). Measurements correspond to the average mean intensity of CamKII reporter expression through a confocal stack of the DA-1 glomerulus (8–10 slices) of 10 adult brains analyzed.

C. elegans germline development (Nakamura et al., 2001; Cougot et al., 2004; Andrei et al., 2005; Coller and Parker, 2005; Kedersha et al., 2005; Eulalio et al., 2007b; Boag et al., 2008; Jud et al., 2008; Noble et al., 2008). In addition it is required for the assembly of P-bodies in yeast, Drosophila and mammalian cells (Andrei et al., 2005; Coller and Parker, 2005; Eulalio et al., 2007b; Hillebrand et al., 2007) and as well for the formation of stress granules in mammals (Wilczynska et al., 2005). For these reasons, the punctate distribution of Me31B in postsynaptic dendrites is likely to indicate its presence in a specific type of synaptic mRNP particle. However, unlike Me31B-positive particles described in neurites of cultured Drosophila neurons, synaptic Me31B foci do not appear to contain the RNA hydrolases Dcp1 and Pcm/Xrn-1. Thus, they may be a distinct class of particle, which localize preferentially to postsynaptic dendrites. These represent early images of candidate mRNA storage particles at synapses in vivo (Christie et al., 2009). A paucity of antibodies and the challenging nature of such high-resolution immunocytochemistry in whole brain tissue has so far made it difficult to more completely characterize other components of synaptic Me31B particles as well as to establish whether Dcp1, Pcm, and Stau coexist on the same or different particle in the adult brain. Indeed even our conclusion that Me31B particles constitute a separate class must be qualified by the possibility that the visualization of two apparently distinct particle types arises from an artifact of incomplete antibody penetration into the neuropil.
It is possible that synaptic Me31B particles could be analogous to recently described granules in the C. elegans germline, which contain translationally controlled mRNAs and CGH-1/Me31B but exclude decapping enzymes and the P-body protein PAT1/PAT1 (Boag et al., 2008; Gallo et al., 2008). Immunoprecipitation and further colocalization studies suggest that these granules can also contain PAB-1, ATX-2, or TIA-1, markers of stress granules, which in other systems, contain translation initiation factors together with mRNAs stalled in translational initiation (Kedersha et al., 2005; Gallo et al., 2008). Thus, it is conceivable that Me31B/CGH-1-containing storage particles contain mRNAs stored in a stress-granule like state, in which the resident mRNAs are available for rapid activation (Rajyaguru and Parker, 2008).

The potential separation of storage and degradative particles leads to an attractive model in which individual mRNAs may transition from being available for translational activation in a storage granule, to being targeted for degradation in a P-body like particle. This is supported as well by observations in dendrites of cultured mammalian neurons where a distinct class of RNPs contain the degradative enzyme Xrn1, which is excluded from RNPs supposedly involved in storage (Cougot et al., 2008).

At synapses, a transition between storage and degradation particles may occur by three, non-exclusive, candidate mechanisms: (a) by the remodeling of a storage mRNP to a degradative one through protein exchange; (b) by the initial exit of mRNA from the storage RNP to a translating pool, followed by its subsequent targeting to a degradative particle; or (c) the fusion of the two particles. Recent studies in Drosophila provide a possible mechanism by which a change of proteins in RNP complexes could alter its function. Two related proteins of the Lsm-family, Enhancer of Decapping 3 (EDC3), which is implicated to play a role in mRNA decay, and Trailer Hitch (Tral), which supposedly is involved in mRNA repression, interact at the same domain with the Me31B protein. This suggests that the function of Me31B complexes might be determined by the interaction with specific binding partners (Trischler et al., 2008, 2009). Some support for the second model is provided by the observation that the synthetically localized Arc mRNA is targeted for degradation after its translation is induced by synaptic activity (Bramham et al., 2008) and also by the observation that RCK-positive particles in dendrites of cultured hippocampal neurons are transiently disassembled following BDNF stimulation (Zeitelhofer et al., 2008). Further studies are required to understand how, when, and even whether these transitions of mRNA state occur in synapses and other biological contexts.

FUNCTIONS OF Me31B IN NEURONAL TRANSLATIONAL REPRESSION

Together with many analogous studies in yeast and mammalian cells, previous observations in Drosophila that Me31B is a repressor of maternal mRNA translation, a component of a repression pathway mediated by the bantam microRNA, and a repressor of growth of terminal dendrites, has led to a strong model that Me31B is a translational repressor protein (Nakamura et al., 2001; Coller et al., 2002; Barbee et al., 2006; Chu and Rana, 2006). In contrast, recent studies in C. elegans and P. falciparum have shown that Me31B orthologs, CGH-1 and DOZI, associate with specific mRNAs and protects them from degradation (Mair et al., 2006; Boag et al., 2008).

Our observations in neurons indicate a function for Me31B in repressing translation of a miRNA regulated, dendritically localized reporter mRNA in vivo. This is consistent with two related lines of data. First, it is consistent with the known function for Me31B in repression of miRNA-target genes in Drosophila wing imaginal cells as well as for its human homolog RCK in mammalian cultured cells (Barbee et al., 2006; Chu and Rana, 2006). Second, the correlation we observe between loss of synaptic Dcp1 puncta and upregulation of the CaMKII reporter, is consistent with observations in hippocampal cultured cells, where observed disassembly of “dendritic P-bodies” induced by synaptic stimulation has been proposed to underlie the temporally coincident translation of localized mRNAs (Zeitelhofer et al., 2008).

Thus, we suggest a simple model in which neuronal Me31B, as well as its homologs in other metazoa, mediates the formation of synaptic mRNP particles that contain locally repressed mRNAs. And that synaptic stimulation-induced disassembly of these particles is one aspect of the mechanism of local translational control.

FUTURE DIRECTIONS

One key goal of future studies will be to understand the composition and dynamics of dendritic mRNPs in vivo. This will be aided by genetic techniques to replace endogenous translational control molecules with genetically encoded, fluorescently tagged variants that retain functional and localization patterns of the endogenous proteins. When coupled with procedures to induce local protein synthesis in dendrites, such reagents will allow analysis of functionally relevant particle dynamics in vivo. In addition, by eliminating the need for antibodies whose use may be associated with artifacts of inclusion and exclusion, such reagents may provide more direct insight into the real nature of synaptic mRNPs in vivo.

A second goal is to understand the mechanism by which Me31B regulates expression of CaMKII reporter levels in vivo. Although Me31B has been shown to be required for the miRNA pathway (Barbee et al., 2006; Chu and Rana, 2006) it is also required for other forms of translational repression, for example in S. cerevisiae that does not have miRNAs. Similarly, although the reporter used here is miRNA regulated, the same UTR also has binding sites for translational regulators that may operate independently of miRNAs (Ashraf et al., 2006). Thus, important and linked goals of future studies are to understand mechanisms by which the CaMKII UTR is regulated in dendrites and how Me31B engages with these mechanisms of neuronal translational control.

ACKNOWLEDGMENTS

We thank Prof Veronica Rodrigues and her lab at the NCBS for generous and extensive advice, Akira Nakamura, Eimear Holohan, Cathal McCann, and members of the Ramaswami lab for discussions, advice, and comments on the manuscript. We acknowledge the Kyoto, Szeged, VDRC, and Bloomington Stock Centers, Akira Nakamura, Veronica Rodrigues, S. G. Cajendria, Liqun Luo, and Rachel Wilson for Drosophila stocks; Shunji Ohsako, Leslie Griffith, Sarah Newbury, Jim Wilhelmn, and Lynn Manseau for fly stocks and antibodies. This work, initiated with funds from the National Institute of Drug Abuse of the NIH, was funded by grants from the Science Foundation of Ireland to Mani Ramaswami and Anil Kokaram.
REFERENCES

Aakalu, G., Smith, W. B., Nguyen, N., Jiang, C., and Schuman, E. M. (2001). Dynamic visualization of local protein synthesis in hippocampal neurons. Neuron 28, 1176–1177.

Anderson, P., and Kedersha, N. (2006). General translational repression by activators of mRNA decapping. Cell 122, 875–886.

Coller, J. M., and Parker, R. (2005). Translation repression in human cells requires RCK/p54. PLoS Biol. 4, e210. doi: 10.1371/journal.pbio.0040210.

Chu, C. Y., and Rana, T. M. (2006). Dynamic visualization of local protein synthesis in human cells by microRNA-induced gene silencing. Roe 35, 489–492.

Cox, H., Broadie, M., and Izaurralde, E. (2007a). P bodies are dynamically linked sites of mRNP remodeling. J. Cell Biol. 169, 871–884.

Cox, H., Broadie, M., and Izaurralde, E. (2007b). P bodies: at the crossroads of post-transcriptional pathways. Nat. Rev. Mol. Cell Biol. 8, 9–22.

Cox, H., Broadie, M., and Izaurralde, E. (2007b). P-body formation is a consequence, not the cause of RNA-mediated gene silencing. Mol. Cell. Biol. 27, 3970–3981.

Dietzl, G., Chen, D., Schnorrer, F., Su, K., Bouwmeester, T., Schubiger, G., Thoma, J., Beese, L., and Teo, G. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448, 151–156.

Dubnau, J., Chiang, A. S., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Budsoc, E., Scott, R., Celis, E., and Budnik, V. (2008). Small GTPase Rac1. Dev. Biol. 318, 38–51.

Euler, L., and Aebi, U. (2004). Lysine residues in RNA helicase required for gametogenesis and protection from physiological germline apoptosis in C. elegans. Development 131, 3221–3232.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.

Enomoto, S., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic Acids Res. 32, 1325–1334.
Hillebrand et al. mRNPs in vivo in the Drosophila brains in the hippocampal neurons. Proceedings of the National Academy of Sciences of the USA 107, 13136–13141.

Richter, J. D., and Klann, E. (2009). Making synaptic plasticity and memory last: mechanisms of translational regulation. *Genes Dev.* 23, 1–11.

Sossin, W. S., and DesGroseillers, L. (2006). Intracellular trafficking of RNA in neurons. *Traffic* 7, 1581–1589.

Steward, O., and Schuman, E. M. (2001). Protein synthesis at synaptic sites on dendrites. *Annu. Rev. Neurosci.* 24, 299–325.

St Johnston, D., Beuchle, D., and Nusslein-Volhard, C. (1991). Staufen, a gene required to localize maternal RNAs in the *Drosophila* egg. *Cell* 66, 51–63.

Stocker, R. F., Lienhard, M. C., Borst, A., and Fischbach, K. F. (1990). Neuronal architecture of the antennal lobe in *Drosophila melanogaster*. *Cell Tissue Res.* 262, 9–34.

Sutton, M. A., and Schuman, E. M. (2006). Dendritic protein synthesis, synaptic plasticity, and memory. *Cell* 127, 49–58.

Takamatsu, Y., Kishimoto, Y., and Ohnaka, S. (2003). Immunohistochemical study of Ca2+/-calmodulin-dependent protein kinase II in the *Drosophila* brain using a specific monoclonal antibody. *Brain Res.* 974, 99–116.

Tritschler, F., Braun, J. E., Eulalio, A., Truffault, V., Izaurralde, E., and Weichenrieder, O. (2009). Structural basis for the mutually exclusive anchoring of P body components EDC3 and Traf to the DEAD box protein DDX6/Met31B. *Mol. Cell* 33, 661–668.

Tritschler, F., Eulalio, A., Helms, S., Schmidt, S., Dohmen, M., Weichenrieder, O., Izaurralde, E., and Truffault, V. (2008). A similar mode of interaction enables Trafler Hitch and EDC3 to associate with DCP1 and Met31B in distinct protein complexes. *Mol. Cell Biol.* 28, 6695–6708.

Tubing, F., Vendra, G., Mikl, M., Macchi, P., Thomas, S., and Kiebler, M. A. (2010). Dendritically localized ribonucleoprotein particles that display fast directional motility along dendrites of hippocampal neurons. *J. Neurosci.* 30, 4160–4170.

Ueda, N., Nakano, R., Ghahramani, Z., and Hinton, G. E. (2000). SMEM algorithm for mixture models. *Neural Comput.* 12, 2109–2128.

Voshall, L. B., and Stocker, R. F. (2007). Molecular architecture of smell and taste in *Drosophila*. *Annu. Rev. Neurosci.* 30, 505–533.

Weston, A., and Sommerville, J. (2006). Xp54 and related (DDX6-like) RNA helicases: roles in messenger RNP assembly, translation regulation and RNA degradation. *Nucleic Acids Res.* 34, 3082–3094.

Wilczynska, A., Aigueperse, C., Kress, M., Dautry, F., and Weil, D. (2005). The translational regulator CPEB1 provides a link between dcp1 bodies and stress granules. *J. Cell Sci.* 118, 981–992.

Ye, B., Petritsch, C., Clark, I. E., Gavis, E. R., Jan, L. Y., and Jan, Y. N. (2004). Nanos and Pumilio are essential for dendrite morphogenesis in *Drosophila* peripheral neurons. *Curr. Biol.* 14, 314–321.

Zabolotskaya, M. V., Grima, D. P., Lin, M. D., Chou, T. B., and Newbury, S. F. (2008). The 5′–3′ exoribonuclease Pacman is required for normal male fertility and is dynamically localized in cytoplasmic particles in *Drosophila* testis cells. *Biochem. J.* 416, 327–335.

Zeitelhofer, M., Karra, D., Macchi, P., Tolino, M., Thomas, S., Schwarz, M., Kiebler, M., and Dahm, R. (2008). Dynamic interaction between P-bodies and transport ribonucleoprotein particles in dendrites of mature hippocampal neurons. *J. Neurosci.* 28, 7555–7562.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
**SUPPLEMENTARY MATERIAL**

**FIGURE S1** | Depleting Me31B in clusters of cells results in the loss of Me31B foci. (A) Confocal images of a WT MARCM clone marking large cell clusters (green). (B) Me31B foci (magenta) can be seen in the cell bodies. (C,D) In Me31B mutant MARCM clones (magenta) Me31B foci (green, D) are invisible. Genotype of imaged flies: wt MARCM: hsFLP; mCD8-GFP; NP225; FRT82B/tubGal80, FRT82B Me31B MARCM: GH146/hsFLP; mCD8-GFP; Me31BΔ2, FRT40A/tubGal80, FRT40A.

**FIGURE S2** | (A) To test the significance of the quantitative data for Dcp1, Pcm, and Staufen coexpression with Me31B, presented in Figure 4, we divided the raw data into two classes, spot distance below 400 nm and spot distance between 400 nm and 1 μm. The graphs in (A) show the percentage of Dcp1, Pcm, and Staufen foci which are found in a distance to the nearest Me31B foci below or above 400 nm. A Student’s t-test was used to analyze the statistical significance of differences between somatic and synaptic coexpression. For all three proteins tested, the difference between somatic and synaptic coexpression is highly significant (P-value <0.0001, n = 20). (B) To analyze the specificity of “Spotnik”, we performed an antibody staining against Me31B using a primary rabbit antibody and two secondary anti-rabbit IgG antibodies (Alexa anti-rabbit 488 and 555). The quantification of coexpression between 488 and 555 in the soma and the neuropil showed a high colocalization (69% of foci below 400 nm apart in the soma and neuropil) and we did not find any significant differences between somatic and synaptic coexpression.
FIGURE S3 | Original single slice confocal images of the false color pictures shown in Figure 7. (A) Representative image of myrGFPCaMKII-3'UTR expression driven in GH146 Gal4; tub Gal80ts flies which restricts reporter expression to adults; Genotype: +/++; GH146-Gal4, UAS-myrGFPCaMKII-3'UTR/+; 2x tub-Gal80ts/+. (B) Representative image of myrGFPCaMKII-3'UTR expression in the antennal lobe of flies expressing two RNAi constructs for Me31B. Genotype: UAS-me31B-RNAi++; GH146Gal4, UAS-myrGFPCaMKII-3'UTR/UAS-me31B-RNAi; 2x tub-Gal80ts/+.