Drosophila Mbm Is a Nucleolar Myc and Casein Kinase 2 Target Required for Ribosome Biogenesis and Cell Growth of Central Brain Neuroblasts

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Proper cell growth is a prerequisite for maintaining repeated cell divisions. Cells need to translate information about intracellular nutrient availability and growth cues from energy-sensing organs into growth-promoting processes, such as sufficient supply with ribosomes for protein synthesis. Mutations in the *mushroom body miniature* (*mbm*) gene impair proliferation of neural progenitor cells (neuroblasts) in the central brain of *Drosophila melanogaster*. Yet the molecular function of Mbm has so far been unknown. Here we show that *mbm* does not affect the molecular machinery controlling asymmetric cell division of neuroblasts but instead decreases their cell size. Mbm is a nucleolar protein required for small ribosomal subunit biogenesis in neuroblasts. Accordingly, levels of protein synthesis are reduced in *mbm* neuroblasts. Mbm expression is transcriptionally regulated by Myc, which, among other functions, relays information from nutrient-dependent signaling pathways to ribosomal gene expression. At the posttranslational level, Mbm becomes phosphorylated by casein kinase 2 (CK2), which has an impact on localization of the protein. We conclude that Mbm is a new part of the Myc target network involved in ribosome biogenesis, which, together with CK2-mediated signals, enables neuroblasts to synthesize sufficient amounts of proteins required for proper cell growth.

A fundamental issue during development of a multicellular organism is to coordinate cell proliferation, the availability of nutrients, and cell growth. Prominent examples are neuroblasts, the progenitor cells of the *Drosophila melanogaster* central nervous system, which proliferate in a highly regulated manner during development (1). Upon selection and specification, central brain neuroblasts proliferate until the end of embryogenesis, when they enter a quiescent state until resuming proliferation with the beginning of larval development (2). Notable exceptions are the neuroblasts generating the mushroom bodies, a paired neuropil structure in the central brain involved in learning and memory processes, which proliferate throughout development. Depending on the neuroblast lineage, proliferation stops at late larval or pupal stages by terminal differentiation or apoptosis (3–6). The embryonic and larval waves of neurogenesis correlate with changes in neuroblast size. Embryonic neuroblasts decrease in size with each cell division until they enter quiescence; resumption of proliferation at the larval stage is preceded by cell growth. In contrast to embryonic neuroblasts, larval neuroblasts maintain their cell size until the end of the proliferation period, which is again accompanied by a decrease in cell size. Exit of neuroblasts from quiescence, and thereby activation of proliferation, depends on the nutritional status of the whole animal and is governed by the insulin receptor (InsR)–phosphatidylinositol 3-kinase (PI3K)–Akt signaling pathway, triggered by insulin-like peptide-producing glia cells, which receive nutritional signals from the fat body (7–9). Maintaining InsR signaling in combination with blocking of apoptosis is sufficient for long-term survival and proliferation of neuroblasts even in the adult fly (6). On the other hand, cellular nutrient sensing is mediated by the target of rapamycin (TOR) pathway, which, together with the InsR pathway, regulates cell growth through a variety of effector proteins at the levels of gene expression, ribosome biogenesis, and protein synthesis (10). Whereas neuroblast

reactivation requires the interconnected InsR-PI3K and TOR pathways (9), neuroblast growth at larval stages is maintained even under nutrient restriction, by anaplastic lymphoma kinase (Alk)-mediated but InsR-independent activation of the PI3K pathway in combination with a direct influence of Alk on TOR effector proteins (11).

Cell growth requires protein synthesis, which depends on a sufficient supply of functional ribosomes. Ribosome biogenesis takes place in the nucleolus and involves transcription of single rRNA units and their processing and modification into 18S, 28S, and 5.8S rRNAs, which assemble with multiple ribosomal proteins to separately form the small and large ribosomal subunits. Upon transport to the cytoplasm, both subunits mature before they build up functional ribosomes (12, 13). In general, one key downstream effector of TOR signaling is the transcription factor Myc, which controls cell growth in part by regulating ribosome biogenesis through transcriptional control of rRNA, ribosomal proteins, and proteins required for processing and transport of ribosomal components (14–16). Genomewide analyses of *Drosophila* Myc transcriptional targets emphasized the role of Myc as a central regulator of growth control but also identified many

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target genes with unknown molecular functions of the corresponding proteins (17–20). One of the Myc-responsive genes with an unknown function was *mushroom body miniature* (*mbm*). The original hypomorphic *mbm*¹ allele was identified in a screen for viable structural brain mutants and showed a pronounced reduction in the size of the adult mushroom body neuropil, which was due at least in part to a reduction in the number of intrinsic mushroom body neurons (21, 22). More severe allelic combinations indicated a general requirement for Mbm in brain development and uncovered a neuroblast proliferation defect as a major cause of the phenotype. However, which step requires Mbm for neuroblast proliferation remains elusive. Homology searches provided no clue about the molecular function of Mbm. Structural features of Mbm include several stretches enriched in certain amino acids, a putative nuclear localization signal, and two consecutive CCHC zinc knuckles (22). In this report, we describe Mbm as a new nucleolar protein. Mbm is highly expressed in neuroblasts and is required for proper cell growth but not for processes controlling asymmetric cell division. Corresponding to the observed cell size defect, evidence is provided that small but not large ribosomal subunit biogenesis is impaired in the mutant, which could be a consequence of defective rRNA processing. Mbm is a transcriptional target of Myc and requires posttranslational modification by casein kinase 2 (CK2) for full functionality, as revealed by mutation of identified CK2 phosphorylation sites. These results provide a new link between Myc and growth control of neuroblasts and also establish a function of CK2 in neuroblasts.

MATERIALS AND METHODS

Fly genetics and transgenes. Flies were maintained at 25°C on standard cornmeal food in a 12-h dark-light cycle. The following fly stocks were used: *mbm*^{SH1819} (23), upstream activation sequence (UAS)-*mRFP::ribosomal protein S6* (*RpS6*) (24), UAS-*GFP::RpL11* (24), UAS-*GFP::NS1* (24), UAS-*GFP::Nol12* (25), *heat-shock promoter (hs)-GFP::Nopp140* (26), UAS-*CK2α-RNAi* (R. Jackson, Boston, MA), *worniu (wor)-Gal4* (27), and *Mz1060-Gal4* (J. Urban, Mainz, Germany) stocks. The wild type was *w*¹¹¹⁸ in all experiments. Myc null mutant clones were induced in male second-instar larvae of the genotype *w dm⁴ tub-FRT-Myc-FRT-Gal4 hs-FLP/Y*; UAS-*GFP* by a single heat shock at 37°C. Third-instar larval brains were scored for green fluorescent protein (GFP)-positive cell clones.

The 4.3-kb HindIII genomic rescue construct *P[TW115]* (22) (renamed *P[mbm^{wt}]* in the following) served as a template to simultaneously replace codons for Ser288, Ser290, and Thr292 in acidic cluster 1 (AC-1) or Thr327, Ser332, and Thr333 in acidic cluster 2 (AC-2) with those for alanine by *in vitro* mutagenesis. Primers used for mutagenesis were as follows (changed codons are underlined): AC-1-for, 5'-CCGACTCCTCAACTGCGGACGCGGACGCGGATGATGAACAGAG-3'; AC-1-rev, 5'-CTCTGTTTCATCATCGGCGTCGCGCTCCGAGTTGAGGAGTCGG-3'; AC-2-for, 5'-CCAGTTTACCATTTGCCGATGAGGAGGAAGCCGCCGAACCTGAAGACG-3'; and AC-2-rev, 5'-CGTCTTCAGGTTCCGGCGCTCTCCTCATCGGCAATGGTAACTGG-3'. All constructs were verified by sequencing and subsequently cloned into the pattB transformation vector to allow for PhiC31-mediated transgenesis at the *attP* site, located at chromosomal position 62B2 on the 3rd chromosome (BestGene Inc.). For each construct, several independent transgenic lines were established and used for rescue experiments.

Immunostainings and EdU and metabolic labeling. For immunostainings, larval brains or imaginal discs were dissected in PBS (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl) and fixed on ice for 30 min in PLP solution (2% paraformaldehyde, 10 mM NaIO₄, 75 mM lysine, 30 mM sodium phosphate buffer, pH 6.8). All washings were done in PBT (PBS plus 0.3% Triton X-100). After blocking in PBT containing 3% normal goat serum for 2 h, tissues were incubated overnight with

combinations of the following primary antibodies: mouse anti-Armadillo (1:100; Developmental Studies Hybridoma Bank [DSHB]), rabbit anti-CK2α (1:400; Stressgen), mouse antifibrillar (clones 72B9 [1:50] and P2G3 [1:250]; U. Scheer, Würzburg, Germany), chicken anti-GFP (1:500; Millipore Upstate), mouse anti-Miranda (1:20; F. Matsuzaki, Kobe, Japan), rabbit anti-Nop5 (1:600; G. Vorbrüggen, Göttingen, Germany), guinea pig anti-Numb (1:1,000; J. Skeath, St. Louis, MO), rabbit anti-phospho-histone H3 (1:2,500; Millipore Upstate), rat anti-Pins (1:500; F. Matsuzaki), rabbit anti-protein kinase C ζ (anti-PKCζ) clone C20 (1:1,000; Santa Cruz Biotechnology), and mouse anti-γ-tubulin (1:100; Sigma). A guinea pig anti-Mbm antibody was generated by immunization with a bacterially expressed and purified glutathione S-transferase (GST)::Mbm (amino acids 1 to 268) fusion protein (Eurogentec) and was diluted 1:100. Secondary antibodies were conjugated with Alexa Fluor 488 (Molecular Probes), Cy3, Cy5, or DyLyte488 (Dianova). After extensive washing in PBT, brains were embedded in Vectashield. For 5-ethynyl-2'-deoxyuridine (EdU) labeling, larval brains were dissected in PBS and incubated with 20 μM EdU in PBS for 2 h. After fixation in 4% paraformaldehyde for 15 min, EdU incorporation into proliferating cells was detected with a Click-iT Alexa Fluor 488 EdU imaging kit (Invitrogen). Protein synthesis within a 1-h interval was analyzed by incubation of dissected third-instar larval brains with the methionine analog L-homopropargylglycine (HPG; final concentration of 50 μM in PBS) followed by fixation in 4% paraformaldehyde for 15 min and signal detection with a Click-iT HPG Alexa Fluor 488 protein synthesis assay kit (Invitrogen). Confocal images were collected with a Leica SP5 or Olympus Fluoview 1000 IX 81 microscope. Image processing was done with ImageJ 1.46r software (NIH, Bethesda, MD).

Cell size and intensity measurements. For calculation of neuroblast area, the length of the apicobasal axis (*d*₁) of mitotic neuroblasts stained for Miranda and atypical PKC (aPKC) and the corresponding orthogonal axis (*d*₂) were measured using the Straight Line tool of ImageJ 1.46r. Cell area (CA) was calculated by the formula $\pi \times d_1/2 \times d_2/2$. The freehand selection tool of ImageJ was used for measurements and calculations of cell and nuclear areas (NA) of the irregularly shaped interphase neuroblasts. The nuclear to cytoplasmic ratio was calculated by the formula $NA/(CA - NA)$. Distributions of variables did not deviate significantly from normality (Kolmogorov-Smirnov test; *P* > 0.2). One-way analysis of variance (ANOVA) was performed for statistical analysis. Axis lengths and areas were considered dependent variables, and the strain (wild type versus mutant) was considered an independent variable. The *t* test was done for the results of the formula $NA/(CA - NA)$. Wing imaginal discs from third-instar larvae were stained for Armadillo to outline cell junctions, and cell sizes were measured in the wing pouch region by using the freehand selection tool of ImageJ. Signal intensities of Mbm were measured as previously described (28), with modifications. Nucleolar and cytoplasmic signals were analyzed separately.

Northern blots. Total RNA was extracted from dissected larval brains by use of TRIzol. Equal amounts (1 μg) of total RNA were resolved in agarose-formaldehyde gels and transferred to nylon membranes according to the DIG Northern kit protocol (Roche). Genomic ribosomal DNA (rDNA) fragments were amplified by PCR, using the same primer pairs as those described previously (29), and cloned into the pSTBlue vector (Novagen). Digoxigenin-labeled RNA probes were synthesized by *in vitro* transcription. Signal detection after hybridization was done according to the DIG Northern kit protocol (Roche).

S2 cell culture, flow cytometry, and Western blots. Schneider S2 cells were cultured at 25°C in Schneider medium (Gibco/BRL) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen or PAA). Double-stranded RNA (dsRNA) synthesis, transfections, and fluorescence-activated cell sorter (FACS) experiments were carried out as described previously (18). Briefly, 1×10^6 S2 cells were seeded into single wells of a 24-well plate. After 2 h, the cells were incubated for 30 min with 2 μg dsRNA in serum-free medium, followed by incubation in complete medium for the indicated time. The transfection procedure for Western

blots was essentially the same, except that 5×10^6 S2 cells were transfected with 10 μ g dsRNA in single wells of a 6-well plate. Samples for Western blots were harvested and further processed according to standard procedures, using rabbit anti-Mbm (1:160) (22), mouse anti-Myc (1:50) (30), and mouse anti- α -tubulin (1:5,000) (Sigma) antibodies for enhanced chemiluminescence (ECL) detection. For proliferation assays, a CellTrace CFSE cell proliferation kit (Molecular Probes) was used.

For Western blot analysis of third-instar larval brain protein lysates, 80 brains were transferred to lysis buffer (25 mM Tris, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1% Nonidet-P40, 1 $\times$ protease inhibitor cocktail [Roche]). After sonication and removal of cell debris by centrifugation, protein lysates were analyzed by Western blotting with anti-Mbm and anti- α -tubulin antibodies.

Luciferase gene reporter assay. Sequences from positions –1265 to –3 (relative to the translation start site of the MbM gene), containing E-box1 (positions –47 to –42) and E-box2 (positions –516 to –511), were amplified by NcoI linker PCR and fused to the firefly luciferase (FLuc) gene open reading frame of the pGL3-Basic vector (Promega). Correspondingly, sequences from positions –503 to –3 (containing only E-box1) were cloned into pGL3-Basic. In the Δ E-box1 mutant, the sequence CACGTG was replaced by GAATTC by using the following complementary oligonucleotides for *in vitro* mutagenesis: 5'-CCCCAATCGGCTCAAGAATTCGCGCGCACTAGGC-3' and 5'-GCCTAGTTGCGGCGGAATTCCTTGAGCCGATTGGGG-3'. Constructs with the E-box-containing promoter sequences of the verified Myc target gene CG5033 (E-box-CG5033) and an E-box-mutated version (Δ E-box-CG5033) fused to firefly luciferase were described previously (18). Correspondingly, Myc-independent expression of *Renilla* luciferase (RLuc) was achieved by using the Δ E-box-CG5033 promoter sequences.

For luciferase assays, 1.3×10^6 S2 cells per well were plated in 24-well plates and transfected with the appropriate combination of plasmids (0.5 μ g for the different E-box-mbm-FLuc and E-box-CG5033-FLuc constructs, 2 μ g Δ E-box-CG5033-FLuc, 0.5 μ g Δ E-box-CG5033-RLuc, 50 ng UAS-HA::Myc, and 0.1 μ g tub-Gal4) and 30 ng Myc dsRNA by use of Cellfectin (Invitrogen). Luciferase assays were carried out as described previously (31), using a dual-luciferase reporter assay system (Promega) and a Glomax luminometer. The experiment was repeated three times, and within a single experiment, each transfection was performed in duplicate.

ChIP. For chromatin immunoprecipitation (ChIP), S2 cells were cross-linked with 1% formaldehyde at 37°C for 10 min, and the reaction was stopped with 50 mM glycine. Sonication was carried out until the majority of fragments showed a nucleosomal size. Chromatin was immunoprecipitated with anti-Myc antibody or species-matched IgG (Sigma) coupled to protein A/G-Dynabeads (Invitrogen). Prior to immunoprecipitation, 1% of the chromatin was put aside as an input control. Qualitatively identical results were obtained in independent experiments with mouse anti-Myc (30) and rabbit anti-Myc (32) antibodies. DNA was purified with phenol-chloroform after elution of the bound chromatin with 1% SDS and reversion of the cross-link. ChIP DNA was either analyzed directly by quantitative real-time PCR or first processed using kits and instructions by Illumina to prepare a library for Illumina sequencing. Real-time PCR was performed with SYBR green PCR master mix (Applied Biosystems). Amplification primers flanking E-box1 were 5'-CCACCAACAATCGGATCTTA-3' and 5'-TGTCCTCCACTGTTGTGCAT-3', and primers flanking E-box2 were 5'-TGTCCACATCCTGGTGTCC-3' and 5'-CGCTCGTTCCACAAGCTACT-3'. Primers used for the positive control, Nop5, and the negative control, Pka-C1, were described previously (31).

Bacterial protein expression and purification. The complete open reading frame of the CK2 α gene was amplified by linker PCR and cloned into the pGEX-5X-1 vector digested with EcoRI and SalI. Correspondingly, a cDNA fragment encoding amino acids 266 to 539 of the MbM protein was cloned as an EcoRI-XhoI fragment into the pGEX-KG vector. This construct served as a template for consecutive rounds of *in vitro*

mutagenesis to replace codons for the putative CK2 phosphorylation sites S288, S290, T292, T327, S332, and T333 with alanine codons, using the same primers as those used for modification of the genomic rescue construct *P[mbm^{wt}]*. All constructs were verified by sequencing. Plasmids were transformed into *Escherichia coli* BL21(DE3), and the GST fusion proteins were purified with glutathione-Sepharose according to standard procedures. Proteins were dialyzed in 30 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT) (GST::CK2 α), and 20 mM HEPES, pH 7.5 (GST::Mbm proteins), for 48 h.

In vitro kinase assays. Kinase reactions were carried out in kinase buffer (20 mM HEPES, pH 7.5, in a total volume of 50 μ l) with 6 μ g of recombinant GST::CK2 α and 30 μ g of the indicated GST::Mbm proteins or GST as a control, in the presence of 20 mM MgCl₂ and ATP (10 μ Ci [γ -³²P]ATP [3,000 Ci/mmol] for radioactive kinase assay and 40 nM ATP for mass spectrometry [MS] analysis), at 30°C for 30 min. Reactions were stopped by addition of Laemmli buffer and incubation at 95°C for 2 min, followed by 9% SDS-polyacrylamide gel electrophoresis and autoradiography. For mass spectrometry analysis, gels were stained with colloidal Coomassie blue, and protein bands corresponding in size to GST::Mbm were cut out.

Mass spectrometry analysis. Proteins were digested in-gel by using either trypsin or chymotrypsin (Roche, Germany) as previously described (33). For chymotryptic digests, a modified incubation buffer was used (100 mM Tris-HCl, pH 7.6, 10 mM CaCl₂). After digestion, the gel pieces were extracted with 50% acetonitrile (ACN)–50% 0.1% formic acid (FA) (vol/vol) for 15 min. The supernatant was collected, and the gel pieces were covered with a 5% volume of FA for 15 min before the same volume of ACN was added. After incubation for 10 min, the supernatant was collected. The pooled supernatants were then dried in a vacuum centrifuge and stored at –20°C. Dried samples were dissolved in 0.1% FA. Nano-liquid chromatography–electrospray ionization–tandem mass spectrometry (nano-LC-ESI-MS/MS) experiments were performed on an Acquity nano-UPLC system (Waters) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Germany). Tryptic and chymotryptic digests of TRP were concentrated and desalted on a precolumn (2 cm by 180 μ m by 5- μ m particle size; Symmetry C₁₈; Waters) and separated on a 25-cm by 75- μ m BEH 130 C₁₈ reversed-phase column (1.7- μ m particle size; Waters). Gradient elution was performed from 1% ACN to 50% ACN in 0.1% FA within 90 min. The LTQ-Orbitrap instrument was operated under the control of XCalibur 2.0.7 software. Survey spectra (m/z = 250 to 2,000) were detected in the Orbitrap instrument at a resolution of 60,000 at m/z = 400. Data-dependent tandem mass spectra were generated for the seven most abundant peptide precursors in the linear ion trap. For all measurements using the Orbitrap detector, internal calibration was performed using lock-mass ions from ambient air as described previously (34).

MS data analysis. The Mascot 2.3 (Matrix Science, United Kingdom) search engine was used for protein identification. Spectra were searched against the *Drosophila* subset of the NCBI protein sequence database, downloaded as FASTA-formatted sequences from <ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz>. Search parameters specified trypsin or chymotrypsin as the cleaving enzyme, allowing four missed cleavages (including cleavage before P), a 3-ppm mass tolerance for peptide precursors, and a 0.6-Da tolerance for fragment ions. Carbamidomethylation of cysteine residues was set as a fixed modification, and S, T, and Y phosphorylation, methionine oxidation, and N-terminal acetylation of proteins were allowed as variable modifications. Proteome Discoverer 1.3 (Thermo Fisher Scientific) was used for data analysis. Phosphopeptide MS/MS spectrum sequence assignments and phosphorylated residues were verified manually.

RESULTS

Mbm is not required for asymmetric cell division. The previously characterized *mbm*¹ mutation is a hypomorphic allele and shows reduced expression of the full-length MbM protein on

FIG 1 Mb m is not required for asymmetric cell division. (A) Genomic organization of *mbm* and flanking transcription units. The coding sequence, the 5' and 3' untranslated regions, and the single intron of the *mbm* transcription unit are represented by dark gray, white, and light gray boxes, respectively. The position of the P-element insertion SH1819 is indicated by a triangle. The genomic rescue construct *P[mbm^{wt}]* is shown below. (B) Western blot of brain lysates from wild-type (wt) and *mbm*^{SH1819} third-instar larvae stained for Mb m and α -tubulin (as a loading control). (C) EdU incorporation (green) after 2 h of pulse labeling. Single EdU-positive wild-type neuroblasts (stained for Miranda [red]; arrow) are associated with 3 or 4 smaller EdU-positive GMCs or neurons (asterisks). For *mbm*^{SH1819} brains, only 1 or 2 EdU-positive cells are associated with a single neuroblast. Representative images from at least 10 brains per genotype are shown. (D) Apical cortical accumulation of aPKC and Pins and basal enrichment of Miranda (Mir) and Numb are not disturbed in *mbm*^{SH1819} metaphase neuroblasts. The orientation of the mitotic spindle, as indicated by centrosomal γ -tubulin relative to apical aPKC, is also unaltered in *mbm*^{SH1819} cells. Phospho-histone H3 (pH3) was used to mark chromatin during mitosis. Ten brains per staining and genotype were analyzed. Numbers in confocal images denote the numbers of analyzed neuroblasts in this and the following figures. "Apical" is upwards in mitotic neuroblasts.

Western blots, which precludes its use for a meaningful loss-of-function analysis (22). We therefore used a recently isolated P-element insertion (SH1819) in the coding region of the *mbm* gene which localizes shortly downstream of the translational start site and therefore should behave as a null allele (Fig. 1A) (23). In contrast to the homozygous viable *mbm*¹ allele, *mbm*^{SH1819} causes lethality around pupal formation, with very rare homozygous escapers with a delayed eclosion time. Lethality can be rescued by the genomic transgene *P[mbm^{wt}]* (Fig. 1A). Lack of Mb m expression in homozygous *mbm*^{SH1819} third-instar larval brains was confirmed by Western blotting. A major protein band, which often

appeared as a doublet of bands, was detected at 80 kDa for the wild type but was absent for *mbm*^{SH1819} (Fig. 1B). *mbm*^{SH1819} brains are considerably smaller than wild-type brains of the same age (data not shown). The small brain phenotype correlates with a general proliferation defect of central brain neuroblasts as determined by pulse labeling with EdU (Fig. 1C). This result confirmed our previous experiments with the strong hypomorphic combination *mbm*¹/*Df(2L)A1* (22). One explanation could be that Mb m is part of the molecular machinery controlling asymmetric cell division of neuroblasts into a new neuroblast and a smaller ganglion mother cell (GMC) as a precursor for a pair of neurons or glia cells. Neuroblast division is controlled mainly by cell-intrinsic processes. An apical-basal polarity axis is established by apical enrichment of the Par protein complex components Bazooka, Par6, and aPKC, which control basal enrichment of the Miranda (Mir)-Brain tumor (Brat)-Prospero (Pros) and Numb-Partner of Numb (Pon) complexes, resulting in their final segregation into the GMC upon division. In mitotic neuroblasts, the Par complex is linked via Inscuteable (Insc) to Partner of Inscuteable (Pins), which in turn controls spindle orientation and asymmetry of cell division. Disturbances of these processes can lead to under- or overproliferation phenotypes (35, 36). Staining for aPKC, Pins, Numb, and Mir as representative members of each complex, together with phospho-histone H3 (pH3) as a mitotic marker, revealed no difference in the localization patterns of these proteins between wild-type and all analyzed *mbm*^{SH1819} metaphase neuroblasts (Fig. 1D). Moreover, orientation of the mitotic spindle along the polarity axis, as determined by visualization of centrosomal γ -tubulin, was unaltered (Fig. 1D). We concluded that Mb m is not required for establishment of cell polarity and spindle orientation as central elements of asymmetric cell division.

Mb m is a nucleolar protein required for cell size control. As previously noticed, Mb m localizes in the nucleus in interphase neuroblasts (22). We extended this analysis by confirming the absence of Mb m staining in *mbm*^{SH1819} neuroblasts (Fig. 2A) and by following the localization pattern of Mb m throughout the cell cycle. Nuclear Mb m was seen from interphase until early prophase and then became redistributed to the cytoplasm during mitosis, when the nucleus was disassembled (Fig. 2A). This analysis also revealed correct segregation of Miranda into the smaller ganglion mother cell in *mbm*^{SH1819} animals, confirming that another major aspect of asymmetric cell division was unaffected (Fig. 2A). Costaining with the nucleolar markers fibrillarin and Nop5 revealed that Mb m is a nucleolar protein (Fig. 2B). Although Mb m was expressed predominantly in neuroblasts, ganglion mother cells, neurons, and glia cells also showed a weak nucleolar Mb m signal (data not shown). Following fibrillarin and Nop5 throughout the cell cycle revealed no difference between wild-type and *mbm*^{SH1819} neuroblasts. Nucleolar accumulation of these proteins was always evident in interphase neuroblasts, and weak homogeneous signals were observed in meta- and telophase neuroblasts (Fig. 2B). Similarly, neuroblast-specific expression of the GFP-tagged nucleolar proteins nucleostemin 1 (NS1) (24) and Nol12 (25) or expression of GFP::Nopp140 under heat shock promoter control (26) in an *mbm*^{SH1819} mutant background provided no evidence for localization defects of these components or changes of the structural integrity of nucleoli (data not shown).

The involvement of the nucleolus in cell growth control raised the possibility that Mb m plays a role in controlling cell growth rather than asymmetric division. We first restricted the analysis to

FIG 2 Mbm is a nucleolar protein. (A) Neuroblasts at different cell cycle stages were stained for Mbm (red), Miranda (green), aPKC, and pH3 (both in blue). Miranda shows cortical localization in interphase neuroblasts, accumulates at the basal cortex in metaphase, and becomes segregated into the future GMC in telophase. In *mbm^{SH1819}* neuroblasts, no Mbm protein can be detected, but asymmetry of cell division and segregation of basal Miranda are not disturbed. Twenty-five brains were analyzed for each genotype. Apical is upwards. (B) Mbm colocalizes with Nop5 and fibrillarin in the nucleoli of wild-type interphase neuroblasts. Nucleolar Nop5 and fibrillarin are also seen in *mbm^{SH1819}* neuroblasts. Weak homogenous signals are observed when neuroblasts enter mitosis. The localization patterns of Nop5 and fibrillarin are not altered in *mbm^{SH1819}* neuroblasts. Shown are representative wild-type metaphase and telophase neuroblasts in comparison to *mbm^{SH1819}* neuroblasts. Differential cortical localization of Miranda was used to distinguish cell cycle phases. At least 10 brains were analyzed for each genotype.

the more globular mitotic neuroblasts stained for Miranda and aPKC to measure the length of the apical-basal axis and the corresponding orthogonal axis to calculate cell area. Analyzing the size distribution revealed a considerable shift toward smaller neuroblasts in *mbm^{SH1819}* brains (Fig. 3A). On average, *mbm^{SH1819}* metaphase neuroblasts were significantly smaller (Fig. 3B). Consistent with the observation that asymmetry of neuroblast division was not affected in *mbm^{SH1819}* larval brains (Fig. 2A), a corresponding decrease of GMC size was observed (Fig. 3E). To address the question of whether loss of Mbm influences cytoplasmic and nuclear sizes to similar degrees, the relative cytoplasmic and nuclear areas of interphase neuroblasts were calculated. A strong impact of *mbm^{SH1819}* on cytoplasmic size but not on nuclear size was observed (Fig. 3C and D).

Finally, we analyzed a putative function of Mbm in size control outside the neuroblast compartment. Neither in *mbm^{SH1819}* wing imaginal discs nor upon knockdown of Mbm in tissue culture S2 cells was a significant reduction of cell size observed (see Fig. S1A to C in the supplemental material). Also, proliferation was not affected in S2 cells (see Fig. S1D in the supplemental material). This indicated a more specific function of Mbm in neuroblasts to maintain proper cell size, despite its expression in other cell types as well.

Loss of Mbm interferes with rRNA processing, small ribosomal subunit biogenesis, and protein synthesis in neuroblasts. Based on the observed cell size defects in neuroblasts and the obvious structural integrity of nucleoli, we considered the possibility of an involvement of Mbm in ribosome biogenesis. The assembly of the large and small ribosomal subunits occurs independently in the nucleolus and is a prerequisite for transport through the nucleoplasm to the cytoplasm, where final assembly of functional ribosomes takes place (37, 38). Failure in ribosomal subunit maturation should lead to retention of the affected subunit in the nucleolus. This has been demonstrated by knockdown of *Drosophila* NS1 in salivary gland cells, which specifically blocked transport of the large ribosomal subunit, as indicated by labeling of the large ribosomal subunit protein RpL11 (GFP::RpL11) and of RpS6 as a component of the small ribosomal subunit (mRFP::RpS6) (24). Upon expression with the neuroblast-specific driver line *Mz1060-Gal4*, GFP::RpL11 accumulated in the cytoplasm of all wild-type and *mbm^{SH1819}* neuroblasts (Fig. 4A and B). Predominant cytoplasmic localization of mRFP::RpS6 was also observed in the majority of wild-type neuroblasts (Fig. 4C) (86% of neuroblasts showed predominantly cytoplasmic localization, and 14% showed cytoplasmic and nucleolar localization; $n = 158$). In contrast, mRFP::RpS6 localization in *mbm^{SH1819}* neuroblasts was severely altered (Fig. 4D). Phenotypes ranged from predominant retention of mRFP::RpS6 in the nucleolus (82%; $n = 123$) to a homogenous cytoplasmic and nucleolar distribution (18%; $n = 123$). Introducing the genomic rescue construct *P[mbm^{wt}]* completely reverted this phenotype (Fig. 4E) (85% predominantly cytoplasmic and 15% cytoplasmic and nucleolar; $n = 92$ neuroblasts). These observations indicate that Mbm is required for either maturation of the small ribosomal subunit or its release from the nucleolus to the nucleoplasm. Cytoplasmic mRFP::RpS6 localization was not affected in *mbm^{SH1819}* GMCs or neurons (Fig. 4D, arrowhead).

The colocalization of Mbm with fibrillarin as a component of the pre-rRNA processing machinery and the observed small ribosomal subunit biogenesis defect raised the possibility that Mbm is

FIG 3 Loss of MbM decreases neuroblast size. (A) Distribution of sizes of wild-type and *mbm^{SH1819}* central brain neuroblasts. The apicobasal and corresponding orthogonal axes of metaphase neuroblasts were measured to calculate the cell area (for the wild type, 109 neuroblasts; and for *mbm^{SH1819}*, 151 neuroblasts). (B) Average cell sizes differ significantly between wild-type and *mbm^{SH1819}* neuroblasts ($P < 0.001$). (C and D) To determine nuclear areas and the nuclear to cytoplasmic ratio, interphase neuroblasts were analyzed. Nuclear areas (NA) were identical between wild-type and *mbm^{SH1819}* neuroblasts (C), but cell areas (CA) and, correspondingly, the nuclear to cytoplasmic (CYT = CA – NA) ratios (D) differed significantly ($P < 0.001$). (E) GMC size was also significantly decreased in *mbm^{SH1819}* brains ($P < 0.001$).

involved in pre-rRNA processing. Therefore, total RNAs isolated from dissected brains from wild-type, *mbm^{SH1819}*, and *mbm^{SH1819}*; *P[mbm^{wt}]* larvae were probed on Northern blots with a fragment which detects pre-rRNA, processing intermediates, and mature 18S and 5.8S rRNAs (Fig. 4F) (for a detailed description of pre-rRNA processing in *Drosophila*, see reference 29). Pre-rRNA, the various intermediates, and mature rRNAs were approximately equivalent between the three genotypes. However, an additional band accumulated in the case of *mbm^{SH1819}* brains, and this band was barely visible for the wild type and completely absent under rescue conditions (Fig. 4F). Whether this intermediate was generated by ectopic cleavage or a failure in normal processing remains to be determined, but it indicates a function of MbM in rRNA processing.

An insufficient number of functional ribosomes in the cytoplasm of neuroblasts could impair protein synthesis. To test this hypothesis, we analyzed newly synthesized proteins in neuroblasts within a 1-h interval by detection of incorporation of the methionine analog HPG. Interphase wild-type neuroblasts showed elevated levels of protein synthesis, and cytoplasmic and nuclear staining was observed. Strikingly, an enhanced nuclear signal was evident in most neuroblasts (81% had an enhanced nuclear signal, and 19% showed equal cytoplasmic and nuclear staining; $n = 92$ neuroblasts) (Fig. 4G). When neuroblasts entered mitosis, the newly synthesized proteins were equally distributed (Fig. 4G). Sig-

nal intensities in *mbm^{SH1819}* neuroblasts appeared generally reduced but in most cases were still slightly higher than in the surrounding tissue. In contrast to wild-type neuroblasts, only 10% of all analyzed *mbm^{SH1819}* neuroblasts ($n = 148$) had an enhanced nuclear signal (Fig. 4H). Although it was not possible to compare absolute intensities between different preparations, the frequent loss of the enhanced nuclear signal provided the first evidence that *mbm* neuroblasts were impaired in the ability to synthesize proteins, which could finally lead to the observed cell size defects.

Mbm is a transcriptional target of Myc. A recent comprehensive transcriptome analysis after downregulation of Myc in *Drosophila* S2 cells identified more than 500 regulated genes, including *mbm*, thus providing a potential link between growth input signals and the growth-regulatory function of MbM in neuroblasts (18). The involvement of *Drosophila* Myc (the corresponding gene is named *diminutive [dm]*) in ribosome biogenesis at the levels of RNA polymerase I- and III-mediated rRNA transcription and RNA polymerase II-driven expression of ribosomal components and modifying enzymes is well established (15). The promoter regions of Myc target genes are characterized by the presence of a hexameric sequence element, the E-box (CACGTG and variations thereof), typically located within the first 100 nucleotides after the transcriptional start site. Based on multiple sequenced cDNAs and transcriptional profiling (39, 40), the transcriptional start site of *mbm* is predicted to be located around position –82 relative to the

FIG 4 Loss of Mbm affects small ribosomal subunit biogenesis. (A and B) Expression of UAS-GFP::RpL11 in neuroblasts with *Mz1060-Gal4* in an otherwise wild-type (A) or *mbm*^{SH1819} (B) background. Interphase neuroblasts from third-instar larval brains stained for Mbm (red) and Miranda (blue) show cytoplasmic accumulation of GFP::RpL11 (green) for both genotypes. (C to E) Visualization of Mbm (green), Miranda (blue), and mRFP::RpS6 (red) expressed with *Mz1060-Gal4*. Loss of Mbm affects cytoplasmic localization of mRFP::RpS6. Shown are a wild-type interphase neuroblast (C) and an *mbm*^{SH1819} neuroblast (D) with strong retention of mRFP::RpS6 in the nucleolus (arrows). Note that cytoplasmic localization was not affected in associated GMCs and neurons (arrowheads). (E) In *mbm*^{SH1819}; *P[mbm^{wt}]* neuroblasts, cytoplasmic localization of mRFP::RpS6 is restored (arrow). For each genotype, at least 12 brains were analyzed.

FIG 5 Myc directly controls MbM expression. (A) *mbm* gene locus with the consensus E-box (E-box1) located in the 5' untranslated sequence (white box) and the degenerate E-box (E-box2) located further upstream. Amplicons for ChIP are indicated above, as solid black boxes. The dark gray boxes represent the *mbm* open reading frame. The diagrams below the locus show the different *mbm*-promoter constructs fused to firefly luciferase. (B) Myc specifically associates with E-box1. Chromatin from S2 cells was immunoprecipitated (IP) with rabbit anti-Myc antibodies or control IgGs. Recovery of the indicated target regions, as assayed by quantitative PCR (qPCR), is indicated relative to the total amount of chromatin contained in the corresponding number of cells, as determined by qPCR with the input control. *Nop5* is bound by Myc through a consensus E-box, whereas *Pka-C1* served as a negative control (18, 31). Amplicons of E-box1-mbm and E-box2-mbm are shown in panel A. Error bars indicate standard deviations for triplicate PCRs with the same biological sample. Three additional biologically independent samples, obtained with either the same rabbit anti-Myc antibody or a mouse anti-Myc antibody, gave qualitatively identical results. (C) Reporter constructs expressing firefly luciferase under the control of the indicated promoter regions were transfected into S2 cells together with a plasmid driving constitutive expression of *Renilla* luciferase. Shown are relative luciferase activities under control conditions, upon depletion of Myc by dsRNA, and under Myc-overexpression conditions (Myc o.e.). E-box-CG5033 and ΔE-box-CG5033 served as positive and negative controls, respectively (18). Data from a representative experiment are shown, and error bars indicate standard deviations for duplicate transfections. (D) A single *Myc* (*dm⁴*) mutant neuroblast (encircled) and its associated cell lineage were detected by GFP expression. In comparison to the case in surrounding wild-type neuroblasts, expression of *Nop5* and MbM is reduced. aPKC was used as a neuroblast marker. A total of 293 wild-type and 81 *Myc* mutant neuroblasts from 15 brains were analyzed.

translational start codon. One canonical E-box sequence is found within the 5' untranslated sequence, from positions -42 to -47 of *mbm* (E-box1), which also conforms to the extended E-box consensus sequence AACACGTGCG (18). A second, noncanonical E-box (E-box2) is located further upstream (positions -511 to -516; CACATG) (Fig. 5A). Both E-boxes are present in the genomic construct *P[mbm^{wt}]*, which is able to rescue all known *mbm* phenotypes, indicating the presence of all essential regulatory elements for *mbm* expression (Fig. 1A) (22). Chromatin immunoprecipitation revealed that Myc strongly binds to E-box1 in S2 cells, whereas no consistent binding of Myc to E-box2 could be detected (Fig. 5B), suggesting that Myc might directly control

mbm expression via E-box1. To determine whether one or both identified E-boxes are functional and are required for *mbm* transcription, we performed gene reporter assays. In E-box1-mbm, a 500-bp genomic fragment upstream of the translational start of MbM, encompassing only E-box1, was fused to the firefly luciferase coding region, such that the translational start of the luciferase gene corresponds to the ATG of MbM. In ΔE-box1-mbm, the E-box1 sequence was mutated to GAATTC, and in rev-E-box1-mbm, the 500-bp fragment was cloned in the opposite orientation to control for the directionality of E-box-mediated transcription. E-box1+2-mbm is a 1,260-bp fragment which includes both E-box sequences (Fig. 5A). As controls, we used the E-box-contain-

(F) rRNA processing is altered in *mbm^{SH1819}* brains. The schematic representation shows the pre-rRNA with external and internal transcribed spacers (ETS and ITS) and derived mature 18S, 5.8S, 2S, and 28S rRNAs. The line represents the probe used for hybridization, which, in addition to pre-rRNA and 18S and 5.8S rRNAs, also detects processing intermediates (a to d) (for a detailed description, see reference 29). Two Northern blots with independently isolated total RNA samples from *mbm^{SH1819}* larval brains are shown. Exposures were reduced in the lower parts to improve the visibility of the prominent mature 18S and 5.8S rRNAs. Compared to the wild-type lane (left blot), an aberrant intermediate (arrow) accumulated in the *mbm^{SH1819}* lane, which disappeared in the presence of the *P[mbm^{wt}]* rescue construct (right blot). Two additional Northern blots with biologically independent samples showed identical results. (G and H) Protein synthesis, as determined by metabolic labeling and detection of HPG-Alexa Fluor 488 (green), is reduced in *mbm^{SH1819}* neuroblasts, which are marked by Miranda (red) and Nop5 (blue). The stronger nuclear HPG-Alexa Fluor 488 signal in wild-type neuroblasts (G) is no longer visible in *mbm^{SH1819}* neuroblasts (H). The single metaphase neuroblast in panel G shows a homogenous HPG-Alexa Fluor 488 distribution. For each genotype, at least 20 brains were analyzed.

FIG 6 CK2 α is a nucleolar protein and affects MbM localization. Interphase neuroblasts from wild-type or *mbm*^{SH1819} animals or from animals expressing a UAS-CK2 α -RNAi construct in neuroblasts, driven by *wor-Gal4*, were stained for CK2 α , fibrillarin, and MbM as nucleolar markers and for Miranda as a neuroblast marker. In comparison to wild-type expression (A), nucleolar expression of CK2 α was greatly reduced in *mbm*^{SH1819} neuroblasts (B). (C) Under CK2 α -knockdown conditions, CK2 α was barely detectable, and MbM localized partially in the cytoplasm (arrow). Eight wild-type brains, 12 *mbm*^{SH1819} brains, and 10 CK2 α -RNAi brains were analyzed.

ing promoter sequences of the verified Myc target gene CG5033 (E-box-CG5033) and the corresponding E-box-mutated version (Δ E-box-CG5033) fused to firefly luciferase, which is no longer responsive to Myc (18). Single constructs were transiently cotransfected with a plasmid constitutively expressing the *Renilla* luciferase into S2 cells and then analyzed under control, Myc-knockdown, or Myc-overexpression conditions. Relative luciferase activities measured 48 h after transfection proved the responsiveness of the E-box1-*mbm* construct for up- or downregulation of Myc (Fig. 5C). Responsiveness was abrogated by mutation of E-box1 or in case of reverse orientation of the *mbm* promoter region. The presence of E-box2 had no additional influence on Myc responsiveness, indicating that E-box1 in the *mbm* promoter region is the major mediator of Myc-induced transcription (Fig. 5C).

We also tested for Myc-dependent expression of MbM in larval neuroblasts. Because of recessive lethality of the *Myc* (*dm*⁴) null mutation, we used a fly line additionally expressing a *tubulin* (*tub*) promoter-driven *Myc* transgene flanked by FLP recombination target (FRT) sites (*dm*⁴ *tub*-FRT-*Myc*-FRT-*Gal4* *hs*-FLP; UAS-GFP) to restore viability. Clonal removal of the FRT-*Myc*-FRT cassette by heat shock-induced flippase (FLP) expression resulted in cells devoid of Myc and instead expressing GFP under *tub*-*Gal4* control. Larval neuroblasts marked by aPKC were analyzed for expression of GFP, MbM, and the nucleolar marker Nop5. As for many genes encoding nucleolar proteins, Nop5 expression is dependent on Myc (18). The analysis showed that both Nop5 and MbM expression was significantly downregulated in GFP-positive, Myc-deficient neuroblasts in comparison to neighboring, GFP-negative, wild-type neuroblasts (Fig. 5D). These results strongly suggest that MbM expression is indeed dependent on Myc *in vivo*.

Posttranslational regulation of MbM by protein kinase CK2. Reversible phosphorylation is a key regulatory mechanism to con-

FIG 7 CK2 phosphorylates MbM. *In vitro* kinase assays of the indicated GST::MbM fusion proteins were performed in the presence (+) or absence (–) of GST::CK2 α . Control reactions were performed with CK2 α alone or in combination with GST. Coomassie blue staining proves the presence of equal amounts of the indicated MbM proteins, GST, and GST::CK2 α in the reaction mixtures. Phosphorylation of MbM proteins was detected by autoradiography. Images representative of three independent experiments are shown. For uncropped images, see Fig. S3 in the supplemental material.

trol protein function in a signal-dependent manner. Recently, MbM was identified in two phosphoproteome approaches in cell culture and embryos (41, 42). Moreover, the *Drosophila* protein interaction map (DPIM), relying on a systematic mass spectrometry analysis of protein complexes copurified with epitope-tagged proteins, identified MbM in a complex with the α -subunit of the protein kinase CK2 (43). The CK2 holoenzyme is a heterotetramer composed of two regulatory β -subunits and two catalytic α -subunits; however, independent functions of both subunits have also been described. CK2-mediated phosphorylation controls the activity, localization, stability, or interactions of a multitude of substrate proteins found in different cellular compartments (44, 45). Further complexity arises by the presence of several CK2 β isoforms in *Drosophila* (46). Therefore, we focused on the single catalytic CK2 α isoform found in *Drosophila* to test for MbM phosphorylation and functional relevance. Staining of larval interphase neuroblasts showed colocalization of CK2 α with MbM in the nucleolus (Fig. 6A). In *mbm*^{SH1819} neuroblasts, nucleolar localization of CK2 α was still evident, although we consistently observed much weaker nucleolar CK2 α expression levels (Fig. 6B). Relying on the copurification of MbM and CK2 α (43), we next tested for MbM phosphorylation. CK2 is an acidophilic kinase which phosphorylates serine or threonine residues, with a strong bias for acidic amino acids in the +1, +2, +3, and +4 positions (42). Three clusters of acidic amino acids, each with several embedded serine and threonine residues, are found in the C-terminal half of the MbM sequence (amino acids 281 to 295, 327 to 338, and 454 to 482, referred to as acidic cluster 1 [AC-1], AC-2, and AC-3, respectively) (see Fig. S2 in the supplemental material). *In vitro* kinase assays performed with bacterially expressed and purified GST::CK2 α showed efficient phosphorylation of a bacterially expressed GST::MbM fusion protein (amino acids 266 to 539) that includes all three acidic clusters (Fig. 7; see Fig. S3 in the supplemental material). MS analysis with the *in vitro*-phosphorylated GST::MbM protein detected three phosphorylated sites in AC-1 (S288, S290, and T292) and three phosphorylated residues in AC-2 (T327, S332, and T333) (see Fig. S2 in the supplemental material). The AC-3 region contains almost exclusively acidic amino acids. Enzymatic digestion of this region with trypsin or chymotrypsin gives rise to large, highly negatively charged pep-

tides, which are hard to analyze by MS. Therefore, no information about CK2 phosphorylation events in the AC-3 cluster was obtained by MS analysis. Alanine substitutions for all phosphosites in AC-1 (S288A, S290A, and T292A; labeled AC-1 Δ P), AC-2 (T327A, S332A, and T333A; labeled AC-2 Δ P), or a combination of both (AC-1+2 Δ P) were introduced. Kinase assays with the purified GST fusion proteins revealed a strong reduction of phosphorylation by CK2 α in the case of GST::Mbm-AC-1 Δ P and GST::Mbm-AC-1+2 Δ P, whereas no or only a minor reduction was observed in the case of GST::Mbm-AC-2 Δ P (Fig. 7). Although it was strongly reduced, in no case was phosphorylation completely abolished. This indicated the presence of additional CK2 phosphorylation sites, which presumably lie in the AC-3 cluster. We concluded that S288, S290, and/or T292 are the primary CK2 phosphorylation sites, at least *in vitro*.

To verify whether CK2 has an impact on MbM *in vivo*, we first performed knockdown experiments for CK2 α by *worniu-Gal4*-driven expression of a UAS-CK2 α -RNAi transgene in neuroblasts. This resulted in a strong reduction of CK2 α expression levels (Fig. 6C). Under these conditions, MbM still accumulated mainly in the nucleolus, but in addition, a slight cytoplasmic MbM signal became visible (arrow in Fig. 6C). The same slight effect on MbM localization was seen with a second, independent UAS-CK2 α -RNAi line (Bloomington Stock Center line 35136) and upon neuroblast-specific expression of a dominant negative variant of CK2 α (UAS-CK2 α^{TK}) (47; data not shown). Given the interaction of both proteins, this suggested that CK2 could influence the stability or localization of MbM by phosphorylation. However, CK2 is required for many cellular functions, so knockdown of CK2 α might only indirectly influence MbM. In order to exclude this possibility and to test the *in vivo* functional relevance of the identified CK2 phosphorylation sites, the triple alanine substitutions in AC-1, AC-2, and a combination of both were introduced into the genomic rescue construct $P[mbm^{wt}]$ (Fig. 1A), generating the corresponding transgenic lines $P[mbm^{AC-1\Delta P}]$, $P[mbm^{AC-2\Delta P}]$, and $P[mbm^{AC-1+2\Delta P}]$. To minimize variations in expression levels, all transgenes were integrated at the same chromosomal position by site-specific recombination. Transgenic lines were first tested for the ability to rescue the homozygous lethality of mbm^{SH1819} . This was the case for the presence of the wild-type transgene $P[mbm^{wt}]$. The $P[mbm^{AC-2\Delta P}]$ transgene provided rescue to a much smaller degree, whereas in the case of the $P[mbm^{AC-1\Delta P}]$ transgene, only very rarely were rescued flies observed. The $P[mbm^{AC-1+2\Delta P}]$ transgene was unable to rescue lethality of mbm^{SH1819} . Corresponding stainings of mbm^{SH1819} interphase neuroblasts expressing the different transgenes were performed. Expression of the wild-type transgene in a homozygous mbm^{SH1819} background restored nucleolar localization of MbM in a manner indistinguishable from that of endogenous MbM in wild-type animals (Fig. 8A to C, with quantification in Fig. 8G). In the case of MbM $^{AC-2\Delta P}$ (Fig. 8D), MbM $^{AC-1\Delta P}$ (Fig. 8E), and MbM $^{AC-1+2\Delta P}$ (Fig. 8F), nucleolar MbM localization was still detected, but pronounced cytoplasmic MbM staining also became evident (quantified in Fig. 8G). The localization defect of the mutated MbM proteins was stronger than the effect seen by CK2 α knockdown (Fig. 6C). This is consistent with residual CK2 α being present in neuroblasts under knockdown conditions (Fig. 6C). In summary, we have identified two clusters of CK2 phosphorylation sites in MbM and verified their importance for MbM function *in vivo*. Although MbM $^{AC-2\Delta P}$ was still highly phosphorylated by

CK2 *in vitro* (Fig. 7), the phosphorylation sites in AC-2 are important for MbM function *in vivo*. The AC-1 cluster contains the major CK2 phosphorylation sites, which are also required for MbM function *in vivo*.

DISCUSSION

Ribosome biogenesis must be regulated tightly to provide cells with sufficient amounts of proteins for repeated divisions, to allow for further differentiation, or to maintain physiological functions of cells. Thus, ribosomal biogenesis must be regulated in a cell context-dependent manner to adjust ribosome production to cellular demands, either directly at the level of expression of ribosomal components or by influencing the expression or functionality of proteins required for coordinated modification, assembly, and transport of ribosomal subunits. In addition, nucleolar function is not restricted to ribosome biogenesis but has also been linked to cell cycle regulation, stress responses, and assembly of other ribonucleoprotein (RNP) complexes (12, 48). This complexity was validated by recent surveys of the human nucleolar proteome. More than 700 proteins were identified; for many, their functions remain to be determined (49–51). Importantly, species-specific differences in pre-rRNA processing factors call into question the concept of a highly conserved eukaryotic ribosome biogenesis machinery (52). In this study, we identified MbM as a new component of the nucleolus which has no obvious homologue outside the *Drosophilidae*. In contrast to the tripartite organization of vertebrate nucleoli in a fibrillar center, a dense fibrillar component (DFC), and a granular component (12), nucleoli of *Drosophila* neuroblasts often appear as a homogenous structure at the ultrastructural level, sometimes with intermingled fibrillar and granular components (53). In neuroblasts, MbM colocalizes with fibrillarin and Nop5. In vertebrates, the methyltransferase fibrillarin is associated with Nop56/58 (corresponding to *Drosophila* Nop5) as part of the C/D type of small nucleolar ribonucleoprotein (snoRNP) complex required for rRNA processing in the DFC (54). Indeed, we observed an aberrant rRNA intermediate in *mbm* on Northern blots, implicating a requirement of MbM in rRNA processing. More specifically, based on the retention of Rps6 in the nucleoli of *mbm* neuroblasts, we propose a function of MbM in small ribosomal subunit biogenesis. The complementary phenotype, failure of large ribosomal subunit nucleolar-to-cytoplasmic transport, was observed upon knockdown of nucleostemin 1 (NS1) (24). Yet the molecular function of MbM remains elusive at this point because of its unique domain composition, with two zinc knuckle structures, several clusters of acidic or basic amino acids, and arginine/glycine-rich sequence stretches (see Fig. S2 in the supplemental material). For example, proteins containing arginine/glycine (RGG) repeats are found in a variety of RNP complexes, including snoRNPs (55). For a detailed biochemical analysis of MbM function in ribosome biogenesis, cellular systems that are more accessible than neuroblasts are required, as these represent only a minor fraction of all brain cells. However, despite expression of MbM in tissue culture S2 cells, neither cell size nor proliferation defects were detected under knockdown conditions (see Fig. S1).

Metabolic labeling of *mbm* neuroblasts indicated lowered protein synthesis rates, which could have been due to the lack of sufficient numbers of functional ribosomes. *mbm* larval brains reach nearly wild-type size, with a delay of several days, indicating that protein synthesis is maintained to at least some degree in

FIG 8 Requirement of CK2 phosphorylation sites for Mbm localization. Neuroblasts of the indicated genotypes were stained for the neuroblast markers Miranda and aPKC, for Mbm, and for Nop5, as an independent nucleolar marker. (A to C) Lack of nucleolar Mbm expression in *mbm*^{SH1819} neuroblasts is completely reversed by the genomic rescue construct *P[mbm^{wt}]*. (D to F) Alanine substitution of identified CK2 phosphorylation sites in acidic cluster 2 (*P[mbm^{AC-2ΔP}]*), acidic cluster 1 (*P[mbm^{AC-1ΔP}]*), or a combination of both (*P[mbm^{AC-1+2ΔP}]*) resulted in partial delocalization of the mutated Mbm proteins in the cytoplasm (arrows). (G) Box plot of nucleolar (N) and cytoplasmic (Cyt) Mbm signal intensities for each genotype. Neuroblasts from at least 8 brains were analyzed for each genotype.

neuroblasts. Since the process of asymmetric cell division itself is not affected in *mbm* flies, this provides one likely explanation for impaired neuroblast growth and proliferation. The importance of sufficient cell growth for repeated division of neuroblasts has been documented for mutations in signaling components (56–58). The comparison of the relative protein expression levels of Mbm and other nucleolar proteins in different cell types showed a more pronounced expression of Mbm in neuroblasts. This is confirmed by a comparative transcriptome analysis between neuroblasts and neurons (59). Altogether, the data suggest a more neuroblast-spe-

cific function of Mbm in ribosome biogenesis. Indeed, whereas most ribosomal subunit components are required in all cells, different isoforms are expressed for some components, with one isoform being required in all cells and the other isoform being required more specifically in stem cell lineages (60, 61). Whereas loss of the generally required components causes early lethality, loss of specifically expressed isoforms is associated with a decrease in neuroblast size and an underproliferation phenotype (61). This emphasizes the specific needs of neuroblasts in ribosome biogenesis and cell growth as rate-limiting steps for self-renewal.

The identification of MbM as a transcriptional target of Myc provides a potential link to systemic and cell-intrinsic growth control of neuroblasts mediated by the InsR-PI3K-Akt and TOR pathways (8, 9). In contrast to other tissues, where Myc is a downstream effector of these pathways (19, 62, 63), information is still largely missing in the case of neuroblasts. Myc is expressed in neuroblasts, and upon knockdown, mild effects on neuroblast size but not neuroblast number were observed (64, 65). Consistent with the role of *Drosophila* Myc in expression of many genes involved in ribosome biogenesis (15), removal of Myc function in single neuroblasts caused corresponding decreases in MbM and Nop5 levels (Fig. 5D).

MbM function is dependent on posttranslational modification by the protein kinase CK2. CK2 is a promiscuous kinase expressed in all eukaryotic cells, with a vast array of substrates with pleiotropic functions. However, CK2 not only acts as a heterotetrameric $\alpha_2\beta_2$ holoenzyme but also exists as free populations of both subunits, with independent functions. Pronounced nuclear or nucleolar localization of CK2 subunits was observed in vertebrate cells. In the nucleolus, CK2 participates in rRNA transcription by phosphorylating different components of the RNA polymerase I transcription machinery (44, 45). Proteins involved in ribosome biogenesis, such as B23 (also known as nucleophosmin), are also CK2 phosphorylation targets. CK2 modulates the ability of B23 to act as a molecular chaperone, its mobility rate and compartmentalization in the nucleolus, and its shuttling between the nucleolus and the nucleoplasm (66–68). MbM and Nopp140 are the only described nucleolar phosphorylation targets of CK2 in *Drosophila* (69). This correlates with the observed nucleolar accumulation of CK2 α in neuroblasts and the copurification of MbM and CK2 α (43). Although MbM proteins with mutated CK2 phosphorylation sites showed cytoplasmic accumulation, nucleolar localization was still evident. Yet they were largely unable to complement the loss of endogenous MbM function, indicating that phosphorylation by CK2 not only is a localization determinant but also is important for proper functioning of MbM in the nucleolus. CK2 is often considered a constitutively active kinase which is not regulated by second messenger signaling cascades. However, there is increasing evidence for regulation of CK2 at the levels of the holoenzyme, the dynamics of localization of individual subunits under different conditions, and interactions with small molecules such as polyamines (70, 71). Overexpression of ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis and a known Myc target gene, increases CK2 phosphorylation activity toward nucleolar B23 in mouse keratinocytes (72). It would be interesting to test for a regulatory influence of CK2 on MbM function under different conditions.

In summary, we consider MbM to be part of the Myc and CK2 regulatory networks for coordination of neuroblast growth and proliferation; however, more information about the molecular function of MbM at the level of small ribosomal subunit biogenesis is still required.

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