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Mammalian Smaug Is a Translational Repressor That Forms Cytoplasmic Foci Similar to Stress Granules*[S]

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Cytoplasmic events depending on RNA-binding proteins contribute to the fine-tuning of gene expression. Sterile α motif-containing RNA-binding proteins constitute a novel family of post-transcriptional regulators that recognize a specific RNA sequence motif known as Smaug recognition element (SRE). The *Drosophila* member of this family, dSmaug, triggers the translational repression and deadenylation of maternal mRNAs by independent mechanisms, and the yeast homologue Vts1 stimulates degradation of SRE-containing messengers. Two homologous genes are present in the mammalian genome. Here we showed that hSmaug 1, encoded in human chromosome 14, represses the translation of reporter transcripts carrying SRE motifs. When expressed in fibroblasts, hSmaug 1 forms cytoplasmic granules that contain polyadenylated mRNA and the RNA-binding proteins Staufen, TIAR, TIA-1, and HuR. Smaug 1 foci are distinct from degradation foci. The murine protein mSmaug 1 is expressed in the central nervous system and is abundant in post-synaptic densities, a subcellular region where translation is tightly regulated by synaptic stimulation. Biochemical analysis indicated that mSmaug 1 is present in synaptoneurosomal 20 S particles. These results suggest a role for mammalian Smaug 1 in RNA granule formation and translation regulation in neurons.

Messenger RNA localization, translation activation, silencing, and controlled degradation contribute to the fine-tuning of gene expression in time and space. All these processes depend on several families of RNA-binding proteins that are of comparable importance to transcription factors in regulating gene expression (1). Sterile α motif (SAM)[3]-containing RNA binding domains define a novel family of RNA-binding proteins that function as post-transcriptional regulators (2). They bind to an RNA sequence motif known as SRE (Smaug recognition element), the *Drosophila* protein Smaug being the first member that was identified (2–5). *Drosophila* Smaug is involved in translational repression of the maternal mRNA encoding nanos, a posterior determinant, and thus plays a role in defining embryo polarity. Smaug recruits Cup, an eIF4E-binding protein that prevents the association of eIF4E with eIF4G, thus blocking initiation of the translation of SRE-containing messengers (6). In addition, it has been reported recently that *Drosophila* Smaug mediates degradation of maternal Hsp83 mRNAs by an independent mechanism that involves the CCR4 deadenylase and does not require Cup nor SRE motifs (7). The yeast homologue Vts1 stimulates degradation of SRE-containing messengers by a similar mechanism (2).

Two Smaug homologous genes of unknown function are present in the mammalian genome (2, 3). Here we show that Smaug 1, encoded in human chromosome 14, represses translation of SRE-containing messengers in fibroblast cell lines. Both hSmaug 1 and *Drosophila* Smaug form cytoplasmic granules when expressed in fibroblasts and colocalize when cotransfected. Furthermore, hSmaug 1 foci contain polyadenylated mRNAs, and their size and number depend on polysome integrity, as described in the cases of stress granules (SG) and processing bodies (PB) (8–10). We found that murine Smaug 1 is expressed in the brain and is abundant in synaptoneurosomes, a subcellular region where translation is tightly regulated by synaptic stimulation (reviewed in Refs. 11–18). Our results suggest a role for Smaug 1 in RNA granule formation and translation regulation of SRE-containing transcripts at post-synaptic sites.

EXPERIMENTAL PROCEDURES

Plasmids and Library Screening—A pCDNA3.0 vector (Invitrogen) encoding *Drosophila* Smaug was generated by subcloning the coding region from a dSmaug cDNA kindly provided by Dr. C. Smibert (University of Toronto, Canada) using the primers 5′-TAAGAACTATCCCGGTACCACAA-3′ and 5′-GATCAAATTTGCTCGAGTTCTCC-3′. Firefly luciferase reporters carrying three copies of either wild type or mutated SRE were constructed by subcloning of the BamHI/HindIII fragment of C145 and C146 plasmids, a generous gift of C. Smibert (19), into a pcDNA3.0 vector. A pCDNA6.0 encoding murine Staufen 1 (GenBank™ accession number AF395842) (20) was used. The predicted coding region of hSmaug 1 from the AK034323 EST was subcloned between HindIII and SacII sites in the pECFP-N1 vector (Clontech) and KpnI and XhoI sites in the pcDNA6.0 vector (Invitrogen).

Screening of the mouse brain, heart, kidney, testis, and embryo cDNA libraries was performed at OriGene Technologies, Inc. (Rockville, MD) using three pairs of primers: 5′-GTGGAGTAGTGATTGCCGCTTG-3′ and 5′-CACTCGTTCCAGCCCTTAAACC-3′; 5′-CAGTCCAACTCCCTCCCAACAG-3′ and 5′-AGTCTCTGCAACCCTGAAGATGG-3′; and 5′-AGACTGTTGCACTGCTGTCG-3′ and 5′-TCCAATCGTGTTGATTGTGG-3′.

Primary Antibody against Mammalian Smaug 1—Rabbit polyclonal antisera were raised against the hSmaug 1 SAM domain, which was prepared as similarly described for the *Drosophila* SAM domain (21).

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[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. S1 and S2.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ278487.

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[3] The abbreviations used are: SAM, sterile α motif; SRE, Smaug recognition element; ECFP, enhanced cyan fluorescent protein; PABP, poly(A)-binding protein; SG, stress granules; PB, processing bodies; SMN, survival motor neuron protein; TIA-1, T-cell intracytoplasmic antigen; TIAR, TIA-1-related protein; PBS, phosphate-buffered saline; RT, reverse transcription; BHK, baby hamster kidney; d, *Drosophila*; h, human; m, murine.

Briefly, XL1-Blue cells were transformed with a pET22b expression vector (Novagen, San Diego) encoding the hSmaug 1-(306–371) fragment. Bacteria were induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 12–16 h at 18 °C. After cell harvesting by centrifugation, recombinant protein was purified using a nickel-nitrilotriacetic acid column (Qiagen, Germantown, MD), recovered by elution in 300 mM imidazole, and then dialyzed against PBS. Animals were bled after two boosters.

Translation and Real Time PCR Assays—The following three plasmids were cotransfected into BHK-21 cells plated in 60-mm dishes (10^6 cells per dish) using Lipofectamine 2000 (Invitrogen) in Dulbecco's modified Eagle's medium (Sigma) without serum and antibiotics: 1) 20–500 ng of pCDNA3.0 vector carrying the firefly-luciferase coding region fused to a SRE tandem repeat, either wild type or mutated (2); 2) 10 μg of pECFP-hSmaug 1 or 6.7 μg of pECFP vector (Clontech); and 3) 50 ng of pRL-CMV (Promega, Madison WI) encoding *Renilla* luciferase. Twenty four hours after transfection, firefly and *Renilla* luciferase activities were quantified using the dual-luciferase reporter assay system (Promega). Cell lysis was performed as indicated by the manufacturers, and measurements were both taken immediately and after freeze-thawing, with no significant variations. Transfections were performed in triplicate, and results were expressed as the average ratio of firefly to *Renilla* luciferase activity.

Real time RT-PCR using SYBR Green (Molecular Probes, Eugene, OR) was performed in triplicates of 1:10 serial dilutions of RNA using the following primers: 5′-tcgcggttgttacttgactg-3′ and 5′-cgatcttgttacaacacccc-3′ for firefly and 5′-ggaaacggatgataactggtcc-3′ and 5′-aggccgcgttaccatgta-3′ for *Renilla* luciferase. Following Equation 2 in Ref. 22, cycle threshold (*CT*) *versus* log [RNA dilution] was plotted. Only dilutions where plot slopes (= 1 + *E* (where *E* is the efficiency of target amplification), according to Ref. 22, were close to 2 and similar for the two templates (difference lower than 5%) were considered, indicating similar amplification efficiencies (22). A linear regression was applied to calculate the Δ*CT* (firefly minus *Renilla*) and the corresponding S.D. The ratio of firefly luciferase mRNA to *Renilla* luciferase mRNA was calculated as $(1 + E)^{\Delta CT}$, where (1 + *E*) was calculated from the slope, and the standard deviation was calculated by error propagation.

Cell Transfection, Drug Treatment, and Immunofluorescence—HeLa, BHK-21, and COS-7 cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Natocor, Córdoba, Argentina), penicillin, and streptomycin (Sigma). Plasmid transfection was performed in subconfluent cells plated onto poly-L-lysine-coated coverslips using Lipofectamine 2000 (Invitrogen). Cells were harvested or processed for immunofluorescence 8–16 h after transfection. Fixation in 4% paraformaldehyde at 37 °C, 4% sucrose in PBS was performed with or without prior extraction of living cells in CSK buffer (CSKB: 25 mM KCl, 1 mM HEPES, pH 6.8, 1 mM EGTA, 5 mM $MgCl_2$) containing 0.25 M sucrose and Triton X-100 0.1% for 1 min. After fixation, cells were permeabilized in 0.1% Triton X-100 in PBS and blocked in 2% bovine serum albumin. Primary antibodies were diluted as follows: anti-mammalian Smaug 1, 1:500 to 1:1000, and RLS1, 1:500; anti-PABP rabbit polyclonal antibody (kindly provided by Dr. Evita Mohr, University of Hamburg, Germany), 1:500; anti-TIA-1 goat polyclonal antibody 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA). Monoclonal antibodies and suppliers are as follows: anti-TIAR, SMN (BD Biosciences); anti-HuR and anti-GW182 (Cytostore, Calgary, Alberta, Canada), anti-hPABP (ImmuQuest, Cleveland; UK), 1:100, and anti-V5 (Invitrogen), 1:500. Secondary antibodies were from Molecular Probes or Jackson ImmunoResearch (West Grove, PA). Cells were mounted in Mowiol 4-88 (Calbiochem, EMD Biosciences, Inc). Images were acquired using an LSM-5 PASCAL confocal microscope (Carl Zeiss, Oberkochen, Germany). Proper equipment adjustment was ensured using 1-μm FocalCheck fluorescent microspheres (Molecular Probes).

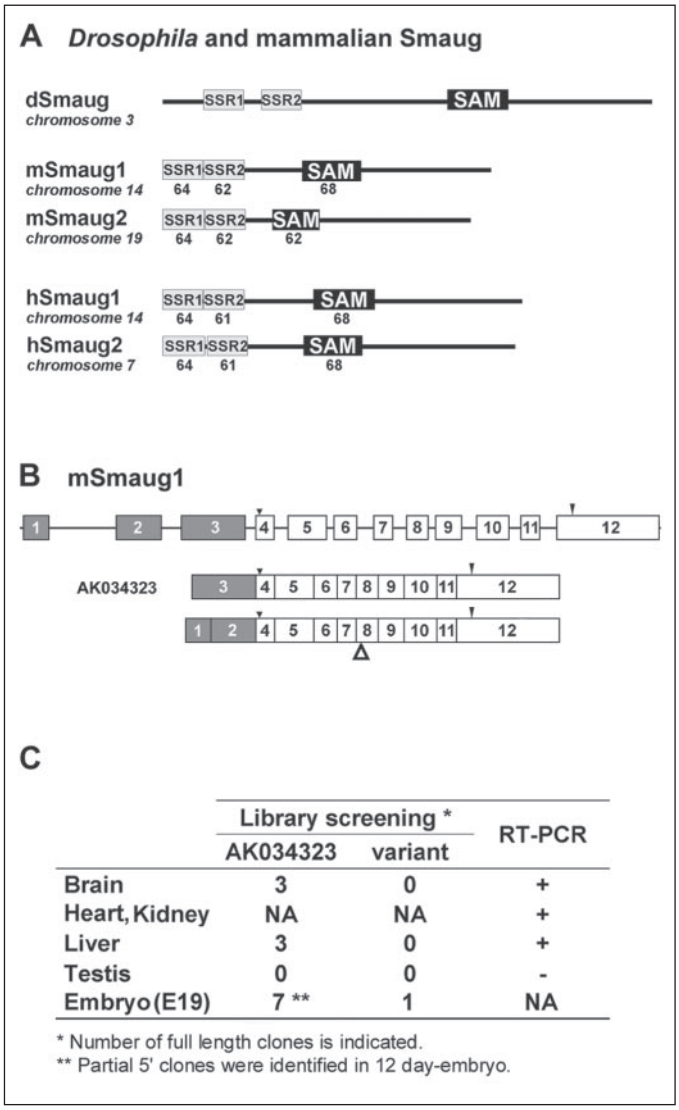
Cycloheximide and puromycin (Sigma) were added to conditioned media from stock aqueous solutions. Treatment with 50 ng/ml leptomycin B (Sigma) was performed in conditioned media for 2 h.

Biochemical Fractionation, Sedimentation Velocity Centrifugation, and Western Blotting—Brains from 6-week-old mice were homogenized in 0.8 M sucrose, in CSKB containing the following protease inhibitors: 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin (all from Sigma). Synaptoneurosomes, post-synaptic densities, and a membrane fraction were isolated as described previously (23). For sedimentation velocity centrifugation, brain post-synaptic densities or post-nuclear extracts from cultured cells were prepared in CSKB containing 1% Triton X-100, 0.25 M sucrose. Samples of 0.5–1 mg of protein (determined by the bicinchoninic acid protein kit assay; Sigma) were loaded onto continuous 13-ml sucrose gradients (20–60, 15–45, or 10–30% w/v in CSKB) and centrifuged at 220,000 × *g* for either 2 or 4 h. When required, 1 unit of β-galactosidase was added as a 20 S marker. The polysomal profile was monitored by absorbance at 260 nm and β-galactosidase activity by colorimetric *o*-nitrophenyl β-D-galactopyranoside reaction. Protein from 1-ml fractions was precipitated in chloroform/methanol (1:2) using 20 μg of lysozyme as carrier and analyzed by Western blot. Briefly, protein was resuspended in Laemmli sample buffer, separated by SDS-PAGE, and electrotransferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). The following primary antibodies were used: anti-mammalian Smaug 1, diluted 1:10,000 to 1:20,000; anti-Staufen 1 RLS1, 1:5000 (20); rabbit anti-S6 (Cell Signaling, Beverly, MA), 1:1000; and anti-*Drosophila* Smaug (generous gift from C. Smibert,), 1:10,000. Detection of peroxidase-conjugated anti-V5 antibody (Invitrogen) and peroxidase-coupled secondary antibodies (Sigma) was performed by chemiluminescence using the LumiGlo system (Cell Signaling).

RESULTS

Mammalian Smaug 1 Represses Translation of SRE-containing mRNAs—The two genes homologous to *Drosophila* Smaug present in mammalian genomes were named as Smaug 1, located in human and mouse chromosome 14, and Smaug 2, located in human chromosome 19 and mouse chromosome 7 (Fig. 1*A*). We performed a screening of mouse brain, liver, testis, and whole embryo libraries and found two mSmaug 1 transcripts. The most frequent of them corresponds to a previously reported EST (GenBank™ accession number AK034323), and the second most frequent corresponds to a novel late embryonic 5′-untranslated region splicing variant (Fig. 1*B*). No splicing variants involving the coding region were found in our screening nor in the available data bases, including human and mouse sequences. We confirmed the presence of mSmaug 1 transcripts in brain, kidney, heart, and liver by RT-PCR analysis (Fig. 1*C*).

We sought to investigate the effect of mammalian Smaug 1 on translation and stability of SRE-containing messengers. Although the direct interaction of hSmaug 1 with SRE-containing messengers was not addressed in this study nor in previous reports, it has been shown previously that SAM domains of vertebrate Smaug homologues specifically recognize SREs (2, 5). BHK cells were cotransfected with plasmids encoding an enhanced cyan fluorescence protein (ECFP) or a hSmaug 1-ECFP chimera together with firefly luciferase-translational reporters bearing either wild type or mutated SREs in a trimeric tandem array

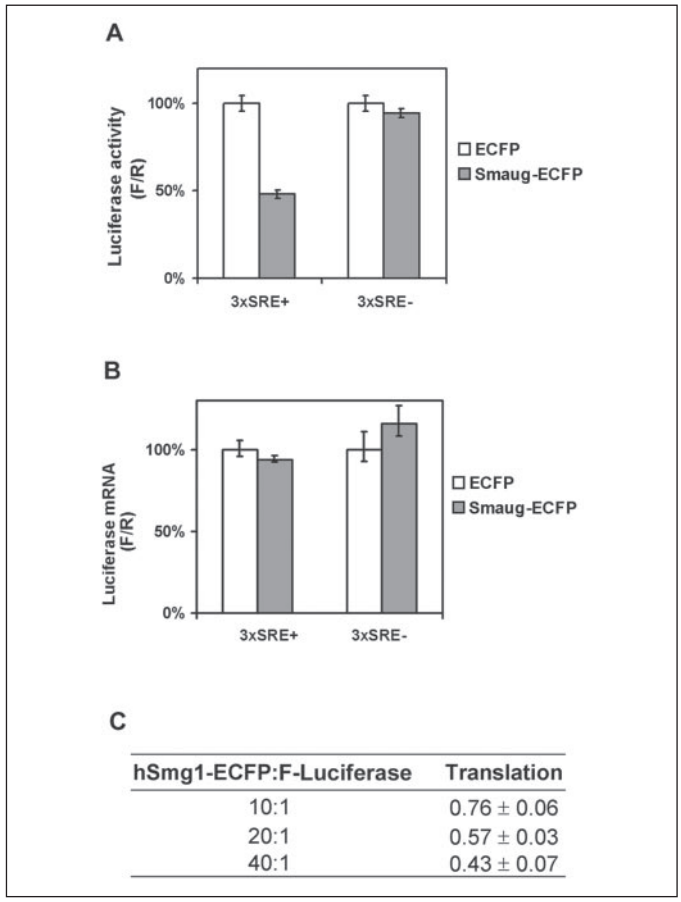


C

	Library screening *		RT-PCR
	AK034323	variant	
Brain	3	0	+
Heart, Kidney	NA	NA	+
Liver	3	0	+
Testis	0	0	-
Embryo (E19)	7 **	1	NA

* Number of full length clones is indicated.
** Partial 5' clones were identified in 12 day-embryo.

FIGURE 1. ***Drosophila* and mammalian Smaug proteins.** *A*, single *smaug* gene with no alternative splicing is present in *Drosophila*. *SSR1* and *SSR2* indicate the Smaug similarity regions described in Ref. 3. The RNA binding domain includes the SAM domain. Two Smaug homologous proteins are present in human and mouse. The percentage of identity with the corresponding *Drosophila* domains is indicated. In all cases, identity among mammalian SSR1, SSR2, or SAM domains is higher than 90%. *B*, the mouse *smaug 1* gene has two alternative splicing variants encoding a single Smaug 1 molecule. Initiation and stop codons are indicated with *arrowheads*. A predicted nuclear localization signal (LTPIKAYSSP) is indicated by an *open arrowhead*. *C*, expression of *smaug 1* was evaluated by library screening and RT-PCR experiments. *NA* indicates not assessed.



C

hSmg1-ECFP:F-Luciferase	Translation
10:1	0.76 ± 0.06
20:1	0.57 ± 0.03
40:1	0.43 ± 0.07

FIGURE 2. **Mammalian Smaug 1 represses translation of SRE-containing mRNAs.** *A*, BHK cells were transfected with hSmaug 1-ECFP or ECFP cDNAs and firefly luciferase reporters in a 40:1 molar ratio. Levels of firefly luciferase (*F*) relative to that of cotransfected *Renilla* luciferase (*R*) are plotted. *B*, mRNA levels were evaluated by real time PCR in the same experiment as in *A*. Calculations were performed as indicated under "Experimental Procedures," and values in the presence of enhanced green fluorescent protein (EGFP) were taken as 100%. *C*, translation inhibition was evaluated in independent experiments where the molar ratio of ECFP reporters to firefly luciferase reporters was 10:1, 20:1, or 40:1. Translation in the presence of hSmaug 1-ECFP (*hSmg1*) relative to that in the presence of ECFP is indicated. Translation of 3xSRE− transcripts was not affected, and SRE containing mRNA levels remained constant (data not shown).

(3xSRE+ and 3xSRE−, respectively) (19). A plasmid encoding *Renilla* luciferase to normalize for transfection efficiency was cotransfected. We found that in the presence of hSmaug 1-ECFP the firefly luciferase activity yielded from 3xSRE+-containing transcripts was reduced to 43% relative to the ECFP control. In contrast, firefly luciferase activity yielded from 3xSRE− reporters was the same in the presence of hSmaug 1-ECFP or ECFP (Fig. 2*A*). When extracts of cells expressing firefly luciferase were preincubated with extracts of cells expressing hSmaug 1-ECFP, no effects on luciferase activity levels were observed (not shown), further indicating that luciferase reporters are not modulated at the protein level by hSmaug 1.

Simultaneously, firefly and *Renilla* luciferase mRNA levels were determined by real time PCR. The abundance of the firefly reporters relative to the coexpressed control *Renilla* mRNA was not affected by the expression of hSmaug 1 (Fig. 2*B*). As expected, the translational repression was proportional to the relative amounts of hSmaug 1 (Fig. 2*C*).

These results indicate that human Smaug 1 represses translation of SRE-containing messengers without affecting their stability. The possibility of an effect on mRNA decay or deadenylation by recruitment of the CCR4 deadenylase, as is the case of the *Drosophila* molecule (7), in a different molecular or cell context was not analyzed, and thus this remains open.

Murine Smaug 1 Is Present in Brain Synaptoneurosomes and Forms Small Particles—An antibody raised in our laboratory against the native SAM domain of hSmaug 1 specifically recognized the human protein in Western blot assays of BHK cells expressing hSmaug 1-V5. Expression of endogenous Smaug 1 was not detected in BHK or other fibroblasts cell lines (Fig. 3*A* and data not shown).

Next, we evaluated the expression of Smaug 1 in the brain, as translational regulation is frequent in neurons (11, 13–15, 17, 24). A protein band of ~70 kDa was observed in blots of mouse and rat brain extracts, in accordance with the predicted molecular weight of mSmaug 1 (Fig. 3*B*). We then examined the distribution of mSmaug 1 in brain subcellular fractions. Murine Smaug 1 was enriched in synaptoneurosomes (Fig. 3*C*), and a further purification indicated that mSmaug 1 concen-

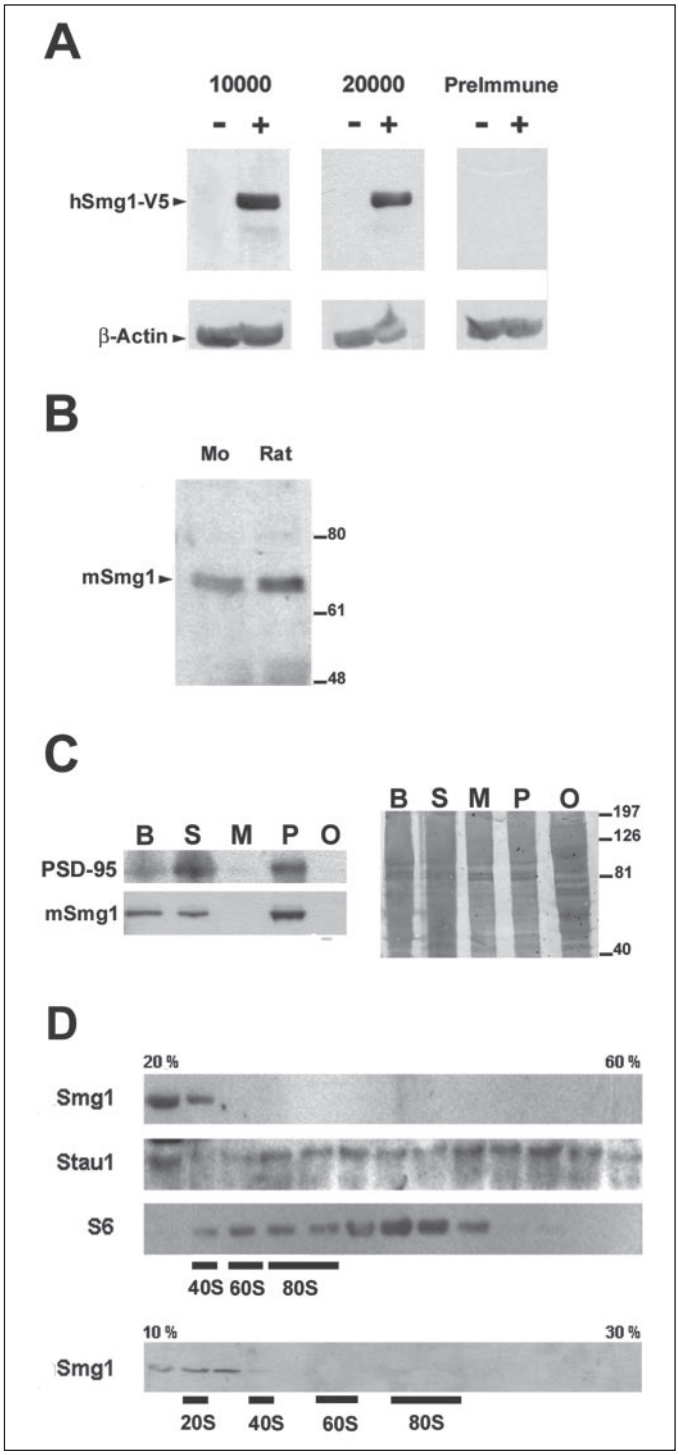


FIGURE 3. **Smaug 1 is present in brain synaptoneurosomes as 20 S particles.** *A*, polyclonal antibody against the SAM domain of the human Smaug 1 molecule (*hSmg1*) was used in Western blot assays. Extracts from BHK cells transfected with a hSmaug 1 construct fused to V5 (+) or nontransfected (−) were analyzed. Equivalent amounts of protein extracts were loaded, and β-actin was detected as a loading control. Dilutions of the primary antibody are indicated. No signal was observed with a preimmune serum. *B*, Western blot analysis of adult mouse (*Mo*) and P0 rat brain. *C*, Western blot analysis (*left*) of total brain (*B*), crude synaptoneurosomes (*S*), membrane from synaptoneurosomes (*M*), postsynaptic densities (*P*), and rat oligodendrocyte primary culture extract (*O*). *Right,* Coomassie Brilliant Blue staining of the same filter. Twenty μg of protein were loaded in total brain, crude synaptoneurosomes, and rat oligodendrocyte primary culture extract, and 5 μg in membrane from synaptoneurosomes and postsynaptic densities. *D*, PSD extract was analyzed in sedimentation sucrose gradients (20–60%, *top panel* or 10–30%, *bottom panel*). Individual fractions were collected, and the presence of mSmaug 1, Staufen 1 (*Stau1*), and the small ribosomal marker S6 were simultaneously detected by Western blot. β-galactosidase was included in the bottom gradient as a 20 S marker.

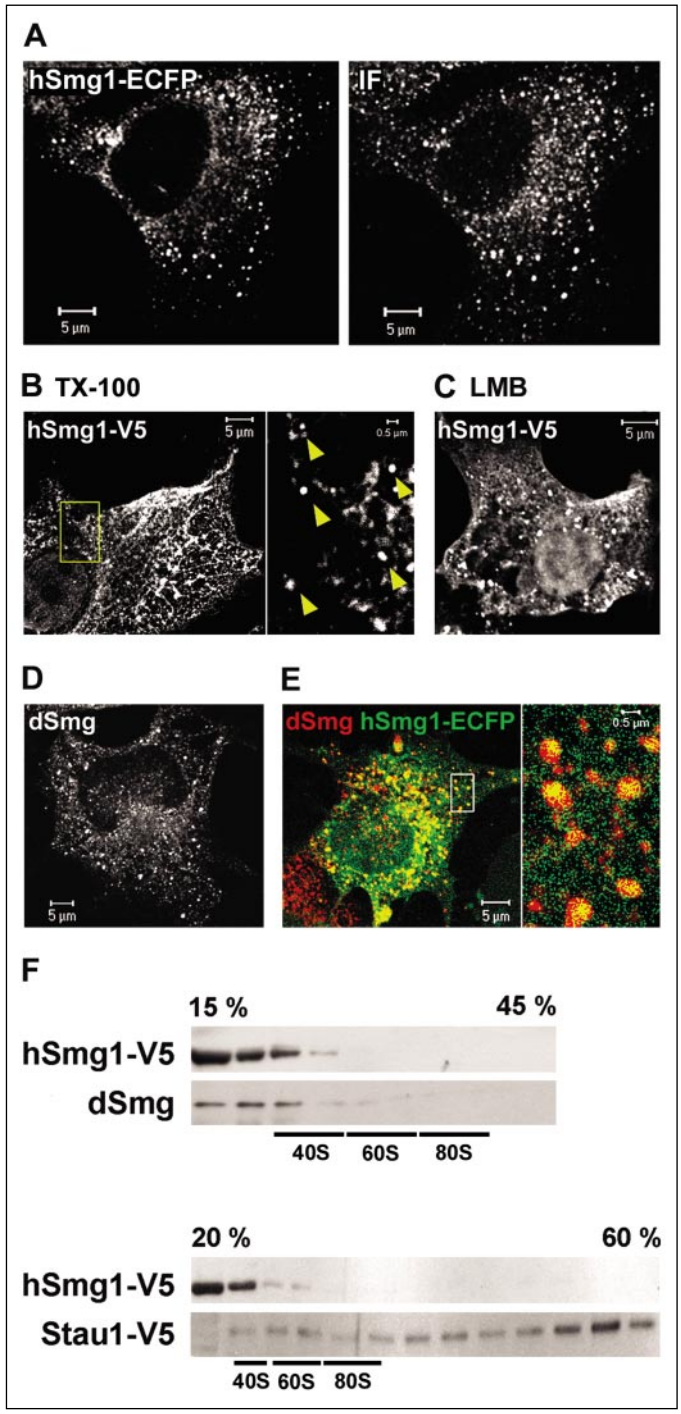


FIGURE 4. ***Drosophila* and mammalian Smaug 1 form cytoplasmic granules in fibroblasts.** *A, left,* cytoplasmic granules containing hSmaug 1 were observed after 8 h of expression of hSmaug 1-ECFP in COS-7 cells. *Right,* immunofluorescence (*IF*) with the polyclonal antibody raised against the hSmaug 1 (*hSmg1*) SAM domain. *B*, hSmaug 1-V5 cytoplasmic foci and a homogeneous nuclear staining are observed after a Triton X-100 (*TX100*) extraction of living cells. *C*, subcellular distribution hSmaug 1-V5 after exposure to leptomycin B (*LMB*). *D*, *Drosophila* Smaug (*dSmg*) forms cytoplasmic granules in COS-7 cells, and complete overlapping was observed when coexpressed with hSmaug 1-ECFP (*E*). *F*, a sedimentation velocity gradient was performed after coexpression of hSmaug 1-V5 and *Drosophila* Smaug (*top*) or hSmaug 1-V5 and murine Staufen 1-V5 in BHK cells (*bottom*).

trates in post-synaptic densities, a fraction from synatoneurosomes almost membrane-free and enriched in cortical cytoskeleton and synapse-associated elements, such as localized mRNAs and polyribosomes and the marker protein PSD-95 (11, 13, 16). In contrast, mSmaug 1 was

TABLE ONE

Presence of TIA-1, TIAR, and PABP in hSmaug 1 foci

The frequency of hSmaug 1 foci and the presence of marker proteins in those foci were analyzed in independent transient-transfection experiments by immunostaining and confocal microscopy. Determinations were performed after 16 h of expression, unless indicated otherwise. *N* indicates number of transfected cells analyzed. Cells with more than four hSmaug1 granules larger than 0.5 μm were considered as containing foci. The percentage relative to total transfected cells is indicated. The percentage of cells showing colocalization with the different markers was relative to the number of granule-containing cells. Cells were scored as positive for colocalization when more than 60% of their hSmaug1 foci contained the analyzed marker. Cells positive for PABP colocalization always showed more than 95% of double-stained foci. No differences in colocalization frequency of the distinct markers were observed between high expressing and low expressing cells (not shown). ND indicates not determined.

Construction	Cell line	*N*	Foci	PABP	TIAR	TIA-1
			%	%	%	%
hSmg1-ECFP	BHK-21[a]	400	66	ND	ND	ND
		58	85	100	ND	ND
		73	76	ND	63	69
		167	83	ND	ND	79
	BHK-21	141	83	ND	65	ND
		114	70	98	52	ND
	COS-7	112	83	94	64	ND
hSmg1-V5	BHK-21	138	80	93	ND	66
		123	81	ND	66	ND
	COS-7	99	83	ND	68	ND
hSmg1-ECFP (after Triton X-100)	BHK-21	90	100[b]	84	77	ND
	COS-7	97	100[b]	92	73	ND

[a] Determinations were performed after 8 h of expression.
[b] Nongranular hSmaug1 signal was washed out after Triton extraction of living cells, and therefore the remaining signal was granular in 100% of the cases.

not detected in the synatoneurosomes membrane fraction (Fig. 3*C*). It also seems to be absent from myelin, as an oligodendrocyte extract gave no signal (Fig. 3*C*). Taken together, these results suggest that mSmaug 1 is localized in neuronal structures associated with dendritic synapses.

Next, we investigated the interaction of mSmaug 1 with the synaptoneurosomal translational apparatus by sedimentation velocity analysis. After centrifugation through a sucrose gradient, mSmaug 1 migrated as a 20 S particle and appeared not to be associated with polyribosomes or with ribosomal subunits (Fig. 3*D*). As expected, the double-stranded RNA-binding protein Staufen 1 was detected in polysomes and in a faster sedimenting fraction (Fig. 3*D*), as described previously (16, 20, 25–28). These results are compatible with a role of mSmaug 1 in inhibition of translation initiation in neurons.

Smaug Forms Cytosolic Granules That Contain Polyadenylated RNA—To analyze further translational repression by mammalian Smaug 1, tagged hSmaug 1 molecules were transiently transfected in fibroblast cell lines, and their subcellular distribution was then analyzed by immunofluorescence and sedimentation velocity gradients.

We found that a large proportion of Smaug 1 concentrated in cytoplasmic granules no larger than 0.5–2 μm (Fig. 4*A*). These granules were observed in COS-7, BHK, and HeLa cells transfected with two different human Smaug 1 chimeras, fused to ECFP or to the small tag His_6-V5 (Fig. 4 and data not shown). Most cells (66–85%) showed the representative phenotype depicted in Fig. 4*A*, granule size, and number as being roughly proportional to expression levels (TABLE ONE). As expected, transfected ECFP presented no granules (data not shown). Human Smaug 1 granules were distributed throughout the cytoplasm and were also observed after Triton X-100 extraction of living cells (Fig. 4*B*), whereas the nonpunctate hSmaug 1 signal was no longer present (see also TABLE ONE). Treatment with the CRM1-inhibitory drug leptomycin B strikingly increased the otherwise minor nuclear hSmaug 1 staining (Fig. 4, *B* and *C*), suggesting that this protein is shuttling between the nucleus and the cytoplasm. Smaug 1 foci were extremely infrequent in the nuclear compartment; the protein showed a homogeneous distribution throughout the nucleoplasm (Fig. 4*C*).

Remarkably, *Drosophila* Smaug also formed cytoplasmic granules when expressed in fibroblast cell lines (Fig. 4*D*). Moreover, the *Drosophila* protein colocalized with mammalian Smaug 1 when coexpressed (Fig. 4*E*), further suggesting a functional similarity between the fly and the mammalian homologues.

We then performed a subcellular fractionation of BHK cells expressing hSmaug 1-V5. Transfected hSmaug 1 was quantitatively recovered in the cytosolic fraction. When post-nuclear extracts were analyzed by sedimentation velocity centrifugation, hSmaug 1 migrated close to the 20 S marker (Fig. 4*F*), similar to the synaptoneurosome-associated mSmaug 1 (Fig. 3*D*). We simultaneously analyzed the distribution of the *Drosophila* protein coexpressed in the same conditions. Most interestingly, fly Smaug also migrates as a small ribonucleoparticle or free protein (Fig. 4*F*). Finally, for comparison, a construct encoding Staufen 1-V5 was cotransfected with hSmaug 1-V5 and analyzed simultaneously. Similarly to the endogenous Staufen 1 (Fig. 3*D*), Staufen 1-V5 comigrated with polysomes (Fig. 4*F*), indicating that overexpression is not perturbing the subcellular distribution of the transfected proteins. Furthermore, the absence of the transfected dSmaug and hSmaug 1 in fast sedimentation fractions, together with the plasticity of hSmaug 1 foci (see below), indicate that Smaug foci were not aggregates of misfolded proteins.

Given that hSmaug 1 repressed the translation of SRE-containing transcripts (Fig. 2), we investigated the presence of messenger RNA in the hSmaug 1 foci. Detection of the general PABP in these structures indicated that Smaug granules likely contain polyadenylated RNA. Remarkably, PABP became granular in all cells bearing hSmaug 1 foci, regardless of their size (Fig. 5, *A*, *B*, and *D*). This was observed in both BHK and COS-7 cells at different expression times and by using polyclonal or monoclonal anti-PABP antibodies (Fig. 5, *A* and *B*). PABP presented a uniform cytoplasmic staining in nontransfected cells (Fig. 5*A*), and expression of ECFP had no effect on PABP distribution (Fig. 5*C*). PABP was retained in hSmaug 1 foci after Triton X-100 extraction (Fig. 5*D*), and strict colocalization was confirmed in all foci in 94% of the cells, regardless of size and subcellular localization (Fig. 5*D*, see also

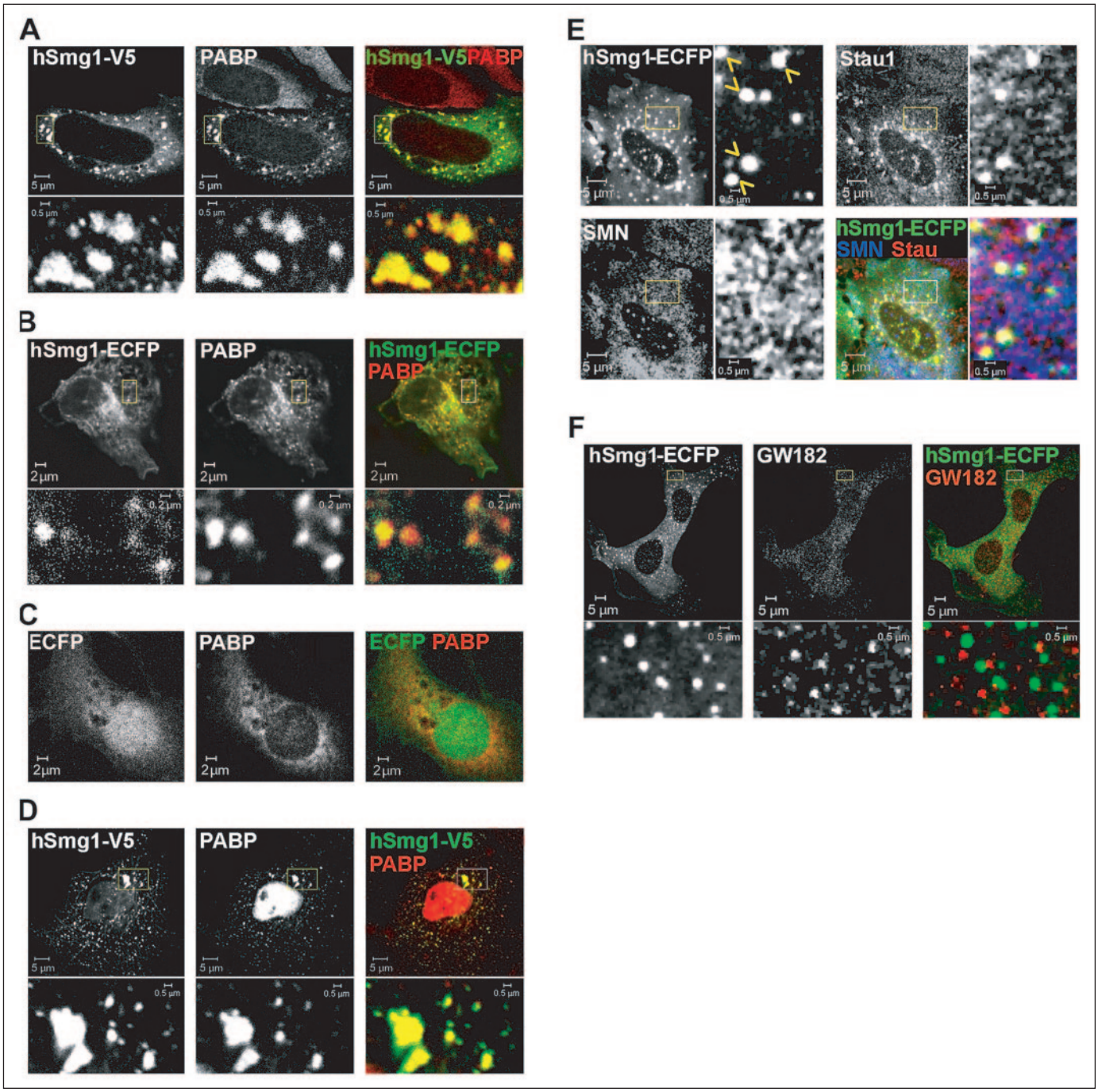


FIGURE 5. **Smaug 1 granules contain PABP and are distinct from SMN granules and degradation foci.** Staining for PABP (*A–D*), SMN, and Staufen 1 (*E*) or GW182 (*F*) was performed after transfection of hSmaug 1 constructs (*hSmg1*) or ECFP in COS-7 cells after 16 h of expression (*A* and *D*); BHK cells after 8 h of expression (*B*); BHK cells after 16 h of expression (*C* and *E*) or in HeLa cells after 16 h of expression (*F*). Polyclonal anti-PABP was used in *A* and *D*, and monoclonal anti-PABP was used in *B* and *C*. Human Smaug 1 granules contain PABP (*A* and *B*), and PABP is retained in the foci after Triton X-100 extraction of living cells (*D*). *E*, Staufen 1 and SMN were detected simultaneously. The presence of Staufen 1 in a number of hSmaug 1-ECFP granules is indicated by *arrows*. *F*, P bodies were identified by staining for GW182.

TABLE ONE). The distribution of SMN and Staufen 1, two RNA-binding proteins that form granules in neurons (26, 29), was also compared. Human Smaug 1 and the endogenous SMN localized in distinct cytoplasmic foci, whereas Staufen 1 was frequently detected in hSmaug 1 granules (Fig. 5*E*). Similar to PABP, Staufen 1 distribution was not affected by ECFP expression, indicating that PABP and Staufen 1 were selectively recruited to hSmaug 1 foci.

Cytoplasmic RNA granules are compatible with degradation foci, which are ubiquitous structures containing proteins involved in RNA decay (30). Thus, we investigated whether GW182, an RNA-binding protein marker of degradation foci (31, 32), was present in hSmaug 1 granules. Complete reciprocal exclusion of GW182 and hSmaug 1 was observed in the foci in two independent experiments in HeLa cells. The overall distribution of GW182 remained unchanged in hSmaug 1-expressing cells (Fig. 5*F*).

We concluded that in fibroblast cell lines both mammalian hSmaug 1 and fly Smaug form 20 S particles that do not contain ribosomal subunits and that cluster into larger granules containing polyadenylated RNA. In addition, cytoplasmic hSmaug 1 granules do not overlap with the previously described degradation foci or SMN granules.

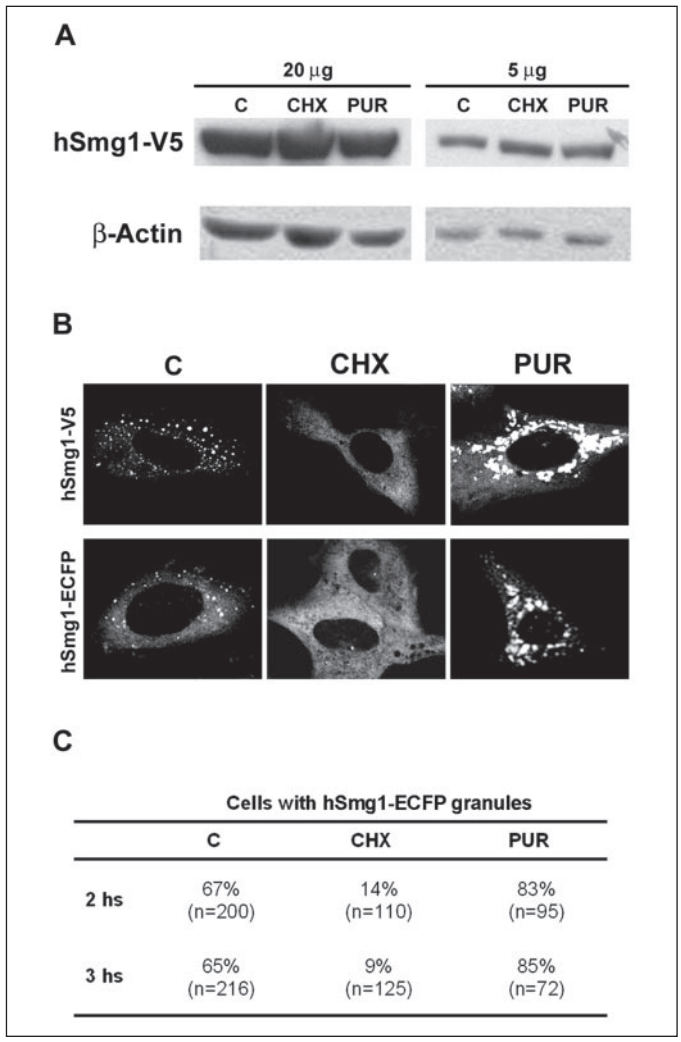
Smaug 1 Foci Are in Equilibrium with Polysomes and Contain SG Markers—As our results indicate that Smaug 1 granules may contain polyadenylated RNA in a silent state, and cytoplasmic granules containing silenced mRNA can be in dynamic equilibrium with translating polysomes (8, 16, 30, 33), we sought to investigate the effect of drugs that affect polysome stability on hSmaug 1 foci. We found that after a strong treatment with the polysome-stabilizing drug cycloheximide, hSmaug 1 granules disappeared almost completely (Fig. 6). The effect was time- and dose-dependent; both granule size and number were always smaller after cycloheximide exposure (Fig. 6, *B* and *C*, and data not shown). Remarkably, puromycin, which inhibits translation by disrupting polysomes, provoked the opposite effect; more hSmaug 1 granules were observed, and these granules were significantly larger (Fig. 6, *B* and *C*). Inhibition of protein synthesis by either cycloheximide or puromycin did not affect hSmaug 1 levels (Fig. 6*A*). The disruption of hSmaug 1 granules by cycloheximide treatment together with the granule-enhancement effect elicited by puromycin indicate that hSmaug 1 foci are dynamic and that their formation and dissolution are linked to polysome integrity.

Smaug 1 foci plasticity is reminiscent to that of SGs that are induced during cell stress and contain transiently silenced mRNAs (8). Remarkably, we found that Staufen 1, which was recently reported as an SG component (20), was recruited into hSmaug 1 foci (Fig. 5*E*). Therefore, we investigated the presence of SG markers in hSmaug 1 foci. We focused on TIAR and TIA-1, two nucleus-cytoplasm shuttling proteins that are present in SG (8, 33, 34). Most hSmaug 1 foci were observed to contain TIAR and TIA-1 in independent transfection experiments performed in BHK or COS-7 cells (Figs. 7 and 8). Colocalization with TIA-1 and TIAR was observed in both the small foci (Fig. 7*A*) as well as in the large foci present at higher expression levels (Fig. 7*B*) and resisted the extraction with nonionic detergents (Fig. 7*C* and TABLE ONE). As reported previously (35), the presence of cytoplasmic granules containing TIAR/TIA-1 was observed in 15–20% of ECFP-transfected cells and was less than 5% in nontransfected cells (data not shown), indicating a minor cellular stress likely due to plasmid transfection and general protein overexpression. HuR, another nuclear protein present in stress granules (34), was also frequently detected in the hSmaug 1 foci (Fig. 8).

In both extracted and nonextracted cells, we observed that about 25% of cells expressing hSmaug 1-ECFP or hSmaug 1-V5 lacked TIAR and TIA in some foci (Fig. 7*C* and TABLE ONE). A similar phenomenon was observed previously for G3BP-induced stress granules (35). We found that these two SG-marker proteins were simultaneously present or absent from hSmaug 1 foci. More importantly, the TIAR/TIA-1-negative foci always contained PABP (Fig. 7, *C* and *D*), thus suggesting that the presence of polyadenylated transcripts was independent of the recruitment of TIAR/TIA-1.

The two previously described foci, SG and PB, were recently shown to interact physically, displaying encapsulation of PB by SG, juxtaposition, or fusion (30, 36, 37). The spatial relationship between hSmaug 1 foci and SG markers was analyzed by confocal slicing (Fig. 8). The intensity profile of the hSmaug 1 signal along a line crossing several foci strictly correlated with that of TIAR (Fig. 8*A*) and HuR (Fig. 8*B*), indicating that these molecules overlapped spatially in the foci. In contrast, the PB marker GW182 did not colocalize with hSmaug 1, although they were frequently observed in adjacent foci (Fig. 5*F* and data not shown).

Altogether, these observations indicate the following: transiently transfected hSmaug 1 forms foci distinct from processing bodies; are in equilibrium with translating polysomes; always contain polyadenylated RNA, and frequently contain stress-granule markers.



Cells with hSmg1-ECFP granules	C	CHX	PUR
2 hs	67% (n=200)	14% (n=110)	83% (n=95)
3 hs	65% (n=216)	9% (n=125)	85% (n=72)

FIGURE 6. **Smaug 1 granules are dynamic structures in equilibrium with translating polysomes.** BHK cells transiently expressing hSmaug 1-V5 for 16 h or hSmaug 1-ECFP for 8 h were exposed to 0.25 mg/ml cycloheximide (*CHX*) or 0.25 mg/ml puromycin (*PUR*). *A*, after 2 h of treatment 20 and 5 μg of total protein were analyzed by Western blot with anti-V5 and β-actin antibodies, showing no differences in the levels of hSmaug 1-V5 (*C* indicates control). *B*, representative cells after 2 h of treatment are shown. *C*, percentages of hSmaug 1-ECFP-transfected cells with more than four granules larger than 0.5 μm after 2 or 3 h of treatment are indicated. Similar values were obtained with the hSmaug 1-V5 construct.

DISCUSSION

We have characterized Smaug 1, a mammalian homologue of *Drosophila* Smaug, an RNA-binding protein defining a novel family of post-transcriptional regulators involved in mRNA silencing and deadenylation (2–4, 7). We found that hSmaug 1 represses the translation of SRE-containing messengers and no effect of human Smaug 1 on mRNA stability was observed in fibroblast cell lines. It is still possible that hSmaug 1 or other mammalian orthologues may have a role in RNA degradation in a different cell context. We found that in the absence of hSmaug 1 constructs, the abundance of SRE-containing transcripts was lower than that of transcripts bearing mutated SRE, and simultaneously, the translation efficiency was stimulated by the presence of SRE motifs (data not shown). This likely suggests the presence of unknown endogenous factors regulating the activation or stability of SRE-containing transcripts that was not analyzed further.

The mechanism involved in mRNA repression by mammalian Smaug 1 has not been addressed in this study, but it is likely to be similar to the one reported for *Drosophila* Smaug; dSmaug binds Cup, which in turn

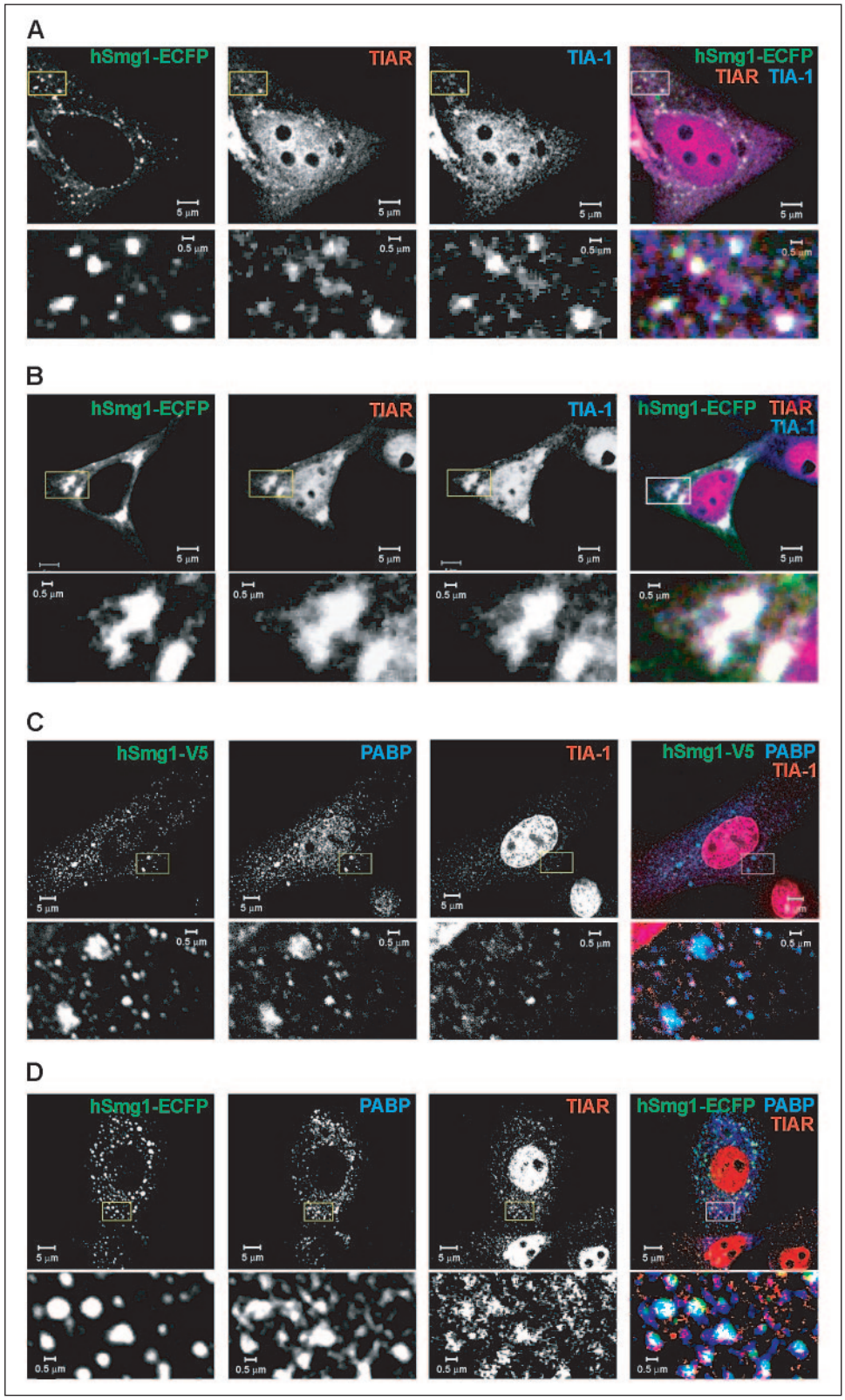


FIGURE 7. **Smaug 1 granules contain SG markers.** Human Smaug 1 (*hSmg1*) constructs were expressed in BHK cells (*A–C*) and COS-7 cells (*D*). Immunostaining for the indicated proteins was performed in nonextracted cells (*A, B,* and *D*) or after Triton extraction of living cells (*C*) after 16 h of expression. TIAR and TIA-1 were simultaneously detected in hSmaug 1 foci, regardless of expression levels and granule size (*A* and *B*). Foci negative and positive for TIA-1 and TIAR coexist inside cells, and all foci contain PABP (*C* and *D*).

binds to eIF4E, thus preventing eIF4G recruitment (6). As a consequence, translation initiation is blocked, and ribosome subunits cannot be recruited. A human Cup homologous protein has been identified, and similar to *Drosophila* Cup, this ubiquitous molecule, known as 4ET, inhibits the interaction of eIF4E with eIF4G (39). Our observation that *Drosophila* and mammalian Smaug form 20 S particles that do not include 40 S ribosomal subunits is compatible with a role for these molecules in translation initiation blockage. These 20 S particles appear to build up large aggregates inside cells, which can be visualized as granules. The formation of hSmaug 1 cytoplasmic granules was observed in several different cell lines, and in all cases, they contained PABP, suggesting the presence of polyadenylated SRE-containing messengers. We

found that hSmaug 1 foci enlarge when polysomes are disrupted, and conversely, they disassemble in the presence of the polysome-stabilizing drug cycloheximide, as reported for stress granules and more recently for processing bodies (8, 16, 30, 33, 37).

In addition to PABP, hSmaug 1 foci frequently contained TIAR, TIA-1, and HuR, three RNA-binding proteins that are normally concentrated in the nucleus and that are recruited to cytoplasmic stress granules (8–10, 34) and Staufen 1, a ubiquitous double-stranded RNA-binding protein recently described to be an SG component (20).

The finding that both fly and mammalian Smaug induce the formation of cytoplasmic granules containing stress granule markers in the absence of cell stressors was unexpected. Nevertheless, this observation is compatible with the fact that dSmaug and hSmaug 1 are translational repressors, given that stress granules are formed when translation initiation is impaired by cellular stress, expression of a phosphomimetic mutant of eIF2α, or translation inhibitory drugs (8, 9, 20, 33, 38, 40). Furthermore, stalled translation initiation complexes have been suggested to function as nucleation centers for SG aggregation (10).

By extending our observations on Smaug, we speculate that SG-related structures would assemble onto core complexes of mRNA and proteins that block translation initiation. Relevantly, the translation inhibitor CPEB was recently shown to form similar granules in the absence of stress when overexpressed (36). Despite the presence of SG markers in hSmaug 1 cytoplasmic foci observed under normal conditions, these foci do not necessarily represent structures identical to the stress granules observed upon induction of cellular stress, as SG formation is a consequence of the activation of a complex signal transduction pathway not expected to be activated by Smaug expression.

As an alternative to the *de novo* formation of granules induced by Smaug, we should consider the possibility of Smaug being recruited to pre-existing cytoplasmic structures, like the PB that are present in all cell type (30, 31, 32, 36, 37). Relevantly, fly Smaug and the yeast homologue Vts1 trigger mRNA deadenylation by direct interaction with CCR4 (2, 7) that localize at degradation foci in mammalian cells (30). In addition, 4ET, a mammalian homologue of Cup, a molecular partner of *Drosophila* Smaug, was recently reported to be present in processing bodies (37, 41). We found that Smaug 1 foci contain PABP in BHK, COS-7, and HeLa cells, likely indicating the presence of polyadenylated mRNA, which is normally excluded from degradation foci (30, 37) but that can be detected when mRNA degradation is impaired (30). Nevertheless, we found that the presence of hSmaug 1 and the degradation foci marker GW182 in cytoplasmic granules are mutually exclusive in HeLa cells.

Thus, Smaug 1 granules containing silenced mRNAs may be expected to be present at the postsynapse, where this protein accumulates. The formation of hSmaug 1 cytoplasmic granules in fibroblasts indicates that neuron-specific factors are not required, thus underscoring the capacity of Smaug as an RNA granule-forming protein and suggesting the presence of similar structures in neurons. Relevantly, *Drosophila* Smaug also concentrates in granules, likely the polar granules, at the posterior of the embryo and is present in the fly central nervous system during embryogenesis (3, 4). Thus, it seems possible that Smaug 1 molecules located at post-synaptic sites specifically sequester SRE-containing transcripts in granules, where translation is suppressed. More importantly, a growing number of RNA-binding proteins thought to be involved in the local regulation of gene expression are present in dendrites and post-synaptic sites forming granules (11, 26, 29, 42–45). Relevantly, some of them, namely Staufen, SMN, fragile X mental retardation protein, and ELAV family members, are also found, under certain conditions, in larger accretions eventually identified as stress granules in a number of cases (20, 46–48).

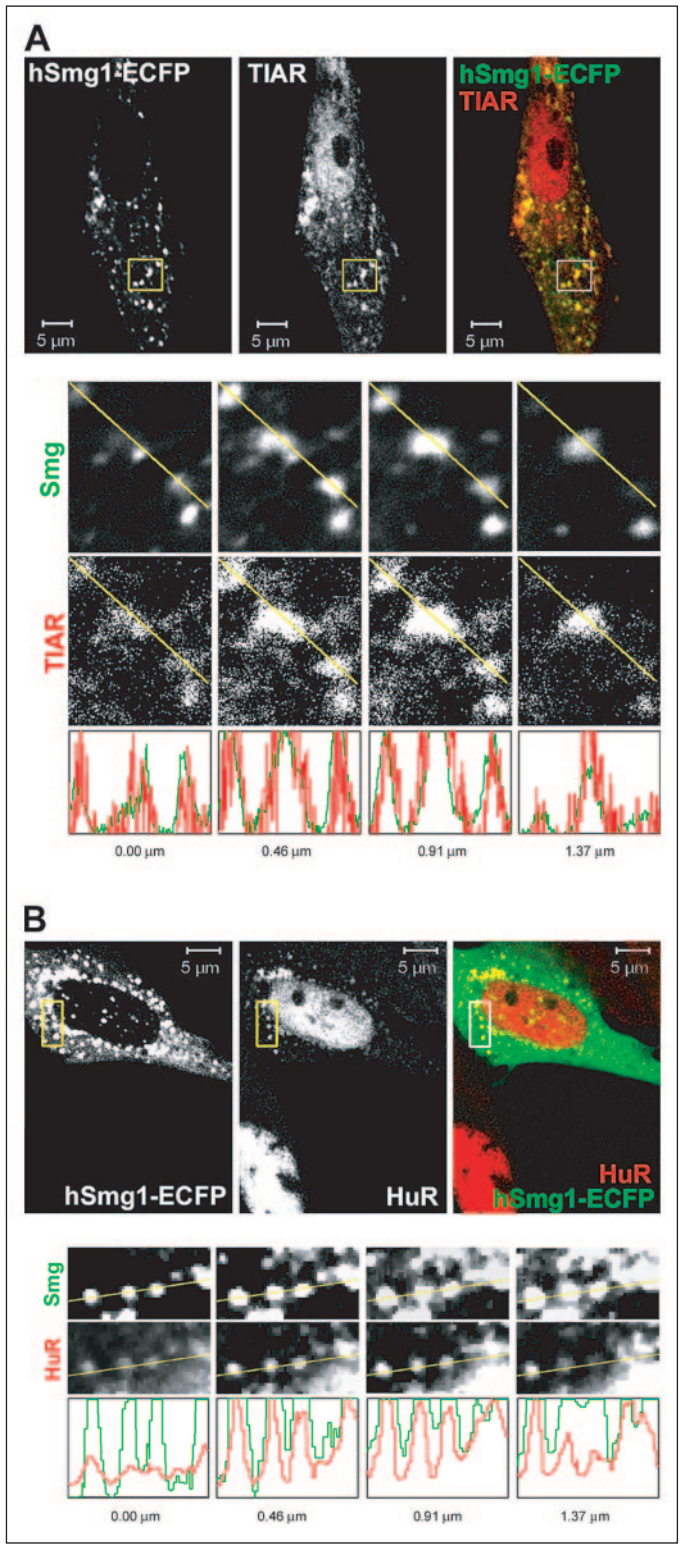


FIGURE 8. **Colocalization of TIAR and HuR in hSmaug 1 foci.** BHK cells expressing hSmaug 1-ECFP were immunostained for TIAR (*A*) or HuR (*B*), and confocal optical slicing of representative hSmaug 1 (*hSmg1*) foci was performed. Intensity profiles for marker molecules on each Z plane were recorded using a line scan. *Bottom panels*, representative analysis of intensity peaks showing correlation of SG marker proteins and hSmaug 1 (*Smg*) signal along the lines in each slice of the Z stack.

Mechanisms for translational regulation contributing to synapse for-

mation and plasticity include CPEB-mediated poly(A) elongation (15), use of an internal ribosome entry site (49, 50), and translation regulation by fragile X mental retardation protein and BC1 (24), among others. In addition, availability and activity of general factors for protein synthesis are also regulated at the synapse (11, 14, 17, 18). Our results suggest the SRE-Smaug system as a novel additional mechanism for local translational control at the synapse. A survey of neuronal messengers containing SREs in data bases yielded a reduced number of mRNAs encoding distinct functions.[4] Some of them are known to be localized at the dendritic compartment, and thus, the possibility exists that Smaug contributes to the regulation of these transcripts. This hypothesis and whether Smaug is controlled by synaptic activity have yet to be confirmed.

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[4] M. V. Baez and G. L. Boccaccio, unpublished observations.