Neuronal RNA granules are ribosome complexes stalled at the pre-translocation state

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Abstract

The polarized cell morphology of neurons dictates many neuronal processes, including the axodendridic transport of specific mRNAs and subsequent translation. mRNAs together with ribosomes and RNA-binding proteins form RNA granules that are targeted to axodendrites for localized translation in neurons. It has been established that localized protein synthesis in neurons is essential for long-term memory formation, synaptic plasticity, and neurodegeneration. We have used proteomics and electron microscopy to characterize nRNAg's isolated from rat brain tissues or human neuroblastoma. We show that ribosome containing RNA granules are morula-like structures when visualized by electron microscopy. Crosslinking-coupled mass-spectrometry identified potential G3BP2 binding site on the ribosome near the eIF3d-binding site on the 40S ribosomal subunit. We used cryo-EM to resolve the structure of the ribosome-component of nRNAg. The cryo-EM reveals that ribosomes in the nRNAg are stalled at the elongation state where tRNA's are in the hybrid A/P and P/E site, and resemble the pre-translocation state ribosomes. We also describe a new kind of principal motion of the ribosome, which we call the rocking motion.

Keywords: cryo-EM/ neuron/ ribosome/ RNA granule/ cross-link

Introduction

Mammalian neurons are the largest cells in the body and are highly compartmentalized. In a highly elongated neuron, these compartments may be tens to hundreds of micrometers apart. Due to the kinetic constraints of long-distance intracellular transport, even when active mechanisms are used, these subcellular compartments must be able to function largely independently, neccessitating separate pre-allocated pools of macromolecules. One important effect of such a functional separation is neuronal protein synthesis. While the bulk of neuronal protein synthesis occurs in the soma, ribosomes in the axo-dendrtic compartments translate specific mRNAs in a precise spatiotemporal manner to ensure a rapid response to signals from neighbouring cells in a neural circuit. This localized protein synthesis in axons and dendrites has attracted considerable attention in recent years, due to its link to synaptic plasticity and thereby long-term memory formation. Translational remodelling of gene expression has been found to underly behavioural plasticity (Sutton and Schuman, 2006), a local translational program directed by specialized ribosomes, required for synaptic plasticity and memory formation.

The ribosome follows the genetic instructions to produce proteins in all organisms. Actively translating ribosomes form polyribosomes where multiple ribosomes occupy a single mRNA molecule. Polyribosome complexes are abundant in neuronal soma but have also been detected in dendrites (Steward & Falk, 1986; Steward & Levy, 1982) and in axons (Koenig *et al*, 2000; Li *et al*, 2005; Ostroff *et al*, 2019; Shigeoka *et al*, 2016; Sotelo *et al*, 1999; Spencer *et al*, 2000; Steward & Ribak, 1986). A prerequisite for such compartment-specific mRNA translation is the transport and storage of ribosomes to distant locations in a neuron. Graber and colleagues proposed that neuronal mRNAs are transported as stably paused polyribosomes, and that this kind of mechanism can bypass the translation initiation process that is the rate limiting step for protein synthesis (Graber *et al*, 2013). Transport of mRNA to distant locations within a neuron followed by local translation is more cost-efficient than transportation and storage of proteins (Spaulding & Burgess, 2017). In growing neurons, mRNA's are targeted to neuronal processes as granules which also contain ribosomal subunits and translation factors (Knowles *et al*, 1996; Olink-Coux & Hollenbeck, 1996; Spaulding & Burgess, 2017). mRNAs together with ribosomes and RNA-binding proteins (RBP) form RNA granules that are targeted to axodendrites for localized translation in neurons (Buxbaum *et al*, 2014; Chu *et al*, 2019; El Fatimy *et al*, 2016; Graber *et al.*, 2013; Liao *et al*, 2019; Ohashi & Shiina, 2020; Ostroff *et al.*, 2019; Sahoo *et al*, 2018). Recently it was proposed that the eIF4G microexons function as a translational brake by causing ribosome stalling in cooperation with RBPs to propagate the cytoplasmic granule formation, and that misregulation of eIF4G microexons causes autism spectrum disorder (Gonatopoulos-Pournatzis *et al*, 2020). The RNA granule components are associated with various neurodegenerative disease, including autism spectrum disorder, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (Alami *et al*, 2014; Bassell & Warren, 2008; Bentmann *et al*, 2013; Bosco *et al*, 2010; Bramham & Wells, 2007; Broadbelt *et al*, 2006; Burguete *et al*, 2015; Conicella *et al*, 2016; Darnell *et al*, 2011; DeJesus-Hernandez *et al*, 2011; Dictenberg *et al*, 2008; Dobra *et al*, 2018; Liu-Yesucevitz *et al*, 2011; Nakayama *et al*, 2017; Russo *et al*, 2017; Salapa *et al*, 2020). Also, upregulation of axonal ribosomes occurs early in the pathogenesis of ALS (Verheijen et al., 2014).

Localized protein synthesis in neurons is essential for long-term memory formation, and synaptic plasticity (Alves *et al*, 2019; Chen *et al*, 2017; Costa-Mattioli *et al*, 2009; Graber *et al*, 2017; Lebeau *et al*, 2011; Miyakawa *et al*, 2001; Nakayama *et al.*, 2017; Ohashi *et al*, 2016; Shepherd *et al*, 2006; Sudhakaran *et al*, 2014; Weng *et al*, 2018). Neuronal RNA

granules serve as the functional units for transport and translational control (Alami *et al.*, 2014; Gopal *et al*, 2017; Liao *et al.*, 2019; Sahoo *et al.*, 2018). The translational activity within the RNA granules is suppressed during their transport from soma and storage in axodendrites by specific RBPs. For example, it has been shown that G3BP1 binds to Nrn1 and Impβ1 mRNAs and attenuates their translation in axons (Sahoo *et al.*, 2018). It is thought that upon synaptic stimulation, RBP-mediated repression of the granules is relieved by the dissociation of the RBP's and translation follows of the synapse-specific mRNA's. Targeting translationally silenced ribosomes in the form of RNA granules into the synaptic compartments could be nature's way of regulating neuronal functions.

Although synapse-specific mRNAs are ubiquitous components of RNA granules, the number and identities of mRNAs within the granules are unclear. A transcriptome analysis of the rat hippocampus revealed 2550 transcripts localized within dendrites and/or axons (Cajigas *et al*, 2012). While several different mRNAs can be carried by the same RNA granule (Kiebler *et al*, 1999), recent quantitative imaging approaches in neurons indicate that some mRNA species can transit in granules harboring just a single mRNA species (Batish *et al*, 2012; Buxbaum *et al.*, 2014; Mateu-Regue *et al*, 2019; Mikl *et al*, 2011; Park *et al*, 2014; Sahoo *et al.*, 2018). This indicates that there are multiple species of RNA granules, each containing distinct populations of mRNAs and RBP's (Elvira *et al*, 2006; Kanai *et al*, 2004). Ribosome profiling of RNA granules extracted from rat brains revealed that the most abundant mRNAs within the granules code for cytoskeletal proteins that are expressed developmentally in neuronal projection and synaptic compartments (Anadolu *et al*, 2021). Also, significant over-representation was seen of mRNA's encoding RNA binding proteins or proteins involved in RNA metabolism (Anadolu *et al.*, 2021).
Local translation requires post-translational modifications (PTM) of the bound RBP, including (de)phosphorylation and methylation that are mediated by locally-hosted enzymes in response to intracellular signaling (Coffee *et al*, 2012; Jobert *et al*, 2009; Narayanan *et al*, 2007; Urbanska *et al*, 2017). Therefore, after depolarization, localized mRNAs, including those involved in plasticity, rapidly shift from the RNA granule state to polysomes (Krichevsky & Kosik, 2001), from an inactive granule state to a translationally active polysome state.

Here we use proteomics and electron microscopy approaches to characterize molecular mechanisms of neuronal RNA granules isolated from Wistar rat brain tissues or a human neuroblastoma cell line. Proteomics detected the presence of important RBP's known to be part of nRNAg (Caprin-1, FMRP, Staufen 1 or 2, G3BP1 or 2, hnRNP's, ELAV-like proteins, Serbp1, and others) (Aschrafi *et al*, 2005; Chu *et al.*, 2019; El Fatimy *et al.*, 2016; Elvira *et al.*, 2006; Krichevsky & Kosik, 2001; Nakayama *et al.*, 2017). Chemical cross-linking experiments conducted with purified nRNAg samples revealed a potential G3BP2 binding site on the ribosome. Post-translational modification analysis of nRNAg shows the presence of phosphorylation and methyl-arginine sites on G3BP1 or G3BP2. The comparison of PTMs of G3BP1 or G3BP2 in nRNAg sample vs. nRNAg unbound sample, revealed differences in the modification patterns that indicate functional significance for identified PTMs for nRNAg assembly or disassembly.

The integrity and gross morphology of the purified nRNAgs was evaluated by negative staining electron microscopy (ns-EM). Differently from polysomes that tend to exhibit circular and zigzag-like morphologies (Myasnikov et al., 2014), RNA granules are known to possess a characteristic morula-like structure where individual ribosomes are tighly packed against each other (Elvira et al., 2006; Krichevsky and Kosik, 2001; El Fatimy et al., 2016).

We observed a similar morula-like morphology in the cortex-hippocampus-derived nRNAg's. and the presence of microtubules/filaments or vesicles in the proximity of nRNAg's, indicating their involvement in the active transport of RNA granules.

The structure of the ribosomes within the RNA granule was resolved by cryo-EM, using either intact nRNAgs or individual ribosomes obtained upon an RNase T1 treatment of the granules. Based on previous studies, ribosomes form the major structural unit of the nRNAg's (El Fatimy *et al.* 2016). The RNase T1 treated nRNAg sample was resolved to a 2.9 Å resolution whereas the intact map was resolved to 5.5 Å. We find that ribosomes are stalled in polysomic state in the nRNAg particles. This finding is consistent with Graber et al, 2014 where the authors used chemical ribopuromycylation assay and concluded that synaptic mRNAs are transported as stably paused polyribosomes. Recent ribosome profiling results of nRNAg support the polysome stalling as well (Anadolu *et al.*, 2021). We show the structural proof that ribosomes in nRNAg's are mainly stalled at polysomic state. The most suprising finding of our cryo-EM data is the discovery of new kind of principal motion inside the ribosome that we call "rocking" motion, where ribosomal small subunit moves around the horizontal axis.

Results

Neuronal RNA granules are morula-like particles

Wistar rat cerebral cortex, cerebellum, and hippocampus were used as sources of RNA granule material, as those tissues are involved in various memory-related tasks (Alves *et al.*, 2019; Chen *et al.*, 2017; Costa-Mattioli *et al.*, 2009; Graber *et al.*, 2017; Lebeau *et al.*, 2011; Miyakawa *et al.*, 2001; Nakayama *et al.*, 2017; Ohashi *et al.*, 2016; Shepherd *et al.*, 2006). To reduce contamination from the surrounding glial cells, the tissues were extracted from the brains of postnatal day 0 to 6 rats of both sexes, as gliogenesis starts from postnatal week 2 (Bandeira *et al*, 2009). RNA granules from tissue homogenates were purified using a combination of ultracentrifugation and size-exclusion chromatography. Briefly, the cytoplasmic extracts of the cortex-hippocampus tissue were first centrifuged through a 60 % sucrose cushion at 246 000 × g, followed by the solubilization of the resulting opalescent pellet (hereafter referred to as "Pellet 1"). According to a negative staining EM analysis, this material was heterogenous, containing filamentous polysome-like structures, as well as tightly packed, round-shaped particles of 100 – 200 nm diameter (Supplementary Figure S1A). The overall size and shape of the spherical-shaped particles resembles density-gradient purified RNA granules (Anadolu *et al.*, 2021; El Fatimy *et al.*, 2016; Krichevsky & Kosik, 2001).

To obtain a more homogenous preparation of the RNA granules, the redissolved sucrose gradient pellet fraction was passed through a Sephacryl S500 size-exclusion resin. Upon size-exclusion chromatography, 20 % - 50 % of the original pelleted material was recovered in the flow-through (referred to as "nRNAg"). EM analysis of the Sephacryl S500-purified material revealed a near-homogenous population of the spherical-shaped particles seen in the original sucrose gradient pellet (Figure 1 or Supplementary Figure S1B). When subjected to treatment with different RNases (RNase T1, MNase) at a physiological potassium concentration (150 mM), a majority of the morula-like particles in an nRNAg preparation falls apart into what appear as monosomes or smaller polysomes (di- or trisomes) (Figure 2; Supplementary Figure S2). However, depending on the type of nuclease, a variable fraction of the large and compact particles persist even after the nuclease treatment, particularly with MNase (Supplementary Figure S2A).

The same purification protocol was used to obtain nRNAg's from hippocampus (P0 to P5 rats) and cerebellum (P6 to P7 rats). The overall morphology of nRNAgs purified from those tissues was similar to the cortex-derived granules (Figure 1B and 1C). Consistent with the smaller number of neurons in hippocampus and cerebellum (Bandeira *et al.*, 2009), the yield of nRNAgs was reduced 10-fold for the cerebellum and 40-fold for the hippocampus relative to the cortex. Since the small amount of material obtained from hippocampus/cerebellum was insufficient for the downstream biochemical analyses, the experiments described below were exclusively performed with the the cortex-hippocampus (CH)-derived nRNAgs.
To make sure that the tightly packed particles in the nRNAg preparation are not an artifact of polysome aggregation due to the pelleting of the particles under high g-forces, we omitted the pelleting of the cortex-hippocampus postmitochondrial lysate and analyzed the sample for the presence of the morula-like particles. In the first experiment, the clarified lysate was directly passed through the Sephacryl-S500 resin and the particles were analyzed by ns-EM. Though larger contaminating complexes (vesicles, membranes) remain in the sample, a few tightly packed particles similar in size and shape to the nRNAg's were still observed (Supplementary Figure S3A – S3C). In the second experiment, the clarified lysate was centrifuged over a double sucrose cushion (90% bottom/ 60% above), so that the nRNAg material remained in the interface of two sucrose cushions (hence no pelleting of the particles) and was subsequently concentrated in a stirred cell unit where particles remain in solution during the concentration. Again, particles similar to the nRNAg's fraction were observed in an ns-EM analysis (Supplementary Figure S3D). Collectively, these observations indicate that the tightly packed complexes in the nRNAg fraction are not pelleting artifacts, but rather represent *bona fide* RNA granules.

Stress-granules (SG) are another type of RNA-protein complexes, whose assembly is similar to neuronal RNA granules in that it requires RBPs containing low complexity (LC) and intrinsically disordered (ID) regions (Kedersha *et al*, 2013). Unlike nRNAg, stress granules are phase-separated liquid-like droplets exhibiting the macroscopic behavior of hydrogels (Gopal *et al.*, 2017; Langdon & Gladfelter, 2018; Shiina, 2019). We observe such liquid-like droplets, when purified recombinant G3BP1 is visualized by ns-EM without additional factors (Supplementary Figure S4A and B). No droplets were observed with an MBP-tagged G3BP1 (Supplementary Figure S4C). MBP-tag is known to keep proteins in their solublized state (Costa *et al*, 2014). These liquid-like droplets of G3BP1 are markedly different from the solid morula-like particles that we obtain from the brain tissue. Additionally, the morula-like particles persist when the extraction and purification of the RNA granules is performed in the presence of cycloheximide, a known inhibitor of stress granule assembly (Bounedjah *et al*, 2014; Buchan *et al*, 2011; Kedersha *et al*, 2000). Below we present mass-spectrometry data for the presence of a full set of r-proteins (including the proteins of the 60S subunit) in the RNA granules as a further argument against the morula-like particles being stress granules, as 60S subunits are excluded form SG (Kimball *et al*, 2003; Wolozin & Ivanov, 2019). Coupled with the fact that the processing of the cryo-EM data (2D and 3D classification) revealed the 80S ribosome as the prevalent component of the granules, these observations collectively indicate that the morula-like particles in the nRNAg fraction are unlikely to be stress granules and rather represent *bona fide* neuronal RNA granules, as observed previously (Anadolu *et al.*, 2021; El Fatimy *et al.*, 2016; Krichevsky & Kosik, 2001).

Proteomic analysis of nRNAg composition

An LC-MS/MS analysis of the cortex-derived nRNAg preparation revealed the presence of a full set of ribosomal proteins, as well as some r-protein analogs. The r-proteins were the most abundant identified proteins based on their iBAQ (intensity based absolute quantification)

value (Supplementary Table S1). We also identified some uncharacterized r-protein analogs in the nRNAg preparation (Supplementary Table S2). We estimated the relative abundance of the identified r-proteins by normalizing the iBAQ value of each individual r-protein to the mean iBAQ value over all identified r-proteins. We chose the iBAQ value as the metric for r-protein abundance since it is commonly used to estimate the relative abundance of proteins from label-free proteomics data (Nagaraj *et al*, 2011; Schwanhausser *et al*, 2011) and has been shown to represent the best correlation between biological replicates, a normal distribution among all protein abundances, and the lowest variation among ribosomal protein abundances (Arike *et al*, 2012). For 66 out of the 80 identified r-proteins in the corticohippocampal nRNAg sample, the fold difference in the relative abundance with respect to the mean did not exceed 2 (Supplementary Table S3 and Supplementary Figure S5). A reanalysis of previously published data on r-protein abundance from mouse embryonic stem cells (Supplementary Figure S5) (Slavov *et al*, 2015) and HEK293-6E cells (Supplementary Figure S6) (van de Waterbeemd *et al*, 2018) revealed a similar "within 2-fold" variation in the relative abundances of the r-proteins. Out of the remaining 14 r-proteins in the nRNAg, the relative abundances of 12 r-proteins were 2 to 11-fold below the mean while the abundance of ubiquitin-60S ribosomal protein L40 (Uba52) was 97-fold below the mean (Supplementary Table 3). With the exception of Rpl37 and Uba52, the low-abundance r-proteins tended to be localized on the ribosomal surface and may therefore have been partially lost during nRNAg isolation. We also note that the number of identified unique + razor peptides was lower for the low-abundance proteins, particularly for Rps27l, Rpl37 (7 peptides) and Rpl39 and Uba52 (3 peptides) (Supplementary Table 3).

The RBPs (Caprin-1, G3BP1 or 2, Staufen2, Serbp1 etc.) were the second class of proteins that were identified in the RNAg preparations by the MS (Supplementary Table S1) or Western blot analysis (Supplementary Figure S7). To estimate the stoichiometry of RBPs relative to the ribosome in the RNAg we used the iBAQ intensity values determined by/obtained from the proteomic analysis of RNA granules. We first calculated the ribosome iBAQ value as an average of the individual iBAQ values of the r-proteins in two biological replicates, see Material and Methods (Supplementary Table S1).This average iBAQ intensity value of r-proteins was taken as 1 equimole of ribosome and the iBAQ values of the RBPs were normalized to it. Based on these estimations, the relative abundance of key RBPs varies from 8 to 123 fold compared to ribosomes (Supplementary Table S4) with Caprin-1 and G3BP2 being the most abundant ones at 9.5 and 8.4 ribosomes per RBP, respectively.

Neuronal RNA granules in a human neuroblastoma cell line

Human neuroblastoma cell line SH-SY5Y is a widely used in vitro model for various neurodegenerative disorders, as SH-SY5Y can be induced to differentiate into neuron-like cells by treatment with differentiation-promoting agents, including all-trans-retinoic acid (ATRA) (Kovalevich & Langford, 2013). We cultivated SH-SY5Y cells in the presence of 10 μM ATRA for 5 days, followed by cell lysis and purification of the post-mitochondrial supernatant identically to the cortical material. An ns-EM analysis of the Sephacryl-S500 purified material from SH-SY5Y revealed the presence of occasional morula-like particles similar to the particles obtained from the rodent cerebral cortex (Figure 3). A full set of r-proteins was detected in the nRNAg preparation in an MS analysis (Supplementary Table S1). Overall, the relative abundances of r-proteins in the RNAgs from rat and SH-SY5Y were significantly correlated (Pearson corrleation coefficient 0.59) (Supplementary Figure S8A). We also noted a significant positive correlation between the r-protein abundances in the SH-SY5Y RNA granules and 80S ribosomes from HEK293-6E (correlation coefficient 0.55) (Supplementary Figure S8C). Moreover, out of the 18 low-abundance r-proteins in the SH-

SY5Y RNA granules, 7 proteins (Rps27a, Rpl10a, Rpl37, Rps26, Rps15, Uba52, and Rps27l) were shared with the low-abundance r-protein set of the rat RNA granules (Supplementary Table S3). Interestingly, two of the low-abundance r-proteins in rat nRNAg (Rplp2, Rpl35) were in contrast present in the high-abundance r-protein set (r-proteins with a relative abundance more than 2-fold higher relative to the mean) in the SH-SY5Y RNA granules (Supplementary Table S3).

Similarly to the rat corticohippocampal nRNAgs, we estimated the relative abundance of RBPs in the SH-SY5Y derived nRNAg. Though the variation of the relative abundance of the RBPs with respect to ribosomis higher between the two replicates of the SH-SY5Y RNA granules the overall trend is that there is lesser amount of RBPs per ribosome in the SH-SY5Y nRNAg compared to the corticohippocampal RNA granules. Caprin-1 together with ELAVL4 and Serbp1 were the most abundant RBPs in the SH-SY5Y RNA granules, with a stoichiometry of one molecule of the respective RBP per 18.6, 7.8 and 8.1 ribosomes (Supplementary Table S4).

However, as shown by the ns-EM analysis, the major component of the nRNAg preparation consisted of lighter-staining particles, which bear close resemblance to glycogen storage granules (Figure 3A) (Besford *et al*, 2012; Sullivan *et al*, 2010). The levels of glycogen are up-regulated in various cancers: breast, kidney, uterus, bladder, ovary, skin and brain cancer cell lines (Zois *et al*, 2014).

We tried to remove the contaminating glycogen granules from the nRNAg preparation by subtractive affinity chromatography on a Ni-Sepharose resin pre-coated with a His-tagged maltose-binding protein (His-MBP) as MBP is known to bind various oligosaccharides (Quiocho *et al*, 1997; Walker *et al*, 2010). The unbound fraction (i.e. material not bound to MBP-Ni-Sepharose) obtained after pelleting the Ni-Sepharose resin consisted mostly of the morula-like stained particles similar to the ones observed in the cortical nRNAg sample (Figure 3B), indicating the ability of MBP to remove the glycogen contamination. However, a potential drawback of the Ni-Sepharose-coupled purification scheme is the ability of the Ni-resin to nonspecifically bind poly(A) containing RNA sequences (Nastasijevic *et al*, 2008). Since the majority of eukaryotic mRNAs contain poly(A) tails, we anticipated that at least a fraction of the mRNA-containing RNA granules may be selectively retained on the resin, thus biasing the downstream biochemical and cryo-EM analysis of the RNA granules. Indeed, when the His-MBP coated Ni-Sepharose resin was incubated with a cortical RNA granule preparation, followed by a wash-out of the resin with buffer and a subsequent elution of the nonspecifically bound material in 8 M urea, a mass-spectrometry analysis of the urea eluate detected 127 proteins, most of them r-proteins, as well as a number of RBP components of RNA granules (Caprin-1, Hnrnpu, Hnrnph1, Hnrnpd, Hnrnpr, Hnrnpf, Ybx1, Npm1, Serbp1, G3BP1, G3BP2, PABPC1, Ncl, Celf2, Cirbp, TDP-43, Elavl2, Stau2) (Supplementary Table S5). The non-specifically bound material was also tested against the anti-eL8, anti-G3BP1 and anti-Caprin-1 antibodies by Western blot analysis (Supplementary Figure S9) that shows the presence of key RBPs. Based on this analysis we conclude that extraction of RNA granules from the neuroblastoma cell line requires further optimization with respect to removing the extensive glycogen contamination, while simultaneously preserving the compositional integrity of the RNA granules. Hence, in the following we restrict our analysis to the brain-derived RNA granules.

nRNAg active transport by microtubules or filaments

In a ns-EM analysis of the cortical RNA granules we occasionally observe very large nRNAg that overstain, but have clearly visible and abundant microtubule/actin filaments associated with them (Figure 4). To our knowledge this is the first time that such a proximity between the nRNAg's and components of the cytoskeleton has been captured by EM. This result supports the notion that nRNAgs are actively transported on the microtubule/actin filaments (Balasanyan & Arnold, 2014; Heisler *et al*, 2011; Hirokawa, 2006). The association of nRNAgs with the components of the cytoskeleton is supported by our proteomic analysis of nRNAg, where we observe a number of microtubule related proteins in the cortical nRNAg preparation, including Tubb4b, Map1b, Tubb2b, Tubb3, Map1a, Mapt, Tubb2a, Tuba1b/Tuba4a, Kif5a, Kif1c, DynII1, DynII2, Vbp1, and Map2 (Supplementary Table S1). Components of the actin filaments were also detected in the nRNAg preparation, including Actb, Actr8, Acta1, Capzb, Dctn2, Fmnl2, Fnbp1 and Capza1 (Supplementary Table S1).

The close association of the cytoskeletal components with nRNAg's is further supported by crosslinking-coupled mass-spectrometry (CL-MS) experiments aimed at identifying interaction networks in RNA granules. Using cross-linkers of varying spacer arm lengths (DSS, DSG, BS3, BS(PEG)5, and Leiker), we were able to identify crosslinks between microtubule components and r-proteins, translation initiation factors, as well as uncharacterized proteins (Table 1). For example, using BS(PEG)5 we observed a cross-link between residue K402 of Dctn2 and K129 of the ribosomal protein eS24. Dctn2 is involved in dynein binding to an organelle and plays a role in synapse formation during brain development (LaMonte *et al*, 2002; Urnavicius *et al*, 2015). Likewise, using DSG we observed a cross-link between residue K143 of eS19 and K2 of kinesin-like protein 1 A (Kif1A), a neuron-specific protein involved in anterograde axonal transport of synaptic vesicle precursors (Okada *et al*, 1995). Residue K724 of another kinesin isoform, 5C (Kif5C) cross-linked to residue K11 of eS19 (Table 1). However, the interactions of cytoskeletal proteins in the RNA granules are not restricted to r-proteins as shown by the BS(PEG)5-specific cross-links of microtubule associated proteins Map1b and Map2 to translation initiation factors eIF5b and eIF3a (Table 1). Additionally, the microtubule associated protein Map1a gave a cross-link to Lrrc49, an uncharacterized protein predicted to be part of neuronal tubulin polyglytamylase complex (Huttlin *et al*, 2020). Hence, our observation of a Map1b-Lrrc49 cross-link provides further evidence that Lrrc49 is indeed involved in the microtubule trafficking complex.
In addition to the microtubule/filament components, the RNA-binding protein Rtraf (hCLE) was detected in nRNAg's in our proteomic analysis. In mature brain, Rtraf is found in mRNA-containing kinesin-associated granules in dendrites (Kanai *et al.*, 2004) whereas in developing brain Rtraf is a component of cytosolic, ribosone-containing RNA granules that transport specific mRNAs to dendrites for local translation (Elvira *et al.*, 2006). Recent evidence indicates that Rtraf in complex with Ddx1, Fam98B and/or Hspc117 (Rtcb) forms an alternative cap-binding complex which may modulate the translation of specific mRNAs in response to particular synaptic signals (Pazo *et al*, 2019). We note in this connection that Ddx1, Rtcb and Fam98A were also detected in nRNAgs in our proteomic analysis.
Together, the identification of cytoskeletal components and regulatory proteins like Rtraf in our nRNAg preparation as well as the physical association of the granules with microtubules/actin filaments seen in ns-EM supports the notion that nRNAg move along the cytoskeleton components.

Lysosome trafficking of nRNAg

When the purified cortical nRNAg's are analyzed by ns-EM, we occasionally observe RNA granules closely apposed to what appears as broken vesicles of a ca. 200 nm diameter (Figure

5). Those vesicles could be derived from e.g. endosomes or lysosomes. Given that our purification scheme includes an initial hypotonic lysis step and that lysosomes are known to be destroyed under hypotonic conditions, in contrast to osmotically insensitive endosomes (Schroter *et al*, 1999), the vesicle remnants are more likely to represent lysosomes. Since endocytic organelles do not penetrate dense sucrose solution during ultracentrifugation (de Araujo *et al*, 2008), the presence of the vesicle remnants in the nRNAg preparations indicates their physical association with the RNA granules prior to and during the purification. Importantly, in a recent study combining fluorescence microscopy and proximity labeling proteomics, lysosomes were shown to serve as vehicles for RNA granule transport with Anxa11 functioning as a tether between RNA granules and lysosomes (Liao *et al.*, 2019). Anxa11 presence was not detected in purified nRNAg's in this study. Instead, Anxa6 was present in our nRNAg sample and may play a similar role in granule trafficking as Anxa11.

This observation fits in with a growing body of evidence indicating a central role for membrane-bound organelles in RNA granule trafficking in different cell types (Pushpalatha & Besse, 2019), including neurons, where the organelles may additionally serve as platforms for localized mRNA translation (Cioni *et al*, 2019). Importantly in this respect, our proteomic analysis identified Ras-related proteins in brain (Rab) GTPases that are molecular switches cycling between a cytosolic GDP-bound inactive form and a membrane-associated GTP-bound active form to regulate membrane trafficking (Stenmark, 2009; Tang *et al*, 2014). Different Rab's localize to distinct membrane-bound cellular compartments in mammalian cells, including the endoplasmic reticulum (ER)-Golgi region (Rab1a, Rab2, Rab6, Rab30, and Rab33b), early and recycling endosomes (Rab4, Rab5, Rab11, Rab15, Rab17, Rab18, Rab22, and Rab25), late endosomes/lysosomes (Rab7 and Rab9), and specialized organelles such as synaptic vesicles (Rab3a and 3c), secretory granules (Rab3D, Rab37), and melanosomes (Rab27) (Junutula *et al*, 2004; Zerial & McBride, 2001). Rab18, Rab11a, Rab11b, Rab7a, Rab1a, Rab6b and Rab2a were detected in our nRNAg preprations (Supplementary Table S1). In addition, Gdi1 was detected that regulates the Rab binding to membranes through GDP/GTP exchange (Oesterlin *et al*, 2012). Our nRNAg proteomics analysis also identified uncharacterized protein (Gene ID: A0A0G2KAW3) that is believed to be part of lysosome trafficking (predicted by Uniprot). The protein sequence is 45% identical to Rab7a.

The crosslinking-coupled proteomics study was unable to detect cross-links between lysosome components and other proteins, probably to do the low abundance of complexes that co-purify with nRNAg.

Hence, the observed nRNAg-adjacent broken vesicles may be remnants of the RNA granule trafficking machinery which co-purifies with the granules.

RBPs interactions with the ribosome

In vivo fluorescence co-localization, together with co-sedimentation and pull-down assays, are commonly used to probe the interaction pattern of RBPs within RNA granules (Kedersha *et al*, 2016; Russo *et al.*, 2017). However, given the central role of RBPs in RNA granule assembly/disassembly, those methods are not sufficient to provide detailed information about the molecular contacts involved in the modulation of the translational activity of the RNA granules by RBPs. This information can potentially be provided by cross-linking or proximity-based labelling techniques (Leitner *et al*, 2016; O'Reilly & Rappsilber, 2018).

To characterize the interactions of RBPs with components of the RNA granule at a molecular level, we subjected the purified RNA granules to cross-linking with various lysine-specific cross-linkers, followed by a chromatographic enrichment of the cross-linked peptides and a

mass-spectrometric identification, essentially as described by Leitner and coworkers (Leitner *et al*, 2012). As expected, the majority of the identified cross-links were between r-proteins as the ribosomes are the main component of the RNA granules (Supplementary Table S6). CL-MS study identified over 50 different intermolecular and over 200 intramolecular CL between r-proteins (rProt). The rProt - rProt crosslinks are within the distance spanned by the cross-linker $BS(PEG)_5$ when mapped on the ribosome structure (5AJ0), validating our cross-linking protocol (Figure 6). However, we managed to identify a number of cross-links between known RBPs and r-proteins or other components of the translational apparatus (Table 1). The most interesting CL to us is a cross-link between G3BP2 (residues 248 or 332) and eS28 (residue 16) (Table 1). This cross-link indicates that in the cortex-derived RNA granules G3BP2 is located close to the ribosomal E-site, hinting at a direct interaction of G3BP2 with the ribosome. The Ras-GAP SH3 domain binding proteins (G3BPs) are well-known RNA-binding proteins whose medical relevance is underscored by the observation that their aberrant expression is common in various types of cancer, misregulated stress granule formation, defects in the translation of axodendritic mRNAs as well as by the fact that they are frequently targeted by viruses (Alam & Kennedy, 2019; Bidet *et al*, 2014; Reineke & Lloyd, 2013; Tourriere *et al*, 2003; White *et al*, 2007). As mammals contain two versions of G3BP (G3BP1, G3BP2), it is of note that the cross-link to eS28 was only detected with G3BP2, despite G3BP1 appearing in the list of proteins identified in our nRNAg preparations. Moreover, the same residue (K16) of eS28 that cross-linked to G3BP2 also gave a cross-link to eukaryotic initiation factor eIF3d (residues 142 and 149) (Table 1). Unfortunately, this cross-link could not be directly mapped to the structure of eIF3d in the 48S complex since the region of eIF3d containing the cross-linked residues is missing from the existing cryo-EM map (PDB id: 6fec or 6zmw) (Brito Querido *et al*, 2020; Eliseev *et al*, 2018). However, in the 48S structure of Eliseev and coworkers containing residues 172 – 536 of eIF3d the overall placement of the protein indicates that the missing region containing the cross-linking sites could indeed approach residue 16 of eS28 (Figure 7). eS28 together with eS26, and uS11 appear to interact with the eukaryotic mRNA and thus line out an alternative path of the mRNA exit (Budkevich *et al*, 2014). G3BP2 binding near the E-site or in the same pocket as eIF3d may indicate that G3BP2 interacts with the 5'-side of the mRNA that exits the ribosome. A binding location near the E-site presents clues about the mechanism of how the ribosomes could be stalled by G3BP2 since many translation inhibitors bind to 40S or 60S subunit E-site: cycloheximide, lactimidomycin, and phyllanthoside in 60S E-site or emetine, pactamycin, cryptopleurine and edeine in the 40S E-site.

Other functionally relevant cross-links between r-proteins and RBPs with a known role in the regulation of ribosomal activity/translational control include Serbp1 (CL to uS4, eS10, eS12, and RACK1), Pa2g4 (CL to uL29), Ttc5 (CL to uL24), Ccdc124 (CL to eS30) and Btf3 (CL to eL31) in our RNA granule preparations (Table 1). Some of those cross-links could be directly mapped to the existing structures of Serbp1/Pa2g4/Ttc5/Ccdc124/Btf3 on the eukaryotic ribosome where the cross-linked residues are seen to lie within the distance spanned by the spacer arm of the cross-linker (Supplementary Figure S10). With Serbp1, the cross-links to eS10 and eS12 are consistent with the protein's placement on the head of the 40S subunit as observed by Anger and coworkers (Anger *et al*, 2013). Likewise, the cross-link of Ttc5 to uL24 agrees with the data of Lin and coworkers showing uL24 as the unique protein binding partner of Ttc5 near the peptide exit site (Lin *et al*, 2020). Similarly, the cross-link between K238 of Pa2g4 and K43 of uL29 places Pa2g4 close to the peptide exit site on the 60S subunit and within an interaction distance of eL19, uL23 and uL29 as was observed by translationally inactive 80S ribosome (Bhaskar *et al*, 2021).

In addition to those RBPs, we identified cross-links between r-proteins and proteins involved in ribosome biogenesis (Pelp1, Gtpbp4, Nop58), ubiquitin-dependent protein degradation (Zer1) as well as RNA splicing (Nop58, Hnrnpc, Snu13, Sf3bf3) and stress granule assembly (Casc3).

Neuronal RNA granules are stalled at a polysomic state

We therefore sought to resolve the structure of an individual ribosomal particle within an nRNAg. For this, a data-set of nRNAg complexes purified from rat cortex-hippocampus tissue and subsequently treated with RNase T1 (to improve the particle separation) was collected. Titan Krios I TEM with Falcon 3 detector in linear mode was used with the magnified pixel size at 1.41 Å. As there was little difference between the cryo-EM maps of intact vs. RNase T1 treated nRNAg, we will discuss the findings of the higher resolution map (RNase T1 treated).
In total, 3644 micrographs were collected with the RNase T1 treated nRNAg sample. Relion 3.1 software (Zivanov *et al*, 2018) was used to process the single-particle cryo-EM data. As seen from the 2D classification step, we were not able to identify any 2D classes of 48S pre-initiation complexes that are thought to be the predominant form of ribosomes in stress granules (Anderson & Kedersha, 2009; Bentmann *et al.*, 2013; Buchan *et al.*, 2011; Kedersha *et al.*, 2016) (Supplementary Figure S11A). Also, proteomic analysis does not show a bias towards the small ribosomal subunit proteins (Supplementary Table S1), further indicating that the purified complexes are not stress granules.

After 2D classification (Supplementary Figure S11A) and multiple 3D classifications (Fig. 8A or Supplementary Figure S12), 3D class Cl2 (62 369 particles) was selected for further data-processing. The other classes (Cl3 and Cl4) did not show significant differences from Cl2 as seen from correlation value and visual inspection (Supplementary Figure S12). The Cl2 was further refined using unmasked and masked refinement, the resolution at FSC 0.143 was reported to be 2.95 Å (Supplementary Figure S11B). Per-particle, reference-based beam-induced motion correction step improved resolution to 2.87 Å and final map is shown in Figure 8B). Since no rat ribosome structure currently exists, we adapted the human ribosome structure (PDB ID 6ole) for refinement as it's conformation is closest to our structure (Figure 9). Phenix real-space refinement (Liebschner *et al*, 2019) was used to refine human 80S ribosome structure (PDB ID 6ole) against the map. The human ribosomal protein sequences were adapted to the corresponding rat sequences in the refined structure by using Coot software (Emsley *et al*, 2010). Total RNA was extracted from the nRNAg and variable regions of 18S rRNA were sequenced to adopt the sequence differences between human and rat rRNA on structure (Supplementary Figure S13). A similar analysis was performed with the 28S rRNA regions (Supplementary Figure S14). The 5S rRNA and 5.8S rRNA sequences are identical between human and rat when compared using nucleotide blastn suite of NCBI.

The refined structure resembles polysomic ribosomes with tRNAs in hybrid A/P and P/E states and contains nascent chain (4 residues long) in peptide exit tunnel (Figure 8C). The overall conformation of the ribosome is similar to Li et al., 2019 human 80S complexes stalled at the elongation state with the compound PF846 (Figure 9) (Li *et al*, 2019) or Budkevich et al., 2011 pre-translocation complex Rotated-2 state (Budkevich *et al*, 2011). This kind of rotated state structure precedes the mRNA and tRNA translocation step that moves them to the next codon, presumably impeding the action of eEF2 (Dever & Green, 2012; Li *et al.*, 2019). These results indicate that the ribosomes in RNA granules are stalled at the polysomic translation elongation state as proposed by (Graber et al., 2013) and more

recently by (Langille *et al*, 2019), where mRNAs are repressed at the elongation stage awaiting translational reactivation upon appropriate synaptic signals. A hybrid A/P state of tRNA may explain why stalled nRNAg complexes can bind puromycin as seen by Graber et al., 2013, as the 60S subunit A-site is not occupied by tRNA. Our results support the idea that the majority of the ribosomes in the complex are in a polysomic state. How the RNA binding proteins stall the ribosomes in nRNAg remains elusive.

As the majority of the ribosomes in our data-set are similar in composition and conformation and as it is known that when macromolecules exhibit continuous molecular motions, it is difficult to separate individual states by classical approaches, as one would need an infinite amount of classes to describe a continuum (Nakane & Scheres, 2021). We assumed that ribosomes in nRNAg should be more heterogenius than what was observed after multiple 3D classification steps (Supplementary Figure S12). The classical approach did not result in classes that significantly differ from the consensus map. The masked or focused refinements provide an efficient way to obtain high-resolution reconstructions. In our case, the masked classification with partial signal subtraction (40S and 60S) did not show any signs of deviation from consensus map.

The 80S ribosomes are intrinsically dynamic and adopt different conformational states during protein synthesis. The process of inter-subunit rotation is one of the principal motions where tRNA's adopt hybrid configurations and it can occur spontaneously (Budkevich *et al.*, 2014). Ribosomal subunits move in respect to each other in a ratchet-like inter-subunit motion (Budkevich *et al.*, 2011; Frank & Agrawal, 2000). The ratchet-like motion is coupled with the movement of tRNAs to intersubunit A/P and P/E hybrid states (Agirrezabala *et al*, 2008; Blanchard *et al*, 2004; Budkevich *et al.*, 2011; Munro *et al*, 2007). We used Relion multibody refinement approach to deal with continuous structural heterogeneity in cryo-EM (Nakane *et al*, 2018; Nakane & Scheres, 2021). The multibody refinement detected the ratcheting motion as the primary motion in our data-set (Movie S1). The ribosomal subunits rotate 12.3^{o} (~ 44 Å) with respect to each other. During the ratcheting step, the tRNA acceptor ends (3' CCA) move from A to P and P to E sites (Movie S2). This amount of movement has also been reported by others and agrees with the notion that the ratcheting is the main principal motion that drives the translocation of the ribosome (Brilot *et al*, 2013; Flis *et al*, 2018).

Whereas the 40S subunit "rolling" motion is described as a 40S subunit rotation ~6° toward the L1 stalk around the long axis of the small subunit (Budkevich *et al.*, 2014). The subunit rolling changes the intersubunit space and causes a reciprocal opening and closing of the A- and E-site regions (Budkevich *et al.*, 2014). It has been reported that the distance between the 40S and 60S subunits on the A-site of the 80S ribosome decreases by about 13–15 Å during subunit rolling from the POST state to the classical-1 PRE state (Budkevich et al., 2014). The multibody refinement on our data also detected the "rolling" motion (Movie S3). We measured the "subunit rolling" range in the A-site. When farthest points (28S rRNA U1961 – 18S rRNA A554) with respect to the rolling axis are selected to measure the distance, the widening of the A-site is ~13 Å, but if more proximal points (28S rRNA U4381 – eS30 K18) are chosen near the A-site tRNA, the opening is 9 Å. The opposite was reported for the E-site, which is narrower in the POST state than in the classical-1 PRE state but due to the smaller distance from the rotation axis, the underlying movements were in the range of 6–7 Å at the E-site (Budkevich *et al.*, 2014). Our measurements show 6 Å difference in the E-site when distance is measured between residues eL42 (P75) – uS7 (N186). Our "rolling" movement results are therefore similar to the previously reported results by Budkevich et al., 2014. However, the question remains as to what state the consensus map represents in the

“rolling” movement landscape? The Relion multibody refienent software outputs 10 maps (called bins) that represent the specific principal motions (movies are made using these 10 maps). The bin1 and bin10 maps represent the largest differences when compared to each other. Our consensus map that is refined to high resolution lies in the middle of these two extremes.

40S head swivel

In addition to the ratcheting and rolling motion of the ribosome, the 40S head can move independently from the rest of the small subunit (body/platform). It was established in a bacterial system that the translocation of a tRNA on the 30S subunit parallels the swiveling of the 30S head and is coupled to the unratcheting of the 30S body (Ratje *et al*, 2010). The head swivel is coupled to the tRNA anticodon stem-loop movement during the translocation step that is mediated by EF-G/eEF2. It was shown in the mammalian system that back-rotation and back-rolling, together with the swivel-like motion of the 40S head, provide a significant shift of the $tRNA_2$ - mRNA module relative to the 60S subunit and the 40S body/platform in the direction of translocation, resulting in compacted TI-POST-1 chimeric ap/P and pe/E hybrid states (Flis *et al*., 2018).

Our multibody refinement detected the head swivel and unratcheting of the 40S subunit as well. For the multibody refinement, 40S subunit was divided into two segments (40S head-tRNA and 40S body/platform) and 60S as third body. Interestingly, the multibody refinement detected two principal movement events associated with head swiveling and 40S unratcheting (Movie S4 and S5). In the first principal motion of the 40S head and body, the head moved only slightly ~ 4.7 - 7.7 Å, whereas body moved as much as 19.4 Å (Movie S4). In the second motion, 40S head moved as much as ~ 28 Å and body ~5.4 Å (Movie S5).

The “rocking” motion

We report a new kind of motion of the ribosome that is opposite to “rolling” motion and call it “rocking” (Movie S6). The “rocking” motion is detected by Relion multibody refinement when the ribosome is divided into 2 bodies (40S-tRNA and 60S). During the “rocking” motion the 40S subunit rotates ~ 5° (11 Å) toward the 60S subunit around the horizontal axis of tRNA 3’CCA-end (Figure 10). The entire 40S subunit moves in a manner where the 40S subunit head moves toward but the 40S bottom part (spur-body) moves away from the 60S subunit or vice versa. The tRNA T-arm (elbow) becomes twisted from the 60S subunit by ~ 2 Å. The tRNA’s (A/P and P/E states) anticodon stem-loops move as much as 6.2 Å but the acceptor-ends remain immobile. The intersubunit bridges B1a and B1b/c stretch during the “rocking” motion (Movie S7). Comparing the motion maps with our consensus map reveals that the consensus map is closest to the bin5 map. The bin1 map is more distinct from the consensus map than bin10 map. The question remains as to what kind of functionality is represented by the “rocking” motion? Is it related to the stalling conformation that is required during transport of RNA granules?

One possibility is that the “rocking” motion represents the 40S head and body motions that cannot be separated by the rigid 40S subunit and therefore these motions are trapped inside the “rocking” motion. Therefore, we conducted the multibody refinement with 3 bodies (40S-head-tRNA/40S-body/60S). As previously, multibody refinement detected ratchet like motion and head swiveling. The fifth principal motion that was detected displayed a “rocking”-like motion where the 40S body behaved similarly to the “rocking” motion but the 40S head only moved slightly compared to the coupled 40S movement (Movie S8 and S9). The 40S body moved as much as ~ 14.4 Å and head ~ 4.3 Å. During the 40S body movement the eS17 helix

(residues 70-82) becomes stretched out (Movie S9). The mRNA entrance channel formed by uS3 and 18S rRNA helix 16 (h16) closes and opens during “rocking” motion.

Discussion

We choose the cortex-hippocampus brain tissue to study neuronal RNA granules as these brain regions are preeminently involved in different memory related tasks. Synaptic plasticity and long-term memory have been linked to the nRNAg components (e.g. Caprin-1) (Nakayama *et al.*, 2017). The hippocampus is involved in recognition and spatial memory and transfers the information into cortical regions to give memories meaning and connection to other memories (Huerta *et al*, 2000; Tsien *et al*, 1996). It is also involved in consolidation where new learned information is transformed into long-term memories (from short-term to long-term memory). It was shown recently that the cerebral cortex is really responsible for long-term memories and not hippocampus (Hasan *et al*, 2013). Therefore the hippocampus provides the necessary environmental cues, which are transmitted to the cortex where learning-dependent associations take place. The cerebellum is involved in procedural memory, and motor learning whereas emotional (e.g. fear memory) memory is associated with amygdala.

The molecular mechanisms of memory storage are still elusive. It is thought that memories are distributed in neural networks (Bassett *et al*, 2013; Ferbinteanu, 2019). Long-lasting memories likely result from structural changes such as the growth of new synapses and associated neural networks (Kolb & Gibb, 2014). Memories that last a lifetime are thought to be stored, at least in part, as persistent enhancement of the strength of particular synapses (Jalil *et al*, 2015). Amnesia and dementia, as well as addiction and post-traumatic stress disorder, are associated with deficient or pathophysiological memory induction and maintenance (Jalil *et al.*, 2015). The fundamental issue here is how memories can be maintained over extended periods of time despite protein turnover and diffusion (Crick, 1984). “Molecular switch” has been proposed as a solution (Tyson *et al*, 2003; Verdugo *et al*, 2013). A local molecular switch could be placed at the level of post-translational modifications (Lisman, 1985; Routtenberg & Rekart, 2005) or at the level of protein synthesis (translation) (Abraham & Williams, 2008; Belelovsky *et al*, 2005), as translation can occur and be regulated locally in dendritic compartments (Batish *et al.*, 2012; Leal *et al*, 2014; Santos *et al*, 2010; Sutton & Schuman, 2006) in the form of neuronal RNA granules.

We show that nRNAg’s purified from the cortices of newborn Wistar rats are morula-like particles that contain ribosomes and RBPs. When analyzed by negative staining electron microscopy, the granules appear morphologically similar to whole brain-derived neuronal RNA granules reported in previous studies (Krichevsky and Kosik 2001; Graber *et al.* 2011; El Fatimy *et al.* 2016) and mostly dissociate into monosomes or smaller polysomes upon RNase treatment (Figure 2). The RNase sensitivity of our cortex-hippocampus-derived RNA granules seems to stand in contrast to the results of Graber *et al.* and El Fatimy *et al.* where the granules were largely resistant to RNase A/MNase treatment under condition similar to the present study. However, a recent study by Anadolu and coworkers indicates that the extent of RNase sensitivity of the granules is affected by *i.a.* monovalent salt concentration. According to Anadolu *et al*., increasing the salt concentration above the physiological level leads to a decompaction of the RNA granules with a concomitant increase in nuclease sensitivity. Though our nuclease sensitivity analysis was only performed at the physiological salt concentration, we note that the particle composition of our nuclease treated samples in ns-EM is broadly similar to that observed by Anadolu *et al.* upon RNase treatment in the absence of a high-salt preincubation of the granules. We therefore surmise that the precise

extent of nuclease sensitivity of RNA granules may depend on factors like the degree of compaction of the RNA granules in a particular preparation and/or the type of nuclease used. Indeed, we observed a somewhat higher fraction of nuclease-resistant particles in an MNase-treated sample compared to RNase T1 treatment (Supplementary Figure S2).

We also purified nRNAg from retinoic acid differentiated human neuroblastoma cell line SH-SY5Y. The major hurdle with neuroblastoma is the glycogen contamination as the particles are similar in size. The glycogen contamination does not interfere the identification of nRNAg components, but interferes cryo-EM analysis. Glycogen granules can be reduced by using pull-down strategies targeting specific component of nRNAg's but this leads to bias toward the specific component.

Caprin-1 together with G3BPs are important RBPs that are present in our nRNAg sample. It has been shown that they work in collaboration in stress granules (Kedersha *et al.*, 2016; Solomon *et al*, 2007). Caprin-1 promotes the assembly of RNA granules and is responsible for the transport of its bound mRNAs in cultured cells (Kedersha *et al.*, 2016; Shiina *et al*, 2005; Shiina & Tokunaga, 2010). Knockdown and knockout (KO) of Caprin-1 in cultured neurons causes a reduction in the synaptic connections on dendrites and the density of neural networks (Shiina & Tokunaga, 2010; Shiina *et al*, 2010). A heterozygous nonsense mutation in the caprin1 gene has been found in a human patient with autism spectrum disorder (ASD), and heterozygous KO of Rng105 gene in mice causes ASD-like behavior (Jiang *et al*, 2013; Ohashi *et al.*, 2016). Caprin-1 has been shown as an essential element of RNA granules for establishing long-term memory, and mediates dendritic localization of mRNAs as an underlying mechanism for AMPA receptor (AMPAR) scaling, synaptic strength and plasticity, and long-term memory formation (Nakayama *et al.*, 2017). Caprin-1 abundance is relatively high compared to other RBPs in our proteomic data based on the calculations of relative iBAQ intensities that are compared between proteins present in nRNAg (Supplementary Table S4). According to this analysis there is 1 molecule of Caprin-1 per 9 molecules of ribosomes in nRNAg. It was proposed that Caprin-1 and G3BP-1 were directly or indirectly associated in a stable complex in HEK293T cell extract pull-down experiments and overexpression induce the stress granule formation (Solomon *et al.*, 2007). The Caprin-1 region 352 to 380 is responsible for the binding to G3BP-1 NTF-2-like domain (residues 1 – 141) (Solomon *et al.*, 2007). We were not able to identify direct cross-links between Caprin-1 and G3BP1 in nRNAg sample, although both of them are present in nRNAg sample (G3BP1 having relative abundance fold value 24 and G3BP2 having 8). As we only identify cross-links between protein amine groups, then one possibile reason, why we do not see the interaction between Caprin-1 and G3BP's, is that there are no available amine groups within the optimal distance on the surface of the proteins to form the covalent bonds with a cross-linker. At the same time, it was shown by others that Caprin-1 and G3BP1 can bind to each other, but the ability to induce SG formation or enter them did not depend on their association with each other (Solomon *et al.*, 2007). Instead, we were able to identify link between Caprin-1 (residue 136) and Pelp1 (residue 1) by using leiker cross-linker (Tan *et al*, 2016). Proline-, glutamic acid- and leucine-rich protein 1 (PELP1) is RBP involved in transcription regulation and 60S ribosomal subunit synthesis and ribosomal RNA transcription (Castle *et al*, 2012; Finkbeiner *et al*, 2011). The two proteins have been shown to be associated with MID1 complex that regulates translation through mTOR and involved in the phosphorylation of tau which hyperphosphorylation induces amyloid-β (Aβ) peptides, and neurofibrillary tangles formation in Alzheimer's disease (Matthes *et al*, 2018). Our cross-linking results show a so far uncharacterized connection between these two proteins in the neuronal RNA granules.

It has been shown that cells lacking both G3BP1 and G3BP2 cannot form SGs in response to eIF2α phosphorylation or eIF4A inhibition (Kedersha *et al.*, 2016). The G3BP1-F33W, a mutant unable to bind G3BP partner proteins Caprin-1 or USP10, can rescue the SG formation (Kedersha *et al.*, 2016). Caprin-1 binding to G3BP's promotes, but USP10 binding inhibits, SG formation (Kedersha *et al.*, 2016). USP10 is also present in our nRNAg sample, although much less abundant than G3BPs and Caprin-1 having 124 fold difference to ribosomes (Supplementary Table S4). It remains unclear if the interaction of G3BP's and Caprin-1 is required for nRNAg formation as we did not see any direct cross-links between these two proteins. It may also indicate that after formation of nRNAg the contacts are no longer maintained. Immunoprecipitation studies show that G3BP1 can interact with 40S ribosomal subunits through its RGG motif, which is also required for G3BP-mediated SG formation (Kedersha *et al.*, 2016). More specifically, small ribosomal subunit proteins eS6 and uS12 co-immunoprecipitated with G3BP1 (Kedersha *et al.*, 2016). Our cross-linking results show that G3BP2 may interact with small ribosomal protein eS28. eS28 is eukaryotic specific r-protein that interacts with mRNA in the exit tunnel. eS28 residue 16 gives cross-links with G3BP2 residues 248 and 332. This cross-link is specific to G3BP2 as we do not detect G3BP1 cross-links with ribosomal proteins. The identity between human G3BP1 and G3BP2a is 64% and G3BP1 and G3BP2b is 59%. The G3BP2 "binding-site" is special as eIF3d subunit can independently bind to this site and regulate translation of sub-set of mRNA's (Lee *et al*, 2016). This may indicate that G3BP2 could also mediate translation of specific mRNAs. Beside inhibition, it has been shown that G3BPs can promote protein synthesis of interferon-stimulated genes that are involved in antiviral activity, and in cancer progression and metastasis (Alam & Kennedy, 2019). The possible G3BP2 binding site on the ribosome needs to be further studied.

The microtubule and filament association with nRNAg's indicate their involvement in transporation that is known phenomenon (Balasanyan & Arnold, 2014; Heisler *et al.*, 2011; Hirokawa, 2006). Lesser-known is the lysosome/vesicle involvement in the transporatation of RNA granules. Recent studies have shown that axonal lysosomes co-traffick with RNA granules labeled with G3BP1 (Sahoo *et al.*, 2018), TDP-43 (Alami *et al.*, 2014; Gopal *et al.*, 2017), and Caprin-1 (Nakayama *et al.*, 2017). We show that lysosomes/vesicles are in close proximity to nRNAg's using ns-EM. The proteomics analysis identified Rab proteins that are markers for different membrane bound organelles (Stenmark, 2009; Tang *et al.*, 2014). The seven Rab proteins identified in this study in nRNAg sample give a clue as to what kind of membrane containing organells are present. Further experiments are required to investigate the involment of these Rab proteins in nRNAg transport or storage in more detail. It is known that some Rab proteins are involved in neuronal processes. The Rab18, Rab11a and Rab11b (were also identified in our study) are part of recycling endosomes (RE) and neuronal RE's regulate retrograde neurotrophic signaling, axonal pathway fixation during protein development, renewal and degradation, vesicle recycling, and synaptic plasticity, among other processes (Dittman & Ryan, 2009; Itofusa & Kamiguchi, 2011; Kennedy & Ehlers, 2006; Rozes-Salvador *et al*, 2020; Winckler & Mellman, 2010). Rab7A is involved in the trafficking of endosomes, autophagosomes, and lysosomes, and regulates tau secretion (Rodriguez *et al*, 2017). In Alzheimer's disease (AD), tau protein accumulation in somato-dendritic compartments is the main cause of neurodegeneration and RAB7A gene expression and protein levels are up-regulated in AD patients (Ginsberg *et al*, 2011; Ginsberg *et al*, 2010). Also, Rab18 is involved in liquid droplet formation and facilitates dengue virus infection (Tang *et al.*, 2014).

Our cryo-EM data indicates that majority of ribosomes are stalled in the state that resembles polysomic pre-translocated state where tRNA's are in A/P or P/E state. The tRNA hybrid states are favored within the mammalian complex (Budkevich *et al.*, 2011). Hybrid tRNA positions are defined by a spontaneous movement of A- and P-site tRNAs in the direction of translocation on the large ribosomal subunit, while the anticodon portions of tRNA remain fixed with respect to the small subunit (A/P and P/E hybrid sites, respectively) (Budkevich *et al.*, 2011; Flis *et al.*, 2018). Hybrid tRNA configurations have been suggested to serve as *bona fide* intermediates in the translocation process by lowering the activation barrier for translocation (Dorner *et al*, 2006; Semenkov *et al*, 2000). The rolling motion of the ribosome converts the POST to the classical PRE state and occurs during the accommodation step of A-site tRNA (Budkevich *et al.*, 2011). We discovered a new kind of ribosome conformational change that we call "rocking" motion. The "rocking" motion has been proposed previously by Brimacombe and coworkers using UV cross-linking assay (Stiege *et al*, 1988). According to their intrepetation of the "rocking" motion was that tRNA binding deep in the cleft of small ribosome in the presence of mRNA causes a "rocking" motion of the head region of the subunit, leading to allosteric protections in the helix 18 region. The rocking motion would likely be accompanied by a corresponding slight opening and closing of the cleft, which would effectively create a molecular turnstile", allowing the tRNA molecules to pass through (Stiege *et al.*, 1988). It is hard to interpretate what kind of motion they are positing as rolling motion opens and closes the A- and E-site regions (Budkevich *et al.*, 2014). The "rocking" motion does not seem to have an impact on the peptidyl transferase center of the ribosome as the tRNA acceptor ends do not exhibit movement. One possibility is that the "rocking" motion plays a role in the E-site tRNA exiting as this kind of motion can loosen the contacts between 40S head and 60S subunit or the L1-stalk. On the other hand, the anticodon stem-loop of P/E site tRNA changes 6 Å, but the movement of tRNA is not toward the exiting site, instead the anticodon part moves perpendicular to exiting. We argue that the "rocking" motion can be part of the stalling mechanism where ribosomes adopt a conformation that does not support the translation due to their inability to bind translation factors. Also, it may reflect the translation factor binding to the A-site that pushes the 40S body in the outside direction. For example, binding of eEF2 to the ribosome during the elongation step induces the power stroke that unlocks the ribosome to initiate translocation (Chen *et al*, 2016; Noller *et al*, 2017). Although, classification and refinement maps to not show any density for A-site bound translation factors.

The main conclusion of our study is that nRNAg's contain polysomic ribosomes that can escape the rate limiting translation initiation step of protein synthesis. We hypothesise that G3BP2 targets the ribosomal E-site to stall or activate translation, dependent of mRNA spices.

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Bank with accession code EMD-13954 (RNase T1 treated) and EMD-13975 (intact). Protein coordinate has been deposited in the Protein Data Bank (PDB) with ID 7QGG. Mass-spectrometry coupled cross-linking data is deposited to ProXL database and can be accessed using URL: https://www.yeastrc.org/proxl_public/viewProject.do?project_id=527

Author contributions

KK and AP performed rat brain dissection, purification of neuronal RNA granules, negative staining EM experiments, and protein purification. KK perfomed the cell culture experiments. KK and AM performed chemical cross-linking experiments. AP prepared the cryo-EM grids. VT, AA and AP performed cryo-EM data analysis. AP conducted the cross-linking data analysis using pLink. AM performed rRNA sequencing assay. AP and KK prepared the manuscript, with contributions and edits from AA, VT and AM.

Conflict of interest

The authors declare that they have no conflict of interest.

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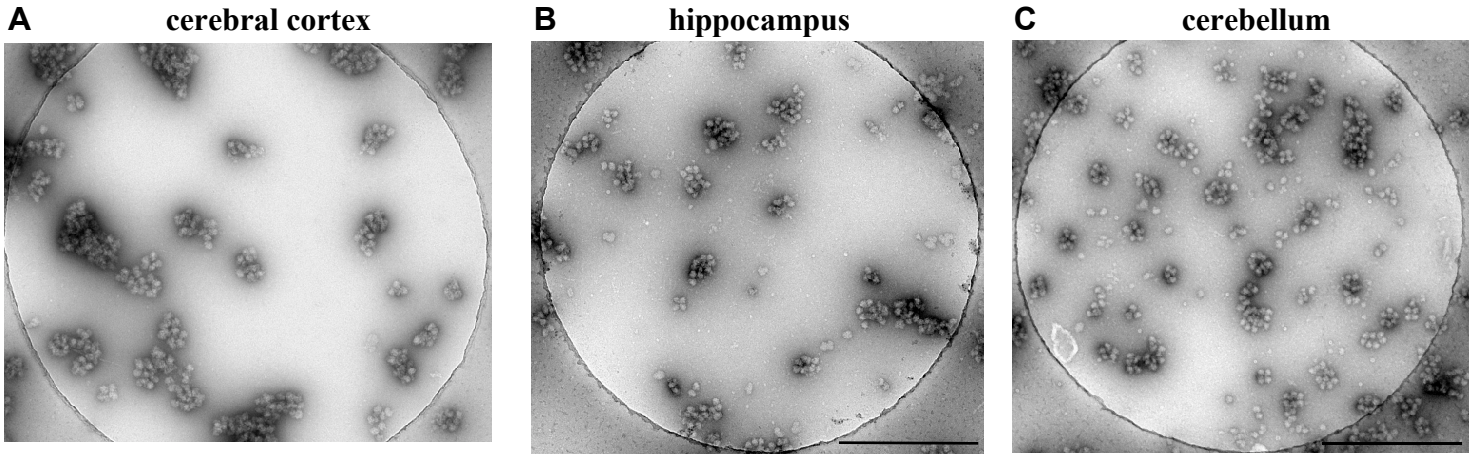


Figure 1.

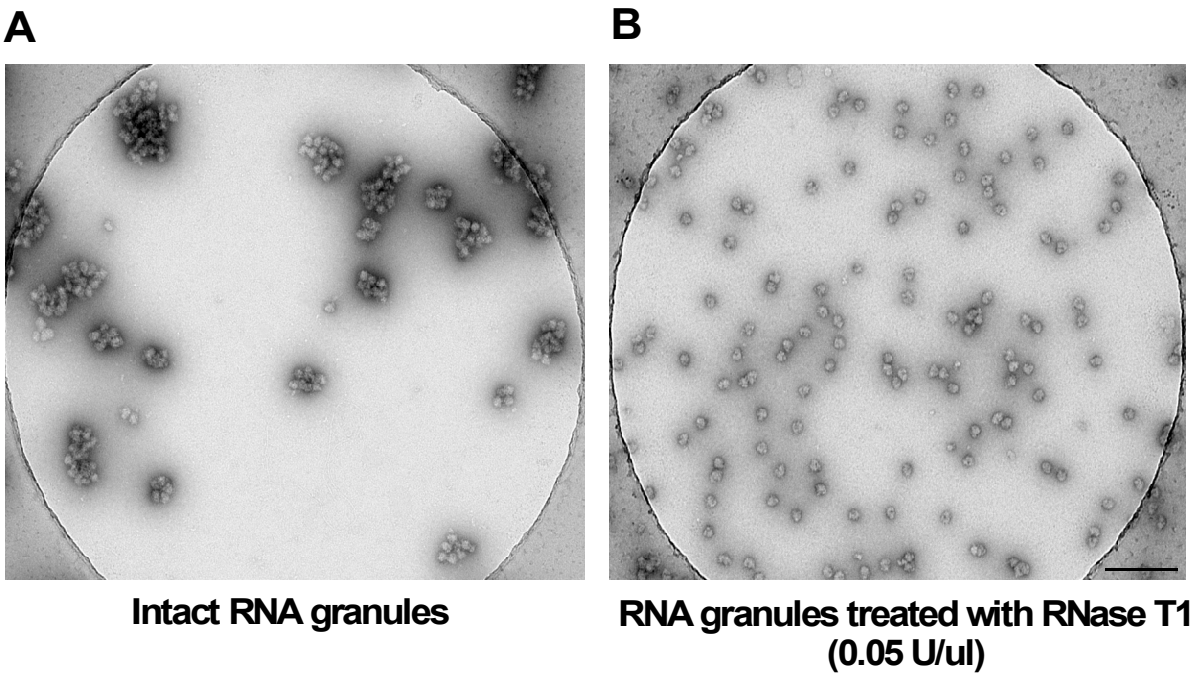

A
B
Intact RNA granules
RNA granules treated with RNase T1
(0.05 U/ul)

Figure 2.

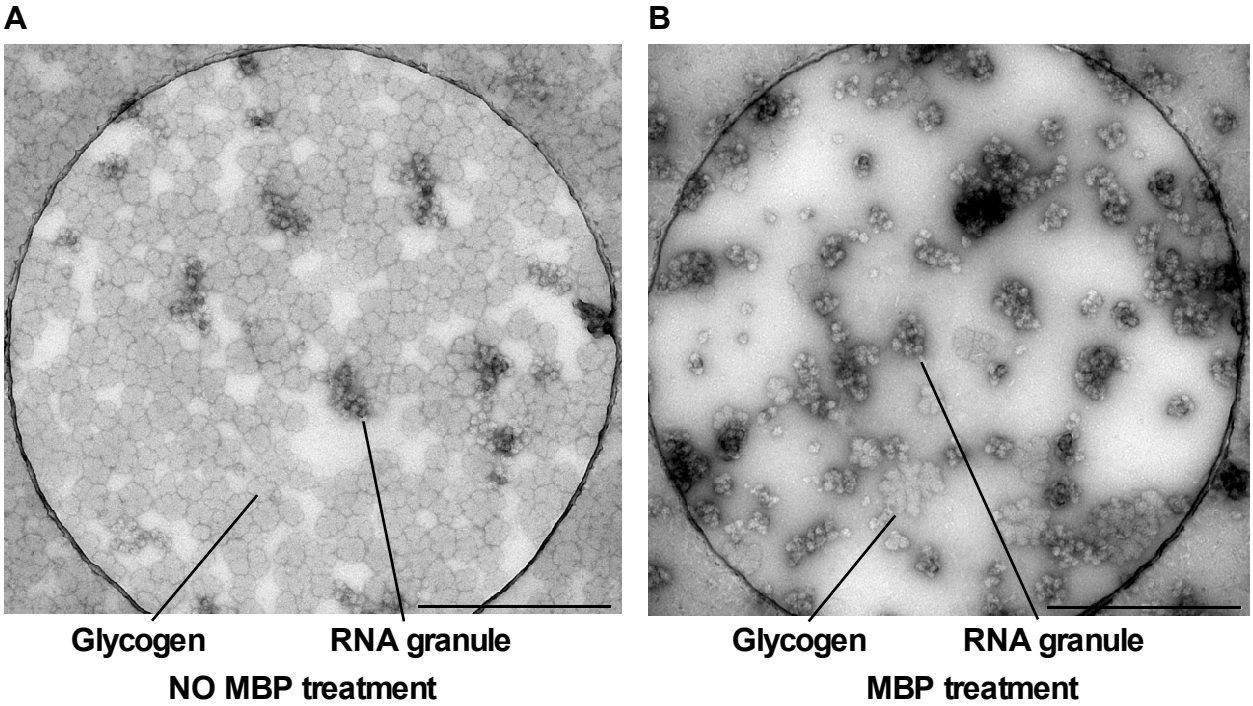
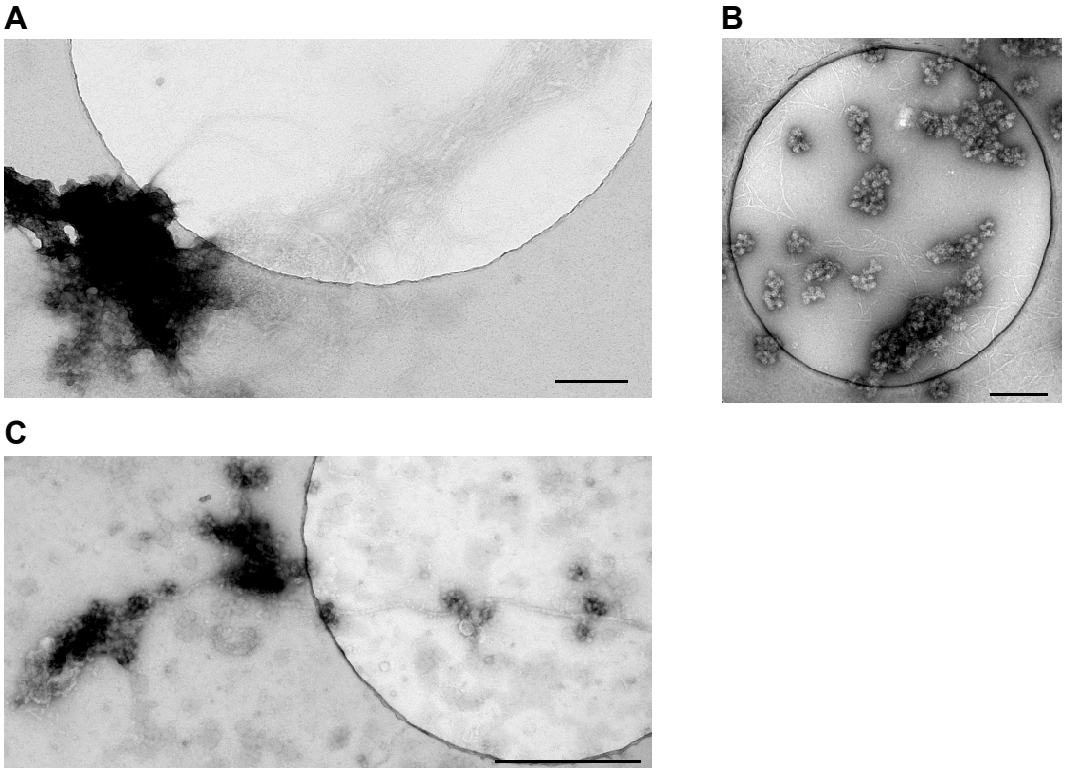

A
B
Glycogen
RNA granule
NO MBP treatment
Glycogen
RNA granule
MBP treatment

Figure 3.



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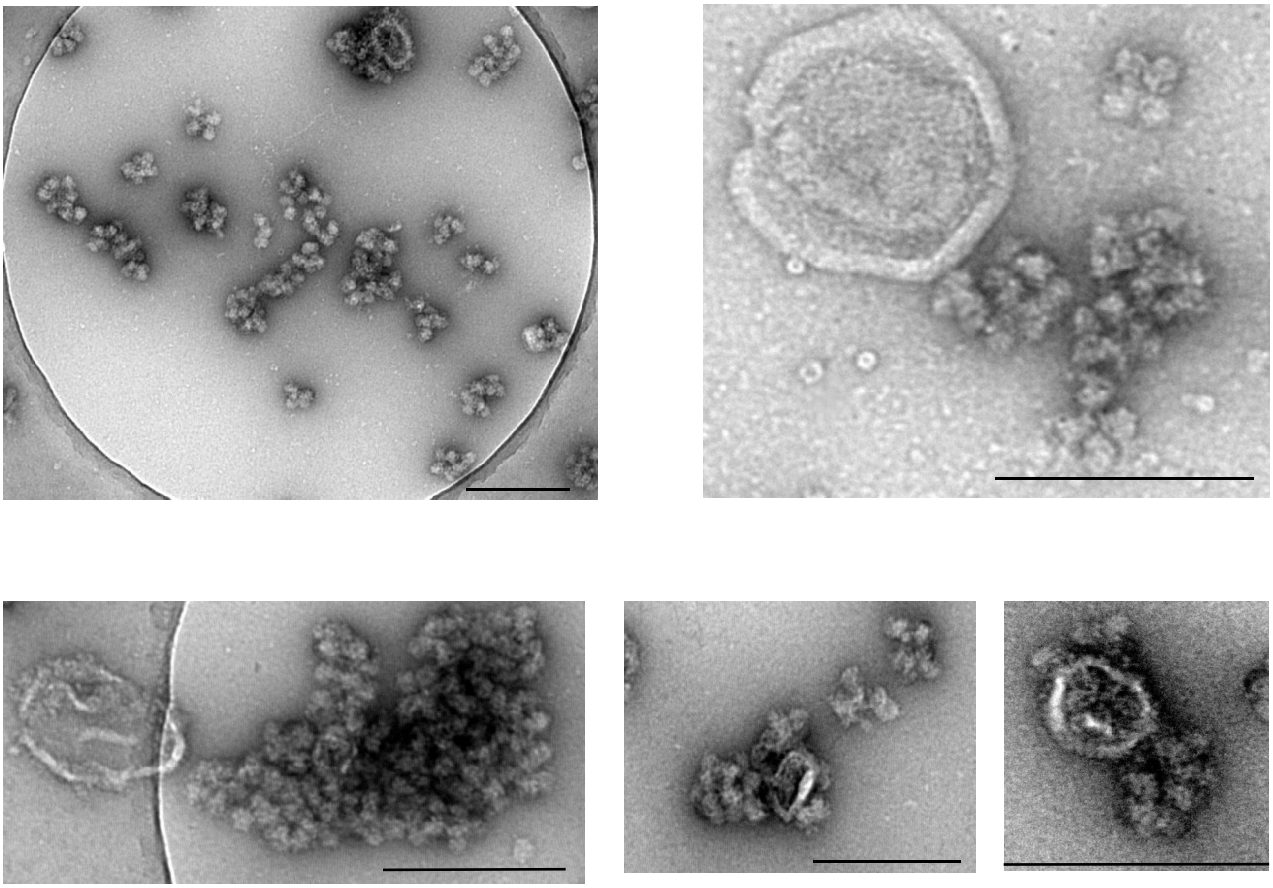

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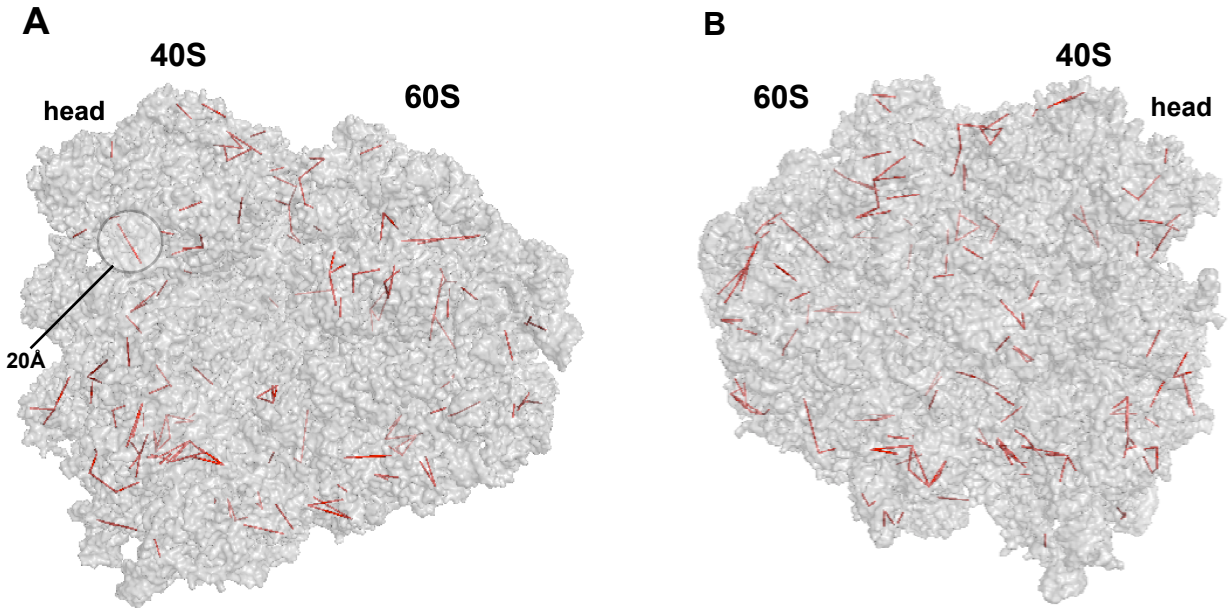

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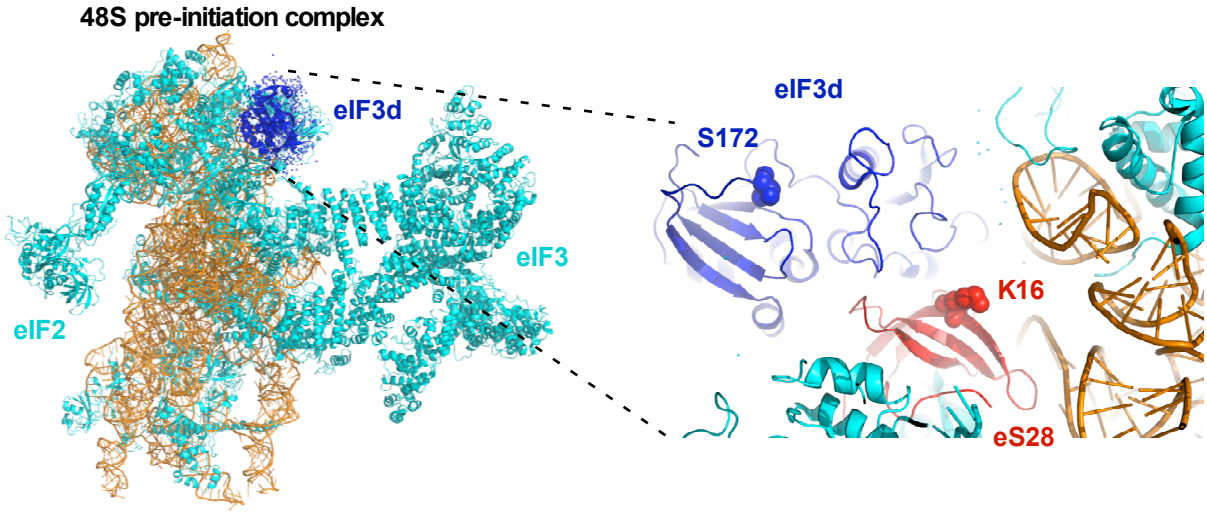
48S pre-initiation complex
eIF3d
eIF3
eIF2
eIF3d
S172
K16
eS28

Figure 7.

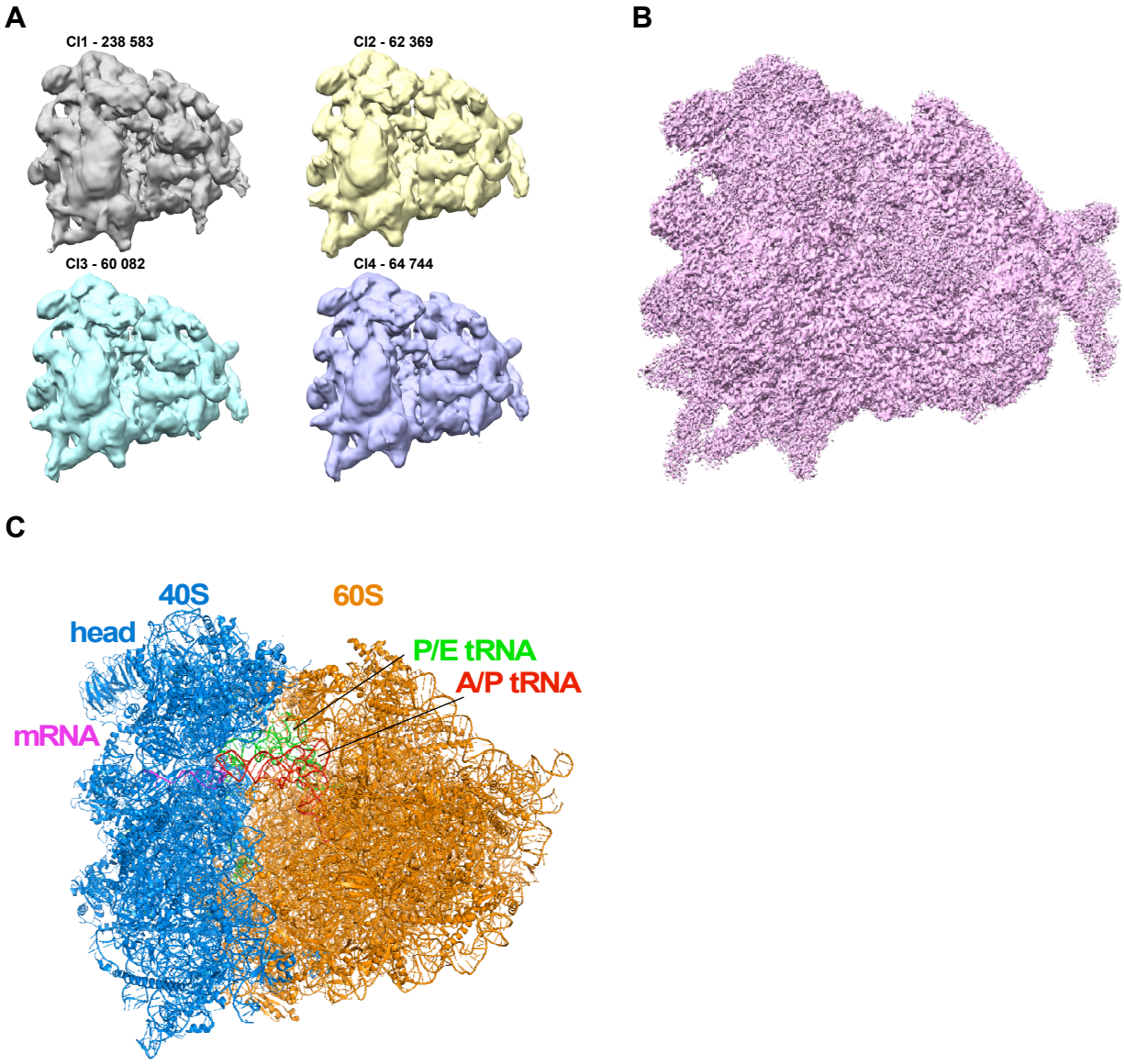
A
Cl1 - 238 583
Cl2 - 62 369
Cl3 - 60 082
Cl4 - 64 744
B
C
40S
60S
head
P/E tRNA
A/P tRNA
mRNA

Figure 8.

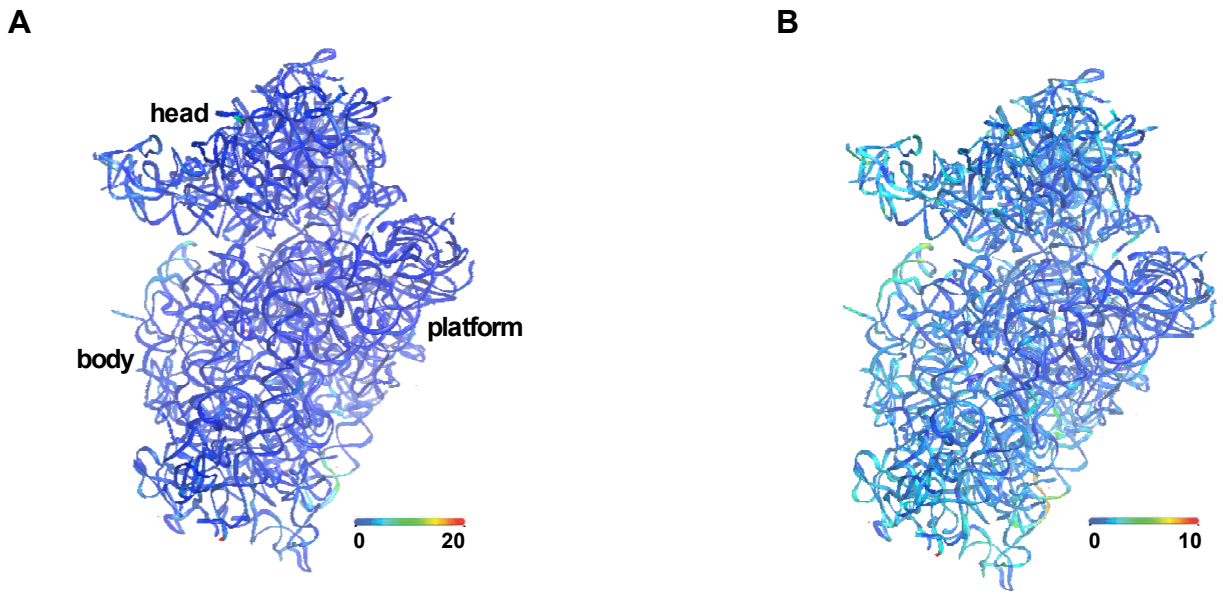

Figure 9

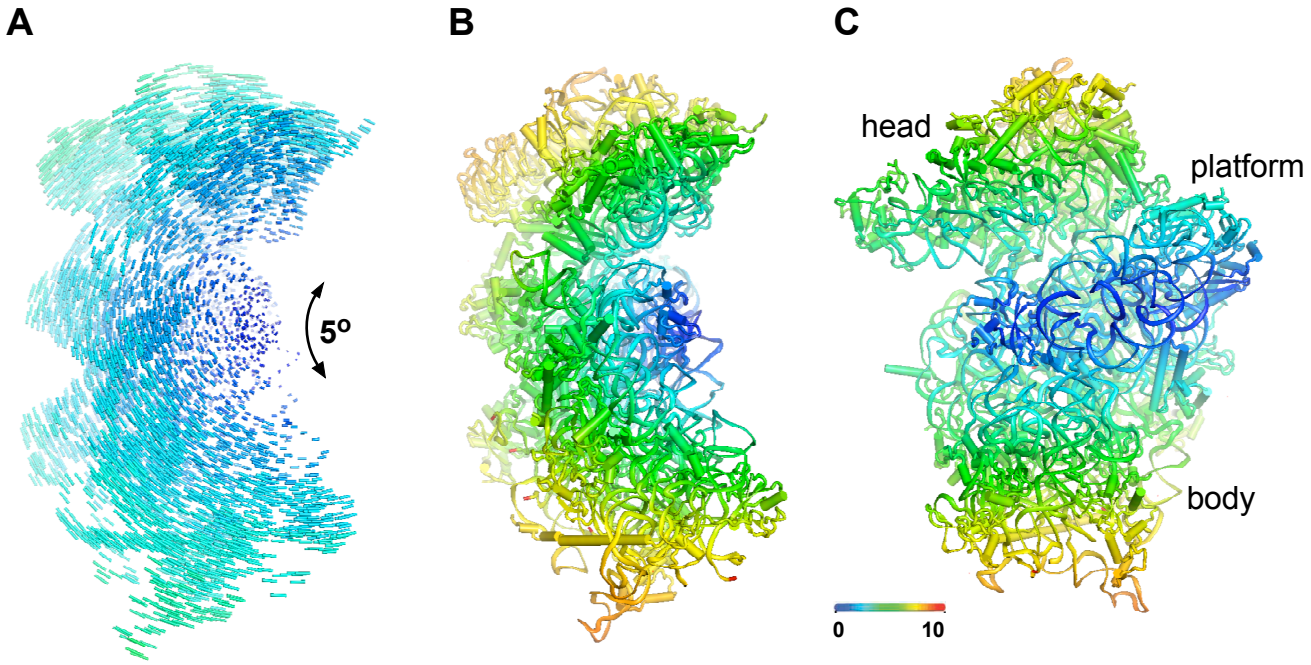

Figure 10

BS(PEG)5	DSG	DSS or BS3	Leiker
RBPs			
Btf3 (13)-Rpl31(70)	Ccdc124(179)-Rps30(1)	Serbp1 (298)-Rps12(116)	Btf3 (23)-Rpl31(70)
Btf3 (13)-Rpl31(91)	Snd1(470)-Rps9(155)	Spats2(95)-Rpl14(156)	Caprin1(136)-Pelp1(1)
Casc3(2)-Rpl22(16)		Ybx1(91)-Lig3(2)	Hnrnpc (98)-Rpl7a(11)
Dctn2(402)-Rps24(129)		Edc3(174)-Rps19(111)	Kars(60)-Rpl38(9)
Edf1(15)-Rps28(16)		Hnrnpa2b1(101)-Hsp90ab1(173)	
Erp29(253)-Rpl14(84)		Lsm14b(317)-RGD1308750(90)	
G3bp2(248)-Rps28(16)			
G3bp2(332)-Rps28(16)			
Pa2g4(238)-Rpl35(43)			
Serbp1 (103)-Rps9(121)			
Serbp1 (298)-Rps12(116)			
Serbp1 (302)-Rps12(129)			
Serbp1 (328)-Rps10(31)			
Serbp1 (389)-Rack1 (183)			
TDP43(192)-Lig3(2) or Pabp1(167)-Lig3(2)			
Ttc5(401)-Rpl26(36)		Ttc5(283)-Rpl26(36)	
Ttc5(401)-Rpl26(41)			
Ubap2(107)-Rpl10(13)			
Translation initiation			
Eif3a (563)-Map2(460)	Dhx29(59)-Rps9(121)	Eif5b(365)-Map1b(2284)	Eif3a (634)-Rpl6(270)
Eif3d (142)-Rps28(16)	Eif3d (142)-Rps28(16)		Eif3j (77)-Rpl38(9)
Eif3d (149)-Rps28(16)			Eif4a1 (226)-Rpl4 (294)
Eif3l (367)-Eif3e (409)			Eif4g1(641)-Rpl31(70)
Eif4a1 (54)-Eif4g1(877)			
Eif5b(365)-Map1b(2284)			
Pre-mRNA splicing			
Scaper(258)-Snrnp200(599)	Srpk1(4)-Rpl31(70)	Sf3b3(974)-Sgo2(25)	
Snu13 (86)-Nop58 (390)	Scaper(568)-Rpl26(41)	Sf3b1(2)-RGD1564606(18)	
Cytoskeleton and membrane binding			
	Atp1b1(22)-Rps14(86)	Lrrc49(1)-Map1a(193)	
	Fnbp1(376)-Rpl5(220)	Tecr (57)-Rps19(23)	
	Kif5c(724)-Rpl7a(11)		
	Kif1a(2)-Rps19(143)		
Proteasomal degradation			
			Zer1(547)-Rpl26(69)
Chromatin remodeling			

Hist1h1a(196)-Rpl18a(11)	Hist1h1a(83)-Rpl23(75)	Hist1h1a(83)-Rpl10(74)	Hist1h1a(196)-Rpl18a(11)
Hist1h1a(83)-Rpl18a(11)	Hist1h1a(83)-Rpl18a(11)	Hist1h1a(83)-Rpl10(82)	Hist1h1b(190)-Rpl14(156)
Hp1bp3(481)-Rpl23(113)	Hist1h1b(31)-Rps4 (233)		Gtpbp4(371)-Rps20(7)
Hist1h1c, subunits(81)-Rps24(129)			Gtpbp4(371)-S10-Unchar-A0A0G2JY70(1)
Gtpbp4(608)-Smarcc1(905)			
Uncharacterized r-proteins			
L6e-Unchar-A0A0G2KBA1(23)-Rpl29(122)	L6e-Unchar-A0A0G2KBA1(23)-Rpl29(132)	L23a-RGD1564606(23)-Lars(196)	L23a-RGD1564606(7)-Rpl36(62)
L6e-Unchar-A0A0G2KBA1(23)-Rpl6(48)	L6e-Unchar-A0A0G2KBA1(23)-Rpl29(140)	L23a-RGD1564606(7)-Rpl36(62)	L23a-RGD1564606(7)-Top1(328)
L6e-Unchar-A0A0G2KBA1(23)-Rpl6(50)	L6e-Unchar-A0A0G2KBA1(23)-Rpl6(42)	L6e-Unchar-A0A0G2KBA1(23)-Rpl6(42)	
uS5-rCG_34378(150)-Rps9(91)			
uS5-rCG_34378(150)-Rps9(93)			
uS5-rCG_34378(32)-Rps3(108)			
uS5-rCG_34378(32)-Rps3(132)			

Table 1. Cross-linking coupled mass-spectrometry results of nRNAg. nRNAg were purified from rat cortices and cross-linkers having various spacer arm lengths were used. Cross-linkers DSG (spacer arm length 7.7 Å), Leiker (9.3 Å), DSS or BS3 (11.4 Å), and $BS(PEG)_5$ (21.7 Å) are indicated on top row and Cl proteins are sorted by functional groups. Only inter-protein cross-links are shown. pLink software was used to process the mass-spectrometry CL data.