Reining in RNA

Workshop on Intracellular RNA Localization and Localized Translation

Patrick C. Gilligan & Karuna Sampath⁺

Temasek Life Sciences Laboratory, National University of Singapore, Singapore

The first joint EMBO/FASEB Workshop on Intracellular RNA Localization and Localized Translation took place between 1 and 6 July 2007, in Il Ciocco, Italy, and was organized by A. Ephrussi, E. Gavis, D. Ish-Horowicz and J. Richter. Originally a biannual FASEB summer research conference, it is anticipated that the joint meeting will in future alternate between sites in Europe and the USA.

& Derisi, 2007). The subjects discussed at this meeting included structural insights into RNA localization, improved methods for live imaging of RNA transport, and the combination of genome-wide screens and biochemical approaches to identify localized RNAs and localization factors. Here, we highlight some of the crucial findings that were presented.

RNA localization: mechanisms and patterns

Drosophila melanogaster has been widely used as a model organism to study mRNA localization and localized translation. This reflects the importance of these processes during *Drosophila* embryogenesis and the success of genetic screens in this organism. Indeed, visual screening methods continue to be used for the identification of new localization factors. For example, in a screen for new factors controlling microtubule organization in the fly oocyte, A. Guichet (Paris, France) found that the phosphatidylinositol-4-phosphate 5-kinase (PIP5K) *Skittles* is necessary for phosphatidylinositol 4,5-bisphosphate (PIP2) production and subsequent microtubule polarization, as well as for actin organization at the cortex. A. Ephrussi (Heidelberg, Germany) used a protein-trap screen to identify *Drosophila* heterogeneous nuclear ribonucleoprotein 1/polypyrimidine tract binding protein 1 (hnRNP1/PTB) as a new component of the *oskar* (*osk*) RNP complex. Oocytes of *ptb* mutants show defects in polarization of the microtubule cytoskeleton, delayed posterior *osk* RNA localization and premature translation of *osk* mRNA. Therefore, Ephrussi proposed that PTB constitutes a new *osk* mRNA translational repressor.

In many organisms, the germ-line progenitor cells (PGCs) are specified in the embryo by the localization and/or localized translation of maternal mRNAs. In *Drosophila*, the germ plasm is localized to the posterior of oocytes and early embryos, which is where germ cells are formed. Analysing expression patterns from microarrays and the Berkeley *Drosophila* Genome Project, R. Lehmann (New York, NY, USA) reported that fewer than 20 of the more than 200 mRNAs present in embryonic PGCs are actively localized. The rest are uniformly distributed in the egg and protected from degradation in early germ cells. Real-time imaging of nanos RNA fused to bacteriophage MS2 RNA at the posterior pole showed that nuclei entering the germ plasm capture the nanos RNA. During subsequent nuclear divisions in some cells, RNA is partitioned asymmetrically and nuclei that receive only small amounts of nanos RNA adopt somatic fates.

Keywords: RNA localization; translational control; RNA transport; RNA-protein interactions; localization factors

EMBO reports (2008) 9, 22–26. doi:10.1038/sj.embor.7401140

Introduction

Intracellular RNA localization and localized translation are involved in diverse processes such as embryonic patterning in *Drosophila*, jellyfish, tunicates, frogs and fish; the asymmetrical partitioning of fates in *Drosophila* neural stem cells and yeast mating types; fibroblast and neuronal growth cone motility; and synapse function (St Johnston, 2005; Du *et al*, 2007; Jambhekar

Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, 117604 Singapore

⁺Corresponding author. Tel: +65 6872 7090; Fax: +65 6872 7007;

E-mail: karuna@tll.org.sg

Submitted 25 September 2007; accepted 19 November 2007;
published online 14 December 2007

H. Krause (Toronto, ON, Canada) discussed a high-throughput *in situ* hybridization screen in *Drosophila* that he has used to detect the expression patterns of approximately 3,500 fly genes (Lecuyer *et al.*, 2007). Interestingly, approximately 71% of the transcripts show some form of subcellular localization, many with unique patterns. A comparison of maternal and zygotic RNAs showed that almost 67% of the genome is expressed maternally, whereas 15% of the genes examined during the first 4 h of embryogenesis are expressed zygotically, with an overlap of approximately 11%. Importantly, Krause found a strong correlation between the subcellular localization of RNAs and their corresponding protein patterns and functions.

E. Houliston (Villefranche-sur-Mer, France) discussed systematic *in situ* hybridization assays in the hydrozoan, *Clytia*, which her group has used to identify localized RNAs. *Clytia* ovaries can be cultured in a simple salt solution, which enables the analysis of gene function by knockdown in oocytes. Houliston's group focused on components and regulators of the Wnt pathway and performed functional studies for two RNAs encoding the Frizzled-related Wnt receptors, CheFz1 and CheFz3, which are localized in opposite regions of the early *Clytia* embryo. They showed that localization of CheFz1 at the animal pole is required to activate canonical Wnt signalling to promote oral fates, whereas vegetal localization of CheFz3 is required to specify aboral fates (Momose & Houliston, 2007). Both RNAs arrive at their target locations in the egg by distinct mechanisms during oocyte development. Thus, this simple hydrozoan can provide valuable insights into understanding the evolution of localization patterns, as well as embryonic patterning by localized factors.

Translational control

Many germ plasm mRNAs are silenced during transport. M.L. King (Miami, FL, USA) showed that the nanos-related Xcat2 RNA in *Xenopus* localizes to the germ plasm and is stored within granules until its translation at a later embryonic stage. Xcat2 RNA is translated efficiently in rabbit reticulocyte lysates, but not in *Xenopus* oocytes or oocyte lysates. Translational repression in oocyte extracts is dependent on the first 90 nucleotides of the Xcat2 open reading frame and cannot be competed out with RNA, which argues against a requirement for an RNA-binding protein. King proposed that Xcat2 repression is mediated through the packaging of Xcat2 RNA into germinal granules for long-term storage, and also through the secondary structure of the 5' end of Xcat2 RNA, which blocks translational initiation.

E. Izaurralde (Tübingen, Germany) has analysed the mechanism of translational repression of target RNAs by microRNAs (miRNAs). Whether a target RNA is degraded or translationally repressed by a miRNA can depend, in part, on the RNA-binding proteins interacting with the target RNA. Izaurralde showed that some proteins—such as GW182, which trigger deadenylation and decapping of target RNAs—are required for miRNA-mediated gene silencing. GW182 is present in P-bodies, which are sites of RNA degradation and translational control. P. Lasko (Montreal, QC, Canada) showed that the RNA-binding protein Bic-C destabilizes target mRNAs, such as bic-C and nanos, by recruiting the CCR4 core deadenylase complex (Chicoine *et al.*, 2007). Deadenylation and the subsequent decay of several maternal mRNAs is known to occur during the mid-blastula transition (MBT) in fly embryos, and is mediated in part by the Smaug protein. H. Lipshitz (Toronto, ON, Canada) showed that several posterior-protected mRNAs in the fly embryo are localized within somatic cells underlying the germ cells. These mRNAs co-localize with Smaug in a structure called the 'S-body',

which is distinct from P-bodies. S-bodies seem to act as sites for translational regulation to ensure that posterior cells maintain their somatic character, even though they express some germ-plasm components. Smaug also functions in the translational repression of nanos RNA in the embryo by forming a complex with the Cup and eIF4E proteins, as reported by C. Smibert (Toronto, ON, Canada). B. Theurkauf (Worcester, MA, USA) showed that, in addition to mRNA degradation and translational repression, Smaug regulates cell-cycle checkpoint activation, cellularization and zygotic transcription during MBT, and therefore acts as a component of a maternal clock that regulates the progression of MBT in the fly embryo.

R. Singer (New York, NY, USA) presented results showing translation of β -actin mRNA at the leading edge in motile fibroblasts. His group were able to visualize newly translated protein by total internal reflection fluorescence (TIRF) microscopy using β -actin containing a tetracycline motif, which binds to the small, membrane-permeable fluorophores FLAsh and ReAsh that fluoresce green and red, respectively. β -Actin mRNA localization and translation are punctate, and coincide with focal adhesion complexes (FACs). Interestingly, mRNAs for zyxin, paxillin and talin also localize to the FAC, and their 3'-untranslated regions (UTRs) contain localization elements ('zipcodes') that are identical to those in β -actin. Singer proposed a model in which the membranes apposed to FACs and podosomes act as anchoring and translation sites for specific mRNAs.

Structural insights to RNA function

One of the crucial themes that emerged from this meeting is that 'structure underlies function'. It is thought that many RNA-binding proteins recognize secondary structures such as hairpins rather than the nucleotide sequence, in part because the narrow major groove in double-stranded RNA might not allow proteins to come into contact with the sides of the bases.

Historically, it has been difficult to crystallize large RNAs either alone or in complexes with proteins. Structural determination using nuclear magnetic resonance (NMR) spectroscopy has also been difficult, as RNA is essentially a repetitive polymer of four nucleosides (Tzakos *et al.*, 2006). Recent technical advances have made it possible to solve structures of larger RNAs, two of which—parts of fs(1)K10 and brain cytoplasmic 1 (BC1) RNA—were presented at the meeting. The NMR solution structure of the fs(1)K10 transport/localization signal (TLS), determined by the groups of D. Ish-Horowicz (London, UK) and P. Lukavsky (Cambridge, UK), reveals a stretch of double-stranded helix with an unusual, widened major groove termed the A'-form, which is reminiscent of the B-form DNA. Mutations that disrupt the B-form-like helix impair signal activity and RNA localization. Ish-Horowicz suggested that the width of the major groove might be recognized by TLS-binding proteins. Lukavsky described a high-resolution solution structure of the BC1 dendritic targeting element (DTE). BC1 is a non-coding RNA implicated in translational control in neuronal dendrites. The elongated BC1 RNA hairpin is capped by a tri-loop and contains an apical, purine-rich internal loop that specifically interacts with the Pur- α protein. A basal internal loop also binds to Pur- α but with less specificity. The identification of BC1 DTE-binding proteins from extracts of rat brain revealed many transport/motor proteins and translational repressors. Lukavsky raised the possibility that BC1 could act as scaffolding to recruit various RNA-binding factors for transport to dendrites.

Fig 1 | Ribbon representation of the *Sulfolobus solfataricus* exosome including a Corey–Pauling–Koltun model of the RNA bound in the structure. A possible path for RNA substrates is indicated by a magenta-coloured line. In this model, the 3' end of the RNA substrate is recruited by the S1 pore and threaded through the neck of the central channel to the processing chamber, where it binds at one of the active sites and is degraded in a processive manner. (Figure reproduced with permission from Lorentzen *et al*, 2007).

In contrast to the idea that RNA-binding proteins mainly recognize RNA secondary structures, T. Hall (Research Triangle Park, NC, USA) described a ‘code for RNA sequence recognition’ in Pumilio-FBF (Puf) proteins. Puf proteins are developmental regulators that control mRNA stability and localized translation by binding to sequences in the 3' UTRs of target mRNAs. The crystal structure of human Pumilio1, in complex with a 10-nucleotide RNA, revealed that each Pumilio repeat binds to an RNA base through a combination of three side chains at conserved positions. Hall and colleagues inferred a code for RNA recognition, and tested this by generating a mutant Pumilio that specifically binds to an AU-rich element (ARE; Cheong & Hall, 2006). This shows the potential to design mutant proteins that bind to and modulate heterologous RNAs on a Pumilio scaffold.

Exosomes are complexes with 3' to 5' exoribonuclease activity that function in RNA processing, decay and quality control. They can either completely degrade or partly trim a given RNA substrate, and they stall at RNA secondary structures. The exosome is composed of nine subunits: a six-member RNase PH-like ring and a three-member S1-domain protein ring that is expected to bind to RNA. E. Conti (Martinsried, Germany) presented co-crystals of an archaeal exosome and an RNA specifically designed to stall the exosome. She showed that the RNA feeds from the S1 ring to the phosphorolytic ring through a narrow channel that is just wide enough for single-stranded RNA to pass through (Fig 1; Lorentzen *et al*, 2007).

Mechanisms of RNA transport

The imaging of RNA localization in live cells was revolutionized some years ago by the creation of reporter RNAs carrying hairpins

from bacteriophage MS2 RNA. The reporter RNA can then be visualized by the addition of MS2 coat protein fused to the green fluorescent protein (GFP; Bertrand *et al*, 1998). This system has been used to follow the localization of Ash1 RNA in yeast cells and, subsequently, in other organisms (Rodriguez *et al*, 2007). E. Gavis (Princeton, NJ, USA) and co-workers have adapted the MS2 system for use in *Drosophila* (Forrest & Gavis, 2003) to visualize bicoid and nanos mRNA movement in live fly oocytes by confocal and wide-field de-convolution microscopy. At the meeting, Gavis reported that these mRNAs are concurrently localized by distinct mechanisms. T. Schupbach (Princeton, NJ, USA) reported that endogenous *gurken*:MS2 stem-loop RNA does not form large transport particles. Furthermore, *gurken* RNA is localized dynamically in early stages in the oocyte, but not after its localization to the dorso–anterior corner of the oocyte.

D. St Johnston (Cambridge, UK) reported that *osk* mRNA particles move both posteriorly and anteriorly, but with a slight bias towards the posterior. Several perturbations—such as slow kinesin heavy chain (KHC) mutants—reveal that essentially all oocyte *osk* transport is mediated by kinesin. As kinesin is plus end-directed, the direction of *osk* movement reveals the polarity of the microtubules present in both orientations, with a slight excess of microtubules with plus ends to the posterior. Nevertheless, this slight bias in microtubule organization is sufficient to localize *osk* mRNA efficiently to the oocyte posterior (Dahlgard *et al*, 2007). Similarly in *Xenopus*, K. Mowry (Providence, RI, USA) reported that vegetal enrichment of plus ends of microtubules—detected by EB1 antibodies—precedes Vg1 RNA localization to the vegetal cortex.

A caveat of using the MS2 coat protein–GFP fusion proteins is that some sequences are not recognized in the appropriate context. The recent development of the λ N/BoxB system (Fig 2) could alleviate this limitation (Baron-Benhamou *et al*, 2004), and also has the potential—in combination with the MS2 system—to allow visualization of several RNAs simultaneously *in vivo*, as reported by R. Jansen (Munich, Germany). Another potential use of these systems is to target RNAs or to tether proteins to specific locations to test their activity.

Movement of RNAs on the actin or microtubule cytoskeleton requires various motor proteins. D. Niessing (Munich, Germany) reported that the efficient translocation of Ash1 RNA along actin filaments in yeast cells requires Myo4—a type V myosin—which interacts with Ash1 RNA through She3. However, it is not clear how several motors work together, how the number of motors affects transport or how the number of motors per cargo molecule is regulated.

S. Gross (Irvine, CA, USA) discussed the number of motors engaged during active transport of cargoes on microtubules. The cytoplasm in fly embryos contains many small lipid droplets that are actively transported on microtubules and are visible under phase contrast microscopy, allowing motor-based transport to be studied *in vivo*. By using optical traps of increasing strength to stall the particles and thereby estimate the force exerted by the motors, Gross and co-workers estimate that nearly all droplets are moved by five or less motors, and more than 60% are moved by two or more motors. Their data contradict an earlier suggestion that the velocity of cargoes is proportional to the number of engaged motors (reviewed by Gross *et al*, 2007), and suggests that viscous drag is not substantial. Interestingly, although two motors can move cargoes continually *in vitro*, movement *in vivo* decays and eventually stops, owing to either viscosity or active termination.

RNA localization in the nervous system

Localized translation is required in the tips of growing neurons—the growth cones—for them to turn in response to cues from intermediate targets. Even when an axon is severed from the cell body, the growth cone can respond appropriately to cues. C. Holt (Cambridge, UK) showed that several RNAs are localized to growth cones. In retinal axons, β -actin mRNA is localized to the growth cones and is bound by the RNA-binding protein Vg1RBP. Vg1RBP granules move into filopodia in response to Netrin, which is an attractive cue in this context. A Netrin gradient asymmetrically activates the translation regulator eIF-4e-binding protein (4EBP), and triggers an asymmetrical rise in β -actin translation on the side of the growth cone closest to the Netrin source before growth cone turning. Conversely, the repulsive cue Slit2 triggers increased synthesis of Cofilin, an actin-severing protein. This suggests a general model in which attractive cues stimulate translation of mRNAs that build up the actin cytoskeleton and stimulate filopodia extension on the side closest to the source, which leads to the growth cone turning towards the source. Conversely, repulsive cues stimulate the translation of actin-depolymerizing/severing proteins, leading to asymmetrical filopodia withdrawal (Lin & Holt, 2007).

Although it is known that long-term memory requires protein synthesis, the mechanisms that regulate this process are unclear. Work by S. Kunes' group (Cambridge, MA, USA) has shown that the miRNA/RNA-induced silencing complex (RISC) pathway controls synaptic protein synthesis during formation of long-term memory in *Drosophila*. Local degradation of the RISC component Armitage relieves miR-mediated silencing of calcium-calmodulin dependent kinase II (CaMKII) mRNA at the synapse (Ashraf *et al*, 2006). Kunes showed that CaMKII expression is regulated by miR280 in the antennal lobe and mushroom bodies of flies. Neural activity induces the degradation of Armitage and a decrease in miR280 biogenesis. Their work suggests that degradative control of the RISC pathway underlies the pattern of synaptic protein synthesis that is associated with stable memory.

H. Tiedge (Brooklyn, NY, USA) described the role of small dendritic RNAs in translational control at the synapse. Synapto-dendritic brain cytoplasmic (BC) RNAs are 100–200 nucleotide long non-coding RNAs that regulate translation initiation. For example, BC1 is thought to regulate translation at the synapse by inhibiting assembly of 48S initiation complexes. Tiedge reported that BC1 RNA inhibits eIF4A, an ATP-dependent helicase, by uncoupling the eIF4A helicase activity from its ATPase activity.

Dendritic branching and dendritic spine morphogenesis requires the function of the double-stranded RNA-binding protein Staufen2, as reported by M. Kiebler (Vienna, Austria). Neurons lacking Staufen2 show reduced dendritic spines and a marked reduction of excitatory post-synaptic currents (Goetze *et al*, 2006). Isolation of coding and non-coding RNAs that are co-enriched in high-molecular-weight Staufen-containing RNPs (Mallardo *et al*, 2003) led to the identification of Septin7, the knockdown of which causes a reduction in dendrite branching and elongated dendritic spines (Xie *et al*, 2007).

The cytoplasmic polyadenylation element binding protein (CPEB) regulates polyadenylation in early vertebrate development and in neurons. J. Richter (Worcester, MA, USA) showed that in neurons, CPEB promotes polyadenylation and translation of CPE-containing mRNAs at post-synaptic sites in response to synaptic activity. Synaptic plasticity and hippocampal-dependent memories are reduced in CPEB mutant mice. Interestingly, embryonic fibroblast cells cultured from CPEB mutant mouse embryos are immortal, and manifest upregulated

Fig 2 | Potential use of a dual-visualization system in live fly egg chambers. In the hypothetical example, posterior localization of oskar RNA tagged with λ N/BoxB is visualized with an amino-peptide:red fluorescent protein (RFP) fusion; gurken RNA, localized later to dorsal, is tagged with MS2 hairpins and visualized with a MS2CP:green fluorescent protein (GFP) fusion protein.

levels of the proto-oncogene, Myc. CPEB mutant mice are more susceptible to cancer-causing agents, suggesting that this protein might also function in cell-cycle regulation in mammals.

Perspectives and conclusions

This meeting highlighted the importance of RNA localization and translational control in a wide variety of cell types and processes. Many new regulatory molecules were identified by genome-wide and biochemical screens, and new mechanisms of action were presented; the challenge now is to understand how these function. Recent improvements in imaging methods can allow more precise and detailed visualization of the dynamics of the various processes, and structural studies of localization elements and RNA-protein interactions can provide fresh insights. With these new tools, the mechanisms and functions of RNA localization and translational control will no doubt be ascertained as never before.

ACKNOWLEDGEMENTS

We thank the meeting participants for allowing us to cite their work. Thanks to M. Kiebler and D. Ish-Horowicz for comments and suggestions. We apologize to the meeting participants whose work we were unable to cite owing to space constraints. P.C.G. is supported by the Singapore Millennium Foundation, and K.S. is supported by the Temasek Life Sciences Laboratory, Singapore.

REFERENCES

- Ashraf SI, McLoon AL, Sclarsic SM, Kunes S (2006) Synaptic protein synthesis associated with memory is regulated by the RISC pathway in *Drosophila*. *Cell* **124**: 191
- Baron-Benhamou J, Gehring NH, Kulozik AE, Hentze MW (2004) Using the λ N peptide to tether proteins to RNAs. *Methods Mol Biol* **257**: 135
- Bertrand E, Chartrand P, Schaefer M, Shenoy SM, Singer RH, Long RM (1998) Localization of ASH1 mRNA particles in living yeast. *Mol Cell* **2**: 437

- Cheong CG, Hall TM (2006) Engineering RNA sequence specificity of *Pumilio* repeats. *Proc Natl Acad Sci USA* **103**: 13635
- Chicoine J, Benoit P, Gamberi C, Paliouras M, Simonelig M, Lasko P (2007) Bicaudal-C recruits CCR4-NOT deadenylase to target mRNAs and regulates oogenesis, cytoskeletal organization, and its own expression. *Dev Cell* **13**: 691
- Dahlgaard K, Raposo AA, Nicolli T, St Johnston D (2007) Capu and Spire assemble a cytoplasmic actin mesh that maintains microtubule organization in the *Drosophila* oocyte. *Dev Cell* **14**: 539–553
- Du TG, Schmid M, Jansen RP (2007) Why cells move messages: the biological functions of mRNA localization. *Semin Cell Dev Biol* **18**: 171
- Forrest KM, Gavis ER (2003) Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in *Drosophila*. *Curr Biol* **13**: 1159
- Goetze B, Tuebing F, Xie Y, Dorostkar MM, Thomas S, Pehl U, Boehm S, Macchi P, Kiebler MA (2006) The brain-specific double-stranded RNA-binding protein Staufen2 is required for dendritic spine morphogenesis. *J Cell Biol* **172**: 221
- Gross SP, Vershinin M, Shubeita GT (2007) Cargo transport: two motors are sometimes better than one. *Curr Biol* **17**: R478
- Jambhekar A, Derisi JL (2007) Cis-acting determinants of asymmetric, cytoplasmic RNA transport. *RNA* **13**: 625
- Lecuyer E, Yoshida H, Parthasarathy N, Alm C, Babak T, Cerochina T, Hughes TR, Tomancak P, Krause HM (2007) Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. *Cell* **131**: 174
- Lin AC, Holt CE (2007) Local translation and directional steering in axons. *EMBO J* **26**: 3729
- Lorentzen E, Dziembowski A, Lindner D, Seraphin B, Conti E (2007) RNA channelling by the archaeal exosome. *EMBO Rep* **8**: 470
- Mallardo M, Deitinghoff A, Muller J, Goetze B, Macchi P, Peters C, Kiebler MA (2003) Isolation and characterization of Staufen-containing ribonucleoprotein particles from rat brain. *Proc Natl Acad Sci USA* **100**: 2100
- Momose T, Houliston E (2007) Two oppositely localised frizzled RNAs as axis determinants in a cnidarian embryo. *PLoS Biol* **5**: e70
- Rodriguez AJ, Condeelis J, Singer RH, Dichtenberg JB (2007) Imaging mRNA movement from transcription sites to translation sites. *Semin Cell Dev Biol* **18**: 202
- St Johnston D (2005) Moving messages: the intracellular localization of mRNAs. *Nat Rev Mol Cell Biol* **6**: 363
- Tzakos AG, Easton LE, Lukavsky PJ (2006) Complementary segmental labeling of large RNAs: economic preparation and simplified NMR spectra for measurement of more RDCs. *J Am Chem Soc* **128**: 13344
- Xie Y, Vessey JP, Konecna A, Dahm R, Macchi P, Kiebler MA (2007) The dendritic spine-associated GTP-binding protein Septin 7 is critical for dendrite branching and dendritic spine morphology. *Curr Biol* **17**: 1746

Patrick C. Gilligan

Karuna Sampath