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Review

Putting RNAs in the right place at the right time: RNA localization in the frog oocyte

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Abstract

Localization of maternal mRNAs in many developing organisms provides the basis for both initial polarity during oogenesis and patterning during embryogenesis. Prominent examples of this phenomenon are found in *Xenopus laevis*, where localized maternal mRNAs generate developmental polarity along the animal/vegetal axis. Targeting of mRNA molecules to specific subcellular regions is a fundamental mechanism for spatial regulation of gene expression, and considerable progress has been made in defining the underlying molecular pathways.

Introduction

Localization of specific RNAs to subcellular domains is a means by which cells restrict protein synthesis in time and space. This post-transcriptional mechanism for gene regulation plays a pivotal role in cell polarity, and such essential and divergent activities as cell fate decisions in development (reviewed in Palacios et al., 2001; Kloc et al., 2002a), directed cell movement (Shestakova et al., 2001), vitellogenesis (Zhou et al., 2003; 2004), myelination of axons (Song et al., 2003), and synaptic plasticity in the brain (Lin et al., 2001). RNA localization probably operates in every cell, including single celled organisms such as yeast (Gonsalvez et al., 2005). In *Xenopus*, as in *Drosophila*, the asymmetric distribution of RNAs during oogenesis sets up the body plan for the embryo and is essential for normal development (Kloc et al., 2002a; Zhou and King, 2004). In this review we will focus on what is known about RNA localization during *Xenopus* oogenesis.

The *Xenopus* oocyte has served as an important model system for understanding RNA localization, and offers several unique advantages. At each of the six stages of oogenesis, the animal/vegetal (A/V) axis can be distinguished and it is along this axis that

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Key words: determinant, germline, oocyte, RNA localization, VegT, Vg1, Xcat2, *Xenopus*.

Abbreviations used: ARH, autosomal recessive hypercholesterolaemia; A/V, animal/vegetal; ER, endoplasmic reticulum; GFM, granulofibrillar material; LE, localization element; LS, localization signal; MC, mitochondrial cloud; METRO, message transport organizer; PGC, primordial germ cell; R1, repeat 1; RBP, RNA-binding protein; RNP, ribonucleic protein; UTR, untranslated region; TCE, translational control element; Xlsirts, *Xenopus laevis* short interspersed repeat transcripts.

Six stages of oogenesis: Oogenesis in *Xenopus* is divided into six stages spent mostly in meiotic I prophase. Stage I oocytes are previtellogenic (before the uptake of yolk from the blood), contain a mitochondrial cloud, and grow to 300 μm in diameter. In stage II oocytes vitellogenesis begins, the oocyte reaching 450 μm in diameter. Vitellogenesis continues during stages III–V and pigment accumulates, becoming polarized along the animal/vegetal axis. Stage VI oocytes are fully grown at 1.2 mm in diameter and are capable of undergoing maturation in response to progesterone.

Animal/vegetal axis: The axis developed during oogenesis that foreshadows the placement of the three primary germ layers in the embryo. The progeny of the animal pole will give rise to ectoderm and neuroectoderm, cells from the vegetal region will form the endoderm, and the middle region contains future mesoderm.

RNAs will localize in the oocyte. Frog oocytes are enormous cells, approx. 100 times larger than a somatic cell, facilitating the microinjection of RNA transcripts. Moreover, the oocyte nucleus is larger than most somatic cells and it is possible to isolate them manually at any oogenic stage. Early stage oocytes are optically clear and are amenable to using *in vivo* confocal analyses as a tool to study the localization process; therefore, both nuclear and cytoplasmic events of RNA localization can be qualitatively and quantitatively assessed. Importantly, the functional significance of RNA localization can also be judged by determining the developmental consequences of molecular mutation or mis-localization of specific RNAs (Zhang et al., 1998; Houston and King, 2000b).

A small subset of maternal RNAs are found differentially distributed at either the animal or vegetal pole (Table 1). At present, little is known about how or when RNAs become enriched in the animal pole or, indeed, if an active localization or retention mechanism is required. We do know that while RNAs at the vegetal pole become highly concentrated there (20 to 50-fold) and are anchored within the cortical cytoskeleton, animal pole RNAs are diffusely distributed in the hemisphere, no more than 3- to 10-fold enriched (King, 1995). The poly(A)$^+$ (polyadenylated) binding protein, PABP, may be unusual in this regard as it appears to be associated with the cortex at the animal pole (Schroeder and Yost, 1996). With the possible exception of xARHα (Zhou et al., 2003; 2004), vegetally localized RNAs are asymmetrically inherited by the cellular progeny that arise there, but animal pole RNAs frequently are not restricted regionally in the embryo. Therefore, the developmental significance of localizing RNAs to the animal hemisphere remains to be demonstrated. Fifteen RNAs localized in the animal hemisphere have now been described and are listed in Table 1. Because of the dearth of information concerning how RNAs become enriched at the animal pole, this review will concentrate instead on the two known vegetal localization pathways.

More than twenty RNAs have now been identified that localize during two major pathways to distinct but overlapping domains within the vegetal cortex (Forristall et al., 1995; Kloc and Etkin, 1995) (Table 1). These two pathways operate sequentially and by different mechanisms to localize different sets of RNAs. In general, RNAs such as Xcat2 and Xdazl, destined to be germ plasm components, localize during the early pathway by associating with a structure called the mitochondrial cloud (MC) or Balbiani body (Heasman et al., 1984). The late pathway begins as the early pathway comes to completion and localizes RNAs such as Vg1 and VegT involved in somatic cell fates (Zhang et al., 1998; Joseph and Melton, 1998). Intriguingly, there is evidence that these two pathways may overlap mechanistically (Kloc and Etkin, 1998). Thus, the vegetal cortex becomes the repository for determinants of the future cell lineages of the embryo, and can be thought of as the first organizing centre in development.

Recent efforts to define a consensus localization signal (LS) and *trans*-acting factors for the vegetal pole pathways have provided new insights into these processes and we will highlight advances in these areas with an emphasis on the well-studied Vg1 and Xcat2 RNAs as representatives of the two vegetal pole pathways.

The oocyte is polarized along an A/V axis

Oogenesis in *Xenopus* occurs over a 4 to 5 month period, and culminates in a 1.3 mm cell, which is polarized along the A/V axis. Melanosomes accumulate within the animal pole cortex beginning in stage III and provide an obvious external marker for the A/V axis. Although endocytosis of yolk protein shows no A/V bias, there is a polarized intracellular translocation of platelets into the vegetal hemisphere resulting in large (older) yolk platelets accumulating there during vitellogenesis and displacing the nucleus into the

Cortical cytoskeleton: An approx. 10 μm thick region associated with the plasma membrane that contains intermediate filaments, microtubules, and a rich network of microfilaments.

Germ plasm: A subcellular domain containing the determinants of the germline. Contains unique mRNAs, and proteins as well as granules, endoplasmic reticulum and mitochondria. Also found in *C. elegans* and *Drosophila*. Cells that inherit sufficient germ plasm will become germ cell precursors.

Mitochondrial cloud (MC) or Balbiani body: A discrete, non-membrane bound structure found in previtellogenic oocytes (stage I) in *Xenopus* that is the site of mitochondrial replication and germ plasm formation. It lies close to the nucleus, associated with it through a cytokeratin network which helps maintain its structure, and can reach 30 μm in diameter. Balbiani body (deriving from Dr E. Balbiani who first described this structure) or yolk nucleus are alternative names for mitochondrial cloud.

Table 1 | RNAs localized during *Xenopus* oogenesis

Localized RNA	Localization	Pathway	Encoded Protein	References*
DEADSouth	Vegetal cortex/Germ plasm	Early/METRO	RNA helicase	1
Fingers	Vegetal cortex/Germ plasm	Early/METRO	Zn finger protein	2
Germes	Vegetal cortex/Germ plasm	Early/METRO	Novel protein; EF-hand, leucine zipper motifs	3
Xcat2	Vegetal cortex/Germ plasm	Early/METRO	Nanos-related; Zn finger protein	4
Xdazl	Vegetal cortex/Germ plasm	Early/METRO	DAZ-related; RNA-binding protein	5
XFACS	Vegetal cortex/Germ plasm	Early/METRO	Fatty Acyl CoA synthetase (FACS)	6
Xlerk	Vegetal cortex/Germ plasm	Early/METRO	Transmembrane Eph family ligand	7
Xlsirts	Vegetal cortex/Germ plasm	Early/METRO	Family of noncoding RNAs	8
Xwnt11	Vegetal cortex/Germ plasm	Early/METRO	Wnt family member	9
Xpat	Vegetal cortex/Germ plasm	Early/METRO	Novel protein	10
Fatvg	Vegetal cortex/Germ plasm	Intermediate	Adipophilin/ADRP-related	11
Hermes	Vegetal cortex/Germ plasm	Intermediate	RNA-binding protein	12
Xotx1	Vegetal cortex	Intermediate	Orthodenticle-related; Transcription factor	13
C3H-3	Vegetal cortex	Late	Zn finger protein	14
VegT	Vegetal cortex	Late	T-box transcription factor	15
Vg1	Vegetal cortex	Late	TGF-β family member	16
xBic-C	Vegetal cortex	Late	Bicaudal-C homologue; RBP	17
Xvelo	Vegetal cortex	Late	Novel protein	18
xARHα	Vegetal hemisphere	Late	ARH-related	19
β-TrCP-2/3	Vegetal hemisphere	Unknown	β-transducin	20
An1	Animal hemisphere	Unknown	Ubiquitin-like fusion protein	21
An2	Animal hemisphere	Unknown	Mitochondrial ATPase subunit	22
An3	Animal hemisphere	Unknown	RNA helicase	23
An4	Animal hemisphere	Unknown	Novel protein	24
β-TrCP	Animal hemisphere	Unknown	β-transducin	25
Ets-1/2	Animal hemisphere	Unknown	ETS-family members; transcription factor	26
Fibronectin	Animal hemisphere	Unknown	ECM component; adhesion protein	27
XLPOU60	Animal hemisphere	Unknown	POU domain; transcription factor	28
PAPB	Animal hemisphere	Unknown	Poly(A)-binding protein	29
XPar-1	Animal hemisphere	Unknown	Serine/threonine kinase	30
Tcf-1	Animal hemisphere	Unknown	Tcf/Lef family member; transcription factor	31
Vg1RBP/Vera	Animal hemisphere	Unknown	RBP	32
xArHβ	Animal hemisphere	Unknown	ARH-related	33
xl21	Animal hemisphere	Unknown	Transcription factor?	34
XGβ1	Animal hemisphere	Unknown	β-subunit of heterotrimeric G protein	35
Xlan4	Animal hemisphere	Unknown	Novel protein	36

*Selected references: 1 (MacArthur et al., 2000); 2 (King, 1995; Kloc et al., 2002b); 3 (Berekelya et al., 2003); 4 (Mosquera et al., 1993; Forristall et al., 1995; Kloc and Etkin, 1995); 5 (Houston et al., 1998); 6 (King, 1995; Kloc et al., 2002b); 7 (Jones et al., 1997; Betley et al., 2002); 8 (Kloc et al., 1993; Zearfoss et al., 2003); 9 (Ku and Melton, 1993; Kloc et al., 1998); 10 (Hudson and Woodland, 1998); 11 (Chan et al., 1999; 2001); 12 (Zearfoss et al., 2004); 13 (Pannese et al., 2000); 14 (De et al., 1999; Betley et al., 2002); 15 (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996); 16 (Rebagliati et al., 1985; Weeks and Melton, 1987b); 17 (Wessely and DeRobertis, 2000); 18 (Claussen and Pieler, 2004); 19 (Zhou et al., 2003; 2004); 20 (Hudson et al., 1996); 21 (Rebagliati et al., 1985; Linnen et al., 1993); 22 (Rebagliati et al., 1985; Weeks and Melton, 1987a); 23 (Rebagliati et al., 1985; Gururajan et al., 1991); 24 (Hudson et al., 1996); 25 (Hudson et al., 1996); 26 (Meyer et al., 1997); 27 (Oberman and Yisraeli, 1995); 28 (Whitfield et al., 1993); 29 (Schroeder and Yost, 1996); 30 (Ossipova et al., 2002); 31 (Roel et al., 2003); 32 (Zhang et al., 1999); 33 (Zhou et al., 2003); 34 (Kloc et al., 1991); 35 (Devic et al., 1996); 36 (Reddy et al., 1992).

animal hemisphere (Danilchik and Gerhart, 1987). Golgi and a large pool of tubulin and microtubules accumulate on the vegetal side of the nucleus, further reflecting the polarized intracellular transport system that exists in the oocyte (Gard, 1991). Distinct domains of endoplasmic reticulum (ER) and islands of germ plasm are found in the vegetal cortical region (Hausen and Riebesell, 1991) along with a relatively small subset of RNAs and proteins. Unique to germ plasm are RNA–protein complexes implicated in specifying germ cell fate. Germinal granules are 250–500 nm in diameter and are associated with a number of early pathway RNAs (Kloc et al., 2002b). The asymmetry of the oocyte along the A/V axis lays the foundation for establishing germ layer identity as well as the germ cell lineage in the embryo, underscoring the importance of the polar organization of the oocyte to both subsequent development and continuity of the species.

Formation of the A/V axis, germ plasm formation and the early pathway

The ultimate purpose of the early RNA-localization pathway is to collect coding and non-coding RNAs essential to germ cell development into a discrete subcellular domain that can be moved first to the vegetal cortex of the oocyte and subsequently partitioned asymmetrically during cleavage into the cells that will become primordial germ cells (PGCs). The RNAs within the germ plasm may not be translated for many months, even years (Houston and King, 2000a, 2000b; MacArthur et al., 1999). Therefore, germ plasm also functions as a long-term storage depot for RNAs during oogenesis. Consistent with this function, many of the early pathway RNAs that have been identified represent different families of RBPs (RNA-binding proteins), such as Xcat2, Xdazl, and DEADSouth (Table 1). Xcat2 encodes an RBP related to Nanos (Mosquera et al., 1993), the product of an essential gene in *Drosophila* and mouse that is required for the acquisition of germ-line fate and for germ-cell migration (Kobayashi et al., 1996; Forbes and Lehmann, 1998; Tsuda et al., 2003; Wang and Lin, 2004). Xdazl encodes an RBP required for PGC differentiation. Depletion studies have shown that PGCs deficient in Xdazl RNA fail to migrate out of the endoderm (Houston and King, 2000b). DEADSouth is an RNA helicase more closely related to eIF4A than to the *Drosophila* germline helicase, Vasa. It is translated during mid-oogenesis and like Xcat2 and Xdazl, is thought to function through its ability to regulate the expression of other germ plasm RNAs (MacArthur et al., 2000). The novel protein Xpat and the non-coding/coding Xlsirts (*Xenopus laevis* short interspersed repeat transcripts) RNAs may have structural functions in the assembly or cortical anchoring of germ plasm (Kloc and Etkin, 1994; Hudson and Woodland, 1998). All of these, and other RNAs (Table 1) are targeted to the germ-plasm-forming METRO (message transport organizer) region of the MC in stage I oocytes. RNA localization then is inextricably linked to the assembly of germ plasm and the generation of the A/V axis.

How is the A/V axis established and germ plasm assembled in the oocyte? Although a definitive answer to this question remains elusive, there is now strong correlative evidence that the process initiates with the organization of the primary oogonium through a structure called the fusome originally described in *Drosophila* (Cox and Spradling, 2003; Kloc et al., 2004). In these cells, the Golgi apparatus, ER, and mitochondria favour one side of the nucleus (Al-Mukhtar and Webb, 1971; Kalt and Gall, 1974). This polarized cellular organization is further elaborated in secondary oogonium where the last four mitotic divisions are incomplete, forming a germ-line cyst of 16 interconnected cells (Coggins, 1973; Kloc et al., 2004). The cluster of mitochondria embedded in an electron dense material (the so-called mitochondrial cement) will become the hub of the early RNA pathway and germ plasm formation (Heasman et al., 1984; reviewed in Houston and King, 2000a; Zhou and King, 2004; Kloc et al., 2001; 2004). Importantly, components of a microtubule organizing centre, complete with γ-tubulin and a pair of centrioles, are found within the mitochondrial mass, suggesting the original asymmetry in mitochondria and ER is established by dynein-directed transport of these and other organelles towards the centrosome (Pepling et al., 1999). Xlsirts RNAs are first detected at the centre of the hub near the centrioles (Kloc et al., 1998). Other components of the germ plasm,

METRO region: Message transport organizer region is a term coined by Dr L. Etkin to indicate that region of the mitochondrial cloud where germ plasm RNAs accumulate.

including Xcat2 and Xpat RNAs, are found in association with granulofibrillar material (GFM) in intimate contact with the mass of mitochondria, which is the precursor to the MC found in stage I oocytes (Kloc et al., 2002b). GFM and the germinal granules are likely derivatives of the mitochondrial cement, but molecular markers linking these different structures have not been described. Interestingly, expression of an Xpat–green fluorescent fusion protein is capable of forming GFM-like material in oocytes, suggesting a role for it in creating this germ plasm matrix (H.R. Woodland, personal communication). It appears that spectrin and hts protein as well as membranous vesicles/tubules, are major components of the fusome, and could provide a stable scaffolding matrix for binding early pathway RNAs (Kloc et al., 2004).

By early diplotene of meiosis I, oocytes contain several aggregates of mitochondria surrounding the nucleus, and these apparently also bear GFM-like material and Xcat2 RNA (Kloc et al., 1998). With the increased synthesis of mRNAs that occurs at this stage, both early and late pathway RNAs are also found homogeneously distributed in a common ooplasm (Melton, 1987; Zhou and King, 1996a; Houston et al., 1998). By stage I, the aggregate of mitochondria that contained the centrosome has grown into a large mass 30 μm in diameter (Heasman et al., 1984). This mature MC abuts the nucleus on the future vegetal side and is the exclusive site for the localization of early pathway RNAs (Kloc et al., 1996; Zhou and King, 1996a). Whether RNAs re-localize from the other aggregates of mitochondria or are less stable there is not known. The MC itself is a polarized structure with the germ-plasm-forming METRO region found in the vegetal portion. Here, hundreds of germinal granules concentrate in a ring, and are not found more interiorly (Kloc et al., 2002b). The MC itself is surrounded and penetrated by a dense network of cytokeratins, which lend structural integrity (Gard et al., 1997).

A detailed three-dimensional ultrastructural analysis of RNA distribution within the germ plasm throughout this period has revealed that Xcat2, Xpat, and DEADSouth RNAs are associated with germinal granules, and only Xcat2 is a granule component. Other RNAs such as Xlsirts and Xdazl are found in the matrix between the germinal granules (Kloc et al., 2002b). Therefore, not only are RNAs targeted to the MC germ-plasm-forming region, but to subdomains in the germ plasm. In stage II oocytes, the MC with the germ-plasm-containing portion is moved into the cortex, thereby marking it as the vegetal pole. The initial polarity developed in the oogonium thus strongly correlates with the future A/V axis of the oocyte.

A late localization pathway

A second pathway, functioning to localize mRNAs to the vegetal cortex, is the so-called late pathway (Table 1). An important function of the late pathway is the localization of germ layer determinants to the vegetal hemisphere. In *Xenopus*, the three primary germ layers are specified along the A/V axis. The animal hemisphere gives rise to ectodermal cell types, while the vegetal hemisphere contains prospective endoderm; mesoderm results from an inductive signal emanating from the vegetal blastomeres (Nieuwkoop, 1969). Thus, the vegetal hemisphere is also a repository of both mesodermal and endodermal determinants. Indeed, one mRNA localized through the late pathway is VegT, a T-box transcription factor (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997) that is required for endoderm and mesoderm specification (Zhang et al., 1998). Vg1, a member of the TGFβ growth factor family (Weeks and Melton, 1987b), has also been implicated in mesoderm and endoderm specification (Dale et al., 1993; Thomsen and Melton, 1993; Joseph and Melton, 1998). Mis-expression of either VegT or Vg1 in the animal hemisphere leads to induction of mesoderm in cells that would normally form ectoderm (Dale et al., 1993; Thomsen and Melton, 1993; Zhang and King, 1996; Joseph and Melton, 1998). Defects in germ layer patterning also result from mis-expression of xBic-C, the *Xenopus* homologue of the *Drosophila* RNA-binding protein, Bicaudal-C (Wessely and DeRobertis, 2000).

RNAs localized by the late pathway are uniformly distributed in the cytoplasm of stage I oocytes, and are excluded from the MC (Melton, 1987; Forristall et al., 1995; Kloc and Etkin, 1995). By late stage II, after delivery of the early pathway RNAs to the vegetal cortex, late pathway RNAs begin to accumulate in a wedge-like region within the vegetal ooplasm (Deshler et al., 1997; Kloc and Etkin, 1998). The 'wedge' region has been characterized as a subdomain of the ER, due to enrichment of specific ER proteins

(Deshler et al., 1997; Kloc and Etkin, 1998). During mid-oogenesis (stages III–IV), the late pathway RNAs are transported to the vegetal cortex, where they remain tightly anchored to the cortical cytoskeleton until the end of oogenesis (stage VI) (Yisraeli et al., 1990; Elinson et al., 1993; Forristall et al., 1995; Kloc and Etkin, 1995; Alarcon and Elinson, 2001). Within the vegetal cortex, the late pathway RNAs adopt a broad distribution relative to that of the early pathway RNAs (Forristall et al., 1995). Spatial and temporal differences notwithstanding, a key distinction between the early and late pathways is reliance on the cytoskeleton. In contrast with the microtubule-independent early pathway (Kloc et al., 1996; Chang et al., 2004), late pathway localization requires an intact microtubule cytoskeleton (Yisraeli et al., 1990). Thus, the late localization pathway, operating during mid-oogenesis, might be best characterized as the microtubule-dependent localization pathway.

Motor-driven RNA transport

Directional transport of localized RNAs within the cytoplasm has long been thought to require motor proteins, but only recently have the identities of some of these motors come to light (reviewed in Tekotte and Davis, 2002). Early evidence for active transport by motor proteins came from the requirement for cytoskeletal elements in RNA transport in a number of organisms, including *Xenopus* (Yisraeli et al., 1990). Studies in yeast, *Drosophila* and vertebrates have uncovered roles in RNA localization for members of the myosin, dynein and kinesin families of motor proteins (reviewed in Tekotte and Davis, 2002). In *Xenopus*, recent work has implicated both kinesin I and kinesin II in transport of Vg1 RNA (Betley et al., 2004; Yoon and Mowry, 2004). Biochemical experiments have identified kinesin I (conventional kinesin) as a component of a cytoplasmic Vg1–RNP (ribonucleic protein) complex, suggesting a link to Vg1 RNA localization (Yoon and Mowry, 2004). For kinesin II (heterotrimeric kinesin), interference experiments using either function-blocking antibodies or expression of a dominant mutant subunit, strongly inhibited localization of Vg1 RNA (Betley et al., 2004). It is possible that each motor protein mediates a distinct step in vegetal RNA transport. Moreover, additional, as yet unidentified, motor proteins may play roles in transport, anchoring or both.

Early pathway mechanism

What is the mechanism utilized to localize early pathway RNAs within the germ-plasm-forming METRO region of the MC? Fluorescently tagged RNAs injected into stage I oocytes reliably localize into the METRO region of the MC and this has become the experimental paradigm in which to address this and other questions. It is formally possible that germline RNAs localize by: (1) directed transport along microtubules/microfilaments, (2) selective protection of RNA within the cloud and degradation elsewhere, or (3) non-directed movement and entrapment within the MC. Available evidence strongly supports the last mechanism.

A non-motor-driven mechanism is suggested by studies on Xcat2 RNA showing that the RNA can concentrate within the MC after cold, nocodazole or cytochalasin B treatment (Kloc et al., 1996; Zhou and King, 1996b; Chang et al., 2004). Moreover, stage I oocytes lack a functional microtubule organizing centre and microtubules are poorly organized in relation to the MC, although remnants of the centrosome persist within this structure (Gard, 1991; Gard et al., 1997). A selective protection/degradation mechanism is also unlikely to be operating (Bashirullah et al., 2001). *Xenopus* germline RNAs are not degraded outside of the germ plasm. Regardless of the oocyte stage at which Xcat2 RNA is injected, it remains stable within the cytoplasm over a period of days (Zhou and King, 1996a; M.L. King, unpublished data). Recently, direct evidence has been obtained for the diffusion/entrapment model using a combination of time-lapse high-resolution confocal microscopy and FRAP (fluorescent recovery after photobleaching) analysis (Chang et al., 2004). These studies show that injected Xcat2 and Xdazl RNAs form particles that freely move within the ooplasm with diffusion coefficients virtually identical to non-localized β-globin RNA. Intriguingly, Xcat2 becomes progressively immobilized and forms large aggregates

Nocodazole: Drug which acts to disassemble microtubule networks by binding subunits and preventing their polymerization.

Cytochalasin B: Drug used to depolymerize microfilaments by capping filament at plus 'growing' ends.

predominantly within the METRO region of the MC and not elsewhere in the cell. Although Xdazl does not form granules, it is also entrapped within this region. Xdazl RNA probably arrives at the METRO region in a separate RNP particle from that of Xcat2 as was shown for Xlsirts and Xcat2 RNAs (Kloc et al., 1996). A similar entrapment mechanism has recently been proposed for nanos RNA, a relative of Xcat2 that localizes to the germ plasm at the posterior pole of *Drosophila* oocytes (Forrest and Gavis, 2003). This apparent conservation in the mechanism of germline RNA localization probably reflects the conservation between vertebrates and invertebrates in how female germline cysts and oocyte polarity is established (Pepling et al., 1999; Kloc et al., 2004).

What entraps germline RNAs within the METRO region? In time-lapse analyses of live stage I oocytes co-injected with a lipophilic fluorescent dye to label the ER-like membranes, these immobilized RNAs showed co-ordinated movement with ER in the METRO region (Chang et al., 2004). Whether this association is direct or indirect remains to be determined, but these results suggest that the smooth ER found within the METRO region is distinct in its ability to trap germline RNAs from the ooplasm, perhaps through associated cytoskeletal adaptor proteins. It will be of great interest to learn the identity of the proteins and/or RNAs that mediate these entrapment events. The aggregation of mitochondria, smooth endoplasmic reticulum (SER)-like membranes, Xlsirts, and the components of the spectrin-containing fusome could provide a scaffolding matrix, analogous to a mobile cortical cytoskeleton, to which germline RNAs associate. Xlsirts, spectrin and cytoplasmic keratins are found uniformly distributed in the germ plasm of late stage oocytes and may be involved in retention of the matrix RNAs (Kloc et al., 1998). Some germline RNAs may encode proteins that associate with both the cytokeratin framework of the MC and germline RNAs either directly or indirectly through other RBPs. For example, Germes contains two leucine zippers and an EF-hand domain, like spectrin, and would be one candidate for such a protein (Berekelya et al., 2003).

An intermediate pathway

While the early and late localization pathways have been characterized as mechanistically distinct, a subset of vegetally localized RNAs have been identified with characteristics of both pathways (Chan et al., 1999; Pannese et al., 2000; Chan et al., 2001; Zearfoss et al., 2004). Those RNAs classified as intermediate (Table 1) are both enriched in the MC during early oogenesis and accumulate in the wedge during mid-oogenesis. These RNAs apparently have also dual functions, both in the germline and in somatic cell patterning. One such RNA is fatvg (Table 1), which is found in the germ plasm but also in the developing fat bodies (Chan et al., 2001). It is not yet apparent whether these RNAs localize through a distinct intermediate pathway or whether those RNAs might use both the early/METRO and late pathways sequentially. It is clear that much remains to be understood about how RNAs are sorted into the two pathways or are competent to use both pathways.

Recognition of localized RNAs

A key issue is where and how the localization machinery recognizes RNAs for localization. The recognition event may take place in the nucleus soon after, or even during, transcription, but an understanding of these early events is only now emerging. In general, these LSs consist of multiple elements, and display considerable functional redundancy. Recognition of RNAs destined for cytoplasmic localization relies on *cis*-acting sequences, generally residing in the 3′ UTRs (untranslated regions) of the localized messages (reviewed in Kloc et al., 2002a). For several early and late pathway RNAs, detailed studies have defined sequence elements that are sufficient to direct proper localization; in some cases these localization elements (LEs) can be surprisingly large. For example, the Vg1 and VegT LEs are 340-nt and 300-nt in length respectively (Mowry and Melton, 1992; Bubunenko et al., 2002; Kwon et al., 2002). The Xvelo LE is considerably shorter, only 75-nt in length (Claussen and Pieler, 2004), and LEs have not yet been mapped for other late pathway RNAs, such as C3H-3 and xBic-C (Table 1). For one of the intermediate RNAs, fatvg, a short 25-nt LE has been defined (Chan et al., 1999). Notable similarities are not evident by sequence comparison among the Vg1, VegT, Xvelo and fatvg LEs, and it will be of significant interest to determine whether these different LEs direct vegetal localization through the same molecular mechanism. However, for Vg1 and VegT, this appears to be the case. The Vg1 and VegT LEs compete for localization *in vivo* and share molecular determinants for

localization in the form of specific *cis*-sequences and *trans*-factors (Bubunenko et al., 2002; Kwon et al., 2002). Within the large LEs of Vg1 and VegT RNAs, multiple redundant sequence elements are evident (Deshler et al., 1997; Gautreau et al., 1997; Bubunenko et al., 2002; Kwon et al., 2002; Lewis et al., 2004). Mutational analysis showed repeated sequences YYUCU (VM1) and A/CYCAC (E2) to be necessary for both Vg1 and VegT localization (Deshler et al., 1997; Gautreau et al., 1997; Bubunenko et al., 2002; Kwon et al., 2002; Lewis et al., 2004). Clustering of either E2 motifs (Betley et al., 2002; Kwon et al., 2002) or VM1 and E2 motifs together (Bubunenko et al., 2002; Lewis et al., 2004) has been suggested to be a key feature for LE function. It is possible that such repeated motifs functionally define an LE by facilitating interactions between different *trans*-acting components of the localization machinery.

For the early pathway, the best characterized RNAs are Xcat2 (Zhou and King, 1996a,b; Kloc et al., 2000; Chang et al., 2004) and the large family of non-coding and coding Xlsirts RNAs (Allen et al., 2003). The LS for Xcat2 has two distinct, non-redundant elements. Adjacent to the 3′ end of the open reading frame is a 240-nt MCLS, which is necessary and sufficient to entrap Xcat2 into the MC. Inclusion of Xcat2 into the germinal granules, an event that cannot occur without the prior functioning of the 240-nt MCLS, is driven by the remaining ~160 nt of the 3′-UTR termed the GGLE (Kloc et al., 2002b). Injected Xcat2 transcripts were found to quickly form aggregates upon entrapment on the ER in the germ-plasm-forming region of the MC, suggesting that germinal granule formation may initiate on ER (Chang et al., 2004). It will be interesting to determine how the GGLE mediates granule formation and what *trans*-acting factors are involved. Within the MCLS are six R1 (repeat 1) motifs (UGCAC), but only the last five appear to be important for localization (Chang et al., 2004). Within the MCLS is a critical 57-nt LE (MCLE) necessary but not sufficient for MC localization. This small MCLE contains three of the five critical R1s, and non-cognate substitutions in any one of these three repeats abolish MC entrapment (Chang et al., 2004). However, single point mutations converting the R1 to the E2 (UUCAC) repeats required for the late pathway did not completely abolish MC localization, but only significantly impaired it. The converse experiment, converting all the E2 in the Vg1 LE to R1s, failed to cause this late pathway RNA to enter the MC. Therefore, R1s are required but not sufficient for MC entrapment, and E2 repeats can partially substitute for R1 function (Chang et al., 2004). Xlsirts RNAs contain varying numbers of 79–81 nt repeats tandemly arrayed in addition to their unique sequences (Allen et al., 2003). A 137-nt Xlsirts LE has been defined which can be folded into one secondary structure by computer analysis, and correlative evidence suggests that the structure could be important for localization (Allen et al., 2003). Repeats are found within the Xlsirts LE that are related to E2 and E4 repeats defined for the Vg1 LE, but the significance of any of these repeats is unknown and await protein binding studies (Deshler et al., 1997). Comparing the signals for Xlsirts and Xcat2 does not reveal common features except for a similar number of CAC-bearing repeats (Allen et al., 2003). It is possible, however, that repeated motifs may not be a conserved feature of most early pathway RNAs. The R1 UGCAC is important for the Xcat2 MCLS ($n = 6$), but only shows up as a repeat in one other early pathway LS, Xpat ($n = 10$). Missing is an understanding of the relationship between secondary structure and what constitutes a consensus protein binding motif for any given protein.

RNP-complex assembly

Cytoplasmic RNA localization relies on interactions between *cis*-acting RNA sequence elements and multiple *trans*-acting protein factors, thus functioning in the context of a RNP complex (Mowry, 1996; Ross et al., 1997; Arn et al., 2003). In *Xenopus* oocytes, biochemical approaches have identified a set of Vg1 RBPs (VgRBPs) with potential roles in RNA localization (Schwartz et al., 1992; Mowry, 1996; Deshler et al., 1997; Deshler et al., 1998; Havin et al., 1998; Cote et al., 1999; Zhao et al., 2001; Kroll et al., 2002; Yoon and Mowry, 2004). Two of these, Vg1RBP/Vera (Deshler et al., 1997; Deshler et al., 1998; Havin et al., 1998) and VgRBP60/hnRNPI (Cote et al., 1999) bind to repeated sequences within the Vg1 LE and are implicated with roles in localization, as base changes within these reiterated motifs both abolish localization *in vivo* and eliminate protein binding *in vitro* (Deshler et al., 1997; Gautreau et al., 1997; Deshler et al., 1998; Havin et al., 1998; Cote et al., 1999). Vg1RBP/Vera binds to the Vg1 LE through

E2 sites (Deshler et al., 1997; Deshler et al., 1998), and is the frog homologue of ZBP1 (Deshler et al., 1997; Deshler et al., 1998; Havin et al., 1998), an RBP involved in β-actin mRNA localization in fibroblasts (Ross et al., 1997). The Vg1RBP/Vera binding is especially intriguing as it is weakly associated with the ER and may link Vg1 RNA to this organelle (Deshler et al., 1997). VgRBP60/hnRNPI is a homologue of mammalian hnRNP I and PTB (polypyrimidine tract-binding protein), which are spliced isoforms of one another and play roles in post-transcriptional RNA metabolism (reviewed in Valcarcel and Gebauer, 1997). VgRBP60/hnRNPI colocalizes with Vg1 RNA at the vegetal cortex and binds to VM1 motifs within the Vg1 LE (Cote et al., 1999; Lewis et al., 2004). Formation of a localization-specific RNP is arguably an early or initiating event in the localization pathway and, until recently, it has been assumed that assembly of the transport RNP occurs in the cytoplasm. Over the last several years a number of findings have pointed to the possibility of nuclear initiation instead (reviewed in Farina and Singer, 2002). Insight into this issue has been recently gained through analysis of RNA–protein interactions in isolated *Xenopus* nuclei (Kress et al., 2004). Both VgRBP60/hnRNPI and Vg1RBP/Vera first associate with Vg1 and VegT RNAs in the oocyte nucleus, indicating that cytoplasmic RNA localization initiates in the nucleus rather than in the cytoplasm (Kress et al., 2004). Thus, the binding of specific RBPs in the nucleus may regulate the ability of an RNA to find its destination in the cytoplasm.

Analysis of the cytoplasmic Vg1 RNP has revealed a remodelling of the complex upon export from the nucleus (Kress et al., 2004). Intriguingly, phosphorylation of VgRBP60/hnRNPI is coincident with its nuclear export (Xie et al., 2003), and might be relevant to recruitment of additional factors in the cytoplasm (Kress et al., 2004). One factor recruited in the cytoplasm is Prrp, which both binds to the Vg1 LE and is colocalized with Vg1 RNA at the vegetal cortex (Zhao et al., 2001). Prrp contains a proline-rich domain that interacts with profilin, a regulator of actin dynamics, and has been proposed to act as a mediator of cortical RNP anchoring (Schlüter et al., 1997; Zhao et al., 2001). Notably, actin filaments are required for anchoring of Vg1 RNA to the vegetal cortex (Yisraeli et al., 1990). Also recruited to the Vg1 RNP in the cytoplasm is XStau, which is the frog homologue of Staufen, an RBP with important roles in RNA localization in *Drosophila* (St Johnston et al., 1991). In *Xenopus* oocytes, expression of a mutant version of XStau disrupts Vg1 localization, and XStau interacts with both Vg1 RNA and Kinesin I (Yoon and Mowry, 2004). It is intriguing that although early steps in the late pathway occur in the nucleus, factors with potential roles in transport and anchoring are recruited in the cytoplasm. Perhaps it is the recruitment of such factors that activates the RNP complex for transport.

Less is known about the *trans*-acting factors involved in targeting germline RNAs like Xcat2 into the MC. To date, no protein has been identified as binding only early pathway RNAs. One would predict that such protein(s) must exist and define the affinity of germline RNAs, but not late pathway RNAs, for the MC. The R1s of the Xcat2 MCLS offer the best entry into identifying such proteins. UV-crosslinking studies have consistently shown that late pathway proteins Vg1RBP/Vera and VgRBP60/hnRNPI can specifically bind to the Xcat2 MCLS in oocyte extracts, but evidence supporting an interaction *in vivo* is lacking (Betley et al., 2002; Chang et al., 2004). Co-localization studies with injected tagged Xcat2 RNA and green fluorescent protein–Vg1RBP/Vera or endogenous Vg1RBP/Vera identified by immunostaining could not reveal any cytoplasmic co-localization and, in fact, Vg1RBP/Vera appeared excluded from the MC (Chang et al., 2004). VgRBP71 (FBP2/KSRP), another nuclear and cytoplasmic protein that binds Vg1 RNA, has been shown to co-immunoprecipitate with Xcat2. However, Prrp does associate with VgRBP71, but not with Xcat2 RNA (Kroll et al., 2002). A picture may be emerging of a core complex of proteins that associates with all localizing RNAs; individual RNAs may thus interact with both unique and common components.

Interplay between the early and late pathways

While germline RNAs become concentrated within the MC, late pathway RNAs such as Vg1 and VegT are uniformly distributed in stage I oocytes. An important question is how are these two classes of RNAs selected differentially while existing in a common cytoplasm? Although Vg1 RNA is excluded from the MC in stage I, addition of Xlsirts RNA LSs can drive Vg1 RNA accumulation within the MC (Kloc and

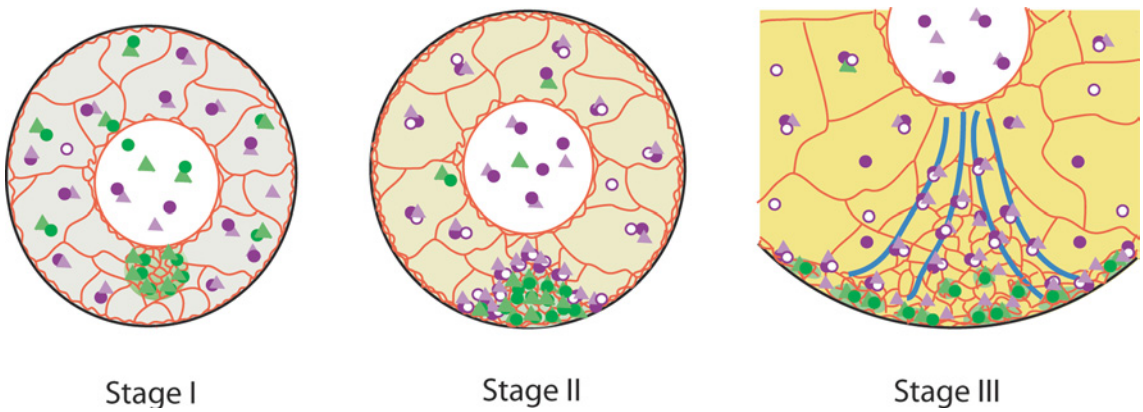
Etkin, 1998). This suggests that the early pathway localization machinery is not normally available to Vg1 RNA, perhaps due to a missing *trans*-acting factor. Movement of the MC to the vegetal cortex may be what triggers initiation of the late pathway, however this event is still a mystery. It is noteworthy that the first steps in Vg1 RNA localization are remarkably similar to the ER-entrapment early pathway mechanism (Chang et al., 2004). As the MC is moved to the vegetal pole, Vg1 RNA co-distributes with a distinct ER subdomain in a microtubule-independent step (Kloc and Etkin, 1998). It is tempting to speculate that it is the same dense network of ER from the MC that could also act as a scaffold to nucleate the late pathway. Vg1RBP/Vera, which has a weak association with ER and binds the Vg1 LE, may participate in this event (Kloc and Etkin, 1998; Deshler et al., 1997). During MC breakdown, the cytokeratin basket that provides the integrity to the MC is likely to be reorganized (Gard et al., 1997), and components derived from the MC, notably the remnants of the centrosome, may further contribute to the establishment of a polarized microtubule network required for the late pathway (Kloc and Etkin, 1998).

Key to understanding how the early and late pathways are separated mechanistically will be a complete description of their complex LSs and associated proteins. Study of the LEs for RNAs competent to use both pathways reveal how closely these two pathways are linked. All early pathway RNAs tested to date, including Xpat, Xcat2 and Xlsirts, can localize using the late pathway if they are injected into stage III/IV oocytes (Hudson and Woodland, 1998; Allen et al., 2003; Zhou and King, 1996b). For Xcat2, the signal both necessary and sufficient for the late pathway is embedded in the first 150 nt of the MCLS (Zhou and King, 1996b). Xlsirts contain a single 137-nt element competent for both pathways, and pathway-specific signals are difficult to separate without impairing either activity (Allen et al., 2003). Normally using both pathways, fatvg contains a small 25 nt LE called FVLE1 that is sufficient for vegetal localization but not MC entrapment. MC entrapment requires additional sequences not yet precisely defined, that lie just downstream of FVLE1 (Chan et al., 1999). This 'dual' competence is likely to be a general one for all early pathway RNAs, and suggests that endogenous RNAs may normally use both pathways. Why would early pathway RNAs maintain competence to use both pathways? This ability may represent a fail-safe mechanism to ensure that germ plasm RNAs that may continue to be transcribed past stage I are not lost from the germline. As mentioned above, these RNAs are stable outside the germ plasm; therefore, this may be a relevant mechanism.

Translational control

Within the cytoplasm, translational repression of localized mRNAs is an important point of regulation to prevent spatially inappropriate expression prior to localization (reviewed in Macdonald, 2001). An example of this in *Xenopus* is Vg1 RNA. Although Vg1 RNA is abundant by the earliest stages of oogenesis (Melton, 1987), the RNA is not translated until after stage IV of oogenesis (Tannahill and Melton, 1989; Wilhelm et al., 2000), after localization to the vegetal cortex. The necessity of repressing Vg1 translation prior to localization is evident from experiments demonstrating that mis-expression of Vg1 in the animal hemisphere results in severe patterning defects (Dale et al., 1993; Thomsen and Melton, 1993). Translational repression of Vg1 RNA is mediated through a *cis*-acting translational control element (TCE) contained within the 3′ UTR, immediately downstream of the Vg1 LE (Wilhelm et al., 2000; Otero et al., 2001). Translation of Vg1 mRNA appears to rely on cap-dependent initiation, and the mechanism of repression does not appear to be linked to cytoplasmic polyadenylation (Otero et al., 2001). As yet, the identity of the factor(s) responsible for TCE-dependent translational repression of Vg1 RNA is unknown. Unlike Vg1 RNA, translation of germline RNAs is repressed not only before but also many months after entry into the germ plasm. Thus, a translational repressor(s) must be a component of the Xcat2 RNP particle, but its identity remains unknown. One promising candidate however, is the RNA binding protein Hermes (Zearfoss et al., 2003). Hermes co-distributes with the germ-plasm material during oogenesis and is degraded during maturation, hours before Xcat2 or Xdazl translation begins in the embryo (Zearfoss et al., 2004; Houston and King, 2000b; MacArthur et al., 1999). The Xcat2 TCE is not within the 3′UTR as substitution of this region for that of the β-globin 3′UTR does not relieve repression (MacArthur et al., 1999). It will be important to elucidate the relationship between the MCLS, the GGLE, and the TCE, as these elements likely

Figure 1 | Model for the relationship between early and late RNA-localization pathways in *Xenopus* oocytes
Early pathway RNAs such as Xcat2 (green triangles), diffuse freely throughout the cytoplasm of the previtellogenic stage I oocyte (left-hand panel), probably as RNP particles formed with specific early pathway proteins (green circles) in the nucleus. These particles are trapped in the MC by direct or indirect association with a dense network of ER (red). During stage II (middle panel), the MC starts to merge with the thickened ER of the oocyte cortex, defining the vegetal pole. During the subsequent vitellogenic stages (right-hand panel), the MC breaks down and early pathway RNAs disperse beneath the vegetal cortex; many of them associated with islands of germ plasm. Late pathway RNAs such as Vg1 (purple triangles) are present from early stages of oogenesis and bind specific proteins such as Vg1RBP/Vera and VgRBP60/hnRNPI (purple circles) in the nucleus. Their localization begins at late stage II, after association with proteins such as XStau and Prrp (purple rings) in the cytoplasm. (The scarcity or absence of these proteins or of essential co-factors prior to this stage may limit the onset of the late pathway.) The late pathway RNP complexes first associate with ER in a 'wedge-shaped' region around the disintegrating MC. From stage III, a polarized organization of microtubules (blue) is established which, with the participation of kinesin motors, is required to move the late pathway RNPs to the vegetal cortex. Residual early pathway RNAs present at stages II and III can bind Vg1RBP/Vera and VgRBP60/hnRNPI and adopt the late pathway. (Figure courtesy of Dr Evelyn Houliston, Observatoire Océanologique, Villefranche-sur-mer, France.)



cooperate to ensure that Xcat2 expression is repressed. Future experiments to identify and characterize the *trans*-acting factors involved in translational control of both early and late pathway RNAs promise to shed light on the co-ordination of RNA localization and translational regulation.

Conclusions and future prospects

Even as understanding of the *cis*-sequences and *trans*-factors utilized by the *Xenopus* vegetal localization pathways emerges, a comprehensive picture of how the pathways are distinguished remains elusive. RNAs that are competent to use both the early and the late pathways underscore how closely these two localization pathways are linked. A common paradigm, exemplified by both Xcat2 and Xlsirts RNAs (Zhou and King, 1996b; Allen et al., 2003), is close proximity of the signals directing localization for the two pathways. Spatial juxtaposition of the LSs, sometimes overlapping, hints at a model where competition among RNAs for *trans*-acting factors could play a role in sorting RNAs into different pathways. Such a model is depicted in Figure 1, showing Xcat2 as an example of an early pathway RNA and Vg1 as a typical late pathway RNA. In this model, Xcat2 enters the MC during stage I because *trans*-acting factors that promote entrapment bind the MCLS in multiple copies with low dissociation constants, while they do not bind the Vg1 LE or do so only weakly. Intermediate pathway RNAs could both enter the MC and stay in the general ooplasm because they bind the putative entrapment factor less efficiently than Xcat2, but more robustly than Vg1. Once the MC migrates vegetally during stage II, the entrapment machinery is only available at the cortex, and the microtubule-based late pathway will be preferred in the vegetal cytoplasm. Late pathway RNAs, along with intermediate RNAs and any remaining germ plasm

RNAs, take that route to arrive at the vegetal cortex during stage III. This model makes specific and testable predictions about binding efficiencies for the *trans*-acting factors participating in the different pathways. Thus, keys to understanding how the early and late pathways are distinguished may be within reach. Future work aimed at obtaining a complete description of complex LSs and associated proteins promises to give new insights into the issue of how RNAs are mechanistically sorted into specific localization pathways.

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