Cell

Resource

Global Analysis of mRNA Localization Reveals a Prominent Role in Organizing Cellular Architecture and Function

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SUMMARY

Although subcellular mRNA trafficking has been demonstrated as a mechanism to control protein distribution, it is generally believed that most protein localization occurs subsequent to translation. To address this point, we developed and employed a high-resolution fluorescent in situ hybridization procedure to comprehensively evaluate mRNA localization dynamics during early *Drosophila* embryogenesis. Surprisingly, of the 3370 genes analyzed, 71% of those expressed encode subcellularly localized mRNAs. Dozens of new and striking localization patterns were observed, implying an equivalent variety of localization mechanisms. Tight correlations between mRNA distribution and subsequent protein localization and function, indicate major roles for mRNA localization in nucleating localized cellular machineries. A searchable web resource documenting mRNA expression and localization dynamics has been established and will serve as an invaluable tool for dissecting localization mechanisms and for predicting gene functions and interactions.

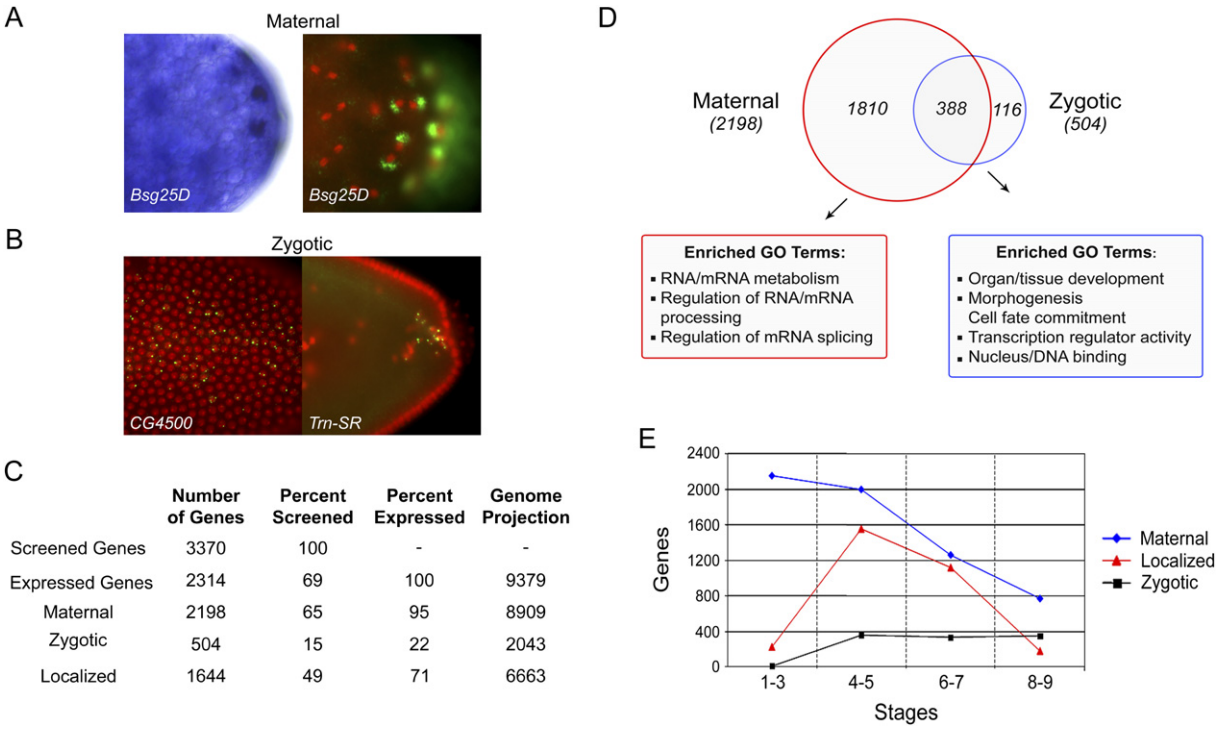
INTRODUCTION

Virtually all cells are polarized, partitioning their contents to a variety of organelles, compartments and membrane interfaces that execute specialized biological and regulatory functions. Since the discovery of the signal peptide by Blobel and colleagues (Blobel and Dobberstein, 1975), the targeting of most proteins to these various subcellular destinations has been thought to occur after translation. More recently, it has been shown that protein localization can also be controlled by localizing the mRNA transcript prior to translation (Bashirullah et al., 1998; Czaplinski and Singer, 2006; Kloc et al., 2002; St Johnston, 2005). A potential advantage of this mechanism is its cost effectiveness. Each localized mRNA can facilitate many rounds of protein synthesis, thereby avoiding the significant energy costs of moving each protein molecule individually (Jansen, 2001). This process also helps to ensure that proteins do not appear where their effects would be detrimental.

Localized mRNAs can serve many biological functions, including the establishment of morphogen gradients (Driever and Nusslein-Volhard, 1988; Ephrussi et al., 1991; Gavis and Lehmann, 1992), the segregation of cell-fate determinants (Broadus et al., 1998; Gore et al., 2005; Hughes et al., 2004; Li et al., 1997; Long et al., 1997; Melton, 1987; Neuman-Silberberg and Schupbach, 1993; Simmonds et al., 2001; Takizawa et al., 1997; Zhang et al., 1998), and the targeting of protein synthesis to specialized organelles or cellular domains (Adereth et al., 2005; Lambert and Nagy, 2002; Lawrence and Singer, 1986; Mingle et al., 2005; Zhang et al., 2001).

While the list of known localized mRNAs has grown steadily over the past two decades (Bashirullah et al., 1998; Czaplinski and Singer, 2006; Kloc et al., 2002; St Johnston, 2005), the prevalence, variety and overall importance of mRNA localization events is unknown. Previous in situ screening efforts in *Drosophila* have established speculative estimates of the proportion of localized mRNAs, ranging from one to ten percent (Dubowy and Macdonald, 1998; Tomancak et al., 2002). However, the detection methods used in past studies were of insufficient resolution to observe intricate subcellular patterns.

To assess mRNA subcellular localization dynamics on a global scale, a high-resolution fluorescent in situ hybridization (FISH) procedure was developed and applied to early developmental stages of *Drosophila* embryogenesis.



	Number of Genes	Percent Screened	Percent Expressed	Genome Projection
Screened Genes	3370	100	-	-
Expressed Genes	2314	69	100	9379
Maternal	2198	65	95	8909
Zygotic	504	15	22	2043
Localized	1644	49	71	6663

Figure 1. Embryonic Gene Expression Dynamics Revealed by High-Resolution FISH

(A and B) The optimized FISH procedure reveals localization patterns not readily discernible with traditional detection methods and enables the unambiguous distinction of maternal and zygotic mRNA populations. Examples of patterns observed are shown for maternal *Bsg25D* transcripts (A), and for zygotically expressed *CG4500* and *Trn-SR* transcripts (B), detected using optimized FISH (mRNAs in green/nuclei in red), or standard alkaline phosphatase-based detection ([A] left panel, image obtained from the BDGP in situ database, Tomancak et al., 2002).

(C) General summary of observed and projected gene expression and mRNA localization events.

(D) Comparison of maternal and zygotic transcripts and their respective gene ontology (GO) term enrichments.

(E) Expression and localization dynamics of maternal and zygotic transcripts during stages 1–9 of embryogenesis.

Of the genes expressed during this developmental window, a surprising 71% were found to encode mRNAs exhibiting clear subcellular distribution patterns. The frequency and variety of localization events suggests that virtually all aspects of cellular function are impacted by RNA trafficking pathways. We conclude that mRNA localization is a major mechanism for controlling cellular architecture and function.

RESULTS

Method Development, Screening, and Localization Database

To circumvent the deficiencies of existing in situ hybridization protocols, considerable effort was made to develop a procedure with optimal subcellular resolution, sensitivity, consistency, throughput and economy. Typical results obtained with the resulting method (Lécuyer et al., 2007), versus traditional alkaline phosphatase-based probe detection, are illustrated in Figures 1A and S1 in the Supplemental Data available with this article online. Reassuringly, control analyses using probes with increasing sequence divergence indicate that the occurrence of false positive signals due to cross-hybridization to mRNAs with similar sequences is highly unlikely (Figure S2).

Following high-throughput FISH, samples were mounted and analyzed using epifluorescence microscopy. For each expressed gene, representative low and high magnification images were captured at key developmental stages and incorporated within a relational database. The first 4.5 hr of *Drosophila* development, spanning embryonic stages 1–9, was chosen for analysis, as this interval is manageable in terms of data annotation and encompasses major developmental landmarks such as the midblastula transition (MBT), gastrulation and the specification of many cell types. The MBT is the period during which developmental regulation switches from control by maternally synthesized gene products to control by zygotically transcribed genes (Tadros et al., 2007b). Importantly, our FISH method enables the unambiguous distinction between maternal and zygotic mRNA populations. Maternally provided transcripts are generally cytoplasmic, exhibit FISH signal intensities above background in stage 1 embryos and decrease in intensity during later stages. Zygotic mRNAs, on the other hand, are always first detected in nascent transcript foci within subsets of

embryonic nuclei and are not generally observed until stage 4. Examples of maternal and zygotic mRNAs are illustrated in Figures 1A and 1B.

An annotation term hierarchy has been created to document stage-specific expression, localization and degradation dynamics of each transcript. In addition, an RNA localization database resource, containing annotation terms and representative images of all transcripts detected, has been established using previously described web-based tools (Tomancak et al., 2002) and can be accessed through a searchable web-browser at: http://fly-fish.ccbr.utoronto.ca.

Embryonic Gene Expression Dynamics

Of the 3370 genes (~25% of genome) successfully screened, 2314 were expressed during the developmental window analyzed (Figure 1C). Of these, 2198 were maternally provided, 504 were zygotically expressed, and 388 were expressed both maternally and zygotically (Figures 1C and 1D). The percentage of genes that we find to be maternally expressed (65%) is more than twice that of earlier predictions (~30%; Arbeitman et al., 2002), although it more closely resembles estimates from a recent microarray-based study (~55%; Tadros et al., 2007a). Our higher values likely reflect the thorough gene-by-gene approach that was used and the improved sensitivity of the detection procedure. Consistent with the importance of transcript degradation in the transition from maternal to zygotic control of embryogenesis (Tadros et al., 2007a), the majority (~65%) of these maternal mRNAs are no longer detectable by stages 8–9 (Figure 1E).

Gene ontology (GO) term enrichment analysis reveals that maternal transcripts are enriched for genes involved in RNA metabolism (Figures 1D, S3, S4A, and Table S1), including, for example, many components of the spliceosome (*crn, prp8, SmD3, snRNP69D, snRNP70K, U2af50*). This finding is consistent with the large and diverse maternal mRNA contingent, and the need to organize aspects of their processing, translation, stability and localization. Interestingly, these terms are more strongly enriched among the more stable maternal mRNA subsets that continue to be detected through stages 6–9 (Figure S4A and Table S1), in agreement with recent observations (Tadros et al., 2007a).

The first major burst of zygotic transcription occurs at stages 4–5 as the *Drosophila* embryo begins to transition from syncytial to cellular growth (Figure 1E). Approximately half (230) of the zygotically expressed genes detected are initiated during stages 4–5 and continue to be expressed throughout the developmental period analyzed (Figure S5A). Interestingly, several of the earliest transcribed mRNAs, which were unexpectedly detected as early as stages 1–2, are encoded by transposable/repetitive elements (*copia, Doc, Ste12DOR*). The significance of these early expression events will be addressed further below. As a group, the zygotically expressed genes are strongly enriched for functions relating to transcriptional regulation, cell fate determination, tissue/organ development and morphogenesis (Figures 1D, S4B, and Table S1), consistent with the rapid patterning and morphogenetic processes that follow the MBT. These functional enrichments are in agreement with a recent study by De Renzis et al. (2007), who identified 1158 putative zygotically expressed genes using microarray analyses of chromosome deletion mutants (De Renzis et al., 2007). However, their dataset does not include 337 of the 504 genes that we unambiguously found to be zygotically expressed. Thus, by extrapolation, we predict the number of zygotically expressed genes during this period to be significantly higher (~2043 versus 1158; Figure 1C). Altogether, our projections suggest that >9000 genes are expressed either maternally or zygotically during the early stages of *Drosophila* embryogenesis (Figure 1C), representing a vast and complex set of regulatory interactions.

Transcript Localization

Of the 2314 mRNAs expressed, a remarkable 71% are subcellularly localized (Figure 1C), with a peak in localization frequency observed between embryonic stages 4–7 (Figure 1E). This peak in localized transcript numbers may reflect a relatively high demand for localization events during the conversion from syncytial to cellular environments. Alternatively, it may be skewed by the relative ease of detection of localization in large embryos versus small cells. Further analyses of transcript localization in other tissues and developmental stages, and using higher resolution microscopy techniques, will undoubtedly reveal many additional localization events. Hence, our numbers represent a conservative estimate of the total number of localized mRNAs encoded in the fly genome.

The 1644 localized transcripts observed were grouped into ~35 localization categories, most of which are listed in Table 1. The following sections will focus on some of the more diverse and striking of these localization patterns and their functional implications.

Subembryonic Localization Patterns

The most prevalent localization patterns observed are the sub-embryonic 'exclusionary' categories, where mRNAs are excluded from parts of the embryo, such as the peripheral apical cytoplasm or the germline pole cells that form at the posterior tip (Table 1). Although these patterns might easily be missed in smaller somatic cells, their importance is clear. For example, the pole cell-excluded subgroup is specifically enriched for transcripts encoding ribosomal constituents and factors involved in mRNA metabolism and processing (Table S2). This is consistent with the general need to prevent transcript synthesis and translation in germline cells during early embryogenesis (Seydoux and Dunn, 1997; Van Doren et al., 1998), and provides new insights into the mechanisms responsible. Similar mechanisms are likely to be used in somatic cells to prevent translation or related processes in portions of the cytoplasm.

Perhaps the most easily detected of the subembryonic patterns are the previously documented anterior and posterior categories. Notably, the number of anterior

Table 1. Summary of mRNA Localization Patterns

Localization Patterns	Number of Genes[a] (Projected[b])	Percent of Expressed	Number of Genes/Stage Range			
			St. 1–3	St. 4–5	St. 6–7	St. 8–9
Expressed	2314 (9379)	100.0	2213	2183	1592	1154
Localized	1644 (6663)	71.0	233	1578	1135	187
Subembryonic patterns	1468 (5950)	63.4	207	1431	1041	147
Exclusionary patterns	1397 (5662)	60.3	123	1371	976	8
Pole plasm/Pole cell exclusion	1272 (5156)	54.9	83	1235	920	
Apical exclusion	1145 (4641)	49.5	NA	1142	747	6
Basal exclusion	256 (1038)	11.1	NA	225	112	
Yolk cortex exclusion	207 (839)	8.9	58	175	6	
Yolk cortex localization	277 (1123)	12.0	9	171	172	3
Posterior localization	198 (803)	8.6	74	195	106	
Pole/Germ cell localization	195 (790)	8.4	NA	192	105	62
Pole plasm localization	70 (284)	3.0	70	NA	NA	NA
Pole buds	54 (219)	2.3	54	NA	NA	NA
RNA islands	44 (178)	1.9	44	NA	NA	NA
Anterior localization	5 (20)	0.2	5	3		
Subcellular localization patterns	366 (1483)	15.8	42	135	270	107
Basal localization	209 (847)	9.0	NA	25	190	50
Nuclei-associated localization	82 (332)	3.5	27	43	36	24
Perinuclear localization	81 (328)	3.5	25	37	35	24
Perinuclear yolk nuclei	59 (239)	2.5	6	25	35	22
Perinuclear cortical nuclei	27 (109)	1.2	8	21	2	
Intranuclear accumulation	14 (57)	0.6	2	14	4	4
Apical localization	78 (316)	3.4	NA	59	54	15
Diffuse apical localization	36 (146)	1.6	NA	29	18	12
Discrete apical foci	27 (109)	1.2	NA	20	18	2
Apical clusters	24 (97)	1.0	NA	15	20	0
Apical in neuroblasts	6 (24)	0.3	NA	NA	NA	6
Cytoplasmic foci	45 (182)	1.9	14	38	14	15
Cell division apparatus	33 (134)	1.4	16	29	1	1
Microtubule-associated	10 (41)	0.4	9	6	1	
Spindle midzone localization	10 (41)	0.4	1	10		
Centrosomal localization	6 (24)	0.3	6	4	1	1
Chromatin-associated	14 (57)	0.6	2	12		
Cell junction-associated	12 (49)	0.5	4	10	9	6
Membrane-associated	8 (32)	0.3	5	5	5	2
Polar body-associated	4 (16)	0.2	4	NA	NA	NA

NA, not applicable to these embryonic stages; St., stages.

[a] All screened genes encoding localized mRNAs in either of the analyzed stages; patterns are not necessarily mutually exclusive.

[b] Projected number of localized transcripts encoded in the *Drosophila* genome, out of a total of 13,659 coding genes.

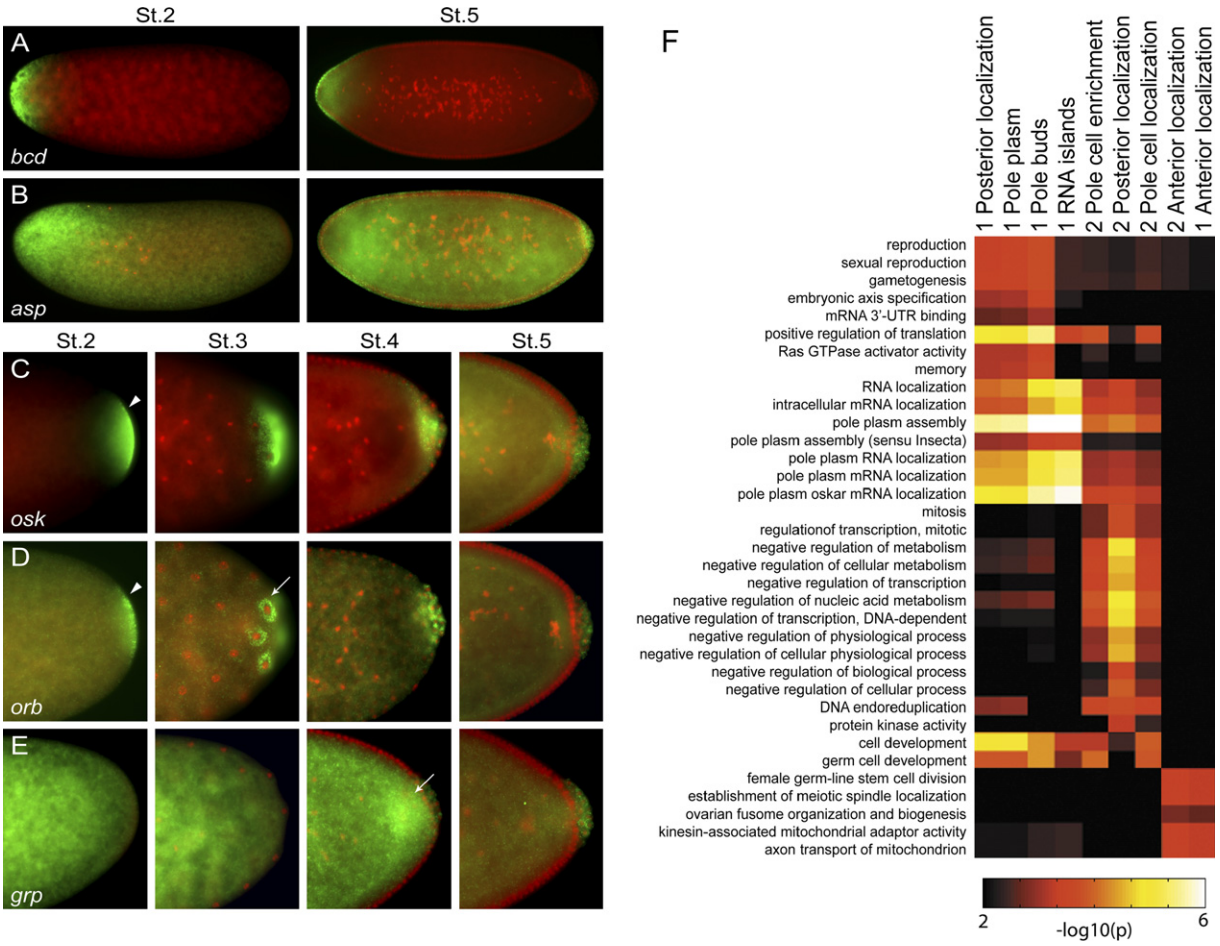


Figure 2. Anterior/Posterior Patterns and Functional Enrichments

(A–E) Sagittal views of entire embryos (A and B) or of the posterior region (C–E) between stages 2–5, following FISH with probes to *bcd* (A), *asp* (B), *osk* (C), *orb* (D), or *grp* (E) transcripts (mRNA green/nuclei red). (A and B) Varieties of anterior patterns, with *bcd* mRNA (A) showing tight anterior localization and *asp* transcripts (B), a more diffuse anterior enrichment. (C–E) Early and late posterior localization patterns. While both *osk* and *orb* transcripts localize to the posterior pole plasm in stage 1–2 embryos ([C and D] arrowheads), *orb* mRNA forms distinctive rings around pole cell nuclei at stage 3 ([D] arrow). In contrast, *grp* transcripts localize in the posterior yolk plasm in early stage 4 embryos ([E] arrow). All of these transcripts localize to the pole cells at stage 5.

(F) GO term enrichments exhibited by transcripts found within annotation categories pertaining to anterior and posterior localization in stage 1–5 embryos (column categories 1 and 2 refer to stages 1–3 and 4–5, respectively). The "hot metal" color scale reflects statistical significance (−log 10 of the p value) of the GO term enrichments.

transcripts is far outnumbered by those that are posteriorly localized (5 versus 198; Table 1). For both of these categories, the sensitivity and resolution of detection enabled the identification of distinct subgroups of patterns (Figure 2). For example, among the anteriorly localized mRNAs, which include *bcd, CycB, lok, milt* and *asp*, only *bcd* exhibits tight anterior localization (Figure 2A), consistent with its extensively characterized function as the primary determinant of anterior cell fate specification (Ephrussi and St Johnston, 2004). The other four mRNAs exhibit a more diffuse gradient of anterior enrichment (Figure 2B). Interestingly, three of these encode proteins involved in cytoskeleton organization and microtubule-based processes (Figure 2F and Table S3).

The posterior group of mRNAs could be clearly subdivided into three categories: (1) transcripts that localize to early pole plasm (Figure 2C), a specialized region of cytoplasm that directs the formation of germline pole cells; (2) transcripts that reside in the pole plasm and then localize further into distinctive rings around pole cell nuclei (Figure 2D); and (3) transcripts that only begin to localize posteriorly in early stage 4 embryos (Figure 2E). The biological significance of these subcategories is underscored by their specific GO term enrichments (Figure 2F and Table S3). For example, the category 1 and 2 pole plasm localized transcripts, which include mRNAs such as *aret, eIF5, gcl, Imp, nos, osk, orb, pAbp, pum, spir*, and *Tm1*, are strongly enriched for cell development,

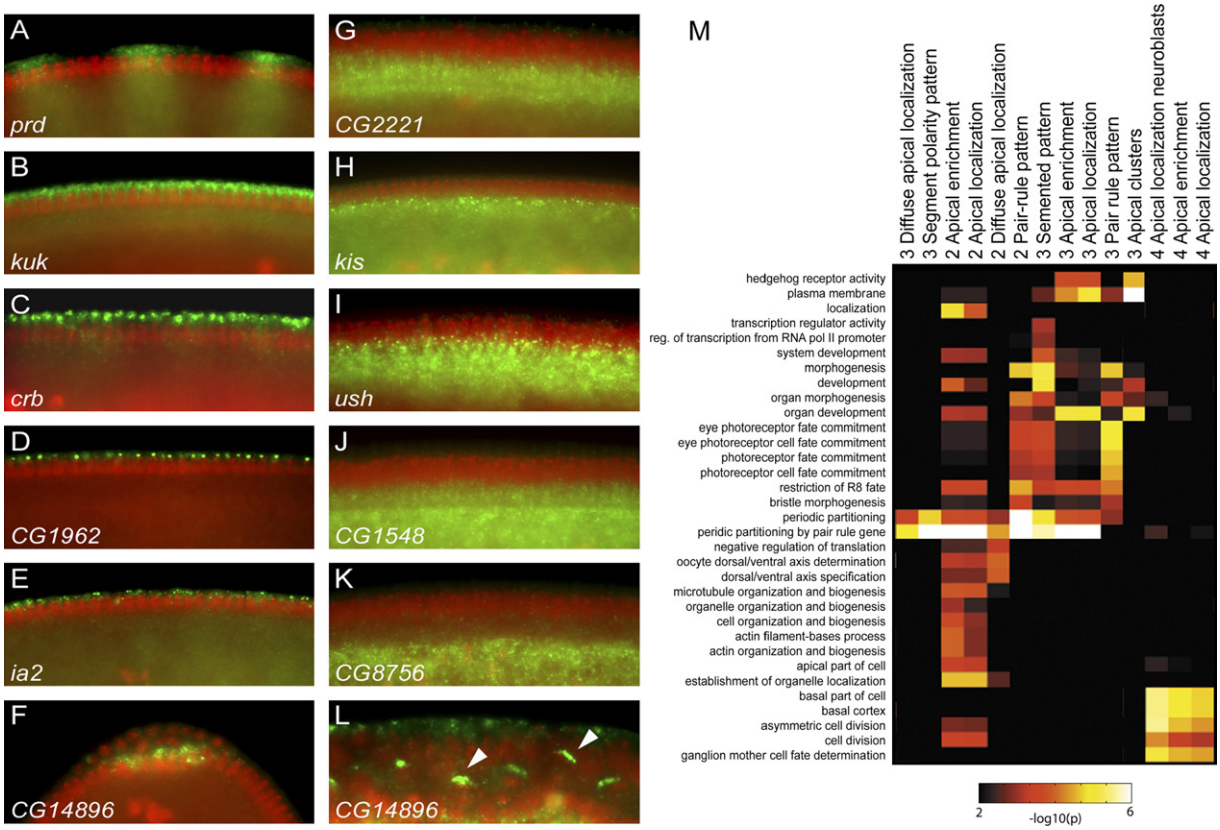


Figure 3. Varieties of Apicobasal Localization Patterns and Their Functional Enrichments

(A–L) Sagittal views through the embryonic epithelium of embryos hybridized with the indicated probes. Several distinctive subcategories of apical (A–E), basal (G–I), or exclusionary (J and K) patterns are shown. (F and L) *CG14896* transcripts are apical in posterior epithelial cells (F) and in later arising neuroblasts ([L] arrowheads). For all images, mRNAs are green and nuclei red.

(M) GO term enrichments observed for different subcategories of apical mRNAs. Enrichment scores are depicted using a hot metal color scale conveying statistical significance (−log 10 of the p value). Column categories 2–4 refer to embryonic stages 4–5, 6–7, and 8–9, respectively.

translation regulation, pole plasm assembly and RNA localization functions (Figure 2F). In contrast, the late posterior group, which includes genes such as *aur, CG14030, CycA, grp, gwl, Rbp-1, Su(var)3-9*, and *ttk*, is enriched for protein kinases and negative regulators of gene expression, again consistent with previous findings that germ cells are transcriptionally silent (Seydoux and Braun, 2006; Seydoux and Dunn, 1997; Van Doren et al., 1998). Taken together, these observations suggest the existence of distinct early and late pathways for posterior transcript localization.

Notably, no maternal transcripts were identified as being either dorsally or ventrally localized. Instead, differential distribution of transcripts along the dorso-ventral axis was always a consequence of localized zygotic transcription. The preponderance of transcripts localized to the posterior pole of the embryo, in comparison to the other embryonic poles, seemingly reflects special requirements for germ cell specification and the sufficiency of existing zygotic mechanisms to define the other coordinates.

Subcellular Categories: Apicobasal Patterns

Besides the subembryonic localization patterns, a large collection of mRNAs, either of maternal or zygotic origin, were found to exhibit intricate subcellular localization patterns. Classic examples include the subset of mRNAs that localize to the apical cytoplasm within the embryonic epithelium (Figure 3). Although apical mRNAs have been characterized previously and considered as a homogeneous group (Davis and Ish-Horowicz, 1991; Simmonds et al., 2001; Tepass et al., 1990), many distinctive subgroups of apical transcripts could be distinguished, ranging from broad gradients of apical enrichment to tightly localized clusters or foci (Figures 3A–3E). Likewise, a large number of basally localized mRNAs were identified, which also fall into a number of subgroups (Figures 3G–3I). Other patterns that vary along the apico-basal axis include transcripts that are excluded from the apical cytoplasm or from the entire blastoderm layer (Figures 3J and 3K).

The functional relevance of these subgroup classifications is underlined by the GO term enrichments observed.

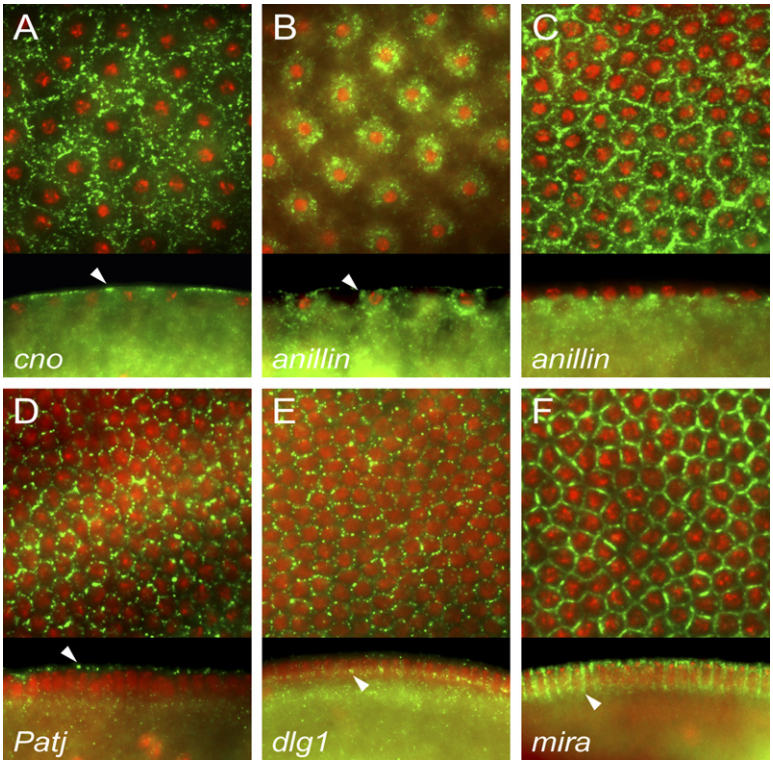


Figure 4. Membrane-Associated Patterns
(A–F) Surface plane (upper panels) and sagittal (lower panels) views of stage 3 (A and B), 4 (C), 5 (E and F), and 6 (D) embryos hybridized with probes for the transcripts indicated in lower panels (mRNA green/nuclei red). *cno* transcripts localize within cortical polygonal networks ([A] arrowhead), while *anillin* mRNA is first perinuclear ([B] arrowhead) and then evolves into a cell junction type pattern (C). (D–F) *Patj, dlg1*, and *mira* transcripts localize at different positions along the lateral membrane (arrowheads).

For example, the apical clusters category, which includes mRNAs such as *Ama, bib, Btk29A, crb, fra, Gp150, htl, Ptr, scb, sog*, and *smo*, is enriched for GO categories for plasma membrane and signaling pathway components (Figure 3M and Table S4). In contrast, the diffuse apical group, which contains several pair-rule gene transcripts (*hairy, odd, prd, run*), is enriched for functions associated with transcriptional regulation and pattern/axis specification.

In addition to the apicobasal patterns detected in the embryonic epithelium, several mRNAs were observed with asymmetric patterns in neuroblasts. This category includes transcripts such as *asp, Gp150, mira, odd, pros*, and *wg*, some of which have been observed previously (Broadus et al., 1998; Schuldt et al., 1998), and not surprisingly, show GO term enrichments for asymmetric cell division functions (Figure 3M). We also identified mRNAs from uncharacterized genes, such *CG14896*, which exhibit apical localization both in the posterior embryonic epithelium underlying the pole cells and in neuroblasts that arise later in embryogenesis (Figures 3F and 3L). This example suggests the likelihood that many localization mechanisms will operate in a variety of cell types.

Membrane-Associated Patterns

Also remarkable are transcripts that localize to membrane-associated structures prior to and following cellularization (Figure 4). For example, *cno* and *anillin* mRNAs (Figures 4A and 4B) associate with the embryonic cortex or perinuclear clouds as early as stage 3, and then evolve into polygonal mosaic networks shortly thereafter (Figures 4A and 4C). These patterns resemble subsequent actin filament distributions and dynamics and precede cell junction formation. Several other mRNAs encoding cell junction components, such as *Patj* (Figure 4D) and *dlg1* (Figure 4E), localize at specific sites along the basolateral membrane. In contrast, *mira* transcripts localize throughout the lateral membrane of embryonic epithelial cells (Figure 4F). Accordingly, this category is enriched for GO terms related to cytoskeleton organization and biogenesis (Table S5). These observations imply a significant role for mRNA localization in the nucleation and positioning of cytoskeletal networks and membrane-associated structures.

Cell Division and Nuclei-Associated Patterns

Many of the most striking subcellular patterns observed occur during nuclear or cellular division (Figure 5). These include transcripts that localize to spindle poles, centrosomes/centrioles, astral microtubules, or along the mitotic spindles themselves during anaphase and telophase (Figures 5A–5H). Furthermore, several mRNAs that are zygotically transcribed in early stage 4 embryos concentrate around metaphase chromosomes during mitosis and often become associated with spindle midbodies (Figure 5D). Intriguingly, many of these mRNAs encode transcriptional regulators (*dpld, nullo, odd, rib, run, stwl, Taf4*). The genes in these categories show GO term enrichments for cell division related processes (Figure S3 and

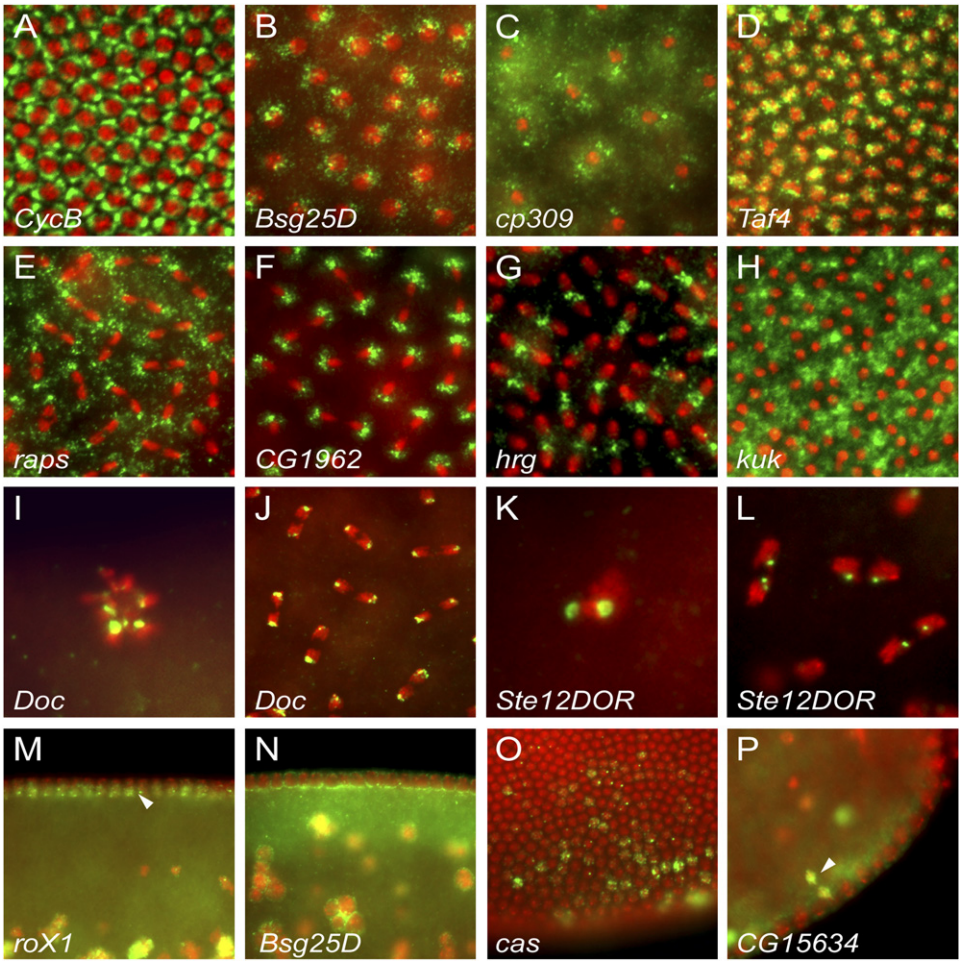


Figure 5. Cell Division and Nuclei-Associated Transcripts

(A–P) Surface plane (A–L and O) or sagittal (M, N, and P) views of stage 1–5 embryos hybridized with the indicated probes (mRNA green/nuclei red). (A–H) Examples of mRNAs that localize to different sections of the cell division apparatus, including spindle poles (A), microtubule networks and centrosomes (B, C, E, and F), the spindle midzone (G and H), or in proximity to metaphase chromosomes (D). (I and J) *Doc*-element transcripts localize to centromeric chromatin regions on polar body chromosomes (I) and during mitosis in diploid nuclei (J). (K and L) *Ste12DOR* transcripts localize in chromatin-associated foci during metaphase (K), which then become telomeric during anaphase (L). (M) *roX1* RNA shows polarized enrichment in the basal portion of blastoderm nuclei (arrowhead). (N) *Bsg25D* transcripts exhibit perinuclear localization around peripheral blastoderm and yolk nuclei. (O and P) Several mRNAs exhibit nuclear retention; (O) *cas* transcripts are retained in groups of ventral nuclei following zygotic expression in stage 4 embryos, and (P) *CG15634* mRNA is retained in nuclei situated just below the peripheral layer (arrowhead).

Table S5), implying important roles for localized mRNAs in the establishment, function and regulation of cell division machineries.

Interestingly, several of the earliest zygotically expressed mRNAs, such as those encoded by the *Doc* and *copia* transposons, exhibit intricate chromatin-associated patterns. Indeed, *Doc*-element RNA localizes in the vicinity of centromeres, either along the 'rosettes' formed by the polar body chromosomes or in dividing diploid nuclei (Figures 5I and 5J). In contrast, mRNA encoded by the *Ste12DOR* gene is found within large chromatin-associated foci that localize to telomeric regions during anaphase (Figures 5K and 5L). Notably, this gene is highly homologous and in close proximity to the tandemly repeated *Stellate* gene cluster on the X chromosome, which has been implicated in the maintenance of male fertility through an RNA interference process involving the *Su(Ste)* gene cluster located on the Y chromosome (Aravin et al., 2001). Finally, *roX1*, a noncoding RNA involved in X chromosome dosage compensation (Park et al., 2002), localizes to the basal side of blastoderm nuclei, where the X chromosome presumably resides (Figure 5M). As has been demonstrated for *roX1*, these assorted DNA-associated RNAs may be functioning to help organize, establish and/or maintain chromatin domains. For *Doc* and *Ste12DOR*, the chromosome-associated RNA may be

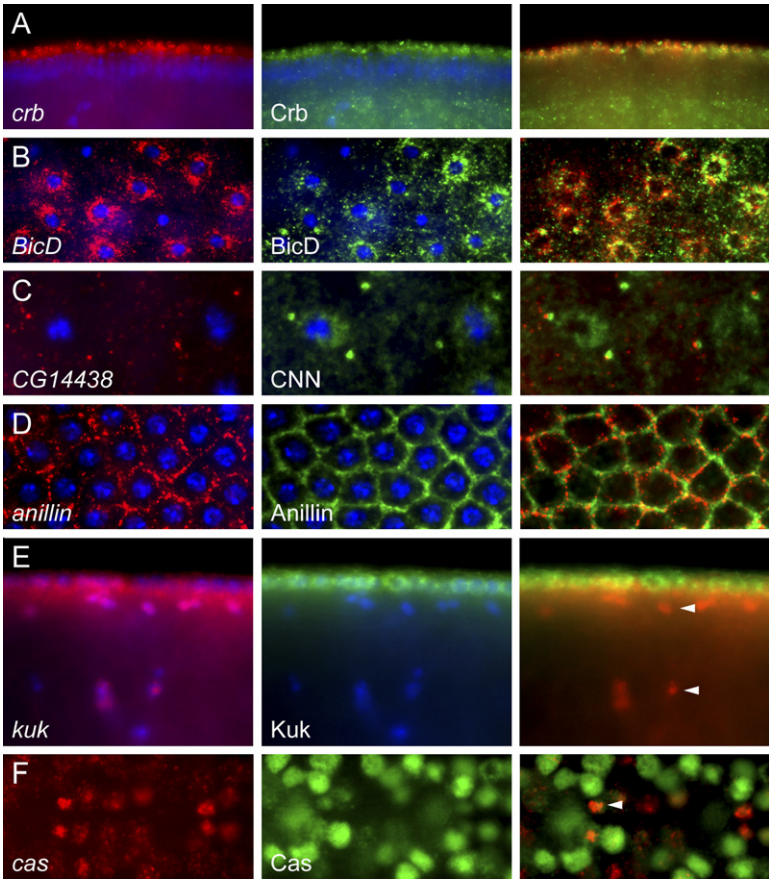


Figure 6. Correlations in mRNA and Protein Distribution Patterns

(A–F) Stage 4 (B–E), 5 (A) and 9 (F) embryos hybridized with probes for the indicated apical (A), cell division-associated (B and C), membrane-associated (D), and nuclear retained (E and F) mRNAs (red signal, left panels) and co-labeled with antibodies against the indicated protein products (green signal, middle panels). Overlaid mRNA and protein signals are shown in the right panels. Nuclei are shown in blue in the left and middle panels in (A)–(E). Arrowheads in (E) and (F) indicate nuclei showing an accumulation of *kuk* and *cas* mRNAs, respectively.

functioning in the 'repeat-associated small interfering RNA' (rasiRNA) pathway, which acts in part to suppress transposable element activity (Slotkin and Martienssen, 2007). If so, this autoregulation would add a new dimension to our understanding of this process.

Finally, other nuclei-associated mRNAs were observed that range from mRNAs with tight perinuclear localization (Figure 5N) to those that appear to be uniformly localized throughout the nucleus (Figures 5O and 5P). These include *cas, CG15634, CG8552, Eip71CD, hb, Jra, kuk, mfas*, and *scw*. Interestingly, some of these are only retained within nuclei that appear to be dropping out of the blastoderm layer (Figures 5P and 6E). As these nuclei are generally observed in pairs, and 'nuclear fallout' may be a consequence of unsuccessful nuclear divisions (Rothwell et al., 1998), this suggests a potential function of these mRNAs in nuclear migration, sorting and/or apoptosis.

Colocalization of RNAs and Proteins

In many of the cases cited above, where details are known about protein localization or gene function, there is a striking correlation between transcript localization and the patterns or functions of the encoded proteins (Table S6). To further illustrate some of these relationships, double-staining for selected transcripts, their protein products or relevant markers was carried out (Figure 6). Examples are shown for mRNAs that are apically localized (Figure 6A), cell division apparatus-associated (Figures 6B and 6C), or that reside at cell junctions (Figure 6D). In each of these cases, mRNA localization is noted prior to the appearance of protein, consistent with the view that transcript localization generally predetermines protein distribution at most subcellular destinations. Another intriguing example is *CG14438* mRNA, which appears to localize at the level of centrioles and is found nestled within structures labeled with the pericentriolar marker CNN (Figure 6C). These types of examples support the notion that localized transcripts, and the proteins they encode, play central nucleation functions within the cell. These observations further suggest that the necessary translation and secretory machineries are generally available at each of these sites.

Interestingly, a reverse correlation exists for mRNAs that show nuclear retention, as shown for *kuk* and *cas* transcripts (Figures 6E and 6F). Indeed, while Kuk protein exhibits robust nuclear envelop localization in cortical nuclei where the mRNA is cytoplasmic, no protein is observed in or around the yolk nuclei in which *kuk* mRNA is retained (Figure 6E). Similarly, in stage 9 neuroblasts,

Cas protein is expressed robustly in cells with diffuse cytoplasmic *cas* mRNA, but shows little or no protein expression in cells where *cas* mRNA is nuclear (Figure 6F). These examples suggest that nuclear mRNA retention serves as a means of precisely timing or coordinating protein expression to related cellular processes (Brandt et al., 2006; Grosskortenhaus et al., 2006; Kambadur et al., 1998; Pilot et al., 2006). Although this mechanism of translational control has only once been documented before (Prasanth et al., 2005), it may prove to be a relatively common form of post-transcriptional gene regulation.

DISCUSSION

The striking diversity and frequency of localized mRNAs observed in this study, and the numerous correlations between mRNA and protein distribution and function uncovered, show that mRNA localization plays a far greater role in coordinating cell physiology and anatomy than ever previously suspected. Over the years, mRNA localization has primarily been thought to coordinate specialized biological processes such as morphogen gradient formation and asymmetric cell division (Kloc et al., 2002; St Johnston, 2005). Our findings, however, necessitate a change in perspective, implicating mRNA localization as a means of regulating a vast number, if not the majority, of cell functions.

mRNAs as Nucleators of Localized Complexes

The major inference of this study is that, since mRNA localization generally occurs prior to encoded protein production, and is so pervasive in scope, it must play a major role in the nucleation and assembly of protein complexes and organelles. One such example is *anillin* mRNA, which encodes an actin-interacting protein and is localized in dynamic patterns that closely resemble actin filament distributions that form subsequently (Field and Alberts, 1995; Karr and Alberts, 1986). Another example is the set of chromatin-associated RNAs, which may be acting like some siRNAs to recruit chromatin remodeling complexes. Other organelles likely to be controlled by mRNA localization include various subcomponents of the cell division apparatus. An example is *CG14438* mRNA, which localizes to centrosome-associated foci that move during cell division to nestle within CNN-containing clusters (Figure 6C). Notably, *CG14438* encodes a protein containing 21 zinc finger domains of the C2H2 type, which could readily serve as a nucleating scaffold for RNA-containing centrosomal/centriolar complexes. Our findings are consistent with recent studies suggesting a role for RNAs in the regulation of centrosome dynamics (Alliegro et al., 2006; Blower et al., 2005; Lambert and Nagy, 2002). Moreover, our study extends these previous findings, by revealing a large collection of cell-division apparatus associated mRNAs that await further characterization.

Localized RNAs as Functional Components of RNP Complexes

In some cases, localized mRNAs are likely to have roles in addition to targeting protein synthesis. For example, they could well serve as structural or catalytic components of RNP complexes, as seen for the many noncoding RNA molecules found in ribosomes and spliceosomes (Eddy, 2001; Erdmann et al., 2001; Prasanth and Spector, 2007). Indeed, a structural function for RNAs that localize to the vegetal pole of *Xenopus* oocytes has recently been uncovered (Kloc et al., 2005), and genetic studies of the *oskar* gene in *Drosophila* have shown that protein null alleles yield only a subset of the phenotypic traits exhibited by RNA nulls (Jenny et al., 2006). This may also be the case for many other genes for which protein null alleles are considered to be genetic knockouts. These additional roles would be consistent with the recent explosion of newly recognized RNA-dependent processes, and with the hypothesis that RNAs preceded proteins evolutionarily in many cellular functions (Gilbert, 1986). Taken further, the high proportion of the genome that is transcribed but noncoding (Mattick, 2004; Willingham and Gingeras, 2006) suggests that we may only now be looking at the tip of an iceberg.

RNA Localization and Disease

As our data suggests that mRNA localization has a key role in targeting various cellular machineries, it can be imagined that the integrity of transcript localization pathways will be essential for ensuring appropriate cell growth and differentiation, while also preventing cellular transformation and tumorigenesis. Indeed, inappropriate targeting of mRNAs would lead to aberrant protein distributions within the cell, interference with normal regulatory pathways and altered complex stoichiometries. In support of this general view, several of the localized mRNAs characterized in this study (ex: *mira, dlg1, raps*) encode well-known regulators of cell polarity and asymmetric cell division with demonstrated tumor suppressor functions (Caussinus and Gonzalez, 2005; Pagliarini and Xu, 2003; Siegrist and Doe, 2005). Other examples include the *CG6522* mRNA, which exhibits an early cell membrane-associated pattern. Homology searches reveal that this mRNA likely encodes the *Drosophila* ortholog of Testin, a cytoskeleton-associated LIM-domain protein that has been identified as a tumor suppressor in mice and humans (Drusco et al., 2005; Mueller et al., 2007). This example typifies the potential usefulness of our database in identifying genes with human disease relevance. Altogether, our findings force a reassessment of many cancer pathways for which modeling has been entirely concentrated on events and interactions occurring at the post-translational level.

Database Usage

The accompanying mRNA localization database will impact many different biological disciplines. For example, it will augment existing resources currently being used to

study functional and regulatory genetic relationships. Since our strategy provides additional levels of detail with regards to spatio-temporal expression dynamics, this dataset will also serve as a powerful tool for deciphering and validating gene regulatory networks.

As there is a tight correlation between mRNA localization and protein function, a more specific use for this resource will be its predictive value in assigning functions for uncharacterized genes. Indeed, 919 of the subcellularly localized mRNAs in this study are encoded by poorly characterized genes designated only by "CG" numbers. For many of these, we can now postulate a function with significantly higher confidence due to our knowledge of where the mRNA is localized in the cell and which other mRNAs, proteins and organelles it temporally and spatially colocalizes with. These predictive values may also suggest new functions for well-characterized genes. Thus, this approach may help identify or confirm many new components of regulatory complexes and pathways. We expect that previously uncharacterized macromolecular complexes will also be discovered, many of which will be spatially and developmentally regulated.

Another area of research that will benefit greatly from this dataset will be the study of RNA *cis*-regulatory elements and *trans*-acting factors that dictate the localization of different mRNA subpopulations. In the past, these studies have been tedious and complicated, due in part to the complexity of RNA regulatory elements, *trans*-acting complexes and the limitations of RNA structure prediction algorithms. Unlike DNA cis-elements, RNA regulatory elements tend to possess complex features of both sequence and structure. The richness of the data set provided here will reveal common sequence and/or structural elements. Together with the growing list of *Drosophila* genome sequences available to assess sequence and structure conservation, rapid advances in the identification of conserved cis-elements and *trans*-acting machineries should be possible. In point of fact, a consensus localization motif for mRNAs in the diffuse apical category has been identified using exactly this approach (dos Santos et al., 2007). Transcripts that colocalize are also likely to share other types of regulatory elements. For example, similarly localized mRNAs may share common regulatory elements used to couple translation and stability control.

Future Prospects and Considerations

An important point that remains to be confirmed is whether the extent and variety of mRNA localization events observed here occur in other tissues and organisms. The amazing level of conservation between *Drosophila* genes and developmental processes, and those of higher organisms, suggests that these mRNA localization frequencies and mechanisms will also be highly conserved. In fact, it may have been the prior existence of these elements and machineries that made early *Drosophila* syncytial development an evolutionary possibility. This would also be consistent with suggestions that RNA-based processes have played a major role in promoting phenotypic variation and complexity in higher eukaryotes (Mattick, 2004).

EXPERIMENTAL PROCEDURES

Probe Production

The *Drosophila* gene collection 1 and 2 (DGC1 and 2) bacterial cDNA libraries (Rubin et al., 2000; Stapleton et al., 2002) were obtained as bacterial glycerol stocks in 96-well plates. Overnight cultures served as templates for PCR using universal primers to amplify library cDNA sequences containing flanking bacteriophage promoter elements (T7, T3, or Sp6). Following PCR product purification on filter plates (Whatman Inc., Clifton, NJ, USA), fragments were concentrated by ethanol precipitation and resuspended in nuclease-free water. Antisense RNA probes labeled with digoxigenin (DIG) were synthesized as described by Lécuyer et al. (2007). The efficiency and accuracy of all PCR amplifications and transcription reactions was systematically verified by agarose gel electrophoresis after each step. Samples for which no PCR product was obtained, or that contained multiple fragments, were excluded from further analysis.

Embryo Collection, Fixation, and Fluorescent In Situ Hybridization (FISH)

Wild-type Oregon R flies were maintained at 25°C and 50% humidity in large plexiglass cages (60 × 60 × 60 cm). Following a 1 hr preclearing step, embryos were collected on fresh food plates for 4.5 hr and processed for fixation and storage as described by Lécuyer et al. (2007). Conditions and methods for FISH and double FISH are also described in detail by Lécuyer et al. (2007). The following antibodies were used for immunostaining: mouse anti-BicD 4C2 and mouse anti-Crumbs Cq4 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), rabbit anti-Anillin (Provided by Dr. Julie Brill, The Hospital for Sick Children, Toronto, ON, Canada), rabbit anti-Cas (Kambadur et al., 1998), rabbit anti-CNN (Provided by Dr. Thomas Kaufman, Indiana University, Bloomington, IN, USA), and rabbit anti-Kuk (Brandt et al., 2006). Mouse and rabbit antisera were used at 1:10 and 1:100 dilutions, respectively.

Sample Imaging Procedure

Samples were analyzed on a Leica DMRA2 epifluorescence microscope equipped with a rotating stage and a Q-imaging Retiga EX digital camera (Quorum Technologies Inc., Guelph, ON, Canada) and Openlab imaging software (Improvision Ltd., Coventry, England). For each positive sample, a combination of low (10×) and high magnification (20× and 40×) images were captured to document transcript dynamics. As often as possible, care was taken to capture images with embryos in the traditional orientation, with anterior to the left and dorsal to the top. Controls probes were included in each experiment to control for experimental variations in staining levels. An autoexposure function was used as a semiquantitative measure of maternal transcript abundance in stage 1–2 embryos, with exposure times used to categorize expression levels as strong (1–20 ms), intermediate (21–40 ms), weak (41–80 ms), or nonexpressed (>81 ms). A maximum exposure time of 80 ms was used at 20× magnification, as this provided a comparative standard, in particular for transcripts that are degraded over time. The autoexposure function was also used for DAPI images. Tyramide-Cy3 and DAPI images were false-colored in green and red, respectively, using Openlab, as this color scheme was found to provide the best contrast. All overlaid images were saved as high-resolution TIF files. Figures were constructed using Photoshop (Adobe Systems Inc., San Jose, CA, USA).

Data Annotation and Database Setup

All of the image and annotation data were organized within a MySQL database using Perl-based annotation tools adapted from a previous study (Tomancak et al., 2002). Images were sorted into appropriate stage ranges (stages 1–3, 4–5, 6–7, 8–9, >10) and saved with a numerical identifier. An annotation term hierarchy was created to document mRNA localization, degradation, and zygotic expression characteristics within each stage range. For each gene, relevant annotation terms were selected and submitted to the database along with specific annotator comments regarding staining quality and/or experimental observations.

Computational Analysis

The annotation data was converted into a binary matrix, containing genes on one axis and localization terms on the other, where the presence of a localization feature for a given gene was indicated numerically as "1," while lack of a feature was annotated as "0." This matrix was then used for GO term enrichment analysis. Functional GO annotations for all genes were downloaded from Flybase (http://flybase.bio.indiana.edu/genes/lk/function/). Annotations were up-propagated using the GO hierarchy (Ashburner et al., 2000), and calculations were restricted to genes that were both GO annotated and analyzed in this study (1651 genes). The hypergeometric distribution was used to calculate probabilities of overlap between each localization category against all GO categories containing three or more genes. The Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) was used to control for multiple testing by computing a P-value threshold corresponding to a false discovery rate (FDR) of 0.25. Transcript subgroups were also analyzed independently for GO term enrichments using EASE (Hosack et al., 2003). EASE scores of less that 0.05 were considered significant, as reported previously (Tadros et al., 2007a).

Supplemental Data

Supplemental Data include five figures and six tables and can be found with this article online at http://www.cell.com/cgi/content/full/131/1/174/DC1/.

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