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Rift Valley fever virus NS_S gene expression correlates with a defect in nuclear mRNA export

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ABSTRACT

We investigated the localization of host mRNA during Rift Valley fever virus (RVFV) infection. Fluorescence in situ hybridization revealed that infection with RVFV altered the localization of host mRNA. mRNA accumulated in the nuclei of RVFV-infected but not mock-infected cells. Further, overexpression of the NS_S gene, but not the N, G_N or NS_M genes correlated with mRNA nuclear accumulation. Nuclear accumulation of host mRNA was not observed in cells infected with a strain of RVFV lacking the gene encoding NS_S, confirming that expression of NS_S is likely responsible for this phenomenon.

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Introduction

Rift Valley fever virus (RVFV) is a mosquito-borne virus in the family *Bunyaviridae*, genus *Phlebovirus*. RVFV encodes seven proteins in its three genome segments L, M, and S. The L segment encodes the RNA-dependent RNA polymerase (Muller et al., 1992, 1994). The M segment encodes the viral glycoproteins G_N and G_C, a nonstructural protein NS_M, and a 78-kDa protein (Collett et al., 1985; Kakach et al., 1988). The S segment encodes the nucleocapsid protein (N) and a nonstructural protein NS_S (Giorgi et al., 1991). The NS_S protein has been shown to interfere with host RNA synthesis by targeting components of the TFIIH transcription factor (Cyr et al., 2015; Kalveram et al., 2011; Le May et al., 2004). We previously demonstrated that NS_S gene expression alters the localization of the translation-related protein polyadenylate binding protein 1 (PABP1) (Copeland et al., 2013).

Our prior studies demonstrated that PABP1 becomes sequestered in the nuclei of RVFV-infected cells, suggesting a block in PABP1 nuclear export during infection (Copeland et al., 2013). PABP1 nuclear export has been shown to occur by mRNA export-dependent (Burgess et al., 2011) and -independent mechanisms (Woods et al., 2005). A change in PABP1 export can therefore be an indicator of a change in mRNA export. To determine if mRNA export was altered during RVFV infection, we measured changes in mRNA localization.

Results and discussion

Host mRNA accumulates in the nuclei of RVFV infected cells

We evaluated the location of host mRNA during infection with RVFV. To determine if changes in mRNA localization occurred during infection with RVFV, we examined infected cells using fluorescence in situ hybridization (FISH) with a Cy3-labeled oligo d (T) probe. In mock-infected cells, polyadenylated RNA produced a diffuse signal in the cytoplasm and a slightly stronger mottled nuclear pattern (Fig. 1A). This pattern is consistent with previously observed mRNA staining in cells (Dias et al., 2010; Faria et al., 2005; Satterly et al., 2007). In contrast, RVFV MP12-infected cells exhibited weak diffuse cytoplasmic staining with strong nuclear aggregates (Fig. 1A). The observed decrease in cytoplasmic signal is not surprising given that RVFV inhibits host transcription (Kalveram et al., 2011; Le May et al., 2004). The nuclear aggregation of mRNA seen during RVFV infection is consistent with the staining pattern observed when polyadenylated RNA export is blocked (Burgess et al., 2011; Satterly et al., 2007). These results reveal a previously undescribed arrest in mRNA export during RVFV infection.

To determine if the aggregated nuclear mRNA was coincident with nuclear PABP1, co-staining was performed with oligo d

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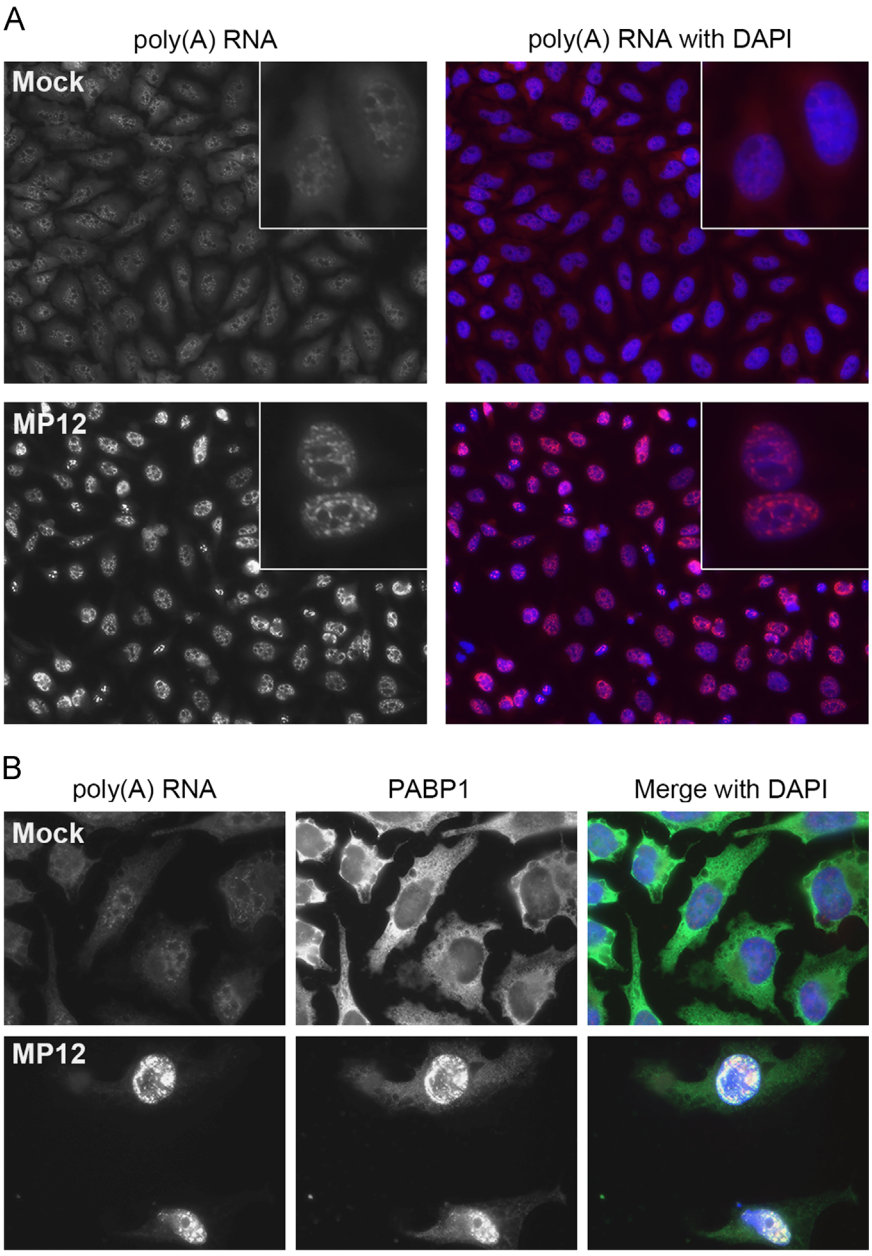

Fig. 1. mRNA localization during RVFV infection. (A) Fluorescence micrographs of cells infected with RVFV MP12 or mock-infected for 24 h and then fixed and stained for polyadenylated RNA and DNA. Insets show an enlarged view (3 ×) of each panel. (B) Fluorescence micrographs of cells infected with RVFV MP12 or mock-infected for 24 h and then fixed and stained for polyadenylated RNA and PABP1.

(T) probe and an antibody specific for PABP1 on mock- and RVFV MP12-infected samples. PABP1 and polyadenylated RNA staining were coincident in the nuclei of RVFV-infected but not mock-infected cells (Fig. 1B). As in our previous study, nuclear relocalization of PABP1 occurred in 80–90% of RVFV infected cells.

NS_S is responsible for the nuclear accumulation of host mRNA during RVFV infection

To examine the roles of individual RVFV proteins in the observed change in mRNA localization, we transfected cells with RVFV M or S segment expression plasmids and subsequently detected mRNA location by FISH. To assess M segment protein influences on the localization of mRNA, we transfected cells with a plasmid expressing the codon-optimized M segment from the fourth start codon. Expression was confirmed by immunofluorescence microscopy using an anti-G_N monoclonal antibody (4D4) (Keegan and Collett, 1986). G_N exhibited a perinuclear staining pattern consistent with its expected localization (Fig. 2). However, no change in mRNA location or signal intensity was observed in transfected cells. To examine a possible role for NS_M, we transfected cells with a plasmid encoding a FLAG-tagged partial NS_M and confirmed its expression with an anti-FLAG antibody. NS_M staining was observed throughout the cytoplasm and nucleus (Fig. 2). As with G_N, we observed no change in mRNA signal or intensity upon NS_M expression.

For assessing the roles of S segment proteins, we used two expression plasmids: one encoding the open reading frame (ORF) of N and one encoding a V5 tagged NS_S. Upon N expression, a monoclonal anti-N antibody (R3-1D8-1-1a) detected N in the cytoplasm (Fig. 2). In cells expressing the N ORF, the mRNA staining pattern was altered, with mRNA aggregating in the cytoplasm at sites of N aggregation (Fig. 2). This was not consistent with the changes in mRNA seen during infection. Therefore while

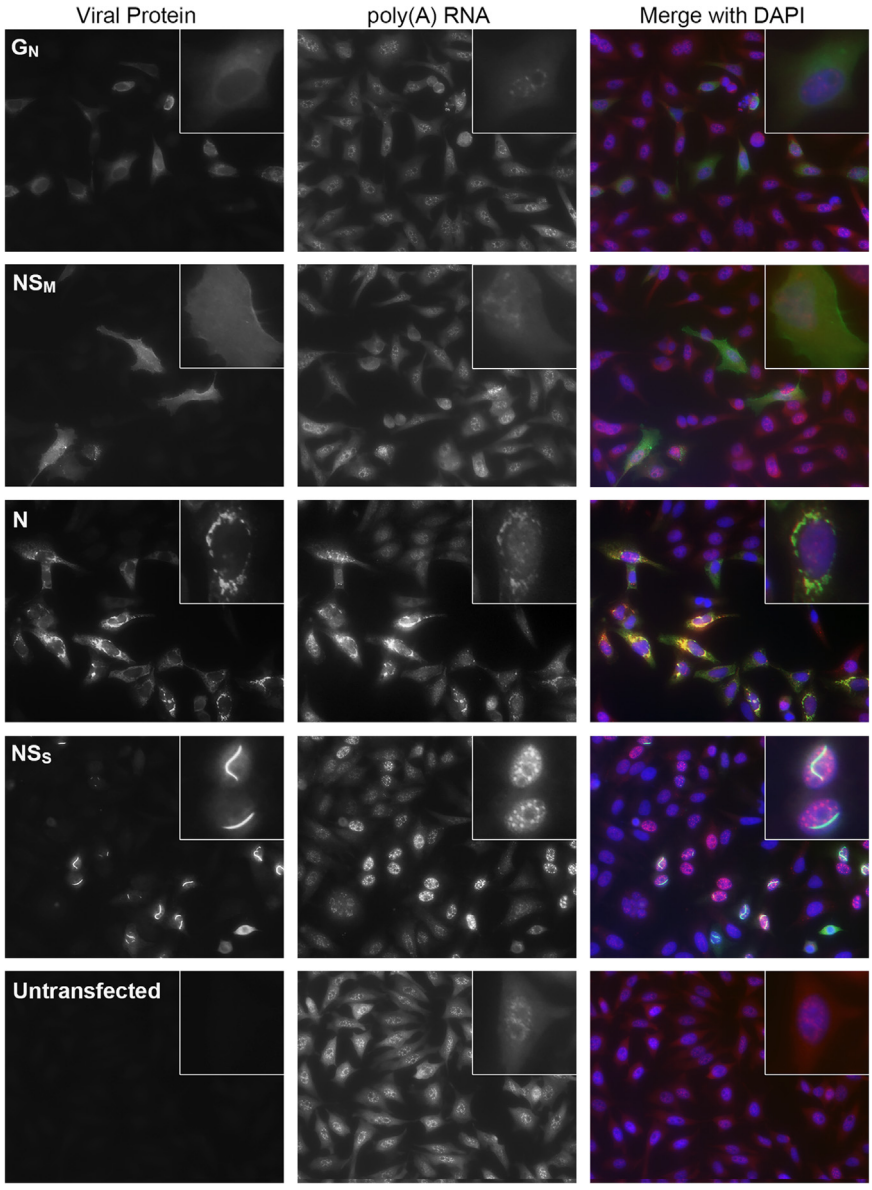

Fig. 2. mRNA localization during overexpression of RVFV genes. Fluorescence micrographs of HeLa cells individually expressing G_N, NS_M, N, and NS_S. Cells were transfected and stained for polyadenylated RNA and the specific viral expression product indicated in the left panels. Insets show an enlarged view (3 ×) of each panel.

expression of N alone appears to be able to alter mRNA localization it does not appear to be responsible for the nuclear accumulation of mRNA seen during infection. We hypothesize that this change is an artifact of overexpression as mRNA-N co-aggregation was not observed during infection (data not shown). Upon expression of the NS_S coding region, an anti-V5 antibody detected filaments of NS_S in the nuclei of transfected cells (Fig. 2). This is consistent with the native distribution of NS_S during infection (Struthers and Swanepoel, 1982; Struthers et al., 1984; Swanepoel and Blackburn, 1977). In cells expressing the NS_S gene, mRNA localization resembled the pattern seen during infection, with weak cytoplasmic staining and bright nuclear aggregates (Fig. 2). The nuclear accumulation of mRNA upon NS_S expression was highly suggestive that NS_S was responsible for the mRNA accumulation.

To confirm the role of NS_S in relocalization of mRNA, HeLa cells were mock-infected, RVFV MP12-infected, or infected with a recombinant RVFV in which the NS_S gene was replaced with a luciferase gene (MP12rLuc) (Ikegami et al., 2006). Upon infection with the MP12rLuc RVFV, no change in mRNA localization was observed (Fig. 3A). To quantify the changes in mRNA signal, Zeiss Zen software was used to measure the mean fluorescence intensity from the mRNA probe in the nucleus and cytoplasm of individual cells (Fig. 3B). No significant difference was seen between MP12rLuc- and Mock-infected samples. Measurements for MP12-infected cells were significantly different from both MP12rLuc-infected and Mock-infected cells. These results indicate that expression of the NS_S gene of RVFV leads to nuclear accumulation of mRNA during RVFV infection.

Our study revealed previously undescribed changes in host mRNA during RVFV infection. mRNA export is crucial for host cells and is targeted by many viruses through a variety of mechanisms (Castello et al., 2009; Faria et al., 2005; Her et al., 1997; Ricour et al., 2009; Satterly et al., 2007; von Kobbe et al., 2000). We were unable to identify the specific mechanism by which NS_S exerts its effects on host cell mRNA relocalization. Studies measuring the effects of NS_S on components of the nuclear export pathway, to include Aly, Nxf1/TAP, Rae1, Nxt1, UAP56, and Gle1 did not yield any measureable changes (data not shown). It is possible that the observed arrest in mRNA export is linked to the arrest in host transcription as transcription, splicing and export are connected

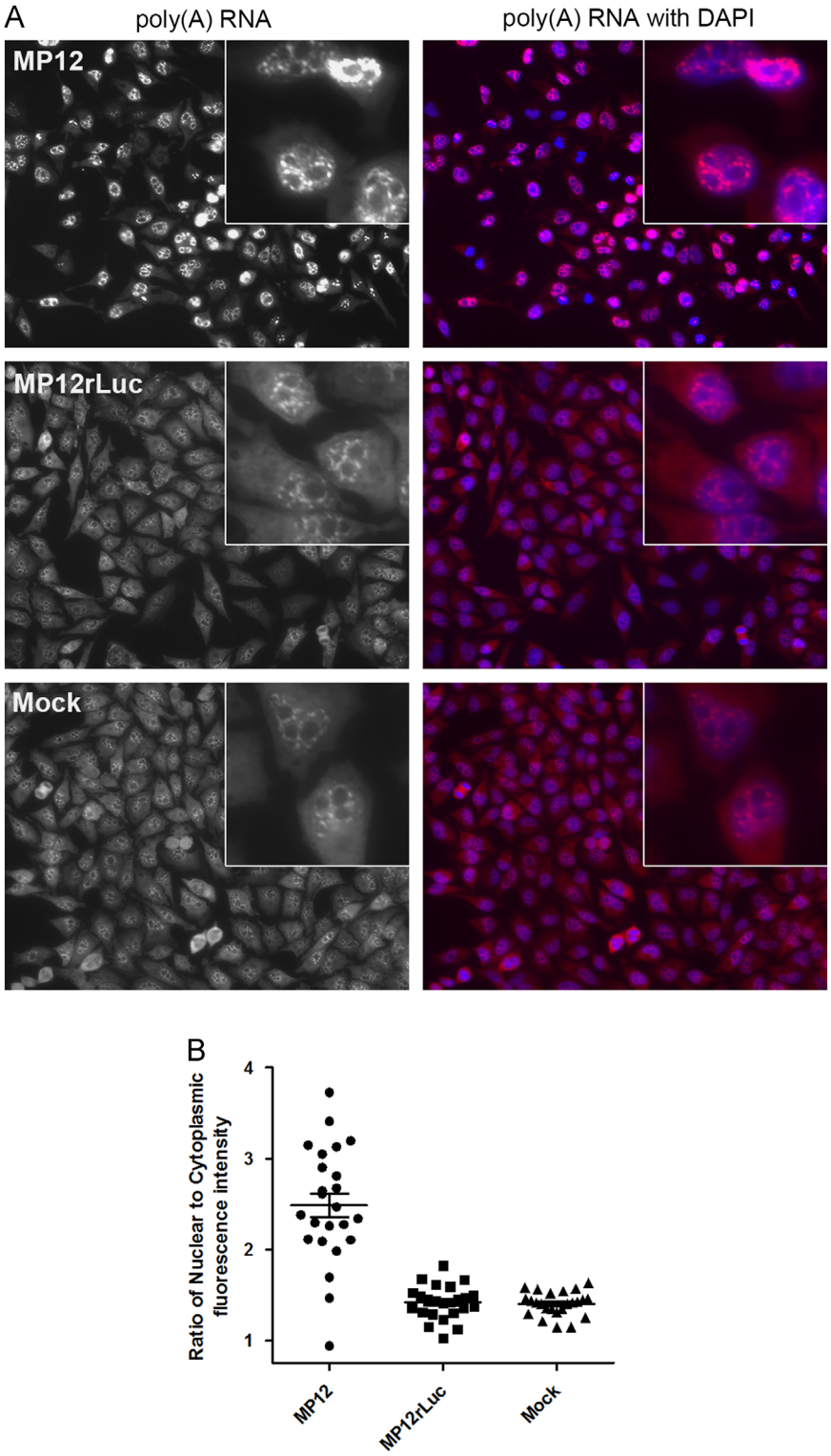

Fig. 3. mRNA localization during RVFV infection. (A) Fluorescence micrographs of cells infected with RVFV MP12, MP12rLuc or mock-infected for 24 h and then fixed and stained for polyadenylated RNA and DNA. Insets show an enlarged view (4 ×) of each panel. (B) Graph of ratio of nuclear to cytoplasmic Cy3 fluorescence intensity. A statistically significant difference was seen between MP12-infected and MP12rLuc-infected cells ($p < 0.0001$) as well as between MP12-infected and mock-infected cells ($p < 0.0001$) as determined by Welch's *t*-test.

(Carmody and Wente, 2009; Reed, 2003; Vinciguerra and Stutz, 2004).

Altered mRNA export provides an explanation for our earlier findings of nuclear accumulation of PABP1 during RVFV infection (Copeland et al., 2013), as previous studies demonstrated that a block in mRNA export can result in nuclear accumulation of PABP1 (Burgess et al., 2011). Further, studies have shown that PABP1 accumulates in the nucleus when cytoplasmic mRNA is depleted due to mRNA antagonizing PABP1's ability to interact with nuclear import factors (Kumar et al., 2011). The observed block in mRNA

export and concurrent reduction in cytoplasmic mRNA identified here would both be expected to induce nuclear accumulation of PABP1, by inhibiting nuclear export of PABP1 and by allowing nuclear import of PABP1 respectively. This study represents the first observation of a defect in mRNA export during infection with a bunyavirus.

Materials and methods

Cells and viruses

All experiments were performed with HeLa cells. Unless otherwise noted, all experiments were performed with the MP12 strain of RVFV. Cells were maintained in modified essential medium (MEM) supplemented with 10% (vol/vol) fetal calf serum (FCS), 75 U/ml penicillin–streptomycin, and 2 mM L-glutamine. Infections were performed by adding virus to cell cultures. Following 1 h of incubation, the inoculum was removed and replaced with fresh MEM. Cells were infected at multiplicities of infection between 2.5 and 5.

Immunofluorescence

RVFV-infected samples and accompanying mock-infected samples were fixed by submersion in 4% (wt/vol) paraformaldehyde (PFA) for 10 min. After fixation, cells were permeabilized by submersion in ice-cold methanol for 5 min. Nonspecific binding sites were blocked for 1 h at room temperature in 5% (vol/vol) goat serum. Primary antibody incubation proceeded for 1 h at room temperature at antibody specific optimized dilutions in 5% (vol/vol) goat serum. Cells were washed three times in 1 × phosphate-buffered saline (PBS). Secondary antibodies (Alexa Fluor-conjuated goat anti-mouse and/or goat anti-rabbit secondary antibodies [Life Technologies]) were added at a dilution of 1:2000 for 1 h at room temperature. Cells were then washed three times in PBS and mounted on slides with mounting medium containing diamidion-2-phenylindole (DAPI) (Prolong Gold, Life Technologies). Slides were allowed to cure overnight at room temperature prior to imaging.

Microscopy and image processing

All fluorescence microscopy was performed with a Zeiss-Axio Observer D1 microscope. Contrast enhancement was performed equally on all areas and panels of Figs. 1 and 2.

Transfection

HeLa cells were grown to 75% confluence in MEM supplemented with 10% (vol/vol) FCS. Plasmid DNA was diluted to 1 μg/50 μl in Opti-MEM. FuGene HD transfection reagent (Promega) was added at a Fugene-to-plasmid ratio of 3 μl to 2 μg. The FuGene-DNA solution was incubated at room temperature for 15 min and then added dropwise to cells. At 24 h posttransfection, cells were fixed for immunofluorescence assay (IFA) as described above.

Antibodies

The following primary antibodies and antibody concentrations were used: RVFV N R3-1D8-1-1a (mouse monoclonal antibody) IFA 1:1000 (J. Smith, United States Army Medical Research Institute of Infectious Diseases [USAMRIID]), PABP1 IFA 1:1000 (ab21060; Abcam), FLAG IFA 1:1000 (F1804; Sigma), V5 IFA 1:200 (P/N 46-0705; Invitrogen), G_N 4D4 (mouse monoclonal antibody) IFA 1:1000, NS_S 3C3 (mouse monoclonal antibody) IFA 1:1000. Alexa Fluor-conjugated secondary antibodies were all used at 1:2000 for IFA (Alexa Fluor 594 goat anti-rabbit, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 488 goat anti-mouse; Life Technologies).

Plasmids

A plasmid encoding the open reading frame of the RVFV ZH-501N protein (pCAGGS RVFV ZH-501N) was provided by Stuart Nichol (Centers for Disease Control and Prevention, Altanta, GA). A V5-tagged NS_S plasmid was provided by Sheli Radoshitzky, Julie Constantino, and Sina Bavari (United States Army Medical Research Institute of Infectious Diseases). A plasmid encoding the RVFV M segment was provided by Robert Doms (University of Pennsylvania). A plasmid encoding a FLAG-tagged partial NS_M was produced by cloning codon optimized ZH-501M segment (aa135-130) into pDEST737 as previously described (Copeland et al., 2013).

Fluorescence in-situ hybridization

Our method was adapted from that of Chakraborty et al. (2006). Cells were washed once in PBS and fixed by submersion in 4% (wt/vol) PFA for 10 min. Cells were washed 3 × with PBS and permeabilized by submersion in 100% ice cold methanol for 5 min at 4 °C. Methanol was aspirated and cells were incubated in 70% (vol/vol) ethanol for 10 min. Ethanol was aspirated and cells were washed twice in PBS. 1 M Tris pH8 was added to cells for 5 min. Cells were then incubated in pre-warmed (37 °C) pre-hybridization buffer (10 μg/ml tRNA, 10% (wt/vol) dextran sulfate, 25% (vol/vol) formamide, 100–200 units/ml RNAsin (Promega N2115), and 2 × SSC (1 × SSC is 150 mM sodium chloride and 15 mM sodium citrate at pH7)) for 15 min at 37 °C. Cells were then incubated overnight at 37 °C in hybridization buffer (pre-hybridization buffer with 5 ng/μl 5′-Cy3-Oligo d(T)30 (Genelink, 26-4330-02) protected from the light. All subsequent steps were performed protected from the light. The following day cells were washed 2 × for 15 min in pre-warmed (37 °C) 2 × SSC. Cells were subsequently washed 2 × for 15 min in pre-warmed (37 °C) 0.5 × SSC. Cells were immediately submerged in 4% PFA for 10 min. Cells were washed 3 × in PBS and either mounted and visualized or stained for host proteins as described above.

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