

**Final SG/PB Protocol**

Grow cells

Stresses: Hypoxia 3 hrs

    Arsenite 500 uM (1:100 dilution) 1 hr

2% PFA/PBS (make fresh, weigh out, put in PBS, 60 degrees 30 min) X 10 min

Aspirate

Ice cold methanol on ice X 10 mins

Rinse with PBS

Block with 5% normal horse serum/PBS x 1 hr

Aspirate

Stain- eIF3 1:1500 in PHEM buffer

    G2BP 1:200 in PHEM

    S6: 1:250 in PHEM

X 2 hr

Aspirate, rinse with PBS

Secondary in 5% NHS X 1 hr

Wash with PBS X 10 mins X 2

Stain with Dapi, coverslip

1  $\mu$ M aresenite or 100 nM Thapsigargin x 1 hr

Aspirate Media

2% paraformaldehyde in PBS for 10 mins RA

Aspirate

Cover with -20 methanol 10 mins

Rinse with PBS x 3 for 5 mins each

Block with PBS/ 5% BSA x 1 hr RT

Incubate with antibody x 1 hr 37 degrees

Wash with PBS x 2 for 5 mins

Incubate with secondary antibody

#### KEDERSHA ANDERSON

Cells were grown, fixed, and stained as described previously (Kedersha et al. 1999  $\ast$ ), with minor modifications as follows. Normal horse serum was used as a blocking agent in lieu of normal goat serum, and secondary antibodies were raised in donkey (ML grade; [Jackson ImmunoResearch Laboratories](#)). Cy3-conjugated antibodies were used instead of Texas red conjugates. In situ hybridization was performed as described previously (Kedersha et al. 1999  $\ast$ ). Cells were viewed using a [Nikon](#) Eclipse 800 microscope, and images were digitally captured using a CCD-SPOT RT digital camera and compiled using Adobe Photoshop<sup>®</sup> software (v5.5).

Cells were briefly rinsed in PBS and incubated for 10 min in 2% paraformaldehyde in PBS. This was removed and the cells were immediately immersed in -20°C methanol for 10 min, rinsed in PBS, and incubated in blocking buffer (5% normal goat serum in PBS) for 1 h before the addition of primary antibodies.

cells were incubated in the diluted antibody for 1–12 h, washed several times in PBS, and incubated for 1 h in diluted isotype-specific secondary antibodies (1/200 for FITC-labeled secondary antibodies; 1/2,000 for Texas red-labeled secondary antibodies) in blocking solution supplemented with Hoechst dye 33258 at 50 ng/ml. Cells were washed three times for 10 min in PBS, mounted in vinyl mounting media (Fukui et al. 1987  $\ast$ ), and viewed through a [Nikon](#) Eclipse 800 microscope equipped with epifluorescence optics and appropriate filters for detection of FITC, Texas red, or Hoechst dye.

#### KIMBALL HARDING

Cells on coverslips were fixed in either 1% or 3% paraformaldehyde, as indicated in the figure legends, for 20 min at room temperature and the coverslips were washed twice with phosphate-buffered saline (PBS). The fixed cells were permeabilized by incubation in 0.2% Triton X-100 in PBS. The coverslips were then incubated for 1 h in 10% bovine serum albumin in PBS containing 0.1% Triton X-100.

#### COUGOT

HEK293 cells were grown in DME Glutamax (Invitrogen) containing 10% FCS on clean glass slides to 70% confluence. The cells were washed with 1x PBS and fixed for 20 min in 4% PFA. After several

washes, cells were permeabilized in 0.1% Triton X-100 for 10 min. Then, cells were saturated for 30 min with 0.2% BSA/1x PBS, and after several washes cells were immunostained for 1 h at 37°C with a rabbit anti-hDcp1a serum (1:1,000) or anti-hTIA-1 mAbs (provided by N. Kedersha, Harvard Medical School, Boston, MA; 1:1,000). After subsequent washes, cells were incubated 1 h at 37°C with secondary antibody, Fluorolink Cy5-labeled goat anti-rabbit IgG (Amersham Biosciences) for anti-hDcp1a serum or goat anti-mouse IgG FITC conjugated (Sigma-Aldrich) for anti-hTIA-1 antibodies (1:1,000). The anti-hDcp1a serum used for immunofluorescence revealed specifically hDcp1a in Western blotting analysis ([van Dijk et al., 2002](#)).

For localization of the GFP fusions, cells were fixed and treated as described in the previous paragraph and nuclei were stained for 30 min in a dark chamber with 5 µg/ml propidium iodide. For the colocalization experiments, HEK293 cells were transiently transfected either with GFP-hCcr4, GFP-hDcp1b, GFP-hLsm1, GFP-hLsm3, or GFP-rck/p54 as described in Cell culture and transfection. Then, cells were stained by immunofluorescence as described in the previous paragraph.

Preparations were analyzed using a confocal microscope (model RCS SP2; Leica) on an inverted stand using objectives HC Plan APO OS 100x oil NA 1.4. Specific excitation of GFP fusions was performed at 488 nm and collection of emitted light at 500–542 nm. Images were prepared using Leica software.

#### GENERAL

- Drain off culture medium and rinse cover slips with PBS.
- Drain off PBS with any of the above mentioned fixation methods.
- Wash in PBS 3-times 5 min.
- Permeabilize with 0.01% Triton X-100 in PBS for 30 sec (if needed).
- Wash in PBS 3-times 5 min
- Incubate in 1% BSA, PBS pH 7.5 for 30 min to block unspecific binding of the antibodies.  
(alternative blocking solutions are: 1 % gelatine, 1 % bovine or horse serum)
- Incubate with primary antibody in 1% BSA, PBS pH 7.5 for 60 min (or over night at r.t. depending on antibody concentration and the accessibility of the antigen).
- Wash with PBS pH 7.5, 3-times 10 min.
- Incubate 2nd antibody in 1% BSA, PBS pH 7.5, 60 min at r.t.; e.g., goat anti-rabbit Texas Red (Accurate), 1:80
- Wash with PBS pH 7.5, 3-times 10 min.