Mitochondria Associate with P-bodies and Modulate MicroRNA-mediated RNA Interference*

Lue Huang†1,2, Stéphanie Mollet†1,3, Sylvie Souquere§, Florence Le Roy§4, Michèle Ernoul Langé§5, Gérard Pierron§, François Dautry§, and Dominique Weil§

From the †UPMC University Paris 06, CNRS-FRE 3402, 9 quai Saint Bernard, 75005 Paris, the ‡LBPA, CNRS, Ecole Normale Supérieure de Cachan, 94230 Cachan, and the §CNRS UMR 8122, Institut Gustave Roussy, 39 Rue Camille Desmoulins, 94800 Villejuif, France

P-bodies are cytoplasmic granules that are linked to mRNA decay, mRNA storage, and RNA interference (RNAi). They are known to interact with stress granules in stressed cells, and with late endosomes. Here, we report that P-bodies also interact with mitochondria, as previously described for P-body-related granules in germ cells. The interaction is dynamic, as a large majority of P-bodies contacts mitochondria at least once within a 3-min interval, and for about 18 s. This association requires an intact microtubule network. The depletion of P-bodies does not seem to affect mitochondria, nor the mitochondrial activity to be required for their contacts with P-bodies. However, inactivation of mitochondria leads to a strong decrease of microRNA-mediated RNAi efficiency, and to a lesser extent of siRNA-mediated RNAi. The defect occurs during the assembly of active RISC and is associated with a specific delocalization of endogenous Ago2 from P-bodies. Our study reveals the possible involvement of RNAi defect in pathologies involving mitochondrial deficiencies.

P-bodies are ribonucleoprotein granules present in the cytoplasm of eukaryotic cells. They contain all proteins involved in the 5′ to 3′ mRNA degradation pathway, such as the decapping enzyme Dcp2, its enhancers Dcp1, Lsm1–7, Edc3, Heddls/Ge1, and the exonuclease Xrn1. This list extends to factors involved in specific degradation pathways, such as RNAi, NMD, and NGD (1, 2). They also contain proteins involved in translational repression, such as eIF4ET, Rck/p54/Dhh1, CPEB1, and the RISC complex. Some of the latter proteins also play a role in mRNA degradation, in particular Rck/p54/Dhh1, which is known as an enhancer of decapping, and the RISC complex when it is guided by a siRNA. Such catalogue of components indicates that P-bodies participate in these two aspects of mRNA metabolism. In addition, P-bodies increase in number and size when free untranslated mRNA accumulates. In mammals, this is observed when degradation is compromised by Xrn1 silencing (3) or when polysomes are disrupted with puromycin or arsenite (4). Taken together, these data support a role of P-bodies in mRNA degradation, mRNA storage, and RNA interference. Yet, their exact participation is unclear, as none of these functions is markedly affected in cells where P-bodies have been depleted (5–9).

Live cell observations show that the number of P-bodies is quite stable over hours, although occasional formation of new P-bodies or fusions of pre-existing ones are observed (10). Nevertheless, P-bodies are dynamic structures within the cytoplasm. Most of the time, they exhibit chaotic movements within a spatially confined area of the cytoplasm. Occasionally, they also perform sudden directional movements over a few micrometers, which, in human U2OS cells, take place along microtubule tracks, at a velocity of 0.5 to 1 μm/s (11). In addition, many P-body components actively traffic in and out of P-bodies. Photobleaching and photoactivation experiments show a continuous shuttling of Dcp1a, Dcp1b, Lsm6, eIF4E, eIF4ET, and TTP proteins between P-bodies and cytosol. This is not observed for other components such as Dcp2, GW182 (TNRC6A), and Ago2 (11–14). Moreover, P-bodies not only uptake mRNA for degradation or storage, but also release stored mRNA when translation resumes, as shown in yeast and mammalian cells (15, 16). Therefore, despite their apparent stability, P-bodies are dynamic granules that actively exchange molecules with their environment.

P-bodies have been shown to interact with other cytoplasmic organelles. In stressed cells, we and others have shown that P-bodies establish contacts with another type of ribonucleoprotein granule, called stress granules (4, 13). These granules form following stresses that strongly repress translation. They contain a fraction of the arrested mRNAs, associated with translation initiation factors, the small ribosomal subunit, and various RNA-binding proteins (17, 18). It was proposed that the contacts between stress granules and P-bodies serve the transfer of mRNPs from stress granules to P-bodies for triggering their degradation (4, 13). In fact, these contacts are close enough for such a transfer, as observed in electron microscopy (18). However, the pool of mRNA transiting through stress granules is not particularly unstable (19). Therefore the nature of the exchange between the two types of granules, and its significance, are still unknown.

In addition, interactions between P-bodies and late endosomes or multivesicular bodies have been reported in Drosophila.
Mitochondria Regulate P-body Functions

ila and mammalian cells (20, 21). Mutations blocking multivesicular body formation or multivesicular body turnover inhibit or stimulate silencing, respectively. As GW182 is highly enriched at the membrane of these vesicles, the authors proposed that miRISC loading or recycling at these membranes could be required for efficient RNAi. Finally, it was recently reported that specific miRNAs were enriched along with the Ago2 protein in mitochondria purified from rat and mouse liver (22, 23). Most of the potential targets of these miRNAs were encoding non-mitochondrial proteins, raising the possibility that the mitochondria are a reservoir for specific miRNAs involved in the regulation of general cellular functions. In fact, whereas the assembly of active RISC complexes has been followed in vitro, enabling the identification of a RISC loading complex (24), the details of the in vivo process are poorly known. Fluorescence correlation spectroscopy has indicated that RISC assembly takes place in the cytoplasm within a few minutes after the microinjection of si- or miRNA, but could not allow a precise localization (25).

Here, we report that P-bodies establish frequent and prolonged contacts with mitochondria. Disrupting P-bodies does not seem to affect mitochondrion morphology and function. However, disturbing mitochondrial activity strongly represses a P-body-associated function, silencing by small RNAs. Our data indicate that the defect occurs during RISC assembly and correlates with a decreased accumulation of Ago2 in P-bodies.

EXPERIMENTAL PROCEDURES

Cell Culture—Epithelioid carcinoma HeLa cells, human embryonic kidney 293, and HEK 293 Tet-On Advanced cells (Clontech) were maintained in DMEM supplemented with 10% fetal calf serum, and human epithelial retina RPE-1 cells in DMEM/F-12 with 10% fetal calf serum. Human umbilical vein endothelial cells (a kind gift of Georges Uzan, IAL, Villejuif, France) were maintained in Endothelial Growth Media-2 (Lonza France) supplemented with 5% fetal calf serum, 0.4% hFGF-B, 0.1% VEGF, 0.4% insulin-like growth factor, 0.1% R3-insulin-like growth factor-1, and 0.1% hEGF (Lonza France) (26), and analyzed at passage 8. Mitochondrial staining was achieved by culture in the presence of 20 μM CMX Ros MitoTracker (Molecular Probes) for 30 min at 37 °C.

To measure the mitochondrial transmembrane potential, trypsinized cells were incubated with 40 nM 3,3’-dihexyloxacarbocyanine iodide for 15 min at 37 °C and analyzed by flow cytometry. Vinblastine (Sigma) was used at 10 μM for a total of 90 min, arsenite (Sigma) at 0.5 mM for 30 min, and CCCP (Sigma) at 20 μM for the indicated period of time. Cellular ATP was measured in duplicates using the ATP somatic cell assay kit (Sigma) and normalized by the number of cells.

Transfection—For microscopy studies, transient transfections were performed with 3 μg of plasmid DNA/60-mm diameter dish using a standard calcium phosphate procedure. For silencing studies, transfections were performed in 12-well plates, with 0.5 μg of plasmid and the indicated concentration of siRNA/well using Lipofectamine 2000 (Invitrogen). Induction of the reporter construct was achieved by adding doxycycline at 1 μg/ml. Cells were analyzed by flow cytometry 24 h after the addition of CCCP, unless otherwise indicated (Fig. 7A). Luciferase assays were performed in 24-well plates transfected with 25 ng of Renilla and firefly expression vectors, using the dual luciferase assay (Promega).

Plasmids and siRNAs—RFP-p54 contains the full open reading frame of human Rck/p54 (4). MtGFP was a kind gift of Rosario Rizzuto (University of Ferrara, Italy) (27). The bidirectional reporter construct pBiFluo was created by inserting the EGFP and the DsRed express cDNAs (Clontech) on both sides of the Tet-regulated bidirectional promoter (Clontech), with a linker allowing for the introduction of regulatory sequences in the 3’ UTR of EGFP. pBiFluo-silet7, pBiFluo-3milet7, and pBiFluo-2CXCR4 were obtained by inserting the corresponding binding sites for let-7 and CXCR4 (28), respectively. Renilla silet7 and 3milet7 constructs were kindly provided by W. Filipowicz (29).

Rck/p54, CPEB1, and Globin siRNAs were previously described (si-p54, si-CPEB1.2, and si-Glo.1 in Ref. 7). Let-7b (5’-UGA GGU AGG UGG UGU GGdT dT-3’) and CXCR4 (5’-UGA CUG GAG UGA AAA CdTdT-3’) siRNAs were purchased from MWG, and pre-miRlet-7b (5’-CGG GGU GAG GUA GGU UGU GUG GUU UCA GGG CAG UGA UGU UGC CCC UCG GAA GAU AAC UAU ACA ACC UAC UGC CUU CCC UG-3’) from Ambion.

Immunofluorescence—Cells grown on glass coverslips were fixed in 4% paraformaldehyde for 10 min, and permeabilized in acetone at −20 °C for 10 min. After rehydration, cells were incubated with the primary antibody for 1 h, rinsed with PBS, incubated with the secondary antibody for 30 min, and rinsed with PBS, all steps were performed at room temperature. Slides were mounted in Citifluor (Citifluor, UK).

Rabbit polyclonal anti-p54 and mouse monoclonal anti-Ge1 antibodies were purchased from Bethyl Laboratories Inc. and Santa Cruz, respectively. Mouse monoclonal anti-Ago2 was a kind gift of Gunter Meister (MPIB, Germany) (30). Secondary antibodies conjugated to TRITC and Cy2 were purchased from Jackson ImmunoResearch Laboratories.

Microscopy—Standard microscopy was performed on a Leica DMR microscope (Leica, Heidelberg, Germany) using a ×63 1.32 oil immersion objective. Photographs were taken using a Micromax CCD camera (Princeton Instruments) driven by Metamorph software. Confocal images were obtained on a Leica TCS-NT/SP1 inverted confocal laser-scanning microscope (Leica, Heidelberg, Germany) using an Apochromat ×63 1.32 oil immersion objective. Fluorescence signals were acquired in 0.16-μm optical sections using Leica software. All images were processed using Adobe Photoshop software. For signal quantification, fluorescence was measured simultaneously for Ago2 and Rck/p54, or Ge1 and Rck/p54, along lines drawn across individual P-bodies using Metamorph software. Their enrichment in the P-body was measured as the difference between the maximal fluorescence in the P-body and the fluorescence in the surrounding cytoplasm.

For video microscopy, cells were grown on glass coverslips and mounted in a POC chamber system with 2 ml of culture medium maintained at 37 °C and 5% CO2. Cells were observed...
on a Zeiss inverted microscope Axiovert (Carl Zeiss SAS, France) equipped with a DG4 Lambda switcher (Sutter Instrument) and a CoolSNAP HQ camera (Roper Scientific), driven by Metamorph software. Timed series were acquired using a ×63 1.32 oil immersion objective.

For electron microscopy, Epon and Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, Germany) embedding and immunolocalization of proteins were performed as described previously (18). Ultrathin sections were analyzed with a FEI Tecnai Spirit. Digital images were taken with a SIS Megaview III CCD camera.

**Flow Cytometry**—Transfected HEK 293 Tet-On Advanced cells were washed once with PBS and collected in 400 µl/well of PBS supplemented with 15% enzyme-free Cell Dissociation Buffer (Invitrogen). Cytometry was performed with a FACSCalibur flow cytometer (BD Biosciences) using FL1 and FL2 to measure EGFP and DsRed fluorescence, respectively. For each sample, 40,000 to 100,000 cells were analyzed. Data were analyzed with the Weasel software (version 2, Walter and Eliza Hall Institute). Silencing values were defined by the change in the mean EGFP fluorescence with respect to the mock-transfected cells.

For analysis of DNA fragmentation, cells were trypsinized and resuspended in ice-cold PBS containing 75% ethanol. After one night at −20 °C, cells were resuspended in PBS containing 50 µg/ml of propidium iodide (Sigma) and 20 µg/ml of RNase A, and incubated at 37 °C for 30 min. The samples were then analyzed after gating on the R0 region to eliminate the cell aggregates but not cell debris, as indicated on the figure.

**Western Blotting**—Cells were scraped in Laemmlı lysis buffer. After quantification by the Coomassie Blue protein assay (Pierce), 75 µg of proteins were separated on a 8.5% polyacrylamide SDS-PAGE gel and transferred to a PVDF membrane (PerkinElmer Life Sciences). After blocking in PBS-T (PBS, 0.1% Tween 20) containing 5% (w/v) nonfat dry milk for 1 h at room temperature, the membrane was incubated with the primary antibody for 1 h at 37 °C, rinsed in PBS-T, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing in PBS-T, immune complexes were detected using the SuperSignal West Pico Chemiluminescent Signal kit (Pierce) and visualized by exposure to CL-XPosure film (Pierce). The membrane was then dehybridized and rehybridized to a mouse monoclonal anti-S6K antibody (Santa Cruz). The secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

**RESULTS**

**Association of P-bodies with Mitochondria**—We have previously studied the dynamics of P-bodies in live cells using a specific component, the DEAD box helicase Rck/p54, fused to RFP (19). In the course of this study, we noted that P-bodies were often located next to filamentous structures, which were reminiscent of mitochondria. For confirmation, RPE-1 epithelial cells were cotransfected with RFP-p54 and mtGFP, a GFP targeted to mitochondria (27), and observed live in phase-contrast and fluorescence microscopy after 36 h (Fig. 1A, left panel). The two regions enlarged in the right panels show the close proximity of P-bodies with mitochondria, which were filamentous in phase-contrast and contained the mtGFP. This observation was further extended to non-transfected HeLa cells. Mitochondria were labeled *in vivo* by culture in the presence of fluorescent MitoTracker for 30 min at 37 °C. After fixation, P-bodies were detected by immunostaining of Rck/p54. The association between P-bodies and mitochondria was visible in wide-field (Fig. 1B) and confocal microscopy (Fig. 1C). It was also observed in HEK 293 epithelial cells (data not shown), as well as in primary cultures of endothelial cells such as human umbilical vein endothelial cells (Fig. 1D). When P-bodies were classified as “associated” or not with mitochondria, as defined in the right panels of Fig. 1, B and C, we found 50 to 70% of the P-bodies associated with mitochondria, depending on the experiment and cell line.

To estimate the significance of this observation, we quantitatively analyzed experiments performed in HeLa cells. We measured in each cell the fraction of the P-bodies associated with mitochondria, and the fraction of the cytoplasm occupied by mitochondria. The results of such an analysis (25 cells, 373 P-bodies) are plotted in Fig. 1E. Despite important variations from cell to cell, the mean percentage of P-body associated with mitochondria (66%) was significantly higher than the mean area occupied by the mitochondria (58%, p = 0.006 in a paired t test). As a second assay, we analyzed the localization of these P-bodies after moving them 1 µm in the x and y axis (Fig. 1E). Their association with mitochondria decreased and became similar to the fraction of the cytoplasm occupied by the mitochondria (60%, p = 0.40). These two analyses establish that the frequent association between P-bodies and mitochondria does not simply result from a random localization of the P-bodies in the cytoplasm, but reflects a preferential association with mitochondria.

**Direct Contacts between P-bodies and Mitochondria**—To determine how close the association between P-bodies and mitochondria, and whether the association is direct or mediated by a third organelle, we analyzed HeLa cells by electron microscopy. Because P-bodies are most recognizable after immunogold detection of Rck/p54 (18), cells fixed in paraformaldehyde were embedded in Lowicryl K4M, and thin sections were incubated with an anti-p54 antibody coupled to gold particles (Fig. 2A). Under these conditions, immunostaining is optimal, but membranes are not stained, and mitochondria appear as large oval electron dense structures.

Close contacts between P-bodies and mitochondria were readily observed at this high resolution. We also performed analysis in conventional electron microscopy. In the absence of labeling, low density of the P-bodies and their small number make it difficult to find them in thin sections. However, this can be circumvented by treatment of the cells with arsenite, which increases the size, density, and number of P-bodies (18). Arsenite-treated HeLa cells were fixed in glutaraldehyde, embedded in Epon, prior to observation of ultrathin sections. Tight contacts between P-bodies and mitochondria were also observed (Fig. 2B). The distance between the two organelles in Fig. 2B is less than 25 nm, which, for comparison, is the size of a ribosome. With both protocols, no close association was observed between P-bod-
Mitochondria Regulate P-body Functions

The first cell contains 11 P-bodies, 7 of them being associated with a mitochondrion at time 0 (Fig. 3A). Three of these P-bodies (PB1, PB2, and PB3) were enlarged on the right panel to illustrate their dynamics. PB1 separates from a mitochondrion at the beginning of the time-lapse, stays apart for 1 min, contacts it again, and separates after 1 min (supplemental Video S1). PB2 is never free, associating back and forth with two different mitochondria (supplemental Video S2). Its localization at the extremity of a mitochondrion is a frequently observed
pattern. PB3 remains attached to one mitochondrion, and slides along it over 90 s (supplemental Video S3). This is rare behavior. The second cell contains 12 P-bodies, 10 on them being distant of mitochondria at time 0 (Fig. 3B). Three of these were enlarged. Although PB4 does not move much, during 35 s, a distant mitochondrion extends twice in its direction up to establishing contact, slides along it, and retracts again (supplemental Video S4). PB5 is immobile, whereas a neighboring mitochondrion associates transiently with it during 30 s (supplemental Video S5). Finally, PB6 is joined by a short mitochondrion that then remains attached to it (supplemental Video S6). Such short mitochondria were often observed associated with P-bodies at the periphery of the cytoplasm. Overall, even when P-bodies and mitochondria are far apart at one time, they frequently establish contacts within a few minutes. These contacts result more often from movements of mitochondria than of P-bodies.

Dynamics of the Association between P-bodies and Mitochondria—We then analyzed the dynamics of the association between P-bodies and mitochondria. First, individual P-bodies (106 P-bodies in 10 cells) were followed frame by frame in the previous experiment. Although only 44% of the P-bodies were associated with mitochondria at time 0, 81% of the P-bodies contacted a mitochondrion at least once during the 3-min movie. The duration of each contact (a total of 200 contact events for 106 P-bodies) was estimated taking into account that frames were 6 s apart (Fig. 4A). The association of P-bodies with mitochondria lasted from 6 s or less (1 frame) to 3 min or more (30 consecutive frames), with a median duration of 18 s (3 consecutive frames). The significance of contacts observed on a single frame should not be underestimated, as they are frequent (30%). Instantaneous contacts occurring only during image acquisition (100 ms) would have a very low probability to be captured and can only represent a minor fraction of them. Overall, 54.5% of the contacts lasted more than 12 s, 26.5% more than 1 min, and 8.5% more than 3 min. In conclusion, the association with mitochondria concerns a large majority of the P-bodies and is long enough to enable molecular traffic between the two organelles.

As both mitochondria and P-bodies can be actively transported along microtubules (11, 31), we investigated if the microtubule network is required for the association between P-bodies and mitochondria. HeLa cells were treated with vinblastine for 1 h, which resulted in the full disruption of the microtubule network and the appearance of characteristic spiral aggregates of microtubules, as controlled by immunostaining of tubulin (Fig. 4B, lower panel). Mitochondria were then stained with fluorescent MitoTracker for 30 min in the presence of vinblastine, fixed, and immunostained with anti-p54 antibodies (Fig. 4B, upper panel). Vinblastine increased the number of P-bodies (from 13 to 20 P-bodies per cell in average), as previously reported (11). It also decreased the frequency of P-bodies associated with mitochondria from 57 to 46% (mean value of three experiments, p = 0.045, Fig. 4C). The significance of this decrease was evaluated by measuring in one experiment (31 vinblastine-treated cells, 661 P-bodies) the volume occupied by the mitochondria, the control cells being the one presented in Fig. 1E (see Fig. 4D). After vinblastine treatment, the mean percentage of P-bodies associated with mitochondria (58%) was similar to the mean area occupied by the mitochondria (60%), which indicated the loss of their preferential association with mitochondria. Therefore, the association between P-bodies and mitochondria in HeLa cells requires an intact microtubule network.

Association of P-bodies to Mitochondria Is Independent of Translation and Mitochondrial Activity—We then sought to investigate the role of P-bodies in mitochondrial functions. Some of the mitochondrial proteins are encoded by the nuclear genome, their mRNAs being translated at the surface of the mitochondria. As P-bodies could participate to the control of these mRNAs, we first investigated the effect of disrupting polysomes. HeLa cells were treated with puromycin or arsenite for 30 min, and fluorescent MitoTracker was then added to label mitochondria for 30 min in the presence of the drug. After fixation, P-bodies were immunostained with anti-p54 antibodies. The percentage of P-bodies associated with mitochondria was not significantly different from controls (Fig. 5A), indicating that the association of P-bodies with mitochondria is not dependent on active translation. As a more general assay, we also investigated if mitochondria were disturbed in the absence of P-bodies. HeLa cells were mock-transfected, or transfected with Rck/p54 and CPEB1 siRNAs, which suppress P-bodies, or with a control Globin siRNA, which does not (7). After 48 h, mitochondria were labeled with fluorescent MitoTracker to observe their morphology by microscopy (supplemental Fig. S1A). In parallel, the mitochondrial transmembrane potential was assessed in flow cytometry using the fluorescent cat-

Mitochondria Regulate P-body Functions
ionic dye 3,3'-dihexyloxacarbocyanine iodide (supplemental Fig. S1B). None of these assays showed differences related to the presence of P-bodies.

Conversely, we searched for a role of mitochondria in the assembly of P-bodies, using a mitochondrial poison. RPE-1 cells were transfected with mtGFP to detect mitochondria in live cells and observed in microscopy. The mitochondrial uncoupler CCCP was added during time-lapse acquisition and maintained for up to 19 h. CCCP had a drastic effect on the morphology of the mitochondria, which became swollen and shorter (Fig. 5B, left panel). It is of note that cell death was negligible even after a long CCCP treatment, as attested by the absence of a significant DNA fragmentation (supplemental Fig. S1C). The cellular ATP remained high for the first 8 h, probably due to the energetic metabolism switching from oxidative phosphorylation to glycolysis (Fig. 5C). However, P-bodies were not affected, neither in terms of number nor size. This absence of effect was also observed in untransfected HEK 293 cells treated with CCCP for 8 and 16 h (Fig. 5D). Moreover, the frequency of P-body contacts with mitochondria was unchanged (Fig. 5B, right panel). Therefore, mitochondrial activity was not required for the maintenance of P-bodies. The next step was to assay the potential role of mitochondria in P-body activity.

Mitochondrial Activity and RNAi—To assess the impact of mitochondrial activity on RNAi we used a fluorescent reporter assay that enables measurement of silencing in individual cells by flow cytometry. Briefly, a bidirectional promoter under control of the tetracycline operator was used to drive the expression of DsRed and EGFP (Fig. 6A). Inserting si- or miRNA recognition sites in the EGFP 3' UTR rendered its expression responsive to silencing by the cognate small RNA, whereas that of DsRed was used to measure the activity of the promoter in

FIGURE 3. Relative movements of P-bodies and mitochondria in live cells. RPE-1 cells transfected with p54-RFP and mtGFP were observed in fluorescence microscopy during 3 min. The two cells chosen for illustration have half of their P-bodies (A) and no P-bodies (B) associated with mitochondria at time 0 (left panels). Bars = 10 μm. The P-bodies indicated by arrows were enlarged on the right panels at the indicated time. PB1 illustrates the repeated contact of one P-body on one mitochondrion, PB2, a P-body skipping between two mitochondria, and PB3, a P-body sliding along a mitochondrion. The corresponding movies are supplemental Videos S1–S3. PB4 illustrates a mitochondrion extending to a distant P-body before retracting, PB5 is the repeated association of a P-body with a mitochondrion, and PB6 is a short mitochondrion joining a P-body. The corresponding movies are supplemental Videos S4–S6.
Mitochondria Regulate P-body Functions

FIGURE 4. Dynamics of the contacts between P-bodies and mitochondria. A, a summary of the contacts. The histogram represents the distribution of contact durations in the experiment described in the legend to Fig. 3, as estimated from the number of successive frames showing a given contact. Long (1.1–2.0 min) and very long (2.1–2.9 min) contacts were pooled. B, disruption of the microtubules with drugs. HeLa cells were cultured in the presence of vinblastine for 1 h 30 min, labeled with MitoTracker (red) during the last 30 min and immunostained with anti-p54 antibodies (green) (upper panel), or immunostained with anti-tubulin antibodies (lower panel). Bars = 10 μm. C, the percentage of P-bodies associated with mitochondria was measured in control (C) and vinblastine-treated cells (Vb) (mean ± S.D. of three experiments, 18 to 30 cells, 225 to 611 P-bodies). D, vinblastine-treated cells were individually analyzed for the percentage of P-bodies associated with mitochondria (PB) and the fraction of the cytoplasm occupied by the mitochondria (Mt). The box-plot represents 31 cells containing 661 P-bodies.

the same cell. When the reporter construct was co-transfected with small RNA into HEK 293 Tet-on cells, a sensitive and robust measurement of silencing was achieved when expression of the construct was induced by doxycycline for at least 16 h. Noteworthily, the measured silencing then represents the summation of the silencing that occurred since the addition of doxycycline.

We first assayed the silencing induced by let-7b on a perfectly matched target using the pBiFluo-silet7 construct as a reporter (Fig. 6B). Co-transfection of pBiFluo-silet7 with increasing doses of let-7b induced a decrease in EGFP expression with a plateau at 85% of silencing reached with 1 nm let-7b. When CCCP was added 3 h prior to transfection, the plateau was reached at the same let-7b concentration, but the silencing leveled off at 70%. Expression of the parental construct pBiFluo or pBiFluo-let7m with a mutated let7 binding site was unaffected by co-transfection of let-7b, whether or not in the presence of CCCP (supplemental Fig. S2A, and not shown). Thus, the efficacy of silencing by let-7b was reduced in the presence of CCCP.

The silencing by miRNA was studied using two well characterized model targets, the 3milet7 (29) and 2CXCR4 (28) motifs (Fig. 6A). Importantly, HEK 293 cells express a low level of endogenous let-7 miRNA but no detectable endogenous CXCR4 activity. In controls, the dose responses to their cognate RNA were similar for both targets, the silencing reaching a plateau at 50 to 60% (Fig. 6, C and D). For both constructs, the silencing was markedly reduced in the presence of CCCP and, again, this reduction could not be circumvented by increasing the dose of silencing RNA (Fig. 6, C and D). In these experiments, perfectly matched RNA duplexes were transfected to provide the miRNA activity. Importantly, the same effect of CCCP was observed when a pre-milet-7b RNA (the stem loop precursor of let-7b) was transfected to silence pBiFluo-silet7 or pBiFluo-3milet7 (supplemental Fig. S2B).

Although the impact of CCCP on silencing was visible from the mean EGFP fluorescence, the presence of DsRed within the reporter constructs allowed for a more detailed analysis. The bidirectional promoter drove expression of similar levels of EGFP and DsRed in individual cells, with expression levels spreading over 3 orders of magnitude (supplemental Fig. S2, C and E). CCCP reduced the expression of the reporter construct, but did not alter this correlation. We therefore gated the analysis on cells expressing a given level of DsRed (Region R1 in supplemental Fig. S2, C and E). In high expressers, where silencing is best measured, the efficiency of silencing by a miRNA mechanism was almost completely abrogated by CCCP (Fig. 6E). Indeed, whereas the average silencing was of the order of 50%, analysis at the level of individual cells revealed that miRNA could repress their target gene by more than 90%, in some of the cells (Fig. 6E). In the presence of CCCP, this maximal silencing was reduced more than 5-fold. The effect was not as dramatic when silencing was induced by a siRNA mechanism (supplemental Fig. S2D). In conclusion, CCCP reduced the silencing by siRNA and miRNA, and this effect was particularly drastic on miRNA silencing.

Mitochondrial Activity Is Required for RISC Assembly—To investigate whether the blockage of mitochondrial activity was acting at a specific step in silencing, we varied the timing of CCCP addition from 3 h before to 8 h after transfection of

8 L. Huang and F. Dautry, manuscript in preparation.
let-7b. pBiFluo-3milet7 expression was induced at 8 h so that in all cases it took place after the addition of CCCP, and cells were analyzed 24 h later (Fig. 7A). Addition of CCCP before or at the time of transfection led to the same reduction of silencing as previously. By contrast, a progressive increase in silencing efficiency was observed when CCCP was added after the transfection, so that, by 8 h, it was almost the same as in the absence of CCCP. At this time, the silencing activity of the transfected RNA was almost maximum (data not shown), indicating that the small RNAs were fully incorporated into active RISC complexes. Therefore, the sensitivity of silencing to CCCP disappeared in relation to the formation of active RISC complexes. This was confirmed in dose-response studies where CCCP had no effect when added at 16 h post-transfection (supplemental Fig. S2F for 3milet7, data not shown for silet7). Thus, once they were formed, RISC complexes remained fully active despite the progressive decrease of ATP occurring during the 24-h CCCP treatment (Fig. 5C). This also suggested that, when added early, CCCP was acting rapidly. To be able to study silencing after shorter CCCP treatments, we turned to a luciferase reporter to measure the silencing at 6 h post-transfection, at a time when ATP was only reduced by 40 to 50% (Fig. 5C). For the 3milet7 and silet7 targets (26) we observed the same reduction of the silencing efficiency of 2 nM let-7b as with the fluorescent reporters at later times ($p < 0.01$, supplemental Fig. S2G). Taken together these results establish that CCCP is acting rapidly on the assembly of active RISC complexes and has no impact on their activity once they are formed.

We then investigated whether localization of RISC components was affected by CCCP treatment. We studied the Ago2 protein, which is involved in both si- and miRNA-associated RISC (32), and for which specific monoclonal antibodies have been raised (30). HEK 293 cells were treated or not with CCCP for 8 and 16 h and immunostained with anti-p54 antibodies. The box plot represents the number of P-bodies per cell (three experiments, 105 to 143 cells).
Mitochondria Regulate P-body Functions

A flowchart is shown, illustrating the effects of mitochondrial activity on P-body functions.

**A** 
DsRed EGFP let-7b pBiFluo-silet7 let-7b let-7b pBiFluo-3milet7 CXCR4 CXCR4 pBiFluo-2CXCR4

**B** 
Residual Expression [%]

**C** 
Residual Expression [%]

**D** 
Residual Expression [%]

**E** 
Cell Counts

---

Fraction of Ago2 was enriched in the P-bodies, whereas the majority of the protein was localized diffusely in the cytoplasm (Fig. 7B, upper panel), as previously reported (14). CCCP treatment reduced the accumulation of Ago2 in the P-bodies, whereas Rck/p54 remained unchanged (Fig. 7B, lower panel). This effect was quantified by measuring Ago2 and Rck/p54 fluorescence in individual P-bodies (see “Experimental Procedures”). P-bodies were classified into 6 classes depending on their content in Ago2 or Rck/p54 (Fig. 7C). In untreated cells, Ago2 was enriched to various extents in 94% of the P-bodies (classes 0–5). After CCCP treatment, Ago2 enrichment was strongly reduced, with 70% of the P-bodies containing almost no Ago2 (class 0), whereas Rck/p54 distribution was similar to the control. The loss of Ago2 in P-bodies was specific, as it was observed neither for Rck/p54 nor Ge1 (Fig. 7D, left panel). It was also observed after 8 h in CCCP, although to a lesser extent (Fig. 7D, right panel). Importantly, a Western blot analysis of Ago2 indicated that the protein was expressed at a similar level after up to a 24-h CCCP treatment (Fig. 7E). Altogether, these results indicate that CCCP inhibits RNAi, by acting at an early step of RISC assembly, and causes the delocalization of the Ago2 protein out of the P-bodies.

**DISCUSSION**

We have observed a preferential association of P-bodies with mitochondria in cell lines of various origins. The contacts between the two organelles are close, as judged by electron microscopy images. They are dynamic, a large majority of them lasting less than 3 min, with a median duration of contact of 18 s. They result more often from mitochondrial than from P-body movements in the cytoplasm. Interestingly, both mitochondria and P-bodies can traffic along the microtubule network (11, 31). Long-range movements (>2 μm) of P-bodies along microtubules are only occasional in RPE-1 cells and have a speed of about 1 μm/s (data not shown), as described in other cell lines (11). In contrast, movements of P-bodies leading to contacts with mitochondria are rather short ranged, disordered, and slow. For instance, PB1 and PB3 move at a maximal speed of 0.08 and 0.06 μm/s, respectively (Fig. 3A). Nevertheless, the complete disruption of the microtubule network with vinblastine suppressed the preferential association of P-bodies with mitochondria, indicating that microtubules are required for the encountering of the two organelles in the cytoplasm. Whether they are then required for maintaining the interaction itself remains to be determined.

Interestingly, there are arguments supporting a similar relationship between P-bodies and mitochondria in budding yeast. The Puf3 protein, which is a member of the Pumilio family, binds preferentially the 3’ UTR of mRNAs of nuclear-encoded mitochondrial proteins (33). It contributes to their localization at the periphery of the mitochondria and to their deadenylation and degradation (34, 35). Puf3 has been reported to accumulate in mitochondria-associated foci (36) and P-bodies (37), strongly suggesting that P-bodies are also associated with mitochondria in yeast.

A number of mitochondrial proteins are encoded by mRNAs transcribed from the nuclear genome. Some of these mRNAs bind to the mitochondrial outer membrane for translation, so that P-bodies could associate with mitochondria to regulate them. Disturbing this regulation would be expected to affect the mitochondrial morphology and function, which are very sensitive to mitochondrial protein expression. We have been unsuccessful at showing that the absence of P-bodies is deleterious for mitochondria, suggesting that P-bodies do not play such a role. However, the argument is not definitive, as the absence of P-bodies similarly does not cause any major deregulation of mRNA degradation and storage in yeast, or the RNAi pathway (5–9). It was speculated that P-body function can still be fulfilled by P-body complexes when they are dispersed in microaggregates.

Alternatively, contacts between P-bodies and mitochondria could be required for functions fulfilled by the P-bodies. Mitochondria are involved in various cellular processes, including
ATP production, Ca\textsuperscript{2+} homeostasis, reactive oxygen species signaling, and apoptosis (38). These functions are associated with specific intracellular localizations, or particular relationships with other organelles. For instance, mitochondria establish close contacts with the endoplasmic reticulum, which enable the generation of high local Ca\textsuperscript{2+} concentrations upon Ca\textsuperscript{2+} release from both organelles (39), as well as oscillations of this concentration by a mechanism involving Ca\textsuperscript{2+} shuttling between the two organelles (40). Mitochondria also accumulate at sites of high energy demand. Localization close to the plasma membrane is thought to be important for ATP-driven ion pumps, whereas mitochondria surrounding the nucleus could provide the energy for nuclear import (38). A number of P-body components are dependent on ATP for their activity: helicases of the SF1 family, like Upf1, and of the DEAD-box family, like Ded1 (41) and Rck/p54. Notably, Rck/p54 is particularly abundant in P-bodies and is required for their assembly in mammalian cells (18, 42). One can speculate that continuous mRNP remodeling by this helicase within the P-body actively consumes ATP, which needs to be reloaded within minutes.

FIGURE 7. Inhibition of mitochondrial activity specifically decreases Ago2 in P-bodies. A, efficiency of pSifluoro-3milet7 silencing by let-7b in function of the timing of CCCP addition. Top, schematic representation of the experimental design. Bottom, histograms of the silencing determined by measuring the mean EGFP expression in the presence and absence of 1 nM let-7b (mean ± S.D. of three experiments). B, CCCP treatment reduces Ago2 localization in P-bodies. HEK 293 cells were treated or not with CCCP for 16 h and immunostained with anti-Ago2 (red) and anti-p54 (green) antibodies. Cells were observed in wide-field microscopy. Bars = 10 μm. C, the enrichment of Ago2 and Rck/p54 in individual P-bodies was quantified in the previous experiment. The histogram represents the distribution of P-bodies in function of their enrichment in Ago2 or Rck/p54 before (blue) or after (orange) CCCP treatment (mean ± S.D. of three experiments). D, Ago2 depletion in P-bodies is specific. Ago2, Rck/p54, and Ge1 were measured in P-bodies before (blue) or after (orange) the indicated times of CCCP. For Ago2 and Rck/p54, the results are shown as the mean ± S.D. of three experiments (22 to 42 P-bodies). The fluorescence in control cells was arbitrarily set at 100. E, the Ago2 protein does not decrease during CCCP treatment. Cells were treated with CCCP for 0 to 24 h, and proteins were successively analyzed by Western blot with anti-Ago2 and S6K antibodies.
Mitochondria Regulate P-body Functions

In addition to Rck/p54, P-bodies contain most components of the RNAi pathway, including the Ago proteins, miRNAs, miRNA-repressed mRNAs, and GW182. We designed a new reporter system to finely quantify silencing, based on the analysis of protein expression at the individual cell level. Notably, it allows the measurement of miRNA and siRNA activity when transfected at nanomolar concentrations. Inactivation of the mitochondrial function with CCCP markedly reduced the silencing by si- and miRNA. The same response was observed when small RNAs were provided as siRNA or pre-miRNA, suggesting that both Ago2 and the non-slicing Ago complexes (32) were similarly affected. The silencing, but not the dose response, was changed in the presence of CCCP, indicating that bioavailability of small RNA was not modified. Thus, either response, was changed in the presence of CCCP, indicating that mitochondrial function with CCCP markedly reduced the accumulation of endogenous Ago2, which is a central component of the RISC complex, in the P-bodies. However, it did not modify the number or size of the P-bodies, as judged by the presence of Rck/p54 and Ge1. The exact role of the P-bodies in RNAi is currently ambiguous, with arguments for either a central or an accessory role in RNAi, and loose links between RNAi and other P-body functions. If P-bodies were storage sites for miRNA-repressed mRNA, the reduction of Ago2 in the P-bodies could be the result of fewer RISC-associated mRNA entering the P-bodies. As a corollary, the Rck/p54 protein, which remains unchanged, would mostly participate to complexes other than RISC, and the P-bodies, which remain intact, would be mainly involved in functions other than RNAi. Alternatively, if the P-bodies were a source of RISC components, the fact that a defect in RISC assembly leads to blockage of Ago2 recruitment to P-bodies would suggest that RISC assembly can take place in P-bodies. In human cell lysates, RISC loading with small RNA duplexes (to form pre-Ago2-RISC) has recently been shown to be greatly facilitated by ATP, whereas the following unwinding of the small RNA (to form mature Ago2-RISC) and the silencing activity were ATP-independent (32). Thus, an intriguing possibility would be that the association of P-bodies with functional mitochondria might stimulate in situ assembly of pre-Ago2-RISC by increasing the local ATP concentration. Because in our study the decrease in intracellular ATP never exceeds 50% during the period of RISC assembly, this would indicate a high sensitivity of this process to ATP levels, which would normally be alleviated by frequent interactions between P-bodies and mitochondria. Alternatively, or in addition, mitochondria could contribute to RISC assembly by other means than ATP supply. In this respect, it is highly interesting that miRNAs and Ago2 proteins were found in purified mitochondria (22, 23). As this accumulation was observed after RNase treatment, these miRNAs and Ago2 are likely to be located in the mitochondria rather than in mitochondria-associated P-bodies. If the mitochondria can play a role of miRNA reservoir, as proposed, the contacts between P-bodies and mitochondria would allow the transfer of miRNAs between the two organelles. To progress on these issues will require finding how to disrupt the contacts between P-bodies and mitochondria for a time sufficient to quantify the RNAi efficiency, making it possible to determine whether the mitochondria participate to RNAi through a mechanism dependent or not on their contacts with P-bodies.

Whatever the mechanism, the link between mitochondrial activity and RNAi revealed by the present study has potentially important corollaries. In human, mitochondrial dysfunctions are observed in various types of mitochondrial disorders, as well as in neurodegenerative diseases, diabetes, cancers, and aging. Mitochondrial activity can also be altered in various pathological conditions, such as cell stresses and ischemia reperfusion (38). Our results raise the possibility that these diseases and pathological conditions lead to some derepression of miRNA-controlled genes. Such a deregulation would be likely to significantly impact the cell metabolism in various tissues and contribute to the pathology.

Interestingly, mitochondria are also associated with P-body-related granules in germ cells of organisms such as Xenopus, Drosophila, and Caenorhabditis elegans (43, 44). Overall, germinal granules are thought to play a role in mRNA degradation and storage, like P-bodies, as well as in the repression of selfish genetic elements (45). Data obtained in Drosophila where they have been best characterized indicate that germinal granules contain a number of proteins identical or functionally related to P-body components. This includes proteins involved in mRNA storage, such as Me31B (Rck/p54/CGH1/Dhh1), 4ET, and Trailer hitch (RAP55/CAR1/Scd6), as well as proteins of the germline RNAi pathway, such as Aubergine, Tudor, and Maelstrom (46). Strikingly, among the 27 genes found in a genome-wide screen to identify genes involved in germinal silencing in C. elegans, 10 were directly involved in mitochondrial functions (47). These similarities strongly suggest that the association with mitochondria plays the same role for germinal granules and for P-bodies.

Acknowledgments—We thank Catherine Delmou for help in the culture of human umbilical vein endothelial cells, and Abbas Hadji and Damien Arnoult for the measurement of 3,3′-dihexyloxacarbocyanine iodide incorporation. This work was performed in the FRE3238, at the Institut André Lwoff, Villejuif, France.

REFERENCES

1. Cole, S. E., LaRiviere, F. J., Merrilh, C. N., and Moore, M. J. (2009) Mol. Cell 34, 440–450
2. Unterholzner, L., and Iazzurralde, E. (2004) Mol. Cell 16, 587–596
3. Cougot, N., Babajko, S., and Séraphin, B. (2004) J. Cell Biol. 165, 31–40
4. Wilczynska, A., Aigueperse, C., Kress, M., Daufrey, F., and Weil, D. (2005) J. Cell Sci. 118, 981–992
Mitochondria Regulate P-body Functions

5. Decker, C. J., Teixeira, D., and Parker, R. (2007) J. Cell Biol. 179, 437–449
6. Eulalio, A., Behm-Ansment, I., Schweitzer, D., and Izaurralde, E. (2007) Mol. Cell. Biol. 27, 3970–3981
7. Serman, A., Le Roy, F., Aigueperse, C., Kress, M., Dautry, F., and Weil, D. (2007) Nucleic Acids Res. 35, 4715–4727
8. Stalder, L., and Mühlemann, O. (2009) RNA 15, 1265–1273
9. Sweet, T. J., Boyer, B., Hu, W., Baker, K. E., and Coller, J. (2007) RNA 13, 493–502
10. Yan, Z., Jakyriaw, A., Wood, M. R., Eystathiou, T., Rubin, R. L., Fritzler, M. J., and Chan, E. K. (2004) J. Cell Sci. 117, 5567–5578
11. Aizer, A., Brody, Y., Ler, L. W., Sonenberg, N., Singer, R. H., and Shavit-Tal, Y. (2008) Mol. Biol. Cell 19, 4154–4166
12. Andrei, M. A., Ingelfinger, D., Heintzmann, R., Achsel, T., Rivera-Pomar, R., and Lührmann, R. (2005) RNA 11, 717–727
13. Federspohl, S., Nystrom, S., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fritzler, M. J., Scheuner, D., Kaufman, R. J., Golan, D. E., and Anderson, P. (2005) J. Cell Biol. 169, 871–884
14. Leung, A. K., Cabarelle, J. M., and Sharp, P. A. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 18125–18130
15. Bhattacharyya, S. N., Habermann, R., Martine, U., Closs, E. I., and Filipowicz, W. (2006) Cell 125, 1111–1124
16. Brengues, M., Teixeira, D., and Parker, R. (2005) Science 310, 486–489
17. Federspohl, S., Chen, S., Gilks, N., Li, W., Miller, I. J., Stahl, J., and Anderson, P. (2002) Mol. Biol. Cell 13, 195–210
18. Souquere, S., Mollet, S., Kress, M., Dautry, F., Pierron, G., and Weil, D. (2009) J. Cell Sci. 122, 3619–3626
19. Mollet, S., Cougot, N., Wilczynska, A., Dautry, F., Kress, M., Bertrand, E., and Weil, D. (2008) Mol. Biol. Cell 19, 4469–4479
20. Gibbons, D. J., Ciaudo, C., Erhardt, M., and Voinnet, O. (2009) Nat. Cell Biol. 11, 1143–1149
21. Lee, Y. S., Pressman, S., Andress, A. P., Kim, K., White, J. L., Cassidy, J. J., Li, X., Lubell, K., Lim do, H., Cho, I. S., Nakahara, K., Preall, J. B., Bellare, P., Sontheimer, E. J., and Carthew, R. W. (2009) Nat. Cell Biol. 11, 1150–1156
22. Bian, Z., Li, L. M., Tang, R., Hou, D. X., Chen, X., Zhang, C. Y., and Zen, K. (2010) Cell Res. 20, 1076–1078
23. Kren, B. T., Wong, P. Y., Sarver, A., Zhang, X., Zeng, Y., and Steer, C. J. (2009) RNA Biol. 6, 65–72
24. MacRae, I. J., Ma, E., Zhou, M., Robinson, C. V., and Doudna, J. A. (2008) Nat. Protoc. Natl. Acad. Sci. U.S.A. 105, 512–517
25. Ohrt, T., Mütze, J., Staroske, W., Weinmann, L., Höck, J., Crell, K., Meister, G., and Schwine, P. (2008) Nucleic Acids Res. 36, 6439–6449
26. Zhang, D. H., Marconi, A., Xu, L. M., Yang, C. X., Sun, G. W., Feng, X. L., Ling, C. Q., Qin, W. Z., Uzan, G., and d’Alessio, P. (2006) J. Leukocyte Biol. 80, 309–319
27. Rizzuto, R., Brini, M., Pizzo, P., Murgia, M., and Pozzan, T. (1995) Curr. Biol. 5, 635–642
28. Doench, J. G., Petersen, C. P., and Sharp, P. A. (2003) Genes Dev. 17, 438–442
29. Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005) Science 309, 1573–1576
30. Tiede, S., Flatley, A., Weinmann, L., Kremmer, E., and Meister, G. (2008) RNA 14, 1244–1253
31. Boldogh, I. R., and Pon, L. A. (2007) Trends Cell Biol. 17, 502–510
32. Yoda, M., Kawamata, T., Paroo, Z., Ye, X., Iwasaki, S., Liu, Q., and Tomari, Y. (2010) Nat. Struct. Mol. Biol. 17, 17–23
33. Gerber, A. P., Herschlag, D., and Brown, P. O. (2004) PLoS Biol. 2, e79
34. Foat, B. C., Housholand, S. S., Olivas, W. M., and Bussemaker, H. J. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 17675–17680
35. Saint-Georges, Y., Garcia, M., Delaveau, T., Jourdren, L., Le Crom, S., Lemoine, S., Tanty, V., Devaux, F., and Jacq, C. (2008) PLoS One 3, e2293
36. García-Rodríguez, L. J., Gay, A. C., and Pon, L. A. (2007) J. Cell Biol. 176, 197–207
37. Lee, S. I., Dudley, A. M., Drubin, D., Silver, P. A., Krogan, N. J., Pe’er, D., and Koller, D. (2009) PLoS Genet. 5, e1000358
38. Kuznetsov, A. V., and Margreiter, R. (2009) Int. J. Mol. Sci. 10, 1911–1929
39. Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, V. Y., and Zorzato, F. (2009) Trends Cell Biol. 195–210
40. Black, C., Hilliker, A., Cziko, A. M., Nouri, A., Ramaswami, M., and Parker, R. (2008) Mol. Biol. Cell 19, 984–993
41. Minshall, N., Kress, M., Weil, D., and Standart, N. (2009) Mol. Biol. Cell 20, 2464–2472
42. Chuma, S., Hosokawa, M., Tanaka, T., and Nakatsuji, N. (2009) Mol. Cell. Endocrinol. 306, 17–23
43. Moser, J. J., and Fritzler, M. J. (2010) Int. J. Biochem. Cell Biol. 42, 828–843
44. Lim, A. K., and Kai, T. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 6714–6719
45. Thomson, T., Liu, N., Arkov, A., Lehmann, R., and Lasko, P. (2008) Mech. Dev. 125, 865–873
46. Vastenhouw, N. L., Fischer, S. E., Robert, V. J., Thijsen, K. L., Fraser, A. G., Kamath, R. S., Ahringer, J., and Plasterk, R. H. (2003) Curr. Biol. 13, 1311–1316