Profilin is associated with transcriptionally active genes

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We have raised antibodies against the profilin of Chironomus tentans to study the location of profilin relative to chromatin and to active genes in salivary gland polytene chromosomes. We show that a fraction of profilin is located in the nucleus, where profilin is highly concentrated in the nucleolus and at the nuclear periphery. Moreover, profilin is associated with multiple bands in the polytene chromosomes. By staining salivary glands with propidium iodide, we show that profilin does not co-localize with dense chromatin. Profilin associates instead with protein-coding genes that are transcriptionally active, as revealed by co-localization with hnrNP and snRNp proteins. We have performed experiments of transcription inhibition with actinomycin D and we show that the association of profilin with the chromosomes requires ongoing transcription. However, the interaction of profilin with the gene loci does not depend on RNA. Our results are compatible with profilin regulating actin polymerization in the cell nucleus. However, the association of actin with the polytene chromosomes of C. tentans is sensitive to RNase, whereas the association of profilin is not, and we propose therefore that the chromosomal location of profilin is independent of actin.

Introduction

β-actin is an abundant cellular protein of molecular weight 42 kDa and a major component of the cytoskeleton in all eukaryotic cells. β-actin is involved in essential biological processes such as maintaining cell shape, vesicle trafficking and cell motility. β-actin exists in two major forms: a globular, monomeric form referred to as “G-actin,” and a filamentous polymer named “F-actin.” The structure and activities of the actin cytoskeleton are regulated by the polymerization dynamics of actin, which in turn is modulated by a large number of proteins. Among them, ADF/cofilins and profilins are major regulators of actin polymerization. Profilin is not only a major cytoskeletal component but also a nuclear protein that plays important roles in gene expression. β-actin is part of chromatin remodeling complexes, it is associated with the transcription machineries, it becomes incorporated into newly synthesized ribonucleoproteins, and it influences long-range chromatin organization (reviewed in ref. 2). Immuno-electron microscopy experiments in the salivary glands of the dipteran Chironomus tentans (C. tentans) have shown that actin is associated with active transcription units. The association of actin with active chromatin was has been demonstrated also in mammalian cells. Chromatin immunoprecipitation experiments using anti-actin antibodies have shown that actin is associated with Pol I and Pol II genes. Several heterogeneous nuclear ribonucleoproteins (hnRNPs) bind actin and mediate its association with nascent mRNA. In C. tentans, actin is associated with the mRNA-binding proteins hp36γ and hp65γ. In mammalian cells, actin associates with several hnRNP proteins, including hnRNP A2, hnRNP A3, CArG-box binding factor (CBF-A) and hnRNP U. Both in insect and mammalian cells, the actin-hnRNP complexes participate in the recruitment of co-activators to active genes.

Nuclear β-actin works in conjunction with different types of actin-binding proteins that regulate actin function and bridge interactions between actin and other nuclear components. NM1, a short-tailed myosin that acts as an actin-dependent ATPase, is found in the cell nucleus associated with actin. Studies of Pol-I transcription have shown that an actin-NM1 interaction is required for Pol-I transcription, which has led to the proposal that an actomyosin motor helps the transcription machinery slide along the rDNA. Studies of gene repositioning and chromatin organization point to the possibility that actin also plays a role in the large-scale organization of the genome. Altogether, these observations suggest that the dynamics of actin polymerization has important implications for chromatin organization and gene expression. Other actin-binding proteins, including α-actinin, filamin, p39Gluγ, paxillin, spectrin, tropomyosin, cofillin and profilin, have also been detected in the nucleus of human cells. Profilins are small proteins with masses in the 15–17 kDa range. They are evolutionarily conserved and play a key role in the regulation of F-actin formation. Profilins promote actin polymerization by favoring the exchange of ADP for ATP in actin. Localization studies using profilin fused to green fluorescent protein (GFP)
Four profilin genes have been identified in mammals: Pfn1, Pfn2, Pfn3 and Pfn4, of which Pfn1 codes for the ubiquitous profilin 1. There is a single gene encoding profilin in the genome of Drosophila melanogaster and from D. melanogaster cells were probed by western blot with anti-profilin antibodies. Ab1 and ab2 were affinity purified from two rabbits immunized with the same peptide. Molecular mass standards are shown to the left in kDa. (C) A cytoplasmic extract (C), a nuclear pellet (NP) and a nuclear soluble extract (NS) were prepared from C. tentans tissue culture cells and probed with antibodies against profilin and actin, as indicated.

We immunized two rabbits with a synthetic peptide containing against total protein extracts of C. tentans (Fig. 1B). The anti-profilin antibodies, and in particular the antibody ab1, were specific and suitable for immunolocalization studies in C. tentans. D. melanogaster profilin, the product of the chickadee gene, was also detected by the anti-profilin antibodies.

Previous studies had shown the existence of profilin in the nucleus of mammalian cells. To determine whether profilin was also a nuclear protein in C. tentans, we performed fractionation experiments using C. tentans cultured cells. The cells were homogenized in a detergent-containing buffer and the nuclei were collected by centrifugation. The supernatant was the cytoplasmic fraction (“C” in Fig. 1C). The nuclear pellet was resuspended in PBS, mildly sonicated to lysate the nuclei, and centrifuged again. The supernatant of this second centrifugation was the nuclear soluble fraction (“NS” in Fig. 1C) containing soluble proteins and mRNPs. The pellet was the nuclear insoluble fraction (“NP” in Fig. 1C) and contained the nuclear envelope, the nucleolus and the chromatin, including the nascent transcripts. We analyzed the presence of actin and profilin in these fractions by western blotting. The fractionation patterns of actin and profilin were very similar. Both proteins were predominantly found in the insoluble nuclear fraction. We next used the anti-profilin antibodies to stain the salivary gland cells of C. tentans by immunofluorescence. Salivary glands were dissected from fourth instar larvae have large polyteny chromosomes and the interchromatin space is free of chromatin. The salivary gland cells are therefore an excellent material to study the location of profilin relative to chromatin and to active genes.
bands in the polytene chromosomes were negative for profilin and vice versa (Fig. 4B and C). We concluded that profilin is excluded from dense chromatin.

We performed double-staining experiments to analyze the localization of profilin relative to transcribed genes. We used antibodies against two different proteins that associate with the nascent mRNA: the hnRNP protein hrp23 and the core proteins of the snRNP complexes. Salivary glands stained with antibodies against profilin and hrp23 are shown in Figure 5A. Profilin and hrp23 co-localized to a large extent, and most profilin-positive bands in the polytene chromosomes were also stained with the anti-hrp23 antibody. However, the patterns of staining along the chromosomes were not identical. A few loci, such as the BR puffs, were predominantly stained by hrp23 with less profilin staining. Some loci were positive for profilin but negative for hrp23.

In another series of experiments, we stained salivary glands with antibodies against Ct-profilin and snRNPs (Fig. 5B and C). Profilin co-localized to a large extent with snRNPs, although the patterns of staining were not identical, as in the case of hrp23. To better compare the distribution patterns of profilin and snRNPs, we immunostained isolated chromosomes. Figure 5D shows an example of chromosome IV immunostained with anti-profilin and anti-snRNP antibodies. The analysis of isolated chromosomes confirmed a partial co-localization. The BR puffs in chromosome IV were positively stained with the anti-profilin antibody in preparations of isolated chromosomes (Fig. 5D). In whole-mount salivary glands, the BR puffs were positive but were not among the most prominently stained loci (see, for example, Fig. 5A). The difference in relative intensity can be explained by

multiple bands in the polytene chromosomes, but was excluded from the nucleolus (Fig. 2B). The Balbiani ring (BR) puffs were stained to a certain degree, but the levels of fluorescence in BR puffs were lower than that in the nucleoplasm (see Figs. 5 and 6). Both affinity-purified antibodies, ab1 and ab2, gave the same pattern of staining. Ab1 was chosen for subsequent studies.

As shown in Figure 1B, the anti-profilin antibody also recognized the product of the D. melanogaster gene chickadee. We immunostained S2 cells with antibodies against profilin to determine whether profilin was also a nuclear protein in D. melanogaster. We used an anti-snRNP antibody as a nuclear marker. The anti-profilin ab1 antibody stained both the cytoplasm and the nucleus of the S2 cells, whereas the anti-snRNP antibody gave a predominantly nuclear staining (Fig. 3A). We also stained S2 cells with a mAb raised against the profilin of D. melanogaster. The pattern of staining observed with this mAb was very similar to the one obtained with our peptide-specific antibodies (compare A and B in Fig. 3).

We performed co-localization experiments in the salivary glands of C. tentans to characterize the association of profilin with the chromosomes. In a first series of experiments, we immunostained salivary gland preparations with the anti-profilin ab1 antibody as in Figure 2, and we counterstained the glands with propidium iodide (PI). PI binds to nucleic acids and stains predominantly dense chromatin in the polytene chromosomes. The rim of the nucleolus was also stained, and the cytoplasm to some extent, due to high concentrations of highly structured rRNA in these locations (Fig. 4A). The extent of co-localization between profilin and PI in the chromosomes was very low. The PI-positive bands in the polytene chromosomes were negative for profilin and vice versa (Fig. 4B and C). We concluded that profilin is excluded from dense chromatin.

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the different permeabilization and fixation conditions. In isolated chromosomes, profilin and snRNPs co-localized in the BRs. The snRNP staining was very intense in BR3, which is consistent with the fact the BR3 pre-mRNA contains a large number of introns, most of which are spliced co-transcriptionally.27 Interestingly, the anti-snRNP antibody stained the entire BR3, whereas profilin had a more internal localization, near the body of the chromosome (see the merged image in Fig. 5D).

The results presented above suggest that profilin is associated with transcriptionally active loci in the polytene chromosomes. We next performed a series of experiments in which we digested the preparations with RNase A before the immunostaining, to determine whether the association of profilin with the chromosomes was mediated by RNA. The glands were double-stained with an antibody against the hnRNP protein hrp45 to monitor the effect of the RNase digestion. The nuclear localization of profilin was not affected by the RNase digestion, while the anti-hrp45 staining was drastically reduced (Fig. 6A). We also performed experiments with RNase digestion of isolated polytene chromosomes. In this case, the preparations were counterstained with the anti-snRNP antibody (Fig. 6B). The snRNP staining was drastically reduced by the digestion, but profilin was still associated with the chromosomes after digestion with RNase A. We concluded that the association of profilin with the chromosomes was not mediated by RNA.

The results presented above suggest that profilin is associated with transcriptionally active loci, but that the association of profilin with the active loci does not depend on RNA. We next asked whether the chromosomal association of profilin depends on ongoing transcription. We incubated salivary glands in the presence of actinomycin D for one hour before fixation and immunostaining. Control glands were treated in parallel without actinomycin D. The glands were double-stained with antibodies against profilin and snRNP. The effect of actinomycin D on the overall nuclear staining was not striking, but the chromosomes were devoid of snRNP staining after actinomycin D treatment, which indicated that the treatment was effective. The anti-profilin antibodies still stained the nucleoplasm, while the chromosomal staining was significantly reduced (Fig. 7).

We concluded that the association of profilin with the chromosomes is transcription-dependent. Actin becomes incorporated into nascent pre-mRNAs and remains associated with snRNP complexes during transport from the gene to the nuclear pores.3 The results presented above suggested that profilin does not

Figure 3. Localization of profilin in S2 cells of D. melanogaster. (A) S2 cells were double-labeled with ab2 anti-profilin (green) and mab Y12 against snRNPs (red). The anti-profilin antibody was positive in both nucleus and cytoplasm, whereas Y12 labeled preferentially the nucleus. (B) S2 cells were labeled with a mAb against the product of the chickdee gene, chic. This antibody also labeled both nucleus and cytoplasm. The bars represent 5 μm.

Figure 4. Profilin is not associated with dense chromosome bands. Salivary glands were fixed and stained with ab1 against profilin and counterstained with propidium iodide to reveal dense chromatin bands in the polytene chromosomes. (A) Overview of salivary gland cells stained with propidium iodide (PI). The bar represents 25 μm. (B) The nucleus of a salivary gland cell stained with anti-profilin antibody (green) and PI (red). (C) The co-localization between profilin and PI was analyzed using the Profile function of the LSM 510 software. The graph shows the relative intensities for each channel, in arbitrary units, along the chromosome axis defined by the arrow. The analyzed chromosome segment is shown in the inset in (B). The opposite staining profiles for profilin and PI reveal that profilin is excluded from dense chromatin.
become incorporated into mRNP complexes. To further analyze this question, we used immuno-electron microscopy (immuno-EM) to analyze the association of profilin with BR mRNPs. The BR mRNPs are large ribonuclereotein particles that can be identified in the nucleoplasm of the salivary gland cells due to their large dimensions and distinctive morphology. Salivary glands were fixed, cryoprotected and cryosectioned. The thin cryosections were incubated with the anti-profilin antibody and with a secondary antibody conjugated to colloidal gold. The immuno-EM analysis failed to reveal any significant association of profilin with BR mRNPs particles (Fig. 8). The density of gold labeling was relatively high in the nucleoplasm, where the labeling was associated with fibrillar material (arrowheads in Fig. 8A), but the BR mRNPs were devoid of labeling (arrow in Fig. 8A). A high density of labeling was observed near the nuclear envelope, on both the nuclear and the cytoplasmic sides. In some cases, profilin was found to decorate structures in the vicinity of the BR mRNPs (Fig. 8B). These structures probably correspond to the connecting fibers described by Maralles et al.\textsuperscript{21} In summary, the immuno-EM experiments confirmed that profilin is a nuclear protein preferentially located in the nucleoplasm and at the nuclear envelope, but not associated with nucleoplasmic BR mRNPs.

**Discussion**

We have identified the profilin of *C. tentans* and found that it is very similar to profilins from other diptera such as Drosophila and Anopheles. Previous studies have shown that profilins are not restricted to the cytoplasm but are also located in the cell nucleus, both in mammalian cells and in insects.^22^\textsuperscript{23,29-31} Moreover, several nuclear proteins have been identified as ligands for profilin, including the survival motor neuron protein (SMN),\textsuperscript{29} which is involved in snRNP biogenesis, and the Myb-related transcription factor p42.\textsuperscript{22,23} However, previous studies showing that profilin is a nuclear protein have not addressed the detailed localization of profilin in relation to chromatin and active genes. The salivary glands of *C. tentans* offer unique advantages when analyzing the association of specific proteins, in this case profilin, with gene loci. The chromatin and interchromatin compartments in the polytene nuclei are easy to distinguish from each other, and the chromatin is confined to the large polytene chromosomes, which leaves the nucleoplasm free of chromatin. The chromosomes, visible using either light or electron microscopy, are composed of dense bands and interbands that represent different degrees of chromatin condensation. In spite of the fact that the general organization of the polytene nuclei differs from that of diploid nuclei, the basic processes of gene expression are the same in both types of cells (reviewed in refs. 32 and 33). We have used the advantages that polytene nuclei offer for in situ studies of gene expression to analyze the association of profilin with chromatin. For this purpose, we raised antibodies against the profilin of *C. tentans*

Using immunofluorescence microscopy, we showed that a fraction of profilin is located in the nucleus of the salivary gland cells of *C. tentans*. Staining of *D. melanogaster* S2 cells also revealed a nuclear localization of profilin. From these results we conclude that profilin is a nuclear protein in insects as it is in vertebrates, and that the nuclear localization of profilin is characteristic of both polytene and diploid cells. A more detailed analysis of the location of profilin in the nucleus of the salivary gland cells of *C. tentans* revealed that profilin is highly concentrated in the nucleoplasm and at the nuclear periphery. Immuno-EM experiments showed that profilin is located on both sides of the nuclear envelope. Moreover, a fraction of profilin is associated with the polytene chromosomes following a
with actinomycin D and DRB, and we have shown that profilin interacts with the rDNA genes. The anti-profilin antibodies did not give any indication that profilin also interacts with genes that are transcribed by RNA polymerase II, although actin is also a component of several chromatin remodelling complexes (reviewed in refs. 35 and 36). In principle, the association of profilin with the chromosomes could be explained by an interaction between profilin and actin molecules that are bound either to the RNA polymerase or to the chromatin factors located at active genes. However, the association of actin with the polytene chromosomes of C. tentans depends on RNA, whereas profilin remains bound to chromosomes digested with RNase. This result suggests that the profilin located at sites of transcription is not bound to actin. Further studies will be necessary to characterize the molecular interactions that tether profilin to actively transcribed genes.

Several studies have supported the idea that nuclear profilin is involved in gene expression, but each of these studies ascribes profilin a different function. Skare et al. reported that an antibody against profilin has an inhibitory effect on pre-mRNA splicing in vitro and suggested a role for profilin in pre-mRNA processing. Lederer et al. showed that profilin binds to the Myb-related transcriptional repressor p42 POP and regulates its activity, maybe by regulating its subcellular localization. Ye et al. proposed that an actomyosin motor is required for transcription by RNA polymerase I, which raises the possibility that profilin functions as a regulator of actin polymerization at transcription sites. Our immuno-localization studies are compatible with these suggested functions. Moreover, our immuno-EM experiments have revealed the existence of a fraction of profilin complexes, and profilin enhances the interaction of actin with exportin 6, which in turn favors the nuclear export of actin. It is unclear how these observations relate to each other and to profilin, but it is clear that actin and actin-binding proteins are implicated, directly or indirectly, in nucleo-cytoplasmic trafficking.

In summary, the work presented here and several previous reports link profilin with gene expression, but in quite divergent ways. It is clear that we are still far from understanding the roles of profilin in the cell nucleus. A key point will be to determine whether nuclear profilin has actin-independent functions or whether it acts primarily as an actin regulator. The associations

Figure 6. The association of profilin with the chromosomes does not depend on RNA. (A) Salivary glands were digested with RNase A before fixation and immunostaining with antibodies against profilin (green) and hpr45 (red). The hnRNP protein hpr45 was used to monitor the efficiency of the enzymatic digestion. Note that the distribution of profilin in the nucleus was not affected by the digestion with RNase A. The arrowheads point at BR puffs intensely labeled by the anti-hrp45 antibody. The bar represents 20 μm. (B) RNase A digestion of isolated polytene chromosomes before immunostaining with antibodies against profilin (green) and snRNPs (red). The bar represents 10 μm.
of actin and profilin to the chromosomes differ in their RNA dependence, which suggests that the fraction of profilin that is bound to the chromosomes has a role that is independent of actin.

Materials and Methods

Culturing conditions. Chironomus tentans was cultured as described by Meyer et al. Salivary glands were isolated from fourth instar larvae. C. tentans tissue culture cells were cultivated at 29°C as previously described in reference 40. Drosophila melanogaster S2 cells were cultured at 28°C in Schneider's Drosophila medium (Invitrogen, 21720-024) supplemented with 10% heat-inactivated fetal calf serum, 50 μg/ml penicillin and 50 μg/ml streptomycin.

Antibodies. A synthetic peptide spanning amino acids 2 to 15 (SWQ DIY DNQ LIA SQC) located in the N-terminal domain of Ct-profilin was conjugated to keyhole limpet hemocyanin and used to immunize two rabbits following standard procedures. Immunization was performed at AgriSer (Vännäs). The antibodies were affinity purified and eluted in two steps at pH 2.5 and pH 7.0. A mAb against the product of the chickadee gene of D. melanogaster was described by Verheyen and Cooley and purchased from the Developmental Studies Hybridoma Bank (University of Iowa). The Y12 antibody is a mAb against the Sm antigen of mRNPs, and was originally produced and characterized by Lerner et al. mAbs against hrp23 and hrp45 were produced and characterized by Sun et al. and Alzhanova-Ericsson et al., respectively.

SDS-PAGE and western blotting. For analysis of antibody specificity, whole cell extracts were prepared from C. tentans tissue culture cells or D. melanogaster S2 cells. The cells were boiled in 2x sample buffer containing 5% β-mercaptoethanol and separated by SDS-PAGE. After electrophoresis, the proteins in the gel were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, IPVH00010) in Tris-glycine buffer containing 0.02% SDS and 4 M urea using a semi-dry electrophoretic transfer cell (BioRad, 170-3940). The membranes were blocked with 10% non-fat dry milk in PBS, and probed with primary antibody at a final concentration of approximately 2 μg/ml, followed by a goat-anti-rabbit secondary antibody conjugated with alkaline phosphatase (DAKO, D0487). The antibodies were diluted in PBS containing 0.05% Tween-20 and 1% milk. The alkaline phosphatase activity was detected with the NBT/BCIP system.

Immunofluorescence of whole salivary glands. C. tentans salivary glands were isolated and fixed in 4% formaldehyde in PBS for 10 min on ice, and incubated 20 min on ice in PBS containing 4% formaldehyde and 5% Triton X-100. The glands were then washed, permeabilized in 10% Triton X-100 in PBS for 5 min at room temperature, fixed with 4% paraformaldehyde for 30 min, and washed three times 5 min each in PBS. Blocking was done in 5% new born calf serum (NBCS) and 5% bovine serum albumin (BSA) in PBS for 30 min. Incubation with primary antibody was done for 2 h in a humid chamber. The primary antibodies were diluted to a final concentration of approximately 1–5 μg/ml in PBS containing 0.5% NBCS and 0.5% BSA. After primary antibody incubation, the glands were washed three times for 5 min each in PBS, followed by incubation with secondary antibodies, either FITC or Texas Red conjugated, for 1 h. After final washes, the samples were mounted in Vectashield mounting medium.

Figure 7. The association of profilin with the chromosomes requires ongoing transcription. (A) Salivary glands were incubated in the presence of actinomycin D (+ActD) before fixation and immunostaining with antibodies against profilin (green) and snRNPs (red). Control glands were incubated in parallel without the drug, as indicated. (B) Fourth-instar larvae were treated with (+DRB) or without (-DRB) DRB before dissection of the glands and immunostaining. The bar represents 10 μm. Note that the banded labeling of the chromosomes is lost after actinomycin D or DRB treatment.
primary antibodies diluted to a final concentration of approximately 5–10 μg/ml in 0.5% BSA in TKM at 4°C overnight. The chromosomes were washed with 0.01% Tween-20 in TKM three times for 10 min each, followed by incubation with secondary antibodies either FITC or Texas Red conjugated. The chromosomes were mounted with Vectashield mounting medium (Vector, H-1000) and examined in an LSM 510 microscope.

**RNase A digestion.** For experiments of RNase A digestion, the glands were washed with TBS after dissection, then incubated in glycerol buffer (20 mM TRIS-HCl at pH 7.2, 5 mM MgCl₂, 0.5 mM PMSF, 0.5 mM EGTA, 25% glycerol) for 3 min, in glycerol buffer containing 0.05% Triton X-100 for a further 3 min, and then washed three times 5 min in transcription buffer (5 mM TRIS-HCl at pH 7.4, 100 mM KCl, 0.5 mM EGTA, 25% glycerol). The glands were incubated in RNase A diluted to 100 μg/ml in TBS for 20 min at 37°C. Control samples were incubated in TBS without enzyme under the same conditions. The glands were then processed for immunofluorescence as described above.

**Isolated polytene chromosomes.** Isolated polytene chromosomes were incubated with 100 μg/ml RNase A for 30 min and washed with TKM before fixation and immunofluorescence as above.

**Propidium iodide staining.** The glands were processed for immunofluorescence as described above. After incubation with the secondary antibody, the glands were washed three times 5 min each in PBS and incubated in 500 mM propidium iodide in PBS for 10 min at room temperature. Then the glands were washed two times 10 min each in PBS and two times 10 min each in PBS supplemented with 0.001% Tween-20. The glands were finally mounted in Vectashield mounting medium (Vector, H-1000) and examined in an LSM 510 laser confocal microscope (Carl Zeiss). In the negative controls, the primary antibody was replaced by an unrelated rabbit antibody against mouse immunoglobulins. Co-localization was analyzed using the “Profile” function of the LSM 510 software by drawing a test line along the region of interest and measuring the relative fluorescence intensity along the line in arbitrary units.

**Immunofluorescence of isolated polytene chromosomes.** C. tentans polytene chromosomes were isolated from the salivary glands essentially as described by Björkroth et al. with some modifications. The salivary glands were permeabilized with 2% NP40 in TKM buffer (10 mM triethanolamine-HCl at pH 7.0, 100 mM KCl and 1 mM MgCl₂) and the chromosomes were isolated by repeatedly pipetting through a siliconized glass micropipette. Isolated chromosomes were transferred to 8-well slides and fixed with 2% formaldehyde in TKM for 30 min. After fixation, the chromosomes were washed in TKM three times for 5 min each, and blocked in 3% BSA in TKM for 30 min. After blocking, the chromosomes were incubated with

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**Figure 8.** ImmunoeM analysis of profilin in the nucleoplasm of the salivary gland cells. Thin cryosections of salivary glands were stained with ab1 against profilin. A secondary antibody conjugated to 6-nm colloidal gold particles was used to visualize the immunolabelling. (A) Overview of a nucleoplasmic region near the nuclear envelope (NE). The arrow points to a BR mRNp particle. The arrowheads point at immuno-gold markers in the nucleoplasm. (B) Three examples of immuno-gold labelling in the vicinity of BR mRNA particles in the nucleoplasm of the salivary gland cells. The bars represent 100 nm.

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**medium (Vector, H-1000) and examined in an LSM 510 laser confocal microscope (Carl Zeiss). In the negative controls, the primary antibody was replaced by an unrelated rabbit antibody against mouse immunoglobulins. Co-localization was analyzed using the “Profile” function of the LSM 510 software by drawing a test line along the region of interest and measuring the relative fluorescence intensity along the line in arbitrary units.**
in 5% milk and 5% BSA in PBS for 1 h. The primary antibody was diluted in 5%, milk and 5% BSA to a concentration of 1–5 μg/ml. Antibody incubations were performed in a humid chamber.

Immunoelectron microscopy. Salivary glands were dissected from fourth instar larvae, fixed for 20–25 min in 2.5% paraformaldehyde and 0.1% glutaraldehyde, cryoprotected with 2.3 M sucrose, frozen by immersion in liquid nitrogen and cryosectioned. Thin cryosections were picked up on drops of 2.3 M sucrose and mounted onto nickel grids coated with formvar and carbon. The grids were floated onto drops of PBS containing 0.1 M glycine and 10% fetal calf serum before incubation with the antibody solutions. The primary antibody was affinity-purified anti-profilin diluted to 17 μg/ml in PBS containing 0.1 M glycine and 10% fetal calf serum. The secondary antibody, conjugated to 6 mol gold particles (Jackson Immunoresearch Laboratories, West Grove, PA), was diluted 1:50 in the same solution.

After immunolabelling, the specimens were stained with 2% aqueous uranyl acetate for 5–5 min and embedded in polymethyl methacrylate (9–10 kDa, Aldrich, 365627). The specimens were examined and photographed in a FEI Tecnai G2 electron microscope at 80 kV.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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