Plakophilin 1 stimulates translation by promoting eIF4A1 activity

Annika Wolf,[1] Malgorzata Krause-Gruszczynska,[1] Olaf Birkenmeier,[2] Antje Ostareck-Lederer,[4] Stefan Hüttelmaier,[3] and Mechthild Hatzfeld[1]

[1]Division of Pathobiochemistry, [2]Institute for Biotechnology, and [3]Department of Molecular Cell Biology, Martin Luther University Halle-Wittenberg, 06097 Halle, Germany
[4]University Hospital Aachen, 52074 Aachen, Germany

Plakophilins 1–3 (PKP1–3) are desmosomal proteins of the p120^{ctn} family of armadillo-related proteins that are essential for organizing the desmosomal plaque. Recent findings identified PKPs in stress granules, suggesting an association with the translational machinery. However, a role of PKPs in controlling translation remained elusive so far. In this study, we show a direct association of PKP1 with the eukaryotic translation initiation factor 4A1 (eIF4A1). PKP1 stimulated eIF4A1-dependent translation via messenger RNA cap and encephalomyocarditis virus internal ribosomal entry site (IRES) structures, whereas eIF4A1-independent translation via hepatitis C virus IRES was not affected. PKP1 copurified with eIF4A1 in the cap complex, and its overexpression stimulated eIF4A1 recruitment into cap-binding complexes. At the molecular level, PKP1 directly promoted eIF4A1 adenosine triphosphatase activity. The stimulation of translation upon PKP1 overexpression correlated with the up-regulation of proliferation and cell size. In conclusion, these findings identify PKP1 as a regulator of translation and proliferation via modulation of eIF4A1 activity and suggest that PKP1 controls cell growth in physiological and pathological conditions.

Introduction

Plakophilins 1–3 (PKP1–3) are members of the p120^{ctn} family of armadillo-related proteins. All three PKPs have been characterized as desmosomal proteins, whereas p120^{ctn} and the closely related δ-catenin, ARVCF, and p0071 play essential roles in stabilizing cadherin-mediated adhesion in adherens junctions (Anastasiadis and Reynolds, 2000; Hatzfeld, 2005, 2007). PKPs interact with desmosomal cadherins, which are stabilized at the plasma membrane in the presence of PKPs, and with the intermediate filament linker protein desmoplakin (Hatzfeld et al., 2000; Chen et al., 2002; Bonné et al., 2003). The three PKPs reveal distinct expression patterns and, although partially redundant in their function, mediate distinct effects on desmosomal adhesion (Hatzfeld, 2007).

Besides a structural role, a function in signaling has been postulated in analogy to other armadillo proteins such as β-catenin, p120^{ctn}, and p0071, which function in transcriptional control (β-catenin and p120^{ctn}) and in Rho signaling (p120^{ctn} and p0071; Behrens et al., 1996; Daniel and Reynolds, 1999; Noren et al., 2000; Anastasiadis and Reynolds, 2001; Perez-Moreno et al., 2006; Wolf et al., 2006; Keil et al., 2007). In contrast to these proteins, the role of PKPs in intracellular signaling remained largely speculative, although both endogenous and overexpressed PKP1 have been prominently detected in the nucleus and the cytoplasm (Hatzfeld et al., 1994; Schmidt et al., 1997; Hatzfeld, 2007).

Recently, several RNA-binding proteins (RBPs) have been shown to partially co-distribute with PKP3 after sucrose gradient centrifugation. After exposure of cells to stress, these proteins were found together with PKP3 or -1 in stress granules (SGs; Hofmann et al., 2006). SGs are cytoplasmic aggregates of stalled translational preinitiation complexes that accumulate together with many RBPs during cell stress (Anderson and Kedersha, 2006).

Correspondence to Mechthild Hatzfeld: mechthild.hatzfeld@medizin.uni-halle.de

Abbreviations used in this paper: BiFC, bimolecular fluorescence complementation; CAT, chloramphenicol acetyltransferase; eIF, eukaryotic translation initiation factor; EMCV, encephalomyocarditis virus; FFL, firefly luciferase; HCV, hepatitis C virus; IRES, internal ribosomal entry site; PKP, plakophilin; qRT-PCR, quantitative RT-PCR; RBP, RNA-binding protein; SG, stress granule; TIAR, TIA-1–related protein.

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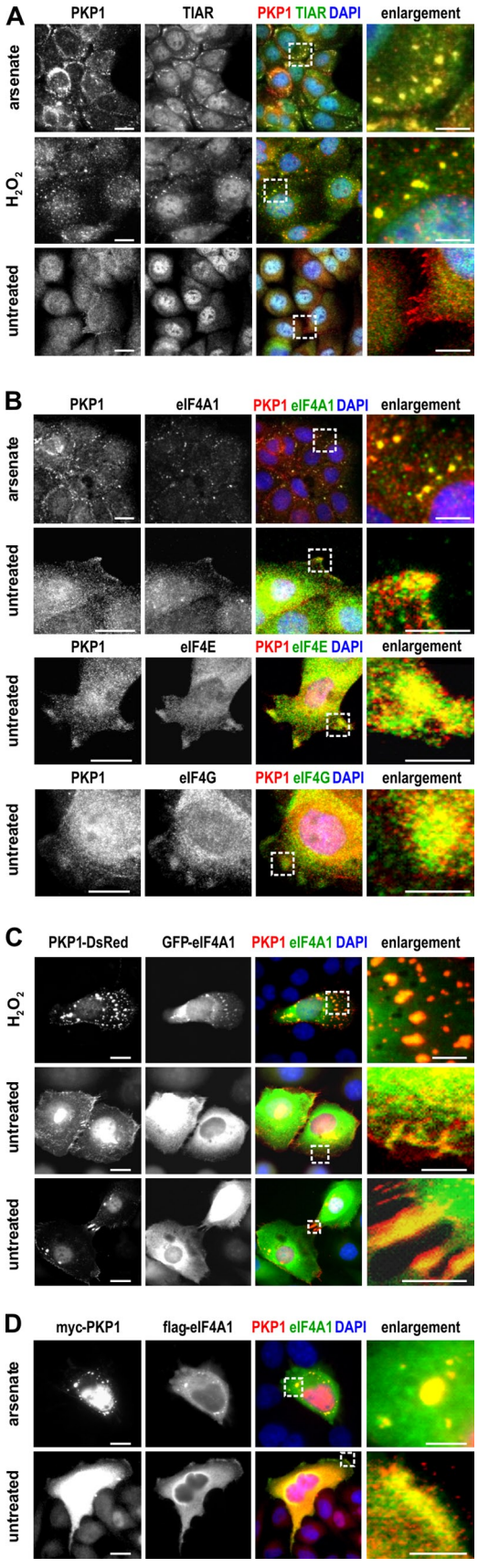

Figure 1. **PKP1 colocalizes with eIF4A1.** (A and B) HaCaT cells were exposed to 1 mM arsenate or 3 mM H_2O_2 for 1-h treatment or were left untreated, fixed, and stained for PKP1 and the SG marker TIAR (A) or for PKP1 and subunits of the initiation complex (eIF4A1, -4E, and -4G; B). (C and D) HaCaT cells were transfected with PKP1-DsRed and GFP-eIF4A1 (C) or myc-PKP1 and Flag-eIF4A1 (D). After 24 h, cells were treated with H_2O_2 (C) or arsenate (D) or left untreated, fixed, and stained with myc and Flag antibodies (D). Nuclei were labeled with DAPI. (A–D) Dashed boxes indicate the enlarged areas. Bars: (A–D, left) 10 µm; (A–D, right) 5 µm.

These findings suggest a role of PKPs in posttranscriptional gene regulation. However, a putative function of PKPs in SGs or in the control of translation remained elusive.

Translation initiation is a multistep process involving the assembly of ribosomes and Met-tRNA at the start codon and is mediated by several eukaryotic translation initiation factors (eIFs; Pestova et al., 2001). The eIF4F complex is responsible for recognition of the mRNA via the 5′ cap and recruitment of ribosomes to mRNAs (Gingras et al., 1999). eIF4F consists of eIF4A, -4G, and -4E. eIF4E binds directly to the cap structure (Goodfellow and Roberts, 2008). eIF4G acts as a scaffold to bridge the mRNA to the 40S ribosomal subunit via its interaction with eIF3 (Prévôt et al., 2003; Hinton et al., 2007). eIF4A reveals ATP-dependent helicase activity and is thought to unwind mRNA secondary structures in the 5′ untranslated region, allowing the 40S ribosomal subunit to bind and scan for the start codon (Svitkin et al., 2001). The activity of free eIF4A is low but stimulated by eIF4B and -4H (Rogers et al., 2001) and is increased in the eIF4F complex (Rogers et al., 2001; Oberer et al., 2005).

In an attempt to characterize a putative role of PKP1 in controlling translation, we identified eIF4A1 as its binding partner. PKP1 associated directly with eIF4A1 and stimulated its activity. In mammalian cells, PKP1 stimulated translation and recruitment of eIF4A1 to translation initiation complexes. Moreover, the PKP1 knockdown was correlated with a downregulation of cell proliferation and cell size. These findings identify PKP1 as a novel regulator of eIF4A1 activity.

Results and discussion

PKP1 associates with eIF4A1

To investigate whether and how PKP1 is involved in controlling protein synthesis, we performed a yeast two-hybrid screen, which revealed an interaction between PKP1 and eIF4A1. To determine whether both proteins colocalized, their distribution was analyzed in the absence or presence of cell stress. Fluorescence microscopy showed that PKP1 colocalized with the SG marker TIA-1–related protein (TIAR) in SGs in arsenate- or H_2O_2-treated cells but did not colocalize with TIAR in unstressed cells (Fig. 1 A; Hofmann et al., 2006). In contrast, eIF4A1 and PKP1 colocalized in stressed cells in SGs but, in addition, revealed overlapping localization in untreated cells preferentially in the perinuclear region and at cell borders (Fig. 1 B). A colocalization in these regions was also detected with eIF4E and -4G, suggesting a colocalization with the initiation complex (Fig. 1 B). After overexpression, PKP1-DsRed and GFP-eIF4A1 or myc-PKP1 and Flag-eIF4A1 were recruited to SGs and localized at cell borders and at cell contacts in unstressed cells (Fig. 1, C and D).

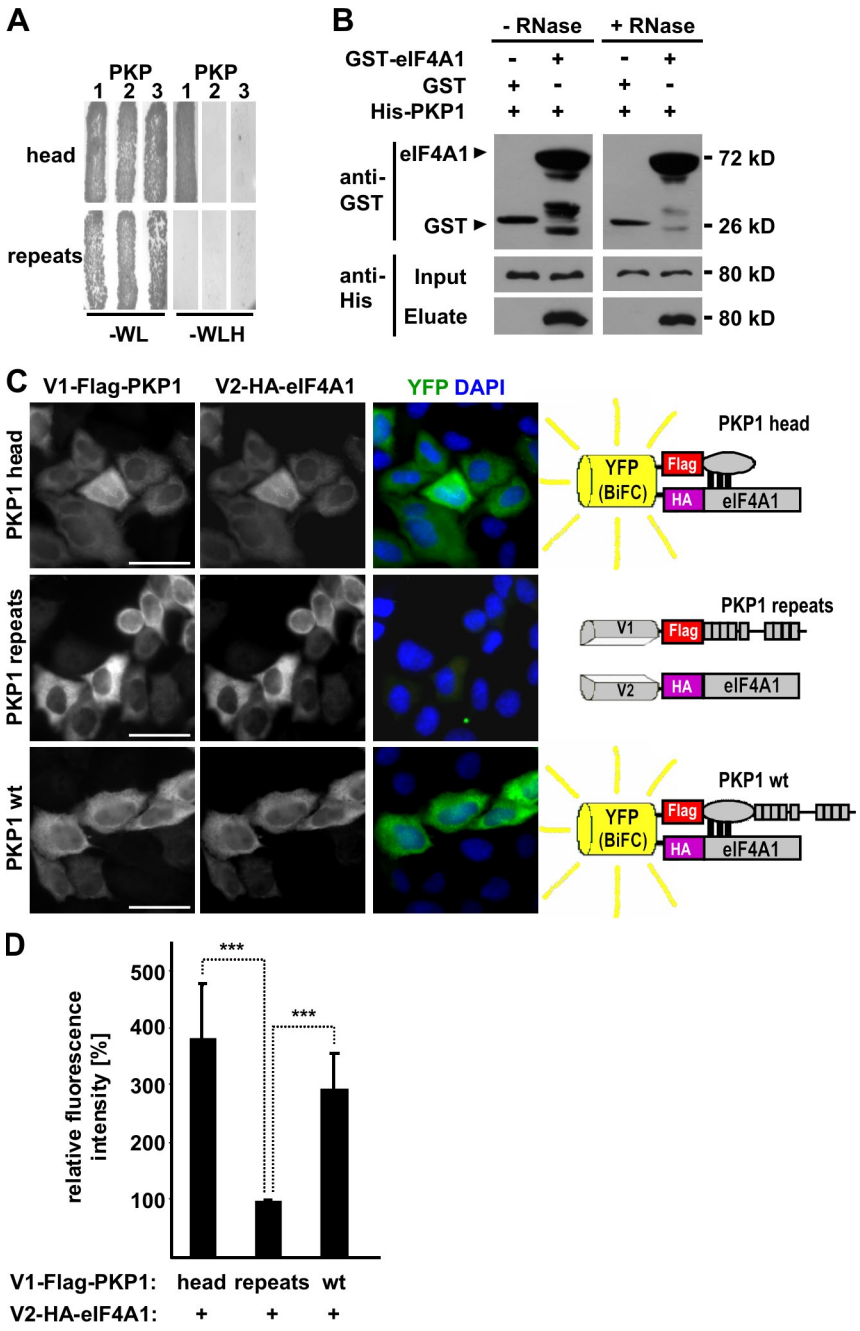

Figure 2. **PKP1 interacts with eIF4A1.** (A) Yeast two-hybrid analysis. YRG2 cells were transformed with PKP1, -2, or -3 constructs and eIF4A1. Transformants were plated on selection plates lacking tryptophan and leucine (−WL) and reporter plates lacking tryptophan, leucine, and histidine (−WLH). (B) GST pull-down assays. GST-tagged eIF4A1 and GST (control) were immobilized on glutathione beads and probed for an interaction with PKP1 in the absence or presence of RNase A. Binding of His-tagged PKP1 to GST-tagged eIF4A1 was determined by Western blotting. (C) BiFC analysis. HeLa cells were cotransfected with the indicated constructs. At 24 h, transfected cells were identified by staining for the Flag and HA epitopes. YFP indicates the BiFC signal. (D) YFP fluorescence intensity was quantified by FACS. Mean values of three independent experiments counting >20,000 cells each are shown. PKP1 repeats were used as reference. Error bars indicate the SD. ***, $P \leq 0.0005$. wt, wild type. Bars, 20 µm.

More detailed yeast two-hybrid experiments revealed an association of eIF4A1 with the N-terminal head domain of PKP1 but not with the C-terminal repeat domain or the N- or C-terminal domains of PKP2 and -3 (Fig. 2 A). A direct association of both proteins was probed in vitro by GST pull-down analyses using recombinant GST-eIF4A1 and His-tagged PKP1. PKP1 copurified with GST-eIF4A1 but not GST alone in the absence or presence of RNase, which is indicative of a specific protein–protein association (Fig. 2 B). To further confirm the association of PKP1 and eIF4A1 in vivo, we used bimolecular fluorescence complementation (BiFC; Fig. 2, C and D; Wolf et al., 2006). These experiments revealed an association of both proteins in the cytoplasm. Quantification of the BiFC signal by FACS confirmed that binding was mediated by the PKP1 head domain (Fig. 2 D). In agreement with the localization of both proteins in SGs, a specific BiFC signal was observed in these structures upon arsenate treatment (Fig. S1, A and B). Although the PKP1 repeats were recruited to SGs and colocalized with eIF4A1 in these structures (Fig. S1, A and B), there was no BiFC signal, which is indicative of a specific interaction of the PKP1 head domain with eIF4A1 in this assay. Recruitment of the PKP1 repeats to SGs appears partially mediated by dimerization with endogenous PKPs because knockdown of PKP3 reduced SG association of the PKP1 repeat domain (Fig. S1 C). However, an association with other RBPs may also contribute to this effect.

Collectively, these results indicate that PKP1 interacts with eIF4A1 in vitro and in vivo. Because of the role of eIF4A1 in translational control, these findings suggested that PKP1

modulates translation via eIF4A1 and/or by acting as a scaffolding factor of SGs.

PKP1 and eIF4A1 are not essential for SG formation

To address a putative role of PKP1 as a scaffolding factor, SG formation was analyzed in response to eIF4A1 and PKP1 knockdown. Knockdown of both factors was efficient and specific, as determined on the mRNA and protein levels, respectively (Fig. 3, A and B). However, SG formation appeared largely unaffected by both siRNAs (Fig. 3, C and D). This suggests that neither PKP1 nor eIF4A1 are essential scaffolding factors required for SG formation. PKP1 may be recruited to SGs via an interaction with other proteins of the translational machinery or via RNAs.

PKP1 stimulates translation

Given the well-established role of eIF4A1 in translation initiation, a putative effect of PKP1 on cap-dependent translation was examined in comparison with PKP2 and -3 by using a firefly luciferase (FFL) reporter assay. To directly measure the effect on protein synthesis and avoid bias by variations in transfection efficiencies or mRNA turnover, luciferase activity was normalized to luciferase mRNA levels determined by quantitative RT-PCR (qRT-PCR). Overexpression of eIF4A1 up-regulated translation ~1.7-fold, whereas PKP1 induced an approximate twofold increase of luciferase activity compared with cells transfected with GFP (Fig. 4 A). In contrast, PKP2 had no effect on translation rates, and PKP3 promoted translation less efficiently than PKP1 (Fig. 4 A). The PKP1 head domain stimulated translation ~1.8-fold, confirming the importance of this domain and its association with eIF4A1, whereas translation remained essentially unaltered after PKP1 repeat overexpression (Fig. 4 B). Because PKP3 did not interact with eIF4A1 in our yeast two-hybrid assay, the mechanism of its function in translation may differ from that of PKP1. In coimmunoprecipitation assays, PKP3 was reported to complex with poly(A)-binding protein (Hofmann et al., 2006), a component of the initiation complex which stimulates eIF4F activity (Bi and Goss, 2000). Thus, PKP3 could stimulate translation via poly(A)-binding protein.

Reporter activity was also measured after eIF4A1 knockdown (Fig. 4 C). Because eIF4A1 is an essential initiation factor, translation was down-regulated in these cells to ~60%. Whereas PKP1 stimulated translation by more than twofold in control siRNA–transfected cells, this effect was reduced to ~1.5-fold in the eIF4A1 knockdown cells, suggesting that the stimulatory activity of PKP1 depends on eIF4A1. The remaining activity was likely mediated via residual eIF4A1 and -4A2, which was slightly up-regulated after eIF4A1 knockdown (Fig. 3 A).

Control of translation by recombinant PKP1 was also analyzed in rabbit reticulocyte lysates. Consistent with the in vivo observation, translation of capped chloramphenicol acetyltransferase (CAT) reporter RNA was enhanced by PKP1 in a concentration-dependent manner, indicating that PKP1 stimulated cap-dependent translation in vivo and in vitro (Fig. 4, D and E). To examine whether the stimulation of translation by

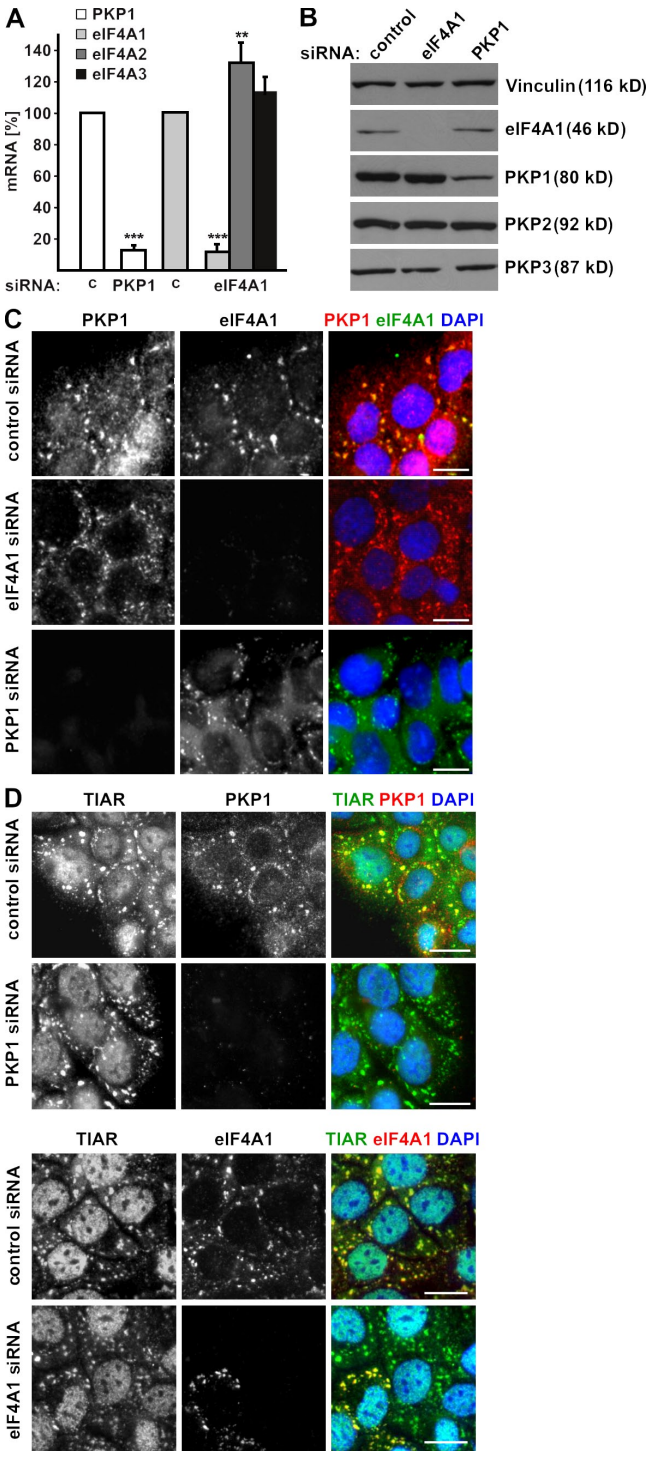

Figure 3. **Depletion of PKP1 or eIF4A1 does not prevent SG formation.** (A and B) HaCaT cells were transfected with siRNAs for PKP1 or eIF4A1 or a control siRNA (c). Knockdown efficiencies were determined at 48 h on the mRNA level by qRT-PCR (mean values of five independent experiments; **, $P \leq 0.005$; ***, $P \leq 0.0005$; A) and on the protein level by Western blot (B). Error bars indicate the SD. (C and D) Cells were treated with 1 mM arsenate for 1 h and stained for eIF4A1 and PKP1 (C) or TIAR and PKP1 or TIAR and eIF4A1 (D). Bars, 20 µm.

PKP1 was indeed eIF4A1 dependent, we analyzed in vitro translation of eIF4A1-dependent (encephalomyocarditis virus [EMCV]) or -independent (hepatitis C virus [HCV]) internal

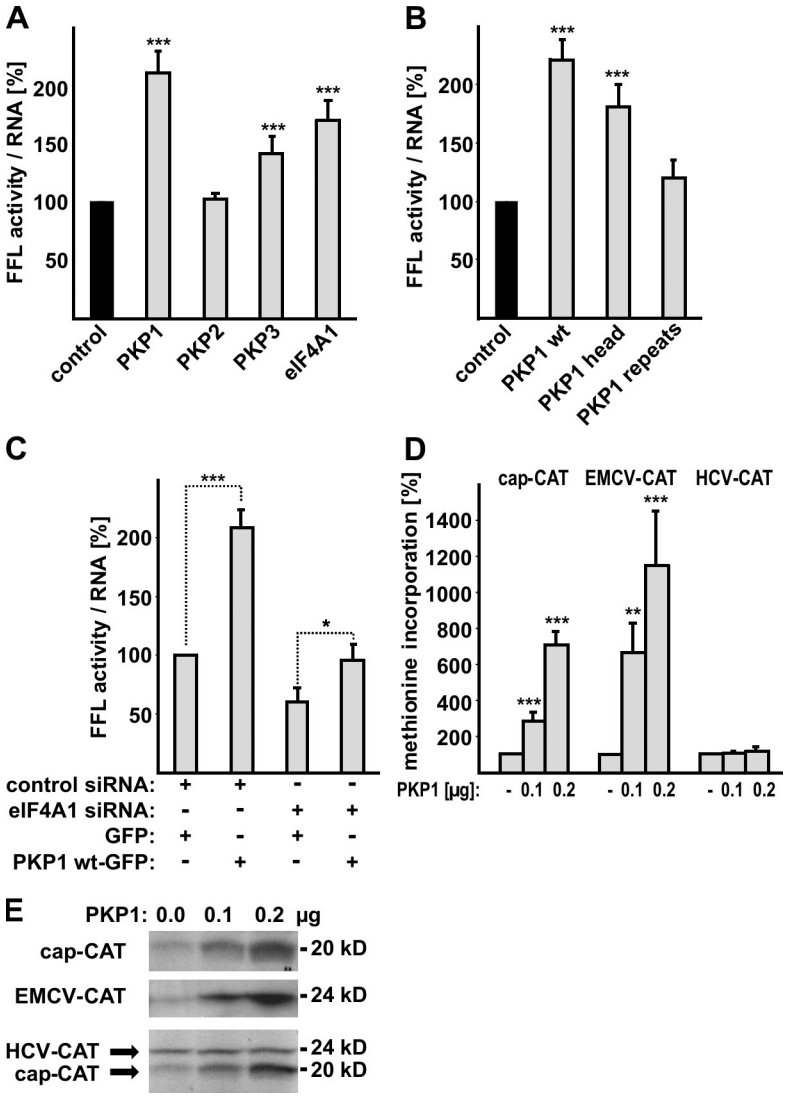

Figure 4. **PKP1 stimulates eIF4A1-dependent translation.** (A–C) The FFL reporter construct was cotransfected with the indicated GFP plasmids and siRNAs into HEK293 cells. Luciferase activity was determined and normalized to the luciferase mRNA level at 48 h. (D and E) In vitro–transcribed reporter mRNAs as indicated were translated in rabbit reticulocyte lysates in the absence or presence of recombinant PKP1. The products were resolved by SDS-PAGE and subjected to autoradiography (E). (D) Newly translated protein was quantified relative to buffer controls (no PKP1). The ^{35}S intensity in the absence of PKP1 was set to 100%. (A–D) Data represent the mean of three independent experiments. Error bars indicate the SD. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$. wt, wild type.

ribosomal entry site (IRES)–containing reporter constructs (Fig. 4, D and E; Pestova et al., 1998). Recombinant PKP1 stimulated EMCV IRES–dependent translation in a dose-dependent manner. In contrast, HCV IRES–mediated translation remained unaffected by the addition of PKP1, whereas cotranslated cap-CAT was increased in the same sample. These results indicated that PKP1 modulates eIF4A1-dependent translation, whereas eIF4A1-independent translation remained completely unaffected. This supported our conclusion that the PKP1–eIF4A1 association is necessary for mediating the effect of PKP1 on translation initiation.

PKP1 stimulates eIF4A1 ATPase activity

We hypothesized that PKP1 could affect translation via eIF4A1 by regulating eIF4A1 activity directly and/or by acting as a scaffold to facilitate initiation factor complex formation. At first, we analyzed the effect of PKP1 on eIF4A1 activity. eIF4A has ATPase and helicase activity, which is thought to be critical for unwinding secondary structures in the 5′ untranslated region to facilitate translation initiation (Svitkin et al., 2001). Using an in vitro ATPase assay, we found that purified recombinant PKP1 showed a modest ATPase activity (Fig. 5 A), which may be the result of contamination with a bacterial ATPase although we used different purification strategies and the recombinant PKP1 appeared essentially pure, as judged by SDS-PAGE (Fig. S2, A and B). Moreover, PKP3 purified according to the same scheme revealed essentially no ATPase activity. ATPase activity of purified eIF4A1 was considerably increased in a concentration-dependent manner after the addition of substoichiometric amounts of PKP1 (Fig. 5, A and B) but not after the addition of PKP3. Assuming that PKP1 and eIF4A1 can form a 1:1 complex, an ∼60 and ∼150% increase after the addition of ∼10 or 20% molar amounts of PKP1 corresponds to an approximately six times higher activity of the eIF4A1–PKP1 complex. This is in good agreement with a two- to sevenfold stimulation of eIF4A activity reported for eIF4B and -4H and the eIF4F complex (Richter et al., 1999; Rogers et al., 1999, 2001; Korneeva et al., 2005; Low et al., 2005). Higher concentrations of PKP1 could not be analyzed because PKP1 aggregated under these conditions and the addition of arginine to assist folding interfered with eIF4A1 ATPase activity. Collectively, these results indicated that PKP1 stimulates eIF4A1 ATPase activity in a

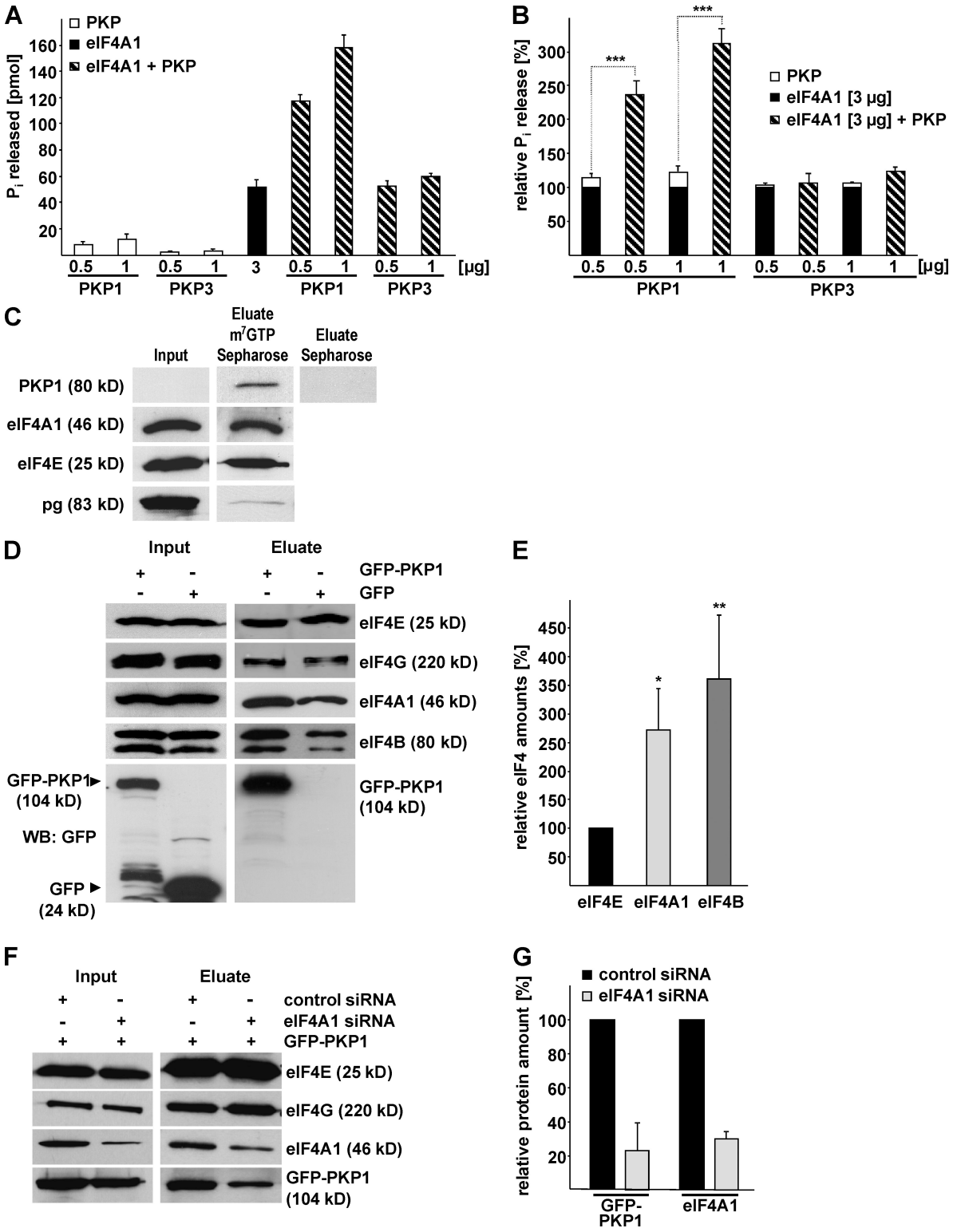

Figure 5. **PKP1 stimulates eIF4A1 activity and its recruitment to the cap-binding complex.** (A) ATPase assays were performed in the presence of 500 µM ATP, 1 µg poly(A) RNA, and PKP1 or PKP3, eIF4A1, or eIF4A1 + PKP as indicated. (B) Activity in the presence of PKP1 and eIF4A1 is presented relative to the sum of the individual activities. eIF4A1 activity was set to 100%. (C–G) Lysates from untransfected HaCaT cells showing endogenous proteins (C) and extracts from HEK293 cells (D–G) transfected with GFP-PKP1 or GFP alone (D and E) or in combination with control or eIF4A1 siRNA (F and G) were incubated with m^7GTP-Sepharose. Bound protein was eluted in SDS buffer and characterized by Western blotting (WB) with the indicated antibodies. Proteins in the eluates were quantified relative to eIF4E (E) or relative to eluates from control siRNA–transfected cells (G). pg, plakoglobin. (A–G) Data represent the mean of three (A–E) and two (F and G) independent experiments. (A, B, E, and G) Error bars indicate the SD. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$.

concentration-dependent manner, whereas no such activity could be measured for PKP3 that did not associate with eIF4A1 in the yeast two-hybrid system.

PKP1 stimulates eIF4A1 recruitment into the m^{7}GTP cap–binding complex

Next, we tested whether PKP1 modulates recruitment of eIF4A1 to the initiation complex. eIF4F complexes were isolated by m^{7}GTP cap–Sepharose affinity purification. As expected, eIF4E and -4A1 were copurified by this approach, as previously reported (Fig. 5 C; Low et al., 2005; Bordeleau et al., 2006). Moreover, we found that PKP1, which is mostly insoluble and thus barely detectable in the soluble fraction, was highly enriched in the purified complex, whereas association with Sepharose beads alone was not observed, indicating specific binding. To further validate the specificity of the PKP1 cap association, we also analyzed the distribution of plakoglobin, another armadillo family protein present in desmosomes. Plakoglobin, although present in much higher amounts in cell lysates, was barely copurified with m^{7}GTP cap–Sepharose (Fig. 5 C). These results suggested a specific association of PKP1 with the cap complex.

We then compared copurification of eIF4F components from GFP (control)- or PKP1-GFP–transfected cells. Elevated amounts of eIF4A1 and -4B, a factor which enhances eIF4A processivity (Rogers et al., 2001), were copurified from cells overexpressing PKP1. In contrast, the amount of copurified eIF4E and -4G remained essentially unaltered after PKP1 overexpression (Fig. 5, D and E). Similar results were obtained by using m^{7}GTP instead of SDS for elution (unpublished data). This indicates that PKP1 promotes recruitment of eIF4A1 and -4B to the preinitiation complex. When eIF4A1 was knocked down before PKP1 or GFP overexpression, strongly reduced amounts of eIF4A1 as well as PKP1 were recruited to the cap complex (Fig. 5, F and G), confirming our conclusion that PKP1 is recruited via eIF4A1 and in turn increases the association of eIF4A1 with the complex.

Recently, it has been shown that eIF4A and -4B can associate with mRNAs not only directly adjacent to the cap structure but also up to 52 bases downstream from the cap (Lindqvist et al., 2008a). These authors proposed a model in which multiple eIF4A and -4B subunits seed the 5′ end of mRNAs to produce a stable mRNA–protein complex. According to this model, PKP1 could potentially enhance translation rates by stimulating eIF4A1 recruitment and activity, which in turn could enhance eIF4B association for further activation.

PKP1 regulates proliferation and cell size

Given the finding that PKP1 positively regulates translation, we investigated the role of PKP1 in controlling cell growth. Upon the knockdown of PKP1 or eIF4A1, cell numbers were significantly reduced at 72 and 96 h after transfection (Fig. S3, A and B). A BrdU incorporation assay to quantify replication rates confirmed the reduction in proliferation rates (Fig. S3 C). The effect was confirmed with a distinct PKP1 siRNA that was less efficient both in reducing PKP1 protein levels and in reducing proliferation (PKP1 #2; Fig. S3, A–C). Additionally, we noticed that PKP1 siRNA–transfected cells were smaller than control siRNA–transfected cells, providing further evidence that PKP1 regulates cell growth (Fig. S3, D and E).

Collectively, we have demonstrated in this study that PKP1 is involved in regulating eIF4A1-dependent translation. This appears to be correlated with a function in controlling proliferation and cell size. To our knowledge, this is the first study characterizing an adhesion-independent function of PKP1 at the molecular level. PKP1 appears to act via two probably interconnected mechanisms: it stimulates recruitment of eIF4A1 to the initiation complex, and it directly regulates eIF4A1 activity, presumably in conjunction with eIF4B.

Our findings have important implications considering putative roles of PKP1 in disease. Up-regulation of translation either by the overexpression of certain eIFs or by up-regulation of signaling pathways controlling translation such as the mTOR (mammalian target of rapamycin) pathway has been observed in many types of tumors (Averous and Proud, 2006). In agreement, the overexpression of some eIFs can promote cell transformation and tumor progression (Shuda et al., 2000; Dong and Zhang, 2006; Graff et al., 2008; Sonenberg, 2008). Thus, it is tempting to speculate that the up-regulation of PKP1 expression could play a role in tumorigenesis. This hypothesis is supported by multiple studies on elevated expression or de novo synthesis of PKP1 in some tumors such as head and neck carcinomas (Villaret et al., 2000) and Ewing sarcoma (Cheung et al., 2007). Interestingly, PKP3 is up-regulated in some tumors as well (Furukawa et al., 2005). In contrast, no such finding has been reported for PKP2, which, according to our results, has no activity in the regulation of protein synthesis. Moreover, our findings may have implications for understanding the molecular mechanism of the genetic disease caused by mutations in PKP1 (McGrath, 1999). Affected individuals exhibit skin fragility with blistering but, in addition, reveal defects in skin appendages such as hair, nails, and sweat glands and also a general failure to thrive. This could possibly be linked to a role of PKP1 in controlling protein synthesis and proliferation.

Materials and methods

Plasmids and antibodies

Human PKP1 constructs have been described previously (Hatzfeld et al., 2000). All PKP constructs and eIF4A1 were amplified by PCR with 5′ EcoRI and 3′ SalI restriction sites for subcloning. PKP1 wild type was subcloned into pDsRed-N1, pEGFP-C2 (BD), pcDNA4-TO-myc, and pRSET (Invitrogen). PKP2 wild type and PKP3 wild type were subcloned into pEGFP-C2. PKP1 head (aa 1–286) and PKP1 repeats (aa 287–726) were cloned into pEGFP-C2 and pGBKT7 (BD). PKP2 head (aa 1–394), PKP3 head (aa 1–342), PKP2 repeats (aa 395–837), and PKP3 repeats (aa 343–797) were inserted into pGBKT7. eIF4A1 was cloned into pEGFP-C2, pcDNA3-Flag, pRSET, pGADT7 (BD), and pGEX-5 × 1 (GE Healthcare). For BiFC constructs, the pEGFP vector backbone was used. EGFP was removed by cutting with NheI and BglII, and the cDNAs corresponding to the YFP fragments (V1: N terminus, aa 1–154; V2: C terminus, aa 155–238) were inserted using the same restriction sites. Flag or HA epitope tags were inserted between BglII and EcoRI sites. PKP1 wild type, head, and repeats were inserted into the EcoRI and XhoI sites of pV1-Flag, and eIF4A1 was inserted into the EcoRI and SalI sites of pV2-HA. Plasmids of cap-CAT–poly(A) and EMCV-CAT were described previously (Ostareck et al., 1997, 2001).

The primary antibodies used for immunostaining and Western blotting were against the PKP1 head domain (Hatzfeld et al., 2000), PKP2 and

-3 (Progen), TIAR, eIF4E and -4G (BD), vinculin, Flag, plakoglobin (Sigma-Aldrich), eIF4A1 (Santa Cruz Biotechnology, Inc.), HA (Rockland), myc (Dianova), and eIF4B (Cell Signaling Technology). Secondary antibodies were obtained from The Jackson Laboratory and Invitrogen.

Yeast two-hybrid analysis
YRG2 yeast cells were cotransformed with PKP1, -2, and -3 constructs in pGBKT7 and eIF4A1 in pGADT7. All procedures were performed according to the yeast protocols handbook (BD).

Cell culture and transfection
HaCaT, HeLa, and HEK293 cells were grown in DME with 10% FCS. The assembly of SGs was induced by 1 mM Na arsenate or 3 mM hydrogen peroxide for 1 h. Plasmids were transfected with Lipofectamine 2000 (Invitrogen). For knockdown analysis in HaCaT cells, siRNAs were transfected by Nucleofector II (Lonza). siRNAs used in this study were as follows: PKP1 #1, 5′-GACCAUGCGUAACUACUCAdTdT-3′; PKP1 #2, 5′-CAAUCGAGGUUCCAUGUAUdTdT-3′; PKP3, 5′-GACUCCGAAAGCUCAUCUUCAdTdT-3′; eIF4A1, 5′-CUGGCCGUGUGUUUGAUAUdTdT-3′; and control, 5′-AAGCUGACCCUGAAGUUCAUCUGCACC-3′. Except when otherwise stated, all PKP1 knockdown experiments were performed using siRNA #1.

Fluorescence microscopy
Cells grown on cover slides were rinsed in PBS and fixed in methanol at −20°C for 10 min, followed by treatment with 0.5% Triton X-100 in PBS for 15 min. Alternatively, cells were fixed in 3.7% formaldehyde in PBS for 15 min at room temperature and permeabilized in 0.5% Triton X-100 in PBS for 15 min. Cells were washed in PBS and blocked for 30 min in PBS containing 1% dry milk before antibody application. Cells were mounted in Mowiol, and images were acquired with a microscope (E600; Nikon) with a 60× objective (Apochromat TIRF; Nikon) connected to a charge-coupled device camera (CCD-1300QLN; VDS Vosskühler) using NIS Elements AR 2.30 software (Nikon). Microscopy and image capture were performed at room temperature, and Photoshop (version 7.0; Adobe) was used for image processing (adjustment of brightness and contrast).

qRT-PCR
RNA was extracted using TRIZOL (Invitrogen), and 2 µg of RNA was reverse transcribed with random primers (Fermentas). qRT-PCR was based on the SYBR green technology using 2xTaq-Master mix (Promega). Real-time PCR quantification was performed according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Recombinant protein purification
His-tagged PKP1 was purified using Ni–nitrilotriacetic acid agarose, followed by Q-Sepharose chromatography (GE Healthcare) or gel filtration chromatography (Superdex2000; GE Healthcare) in 50 mM Tris, pH 8, 100 mM NaCl, 250 mM arginine, 2 mM DTT, and 1 mM EDTA. Recombinant purified PKP3 was provided by C. Kiessling (Martin Luther University Halle-Wittenberg, Halle, Germany). Recombinant eIF4A1 was purified using Ni–nitrilotriacetic acid agarose and Q-Sepharose chromatography as described previously (Lindqvist et al., 2008b). The eluted protein was dialyzed against 20 mM Hepes, pH 7.5, 100 mM KCl, 5% glycerol, 1 mM DTT, and 1 mM EDTA.

GST pull-down
eIF4A1-GST was coupled to glutathione agarose (Thermo Fisher Scientific), and 3 µg of purified PKP1 was added and incubated for 2 h at 4°C with or without 10 µg/ml RNase A. Proteins were eluted in SDS-PAGE buffer and analyzed by Western blotting.

BiFC analysis
HeLa cells were cotransfected with pV2-HA-eIF4A1 and a vector expressing PKP1 head, repeats, and wild type in pV1-Flag. At 24 h, fluorescence was detected in the E600 microscope (see Fluorescence microscopy) and quantitated using FACS analysis. Transfected cells were identified by antibody staining against the Flag and HA tags.

Reporter assays
For cap-dependent in vivo translation assays, FFL-pcDNA3 and GFP (control) or GFP-PKP constructs (wild type, head, repeats) or GFP-eIF4A1 constructs were cotransfected into HEK293 cells. Luciferase activity was measured at 48 h and normalized to FFL-mRNA levels.

For in vitro reporter assays, cap-CAT–poly(A) , EMCV-CAT, and HCV-CAT mRNAs were transcribed (MEGAscript T7 kit; Applied Biosystems) and translated in reticulocyte lysates (Promega) with [^{35}S]methionine in the presence of 0–200 ng of purified PKP1. The products were resolved by SDS-PAGE and subjected to autoradiography.

m^{7}GTP-Sepharose affinity purification
m^{7}GTP-Sepharose affinity purification was performed essentially as described previously (Willett et al., 2006).

ATPase assay
ATP hydrolysis was determined by colorimetric measurement of phosphate release (Chan et al., 1986). Reactions were incubated at 37°C for 1 h and contained 15 mM Hepes, pH 7.5, 80 mM KCl, 2.5 mM magnesium acetate, 1 mM DTT, 500 µM ATP, 1 µg poly(A), and eIF4A1 and PKP1 or -3 as indicated. The quantity of free phosphate was determined by the addition of malachite green/molybdate reagent (AnaSpec). ATP hydrolysis in the absence of protein was subtracted as background.

Proliferation assay
Proliferation was determined using a BrdU labeling and detection kit (Roche). To avoid influences caused by experimental variation such as variability in cell seeding, all data were normalized to values determined at 24 h.

Online supplemental material
Fig. S1 shows that eIF4A1 associates with PKP1 in SGs, as demonstrated by BiFC analysis. Fig. S2 demonstrates the quality of purified recombinant PKP1 by Coomassie staining. Fig. S3 shows that PKP1 regulates proliferation and cell size. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200908135/DC1.

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