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Paip2 cooperates with Cbp80 at an active promoter and participates in RNA Polymerase II phosphorylation in *Drosophila*

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Abstract

The Paip2 protein is a factor regulating mRNA translation and stability in the cytoplasm. It has also been found in the nuclei of several cell types in *Drosophila*. Here, we aim to elucidate the functions of Paip2 in the cell nucleus. We find that nuclear Paip2 is a component of an ~300 kDa protein complex. Paip2 interacts with mRNA capping factor and factors of RNA polymerase II (Pol II) transcription initiation and early elongation. Paip2 functionally cooperates with the Cbp80 subunit of the cap-binding complex, with both proteins ensuring proper Pol II C-terminal domain (CTD) Ser5 phosphorylation at the promoter. Thus, Paip2 is a novel player at the stage of mRNA capping and early Pol II elongation.

Keywords

transcription; promoter; capping; Paip2; Cbp80; protein complex

INTRODUCTION

The Paip2 protein (Polyadenylate-binding protein-interacting protein 2) is a metazoan-specific factor controlling translation efficiency and mRNA stability in the cytoplasm [1–6]. Paip2 protein is thought to function in translational regulation by interacting directly with the cytoplasmic polyA-binding protein (PABP). Paip2 association with PABP inhibits PABP binding to polyA tails of mRNA, and this leads to downregulation of mRNA translation. In

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AUTHOR CONTRIBUTIONS

Z.M.K. planned and performed the experimental work and wrote the paper; L.A.L., A.V.S., J.J.M., and J.R.Y. performed the experimental work; P.S. and Y.V.Sh. planned the experiments and wrote the paper.

COMPETING INTERESTS

The authors declare that they have no competing interests.

mammals, Paip2 proteins have important functions in spermatogenesis, oogenesis, and in the functioning of the nervous system. Paip2a is expressed in late spermatids, while its paralogue Paip2b shows stronger expression in oocytes [7]. Paip2a-knockout mice are male sterile, indicating an important role for this protein as a translational regulator during sperm maturation [8]. In neurons, Paip2 is a major factor in synaptic plasticity, learning, and memory formation. In stimulated neurons, Paip2a is degraded by a calpain-dependent mechanism, which results in enhanced expression of memory-related genes [9]. This protein has a role not only in normal development and differentiation: changes in the levels of Paip2 are associated with leukoplakia and oral squamous cell carcinomas [10]. Paip2 also suppresses cellular transformation mediated by the *hRas V12* oncogene in NIH 3T3 cells [11]. There is a Paip2 homologue in *Drosophila*. Like its mammalian counterparts, the *Drosophila* Paip2 protein is broadly expressed, interacts with PABP in the cytoplasm, and inhibits PABP binding to mRNA polyA tails. Moreover, overexpression of Paip2 in proliferating cells of the wing discs results in a size reduction phenotype due to a decrease in cell number [12].

We have recently shown that *Drosophila* Paip2 is found not only in the cytoplasm but also in the nucleus [2], with its nuclear localization being detected in embryos, salivary glands, testes, and S2 tissue culture cells. In salivary gland nuclei, Paip2 is associated with chromosomes, where it is localized mainly to interbands and puffs. Paip2 in S2 cells may be crosslinked to the promoter regions of actively transcribed genes. We have found that chromatin association is RNA dependent, and our experiments indicate that Paip2 is bound to RNA sequences at or near the 5’ end of nascent transcripts. To learn more about its association with promoters and nascent transcripts, we have used a multistep procedure to identify Paip2-associated proteins. The proteins we have identified include the cap-binding protein 80 (Cbp80), which is one of the components of the nuclear cap-binding complex. We have found that both Cbp80 and Paip2 modulate Pol II CTD Ser5 phosphorylation status.

MATERIAL AND METHODS

Ethics statement

Animal handling for the antibody production was carried out strictly according to the procedures outlined in the NIH (USA) Guide for the Care and Use of Laboratory Animals. The protocols used were approved by the Committee on Bioethics of the Institute of Gene Biology, Russian Academy of Sciences. All procedures were performed under the supervision of a licensed veterinarian, under conditions that minimize pain and distress.

Experiments with S2 cell culture

Drosophila Schneider cell line 2 (S2) was maintained in SFX medium (HyClone) at 25°C. To activate the ecdysone cascade, the cells were treated with 1 μM ecdysone overnight. RNAi experiments and immunostaining were performed as described previously [2].

Antibodies

Affinity purified antibodies against full-length Paip2 [2], Cbp80 (127–261 aa fragment), and immune serum against Cdk7 (1–100 aa fragment) (Suppl. Fig. 1) were raised in rabbits.

Antibodies against Pol II (ab817, clone 8WG16), Pol II-S5P (ab5131), Pol II-S2P (ab5095), and PAR (ab14459) were from Abcam. The monoclonal antibody JLA20 against actin, raised by J.J.-C. Lin, was obtained from the Developmental Studies Hybridoma Bank created by the National Institute of Child Health and Human Development and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

ChIP and Quantitative (q) PCR Analysis

The protocol for ChIP with S2 cells was described previously [13]. Measurements in an intergenic region and ChIP with antibody-free Sepharose beads (background level) were used as negative controls in each experiment. The sequences of the primers are given in the Supplement. Each experiment was performed in three replicates, mean value and standard deviation are shown on diagrams. Comparisons between experimental groups were made using Student's *t*-test, asterisks indicate that the data are statistically significant at $p < 0.05$.

Western blotting and Immunoprecipitation

Antibodies used in WB were diluted 1:500, each WB experiment was repeated at least twice. To extract proteins, S2 cells were lysed in lysis buffer [10 mM HEPES, pH 7.9 with 5 mM $MgCl_2$, 0.5% Nonidet P-40, 0.45 M NaCl, 1 mM DTT, and complete protease inhibitor mixture (Roche)]. IP was performed as described [14]. DNase I (1 U/μL) and RNase A (10 μg/μL) (Thermo Fisher Scientific) were added to IP buffer in 1/1000 dilution. Image acquisition and quantification were performed by ChemiDoc imaging system and ImageLab software (Bio-Rad).

Nuclear extract and gel filtration

Nuclear extracts from 0–12 hour *Drosophila* embryos were prepared as described [15]. Samples of the extract (10 mg protein) were fractionated by gel filtration on a Superose 6 HR 10/30 column (GE Healthcare) in HEMG buffer [25 mM HEPES-KOH, pH 7.6 with 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and complete protease inhibitor mixture (Roche)] containing 150 mM NaCl (HEMG-150). The column was calibrated with an HMW Calibration Kit (GE Healthcare). The void volume of the column was 7.0 mL, and the volume of each fraction was 0.5 mL.

Purification of Paip2-associated proteins and data analysis

Paip2-containing fractions from three gel filtration runs were pooled and loaded onto an affinity column prepared by coupling anti-Paip2 antibodies to protein A Sepharose beads (Sigma) according to the published protocol [16] and equilibrated with HEMG-150 buffer. The loaded column was incubated for 2 hours, washed with HEMG-500 containing 0.1% Nonidet P-40, and eluted with 0.1 M glycine at pH 2.5. The eluted proteins were precipitated with trichloroacetic acid, then reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (Sigma-Aldrich, C4706), and alkylated. Proteins were digested with a solution containing 2 M urea, 1 mM $CaCl_2$, and 2 μg trypsin (Promega, V5111) in 100 mM Tris, pH 8.5, at 37°C for 18 hours. Analysis was performed using an Agilent 1200 quaternary pump and a Thermo LTQ Orbitrap Velos mass spectrometer using a home-built electrospray stage [17].

Protein and peptide identification and quantification analysis were performed with Integrated Proteomics Pipeline (IP2) (Integrated Proteomics Applications, Inc., San Diego, CA. http://www.integratedproteomics.com/). Tandem mass spectra were extracted from raw files using RawExtract 1.9.9 [18] and searched against the UniProt *Drosophila melanogaster* database with reversed sequences using ProLuCID [19, 20]. The search space included all fully-tryptic and half-tryptic peptide candidates for the tryptic digest with a static modification of 57.02146 on cysteine. Peptide candidates were filtered using DTASelect, with the following parameters: -p 2 -y 1 --trypstat --pfp .01 --extra --pI -DM 10 --DB --dm -in --brief --quiet [18, 21].

Data processing was performed using the PattenLab for Proteomics platform (http://patternlabforproteomics.org) [22]. For peptide identification, we used the *Drosophila melanogaster* protein database from Uniprot (www.uniprot.org) with common contaminants and reversed proteins added. Data comparisons were made using the ACFold module in PatternLab.

RESULTS

Paip2 is associated with factors localized on active promoters

With the aim of gaining insights into the roles Paip2 might play in the nucleus, we sought to identify interacting nuclear factors. We first asked whether Paip2 functions on its own or is assembled into a multiprotein complex. For this purpose, we size-fractionated the nuclear extracts from 0–12 hour embryos on a Superose 6 HR 10/30 gel filtration column. The predicted molecular weight of the Paip2 protein is approximately 14 kDa, and it should have eluted in the very last column fractions. Instead, we found that most of the Paip2 protein was assembled into a large macromolecular complex with an estimated molecular weight of about 300 kDa (Fig. 1a). Although Paip2 association with promoter regions in S2 cell chromatin is RNA dependent [2], RNase A and also DNase treatment of the nuclear extract prior to fractionation had no effect on the elution profile. Thus, it appears that Paip2 is incorporated into this complex via a protein–protein interaction.

We used mass spectrometry to identify components of the complex as well as other potential Paip2 protein partners. To maximize our chances of detecting components of this complex, we used a stepwise purification (Fig. 1b). The embryonic nuclear extract was loaded onto a Superose 6 HR 10/30 gel-filtration column, and the eluted Paip2-containing fractions were then fractionated on a protein A-Sepharose column carrying pre-bound Paip2 antibodies. Samples eluted from the antibody affinity column were then used for mass spectrometry analysis. In control experiments, antibody-free protein A-Sepharose was used. As shown in Fig. 1c, only a subset of the proteins present in the input from the Superose 6 HR 10/30 were recovered after immunoaffinity purification with the Paip2 antibody, with these proteins being largely absent in mock sample. The purification procedure was performed four times, and the results were pooled for analysis.

Altogether, 419 proteins were detected by mass spectrometry at a statistically significant level ($p \leq 0.05$) (Suppl. Table 1). Paip2 was detected in the sample both by mass spec

analysis and by Western blotting (Fig. 1c). Its known cytoplasmic partner PABP was detected as well (Fig. 1d).

Since our previous experiments indicated that nuclear Paip2 binds to sequences in nascent pre-mRNAs located close to the promoter, we searched the list of Paip2 associated proteins for factors involved in early steps of mRNA biogenesis. The cap-binding protein 80 (Cbp80) [23] was detected with the highest significance ($p = 0.00001$) (Fig. 1d). In addition, we detected importins alpha and beta, which have a role in the regulation of the cap-binding complex activity [24, 25]. We also detected CG11399, which is a homologue of mammalian PCIF1 (PDX1 C-Terminal Inhibiting Factor 1). PCIF1 is a S5P CTD Pol II-associated cap-specific adenosine methyltransferase responsible for N(6)-methylation of newly synthesized mRNAs [26, 27].

In addition to proteins involved in mRNA biogenesis, we also detected a number of factors that participate in early transcriptional events on active promoters (Fig. 1d). In particular, these were two subunits of RNA Polymerase II, including Rpb1 possessing the C-terminal domain (CTD); preinitiation transcription complex factors Trf2 (TBP-related factor 2) and TFIIH subunit Cdk7; Poly(ADP)-ribose polymerase (PARP), which has functions in promoter-proximal pausing and transcription elongation [28]; Pol II-associated factor Spt6 controlling CTD phosphorylation [29, 30]; and two components of nuclear pore complex that impact transcription [31].

Among the Paip2 associated proteins, Cbp80 appeared to be the most probable candidate for the RNA-dependent recruitment of Paip2 to active promoters. Its presence had the highest statistical significance among all the Paip2-associated proteins, and sequence coverage in the mass spectrometry analysis was also high. In addition, there was a considerable overlap in the elution profiles of Cpb80 and Paip2 (Fig. 1a).

Paip2 and Cbp80 have an effect on Pol II CTD Ser5 phosphorylation

To investigate the possible functions Paip2 and Cbp80 in promoter activity, we used RNAi knockdown in S2 tissue culture cells. We found that knockdowns of Cbp80 and Paip2 substantially reduced the total levels of each protein in Western blots (Fig. 2a). Unexpectedly, however, immunostaining of S2 cells revealed that Paip2 depletion was not complete and that the protein could still be readily detected in the nuclei of S2 cells after RNAi knockdown (Fig. 2b). If this “RNAi-resistant” pool of nuclear Paip2 is still recruited to active promoters, it would not be unreasonable to expect that the effects of the knockdown on gene activity would be at least somewhat mitigated. [There are precedents for the preferential retention of proteins at specific sites after depletion (e.g., as in the case of incomplete CTCF depletion at specific chromosomal loci in null mutant pupae – [32], Fig. 6).]

To study the effects of Paip2 and also Cpb80 knockdown on gene activity, we used ChIP to examine the recruitment of proteins to the promoters of the ecdysone-inducible *DHR3* and *DHR4* genes and heat shock-inducible *hsp70* gene. Promoters of these genes are occupied by Paip2 and Cbp80 upon induction [2]. As expected from our imaging of S2 cells, the effects of the RNAi knockdowns of Paip2 on the occupancy levels of this protein at these

promoters were rather modest (Figs. 2c-2e). While Paip2 occupancy at *DHR3* is clearly reduced, there are only minimal reductions at the *DHR4* and *hsp70* promoters. Knockdowns of Paip2 had little or no effect on Cpb80 occupancy, and vice versa (Figs. 2b-2d). While these results suggest that the recruitment of Cpb80 to promoters is independent of Paip2, the incomplete depletion of both factors from chromatin does not allow us to make unequivocal conclusions regarding the joint or independent recruitment of these two factors.

Since the Rpb1, Rpb2 subunits were on the list of Paip2-associated proteins (Fig. 1d), we tested whether Pol II recruitment depends upon either Paip2 or Cbp80. Western blot analysis showed that neither Paip2 nor Cbp80 knockdown had any detectable effect on the total levels of Pol II (Fig. 2a). Despite the modest effects of the knockdowns on Paip2 or Cbp80 occupancy, Pol II association with the *hsp70* promoter was reduced after Paip2 or Cbp80 knockdown (Fig. 2e). In the case of the *DHR3* and *DHR4* promoters, Cbp80 knockdown was accompanied by a clear reduction in Pol II recruitment, while Paip2 knockdowns had more modest effects on Pol II occupancy (Figs. 2c, 2d). In this context, it is interesting that the effects of the Paip2 and also Cbp80 knockdown on Pol II occupancy are correlated with polymerase recruitment at each promoter in wild type cells. Overall promoter occupancy is highest at the *hsp70* promoter in the absence of knockdown, and for this promoter knockdowns of Paip2 or Cbp80 have the greatest effects on Pol II occupancy. Much smaller effects on Pol II occupancy are observed for the *DHR4* promoter after knockdown, and this promoter has the lowest level of occupancy prior to knockdown.

Capping of nascent transcripts is closely coupled to Ser5 phosphorylation of the heptad repeats in the CTD domain of the Pol II Rpb1 subunit. Since the CTD Ser5 kinase Cdk7 was found to be associated with Paip2 (Fig. 1d), we measured the relative amount of Ser5-phosphorylated Pol II after RNAi knockdown. Unexpectedly, while total amount of Pol II at the three promoters decreased to a greater or lesser extent after knockdown, the level of Pol II-S5P increased in all three cases. The most significant effects were observed for the two promoters that had the highest Pol II occupancy levels in untreated cells, *hsp70* and *DHR3*. For both of these promoters, the level of Pol II-S5P increased approximately twofold after Paip2 or Cbp80 knockdown. More modest effects of S5P were observed for *DHR4*. The alterations in the level of the S5P isoform also appeared to be specific, as they were not observed for the elongation CTD phosphoisoform S2P on the promoters that we tested.

We also examined the effects of Paip2 and Cpb80 knockdown on Pol II occupancy at the *DHR3* promoter without ecdysone induction. In the uninduced state, Pol II levels (and also Paip2 [2]) were significantly lower than those observed after ecdysone induction (compare Figs. 2c and 2f). Under conditions of minimal ongoing transcription, the knockdown of either Paip2 or Cpb80 had little or no effect on either Pol II occupancy of S5P - Pol II levels.

One mechanism that could explain the increase in CTD S5P levels is that Cdk7 occupancy is elevated when Paip2 or Cbp80 are knocked down. However, ChIP analysis showed that the levels of Cdk7 on all three promoters did not change significantly upon Paip2 knockdown (Fig. 2g), and Cbp80 knockdown had the same effect. From this result, we may conclude that the changes in Ser5 phosphorylation are not due to the recruitment of Cdk7 to the

promoter and that some other mechanism(s) is likely responsible for the increase in CTD S5P.

To determine if Paip2 or Cbp80 are required for the gene activity, we measured the levels of *DHR3, DHR4* and *hsp70* mRNA after Paip2 or Cbp80 knockdown. In both cases, we detected no significant reduction in the total amount of these genes' mRNAs following induction (Fig. 2g). Moreover, nascent mRNA synthesis did not appear to be altered by the knockdowns as well (data not shown). These results indicate that RNAi depletion of Paip2 and Cbp80 is not sufficient to block ecdysone induction of the *DHR3* and *DHR4* genes.

To conclude, our experiments revealed high correlation of the effects of both Paip2 and Cbp80 knockdowns, what indicates functional cooperation of these factors.

Paip2 is subject to PAR modification

Since PARP is found associated with Paip2 in the nuclear extracts, we tested whether Paip2 is a target for this enzyme. During electrophoresis of the extract from tissue culture cells and embryos, the ~14-kDa Paip2 protein migrated as a doublet with an apparent size of about 25 kDa (Fig. 2f). To test whether Paip2 is PARylated, we immunoprecipitated Paip2 from the S2 nuclear extract and probed Western blots with either anti-Paip2 or anti-polyADPribose antibodies. The upper band of Paip2 proved to coincide with the band detected by anti-PAR antibodies (Fig. 2f), indicating that Paip2 was partially PARylated.

To further investigate this issue, we performed *PARP* knockdown in S2 cells and then calculated the intensity ratio of the two Paip2 bands on Western blot (Fig. 2j). Quantification of the blot showed that the relative intensity of the upper band decreased upon PARP depletion. The effect was quite modest as *PARP* knockdown efficiency very moderate: only 3-fold drop of the *PARP* transcript level. Thus, our experiments indicate that PAR modification could be responsible for the formation of two Paip2 bands.

DISCUSSION

We have shown previously that the translation factor Paip2 is associated with the promoters of active genes in *Drosophila* [2]. Promoter association is RNA dependent, indicating that Paip2 is likely to be bound to sequences in nascent RNAs that are close to the start of transcription. Since the Paip2 protein lacks an RNA binding domain, this association likely involves interactions with proteins that load onto nascent RNAs at an early step of transcription. Consistent with this expectation, we found that the 14-kDa Paip2 protein in the nuclear extract is incorporated into a large 300-kDa complex. Mass spectrometry suggests that this complex contains mRNA capping protein Cbp80. In addition, we observed co-purification of Paip2 with chromatin-bound factors involved in transcription initiation and early elongation, including Pol II subunits, and the Cdk7 subunit of TFIIH. It is known that mRNA capping machinery and early elongation factors have a tight functional connection with each other, which is important for the capping checkpoint at which Pol II pauses and can start productive elongation only after the completion of mRNA capping [33–35].

Such a link between these machineries is potentially important in two respects. First, a specific pattern of Pol II CTD phosphorylation reflects the stage of the transcription process [36], and the recruitment and functioning of capping enzymes depend on this pattern [37]. In particular, CTD phosphorylation at Ser5 stimulates capping activity in mammals [38–40]. In yeast, Ser5-phosphorylated Pol II has been shown to co-purify with the capping machinery (and some other RNA-binding factors) and TFIIH kinase [41]. The Cdk7 subunit of TFIIH is a major kinase responsible for Ser5 phosphorylation. Inhibition of human Cdk7 activity results in reduced capping enzyme recruitment, with consequent decrease in mRNA capping and increased Pol II promoter-proximal pausing [42–44].

Second, the capping machinery can influence the process of transcription. For example, yeast mRNA cap methyl-transferase Abd1 has a gene-specific stimulatory effect on Pol II recruitment onto the promoter, and its depletion results in hyperphosphorylation of CTD Ser5 [45]. The cap-binding complex (CBC) is associated with early transcription events as well. CBC participates in the recruitment of transcription factors and regulation of the transcription process [23]. CBC in yeast interacts with CTD kinases, and its depletion affects phosphorylation level of CTD Ser2 and Ser5 [46].

Thus, the mRNA capping machinery has a tight functional connection with the machinery responsible for promoter escape and early Pol II elongation. Paip2 appears to be involved in the network of interactions between these machineries (Fig. 3).

As shown in knockdown experiments, Paip2 depletion leads to an increase in the level of Pol II CTD Ser5 phosphorylation, a transcription initiation mark, and similar effects on CTD phosphorylation are also observed after Cbp80 knockdown. A plausible interpretation of these results is that the Paip2 and Cbp80 proteins facilitate the transition from the initiation phase of transcription to the elongation phase. In this model, when the levels of these two proteins are reduced, the transcription machinery would initiate transcription, but stall temporarily prior to promoter escape and Pol II elongation. This stalling would then result in an increase in the levels of CTD S5P. One might also expect that Pol II stalling after Paip2 or Cbp80 knockdown would lead to a reduction in the overall levels of mRNA; however, we have been unable to detect any effect on mRNA production. One potential explanation for this discrepancy is that RNAi knockdown does not reduce the levels of nuclear Paip2 (or Cbp80) to a point where stalling is of sufficient duration to produce a detectable reduction in transcription efficiency. Consistent with this explanation, only small changes in Paip2 or Cbp80 promoter occupancy were detected in ChIP experiments after we knocked down these proteins. While these changes are apparently sufficient to alter the levels of CTD S5P, they do not seem to impact mRNA production. It is clear that a more comprehensive understanding of how Paip2 (and Cbp80) impact the process of transcription will require experimental approaches that are more effective in eliminating promoter-associated Paip2 (and Cbp80) protein. In spite of these limitations, our studies point to a potential role for Paip2 in a capping-dependent Pol II checkpoint.

Purification of Paip2-containing protein complex revealed its association with Poly(ADP-ribose) polymerase (PARP). Our tests indicate that Paip2 is probably modified by this enzyme, which may result in the formation of the upper Paip2 band. Interestingly,

PARylation in living cells was found to play an important role in transcription [47] and translation [48, 49]. Since Paip2 is involved in both processes, this modification may be important for its functioning both in the nucleus and the cytoplasm.

In addition to participation of Paip2 in transcription events, purification of Paip2-associated proteins resulted in a few more interesting findings. Paip2 does not carry conventional NLS in its sequence. However, a considerable amount of importins co-purified with Paip2. One could suggest that Paip2 is imported into the nucleus as part of the Paip2–CBC–importin alpha complex [24]. Association of Paip2 with Nups could explain its localization at the nuclear periphery [2]. Finally, we detected co-purification of TBP-related factor 2 (Trf2) with Paip2. Trf2 was located in regions differing from those occupied by TBP [50–52]. In particular, Trf2 is recruited onto ecdysone-induced genes as well as is Paip2. Moreover, the pattern of Trf2 localization in the testes [50] substantially coincides with that of Paip2 [2]. It appears that Paip2 is predominately associated with Trf2-dependent promoters, but further studies are needed to test this idea.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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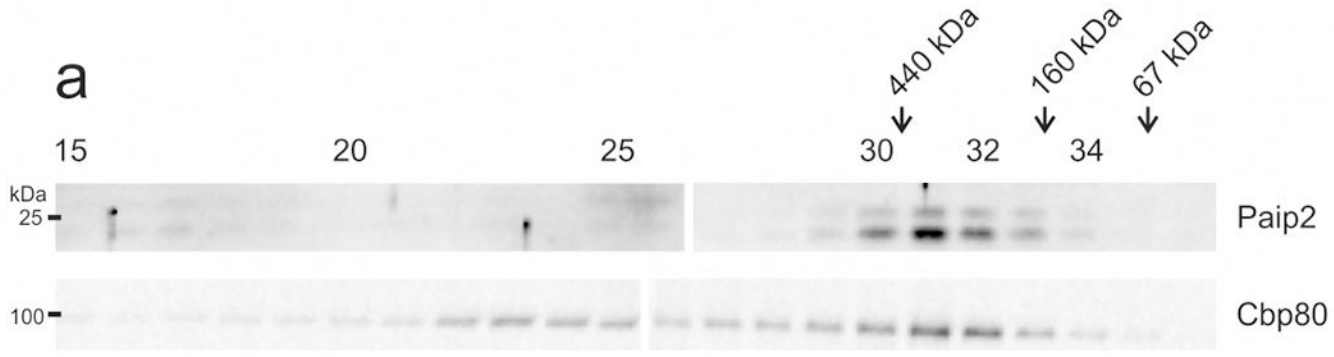

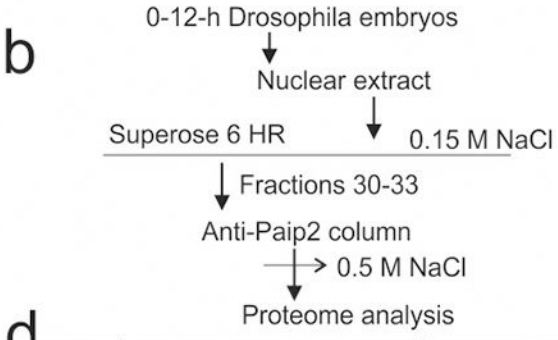

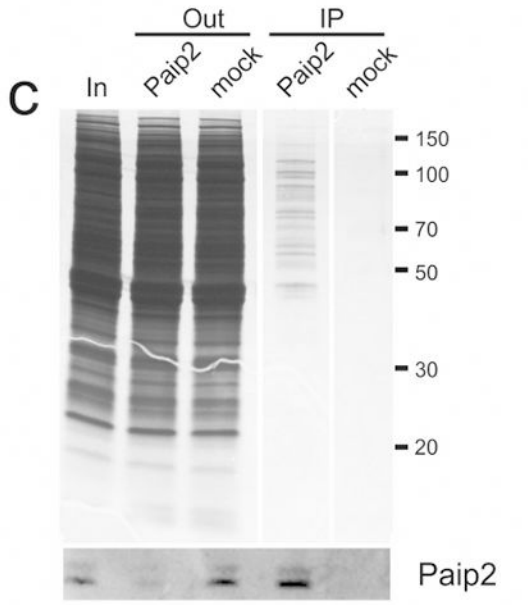

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protein	*spectral count*		*seq coverage*	*p*
	anti-Paip2	mock	**anti-Paip2**	
Paip2	**15.3 ± 13.7**	0 ± 0	**23%**	**0.002**
PABP	**84.8 ± 55.4**	29.3 ± 30.5	**26%**	**0.04**
Cbp80	**83.5 ± 2.4**	4.5 ± 6.6	**23%**	**0.00001**
CG11399	**16.3 ± 7.7**	2.3 ± 2.6	**8%**	**0.02**
Rpb1	**8.5 ± 4.2**	0 ± 0	**3%**	**0.02**
Rpb2	**13.0 ± 6.7**	0 ± 0	**7%**	**0.004**
Cdk7	**9.8 ± 6.7**	0 ± 0	**9%**	**0.02**
Spt6	**51.5 ± 40.3**	10.8 ± 14.4	**13%**	**0.002**
Trf2	**24.3 ± 23.8**	1.8 ± 3.5	**4%**	**0.0004**
PARP	**202.0 ± 75.0**	46.8 ± 32.3	**34%**	**0.00001**
ImpA2	**201.8 ± 195.6**	35.5 ± 34.2	**34%**	**0.00001**
ImpA3	**56.8 ± 38.3**	7.8 ± 11.8	**21%**	**0.00003**
ImpB	**8.3 ± 6.6**	0 ± 0	**6%**	**0.02**
Nup154	**48.0 ± 29.3**	**9.0 ± 10.9**	**13%**	**0.002**
Nup75	**6.8 ± 8.6**	**0 ± 0**	**9%**	**0.05**

Figure 1. Paip2 is a component of protein complex.

(A) Elution profiles of Paip2 and Cbp80 after gel filtration of *Drosophila* embryonic extract on Superose 6. Numbers of fractions and elution points of MW markers are shown above.

(B) Scheme of purification of Paip2-associated proteins.

(C) SDS-PAGE with silver staining (above) and Western blotting with anti-Paip2 antibodies (below) of proteins at different steps of purification procedure: In, material eluted from gel filtration column; Out, material eluted from anti-Paip2 antibody affinity column and from control column with empty beads; IP, material immunoprecipitated by immobilized anti-Paip2 antibodies and mock control. The image is combined from parts of the same gel.

(D) List of mRNA metabolism factors and transcription factors identified in the sample of purified Paip2-associated proteins: PABP, direct cytoplasmic partner of Paip2 (positive control); Cbp80, subunit of cap-binding complex; CG11399, homologue of PCIF1 (phosphorylated CTD-interacting factor 1); Rpb1, and Rpb 2, subunits of Pol II; Cdk7, subunit of TFIIH; Spt6, Pol II-associated transcription factor; Trf2, TATA-related factor 2; PARP, poly(ADP-ribose) polymerase; ImpA and ImpB, importins alpha and beta; Nup154 and Nup75, components of the nuclear pore complex. Mean value and standard deviation for spectral count and sequence coverage are given for each protein.

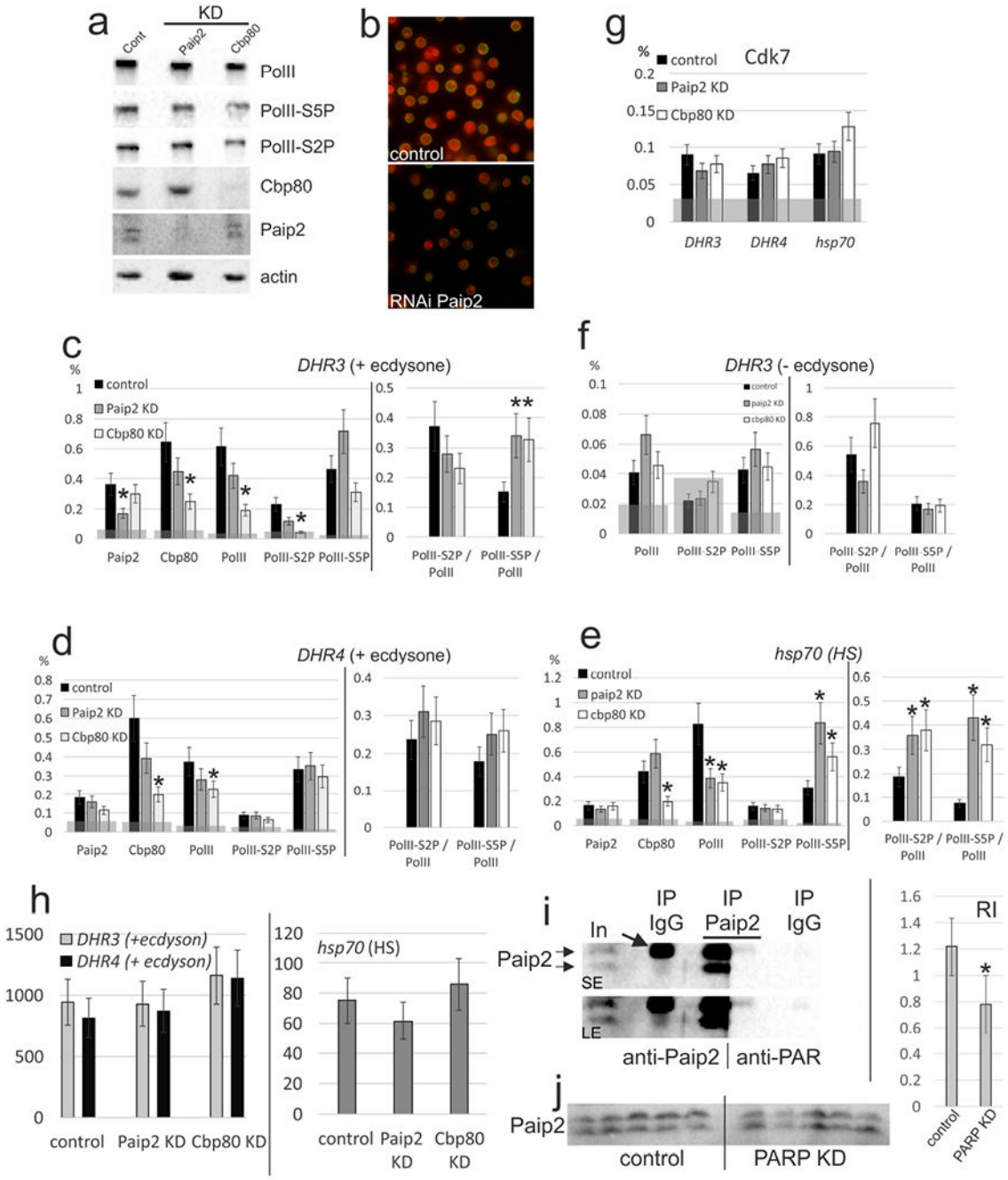

Figure 2. Paip2 functions together with Cbp80.

(A) Western blot analysis of S2 cell lysates after Paip2 or Cbp80 knockdown (KD) or control GFP dsRNA treatment (Cont). Actin was used as a loading control.

(B) Immunostaining of S2 cells after Paip2 knockdown (RNAi Paip2) or control GFP dsRNA treatment (control). Antibodies against Paip2 (red) and lamin (green) were used.

(C-F) ChIP analysis of Paip2, Cbp80 and Pol II recruitment onto (C, F) *DHR3,* (D) *DHR4,* and (E) *hsp70* promoters. Treatment with ecdysone or heat shock (HS) is indicated, normal cells (black bars) and cells with Paip2 (grey bars) or Cbp80 (white bars) knockdown were

used. The levels of Paip2, Cbp80, Pol II, and Pol II-S2P, Pol II-S5P are shown as a percentage of Input (cross-linked chromatin). The levels of Ser5- and Ser2-phosphorylated Pol II forms are also shown as a ratio to the total Pol II level (amounts of S5P-Pol II reduced 2-fold, S5P-Pol II/Pol II reduced 10-fold). Grey filling shows the level of these proteins in intergenic spacer. Asterisks indicate statistically significant differences from the control ($p < 0.05$).

(G) ChIP analysis of activated *DHR3, DHR4* and *hsp70* promoters in normal cells (black bars) and cells with Paip2 (grey bars) or Cbp80 (white bars) knockdown. The level of Cdk7 is shown as a percentage of Input. Grey filling shows the level of Cdk7 proteins in intergenic spacer.

(H) Levels of *DHR3* and *DHR4* mRNAs (after ecdysone treatment) and *hsp70* mRNA (after heat shock) in control and Paip2- or Cbp80-knockdown cells. Uninduced level of transcription for each gene was taken as unity.

(I) PARylation of Paip2. Western blot analysis of (In) S2 cells lysate and (IP) material immunoprecipitated by immobilized anti-Paip2 and anti-PAR antibodies (indicated below) and immobilized preimmune IgG. The band indicated with an arrow corresponds to IgG light chain. The results obtained with different exposure times are shown: SE, short exposure; LE, long exposure.

(J) Analysis of relative intensity (RI) of two Paip2 forms in Western blot. Five replicates of cells after PARP knockdown (RNAi PARP) or control GFP dsRNA treatment (control) were analyzed to quantify the intensity of the upper band relative to the lower band (diagram on the right), which proved to decrease upon PARP knockdown ($p = 0.042$).

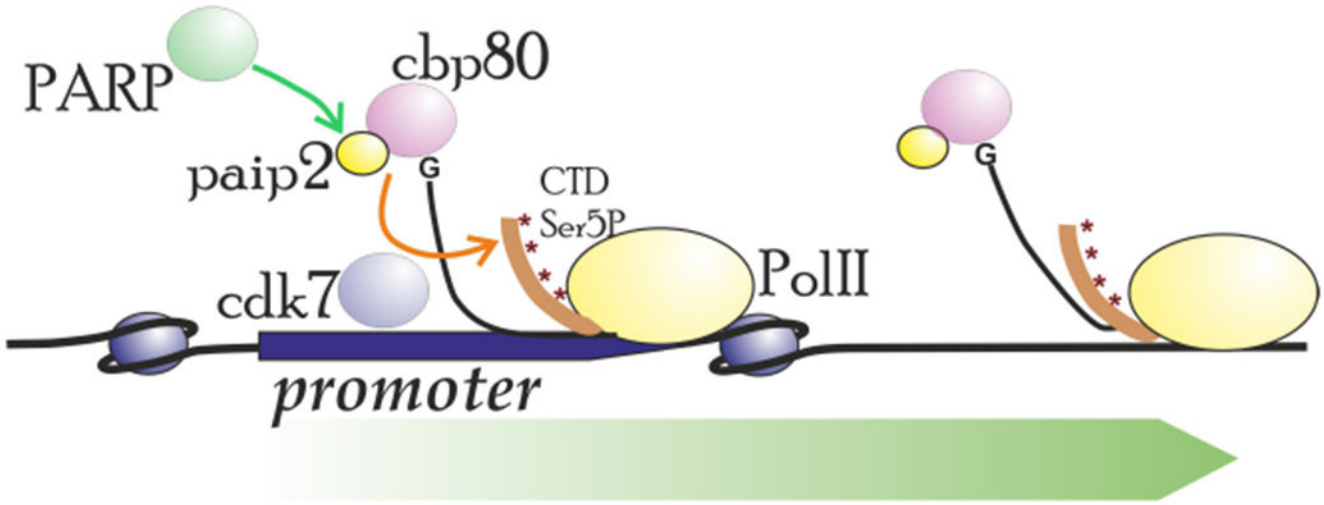

Figure 3. Model of Paip2 functioning on active promoter.

Paip2 interacts with Cbp80, and both proteins participate in the capping checkpoint, which is important for proper CTD Ser5 phosphorylation on the promoter. At subsequent steps of transcription, Paip2 remains associated with the nascent transcript (presumably via the interaction with CBC).