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DNA replication and cytokinesis in Drosophila melanogaster

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DNA REPLICATION AND CYTOKINESIS IN *DROSOPHILA MELANOGASTER*

by

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ABSTRACT

DNA replication and cytokinesis are two important parts of the cell division process. Our studies address different aspects of both of these processes and potential links between them. One of the best approaches for studying DNA replication in eukaryotes has been an analysis in cell free *in vitro* systems. The existing cell free replication systems for *Drosophila* are relatively ineffective. In the first part of this work, we have developed an optimized *in vitro* system to study DNA replication using early egg extracts of *Drosophila*. Our studies indicate that this system proves to be an extremely useful tool for a functional dissection of the processes and factors involved in DNA replication in metazoans. The central component of eukaryotic DNA replication, a six-subunit origin recognition complex (ORC), is known to be involved in non-replicative functions as well. In the second part of this thesis, we analysed the interaction of Orc6, the smallest subunit of ORC, with the septin protein Pnut and the whole *Drosophila* septin complex. Our results suggest an active role for Orc6 in septin complex function. Orc6 might be part of a control mechanism directing the cytokinesis machinery during the final steps of mitosis. Another control mechanism of septin complex activity may be related to post-translational modifications of septins. In the final part of the thesis, we have identified and characterized a phosphorylated form of Pnut protein which can be found in the embryonic extracts in the early stages of development. We propose that phosphorylation of Pnut may inhibit activity of the septin complex to prevent cytokinesis during development of syncitial blasto-

derm. Together, our studies provide further characterization of key components of the replication and cytokinesis machinery, identify new regulatory mechanisms of these processes and the functional link between two major events of cell division.

Keywords: ORC, DNA replication, cytokinesis, septins, phosphorylation.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

DROSOPHILA ORC6 FACILITATES GTPASE ACTIVITY AND FILAMENT FORMATION OF THE SEPTIN COMPLEX

STUDY OF PNUT PHOSPHORYLATION

LIST OF FIGURES

Figure Page

INTRODUCTION

STUDY OF DNA REPLICATION IN *DROSOPHILA* USING CELL FREE *IN VITRO* SYSTEM

DROSOPHILA ORC6 FACILITATES GTPASE ACTIVITY AND FILAMENT FORMATION OF THE SEPTIN COMPLEX

LIST OF FIGURES (continued)

Figure Page

STUDY OF PNUT PHOSPHORYLATION

INTRODUCTION

DNA replication in eukaryotic cells is characterized by its high accuracy. Each genomic region should be replicated once and only once per cell cycle to avoid genomic instability. To assure such a precision of genome duplication, eukaryotes have evolved a mechanism for the initiation of DNA replication that involves multiple origins of replication (*ori*) along the chromosomal DNA.

The basis of our current views on replication initiation stems from the replicon model (Jacob and Brenner, 1963). In this model, a replicon, defined as a DNA molecule capable of autonomous replication, is characterized by two functional components: initiator and replicator. A *trans*-acting initiator protein binds to a specific sequence, the DNA element called replicator. Upon binding, the initiator is transformed into a replication-competent form which is followed by replication initiation at the replicator site.

The replicon model was first validated in prokaryotic organisms. In *Escherichia coli*, initiator protein DnaA binds specifically to the 250-bp AT-rich replicator sequence *oriC*, resulting in local ATP-dependent DNA unwinding and replicative DNA helicase (DnaB) loading (Tomizawa and Selzer, 1979; Fuller and Kornberg, 1983; Bramhill and Kornberg, 1988). This is followed by the assembly of replication fork components, including DNA polymerases**,** and replication initiation.

The replicon model proved to be true for eukaryotes as well. The best- studied and characterized eukaryotic replication origins are found in the genomes of yeast cells.

In budding yeast *Saccharomyces cerevisiae* sites of replication initiation were originally identified in a screen for genomic regions supporting replication of extrachromosomal plasmids which carry them (Stinchcomb *et al.*, 1979). These regions were termed autonomously replicating sequences (ARS) and were shown to have a significant sequence similarity with each other. A typical ARS has size of approximately 100 bp and consists of 11-bp ARS-consensus sequence (ACS or A element) and another less conserved 10- to 15- bp element (B element) essential for origin function (Marahrens and Stillman, 1992).

Unlike budding yeast, the origins in other eukaryotic species are significantly less defined. Even in fission yeast *Schizosaccharomyces pombe* origins are much larger (500-1000 bp) and are not conserved in sequence, except for being AT-rich (Clyne and Kelly, 1995). Difference from budding yeast origin structure is even more obvious in metazoan species where origins are large and do not exhibit any sequence conservation: no sequence elements analogous to yeast ARS were found. The situation is even more complicated by the large size and complexity of the genomes in higher eukaryotes, and consequently, by a significant number of replicons and replication initiation sites. Distribution and localization of origins change during development in metazoans, which further obscures the study and an understanding of origin-defining factors in these species (Stambrook and Flickinger, 1970).

Some of the most characterized metazoan origins are found in *D. melanogaster.* These origins control an amplification of two clusters of chorion genes in somatic follicle cells that surround the developing oocyte (Calvi *et al.*, 1998). Two *cis*-regulatory elements important for the amplification were identified within the chorion gene cluster: the

400 bp ACE3 (Amplification Control Element 3) and ~1 Kb oriβ (Orr-Weaver *et al.*, 1989). It was shown that *Drosophila* ORC localizes to the chorion amplification gene cluster in follicle cells, and that ACE3 and oriβ are sufficient to drive this localization of ORC (Austin *et al.*, 1999; Royzman *et al.*, 1999).

The second major component of the replicon model, the initiator which binds to the replicator and specifies the site of replication initiation, is represented in eukaryotes by ORC. ORC was first identified in *S. cerevisiae* as a factor that specifically bound to the yeast ARSs (Bell and Stillman, 1992). Yeast ORC is six-subunit protein complex with subunit sizes ranging from 104 kDa (Orc1) to 50 kDa (Orc6). ORC plays a central role in the initiation of DNA replication and recruitment of other essential replication factors to the origin to form the pre-replicative complex (pre-RC) thus 'licensing' the origin for further replication activity (Dutta and Bell, 1997; Bell and Dutta, 2002).

ORC has been conserved in eukaryotic evolution. Genes of ORC subunits have been found in various eukaryotic organisms from *S. pombe* to *Homo sapiens* (reviewed in Dutta and Bell, 1997). Such conservation of ORC suggests the existence of common mechanisms for the initiation of DNA replication in all eukaryotes, despite dramatic differences in the structure of eukaryotic origins.

ORC proteins also share homology with another component of pre-RC – Cdc6 (Figure 1) (Bell *et al.*, 1995). This protein is well-conserved among eukaryotes and may be a paralog of Orc1 (Giraldo, 2003). Moreover, Orc1 can be more related to Cdc6 than to other ORC subunits. The structural data indicate that ORC and Cdc6 may form a ring-like structure around the DNA reminiscent of the MCM helicase ring (Speck *et al.*, 2005).

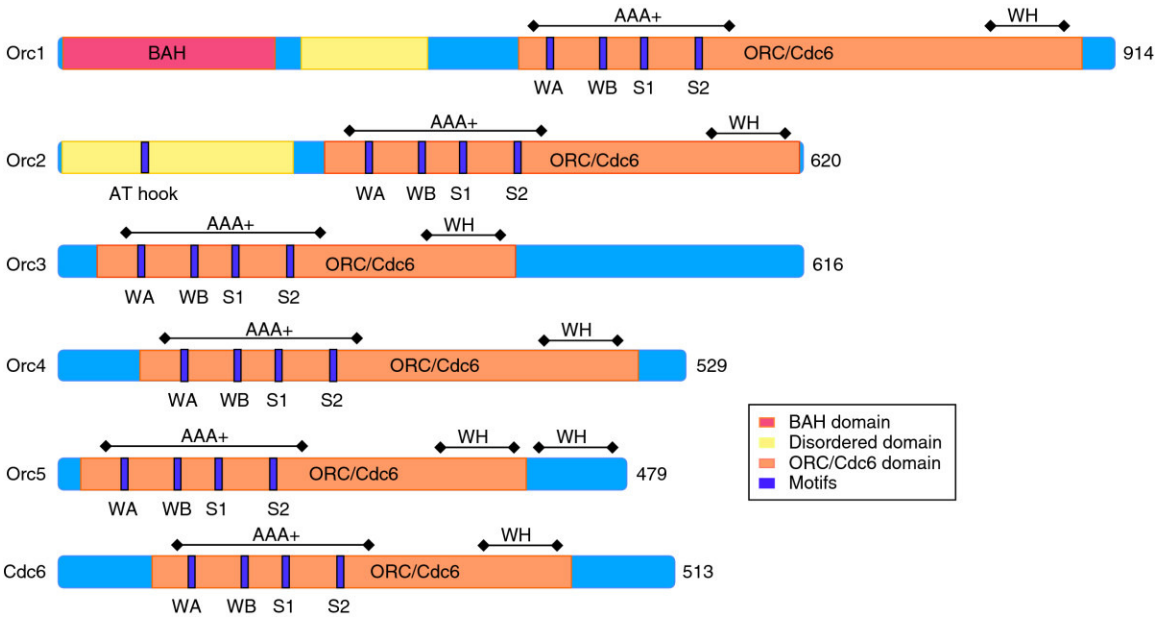

Figure 1. Comparison of domains for Orc1-5 and Cdc6 from *S. cerevisiae*. Orc1, Orc4, Orc5, and Cdc6 each contain an AAA+ domain as part of a larger ORC/Cdc6 domain (orange). Motifs within the AAA+ domain include Walker A (WA), Walker B (WB), Sensor-1 (S1) and Sensor-2 (S2). The carboxy-terminal region of ORC/Cdc6 is predicted to contain a winged-helix domain (WH), involved in DNA binding. Orc1 contains an additional BAH (bromo-adjacent homology) domain (pink). Orc1 and Orc2 have regions of disorder (yellow); a DNA-binding AT-hook motif is identified in *S. cerevisiae* Orc2, and several of these have also been identified in disordered regions in *S. pombe* Orc4. The number of amino acids for each protein is indicated at the right.

Note: Aadapted from "The origin recognition complex protein family" by B. P. Duncker, I. N. Chesnokov and B. J. McConkey, 2009, *Genome Biology*, *10,* p. 214. Copyright 2009 by the BioMed Central Ltd. Adapted with permission.

Subunits 1-5 of ORC as well as Cdc6 contain conserved Walker A and B ATP-binding domains within the AAA+ fold (Marchler-Bauer *et al.*, 2007). These features are characteristic of the proteins which from ring-shaped complexes and bind DNA in the central channel of the ring. The N-terminus of Orc1 contains bromo-adjacent homology (BAH) domain which is important for protein-protein interaction and provides a structural basis for ORC functions in heterochromatin (Bell *et al.*, 1995; Callebaut *et al.*, 1999). Orc6, on the other hand, does not share any of the structural features of Orc1-5 and has its own characteristic domains: unique conserved Orc6 protein fold domain at the

N-terminus and the predicted coil-coiled motif at the C-terminal part found in some metazoan species (Figure 2) (Finn *et al.*, 2008; Lupas *et al.*, 1991). This coil-coiled region in *Drosophila* and human Orc6 is important for cytokinetic functions of Orc6 (see below).

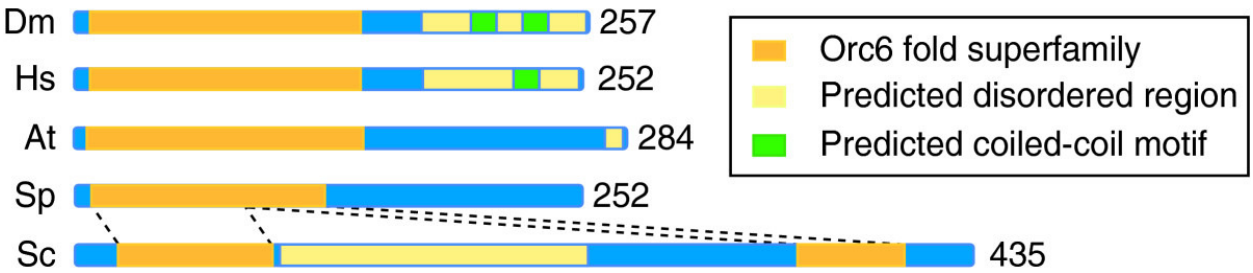

Figure 2. Homology between Orc6 in representative species *D. melanogaster* (Dm), *H. sapiens* (Hs), *A. thaliana* (At), *S. pombe* (Sp), and *S. cerevisiae* (Sc). The number of amino acids for each protein is indicated at the right.

Note: Aadapted from "The origin recognition complex protein family" by B. P. Duncker, I. N. Chesnokov and B. J. McConkey, 2009, *Genome Biology*, *10,* p. 214. Copyright 2009 by the BioMed Central Ltd. Adapted with permission.

Binding of ORC to DNA specifies sites where initiation of replication will occur, and this process is ATP-dependent. Experimental data suggest that it is the ATP binding by ORC, not the ATP hydrolysis, which is important for DNA binding by the ORC (Chesnokov *et al.*, 2001; Giordano-Coltart *et al.*, 2005; Klemm *et al.*, 1997). The coordination of DNA and ATP binding by ORC probably involves allosteric interactions among the subunits. It seems that ORC can exist in alternative conformational states induced by stable interaction of ORC with ATP and origin DNA.

Even though ORC is bound at specific chromosomal regions containing origins of replication in both differentiated *Drosophila* and human somatic cells *in vivo*, little is known about how ORC complex finds these sequences. Several mechanisms which target

ORC to the specific sites on the DNA were proposed. DNA topology may be one of the factors determining a choice of the binding site by ORC. *Drosophila* ORC demonstrates significant preference for negatively supercoiled DNAs as compared to linear or relaxed DNA fragments (Remus *et al.*, 2004). This is closely related to another important factor – a chromatin structure of the binding site. Open chromatin state in the control regions of the genes may be important for the replication initiation. Many yeast origins are known to be located in the proximity of promoters (Gomez and Antequera, 1999). Moreover, direct interactions between ORC and transcription factors important for chorion gene expression, such as E2F1, Rb and Myb complex, are described in *Drosophila*. Mutations in these genes affect localization of ORC and the function of chorion cluster amplification origin (Bosco *et al.*, 2004; Royzman *et al.*, 1999). Replication factors such as Cdc6 can also participate in the origin specification by ORC. In budding yeast, interaction between ORC and Cdc6 is facilitated by origin DNA and reduces the dissociation rate of ORC from the origin (Mizushima *et al.*, 2000). In metazoan species, Cdc6-ORC interaction might also enhance DNA binding by ORC (Harvey and Newport, 2003). It is possible that Cdc6 and transcription factors act in combination to specify the interactions between ORC and DNA.

ATP-dependent ORC binding to DNA provides the basis for the sequential assembly of a number of additional replication factors which together form the pre-RC (Figure 3) (reviewed in Bell, 2002; Bell and Dutta, 2002; Machida *et al.*, 2005). Cdc6 and Cdt1 are recruited first, which is followed by the repeated loading of Mcm2-7, a hexameric helicase complex responsible for DNA unwinding. Coordinated ATPase activity of ORC and Cdc6 facilitates tighter loading of already bound MCM2-7 helicase onto

the origin DNA, along with recruitment and binding of additional MCM2-7 complexes (Bowers *et al.*, 2004).

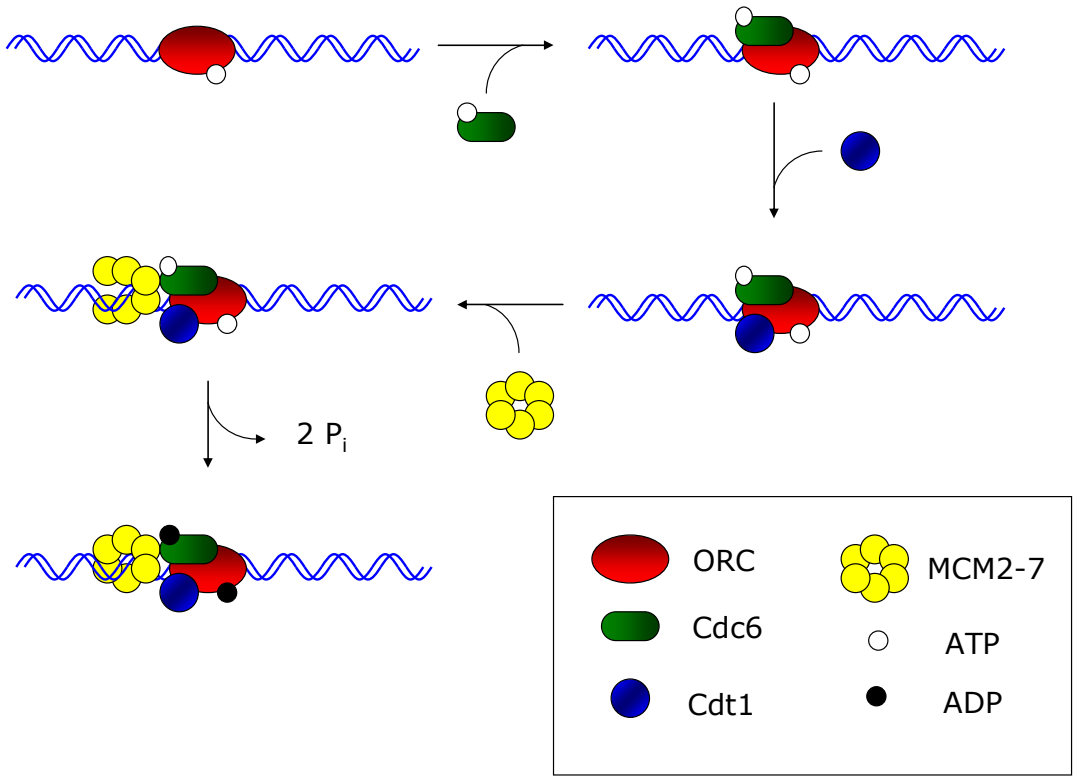

Figure 3. Assembly of the pre-RC. Binding of ORC to the replication origins marks the beginning of pre-RC assembly. On the next step, Cdc6, and then Cdt1, join origin-bound ORC. These proteins together facilitate initial association of MCM2-7 helicase with chromatin. Hydrolysis of ORC- and Cdc6-bound ATP results in loading of MCM2-7 onto DNA.

MCM2-7 loading concludes formation of the pre-RC. At this point, the origin is licenced for further activity and ready for recruitment of additional replication factors, including cyclin dependent and Dbf4 dependent kinases (CDKs and DDK), Cdc45, Mcm10, GINS complex, the three eukaryotic DNA polymerases, and the eukaryotic ssDNA binding protein, RPA (Bell and Dutta, 2002; Machida *et al.*, 2005). Presence of ORC is required for the assembly of pre-RC, even though direct interactions with ORC

has been shown only for a small subset of these factors. Apparently, ORC plays the role of a platform or a landing pad for assembly of the pre-replicative complex (Bell, 2002).

Current understanding of eukaryotic origin structure, selection, and ORC activities was achieved by the employment of a broad array of *in vitro* and *in vivo* methods. These techniques include chromatin immunoprecipitation followed by analysis on microarrays (ChIP-Chip) or direct high-throughput sequencing (ChIP-seq), DNA footprinting, nascent DNA strand assay, 2D gel analysis and DNA fiber-based approaches (Schepers and Papior, 2010; Tuduri *et al.*, 2010). Especially important methods for study of replication and ORC activity are the assays based on cell free replication systems, since they can be used for functional dissection of the processes and factors involved in these events. Such *in vitro* systems are described for human, *Xenopus* and *Drosophila* (Krude *et al.*, 1997; Blow and Laskey, 1986; Crevel and Cotterill, 1991). The system is based on cytosolic cell extract which is able to support semi-conservative replication of the chromatin added to this extract. The first part of this thesis is dedicated to development of an optimized protocol for the study of DNA replication in Drosophila cell-free system and functional characterization of several replication factors in this system.

Many studies indicate that the functions of ORC extend beyond DNA replication. The first discovered non-replication function for ORC was its participation in the establishment of transcriptionally silent chromatin domains at the budding yeast silent mating type loci (HMR and HML) through the interaction with the silent chromatin protein Sir1 (Bell *et al.*, 1995; Zhang *et al.*, 2002). ORC's function in the establishment of transcriptionally repressed regions appears to be a conserved feature, as both *Drosophila* and mammalian ORC interact with HP1, a well known modifier of position effect variegation

(Pak *et al.*, 1997; Prasanth *et al.*, 2004). Mutations in the *Drosophila* Orc2 gene suppress position effect variegation and alter the localization of HP1 (Pak *et al.*, 1997; Huang *et al.*, 1998). siRNA knockdown of Orc2 in human cells results in de-localization of HP1 (Prasanth *et al.*, 2004). Human ORC also interacts with the histone acetyl transferase, HBO1, suggesting that histone acetylation around origins is an active process in which chromatin is remodeled by replication initiators (Iizuka and Stillman, 1999; Burke *et al.*, 2001).

Another non-replication function of ORC is related to mitotic events in the chromosomes. Different ORC subunits in yeast, *Drosophila* and human cells are involved in events such as sister chromatid cohesion, chromosome condensation and chromatid segregation in mitosis, and mutations in ORC subunits cause defects in these processes (Loupart *et al.*, 2000; McHugh and Heck, 2003; Pflumm and Botchan, 2001; Prasanth *et al.*, 2004; Suter *et al.*, 2004).

In both *Drosophila* and human cells, the smallest subunit of ORC, Orc6, has been implicated in coordinating cytokinesis with pre-RC formation and chromosome segregation, a role that it performs independently of the rest of the complex. Orc6 has been found at the cell membranes and cytokinetic furrow in *Drosophila* and mammalian cells (Chesnokov *et al.*, 2003; Prasanth *et al.*, 2002). RNAi/siRNA knockdown of Orc6 in either *Drosophila* or human cells results in reduction of DNA synthesis, but also in the prominent appearance of cells that have completed mitosis without cytokinesis (multinucleate cells), a phenotype that is not seen following knockdown of other ORC subunits. In *Drosophila* Orc6 interacts and co-localizes with the Pnut protein, a member of the septin

complex which is required for cytokinesis and other processes that involve spatial organization of the cell cortex (Chesnokov *et al.*, 2003).

Septins are guanine nucleotide-binding proteins which are capable of polymerizing (Kinoshita, 2003). All the members of this family have GTP-binding domain, and most of them also have a predicted coiled-coil domain at the C-terminus (Field & Kellogg, 1999). Septins are thought to be a part of the cytoskeleton and participate in many processes related to intracellular dynamics such as chromosome segregation in mitosis, vesicular transport, exocytosis and others (Kusch *et al.*, 2002, Dent *et al.*, 2002, Spiliotis *et al.*, 2005, Spiliotis & Nelson, 2006). The best known function of septins is related to cytokinesis where they are important for the formation of the contractile ring which separates the cytoplasm of the daughter cells (Kinoshita *et al.*, 1997, Neufeld & Rubin, 1994, Dobbelaere & Barral, 2004).

Septins are found in many eukaryotic organisms from fungi to mammals, but not in plants or protozoa (Kinoshita, 2003). In *Drosophila melanogaster*, five septin genes are known: Sep1, Sep2, Sep4, Sep5 and Pnut (peanut) (Neufeld & Rubin, 1994, Fares *et al.*, 1995, Field *et al.*, 1996, Adam *et al.*, 2000). Protein products of three of them – Sep1, Sep2 and Pnut – form a hexameric septin complex which is capable of GTP binding and hydrolysis and *in vitro* forms long filaments detectable by electron microscopy (Field *et al.*, 1996).

Of the three proteins constituting the *Drosophila* septin complex, Pnut was studied most thoroughly. This protein is localized to the cell membrane in interphase and to the cleavage furrow (front of membranes moving inward of a dividing cell to separate daughter cells) in cytokinesis. Pnut deletion leads to formation of multinucleated cells as

a result of impaired cytokinesis. Such mutants die shortly after pupation and thus are unable to develop into adult flies (Neufeld & Rubin, 1994). The function of Pnut in this sense seems to be very important for normal cell division.

The interaction of Pnut with Orc6 may reflect a functional link coordinating replication with cytokinesis and thus play significant role in regulation of septin functions. The second part of this thesis is aimed at the detailed characterization of the Pnut domain needed for interaction with Orc6 and study of Orc6 influence on biochemical activities of the septin complex.

Another aspect of regulation of septin functions is post-translational modification of septins. Phosphorylation is known to play important role in the activities of the septin complex. Phosphorylation of yeast septin protein cdc3p by cdc28p kinase is required for disassembly of the septin ring (Tang & Reed, 2002). Kinase Cla4 phosphorylates yeast septin cdc10p and this process is significant for septin filaments assembly (Versele & Thorner, 2004). Mammalian septins Sept3 and Sept5, which are expressed predominantly in neurons, undergo phosphorylation which may regulate their subcellular localization and activity in exocytosis (Xue *et al.*, 2004, Taniguchi *et al.*, 2007). The third part of this thesis involves ithe dentification and study of a newly-identified phosphorylated form of Pnut which is present at certain stages of *Drosophila* embryogenesis.

STUDY OF DNA REPLICATION IN *DROSOPHILA* USING CELL FREE *IN VITRO* SYSTEM

by

ANTON SVITIN AND IGOR CHESNOKOV

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Format adapted and errata corrected for dissertation

Abstract

Using *Drosophila* early egg extracts we have developed an optimized cell free system to study DNA replication. The efficiency of replication depends on a cold treatment of *Drosophila* embryos before the extract preparation and a formation of nuclei facilitated by the addition of membrane fractions to the extracts. *In vitro* DNA replication is ORC and CDC6 dependent, as a removal of these proteins from the extracts abolishes DNA replication. The N-terminal part of Orc1 protein, which is important for non-replicative functions of ORC, is dispensable for the replication *in vitro*. We also show that the conserved ATPase motif of CDC6 is crucial for the replication. Our studies indicate that a *Drosophila* cell free system proves to be an extremely useful tool for a functional dissection of the processes and factors involved in DNA replication in metazoans.

Key words: ORC, DNA replication, CDC6, *Drosophila*.

Introduction

Eukaryotic cells duplicate their genomes with remarkable precision during the course of growth and division. This process depends on stringent regulatory molecular mechanisms that couple DNA replication and cell cycle progression. In the last two decades a model has emerged that explains the coupling of initiation of DNA replication to the cell cycle [1], [2], [3], [4]. During the G1 phase of the cell cycle, the replication initiation factors including ORC (Origin Recognition Complex), Cdc6, Cdt1, MCMs and others form a multi-protein pre-replication complex (pre-RC). Pre-RC formation is restricted to G1 phase and marks potential origins of replication. Origin activation occurs after cells enter the S phase and requires the action of kinases, which modify the pre-RC components and

other replication factors resulting in recruitment of the DNA synthesis machinery. During this transformation the pre-RC is disassembled to prevent a new round of initiation within the same cell cycle [4]. The mechanisms by which the pre-RC is transformed into an active replication fork and the specific biochemical roles of initiation factors are the areas of intense interest.

The best approaches for studying DNA replication in eukaryotes have been genetics and the analysis in cell free *in vitro* systems. To this date the cell free systems have been described for *Xenopus* [5], *Drosophila* [6] and human cells [7]. *Xenopus* egg extracts represent a powerful biochemical system to study DNA replication [5]. These extracts assemble added sperm chromatin into nuclei that undergo a complete round of semiconservative DNA replication. The replication in egg extracts requires initiation factors such as ORC, Cdc6, Cdt1, MCMs, Cdc45 etc [2], [4]. A variation of the egg extract system has also been developed in which nuclear assembly is not required for replication [8]. A cell free system to study DNA replication has been described for *Drosophila* [6] which mimics protocols described for *Xenopus* egg extracts. In our earlier work we used *Drosophila* egg extracts to study ORC dependent DNA replication [9]. The DNA synthesis in these extracts was, in our hands, at least 10 times less efficient than that by synchronized *Xenopus* egg extracts. In the current work we present an optimized protocol for studying DNA replication in a *Drosophila* cell free system. DNA replication in *Drosophila* egg extracts depend on replication initiation factors and can be used for biochemical characterization of replication factors complementing the genetic approach in *Drosophila*.

Results and Discussion

The cell free *in vitro* system to study DNA replication has been described for *Drosophila* [6], however, it proved to be difficult to reproduce in our hands. In this study we optimized the published protocol and show that this optimized assay can be used to elucidate the functions of the proteins involved in the initiation of DNA replication. Our main problem with the published protocol was the inability for *Xenopus* sperm DNA to facilitate a nuclei formation in *Drosophila* early 0-2 hours egg extracts, which resulted in extremely low levels of replication. Young *Drosophila* embryos should have large stores of the components required to build nuclei, since they have to go through the multiple rounds of DNA replication during initial cycles of development. Centrifugation is required to clear the extract from debris and endogenous egg nuclei. However, spinning the extract with high RPM (24,000g as indicated in a previously published protocol [6]) resulted in our hands in a very low extract replication efficiency (Figure 1A). We found that the optimal speed for centrifugation is ~14,000g which corresponds to 14,500 RPM in TLS 55 Beckman rotor. Centrifugation with a lower speed (less than 10,000g) resulted in a residual presence of the *Drosophila* egg nuclei in the extract leading to inability to perform immunodepletion and rescue add back experiments (data not shown). Spinning extracts with higher speed (more than 20,000g) significantly decreased replication efficiency of the extract (Figure 1A). *Xenopus* sperm chromatin, incubated in these extracts, was not able to facilitate the formation of pseudonuclei important for DNA replication. Consistent with this conclusion, the microscopic analysis of *Xenopus* sperm after incubation in egg extract revealed only modest degree of chromatin decondensation (Figure 1E).

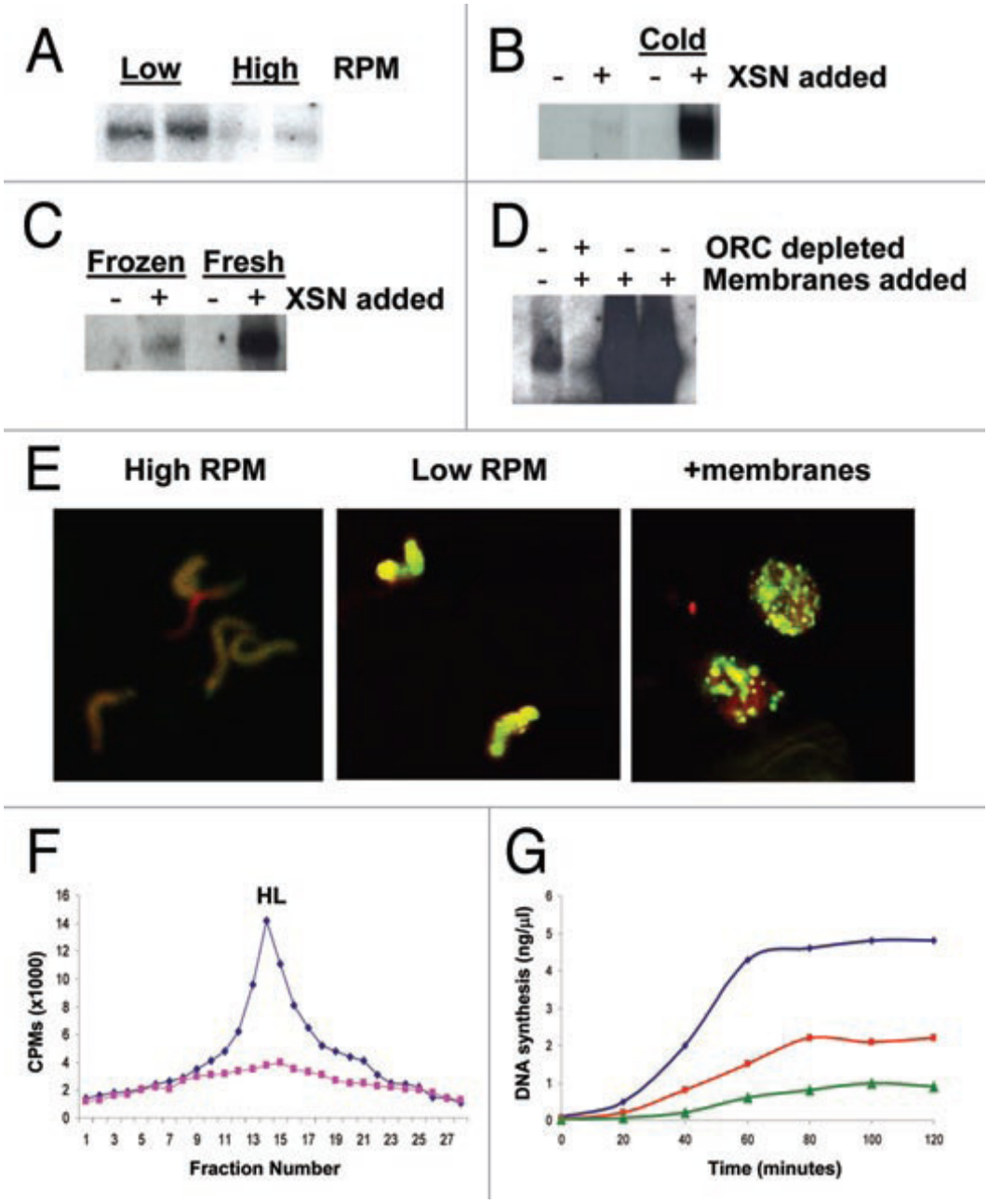

Figure 1. *In vitro* DNA replication in *Drosophila* egg extracts. The effects of centrifugation force (A), cold shock (B), freezing of extracts (C) and the effect of an addition of membranes (D) are shown. (E) Visualization of *in vitro* DNA replication in *Drosophila* egg extracts prepared after high and low RPM centrifugation. The replication in *Xenopus* sperm nuclei after addition of membrane fraction is also shown. Merged confocal images are presented. (F) DNA replication in *Drosophila* egg extracts is ORC dependent as shown by density substitution analysis of replicated DNA. *Xenopus* sperm DNA in *Drosophila* egg extract (blue) and in ORC-depleted *Drosophila* extract (red) are presented on the density profiles. (G) The time course of DNA replication is shown as measured by TCA precipitation. DNA synthesis is shown in extracts after high (green)

(Figure 1, continued) and low (red) speed centrifugation and in extracts supplemented by the addition of the membranes (blue).

Similarly to a published protocol [6] we found that the incubation of eggs on ice necessary for embryo synchronization greatly increased the ability of the extract to replicate DNA (Figure 1B). The cold treatment of *Drosophila* cells has been shown to disrupt the centrosome causing a metaphase block [10], which results in cell synchronization. The extracts prepared from these synchronized embryos facilitated nuclei formation and supported DNA replication. Up to 25-30% of template *Xenopus* sperm nuclei became decondensed rather than the 2-3% obtained from the incubation in the extracts prepared from untreated embryos. We conclude that the cold treatment results in synchronization of young *Drosophila* embryos and facilitates further decondensation of template DNA leading to the elevated levels of nuclear formation and DNA replication.

We also found that the addition of glycerol (10%), suggested by earlier studies [6] for the prolonged storage of the extract, significantly reduced the replication ability of the extract. Extracts, frozen in the presence of glycerol and subsequently thawed, consistently displayed only 10-20% of the activity of the freshly prepared extracts which did not contain glycerol (Figure 1C). Therefore in our studies we always used freshly prepared extracts. No more than 5% of glycerol was used for prolonged storage of the extracts in liquid nitrogen.

Finally, we found that the addition of *Xenopus* egg membranes further increases DNA replication ability of extract up to 10 fold (Figure 1D). Microscopic analysis revealed that under these conditions *Xenopus* sperm nuclei undergo complete

decondensation and form pseudonuclei with a high level of DNA replication (Figure 1E). Figure 1F shows the analysis of replication products by density substitution experiments and proves that DNA replication observed in prepared *Drosophila* egg extracts is ORC dependent. In a parallel experiment DNA synthesis was measured by TCA precipitations in a time course manner (Figure 1G). As for the gel analysis DNA was incubated in the extracts at the concentration ~20 ng/µl. After the incubation DNA was isolated, precipitated with TCA and analyzed for the incorporation of labeled nucleotide as described [5]. A low rate of DNA synthesis observed in the extracts isolated after high speed centrifugation increase 2 to 3 fold when extracts were isolated with a low speed (Figure 1A and G). The efficiency of DNA replication increased even further when membrane fractions were added to the replication extracts prepared with a low speed (Figure 1D and G). The important role for the nuclear envelope in activating DNA replication was suggested by the experiments performed in *Xenopus* egg extracts [11], [5], [12]. It was also shown that any perturbation of the nuclear envelope disrupts DNA replication [12], [13]. The role of the nuclear envelope is to create, through selective nuclear transport, an intranuclear environment that is permissive for DNA replication [14], [8]. The nuclear envelope also plays a more direct role in potentiating DNA replication. Using the *Xenopus* cell free system, Lemaitre and colleagues [15] have shown that at S phase entry in early development, chromatin is organized into short loops and replicons, allowing recruitment of a large amount of ORC protein. Loop size increases progressively during the S phase. Subsequent mitosis reprograms nuclei so that their chromatin again includes short loops and small replicons, enabling the rapid DNA replication in the early embryo [15].

The hexameric Origin Recognition Complex (ORC) is an important component for eukaryotic DNA replication. It was originally discovered in budding yeast *S. cerevisiae* and subsequent studies both in yeast and higher eukaryotes laid the foundation for understanding the functions of this important key initiation factor. ORC binds to origin sites in an ATP dependent manner and serves as a scaffold for the assembly of other initiation factors [16]. ORC also directly participates in the loading of initiation factors [17], [18]. ORC localization and origin selection involve many elaborate pathways with many regulators intervening upstream and downstream of ORC chromatin association [16], [19], [20], [21], [22], [23].

The extensive studies in both *Drosophila* and mammalian systems indicate that Orc1 is more loosely associated with other subunits and is degraded during G2 and M phases of the cell cycle [24], (reviewed in [25]). This process is one of the mechanisms to prevent re-replication in metazoan cells [25], [22]. The cellular levels of Orc1 in *Drosophila* tissues change dramatically throughout the development and are controlled by E2F [26]. In our earlier work we consistently observed two peaks of ORC activity during ORC purification from *Drosophila* embryos [27]. The highest apparent molecular weight peak contained all ORC subunits. The smaller complex was also detected that was apparently without Orc1 subunit. Both complexes can be reconstituted *in vitro* and purified. Figure 2A shows a silver stained gel containing both ORC(1-6) and ORC(2-6) recombinant complexes used in this study. The wild type baculovirus expressed recombinant ORC containing all six subunits can rescue DNA replication in ORC depleted egg extracts (Figure 2C, D and E and [9]). In contrast, recombinant ORC(2-6) complex lacking the largest ORC subunit, Orc1, was not able to restore DNA replication in ORC-depleted

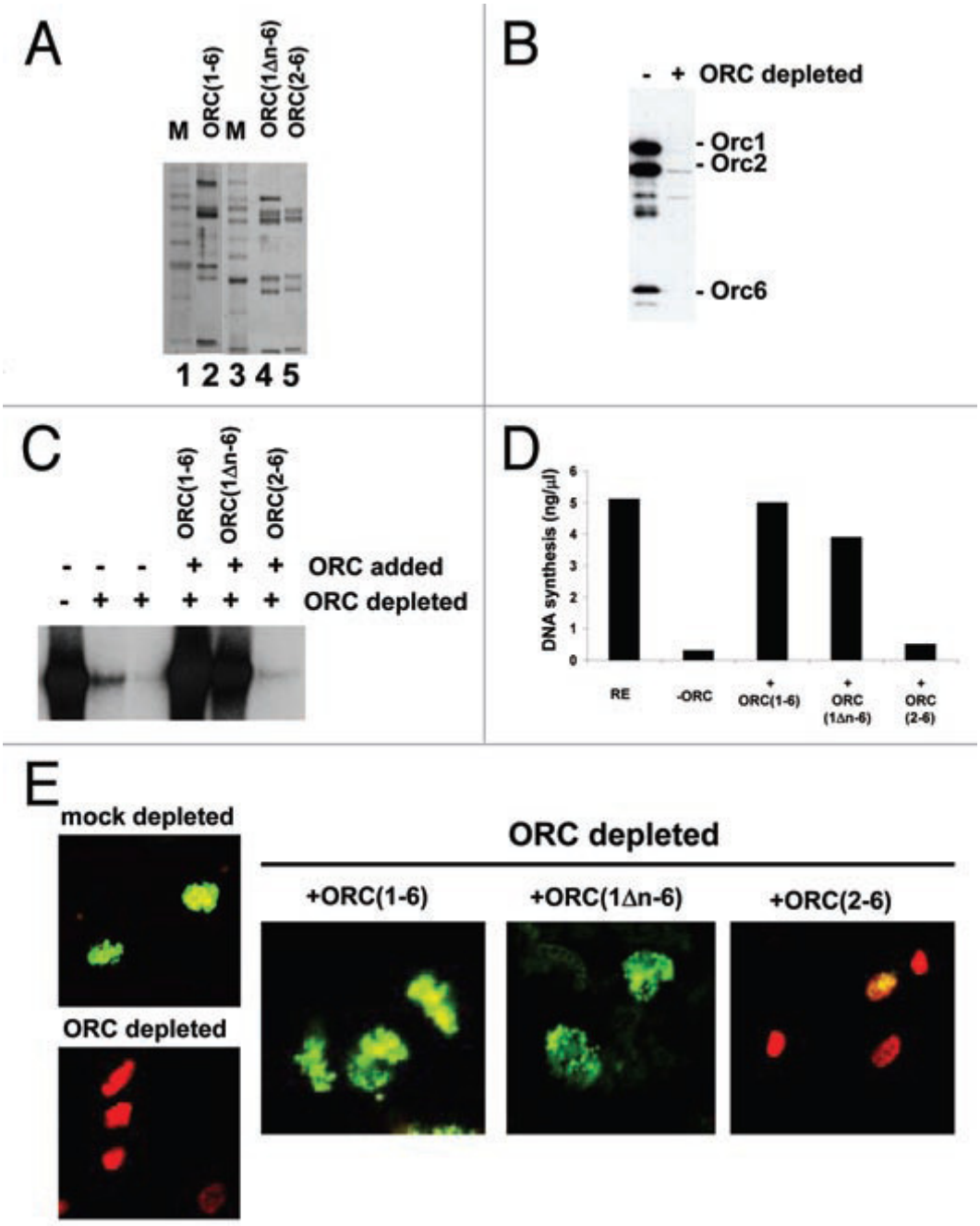

Figure 2. *In vitro* DNA replication in *Drosophila* extracts is ORC dependent. (A) Silver stained gel of wild type ORC(1-6) – lane 2, ORC(1Δn-6) containing truncated at N-terminus Orc1 subunit (lane 4) and ORC(2-6) lacking Orc1 subunit (lane 5). Marker proteins are present in lanes 1 and 3. (B) Western immunoblotting analysis of *Drosophila* egg extract depleted of ORC using antibodies against Orc1, Orc2 and Orc6 subunits. (C) *In vitro* DNA replication in ORC depleted *Drosophila* extracts can be rescued by the addition of recombinant wild type ORC(1-6) and by the addition of ORC(1Δn-6) complex containing truncated at N-terminus Orc1 subunit, but not with ORC(2-6) lacking Orc1 subunit. In a parallel experiment (D) DNA replication was measured by TCA

(Figure 2, continued) precipitation. (E) Visualization of *in vitro* DNA replication in *Drosophila* egg extracts after immunodepletion of ORC and add back rescue experiments with recombinant wild type ORC(1-6), ORC(1Δn-6) and ORC(2-6) complexes.

extract (Figure 2C, D and E). Orc1 in *Drosophila* consists of a highly conserved C-terminal domain (amino acids 555-927) which bears a homology with CDC6 protein and a variable N-terminal domain (amino acids 1-555) which does not display significant homology between Orc1 subunits derived from different species [28], [16]. We asked if the deletion of the N-terminal domain of Orc1 would have an effect of DNA replication activity of ORC. Complete deletion of the N-terminal domain resulted in an inability of truncated Orc1 to form a complex with other subunits (not shown). The N-terminal domain of Orc1 in *Drosophila* contains a motif (amino acids 119-327) responsible for the interaction with Hp1 protein [28]. We found that Orc1Δn subunit missing amino acids 1-327 readily entered ORC complex (Figure 2A). The resulting ORC(1Δn-6) complex was also able to rescue DNA replication *in vitro* in ORC depleted extracts (Figure 2C, D and E). For the experiments described above, early (0-2 hr) egg extracts were immunodepleted of ORC using antibody raised against *Drosophila* Orc1, Orc2 and Orc6 subunits. The efficiency of immunodepletion was tested by western blotting (Figure 2B). In add back experiments increasing amounts of recombinant, baculovirus produced wild type and mutant ORC proteins were added to ORC depleted extracts. Replication efficiency was analyzed by *in vitro* DNA replication assays (Figure 2C), followed by a measurement of DNA synthesis by TCA precipitation (Figure 2D) and microscopy analysis (Figure 2E). We conclude that *Drosophila* ORC, like budding yeast ORC, can not function without its largest Orc1 subunit. The N-terminal domain of Orc1 is important for the interaction with other ORC subunits however, the deletion of the N-

terminus responsible for the non-replicative functions of Orc1 had little or no effect on ORC-dependent DNA replication *in vitro*.

CDC6 protein is an important component for DNA replication [3], [4]. One function of Cdc6 homologues is to load the MCMs onto chromatin, a critical step in licensing the DNA for replication. Similar to many proteins involved in DNA replication CDC6 contains motifs important for binding and hydrolyzing ATP. To date, some work has probed the requirement for these sequences in yeast, *Xenopus* and human Cdc6. Cdc6 proteins containing disrupted Walker A motif are nonfunctional in these organisms [29], [30], [31], [32] suggesting that Cdc6 requires ATP binding to form a productive and stable interaction with ORC and MCMs at replication origins. Microinjection of Walker A mutant human Cdc6 (HsCdc6) into HeLa cells results in a dominant negative phenotype characterized by a block in replication [33], suggesting that the human Walker A mutant protein disrupts the ability of endogenous wild-type Cdc6 to load MCMs stably onto chromatin. As expected, removal of CDC6 (Figure 3B) from replication extract by immunodepletion resulted in the inability of the extracts to replicate DNA, as shown by gel electrophoresis of radioactively labeled replication products (Figure 3A). This result was confirmed by TCA precipitation of replicated DNA (Figure 3C), fluorescence microscopy (Figure 3D) and density substitution experiments (Figure 3E). Again, replication can be rescued by the addition of recombinant CDC6 to CDC6-depleted extracts, but not with a recombinant CDC6(KA) which carries a mutation in ATP binding domain of the protein (Figure 3A, C and D). Interestingly, the peak corresponding to the position of heavy-heavy chains of DNA can be detected during rescue experiments in a density substitution assay suggesting that the excess of CDC6 may force re-replication of DNA in our *in vitro*

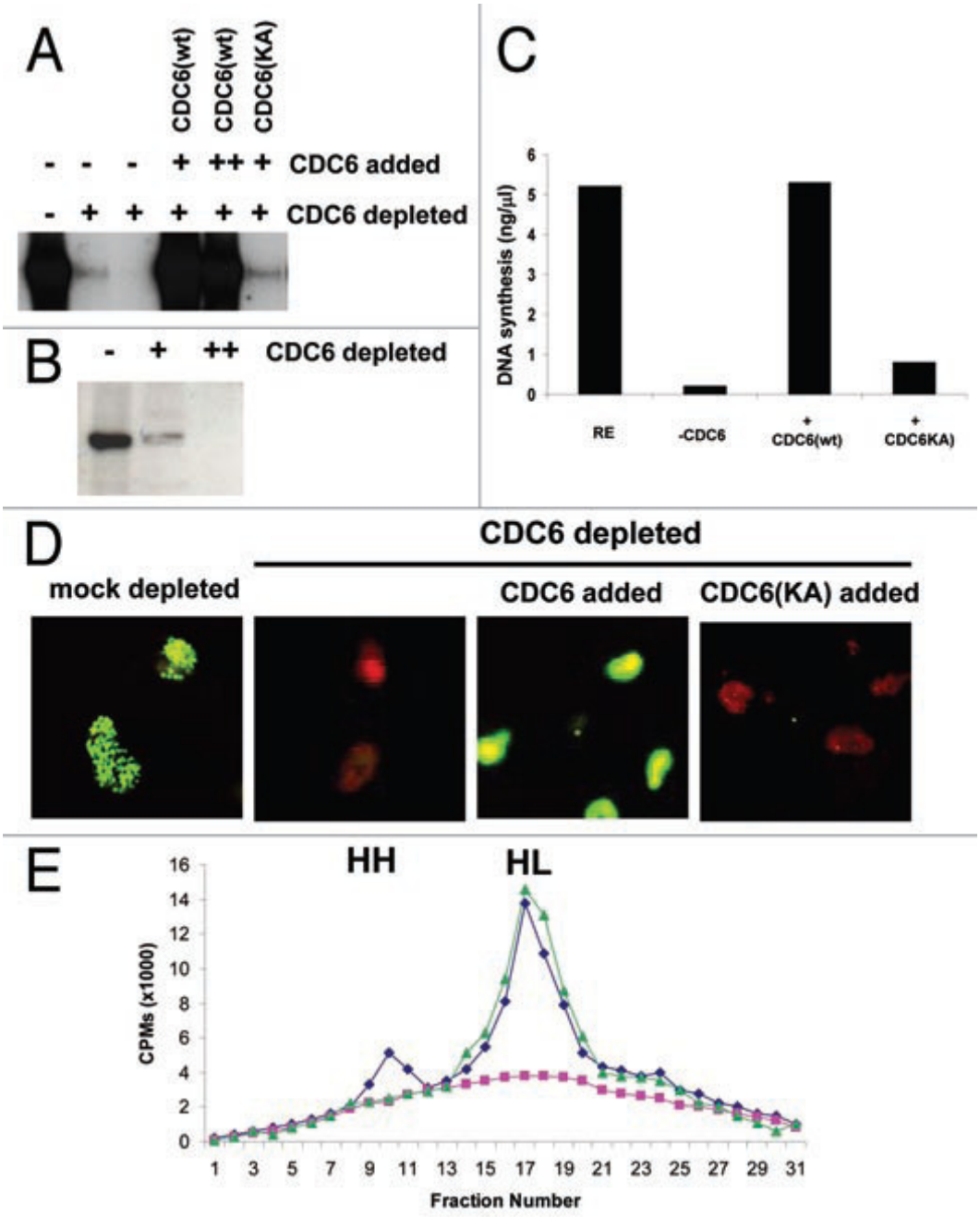

Figure 3. *In vitro* DNA replication in *Drosophila* extracts is CDC6 dependent. (A) *In vitro* DNA replication in CDC6 depleted *Drosophila* extracts can be rescued by the addition of 100 ng (+) or 200 ng (++) recombinant wild type CDC6 but not by 100 ng CDC6(KA) containing mutation that disrupts ATPase motif of CDC6. (B) Western immunoblotting analysis of *Drosophila* egg extract depleted of CDC6 (one (+) and two (++) rounds of depletion were used) using antibodies against CDC6. (C) CDC6 dependent DNA synthesis in the extracts was also measured by TCA precipitations. (D) Visualization of *in vitro* DNA replication in *Drosophila* egg extracts after immunodepletion using anti-CDC6 antibody and add back rescue experiment with 100 ng of either recombinant wild type CDC6 or mutant CDC6(KA) proteins. (C) DNA replication in *Drosophila* egg

(Figure 3, continued) extracts is CDC6 dependent as shown by density substitution analysis of replicated DNA. The replication of *Xenopus* sperm DNA in *Drosophila* egg extract (green) and after addition to CDC6-depleted extract of 100 ng of recombinant purified wild type CDC6 (blue) or CDC6(KA) mutant (red) are presented on the density profiles.

assays.

In conclusion, the experiments presented herein provide further evidence that Walker A is an essential motif of Cdc6 that is required for proper pre-RC assembly. Disruption of ATP binding in CDC6(KA) mutant results in an inability of the mutant protein to support DNA replication *in vitro*, most likely due to the assembly of a nonproductive pre-RC.

Overall, the described cell free system that uses *Drosophila* extracts is a very useful complement of the analogous *in vitro* system in *Xenopus* and will help in understanding the process of DNA replication. The efficiency of DNA replication observed in our experiments was comparable to the *Xenopus in vitro* system. Moreover, the amenability of *Drosophila* to genetic manipulations should open new approaches not possible with studies using *Xenopus*, such as the potential to use specific fly mutants and/or transgenic animals with a gene that is expected to have a clear phenotype in the *in vitro* replication assay. Our findings also provide a biochemical framework with which to dissect further the role of ORC, Cdc6 and other initiator proteins in initiating DNA replication.

Materials and Methods

Cloning and Mutagenesis

cDNAs for Orc1 and CDC6 were cloned by PCR from a *Drosophila* embryonic library (Clontech MATCHMAKER library). N-terminal deletion of Orc1 - Orc1(320-

927), the addition of GST-tag and the His-tag were generated with standard PCR technique. The Lysine to Alanine substitution at the position of 303 within the ATPase domain (GKT) of CDC6 – CDC6(KA) was introduced with Stratagene's site-directed mutagenesis protocol (http://www.stratagene.com/manuals/ 200516.pdf). All constructs were analyzed by sequencing. cDNAs were subcloned into desired vectors with standard molecular biology techniques.

Purification of recombinant Drosophila proteins

Recombinant baculoviruses were generated by using Bac-to-Bac expression system (GIBCO/BRL) as described [27]. For wild type ORC(1-6) complex containing all six ORC subunits, Orc1 gene was fused with 6x His N- terminal tag. Orc2 subunit was tagged to facilitate purification of ORC(2-6) complex which lacks Orc1 subunit. CDC6 gene was similarly fused with 6x His N- terminal tag to facilitate purification of the protein. Cell infections and protein purification was performed as described earlier [27].

In vitro replication in Drosophila egg extracts

The preparation of egg extracts was based on a procedure described previously [6]. *Drosophila* embryos (0-2 h) were washed with extraction buffer, cold treated, and homogenized. The homogenate was centrifuged for 20 min at 11,000-15,000 rpm in TLS 55 Beckmann rotor. The middle layer was collected and re-centrifuged. For long-term storage in liquid nitrogen the supernatant was adjusted to 5% with respect to glycerol and 1 mM to ATP. The extract was frozen in 20 µl beads in liquid nitrogen. Demembraned

sperm chromatin was prepared essentially as described [5]. The membrane fraction was prepared as described [34].

Before the experiment egg extract was supplemented with an ATP regeneration system (60 mM phosphocreatine and 150 μg/ml creatine phosphokinase) and membrane fraction (1 μl). *Xenopus* sperm DNA was added and incubated in extracts for 1 hr at a concentration of 2-5 ng/μl in the presence of [α^{32} P]dCTP. The reactions were stopped with stop solution, DNA was extracted and ethanol precipitated, resuspended in TE buffer and submitted to electrophoresis in a 0.8% agarose gel. The gel was dried and autoradiographed.

Microscopy and indirect immunofluorescence experiments were performed as described previously [35]. For these experiments *Xenopus* sperm DNA (10 ng/μl) was incubated in *Drosophila* egg extracts in the presence of biotin–16-UTP. The extracts were applied to cover slips and subjected to low speed centrifugation, nuclei were fixed and stained for detection of the incorporated UTP analog with fluorescein-conjugated streptavidin. DNA was counterstained with propidium iodide. Merged confocal images are presented.

For immunodepletion experiments affinity purified polyclonal rabbit antibodies against Orc1, Orc2, Orc6 and Cdc6 were used as described [9]. Completeness of immunodepletions was monitored by immunoblotting. To rescue DNA replication, add back experiments were performed by the addition of increasing amounts of baculovirus expressed reconstituted ORC or Cdc6.

Density substitution analysis of replicated DNA

Demembraned *Xenopus* sperm DNA was incubated for 1 hr in *Drosophila* egg extract at a concentration of 10 ng/μl in the presence of BrdUTP and [α-^{32}P]dCTP. DNA was extracted and subjected to centrifugation through a gradient of CsCl. For density substitution experiments [5], in addition to labeled dCTP, BrdUTP was added to a concentration of 1 mM. The reactions were stopped, and DNA was extracted and loaded onto a CsCl density gradient. The gradient was spun in a Beckman 50Ti rotor at 36,000 rpm at 2°C for 40 h. Fractions were collected and counted by Cerenkov radiation. Positions of LL and HL peaks were determined in parallel experiments using single-stranded M13 DNA as a template as described [9]. TCA precipitations and measurements of the DNA synthesis were performed as described [5].

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DROSOPHILA ORC6 FACILITATES GTPASE ACTIVITY AND FILAMENT FORMATION OF THE SEPTIN COMPLEX

by

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Abstract

The Origin Recognition Complex or ORC is a six subunit protein important for DNA replication and other cell functions. Orc6, the smallest subunit of ORC, is essential for both replication and cytokinesis in *Drosophila*, and interacts with the septin protein Pnut, which is part of the *Drosophila* septin complex. In this study we describe the analysis of the interaction of Orc6 with Pnut and whole *Drosophila* septin complex. Septin complex was purified from *Drosophila* embryos and also reconstituted from recombinant proteins. The interaction of Orc6 with the septin complex is dependent on the coiled-coil domain of Pnut. Furthermore, the binding of Orc6 to Pnut increases the intrinsic GTPase activity of the *Drosophila* septin complex, while in the absence of GTP it enhances septin complex filament formation. These results suggest an active role for Orc6 in septin complex function. Orc6 might be a part of a control mechanism directing the cytokinesis machinery during the final steps of mitosis.

Introduction

The Origin Recognition Complex (ORC) plays a central role in the initiation of DNA replication and the recruitment of essential replication factors to the origins of DNA replication in eukaryotes (Dutta and Bell, 1997; Bell, 2002; Bell and Dutta, 2002; Machida *et al.*, 2005). In addition to initiating DNA replication ORC is involved in other functions (for reviews see (Bell, 2002; Chesnokov, 2007; Sasaki and Gilbert, 2007). Some of these activities link cell cycle progression to DNA replication, whereas other functions seem distinct from replication. In *Drosophila,* the smallest subunit of ORC, Orc6, is an essential component of the complex and directly involved in DNA binding

(Balasov *et al.*, 2007). Orc6 is also essential for cytokinesis in both *Drosophila* (Chesnokov *et al.*, 2003) and human cells (Prasanth *et al.*, 2002) as ablation of Orc6 from cells by RNAi results in cytokinesis defects. In *Drosophila*, Orc6 colocalizes and interacts with the septin protein Pnut. This interaction is mediated by the C-terminus of Orc6 that contains a predicted amphipathic α-helical domain (Chesnokov *et al.*, 2003).

Pnut is a member of the septin family of polymerizing GTPases, which are required for cytokinesis and other processes that involve spatial organization of the cell cortex. Identified originally in yeast, septins are now found in many fungi and animals (Kinoshita, 2003; Pan *et al.*, 2007). Septins are essential for cytokinesis (Hartwell, 1971; Neufeld and Rubin, 1994; Kinoshita *et al.*, 1997), however, they have also been implicated in other processes such as polarity establishment, cell cycle checkpoints, formation of diffusion barrier, spindle alignment, chromosome segregation, vesicle trafficking and exocytosis (Gladfelter *et al.*, 2001; Kusch *et al.*, 2002; Dobbelaere and Barral, 2004; Martinez *et al.*, 2004; Spiliotis *et al.*, 2005; Spiliotis and Nelson, 2006)

Septins are found as heteromeric complexes that can assemble in larger filaments (Kinoshita, 2006). All septins have in common a GTP binding domain that consists of the binding motifs G1 (P-loop), G3, and G4. Most septins contain a predicted coiled-coil domain at the C-terminus (Field and Kellogg, 1999). In many cases septins appear to serve as a scaffold or matrix for other proteins (Longtine *et al.*, 1998; Field and Kellogg, 1999; Barral *et al.*, 2000; Longtine *et al.*, 2000; Takizawa *et al.*, 2000). Therefore, progress in understanding septins' functions will depend in part on identifying the proteins with which the septins interact.

Drosophila melanogaster has at least five septin proteins, named Pnut, Sep1, Sep2, Sep4 and Sep5, whose functions are not yet well understood (Neufeld and Rubin, 1994; Fares *et al.*, 1995; Field *et al.*, 1996; Longtine *et al.*, 1996; Field and Kellogg, 1999; Adam *et al.*, 2000; Kinoshita, 2003). Of these 5 *Drosophila* septins, Pnut, Sep1 and Sep2 form a heteromeric six subunit complex consisting of two of each septin subunits (Field *et al.*, 1996). The complex binds and hydrolyses GTP. This GTPase activity is characterized by a faster GTP to GDP conversion but a very slow exchange of bound nucleotide. Pnut and Sep1 colocalize at the cleavage furrows of dividing cells during cytokinesis, to the intercellular bridge that connect post mitotic daughter cells, neurons, the leading edge of epithelial sheaths and the early embryo cortex (Neufeld and Rubin, 1994; Fares *et al.*, 1995). Larvae, homozygous null for Pnut, die shortly after pupation and *pnut*$^{-}$ tissues contain multinucleated cells indicating an involvement for this protein in cytokinesis (Neufeld and Rubin, 1994). Both Pnut (Neufeld and Rubin, 1994) and Orc6 (Chesnokov *et al.*, 2003) are essential for cytokinesis and their interaction might be critical for their tasks in the cell.

In this study, we describe the role of the interaction of Orc6 protein with functionally active *Drosophila* septin complex. Our data revealed that the C-terminal coiled-coil domain of Pnut is essential for both septin complex formation and interaction with Orc6. However, different motifs within the C-terminal domain are responsible for these functions. Orc6 increased GTPase activity of wild type septin complex but not of complex containing coiled-coil domain mutants of Pnut defective for interaction with Orc6. The C-terminal deletion mutant of Orc6, which fails to interact with Pnut, also had no effect on the GTPase activity of the complex. These results indicate that binding of Orc6

via its C-terminal domain to the coiled-coil domain of Pnut is directly related to the effect of Orc6 on septin complex GTPase activity. Orc6, but not Orc6 C-terminal deletion mutant, enhanced filament assembly of recombinant septin complex. In the presence of GTP, the effect of Orc6 on filament formation was significantly decreased or absent. This suggests a dual role for Orc6 in its interaction with the septin complex. In the absence of GTP Orc6 promotes assembly of septin filaments, while in the presence of GTP Orc6 enhances septin complex GTPase activity, resulting in the disassembly of filaments. The data presented here indicate that septins are regulated by additional factors involved in cytokinesis such as Orc6.

Materials and Methods

Cloning and Mutagenesis

cDNAs of Sep1 and Sep2 were cloned by PCR from a *Drosophila* embryonic library (Clontech MATCHMAKER library). C-terminal deletions of Pnut (Pnut1-508, Pnut1-460, Pnut1-427), the C-terminus of Pnut (Pnut427-539), the GST-tag and the FLAG-tag were generated with standard PCR technique. Triple leucine to alanine substitutions in the coiled-coil domain of Pnut (L463A,L468A,L470A and L481A,L483A,L488A) were introduced with Stratagene's site-directed mutagenesis protocol (http://www.stratagene.com/manuals/ 200516.pdf). All constructs were analyzed by sequencing. cDNAs were subcloned into desired vectors with standard molecular biology techniques except for the yeast constructs (see below).

Proteins and Antibodies

Purification of *E.coli* derived recombinant His-tagged wild type Orc6 and Orc6-200 mutant has been described (Balasov *et al.*, 2007). Pnut cDNA was cloned into pET-Duet expression vector (Novagen) using the NdeI and KpnI restriction sites. His-tagged Pnut was produced by expression from the plasmid in *E.coli* strain BL21 DE3. After induction with IPTG, His-tagged protein was isolated with Ni-NTA beads (Qiagen). Purified antigen was used to generate rabbit polyclonal antibodies (Cocalico Biologicals). Antibodies were purified by affinity chromatography as described (Harlow and Lane, 1999). Mouse anti-FLAG (M2 clone) was obtained from Sigma.

RNAi assay

Double stranded RNA (dsRNA) was obtained by using the Megascript kit from Ambion. Pnut primers (5'-CGGCCAGTGAATTGTTTAATACGACTCACTATAGGGA ATAGTCCTCGCTCGAACGCG-3" and 5'-CGGCCAGTGAATTGTTTAATACGACT CACTATAGGGTTAGAACAGACCCTTCTTTTTC-3') flanked with T7 promoter were used. The Orc6 primers used have been described (Chesnokov *et al.*, 2003). L2 cells were cultured at 27ºC in Shields and Sang M3 medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum. For RNAi experiments 1 x 10^6 *Drosophila* L2 cells seeded on a cover slip in a well of a six well dish were inoculated with 30 µg of dsRNA in 1 ml serum-free M3 medium. After 1 hour incubation 1 ml of medium supplemented with 10% fetal bovine serum was added to the culture. After 72 hours cells were fixed with 2% formaldehyde in PBS. Cells were stained for Pnut and Orc6 and counterstained with DAPI. Cover slips were mounted with 80% glycerol 20% 1x PBS, 2% N-propyl-gallate

and analyzed with fluorescence microscopy. RNAi efficiency was tested by immunoblotting with anti-Pnut antibody.

Yeast two-hybrid assays

The Cytotrap yeast two-hybrid system (Stratagene) was used to analyze the interaction between Orc6 and wild type or mutant Pnut proteins. The system has been described (Aronheim *et al.*, 1997). In brief; the system uses the yeast temperature-sensitive mutant strain cdc25H. The *cdc25* mutation prevents growth at 37ºC but allows growth at the permissive temperature (25ºC). The Cytotrap system is based on the ability of the hSos protein to complement the *cdc25* defect and to activate the yeast Ras-signaling pathway. Expression of hSos and its subsequent localization to the plasma membrane allows the cdc25H yeast strain to grow at 37ºC. The localization of hSos to the plasma membrane occurs through the interaction of its fusion protein encoded by the pSos vector with a myristylated protein encoded by the pMyr vector.

A gateway cloning cassette has been introduced into the pMyr and pSos vectors in order to introduce the cDNAs of interest from pENTR/D-TOPO plasmids with CL clonase (Invitrogen) into the yeast expression vectors. PCR products of Orc6 and Pnut cDNAs were cloned into the pENTR/D-TOPO vector according to protocol. These constructs were used to clone Orc6 and Pnut coding sequences into the pMyr and pSos vectors, respectively. All yeast transformations were performed with the standard lithium acetate method. The expression of pSos-fusion proteins is constitutive in the Cytotrap system with low expression levels. To verify that the pSos-Pnut fusion proteins were properly expressed, yeast lysates were immunoprecipitated with anti-hSos antibody

(Santa Cruz) bound to protein A Sepharose 4B beads (GE Healthcare) and the precipitate analyzed by SDS-PAGE followed by immunobloting with mouse anti-Pnut antibody.

For the yeast two hybrid assay, individual colonies expressing both myristylated Orc6 and hSos-Pnut fusion proteins were picked from the plate and resuspended in 35 µl of TE. 2.5 µl of the suspension was applied on gal/raff plates. Plates were grown at either the permissive (25ºC) or restricted (37ºC) temperature.

Immunoprecipitation studies with de novo synthesized proteins

For the *in vitro* expression of proteins a modified pBluescript KS plasmid was used. A T7 terminator signal that originated from pET15b vector was cloned into the BamHI and HindIII sites of pBluescript KS. Coding sequences of wild type and mutant Pnut proteins as well as Orc6 were cloned into this vector within the XbaI site downstream from the T7 promoter region. GST tag was cloned into the SacI and XbaI sites downstream from the T7 promoter region, and Pnut(427-539) was subsequently cloned into the XbaI site to produce a fusion protein. Expression of proteins and immunoprecipitation reactions were performed as described (Pak *et al.*, 1997). Septin complexes consisting of Sep1, Sep2 and wild type or mutant Pnut protein were expressed in one reticulocyte reaction while Orc6 was generated in a separate reaction. 5 µl of a 50 µl reaction was analyzed by SDS-PAGE for synthesis efficiency. 25 µl of the reactions containing septin proteins and, if necessary, Orc6 protein were used for interaction studies and subsequent immunoprecipitations. Half of the precipitated material was analyzed by SDS-PAGE followed by autoradiography.

Expression of FLAG-tagged Pnut in L2 cells

Flag-tagged Pnut proteins were expressed in L2 cells under control of the Pnut promoter from pCasper vectors containing the 1.7 kB genomic region directly upstream from the start codon of Pnut isolated from *Drosophila* Canton S strain. L2 cells were cultured at 27ºC in Shields and Sang M3 medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum. 3 x 10^6 *Drosophila* L2 cells seeded on a cover slip in a well of a six well dish were transfected with 1 µg of DNA using Insect Genejuice according to the manufacturer's recommendations (Novagen) in the presence of 5% serum. 24 hours post-transfection medium containing the transfection mix was replaced for regular medium. 72 hours post-transfection cells were fixed with 2% formaldehyde in PBS. Cells were stained for FLAG and counterstained with DAPI. Cover slips were mounted with 80% glycerol 20% 1x PBS, 2% N-propyl-gallate and analyzed with fluorescence microscopy. Expression of FLAG-tagged Pnut proteins was analyzed by immunoblotting with anti-FLAG antibody.

Purification of native septin complex

Cytosolic embryonic extracts were isolated from the wild type Canton S strain of *Drosophila melanogaster* using a modified method initially designed for the isolation of nuclear extracts of *Drosophila* embryos (Heberlein and Tjian, 1988). Dechorionated 0-12 hour embryos were homogenized in lysis buffer (15 mM Hepes-KOH pH 7.6, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 350 mM sucrose, 1 mM DTT, 1 mM sodium metabisulfite, 0.2 mM PMSF, 3 ml per gram of embryo). Nuclei and yolk were removed from the lysate by low speed centrifugation at 9,800 x g in a JA-14 rotor (Beckman) at 4ºC for

15 min. The crude cytosolic lysate was then cleared from any remaining debris by high speed centrifugation at 142,000 x g in a Ti 45 rotor (Beckman) at 4ºC for 1 hour. Proteins in the cleared cytosolic lysate were concentrated by ammonium sulfate precipitation (30%) followed by centrifugation. Subsequently, the precipitated proteins were dissolved in HEM buffer (25 mM Hepes-KOH pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$) containing 100 mM KCl, 1 mM DTT, 0.5 mM sodium metabisulfite, 0.1 mM PMSF and dialyzed overnight to remove the ammonium sulfate. After dialysis the extract was centrifuged to remove precipitated material, glycerol was added to a concentration of 10% and the cleared extract flash frozen in liquid nitrogen and stored at -80ºC untill further use. 20 ml of the cleared extract was applied to a 50 ml Heparin-Sepharose column (GE Healthcare) in HEMG (HEM, 5% glycerol) buffer containing 100 mM KCl, 0.005% NP40, 1 mM DTT, 0.1 mM PMSF. The flowthrough of this step was concentrated by precipitation with 30% ammonium sulfate, resuspended in 10 ml HEMG buffer containing 100 mM NaCl, 0.005% NP40, 1 mM DTT, 0.1 mM PMSF and subsequently dialyzed overnight. The dialyzed product was fractionated on a Sephacryl S-300 HR 26/60 column (318 ml, GE Healthcare). Fractions containing the majority of Pnut as detected by Western blot were pooled and applied to a MonoQ10/100 GL column (7.8 ml, GE Healthcare). The flowthrough of this step was diluted to 50 mM NaCl and adjusted to pH 8.0 and applied to a 1 ml MonoQ5/50 GL column (GE Healthcare). Peak fractions containing septin complex were pooled, concentrated with a Centricon YM-100 (100 kDa cut-off, Millipore) and loaded on top of a 4 ml 15-35% glycerol gradient in HEM buffer (pH 8.0) with 200 mM NaCl, 0.005% NP40, 1 mM DTT, 0.1 mM PMSF. The gradient was centrifuged in a MLS-50 swinging bucket rotor at 227,000 x g at 4ºC for 16 hours in an Optima

MAX-E tabletop ultracentrifuge (Beckman). ~100 µl fractions were collected and analyzed by SDS-PAGE followed by silver stain or colloidal Coomassie blue stain. Septin proteins were identified by LC-MS as described below.

LC-MS confirmation of native septin complexes

Individual protein bands (~70 kDa, 50 kDa, and 41 kDa) from the glycerol gradient fractions were excised from the gel with a razor blade and subjected to in-gel tryptic digestion (with reduction by 10 mM dithiothreitol and alkylation with iodoacetamide). The tryptic digests were loaded onto a LC-MS system composed of a MicroAS autosampler, 2D LC nanopump (Eksigent, Dublin, CA), and a linear ion trap-Fourier transform ion cyclotron resonance hybrid mass spectrometer (LTQ FT, Thermo Scientific, San Jose, CA). The pulled tip was packed with Jupiter 5 µm C18 reversed phase beads to give a 100 µm diameter, 11 cm column. A gradient of acetonitrile with 0.1% formic acid was run from 5-30% in 60 minutes at 500 nl min^{-1}. LTQ FT parameters were set as previously described (Renfrow *et al.*, 2007). *Drosophila* tryptic peptides were identified by use of the TurboSEQUEST algorithm within Bioworks 3.2 (Thermo Scientific) with a mass accuracy of 2.0 ppm or better. Pnut, Sep2, and Sep1 were each unambiguously identified within their respective excised gel band with >10 unique tryptic peptides.

Reconstitution and purification of recombinant Drosophila septin complex

cDNA encoding for Pnut was cloned into the pET-Duet expression vector to generate a His-tagged protein. cDNAs encoding Sep1 and Sep2 were cloned into the pCDF-Duet expression vector resulting in full length (untagged) proteins. Plasmids were trans-

formed into *E.coli* strain BL21 DE3. Colonies were grown in Miller's LB Broth with 50 μg/ml ampicillin and 50 μg/ml spectromycin to an OD_{600} of 0.6, after which protein expression was induced with 1 mM IPTG for three hours. Cells were lysed in a French press after which protein was isolated with Ni-NTA according to the manufacturer's recommendations (Qiagen). The eluate fractions containing recombinant septin complex were diluted to 50 mM NaCl with dilution buffer (25 mM Hepes-NaOH pH 8.0, 5% glycerol) and applied to a 5 ml HP Q column (GE healthcare). Protein was eluted from the column using a gradient of 50 mM to 1 M NaCl in 25 mM Hepes-NaOH pH 8.0, 5% glycerol, 1 mM DTT. Fractions containing septin complex were further purified by centrifugation on a 15%-35% glycerol gradient in 25 mM Hepes-NaOH pH 8.0, 200 mM NaCl, 1 mM DTT. Centrifugation was carried out as mentioned above.

Recombinant baculoviruses were generated using the Bac-to-Bac expression system (Invitrogen). Viruses carrying Sep1 and Sep2 were mixed with either wild type or mutant N-terminally His-tagged Pnut baculovirus constructs. P3 viral stocks were used to express protein in High Five cells. Cells were infected with the viral stocks of all three septins. 72-90 hours post transfection cells were isolated, and lyzed with a Dounce homogenizer in lysis buffer (10 mM Hepes-KOH pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM β-mercaptoethanol, and the protease inhibitors leupeptin, aprotinin, pepstatin). NaCl was added to a final concentration of 300 mM and nuclei precipitated by low speed centrifugation at 1,800 x g in a JS-4.2 rotor with a JH-4B centrifuge (Beckman) at 4ºC for 10 min. Supernatant was adjusted to 10 mM imidazole and added to Ni-NTA beads (Qiagen). Bead suspension was incubated at 4ºC for 2 hours. Subsequently beads were washed with 50 volumes of wash buffer (10 mM Hepes-KOH pH 7.6, 300

mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM β-mercaptoethanol, 20 mM Imidazole). Proteins were eluted with elution buffer (10 mM Hepes-NaOH pH 8, 300 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM β-mercaptoethanol, 250 mM Imidazole, 5% glycerol). Eluate fractions containing septin complex were diluted to 50 mM NaCl and further purified with anion chromatography on a 1 ml MonoQ 5/5 or 1 ml HiTrap HP Q column using a gradient of 50 mM to 1 M NaCl in 25 mM Hepes pH 8, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 1 mM DTT. Septin complex eluted at 200 mM NaCl.

Electron Microscopy

Septin filament formation was analyzed essentially as described (Field *et al.*, 1996). Purified septin complex was applied to nickel formvar carbon-coated grids and incubated at room temperature for 4 min. Excess solution was removed from the grids and proteins were fixed with 1% uranyl acetate in 30% ethanol for 30 sec. Fixative was removed and grids dried after which images were taken on a Philips FEI Tecnai Spirit TEM at 60 kv.

GTPase assays

GTP hydrolysis assays were based on the method described by Field *et al.* (Field *et al.*, 1996). 2.5 µg of septin complex was incubated with affinity purified Rabbit-anti-Pnut antibody and 5 µl protein A sepharose 4B beads in a total volume of 100 µl GTPase buffer (20 mM Hepes-KOH pH 7.6, 3 mM $MgCl_2$, 1 mM EGTA, 50 mM KCl, 1 mM DTT) at 4ºC for 30 min. Beads were isolated, washed with GTPase buffer and finally resuspended in 50 µl GTPase buffer. 0.5 µCi [α-^{32}P]GTP (3000 Ci/mmol, GE Healthcare)

and cold GTP were added to a final concentration of 2 μM. 0.5 μg of purified recombinant Orc6 protein was included in the mixture when indicated. After incubation at 21-22ºC with continuous agitation, beads were washed four times with 0.5 ml cold GTPase buffer. Nucleotides bound to the septin complex were eluted with 10 μl of 8 M urea, 5 mM EDTA, 20 mM Tris-HCl pH 7.5. 2 μl of the eluate was analyzed for total bound nucleotide by liquid scintillation counting. 4 μl of the eluate was analyzed for nucleotide by thin layer chromatography on polyetheleneimine (PEI)-cellulose plates (Selecto Scientific) developed with 0.85 M KH_2PO_4 pH 3.4. Radioactive GTP and GDP spots were quantified by phosphorimaging.

Results

Pnut depletion by RNAi results in loss of cytoplasmic Orc6 localization.

Pnut mutations in *Drosophila* result in cytokinesis defects (Neufeld and Rubin, 1994). To address the potential role of Pnut in Orc6 localization L2 cells were depleted of Pnut by RNAi and Orc6 localization analyzed by immunohistochemistry. Immunoblot analysis of Pnut dsRNA treated L2 cells revealed that the level of Pnut was greatly reduced by 72 hours, while cells transfected with Orc6 dsRNA showed the same levels of Pnut as control, untransfected cells (Figure 1A). Analysis of cells treated with Pnut dsRNA for 72 hours revealed that, depending on the experiment, up to 35% of the cells showed a binucleated phenotype as measured among a population of 100-300 cells per experiment. Figure 1B shows that in cells lacking Pnut due to the dsRNA treatment the

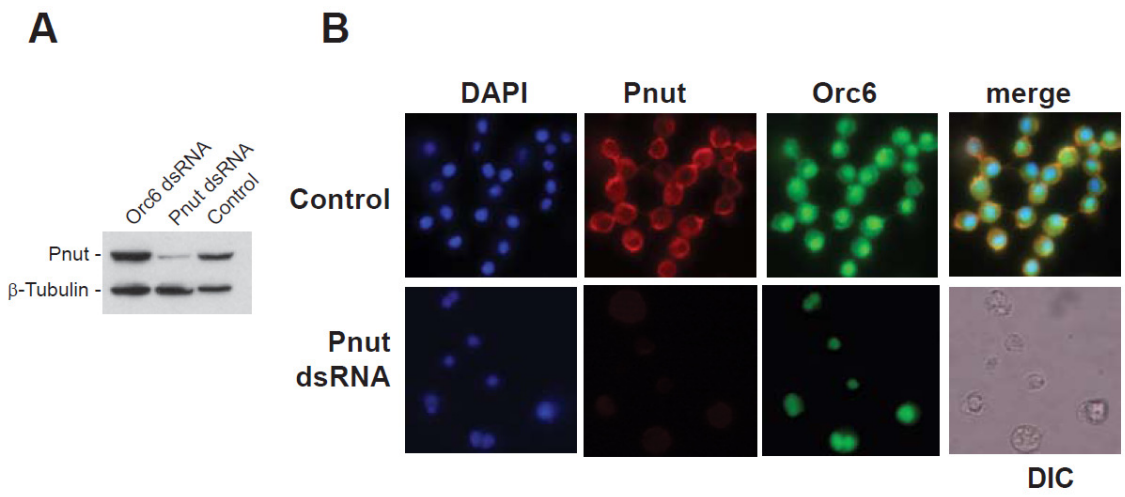

Figure 1. Silencing of Pnut in *Drosophila* L2 cells by dsRNA causes multinucleation and Orc6 mislocalization. (A) Immunoblotting with anti-Pnut antibody of whole cell extract *Drosophila* L2 cells transfected with either Orc6 dsRNA or Pnut dsRNA for 72 hours, or non-transfected (control). Tubulin was used as a loading control. (B) *Drosophila* L2 cells, either control or transfected with Pnut dsRNA were fixed 72 hours post-transfection, and stained as described in the Materials and Methods section. For the Pnut dsRNA treated cells the differential interference contrast (DIC) is also shown.

majority of Orc6 was confined to the nucleus, with some residual cytoplasmic staining, whereas in untreated cells Orc6 and Pnut colocalized at the plasma membrane and at cleavage furrows of dividing cells. These results suggest that Pnut is important for recruiting Orc6 to the plasma membrane of *Drosophila* cells.

The predicted coiled-coil domain of Pnut is essential for its interaction with Orc6

Pnut contains a predicted coiled-coil domain at the C-terminus. The importance of the coiled-coil domain of Pnut for its interaction with Orc6 was first tested in a yeast two hybrid assay. C-terminal deletions that disrupt the coiled-coil domain of Pnut were analyzed. The importance of regularly spaced leucine residues that often form the hydrophobic core of the coiled-coil domain were also examined. Figure 2A shows that the coiled-coil domain of Pnut is essential for its interaction with Orc6. The Pnut1-508 mutant lacks

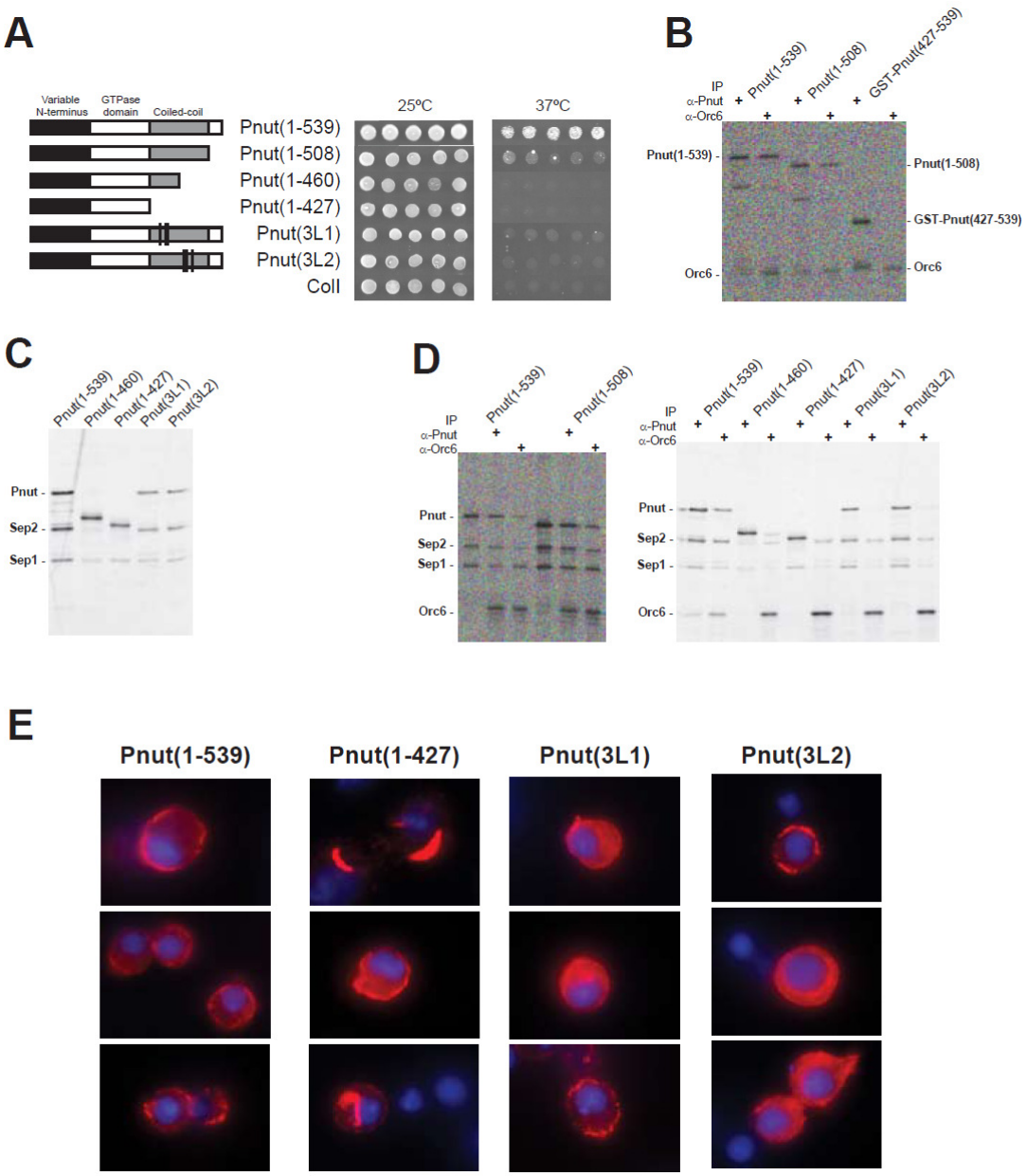

Figure 2. Interaction of Orc6 with Pnut and septin complex. (A-B) The coiled-coil domain of Pnut is essential for its interaction with Orc6. (A) Results from the Cytotrap yeast two hybrid system are presented. Individual colonies expressing both myristylated Orc6 and hSos-Pnut fusion proteins were picked and resuspended in 35 µl of TE. 2.5 µl of the suspension was applied on gal/raff plates which were incubated at either the permissive (25ºC) or restricted (37ºC) temperature. hSos-ColI is a fusion protein that serves as a negative control. A schematic representation is given for each mutant of Pnut tested. The lines for the representations of Pnut(3L1) and Pnut(3L2) represent the relative locations of the mutated leucines which are Pnut(L463A,L468A,L470A) and Pnut(L481A,L483A,L488A), respectively. (B) Direct interaction studies of in *vitro* synthesized Pnut and Orc6 proteins. Proteins were synthesized individually in an *in vitro*

(Figure 2, continued) transcription translation reaction containing ^{35}S-methionine. Reactions containing wild type or mutant Pnut protein were mixed with Orc6. Proteins were immunoprecipitated with either anti-Pnut antibody or anti-Orc6 antibody and further analyzed by SDS-PAGE followed by autoradiography. The GST-Pnut(427-539) synthesis reaction contained a degradation product of slightly bigger size than Orc6 that was pulled down with anti-Pnut antibody only. (C) The coiled-coil domain of Pnut is essential for septin complex formation. Sep1, Sep2 and wild type or mutant Pnut protein were simultaneously synthesized in an *in vitro* transcription translation reaction containing ^{35}S-methionine. Proteins were immunoprecipitated with anti-Pnut antibody and further analyzed by SDS-PAGE followed by autoradiography. Wild type (Pnut1-539) and deletion mutants (Pnut1-460, Pnut 1-427) as well as the triple leucine mutants within the coiled coil domain Pnut(3L1) and Pnut(3L2) are shown. (D) Orc6 interacts with the septin complex via Pnut. Sep1, Sep2 and wild type or mutant Pnut protein were simultaneously synthesized in an *in vitro* transcription translation reaction containing ^{35}S-methionine, while Orc6 was synthesized individually. Reactions were mixed and proteins immunoprecipitated with either anti-Pnut antibody or anti-Orc6 antibody. Immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. (E) Expression of Pnut mutants under control of native promoter in *Drosophila* tissue culture cells. *Drosophila* L2 cells were transfected with pCasper constructs expressing FLAG-tagged Pnut under control of the Pnut promoter. Cells were fixed 72 hours posttransfection, and stained with anti-FLAG antibody, and counterstained with DAPI. Columns of 3 representative images for wild type Pnut and Pnut mutants are shown.

the last C-terminal 31 amino acids but leaves the predicted coiled-coil domain intact. Although significantly reduced compared to wild type (Pnut1-539), yeast growth was still supported at the restrictive temperature, indicating that Orc6 did interact with this Pnut deletion mutant. This was confirmed by direct interaction between Orc6 and the Pnut(1-508) deletion mutant from *in vitro* transcription-translation reactions as shown in Figure 2B. Yeast two-hybrid assay further revealed that the removal of half of the coiled-coil domain of Pnut (Pnut1-460) or the whole coiled-coil domain of Pnut (Pnut1-427) resulted in complete abolishment of growth at the restrictive temperature. Pnut proteins containing triple alanine substitutions for leucine residues at positions L463A, L468A, L470A - Pnut(3L1) and L481A, L483A, L488A - Pnut(3L2) in the coiled-coil domain were severely impaired in their ability to interact with Orc6. The yeast two hybrid results were

confirmed by direct interaction between Orc6 and Pnut deletion and substitution mutant proteins from *in vitro* transcription-translation reactions (data not shown). Our data suggest that the coiled-coil domain of Pnut and the leucine residues herein are important for the interaction with Orc6. Direct interaction between the coiled-coil region of Pnut (Pnut427-539) and wild type Orc6 could not be confirmed with proteins from *in vitro* transcription-translation reactions by either immunoprecipitation (Figure 2B) or the use of glutathione or Chitin beads with GST- or Chitin Binding Domain-fusion proteins, respectively (data not shown). This indicates that although essential the coiled-coil domain of Pnut is not sufficient for the interaction of this protein with Orc6.

The coiled-coil domain of Pnut is important in septin complex formation

The C-terminal regions of most septins contain a predicted coiled-coil domain, which is important for protein-protein interaction (Mason and Arndt, 2004). To test if this domain of Pnut is important for septin complex assembly we analyzed the ability of C-terminal deletion Pnut mutants to form septin complex with Sep1 and Sep2. Sep1, Sep2 and wild type or mutant Pnut proteins were expressed simultaneously *in vitro* using a transcription-translation assay. Pnut was immunoprecipitated with a polyclonal anti-Pnut antibody and pulled-down material was analyzed by SDS-PAGE. Figure 2C shows that the synthesized proteins Sep1 and Sep2 formed a complex with wild type Pnut. However, in the presence of a Pnut mutant lacking half of the coiled coil domain (Pnut1-460), the pulled down material contained little to no Sep2 and low amounts of Sep1 resulting in non-stoichiometric ratios of Sep1:Sep2:Pnut. This indicates that complex formation was significantly impaired. The deletion of the whole coiled-coil domain (Pnut1-427) pro-

duced the same results. Moreover, Pnut mutants lacking the coiled-coil domain were not able to reconstitute into septin complex in baculovirus expression system (data not shown). Interestingly, the triple leucine mutants of Pnut (3L1 and 3L2) which were unable to interact with Orc6 did not inhibit the formation of septin complex (Figure 2C). Figure 2D shows that the C-terminal mutant Pnut(1-508), which contains the whole predicted coiled-coil domain, was able to assemble into a stoichiometric septin complex, indicating that within the C-terminus of Pnut specifically the coiled-coil region is important for septin complex formation.

In vivo localization of Pnut mutants

To investigate the effect of the mutations on Pnut localization Flag-tagged Pnut proteins were expressed in L2 cells. Overexpression of Pnut with inducible promoters was not successful as it resulted in aggregated rods and spirals of the expressed protein (Figure S4). In order to obtain lower expression levels of wild type and mutant FLAG-tagged Pnut proteins the native Pnut promoter was used. Overall, the expression of FLAG-tagged Pnut proteins was modest but detectable as immunoblot analysis confirmed expression of correct size proteins (data not shown). Localization of the expressed proteins was analyzed by immunohistochemistry. Figure 2E shows that wild type FLAG-tagged Pnut localized to the plasma membrane of L2 cells similar to endogenous Pnut. The FLAG-tagged coiled-coil deletion mutant of Pnut, Pnut(1-427) which failed to assemble into septin complex *in vitro*, had the tendency to accumulate into crescent shaped aggregates, although plasma membrane localization was observed in rare cases. Ex-

pressed FLAG-tagged triple leucine mutants of Pnut, Pnut3L1 and Pnut3L2, displayed mainly diffuse cytoplasmic staining but also some plasma membrane localization.

Interaction of Orc6 with the septin complex

Immunofluorescence studies on septins imply that they form a complex *in vivo* (Fares *et al.*, 1995). Orc6 and Pnut interact and colocalize at cleavage furrows of dividing cells (Chesnokov *et al.*, 2003). These results suggest that *in vivo* Orc6 interacts with Pnut as part of the septin complex. To investigate this possibility immunoprecipitation experiments were set up with *in vitro* synthesized septin complex and Orc6. Proteins were immunoprecipitated with either anti-Pnut antibody or anti-Orc6 antibody. Figure 2D shows autoradiograms of the precipitated proteins after separation by SDS-PAGE. Again, coiled-coil deletion mutants of Pnut were not able to incorporate into septin complex, suggesting an important role for the coiled-coil domain of Pnut in complex formation. Furthermore, Orc6 did not interact with Pnut mutants lacking part of the coiled-coil domain. The triple leucine mutants of Pnut, Pnut(3L1) and Pnut(3L2), were able to integrate into septin complex but little to none Orc6 precipitated with the complex. The anti-Orc6 antibody pull down experiments show that indeed Orc6 interacted with the complex when wild type Pnut was present. However, in the case of the Pnut triple leucine mutants Orc6 was not able to pull down significant amounts of complex. Interestingly, immunoprecipitation reactions with anti-Orc6 antibody also pulled down a significant amount of Sep2. This was also confirmed by direct interaction between the two proteins (data not shown). This result suggests that Orc6 can interact with a subset of Sep2 not integrated into the septin complex.

Purification, Reconstitution and characterization of Drosophila septin complex.

In order to study the interaction of Orc6 with the septin complex in more detail septin complex was purified from *Drosophila* embryonic extracts. A new purification method was developed that avoids the previously reported immunoaffinity technique (Field *et al.*, 1996) and uses only chromatography steps as described in the Materials and Methods section. The procedure is not detrimental to the septin complex and has the advantage that it could be used to isolate native complex from other organisms for which no (epitope specific) antibodies against subunits of the septin complex are available. The protein fractions containing Pnut protein were determined by western blotting using anti-Pnut antibodies. At the final stage of purification, samples containing the septin complex were subjected to a 15%-35% glycerol gradient centrifugation. Figure 3A shows a silver stained gel of glycerol gradient fractions containing *Drosophila* septin complex. The proteins in this fractions consisted of Pnut (~70 kDa), Sep2 (~50 kDa) and Sep1 (~41 kDa). The protein identities were verified by western blotting and reversed-phase C18 liquid chromatography-mass spectrometry (LC-MS) as described (Renfrow *et al.*, 2007).

With complete cDNAs for each of the *Drosophila* septin complex subunits available we wanted to determine if coexpression of the genes would be sufficient for complex formation. The reconstitution of the septin complex would also allow the purification of the complex in high amounts for subsequent biochemical assays. Both *E.coli* and baculovirus expression systems were used for reconstitution of the *Drosophila* septin complex. Upon coexpression of all vectors carrying Pnut, Sep1 and Sep2, all proteins remained soluble and readily formed a complex. His-tagged Pnut facilitated isolation of the recombinant septin complexes with Ni-NTA beads. Complex was further purified as de

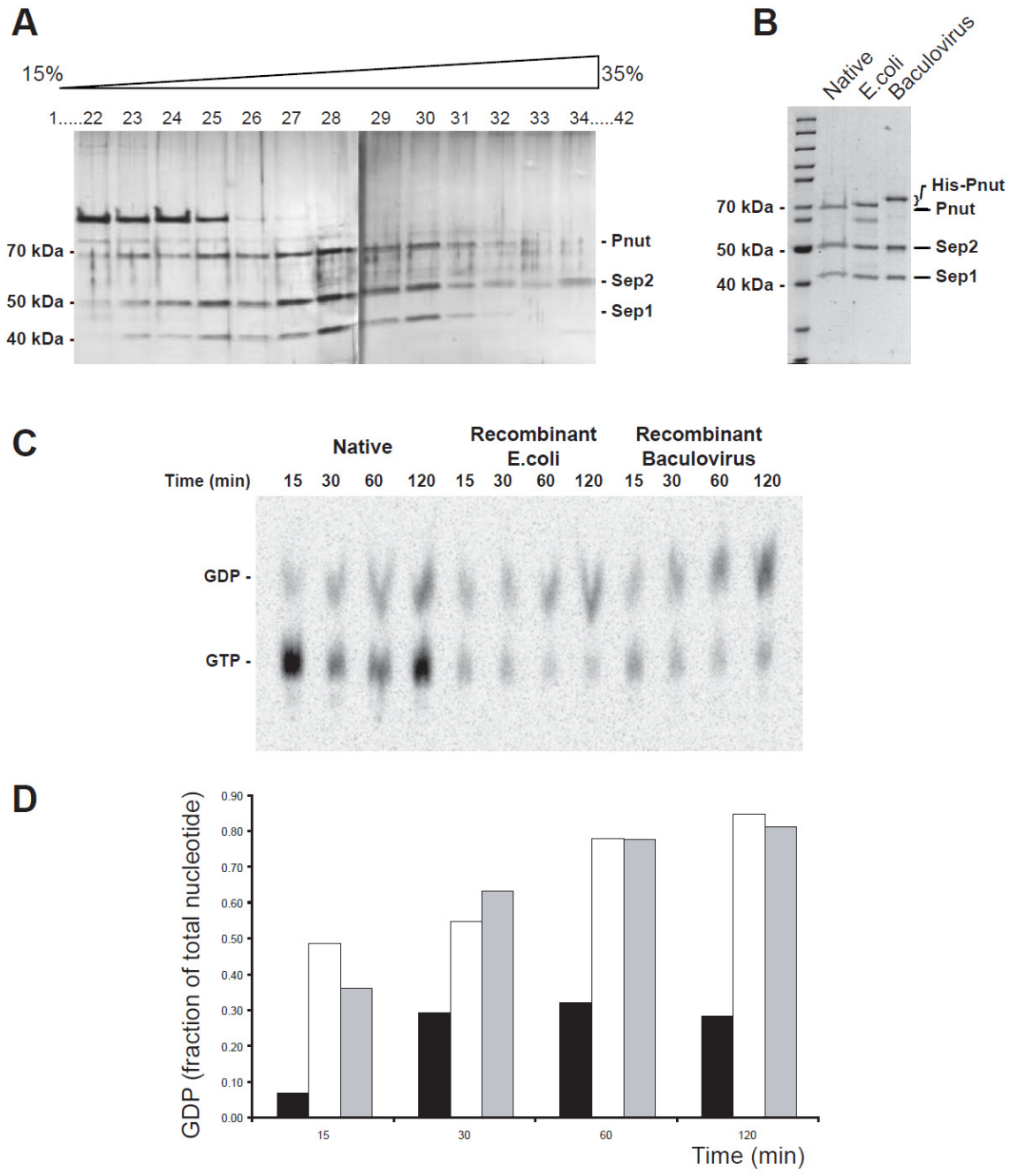

Figure 3. Purification of native and recombinant *Drosophila* septin complexes. (A) SDS-PAGE gel stained with silver of glycerol gradient fractions. In this last step in the purification protocol of septin complex from *Drosophila* embryos samples enriched for septin proteins were fractionated by glycerol gradient centrifugation. Fractions of the gradient were analyzed by SDS-PAGE followed by silver stain for the presence of septin proteins as depicted for a typical purification. (B) SDS-PAGE gel stained with colloidal Coomassie depicts purified reconstituted septin complexes next to purified native complex for comparison. His-tagged Pnut runs slightly higher than native Pnut. The *E.coli* derived complex preparation contains some degraded Pnut protein. (C) Phosphorimaging

(Figure 3, continued) picture of TLC plate containing separated nucleotides eluted from native and recombinant septin complex incubated with GTP at 21ºC for the time points indicated. The conversion of GTP to GDP was traced with α-^{32}P-GTP. (D) GTPase activity of purified septin complex. Graph depicts the amount of GDP generated as a fraction of the total of GTP and GDP measured on the TLC plate. The origin of the septin complex is: black bars – native; white bars - *E.coli*; grey bars - baculovirus.

scribed in the Materials and Methods section. The protein pattern observed in peak fractions is shown in Figure 3B for both *E.coli* and Baculovirus derived complexes. His-tagged Pnut runs slightly higher than native Pnut during electrophoresis.

GTPase activities of native as well as recombinant septin complexes were assayed essentially as described by Field et al. (Field *et al.*, 1996). Purified complex was bound to protein A beads via anti-Pnut antibody and used in a GTPase assay. A control experiment in which pulled down material was analyzed for Pnut by western blot confirmed that despite different sources of *Drosophila* septin complex equal amounts of complex were precipitated and used throughout the GTPase assay (Figure S1A). The hydrolysis of GTP to GDP was analyzed by TLC. Figure 3C shows representative phosphorimaging pictures of TLC plates with separated nucleotides eluted from both native and recombinant septin complexes. Graphic representation of the data is shown in Figure 3D. For native septin complex about 50% to 80% of newly bound nucleotide was retrieved as GDP after a 2 h incubation in individual experiments, depending on the batch of isolated protein. For the recombinant septin complexes this number was around 75 to 80%. This is in good agreement with the results reported previously (Field *et al.*, 1996). Recombinant septin complex seems to have a higher initial hydrolysis rate. Potential post-translational modifications might be the cause of the lower GTPase activity displayed by the purified native septin complex.

Septins are polymerizing proteins and purified septin complexes are known to form filaments *in vitro* (Field *et al.*, 1996). Preparations of both native and recombinant (baculovirus) complexes were analyzed with negative-stain EM for filament formation. Figures 4, A-D show filaments formed by native septin complex. As the concentration of the proteins is relatively low in the glycerol gradient fractions large aggregates were not observed (Figure 4, A-B). The addition of 1.5% PEG to the sample resulted in aggregation of the filaments (Figure 4C). Concentration of the proteins in the glycerol gradient samples using a Centricon concentrator also resulted in the formation of larger filaments (Figure 4D). The concentration dependency of septin complex solutions to form larger aggregates has also been described previously (Field *et al.*, 1996). This process involves a lateral association of filaments. The samples containing a highly concentrated, baculovirus produced reconstituted septin complex formed many larger cable-like aggregates as shown in Figures 4, E-F. Overall, recombinant septin protein complex behaved in a similar fashion as purified native complex.

Orc6 increases the GTP hydrolysis rate of the septin complex

The GTPase activities of native and recombinant, baculovirus derived, septin complex were measured in the presence of purified wild type and C-terminal mutant Orc6 proteins. Proteins were prepared as described (Balasov *et al.*, 2007). Figure 5 shows the TLC analysis of a typical experiment with native and recombinant (baculovirus) septin complexes. Quantification of TLC data for all septin complexes used is summarized in Table 1. Both for native and recombinant septin complex the addition of Orc6 increased the GDP over GTP ratio of bound nucleotide significantly compared to the con-

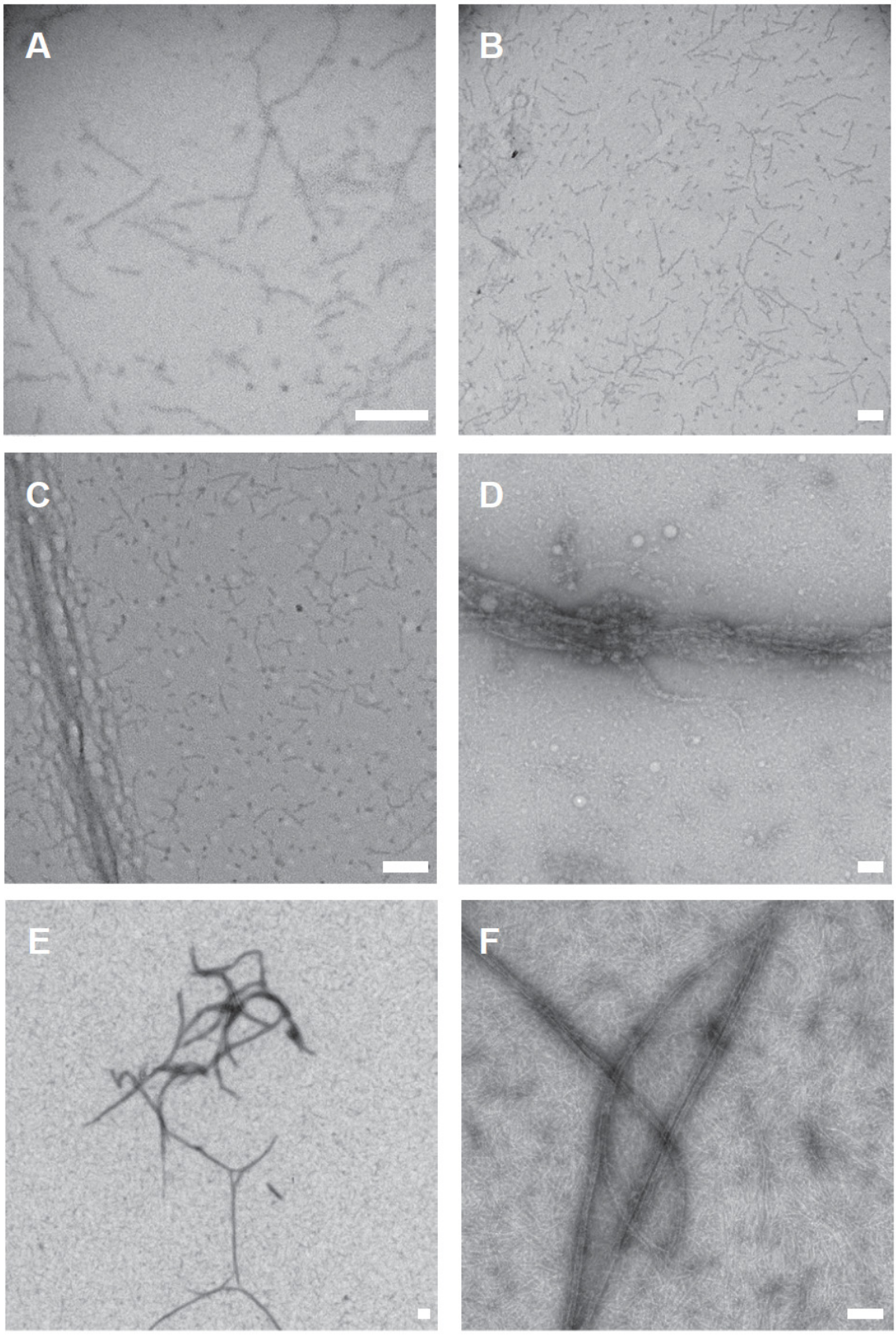
A
B
C
D
E
F

Figure 4. Negative-stain electron microscopy of filaments of native (A-D) and recombinant septin complex (E-F). Native complex shows smaller filaments due to the lower concentration of protein (A-B). The addition of 1.5% PEG 8000 (C) or concentrating the sample (D) induces larger cable-like aggregates. Cable-like aggregates are also present in the concentrated recombinant protein complex (E-F). Images taken at different magnifications, bars depict 100 nm.

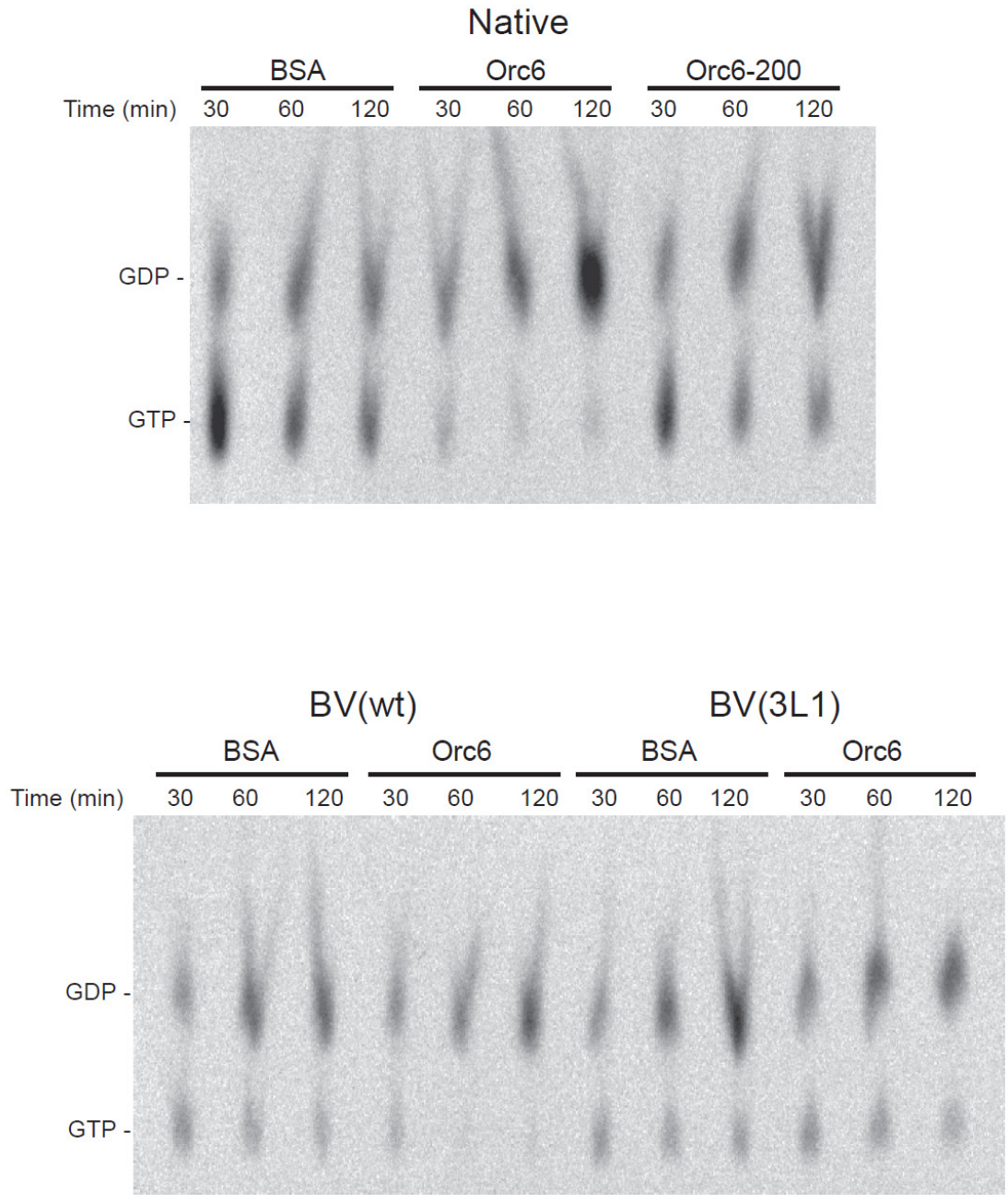

Figure 5. Orc6 increases GTP hydrolysis of septin complex. Phosphorimaging picture of TLC plate containing separated nucleotides eluted from either native or recom-

(Figure 5, continued) binant baculovirus septin complex incubated with GTP at 21ºC in the presence of BSA, wild type Orc6, and the Orc6-200 mutant for the time points indicated. BV(wt) – Baculovirus derived recombinant septin complex containing wild type Pnut. BV(3L1) – Baculovirus derived recombinant septin complex containing 3L1 triple leucine mutant of Pnut.

trol experiment with BSA. An Orc6-200 mutant that is unable to interact with Pnut (Chesnokov *et al.*, 2003) did not have an additional effect on GTPase activity of the septin complex. In fact the addition of Orc6-200 or BSA resulted in similar GDP to GTP ratios of the complex. Overall amount of newly bound nucleotide was in the same range for all septin complexes and resulted in ~ 0.05-0.1 mol nucleotide / mol Pnut after a 2 hour incubation, in good agreement with previously reported data (Field *et al.*, 1996). The increased GTPase activity is not detrimental to the integrity of the septin complex. SDS-PAGE analysis followed by silver stain revealed that after a two hour incubation of septin complex with Orc6 and GTP a stoichiometric septin complex with bound Orc6 could be retrieved (Figure S1B). Table 1 further shows that Orc6 did not have an effect on the GTPase activity of purified baculovirus recombinant septin complex containing triple leucine mutants of Pnut, which are not able to interact with Orc6 (Figure 2D). Septin complex containing wild type Pnut, either native or reconstituted, showed a 1.5 to 2 fold higher bound GDP over GTP ratio for wild type Orc6 than for the Orc6-200 mutant protein. For both wild type and C-terminal deletion mutant of Orc6 equal ratios were observed with septin complex containing a triple leucine mutant of Pnut. These data demonstrate that the effect of Orc6 on septin GTPase activity is via its direct interaction with Pnut.

Table 1. Amount of bound nucleotide converted to GDP on septin complex incu bated with the protein indicated. Values are means ± st. dev.

Septin	Incubation	Protein added to septin complex		
complex	Time (min)	BSA[a]	Orc6-200	Orc6 (wt)[b]
native			n =5	n =4
	30	1.00	1.04 ± 0.21	2.48 ± 0.79[c]
	60	1.00	1.03 ± 0.11	1.66 ± 0.31[c]
	120	1.00	1.06 ± 0.14	1.39 ± 0.35
Baculovirus			n =4	n =3
	30	1.00	0.97 ± 0.22	2.99 ± 1.30[d]
	60	1.00	1.03 ± 0.07	1.93 ± 0.21[c]
	120	1.00	0.97 ± 0.09	1.40 ± 0.40
3L1			n =3	n =3
	30	1.00	1.00 ± 0.04	1.03 ± 0.02
	60	1.00	1.03 ± 0.02	1.01 ± 0.02
	120	1.00	1.01 ± 0.01	1.04 ±0.05
3L2			n =3	n =3
	30	1.00	0.94 ± 0.02	0.89 ± 0.14
	60	1.00	1.00 ± 0.02	0.92 ± 0.15
	120	1.00	1.00 ± 0.01	1.04 ± 0.01

[a] Data is normalized to the amount of GDP associated with septin complex incubated with BSA. [b] Wild type Orc6. Student's T test probabilities for wild type Orc6 compared with Orc6-200 assuming the null hypothesis are: [c] $p < 0.01$, [d] , $p < 0.05$

Orc6 enhances filament assembly of the septin complex

GTP binding has been reported to induce filament formation of the recombinant expressed *Xenopus* septin protein Sept2 (Mendoza *et al.*, 2002). GTP hydrolysis was not important for filament formation and even seemed to be inhibitory as incubating the septin protein with non-hydrolyzable GTP-γ-S instead of GTP resulted in a higher degree of polymerization. These results were obtained for the single *Xenopus* septin protein. Filament formation of septin complexes isolated from yeast (Frazier *et al.*, 1998), *Drosophila* (Field *et al.*, 1996), or from reconstituted human septins (Kinoshita *et al.*, 2002) did not show a dependency for GTP. It was suggested that the GTPase activity of septin complexes might promote disassembly of the heteromeric complex (Mitchison and Field, 2002). In our experiments a 2 hour incubation of wild type recombinant septin complex in the presence of GTP and Orc6 did not seem to induce complex disassembly *in vitro* (Figure S1B).

In order to investigate the effect of Orc6 on septin filaments, EM was used to analyze septin filament formation under the conditions of the GTPase assay. Recombinant (baculovirus) septin complex was incubated with or without Orc6 in GTPase buffer for 2 hours at room temperature and analyzed for filaments. The effect of wild type Orc6 and Orc6-200 deletion mutant protein on septin filament assembly/disassembly was tested in the presence or absence of nucleotide. When diluted in GTPase buffer septin complex did not exhibit large filaments as is shown in Figure 6A. Very small filaments or individual complexes were not detected under the conditions used for EM. The addition of either 2 μM GDP, GTP, or GTP-γ-S had no detectable effect on filament formation (Figure S2). The addition of Orc6 significantly increased filament length as shown in Figure 6B.

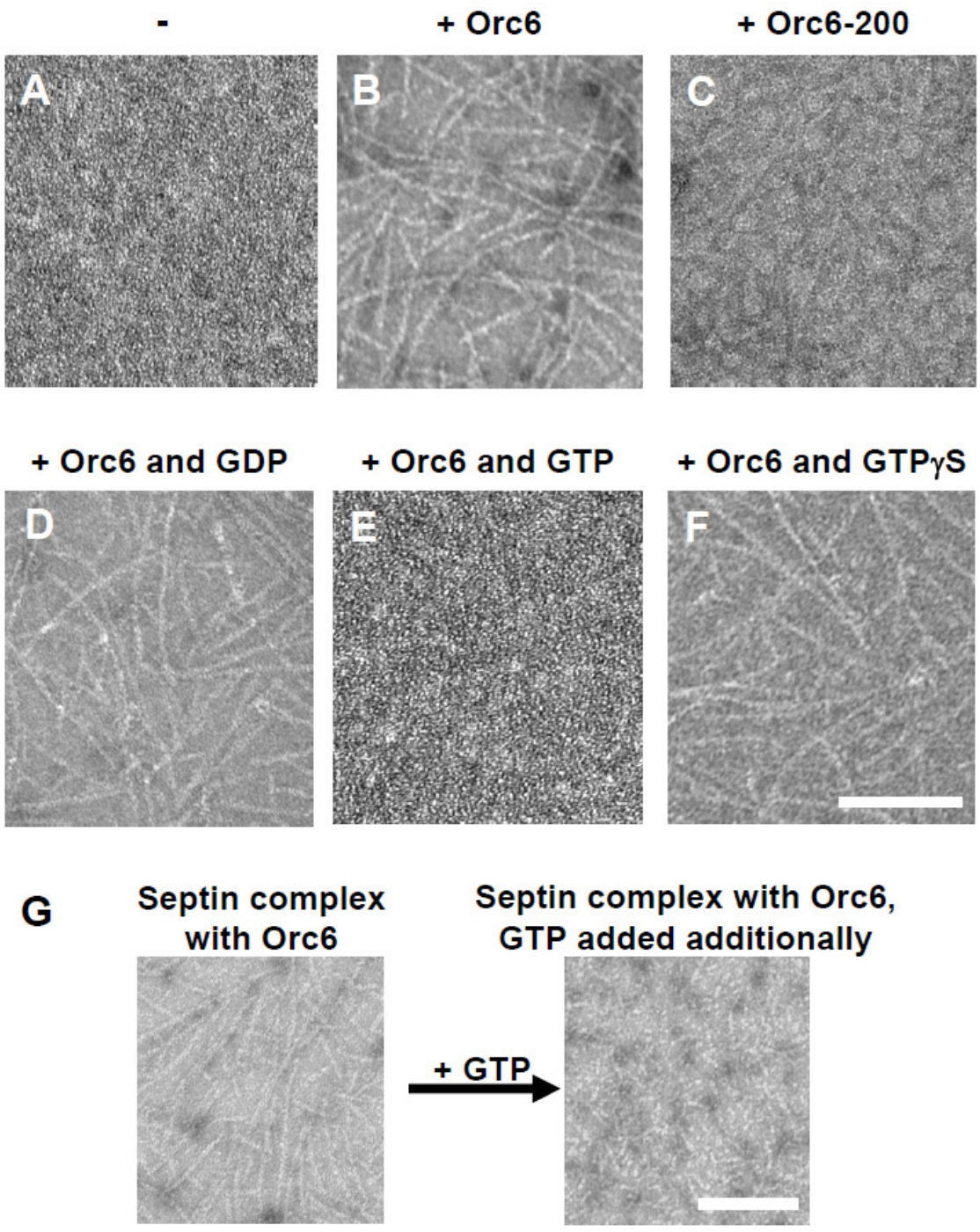

Figure 6. Orc6 enhances septin filament assembly. Recombinant baculovirus derived wild type septin complex was diluted in GTPase buffer to 80 ng Pnut/µl, and incubated with 80 ng/µl Orc6 protein and 2 µM nucleotide where indicated. Samples were incubated at room temperature for 2 hours after which they were prepared for EM. Proteins incubated are: (A) recombinant septin complex, (B) recombinant septin complex and Orc6, (C) recombinant septin complex and Orc6-200, (D) recombinant septin complex and Orc6 in the presence of GDP, (E) recombinant septin complex and Orc6 in the

(Figure 6, continued) presence of GTP, (F) recombinant septin complex and Orc6 in the presence of GTP-γ-S. (G) Recombinant baculovirus derived wild type septin complex was diluted in GTPase buffer to 80 ng Pnut/µl, and incubated with 80 ng/µl wild type Orc6 at room temperature for 2 hours. At this point half the sample was used for EM analysis, confirming filament formation. Subsequently 2 µM GTP was added to the remaining sample after which incubation at room temperature was continued for an additional two hours. Sample was processed and analyzed with EM. Bars depict 100 nm.

However, in the presence of GTP this effect of Orc6 on septin filament formation was greatly reduced (Figure 6E). On the other hand, incubation of Orc6 with septin complex in the presence of either GDP (Figure 6D) or GTP -γ-S (Figure 6F) still resulted in the formation of long filaments. One could argue that the presence of GTP merely prevented assembly of long filaments by Orc6 rather than aiding in their disassembly. However, in a follow-up experiment the filaments formed by the incubation of Orc6 with septin complex were significantly reduced in length when the incubation was continued after the addition of 2 µM GTP (Figure 6G). This result suggests that GTP hydrolysis leads to filament disassembly. The Orc6-200 mutant was not able to enhance filament formation as compared to wild type Orc6 (Figure 6C). No filament formation was detected in GTPase buffer containing Orc6 but no septin complex (data not shown). Furthermore, while septin complexes containing the triple leucine mutants of Pnut are able to form higher order filaments in concentrated samples (Figure S3A), filament formation of these complexes was not dramatically affected by the addition of Orc6 under GTPase assay conditions (Figure S3B). This indicates that the larger filaments are formed from the septin complex and that the assembly is induced/enhanced by its interaction with Orc6.

Discussion

Septin proteins have been discovered throughout the animal kingdom as well as in fungi. The *Drosophila* septins are essential for cytokinesis and are likely involved in other functions as well (Adam *et al.*, 2000). Studies in yeast suggested that a primary function of the septins is to serve as a matrix or scaffold for the organization of other proteins at the cell surface (Field and Kellogg, 1999; Versele and Thorner, 2005). Therefore, the identification of the septin-interacting proteins should be critical to the elucidation of septin functions. Earlier we found that the smallest subunit of *Drosophila* ORC complex, Orc6, is important for cytokinesis and interacts with the septin protein Pnut (Chesnokov *et al.*, 2003).

Both in *Drosophila* (Chesnokov *et al.*, 2001) and human cells (Prasanth *et al.*, 2002) a considerable pool of Orc6 is cytoplasmic and the protein is either associated with or proximal to the plasma membrane and cleavage furrows of dividing cells. In *Drosophila*, Orc6 and Pnut colocalize *in vivo* at cell membranes and cleavage furrows of dividing cells, and during cellularization in *Drosophila* early embryos (Chesnokov *et al.*, 2001). The C-terminal domain of Orc6 is necessary for this colocalization with Pnut. Moreover, Orc6 RNAi results in cytokinesis defects in *Drosophila* tissue culture cells (Chesnokov *et al.*, 2003), while Pnut RNAi disrupts the localization of Orc6 to the plasma membrane (Figure 1B).

Analysis of the cells treated with Pnut dsRNA revealed an elevated number of binucleated cells (5-30 fold increase, depending on the experiment). This is in contrast with previous reported data (Somma *et al.*, 2002) in which no elevated numbers of binucleated cells were detected in cultures treated with Pnut dsRNA. Differences in culture condi-

tions, amount of dsRNA used, as well as cell preparation protocols for analysis might have contributed to the discrepancies between the two studies.

Deletion of part of the predicted coiled-coil domain of Pnut impaired its ability to form a complex with both Sep1 and Sep2 together, while still able to interact with Sep1 (Figure 2C). Previous studies (Adam *et al.*, 2000) proposed that different septin complexes may exist within *Drosophila*. Our data suggests that Pnut and Sep1 might form a precomplex which joins with Sep2 to form the complete septin complex. The interaction of Orc6 with Pnut is also disrupted in C-terminal deletion mutants of Pnut suggesting that the coiled-coil domain of Pnut is important for both binding with Orc6 and for the formation of the septin complex. However, alanine to leucine substitutions within the coiled-coil domain of Pnut prevent Orc6 binding but do not inhibit complex assembly, indicating that these protein interactions are based on different structural moieties within the C-terminus. Furthermore, direct interaction studies (Figure 2B) revealed that the coil-coil domain of Pnut is not sufficient for the interaction of this septin with Orc6 and that other structural features may also be important for the interaction between the two proteins.

To study the effect of Pnut mutations *in vivo* in *Drosophila* tissue culture cells we used various expression systems including heat shock and metallothionein promoters. In all cases expression of GFP-Pnut in L2 cells resulted in rod- and spiral-like structures present throughout the cytosol (Figure S4). Expression of either N-terminal or C-terminal GFP fusions to Pnut, or a FLAG-Pnut protein also resulted in the same aberrant structures, compromising the *in vivo* analysis of Pnut mutants. However, when under control of the native Pnut promoter, proteins could be expressed in L2 cells at lower levels as is shown in Figure 2E. This figure further shows that the coiled-coil domain of Pnut, which

is important for *Drosophila* septin complex assembly, also is essential for the *in vivo* localization of Pnut, as FLAG-Pnut(1-427), lacking the coiled-coil domain, had the tendency to accumulate into crescent shaped aggregates. The FLAG-tagged triple leucine mutants of Pnut exhibited mainly diffuse cytoplasmic staining when expressed in L2 cells, although some plasma membrane staining was observed. It is possible that due to the mutations in the coiled-coil domain these Pnut mutants do not interact properly with other proteins (as shown for Orc6) resulting in a release from the plasma membrane at specific cell stages.

Native septin complex as well as reconstituted septin complexes exhibit the characteristic properties of filament formation and GTPase activity, indicating that they are functional complexes. As insect cell lines are closely related to *Drosophila* we used baculovirus derived reconstituted septin complex for further biochemical studies.

The human SEPT2-SEPT6-SEPT7 complex can be formed from recombinant proteins all lacking their predicted coiled-coil domains, suggesting that their C-termini are dispensable for complex formation (Sirajuddin *et al.*, 2007). Structural analysis of crystals of the human septin complex revealed that the filaments consist of an assembly of GTP-binding domains. However, the coiled-coil domains of SEPT6 ands SEPT7 do interact directly with each other (Low and Macara, 2006), suggesting that although not required for the human septin complex, coiled-coils may further stabilize filament formation (Sirajuddin *et al.*, 2007). The GTP-binding domains of human septin proteins can also interact with coiled-coil structures within the multiple subunit complex (Low and Macara, 2006). This might also occur with the *Drosophila* septins when they assemble

into complex. However, the interaction of Orc6 with the septin complex seems strongly dependent on the coiled-coil domain of Pnut.

One possible role of the interaction of Orc6 with the septin complex could be the regulation of the GTPase activity of the complex during cytokinesis. Orc6 reproducibly increased GDP to GTP ratio of bound nucleotide of the whole septin complex but no significant increase of total nucleotide bound to complex was detected. It has been hypothesized that GTP hydrolysis might promote disassembly of the septin complex (Mitchison and Field, 2002). However, purified recombinant septin complex was retrieved intact with bound Orc6 after 2 hour incubation in the presence of GTP, although potentially the disassembly of a small amount of complex might have occurred. We observed that many larger filaments present in concentrated recombinant septin complex samples were not detected under GTPase assay conditions (see Figure 6A), most likely due to the dilution of concentrated sample. No differences in filament size were observed for septin complexes incubated either in the presence or absence of GTP. However, due to limitations of our EM set up subtle changes in small filament size could not be detected.

Although GTP hydrolysis by septin complex was accelerated by Orc6 binding (as the presence of a non-binding mutant of Orc6 had no additional affect on hydrolysis) no significant changes could be detected in turnover rate. The higher turnover rates reported for individual Xenopus (Mendoza *et al.*, 2002) mouse (Kinoshita *et al.*, 1997) and human (Sheffield *et al.*, 2003; Huang *et al.*, 2006) recombinant septin proteins do not exclude a regulatory function for these subunits *in vivo* when not assembled in complex. A structural rather than regulatory role for septin complex-bound GTP and GDP was proposed from the results obtained with yeast septins (Vrabioiu *et al.*, 2004). No turnover of yeast

septin-bound GTP and GDP could be detected during a cell cycle *in vivo*. Furthermore, *in vitro* experiments revealed that GTP hydrolysis of yeast septin complex was limited by its slow binding or exchange activity, similar to the properties described initially for the *Drosophila* septin complex (Field *et al.*, 1996). The role of Orc6 in GTP hydrolysis and filament disassembly of septin complex also suggests that in the case of *Drosophila* the guanine nucleotides bound to septins may contribute to the structural properties of the complex. Additionally, the importance of the GTP-binding domains for the assembly of the human septin complex and potentially filament formation (Sirajuddin *et al.*, 2007) also indicates a role for guanine nucleotide in septin complex structure.

The addition of Orc6 to septin complex in the absence of GTP on the other hand greatly induced filament formation, while in the presence of GTP the effect was not observed. This indicates that Orc6 exhibits two opposite effects in its interactions with the septin complex, which are described in the model outlined in Figure 7. Based on the sequence homologies between human or *Drosophila* septins (Pan *et al.*, 2007) hexamers are depicted as a linear protein similar to the crystal structure of the human septin complex (Sirajuddin *et al.*, 2007) with Pnut at either end of the complex, as shown in Figure 7A. In the absence (or low concentration) of GTP Orc6 binding to Pnut enhances linear filament assembly (Figure 7B), potentially due to conformational changes in either Pnut or other septin subunits. Orc6 stabilizes the formation of the filaments by protein-protein interactions. It is interesting to note that purified recombinant Orc6 protein behaves as a dimer in biochemical assays (Chesnokov, unpublished). In the presence of GTP, Orc6 increases the GTPase activity of the septin complex, at the same time resulting in filament disassembly (Figure 7C). Increased GTPase activity may lead to conformational

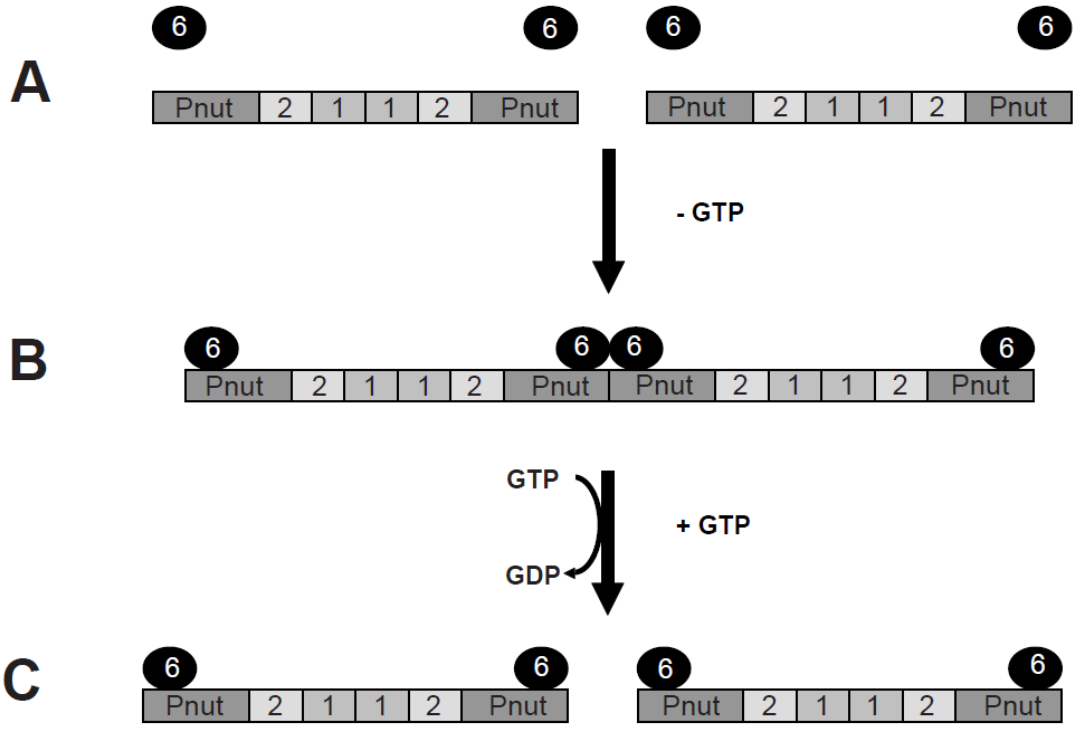

Figure 7. Model for the interaction of Orc6 with the septin complex. Pnut subunits within the linear complex are marked. Sep1 is marked as S1, Sep2 is marked as S2, Orc6 is marked as O6. For details see text.

changes in the septin complex (not shown), causing disassembly of septin filaments. Our results suggest that Orc6 may regulate either assembly or disassembly of septin filaments. Whether Orc6 actively induces filament disassembly in the presence of GTP or this process is a result of the increased GTPase activity of the septin complex remains to be investigated.

The septins are important for cytokinesis but molecular mechanisms of their functions in this process are not completely understood. Our data on interactions between *Drosophila* Orc6 and the septin complex reveal some new aspects for these proteins. Orc6 has an effect on both GTPase activity and filament formation of the septin complex,

suggesting that Orc6 might have a direct role in septin complex functions during the last stage of mitosis.

Acknowledgements

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Supplemental Figures

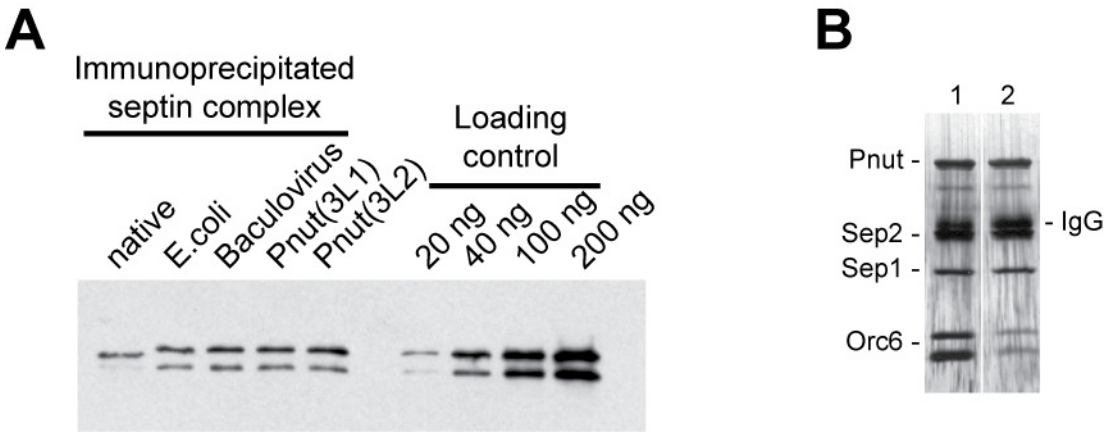

Figure S1. Septin complex used in GTPase assays is equal for different sources and remains intact under the assay conditions. (A) Different sources of Drosophila septin complex were incubated with anti-Pnut-Protein A-beads in GTPase buffer for 30 min at 4ºC. Beads were collected and washed 4 times with GTPase buffer. Beads were boiled in SDS sample buffer and analyzed with SDS-PAGE followed by immunoblotting with anti-Pnut antibody. Purified recombinant baculovirus derived septin complex was used as a loading control. The amount of immunoprecipitated septin complex loaded on the gel corresponds to the amount used in the GTPase assays. Lower band on blot indicates some Pnut degradation. (B). Recombinant baculovirus derived septin complex bound to protein A beads was incubated with 2 µM GTP and E.coli derived recombinant Orc6 in GTPase buffer for 2 hours at 21ºC. Beads were recovered (lane 1) and washed 5 times with GTPase buffer (lane 2) and further analyzed by SDS-PAGE followed by silver stain. Both lanes indicate that complex remains intact and that Orc6 is bound to the complex under GTPase assay conditions. The lower two bands represent Orc6 and equal the input of the purified protein (Balasov *et al.*, 2007).

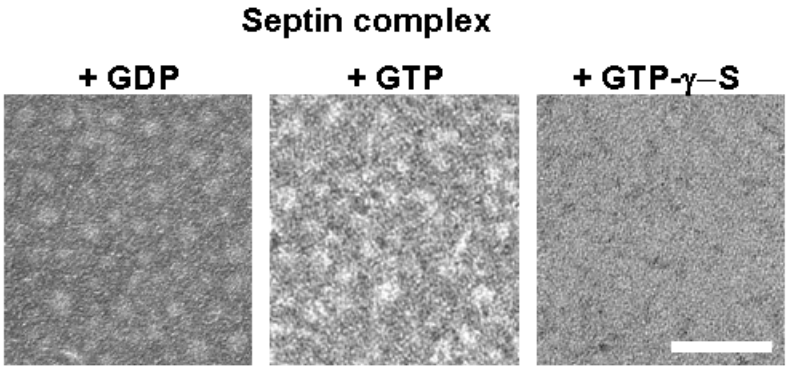

Figure S2. Effect of guanine nucleotides on septin complex filaments. Recombinant baculovirus derived wild type septin complex was diluted in GTPase buffer to 80 ng Pnut/µl, and incubated with 2 µM of the indicated nucleotide at room temperature for 2 hours after which they were prepared for EM. Bar depicts 100 nm.

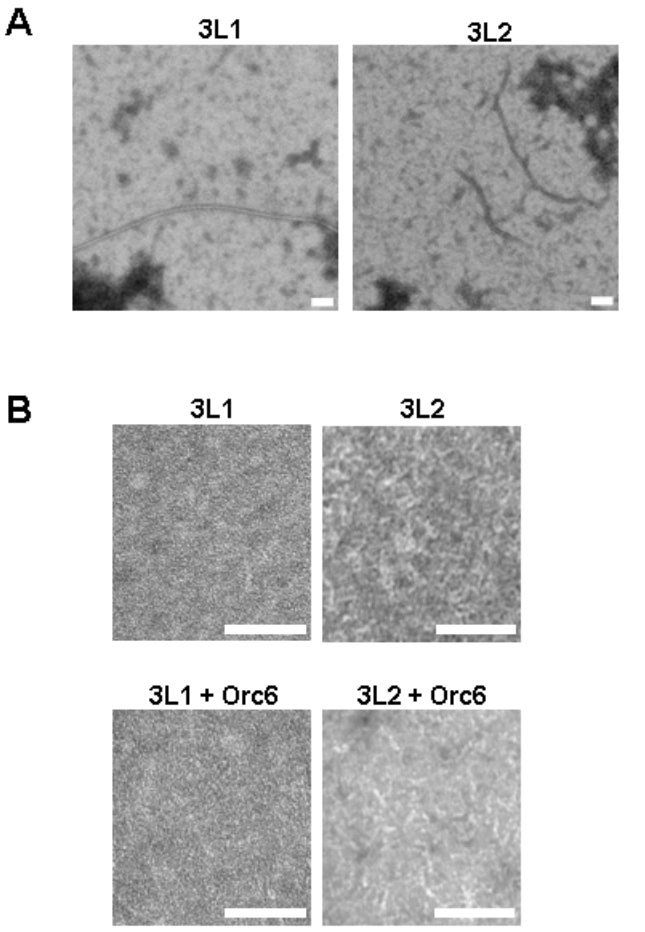

Figure S3. Filament formation of septin complexes containing Pnut(3L1) or Pnut(3L2). (A) Purified recombinant baculovirus derived septin complexes consisting of wild type Sep1 and Sep2 and either Pnut(3L1) or Pnut(3L2) mutant proteins show large filaments including some higher order structures. (B) Pnut(3L1) or Pnut(3L2) containing septin complex was diluted in GTPase buffer to 80 ng Pnut/µl, and incubated with 80 ng/µl Orc6 protein when indicated.

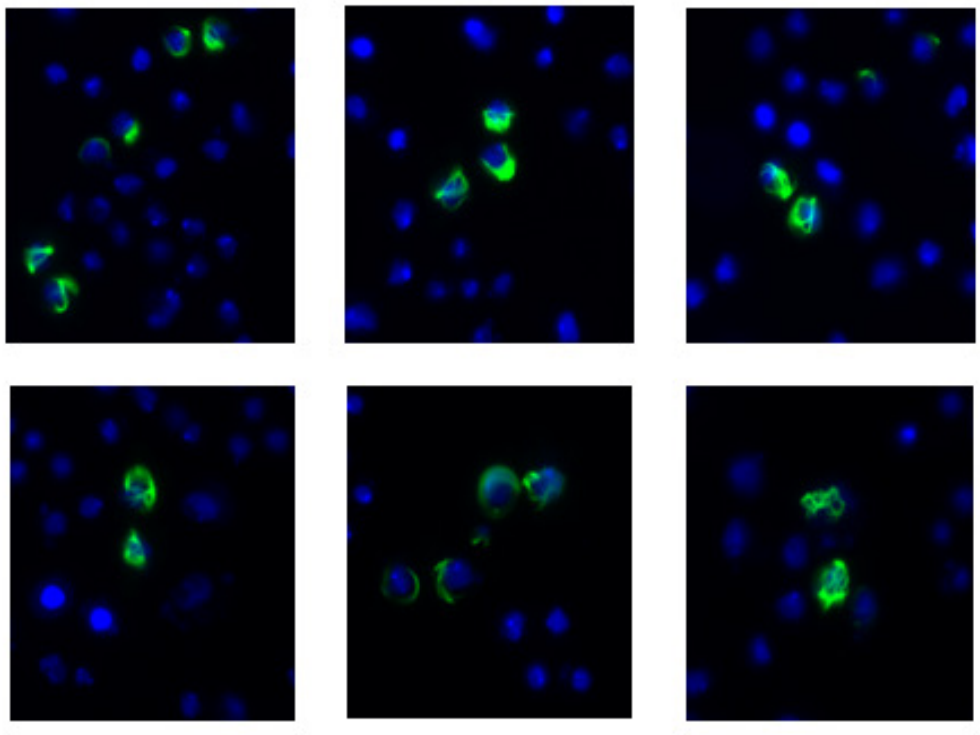

Figure S4. Ectopic expression of GFP-Pnut results in artificial structures within the cytoplasm. 3 x 10^6 *Drosophila* L2 cells seeded on a cover slip in a well of a six well dish were transfected with 1 μg of DNA using Insect Genejuice (Novagen) according to the manufacturer's recommendations. The transfected DNA construct consisted of the GFP-Pnut fusion protein under control of the metallothionein promoter. 24 hours post-transfection expression of GFP-Pnut was induced with 0.5 mM copper sulfate. 24 hours post-induction cells were was fixed with 2% formaldehyde in PBS, counterstained with DAPI, and mounted with 80% glycerol, 20% 1 x PBS, 2% N-propyl-gallate. Cells were analyzed with fluorescence microscopy. Blue: DAPI, Green: GFP-Pnut.

STUDY OF PNUT PHOSPHORYLATION

by

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Introduction

Septins are a family of polymerizing GTPases, which are required for cytokinesis and other processes that involve spatial organization of the cell cortex (Weirich *et al.*, 2008). The septin genes were originally discovered through genetic screening for budding yeast mutants defective in cell-cycle progression (Hartwell 1971) and were later found in other fungi, nematodes, flies, and mammals. Vigorous studies on septin structure and functions performed since their discovery confirmed their essential roles in cytokinesis (Longtine *et al.*, 1996; Field and Kellogg, 1999; Versele and Thorner, 2005).

Septins are found as heteromeric complexes that can assemble in larger filaments (Kinoshita, 2006). Most of these gene products have a set of GTPase motifs near the N-terminus (G-1, G-3 and G-4) and coiled-coil domains localized at the C-terminus. GTP-binding and GTP-hydrolyzing activities of the purified and recombinant septin complexes or polypeptides have been demonstrated *in vitro,* however, the biochemical and biological significance of septin GTPase activity remains unclear (Field *et al*., 1996; Kinoshita *et al.*, 1997; Casamayor and Snyder, 2003). Structural studies and functional analysis of septins performed in recent years strongly suggest that the purpose of having different nucleotide states in septins is to allow structural transitions and recycling of septin filaments during particular stages of the cell cycle (Vrabioiu *et al.*, 2004; Sirajuddin *et al*., 2007; Sirajuddin *et al*., 2009; McMurray and Thorner**,** 2009). Septins polymerize to form rod-shaped hetero-oligomeric complexes, which in turn can form larger filaments. These filaments can assemble *in vitro* into even higher-order structures by self-assembly and templated assembly. Repeating unit complexes consisting of Cdc3p, Cdc10p, Cdc11p, and Cdc12p in budding yeast; Sep1, Sep2 and Pnut in flies; and Sept2, Sept6, and Sept7

in mouse and human have been purified and characterized (Field *et al*., 1996; Sheffield *et al.*, 2003; Frazier *et al.*, 1998; Huijbregts *et al.*, 2009). Although GTP binding renders septin complexes competent for polymerization, additional factors, such as post-translational modifications and interacting partners, might also regulate the dynamics of septin filament formation and breakdown in cells.

Phosphorylation of septins plays a prominent role in initiating and/or stabilizing filament assembly during collar formation and emergence of the bud in yeast (Versele and Thorner, 2004; Weiss *et al*., 2000; Schmidt *et al*., 2003; Kadota *et al*., 2004; Mortensen *et al*., 2002; Dobbelaere *et al*., 2003; Longtine *et al*., 1998) and possibly in mammals (Joberty *et al*., 2001; Tang and Reed, 2002). However, the details and mechanisms of these processes remain unclear and we are just beginning to understand how phosphorylation of septins (and septin-associated proteins) influences the different facets of septin organization, dynamics and function.

In this study we have found that Pnut is phosphorylated in embryonic extracts. This form is present at early stages of embryonic development which suggest that it may play role in the regulation of septin complex activities before and during cellularization. The detailed study of this post-translational modification of Pnut may therefore lead to better understanding of the septins functions and regulatory role in cell division and embryogenesis.

Materials and Methods

Cloning and Mutagenesis

Substitutions in the Pnut phosphorylation sites (T509A, T509E, S517A, S517E, T509A S517A and T509E S517E) were introduced with Stratagene's site-directed mutagenesis protocol (Stratagene, La Jolla, CA; http://www.stratagene.com/manuals/200516.pdf). All constructs were analyzed by sequencing. cDNAs were subcloned into desired vectors with standard molecular biology techniques.

Proteins and Antibodies

Sep1 cDNA was cloned into pET-Duet expression vector (Novagen) using the BamHI and KpnI restriction sites. Sep2 cDNA was cloned into pGEX4T-1 expression vector (Qiagen) using the BamHI and NotI restriction sites. His-tagged Sep1 and GST-tagged Sep2 were produced by expression from the plasmids in *E.coli* strain BL21. After induction with IPTG, His-tagged Sep1 protein was isolated with Cobalt beads (Pierce) and GST-tagged Sep2 protein was isolated with Glutathione beads (Amersham). Purified antigens were used to generate rabbit polyclonal antibodies (Cocalico Biologicals). Antibodies were purified by affinity chromatography as described (Harlow and Lane, 1999). Rabbit polyclonal anti-Pnut antibodies have been described previously (Huijbregts *et al.*, 2009). Mouse anti-FLAG (M2 clone) was obtained from Sigma. Mouse anti-β-tubulin tubulin antibody (clone E7) is from Developmental Studies Hybridoma Bank.

Embryonic extracts, immunoprecipitation reactions and phosphatase treatment

Drosophila early embryonic extracts were prepared as described (Svitin and Chesnokov, 2010). Immunoprecipitations were performed with anti-Pnut, anti-Sep1 and anti-Sep2 antibodies bound to protein A Sepharose 4B beads (GE Healthcare). Were appropriate, precipitates were treated with phage λ protein phosphatase (NEB) for 1h at 30 ºC. Immunoprecipitated material was analyzed by SDS-PAGE followed by immunoblotting or silver staining.

RNAi assay

Double stranded RNA (dsRNA) was obtained by using the Megascript kit from Ambion. Pnut primers complementary to 3'-UTR of Pnut transcript (5'- CGGCCAGT GAATTGTTTAATACGACTCACTATAGGGACGCTCAAAACCCCCATTCCC-3' and 5'-CGGCCAGTGAATTGTTTAATACGACTCACTATAGGGTCGCCTCGCAC TCGTACATTC-3') flanked with T7 promoter were used. Pnut primers complementary to ORF of Pnut transcript have been described (Huijbregts *et al.*, 2009). L2 cells were cultured at 27ºC in Shields and Sang M3 medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum. For immunomicroscopy RNAi experiments 1 x 10^6 *Drosophila* L2 cells seeded on a cover slip in a well of a six well dish were inoculated with 15 µg of dsRNA complementary to Pnut 3'-UTR in 1 ml serum-free M3 medium. After 1 hour incubation 1 ml of medium supplemented with 10% fetal bovine serum was added to the culture. After 120 hours cells were fixed with 2% formaldehyde in PBS. Cells were stained for FLAG, Sep1 or Sep2 and counterstained with DAPI. Cover slips were mounted with 80% glycerol 20% 1x PBS, 2% N-propyl-gallate and analyzed with fluo-

rescence microscopy. RNAi efficiency was tested by immunoblotting with anti-Pnut and anti-FLAG antibody. For protein stability experiments, 2 x 10^5 *Drosophila* L2 cells seeded in a well of a twenty four well dish were inoculated with 6 µg of dsRNA complementary to Pnut ORF in 0.2 ml serum-free M3 medium. After 1 hour incubation 0.2 ml of medium supplemented with 10% fetal bovine serum was added to the culture.

Immunoprecipitation studies with de novo synthesized proteins

Construction of plasmids for the *in vitro* expression of proteins was performed analogously to described previously (Huijbregts *et al.*, 2009). Expression of proteins and immunoprecipitation reactions were performed as described (Pak *et al.*, 1997; Huijbregts *et al.*, 2009).

Obtaining stable cell lines expressing FLAG-tagged Pnut

L2 cells were transfected with a pCasper-based plasmid carrying wild type or modified FLAG-tagged Pnut together with the plasmid pCoHygro using Insect Genejuice transfection reagent (Novagen) as described previously (Huijbregts *et al.*, 2009). 16 hours post-transfection cells were divided over ten 100 mm dishes and allowed to settle overnight. From this point cells were cultured in media containing 50 µg/ml Hygromycin B. After 3-4 weeks colonies are isolated, expanded, and analyzed for protein expression by immunoblotting and immunofluorescent microscopy with anti-FLAG antibody.

MS identification of Pnut phosphorylation sites

Individual protein bands (upper and lower) from the immunoprecipitation reactions were excised from the gel with a razor blade. Gel bands were subsequently reduced with 10mM dithiothreitol for 45 minutes at 37°C, alkylated with 50mM iodoacetamide for 45 minutes at 37°C and digested overnight with both trypsin (Pierce) and AspN (Sigma) at 37ºC. Peptides were extracted from the gel using 50% acetonitrile and concentrated in a speedvac. Recovered peptides were loaded onto a 100-µm diameter, 10.5-cm pulled tip packed column with Jupiter 5-µm C18 reversed-phase beads (Phenomenex) using a Micro AS autosampler and LC nanopump (Eksigent). Parallel runs for both digests were analyzed via a linear ion trap-Fourier transform ion cyclotron resonance hybrid mass spectrometer (LTQ FT, Thermo Scientific) using either CID or ECD fragmentation. A gradient of H_20-acetonitrile in 0.1% formic acid was run from 5-40% over the course of 50 minutes at 650 nl min^{-1} (% H_20/acetonitrile). LTQ FT CID parameters were set as previously described (Renfrow *et al.*, 2007). ECD parameters were set with the Xcalibur software under the following arbitrary parameters and millisecond durations; 100ms ECD at power level 2.25 and a 90ms delay before IRMPD stimulation for 60ms at power level 15. *Drosophila* peptides from both the tryptic and AspN digests were initially identified with SEQUEST (Eng *et al.*, 1994), MASCOT (Perkins *et al.*, 1999), and Protein Prospector (Clauser *et al.*, 1999) software prior to manual inspection of modified and unmodified peptides. Pnut was unambiguously identified within its excised gel bands with >20 unique peptides.

In vivo radioactive labeling in tissue culture

Labeling of Drosophila L2 cells with $^{32}P_i$ was performed as described previously (Remus *et al.*, 2005), except no TBB was added. Pnut was immunoprecipitated from labeled whole-cell extract using affinity-purified anti-Pnut antibody. Half of each sample was treated with phage λ protein phosphatase (NEB). Samples were fractionated by SDS-PAGE and transferred to a poly(vinylidene difluoride) (PVDF) membrane. After exposure of the membrane to autoradiography film, the membrane was probed with antibodies against Pnut.

Protein degradation assay

Stable L2 cell lines expressing FLAG-tagged Pnut wild type or mutant protein along with non-transfected original L2 line were treated with dsRNA complementary to Pnut ORF as described above. Following RNAi treatment, cells were collected at time points with 12 h intervals. Cell were lysed and the lysate was fractionated by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with antibodies against Pnut (for non-transfected original L2 cell line) or FLAG (for transfectant cell lines) paralleled by probing with anti-β-tubulin antibody as a loading control. Bands were scanned and quantified using UN-SCAN-IT gel software (Silk Scientific Corporation). Level of Pnut or FLAG signal was normalized relative to level of tubulin signal, and the log2 of ratio of normalized signal level at each time point versus signal level at initial (0 h) time point was plotted against time.

Transgenic animals and rescue experiments

FLAG-tagged wild type and phosphorylation site mutant alleles of Pnut in described previously (Huijbregts *et al.*, 2009) pCasper-based vector under control of native Pnut promoter were sequenced, purified, and injected into w^{1118} fly embryos. The expression of fused products was verified by Western blot analysis with anti-FLAG antibody. In rescue experiments of the *pnut* mutant, progeny from heterozygous *pnut*/*Cy*; *FLAG-Pnut*/+ were analyzed for the presence of *pnut*/*pnut*; *FLAG-Pnut* flies. The percentage of rescued flies was calculated based on the expected segregation.

Results and Discussion

During isolation of septin complex from embryo extracts of different stages of development by immunoprecipitation with rabbit polyclonal antibody against Pnut protein, we have identified a novel form of Pnut protein which differed in size from regular Pnut. This form could potentially result from post-translational modification, such as phosphorylation (Figure 1, left panel). To check whether presence of this Pnut isoform is caused by phosphorylation we treated immunoprecipitates containing the novel form of Pnut with phage λ protein phosphatase. Such treatment led to disappearance of the upper Pnut band suggesting that it was a phosphorylated form of the protein (Figure 1, right panel).

Using tandem mass spectrometry to analyze the Pnut from the upper band, we found two phosphorylation sites located at the very C-terminus of the Pnut protein, downstream from the putative coiled-coil domain – T509 and S517 (Figire 2). We compared these regions with known consensus sequences of phosphorylation sites. Programs

Prosite (http://www.expasy.ch/prosite/), PredPhospho

(http://pred.ngri.re.kr/PredPhospho.htm)

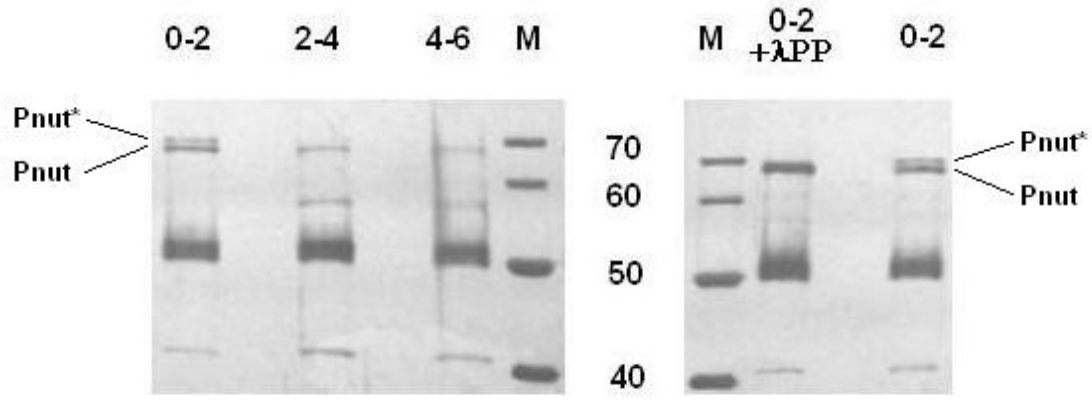

Figure 1**.** Phosphorylation of Pnut. Left panel: Pnut was immunoprecipitated from embryonic extracts made at different time points (0-2, 2-4 and 4-6 hours of development) with anti-Pnut antibody. Additional upper band of Pnut (designated as Pnut*) appears in 0-2 h extracts. Right panel: after treatment with λ protein phosphatase additional Pnut band is gone, compared to untreated sample. M - marker of molecular weight: 40, 50, 60 and 70 kDa.

and Phosida (http://www.phosida.com/) have identified T509 as a putative phosphorylation site for casein kinase 2, and S517 as a putative site for several kinases including protein kinase A, protein kinase G and kinases of the Aurora family (Table 1). In this relation it is interesting to note that human kinase Aurora-B interacts and partially colocalizes with Septin1 (Qi *et al.*, 2005).

481 LELQIRELEE KRREFEREKK EWEDVNHV**T**$_{509}$L 510

511 EELKRR**S**$_{517}$LGA NSSTDNVDGK KEKKKKGLF 539

Figure 2. Phosphorylation sites in the C-terminal region of Pnut. Phosphorylation sites are in red. Aminoacid positions of shown fragments are indicated on the left and right side. 539 is the C-terminal aminoacid residue in Pnut.

Table 1. Results of Pnut phosphorylation site motif search in different databases and algorithms.

Database	Site	
	T 509	S 517
Prosite (http://www.expasy.ch/prosite/)	Casein kinase II phosphorylation site (TleE)	cAMP- and cGMP-dependent protein kinase phosphorylation site (KRrS)
PredPhospho (http://pred.ngri.re.kr/PredPhospho.htm)	CK2 (X-[**S/T**]-X-X-[D/E])	PKA ([K/R]-[K/R]-[**S/T**]) PKG ($[R/K]_{2-3}$-X-[**S/T**]-X)
Phosida (http://www.phosida.com/)	CK2 (**S/T**-X-X-E)	PKA (R-X-**S/T**) Aurora (R/K-X-**S/T**-I/L/V) Aurora-A (R/K/N-R-X-**S/T**-M/L/V/I)

We also asked if this phosphorylation event is unique for early embryonic development. In order to detect phosphorylated form of Pnut in cultured cells we incubated *Drosophila* growing L2 cells in a media containing radioactive orthophosphate. Extracts were prepared from these cells and subjected to immunoprecipitation analysis using antibodies against Pnut. As shown in Figure 3, phosphorylated forms of Pnut are easily detected by autoradiography.

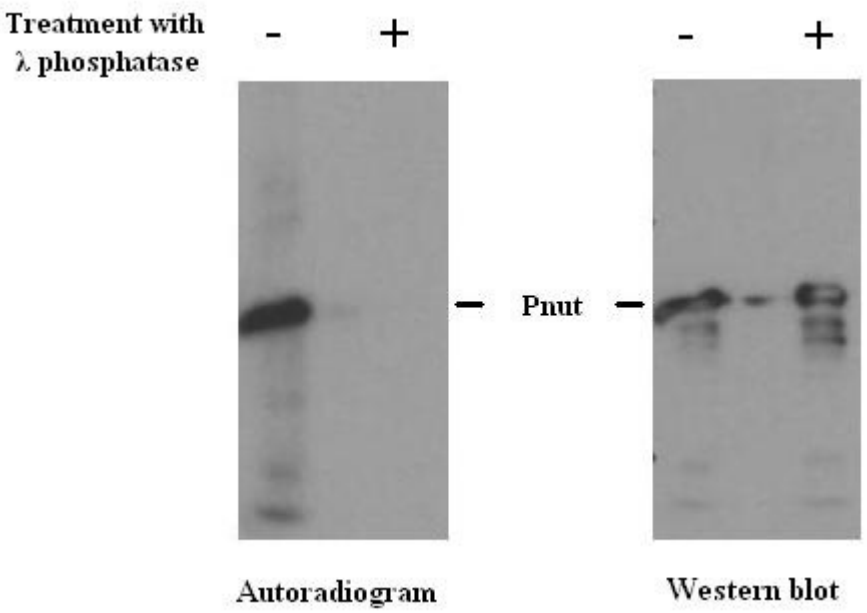

Figure 3. Pnut is phosphorylated in *Drosophila* cultured cells. Pnut was immunoprecipitateded from extracts prepared from cells incubated in a presence of radioactive orthophosphate. Immunoprecipitated material was treated by λ phosphatase (right lanes in both panels). Left panel: autoradiogram.Right panel: the presence of Pnut was confirmed by western blotting using anti-Pnut antibody.

Several hypotheses can be proposed to explain the role of Pnut phosphorylation. Phosphorylation may be involved in the regulation of septin complex activity in development, or alternatively, have a role at specific stages of the cell cycle. Septin complex activity may be regulated by phosphorylation at different steps, such as complex assembly or Pnut protein stability.

Phosphorylation may be needed for regulation of septins in early embryogenesis. The phosphorylated form of Pnut only appears in the extracts prepared from embryos at 0-2 h of development and disappears in later stages (Figure 1, left panel). 2 hour time point in *Drosophila* development is important because at that moment the character of cell divisions in the embryo significantly changes: before this point nuclear divisions are not followed by cytokinesis, and all nuclei of the embryo share a common cytoplasm

(syncitial blastoderm), but at approximately 2 hours of development (mitotic cycle 14) massive invagination of the embryonic plasma membrane in between nuclei leads to partitioning of nuclei into separate cells and cellularization of blastoderm (reviewed in Miller and Kiehart, 1995). We propose that phosphorylation of Pnut may inhibit the activity of the septin complex to prevent cytokinesis during development of syncitial blastoderm.

One potential mechanism of inhibition of septin functions by Pnut phosphorylation could be the disruption of septin complex formation. To check this possibility we analyzed septin complex formation in *in vitro* transcription-translation assay (Figure 4). We have constructed non-phosphorylatable (substitution of serine or threonine to alanine) and phosphorylation-mimicking (substitution of serine or threonine to glutamate) mutations of Pnut. The septin complex containing wild type or mutant Pnut was synthesized *in vitro* and immunoprecipitated with antibody against Pnut. We found that both types of Pnut phosphorylation site mutants coimmunoprecipitate with Sep1 and Sep2 which means that formation of septin complex *in vitro* is not disrupted by these mutations.

We also asked whether ability of Pnut phosphorylation site mutants to participate in formation of septin complex *in vitro* is paralleled by the joining of phosphorylated form of Pnut to the septin complex *in vivo*. To investigate this, we performed isolation of septin complex from embryo extracts of 0-2 h of development by immunoprecipitation with affinity-purified rabbit polyclonal antibodies against Sep1 and Sep2 proteins. Presence of additional high molecular weight form of Pnut in these precipitates indicated that phosphorylated Pnut interacts with both Sep1 and Sep2 and thus is a part of septin complex *in vivo* (Figure 5).

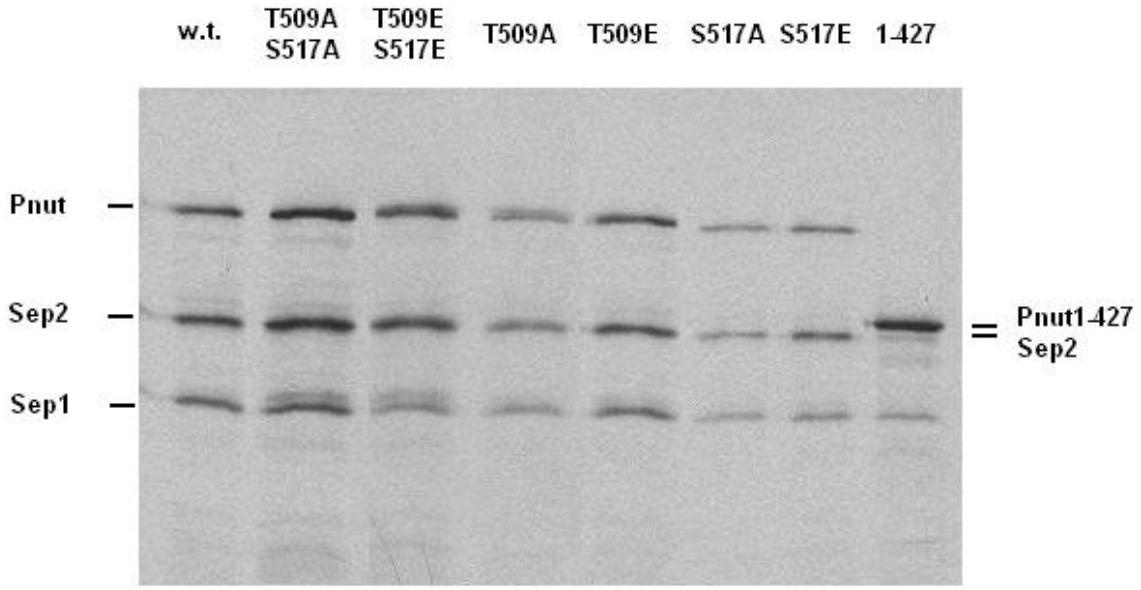

Figure 4**.** *In vitro* studies of septin complex assembly with Pnut protein containing mutated phosphorylation sites. Autoradiogram of immunoprecipitates with anti-Pnut antibody from coupled transcription-translation (TNT, Promega) reactions. Variants of Pnut proteins used in particular reactions are indicated on top. W.t. – wild type. 1-427 – negative control for septin complex assembly: Pnut1-427 mutant weakly (non-stoichiometrically) interacts with Sep1 and does not interact with Sep2.

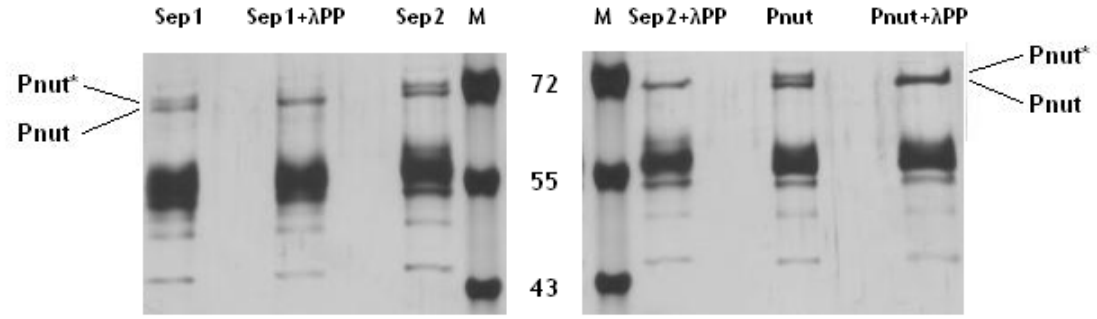

Figure 5. Phosphorylated form of Pnut is present in septin complex *in vivo*. Pnut was immunoprecipitated from embryonic extracts made at 0-2 hours of development with affinity-purified anti-Sep1, anti-Sep2 and anti-Pnut antibodies. Phosphorylated form of Pnut (designated as Pnut*) appears in in all precipitates except those which were treatmed with λ protein phosphatase. M - marker of molecular weight: 43, 55 and 72 kDa.

The ability of phosphorylated Pnut to join the septin complex *in vivo* and the lack of effect of Pnut phosphorylation site mutations on complex formation *in vitro* suggests that phosphorylation does not participate in the regulation of septin complex assembly.

We also considered the possibility that phosphorylation might have an effect on Pnut stability. To address this possibility we quantified half life of the wild type and mutant Pnut (either double knockout or double mimicking) when expressed in *Drosophila* tissue culture cells. We obtained stable cell lines expressing these Pnut phosphorylation mutants. Transfected Pnut construct was tagged with FLAG tag to distinguish the product from the endogenous protein. Expression of Pnut was repressed by treatment of culture with dsRNA followed by collection of cells at different time points, isolation of the proteins and western blotting analysis. As shown in Figure 6, we found that Pnut protein carrying double phosphorylation-mimicking mutation (Pnut T509E S517E) is significantly less stable than wild type Pnut or Pnut with double non-phosphorylatable mutation (Pnut T509A S517A). Calculated half-lives for Pnut wild type, Pnut T509A S517A and Pnut T509E S517E proteins were 17.7 h, 28.7 h and 12.2 h, respectively. This suggests that phosphorylation of Pnut decreases its stability and in this way may affect activity of the septin complex. Shorter half-life of phosphorylated Pnut may attenuate its activity and thus prevent cytokinesis during syncitial divisions. At the time of cellularization, dephosphorylation of Pnut may in turn stabilize this protein and activate septin complex which results in the on the onset of massive cytokinesis.

In this regard it is interesting to note that position of two closely spaced phosphorylation sites of Pnut located on C-terminus of the protein is somewhat similar to the arrangement of phosphorylation sites for Archipellago proteins such as Cyclin E and

dMyc (Hao et al., 2007; Moberg et al., 2001; Moberg et al., 2004). Archipelago binds to phosphorylated forms of these proteins and functions as adapter for ubiquitin-ligase targeting these proteins to degradation. It is possible that similar mechanism takes place in case of Pnut. Phosphorylation of Pnut may result in binding some adaptor protein to it and channeling Pnut to degradation pathway.

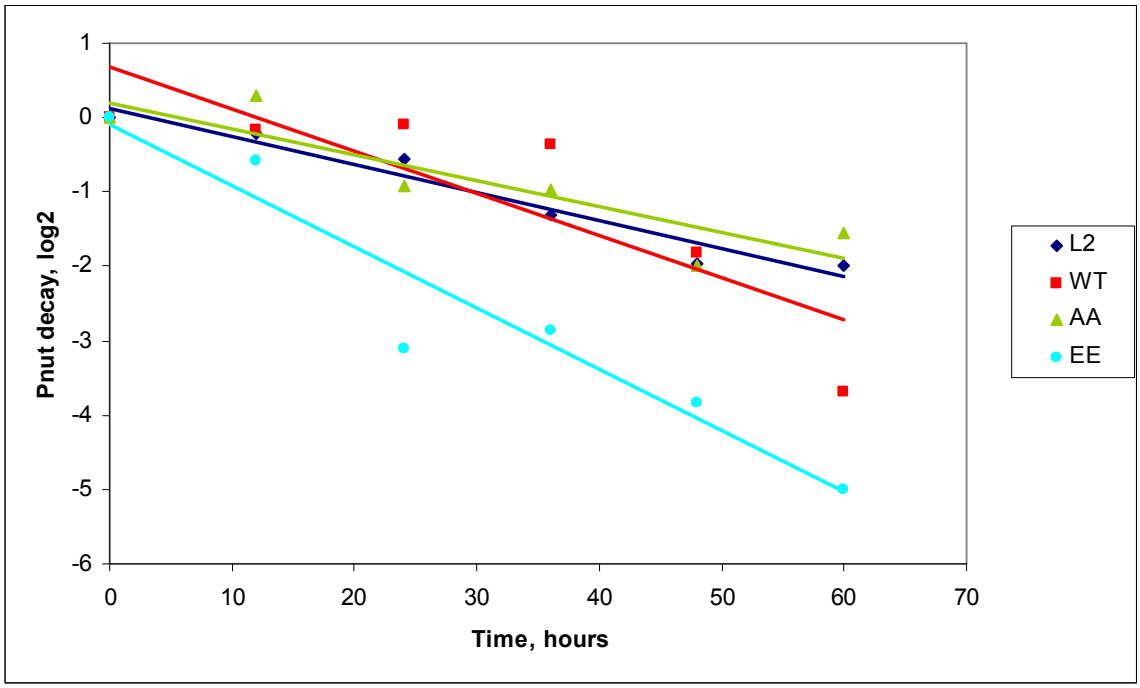

Figure 6. Protein stability assay of Pnut wild type and phosphorylation site mutants. Log2 of the ratio of Pnut level at given time point vs. level at 0 h point is plotted against time. L2 – non-transfected control (level of endogenous Pnut protein is measured), WT – flag-Pnut wild type protein, AA - flag-Pnut T509A S517A protein, EE - flag-Pnut T509E S517E protein (level of transgenic protein is measured for latter three series).

Regulatory role of Pnut phosphorylation may be specific for certain stage of the cell cycle. The functions of septin proteins in different organisms are often regulated by kinases and phosphatases that are active only at a specific time during the cell cycle. For example, in budding yeast direct phosphorylation by Cdc28 of the extreme C-terminus of

Cdc3 has been implicated in septin disassembly (Tang and Reed, 2002). It is possible that Pnut might be phosphorylated during a short period of time during final stages of cytokinesis that allows a disassembly of the complex.

To explore this possibility, we have tested the functional significance of Pnut non-phosphorylatable and mimicking mutants by expression in stable *Drosophila* L2 cell lines. Use of stable cell lines provided an opportunity to perform allele specific RNAi to study the functions of mutants in cells in the absence of endogenous proteins. We have found that treatment of cells with dsRNA complementary to 3'-UTR of Pnut results in an ablation of the endogenous proteins. If phosphorylation of Pnut is important for normal cell cycle progression, we would expect the expression of some of these mutants in *Drosophila* cells to result in defects in cytokinesis and altered cell morphology, particularly multinucleation, which can be easily detected by immunofluorescence microscopy. However, we did not detect significant differences in the number of multinucleated cells in any of cell lines expressing mutations in Pnut phosphorylation sites as compared to the line expressing wild type Pnut (Table 2). At the same time, we observed elevated number of multinucleated cells in original non-transfected L2 cell line treated with dsRNA complementary to 3'-UTR of Pnut under same experimental conditions which confirms that dsRNA-mediated ablation of endogenous Pnut was effective. These results suggests that Pnut non-phosphorylatable and mimicking mutants are able to effectively compensate for the absence of endogenous Pnut protein in tissue culture and thus are functionally intact.

Raw numbers of cells as well as percentages are indicated. Non-transfected original L2 cell line was used as a control. Differences in number of multinucleated cells are only significant between control and transfectant lines ($P<0.0005$), but not between wild

type and any of mutant transfectant lines, as shown by Fisher's exact test with two-sided P value using InStat program (GraphPad Software).

Table 2. Phenotype of L2 stable transfectants with Pnut phosphorylation site mutations under RNAi treatment.

	Wild type	T509A	T509E	S517A	S517E	T509A S517A	T509E S517E	Control L2
Multinucleated cells	1% 2	2.2% 7	3.8% 12	2.7% 6	2.6% 9	1.7% 5	0.9% 2	13.5% 23
Total	100% 197	100% 316	100% 316	100% 221	100% 351	100% 295	100% 221	100% 171

It is possible that the effect of Pnut mutations might take longer than 5 days (time frame between dsRNA treatment and phenotype assessment by microscopy in our experiments) to develop visible manifestation. RNAi experiments with prolonged time of dsRNA treatment may be able to reveal effects of mutations in Pnut phosphorylation sites on cell cycle progression. Also, using synchronized cell cultures will help to find out whether Pnut phosphorylation is specific to the certain stage of the cell cycle which may suggest function for this modification of Pnut.

Study of Pnut phosphorylation site mutation in tissue culture was accompanied by *in vivo* experiments in transgenic flies. We obtained flies expressing Pnut non-phosphorylatable and mimicking double mutants under the control of Pnut native promoter. These flies were used for viability rescue experiments: Pnut alleles were tested for

their ability to rescue lethality associated with *pnut* deletion. We found that both double non-phosphorylatable and double mimicking alleles of Pnut are sufficient for animal survival (Table 3). Thus *in vivo* results parallel experiments in tissue culture demonstrating that mutations in Pnut phosphorylation sites do not affect functional activity of the protein, at least under the conditions tested.

Table 3. Rescue of *pnut* mutants with Pnut transgenes.

Transgenic construct	Total progeny (rescue %)
flag-Pnut wt	716 (33%) 431 (38%) 386(32%)
flag-Pnut T509A S517A	824 (44%) 793 (55%)
flag-Pnut T509E S517E	754 (56%) 937 (0%) 806 (41%)

Importantly, it was recently shown that *pnut* germline clone females produced eggs, indicating that stem cell and cystoblast divisions in the female germline do not require Pnut (Adam et al., 2000). These Pnut deficient embryos completed syncytial divisions but failed at the stage of cellularization in agreement with our hypothesis that maternally deposited Pnut is inactivated by phosphorylation. This might mean that regulatory role of Pnut phosphorylation in development can be elucidated by testing the requirement of this phosphorylation in the absence of maternally deposited Pnut protein. We cannot directly address this question using transgenic flies described above since all rescued animals, including those in which rescue was performed with Pnut wild type

gene, were sterile. However, it can be done by creation of germ line and embryonic clones of Pnut and testing them for developmental abnormalities.

Our preliminary data also indicate that phosphorylated form of Pnut might interact with 14-3-3ε protein. 14-3-3 dimers are known to bind phosphorylated epitopes on other proteins, presumably stabilizing this modification (reviewed in Tzivion and Avruch, 2002). This protein also works as a functional adapter of phosphorylation: the properties of the target protein are modified upon 14-3-3 binding which results in functional switch as effect of phosphorylation. Binding of 14-3-3 to its target may stabilize conformational changes, leading to activation or deactivation of the target. It may also mask or expose interaction sites, leading to changes in binding partners and subcellular localization. In this context, the possibility of interaction between the phosphorylated form of Pnut and 14-3-3 is certainly interesting, since it may help to understand the regulatory role of Pnut phosphorylation and the mechanism of this regulation.

Additionaly, other potential mechanisms of Pnut phosphorylation influence on septin complex functions may also be addressed in the future. Those include the effect of phosphorylation on GTPase activity and filament formation by the septin complex, as well as Orc6 influence on these activities and interaction of septin complex with Orc6. These experiments might reveal new properties of Pnut phosphorylated form and point to the potential functional role of this modification.

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CONCLUSIONS

Both DNA replication and cytokinesis are parts of the cell division process which ensures continuity of life and transmission of genetic information between generations. In this thesis we have studied several aspects of replication and cytokinesis in *Drosophila* cells including those which may serve as link between these two components of mitosis.

First, we have developed an optimized *in vitro* replication assay using a *Drosophila* embryo extract. Using low centrifugation speed and cold treatment of embryos at the stage of extract preparation and addition of *Xenopus* sperm membranes into the reaction have increased *in vitro* replication to a level comparable with reactions in *Xenopus* extracts. In combination with powerful genetic research tools available in *Drosophila*, this optimized *in vitro* replication system will be a valuable instrument in dissection of processes and players participating in replication.

This optimized *in vitro* replication assay was used to characterized two components of pre-RC. We have shown that whereas Orc1 is indispensable for replication, its N-terminal region, which has non-replicative function, is not required for successful DNA replication. Some properties of another member of pre-RC, Cdc6, were also studied in the *in vitro* replication system. Like Orc1, omission of Cdc6 from replication reaction resulted in lack of DNA synthesis. Furthermore, a single point mutation in the Walker A domain of Cdc6 which disrupts ATP binding also had a detrimental effect on replication, which suggests that ATP binding by Cdc6 is crucial for its function in the pre-RC.

Another component of replication machinery, Orc6, was previously shown to interact with septin protein Pnut (Chesnokov *et al.*, 2003). In the second part of this thesis we looked into interaction of these two protein and its possible role. We found that the coiled-coil domain of Pnut is necessary for Orc6 binding, but not sufficient for this process. Triple leucine substitutions in the C-terminal region of Pnut also disrupt Pnut-Orc6 interaction. We have reconstituted the septin complex from recombinant proteins and tested its GTPase activity and filament formation which allowed us to find a functional interaction between Orc6 and the septin complex. In the absence of GTP, Orc6 stimulated filament formation by the septin complex, whereas in the presence of GTP the intrinsic GTPase activity of the complex was increased in the presence of Orc6. We propose that Orc6 plays a regulatory role in the activity of the septin complex, working as a functional switch which stimulates either assembly or disassembly of filaments depending on the current conditions. Orc6-septin complex interaction thus can be a link coordinating replication and cytokinesis on the one hand by redistribution of Orc6 between its cytosolic (cytokinetic) and nuclear (replication) fractions and on the other hand by modulating septin filament formation and stability.

One more potential regulatory mechanism of septin complex activity was studied in the third part of this work. We have identified phosphorylated form of Pnut which is present at the early stages of embryogenesis, and mapped this phosphorylation event to two nearby sites on the C-terminus of the protein. The fact that decrease of Pnut phosphorylation coincided with the onset of cellularization prompted us to propose that this modification of Pnut may have a regulatory role. We hypothesized that phosphorylation somehow decreases activity of the septin complex during the first syncitial divisions of

embryo and allows cytokinesis to be delayed untill the start of cellularization. In search of mechanism for this proposed role of Pnut phosphorylation we have found that stability of Pnut is decreased when both phosphorylation sites are mutated into phospho-mimicking state. This may mean that phosphorylation destabilizes Pnut and thus decreases activity of the septin complex. Interestingly, location of phosphorylation sites in Pnut is somewhat similar to the ones which are important for recognition by ubiquitin-ligases in several proteins with cell cycle-related functions. That raises the possibility of Pnut phosphorylation being indeed a degradation signal thus providing a new regulatory mechanism for septin complex activity.

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