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High Fcp1 phosphatase activity contributes to setting an intense transcription rate required in *Drosophila* nurse and follicular cells for egg production

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ABSTRACT

During transcription cycles serine side chains in the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II undergo dynamic phosphorylation–de-phosphorylation changes, and the modification status of the CTD serves as a signal for proteins involved in transcription and RNA maturation. We show here that the major CTD de-phosphorylating enzyme Fcp1 is expressed at high levels in germline cells of *Drosophila*. We used transgene constructs to modify the Fcp1 phosphatase level in *Drosophila* ovaries and found that high levels of Fcp1 are required for intensive gene expression in nurse cells. On the contrary, low Fcp1 levels might limit the rate of transcription. Fcp1 over-expression results in increased expression of microtubules in nurse cells. Our results show that tightly controlled high level Fcp1 expression in the nurse cells of *Drosophila* ovaries is required for proper egg maturation.

1. Introduction

The carboxy terminal domain (CTD) of the largest subunit of the eukaryotic RNA polymerase II (RNAPII) enzyme is comprised of tandem repeats of heptapeptides. In mammalian RNAPII 52 heptapeptide copies which have a Tyr-Ser-Pro-Thr-Ser-Pro-Ser consensus sequence are present. In the *Drosophila* RNAPII CTD 45 less conserved repeats of a similar heptapeptide can be found (Corden et al., 1985; Corden, 1990). Post-transcriptional modifications at side chains of the CTD heptapeptides play a key role in the regulation of RNA biogenesis. Among several types of modifications the phosphorylations and de-phosphorylations of serines located at the 2nd, 5th and 7th positions of the heptapeptides are the most important in determining polymerase activity (Zhang and Corden, 1991; Baskaran et al., 1993). Preinitiation complex (PIC) formation requires unphosphorylated CTD. Initiation starts with CTD Ser5 phosphorylation and modification of this serine occurs mostly at promoter proximal regions. During transcript elongation, the pattern of phosphorylation changes, and CTDs with Ser2 phosphate groups are more characteristic of polymerases at distal regions of transcription units. The phosphorylation status of the CTD serves as a signal for enzymes involved in RNA biogenesis. Phosphorylated form of the CTD is required for the formation of the 5′ cap structure as well as to facilitate splicing and binding of enzymes that synthesize the polyA tail. Based on the ability to control versatile functions, the various modification forms of the heptapeptide repeats were suggested to constitute a code (CTD code) used by the cellular machinery in the regulation of steps of RNA biogenesis (Buratowski, 2003; Corden, 2007; Egloff and Murphy, 2008). At the end of the transcription cycle, when elongation is completed, phosphate groups from the CTD are removed. This allows RNAPII to enter into a new PIC and start a new round of transcription (Svejstrup et al., 1996; Kobor and Greenblatt, 2002; Lin et al., 2002a, 2002b). Thus, during rounds of transcription RNAPII cycles between hypo- and hyperphosphorylated states. These two forms of the largest subunit (Rpb1) of RNAPII are referred to as IIA and IIO, respectively. Because of their potential to modify RNA biogenesis through CTD modifications the kinases and phosphatases that post-translationally modify amino acids of the CTD attract a lot of attention. Several kinases which modify specific residues at various stages of the transcription process have been identified (Egloff and Murphy, 2008). In contrast, only very few CTD-specific phosphatases have been identified and characterized so far. Among these, Fcp1, a TFIIF-stimulated phosphatase is clearly one of the major CTD de-phosphorylating enzymes. Fcp1 from several organisms has been identified and characterized (Archambault et al., 1998; Palancade et al., 2001; Hausmann et al., 2004). The enzyme is conserved throughout eukaryotes. Its family of phosphatases are characterized by a C-terminal BRCT (Brca-1 C-terminal) domain and an N-terminal phosphatase domain (CPD) with a DxDxT signature motif (Chambers and Dahmus, 1994; Archambault et al., 1998; Cho et al., 1999; Kobor et al., 1999).

Abbreviations: CTD, carboxyl terminal domain; Fcp1, TFIIF-interacting CTD phosphatase; RNAPII, RNA polymerase II; PIC, preinitiation complex; Ser, serine; TFIIF, general transcription factor II F; BRCT, Brca-1 C-terminal; Scp, small CTD phosphatase; Q-RT-PCR, quantitative-reverse transcription-polymerase chain reaction; UAS, upstream activating sequence; GFP, green fluorescent protein; RPB1, RNA polymerase B subunit 1; FCN, follicular cell nucleus; NCN, nurse cell nucleus.

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Recent determination of the *Schizosaccharomyces pombe* Fcp1 crystal structure has revealed that an acylphosphatase domain of Fcp1 is located at the base of a deep canyon within the Y-shaped protein (Ghosh et al., 2008). Fcp1 interacts with the winged helix domain of RAP74, a subunit of TFIIF both through its central and C-terminal domains. This interaction, which interestingly involves the intrinsically disordered C-terminal of Fcp1, stimulates the CTD-phosphatase activity of the enzyme (Wostenberg et al., 2011).

Fcp1 is undoubtedly the major CTD-specific phosphatase. It presence is essential both in yeast and *Drosophila* cells and partial Fcp1 deficiency causes severe developmental disorder in humans. Although a small CTD phosphatase (Scp1) has been identified in some eukarya its phylogenetic distribution and function seems to be more narrow than those of Fcp1 (Yeo et al., 2003). Fcp1 is generally considered to be a CTD-specific phosphatase which shows higher affinity for Ser2P while is able to de-phosphorylate both Ser2P and Ser5P within the CTD heptades. Despite numerous studies, some controversy on the specificity of Fcp1, remains: Cho et al. reported that CTD phosphorylation on Ser2 was increased in *fcp1* mutant *Saccharomyces cerevisiae* and in the same mutant extracts phosphorylation at Ser5 was only mildly increased (Cho et al., 2001). In contrast, Kong et al. found that highly purified Fcp1 of *Saccharomyces cerevisiae* de-phosphorylated Ser5, but not Ser2 (Kong et al., 2005). Fcp1 from the fission yeast *Schizosaccharomyces pombe* was found 10-fold more active in de-phosphorylating Ser2 than Ser5 (Hausmann and Shuman, 2002). Mammalian Fcp1 on the other hand de-phosphorylated both Ser2 and Ser5 *in vitro* in the context of native RNAPII (Lin et al., 2002a).

We have identified the fly *fcp1* gene and, using genetic experiments we have shown that in *Drosophila melanogaster* the level of Fcp1 expression is tightly controlled. Both RNAi silencing and overexpressing Fcp1 resulted in apoptosis and aberrant phenotypes or lethality depending on whether the phosphatase level was altered in vital or non-essential organs (Schauer et al., 2009; Tombacz et al., 2009). Recently we have observed that transgenes carrying LacZ under the control of the *fcp1* promoter were intensely expressed in female ovaries. In contrast, the same reporter genes did not indicate detectable Fcp1 expression during the stages of embryogenesis and in larval tissues. Since no report has been published so far on the role of Fcp1 in the germline, these observations prompted us to ask questions on Fcp1 function in the ovaries.

In Drosophila oocytes develop in egg chamber consisting of cysts of 16 germline cells, surrounded by an epithelium of somatic follicle cells (Spradling, 1993). Each 16-cell cyst is generated from a single cell by four synchronized mitoses. Cytokinesis is incomplete during these divisions and as a result cytoplasmic bridges called ring canals are formed between the cells of a cyst. One cell in each 16-cell cyst is selected to become an oocyte, while the remaining 15 synthesize and through the ring canals supply the maternal dowry to this cell. During stages 7–10 of oogenesis the volumes of the nurse cells double once every 4–5 h, while the oocyte doubles its volume every 2 h (Dapples and King, 1970). To be able to fulfill their primary function, which is to provide a quantity of proteins and RNAs which will permit the start of embryogenesis in the transcriptionally silent zygote after fertilization, the 15 nurse cells undergo partial endo-polyploidization and are extremely active transcriptionally. This led us to ask whether high level Fcp1 is indispensable for egg maturation, or if Fcp1 is synthesized in nurse cells in high concentrations because it will be required to start embryogenesis. An earlier report that in *Xenopus* eggs fertilization triggered CTD de-phosphorylation (Palancade et al., 2001) further increased our interest in studying the role of Fcp1 in oogenesis. To answer the above questions we modified the level of Fcp1 in germline cells of *Drosophila melanogaster in vivo* and analyzed the influence of the altered Fcp1 concentration on CTD phosphorylation and gene expression in the ovaries. Our data show that high level Fcp1 is required for proper egg maturation.

2. Materials and methods

2.1. Recombinant DNA constructs

A 676 bp long fragment upstream of the translational start codon of *fcp1* was selected as a putative promoter and amplified with the following PCR primers: Fcp1promFw: CTATGGATCCTCGCGGTGCATG TAGC and Fcp1promRev: GCATGTCGACTCGCAAGGCTTACGTT. Subsequently these fragments and its shorter derivatives were cloned to expression vectors to analyze promoter activity and into pCaSPeR-AUG-LacZ for transgene construction (Thummel et al., 1988). A transgene construct carrying the full length fragment was injected into w^{1118} *Drosophila melanogaster* embryos to create transgenic lines.

For Fcp1 expression in germline cells a genomic fragment corresponding to the Fcp1 coding region and 676 bp 5′ upstream region was inserted into pPTWF vector using the GATEWAY Cloning System (Invitrogen).

2.2. Expression pattern of the promoter-LacZ fusion transgenes

To reveal Fcp1 expression pattern β-galactosidase reporter activity was detected in pCaSPeR-AUG-LacZ transgene carriers by X-gal staining of embryos, dissected third instar larvae and ovaries as described in (Ashburner, 1989). For staining tissues, the larvae were "blown up" with the fixative and cut open along the dorsal midline in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) (Szabad et al., 1979). The organs were dissected and post-fixed for 10 min in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. The tissues were rinsed in PBS and stained in 1% X-gal containing buffer (5 mM $K_3Fe(CN)_6$, 5 mM K_4 $Fe(CN)_6$, 2 mM $MgCl_2$ in PBS) in dark at 37 °C. The organs were prepared and analyzed using a light microscope.

2.3. Fly stocks

To study the effects of the lack of Fcp1 in the germline *UASt-Fcp1-Flag*/+; *tub-gal4*/+; $fcp1^{null}$/− flies were generated. The $fcp1^{null}$ stands for a P-element insertion line obtained from the Bloomington stock center (stock number: 23609). The " − " symbol stands for a deficiency that removes *fcp1* and a few neighboring genes (Bloomington stock center; stock number: 9068). The construction of *UASt-Fcp1-Flag* transgene has been described (Tombacz et al., 2009). *UASp-Fcp1-Flag* transgenes carriers were generated by the standard germline transformation procedures. These were crossed to the germline specific *tub67C-gal4* driver (Hacker and Perrimon, 1998) carriers to overexpress Fcp1 in the germline. $Ketel^{GFP}$ and $Jupiter^{GFP}$ reporter lines were used to highlight the nuclear envelope and microtubules, respectively (Karpova et al., 2006; Villanyi et al., 2008). To study the effects of Fcp1 overexpression on reporter gene expression in ovaries we generated *UASp-Fcp1-Flag*/$Ketel^{GFP}$; *tub67C-gal4*/$Jupiter^{GFP}$ females and their controls *UASp-Fcp1-Flag*/ $Ketel^{GFP}$; +/$Jupiter^{GFP}$ and also *UASp-Fcp1-Flag*/ $Ketel^{GFP}$; *tub67C-gal4*/+ females and their controls *UASp-Fcp1-Flag*/ $Ketel^{GFP}$. (For an explanation of the genetic symbols see the FlyBase at http://flybase.bio.indiana.edu.)

2.4. Immunological techniques

For microscopy, embryos were rinsed in PBST (0.1% (v/v) Tween 20 in PBS). Ovaries were fixed in 4% paraformaldehyde. To block nonspecific staining, embryos were incubated in 1% BSA (Sigma) in PBST for 120 min at 4 °C. For detection of microtubules or flag epitope, ovaries were incubated with DM1A mouse monoclonal anti-α-tubulin antibody (1:1000, overnight at 4 °C), or with M2 anti-Flag antibody (Sigma F3165) in 1:1000 dilution. The primary antibodies were applied in 1% BSA in PBST. After several rinses in