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Calpain A controls mitotic synchrony in the Drosophila blastoderm embryo

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ABSTRACT

The beautiful mitotic waves that characterize nuclear divisions in the early Drosophila embryo have been the subject of intense research to identify the elements that control mitosis. Calcium waves in phase with mitotic waves suggest that calcium signals control this synchronized pattern of nuclear divisions. However, protein targets that would translate these signals into mitotic control have not been described. Here we investigate the role of the calcium-dependent protease Calpain A in mitosis. We show that impaired Calpain A function results in loss of mitotic synchrony and ultimately halted embryonic development. The presence of defective microtubules and chromosomal architecture at the mitotic spindle during metaphase and anaphase and perturbed levels of Cyclin B indicate that Calpain A is required for the metaphase-to-anaphase transition. Our results suggest that Calpain A functions as part of a timing module in mitosis, at the interface between calcium signals and mitotic cycles of the Drosophila embryo.

1. Introduction

The eukaryotic cell cycle is a spatially and temporally coordinated process that has evolved several checkpoints to ensure viability of daughter cells. Failure in this process may lead to proliferation defects, improper chromosome segregation, cell death, and cancer. The basic cell cycle includes mitosis (M), DNA synthesis (S), and two intervening growth phases (G1 and G2). Entry into mitosis, mitotic phase transitions, and exit from mitosis are driven by the sequential activation and degradation of evolutionarily conserved protein complexes composed of mitotic Cyclins (A or B) and Cyclin-dependent kinases (Murray, 2004). In the early Drosophila embryo, mitotic cycles occur in a syncytial cytoplasm and feature oscillations between M and S phases only. These divisions run independent of gene transcription, determined by the rate of translation and degradation of regulatory molecules. The three Drosophila mitotic Cyclins are degraded in succession during these stages: Cyclin A (CycA) in metaphase, Cyclin B (CycB) at the metaphase–anaphase transition, and Cyclin B3 (CycB3) during anaphase (Sigrist et al., 1995). It was proposed that Cyclins may work as a clock to time cell cycle progression (Murray and Kirschner, 1989). Nonetheless, in the early Drosophila embryo, Cyclin accumulation does not time mitotic entry (McCleland et al., 2009), although the destruction schedule of Cyclins may guide exit from mitosis (Yuan and O'Farrell, 2015).

The first 13 cycles in the Drosophila embryo take place nearly synchronously. After nuclei migrate to the periphery at cycle 9, a 30 s mitotic wave is observed starting at one or both anterior and posterior (AP) poles, swiping the nuclei at 9–20 min intervals (Foe and Alberts, 1983). Disturbance of this synchrony has been reported. It may result from a decrease in spindle checkpoint activity (Perez-Mongiovi et al., 2005) or from slowing of centrosomal and nuclear division cycles (Brunk et al., 2007). Subcortical and nuclear calcium waves in phase with mitosis throughout cycles 10–13 were reported, suggesting that calcium transients regulate mitosis in the early embryo (Parry et al., 2005; Whitaker, 2006). Despite the considerable amount of information on the involvement of calcium transients in mitosis, it is still debated how calcium signals provide input to regulate mitotic divisions. Putative downstream targets of calcium signals operating during mitosis include calcium-dependent proteases, kinases, and phosphatases such as the Calmodulin-dependent protein phosphatase calcineurin and Calmodulin-kinase II (Patel et al., 1999; Santella, 1998).

The neutral Calpain proteases are calcium-dependent modulatory enzymes that cleave substrates in a limited fashion, generating novel activities by substrate proteolysis (Friedrich and Bozoky, 2005; Sorimachi et al., 2011). Calpains modulate many cellular substrates and have been implicated in the regulation of several steps of the cell cycle (Janossy et al., 2004; Magnaghi-Jaulin et al., 2010; Panigrahi et al., 2011). Four calpains have been described in *Drosophila*: Calpains A, B, C, and Sol (Friedrich et al., 2004). The classical Calpains A and B (*CalpA* and *CalpB*) are expressed maternally and their products are detected in the early embryo (Emori and Saigo, 1994; Jekely and Friedrich, 1999; Park and Emori, 2008; Theopold et al., 1995). CalpA presents a unique

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Table 1
Embryo viability is decreased due to loss of maternal *CalpA* function.

Maternal genotype	Unhatched embryos (%)	n
matα/UAS-Dicer2; UAS-CalpA KD/+	27* and **	558
matα/+; UAS-CalpAGFP/+	19	300
matα/+	17*	840
CalpA[3979]/ *CalpA*[3979]	20**	200
CalpA[3979]/*Df(ED3716)*	60	100
CalpA[3979]/+	6	140
wild type	5	200

* Unpaired t test ($p < 0.05$).
** 51% of unhatched embryos from maternal *CalpA* KD and 64% of unhatched embryos from *CalpA*[3979] homozygous mothers do not complete embryonic development.

hydrophobic domain that favors association to membranes (Theopold et al., 1995), consistent with subcortical localization during interphase in syncytial stage embryos (Emori and Saigo, 1994; Fontenele et al., 2013). We have previously reported that CalpA regulates activation of an NFκB transcription factor during interphase. This role requires cortically localized CalpA and is dependent on the levels of the NFκB inhibitor encoded by *cactus* (Fontenele et al., 2013). Here we show that CalpA localizes to the mitotic spindle during mitosis and that loss of *CalpA* function results in a series of mitotic defects. This indicates that CalpA may translate the information from calcium waves into synchronized changes during mitosis. We also propose that Cyclin B is a direct target of CalpA proteolysis, disclosing part of the defects associated to *CalpA*.

2. Results

2.1. Calpain A controls mitotic divisions in the Drosophila embryo

The effect of Calpain inhibitors and Calpain overexpression in vertebrate and insect cells suggested that Calpains regulate mitosis (Janossy et al., 2004; Schollmeyer, 1988). Using endogenous loss-of-function assays, we have investigated Calpain function in Drosophila embryos. A significant percentage of embryos from hypomorphic (*CalpA*[3979]) or knockdown (KD) *CalpA* mothers are lethal and do not complete embryogenesis (Table 1). Lethality is increased as maternal *CalpA* function is reduced (*CalpA*[3979]/Df[ED376]). Close examination of early embryonic stages indicates that the developmental arrest in *CalpA* KD (attained by Gal4/UAS induction or double-stranded RNA injection) or *CalpA* hypomorphs (*CalpA*[3979]) may result from abnormal mitotic divisions.

In the Drosophila syncytial blastoderm, each cortical nuclear cycle takes between 9 and 20 min, with mitosis taking roughly half this time (Foe and Alberts, 1983). Mitosis in all nuclei is nearly synchronous and generates a wave of nuclear divisions that sweeps the embryo starting at one or both poles. Due to this wave, it is possible to see, in fixed embryos, two different mitotic phases in a same embryo. This is noticeable when mitotic chromosomes are highlighted with antibodies against phospho-Histone *H3* (pH 3). In control embryos undergoing mitosis, either all nuclei appear in the same phase or a mitotic wave is observed along the AP axis (Fig. 1A,B,D). In *CalpA* KD, asynchrony between divisions is seen without any clear directionality (Fig. 1C,E). To achieve a rigorous characterization of mitotic asynchrony due to altered *CalpA* function, we quantified the frequency of embryos undergoing mitosis and how many of these displayed nuclei in one, two, or more mitotic phases (Fig. 1F). The frequency of embryos in interphase versus those undergoing mitotic divisions in maternal *CalpA* KD or hypomorphs was not significantly different from control embryos. However, the number of embryos displaying nuclei in more than 3 mitotic phases (hereafter referred to as desynchronized embryos) was significantly increased. This indicates that either mitosis is taking longer or that in some nuclei the mitotic process is delayed due to impaired *CalpA* function.

Another indication that mitosis does not progress normally in *CalpA* loss-of-function is the high frequency of nuclear "fallout." It has been shown that nuclei harboring chromosome segregation defects are eliminated from the blastoderm by "falling" into the central vitellum (Postner et al., 1992; Sullivan et al., 1990). This mitotic catastrophe event allows the elimination of defective nuclei from the embryonic precursor pool (Takada et al., 2003). The remaining nuclei adjust their position after nuclear fallout to maintain an equidistant distribution. Falling nuclei can be seen in *CalpA* KD-fixed embryos due to their characteristic condensed appearance (Fig. 1C′,E′). When the number of abnormal nuclei is too large (as in Fig. 1E′), the remaining nuclei can no longer compensate and embryonic development is likely aborted. These observations indicate that the mitotic process is delayed and abnormal when *CalpA* function is decreased.

In order to identify the source of the mitotic delay in *CalpA* KD embryos, we first examined the morphology of structures that accompany mitotic progression. We examined microtubules of the mitotic spindle and the arrangement of chromosomes during mitosis. We observed an increase in the number of defective metaphase figures exhibiting condensation defects or chromatid misalignment in embryos from *CalpA* KD mothers (Fig. 2C, D, and E). Lagging chromosomes were also observed during anaphase. Furthermore, spindle microtubules aligned in anaphase were reduced, particularly in the spindle mid-body (Fig. 2B,D). The defective metaphase and anaphase figures observed are consistent with an impaired metaphase–anaphase transition. To obtain an accurate description of mitotic progression in embryos from mothers with altered *CalpA* function, we quantified the relative frequency of mitotic phases in fixed cycle 10–13 embryos. We scored embryos according to which and how many cell cycle phases were represented in each embryo as readout for the relative time spent during each phase (Fig. 2F). We observed that metaphase and anaphase figures were overrepresented in *CalpA* KD and *CalpA*[3979] desynchronized embryos. We also detected decreased levels of γ-tubulin at centrosomes resulting from loss of *CalpA* function (Fig. 2G,H). These results indicate that *CalpA* plays a role in regulating mitotic progression in the Drosophila embryo.

2.2. Calpain A associates to mitotic spindles

Calpains are traditionally known as cytosolic proteases. However, Calpains can be found in the nuclear compartment as well as in association with the cell membranes (Sorimachi et al., 2011). In accordance, we and others have shown that Drosophila CalpA is apically enriched at the cell cortex of the embryo syncytium during interphase (Emori and Saigo, 1994; Fontenele et al., 2009). Upon mitotic entry, CalpA is redistributed to the cytoplasm and nuclear domains (Fontenele et al., 2013; Fig. 3). Using fixation conditions that best preserve the mitotic spindle, we have examined CalpA distribution throughout mitosis. Endogenous CalpA protein progressively redistributes to the cytoplasm and perinuclear domains until it concentrates along microtubules of the mitotic spindle during metaphase (Fig. 3C) and anaphase (Fig. 3D). GFP-tagged CalpA expressed in response to maternal control

Fig. 1. Calpain A disrupts mitotic synchrony. A–E) Syncytial blastoderm embryos showing *anti*-phospho-Histone *H3* staining for mitotic nuclei (red, pH 3) and anti-vasa staining for pole cells (green, vasa) in the wild type (A,D), maternal *CalpA* knockdown (C,E) or maternal *CalpB* knockdown (B). Embryos displayed are <cycle 12 (A–C) or >cycle 12 (D,E). DNA is in blue. Arrows indicate highly condensed nuclei in the process of leaving the embryo cortex. F) Mitotic index defined by quantifying the fraction of embryos presenting interphase nuclei only (interphase), all one phase mitotic nuclei (mitosis), or nuclei in 2 (Mito2) or more mitotic phases (Mito3>). Embryos quantified were from the following maternal genotypes: wild type (WT), *CalpA* knockdown (mat > *CalpA* KD), homozygous *CalpA*[3979] mothers (*CalpA*[3979]), embryos injected with buffer (Buff inj), embryos injected with double-stranded RNA for *CalpA* (0.5 μg/ml *CalpA* dsRNA), embryos from *cact*[E10]/*cact*[A2] and from *cact*[E10]/*cact*[011] mothers. Distribution among classes was significantly different between WT ($n = 146$) and mat > *CalpA* KD ($n = 136$) ($p < 0.05$), *CalpA*[3979] ($n = 74$), *cact*[E10]/*cact*[A2] ($n = 155$) ($p < 0.0001$) and *cact*[E10]/*cact*[011] ($n = 98$) ($p < 0.05$), and between buffer injected ($n = 96$) and *CalpA* dsRNA ($n = 71$) ($p < 0.001$).