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Calcineurin Silencing in *Dictyostelium discoideum* Leads to Cellular Alterations Affecting Mitochondria, Gene Expression, and Oxidative Stress Response

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Calcineurin is involved in development and cell differentiation of the social amoeba *Dictyostelium discoideum*. However, since knockouts of the calcineurin-encoding genes are not possible in *D. discoideum* it is assumed that the phosphatase also plays a crucial role during vegetative growth of the amoebae. Therefore, we investigated the role of calcineurin during vegetative growth in *D. discoideum*. RNAi-silenced calcineurin mutants showed cellular alterations with an abnormal morphology of mitochondria and had increased content of mitochondrial DNA (mtDNA). In contrast, mitochondria showed no substantial functional impairment. Calcineurin-silencing led to altered expression of calcium-regulated genes as well as mitochondrially-encoded genes. Furthermore, genes related to oxidative stress were higher expressed in the mutants, which correlated to an increased resistance towards reactive oxygen species (ROS). Most of the changes observed during vegetative growth were not seen after starvation of the calcineurin mutants. We show that impairment of calcineurin led to many subtle, but in the sum crucial cellular alterations in vegetative *D. discoideum* cells. As these alterations were not observed after starvation we propose a dual role for calcineurin during growth and development. Our results imply that calcineurin is one player in the mutual interplay between mitochondria and ROS during vegetative growth.

Key words: mtDNA; mitochondrial remodelling; calcium; oxidative stress; phototaxis.

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Introduction

Calcium ions are important second messengers in all eukaryotes regulating a plethora of cellular functions (Berridge 2012). In the social amoeba *Dictyostelium discoideum* calcium has been shown to be an important signal for cell differentiation, asexual and sexual development, chemotaxis, and others (Gross 2009; Jaffe 1999; Kubohara et al. 2007; Malchow et al. 1996; O'Day and Keszei 2012; Scherer et al. 2010). Calcium-binding proteins like calmodulin or calcineurin can sense a change in the intracellular Ca^{2+} concentration and can transfer the Ca^{2+} signal to further target proteins. The protein phosphatase calcineurin is composed of two subunits: the catalytic calcineurin A (CNA) subunit and the regulatory calcineurin B (CNB) subunit. Activation of calcineurin occurs after Ca^{2+}-mediated binding of calmodulin and the regulatory CNB-subunit to the catalytic CNA-subunit (Li et al. 2011). In *D. discoideum* each subunit is encoded by a single gene, *canA* for CNA and *cnbA* for CNB, respectively, and expression of both genes is developmentally regulated (Aichem and Mutzel 2001; Dammann et al. 1996). Using pharmacological approaches and RNAi-silencing it has been shown that calcineurin plays a role in *D. discoideum* for stalk cell differentiation and tip dominance during asexual development as well as for cation homeostasis (Boeckeler et al. 2006; Horn and Gross 1996; Thewes et al. 2014). Classical knockouts of either calcineurin encoding genes failed, pointing towards an essential role for the phosphatase during vegetative growth. Additionally, expression of the catalytic CNA subunit was shown to be highest in vegetative cells (Dammann et al. 1996), but the exact role of calcineurin during vegetative growth in *D. discoideum* is not understood yet. The importance of calcineurin for growth in *D. discoideum* is in accordance with mouse studies, where knockouts are also lethal (Graef et al. 2001), but is in contrast to other lower eukaryotes like *Saccharomyces cerevisiae*, where calcineurin is dispensable for growth under normal conditions (Cyert 2003).

Given the lethality of calcineurin knock-outs in mammalian cells, researchers investigated the function of calcineurin either by pharmacological inhibition or by overexpression of constitutive active calcineurin. Overexpression of calcineurin led to mitochondrial dysfunction and elevated production of reactive oxygen species (ROS) in transgenic mice (Sayen et al. 2003). Similar results were observed with overexpression of the calcineurin-regulating protein RCAN1 leading as well to mitochondrial dysfunction (Chang and Min 2005; Ermak et al. 2012). Further it was demonstrated that calcineurin is involved in mitochondrial fission and fusion (Kasahara et al. 2013; Pennanen et al. 2014; Slupe et al. 2013; Wang et al. 2011). In mammalian cells, calcineurin can dephosphorylate the master regulator of mitochondrial fission Drp1 (dynamin-related protein 1) leading to translocation of Drp1 from the cytosol to the mitochondria (Cereghetti et al. 2008; Cribbs and Strack 2007; Elgass et al. 2013). In lower eukaryotes like the filamentous fungus *Aspergillus nidulans* calcineurin is involved in the regulation of number and function of mitochondria (Colabardini et al. 2014; Malavazi et al. 2009).

Nevertheless, results obtained with calcineurin inhibitors cyclosporin A (CsA) and FK506 have to be considered carefully, as both inhibitors interact with cyclophilins (Wang and Heitman 2005) and not only block the activity of the phosphatase but – as in the case of CsA – can also inhibit the activity of e.g. the mitochondrial permeability transition pore (mPTP), which is involved in calcium homeostasis of the mitochondria (Broekemeier and Pfeiffer 1995; Broekemeier et al. 1989; Szabo and Zoratti 1991). These side effects of the drugs make it difficult to specify the role of calcineurin for the mitochondria.

The aim of our study was to investigate the role of calcineurin during vegetative growth of *D. discoideum*. We exploited the advantage of having RNAi-silenced CNA and CNB cell lines of *D. discoideum* (Boeckeler et al. 2006; Thewes et al. 2014). We could directly investigate the effects of calcineurin-impairment without the need to consider putative side effects of pharmacological calcineurin-inhibitors. CNA- and CNB-RNAi mutants showed cellular alterations with abnormal mitochondrial morphologies and increased mitochondrial DNA (mtDNA) content. These effects were probably due to an increased intracellular level of reactive oxygen species (ROS). As increasing evidence suggests a role for mitochondria for development and differentiation of *D. discoideum* (Maeda and Chida 2013) and as calcineurin is involved in development and cell differentiation (Boeckeler et al. 2006; Horn and Gross 1996; Thewes et al. 2014; Weissenmayer et al. 2005), we investigated the putative connection between calcineurin and mitochondria during early development too.