Actin dynamics regulate subcellular localization of the F-actin-binding protein PALLD in mouse Sertoli cells

Bryan A Niedenberger¹, Vesna A Chappell¹, Carol A Otey³ and Christopher B Geyer^{1,2}

¹Department of Anatomy and Cell Biology, Brody School of Medicine, Greenville, North Carolina 27834, USA,

²East Carolina Diabetes and Obesity Institute, Greenville, North Carolina 27834, USA and ³Department of Cell Biology and Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Correspondence should be addressed to C B Geyer; Email: geyerc@ecu.edu

Abstract

Sertoli cells undergo terminal differentiation at puberty to support all phases of germ cell development, which occurs in the mouse beginning in the second week of life. By ~18 days *postpartum* (dpp), nearly all Sertoli cells have ceased proliferation. This terminal differentiation is accompanied by the development of unique and regionally concentrated filamentous actin (F-actin) structures at the basal and apical aspects of the seminiferous epithelium, and this reorganization is likely to involve the action of actin-binding proteins. Palladin (PALLD) is a widely expressed F-actin-binding and bundling protein recently shown to regulate these structures, yet it is predominantly nuclear in Sertoli cells at puberty. We found that PALLD localized within nuclei of primary Sertoli cells grown in serum-free media but relocated to the cytoplasm upon serum stimulation. We utilized this system with *in vivo* relevance to Sertoli cell development to investigate mechanisms regulating nuclear localization of this F-actin-binding protein. Our results indicate that PALLD can be shuttled from the nucleus to the cytoplasm, and that this relocation occurred following depolymerization of the F-actin cytoskeleton in response to cAMP signaling. Nuclear localization was reduced in *Hpg*-mutant testes, suggesting the involvement of gonadotropin signaling. We found that PALLD nuclear localization was unaffected in testis tissues from LH receptor and androgen receptor-mutant mice. However, PALLD nuclear localization was reduced in the testes of FSH receptor-mutant mice, suggesting that FSH signaling during Sertoli cell maturation regulates this subcellular localization.

Reproduction (2014) **148** 333–341

Introduction

Sertoli cells reside within the seminiferous epithelium and play critical roles in germ cell development. These include support of the spermatogonial stem cell niche, maintenance of the blood–testis barrier (BTB), remodeling of spermatid nuclei, reduction of spermatid cytoplasm, and release of spermatids into the tubular lumen. During the second week of life in the mouse, Sertoli cells respond to hormones including follicle-stimulating hormone (FSH), testosterone, and thyroid hormone to mature into an adult appearance (Walker 2003, Walker & Cheng 2005). In particular, they stop dividing, become polarized, and span the epithelium to support all phases of germ cell development. These dramatic changes in the Sertoli cell cytoskeleton accompany terminal differentiation, with regional concentrations of filamentous actin (F-actin) at dynamic structures termed the basal and apical ectoplasmic specializations (ESs) respectively (shown in [Supplementary Figure S1](#), see section on [supplementary data](#) given at the end of this article, thoroughly reviewed in [Vogl *et al.* \(2008\)](#) and [Lie *et al.* \(2010\)](#)). The basal ES is

a homotypic tight junction between adjacent the Sertoli cells forming the BTB that partitions the seminiferous epithelium into two distinct compartments, the basal and adluminal, and it must restructure during stages VIII–IX to allow for the translocation of preleptotene spermatocytes into the adluminal compartment to enter meiosis ([Russell 1977](#)). By contrast, the apical ES is a heterotypic adherens junction between Sertoli cells and associated elongating and condensing spermatids, and serves to anchor the spermatid until spermiation ([Ross 1976](#), [Romrell & Ross 1979](#)).

After many years of controversy, it is now accepted that the nucleus contains abundant G-actin, although F-actin has not been detected ([Gettemans *et al.* 2005](#), [Pederson 2008](#), [Zheng *et al.* 2009](#), [Visa & Percipalle 2010](#), [de Lanerolle & Serebryanny 2011](#), [Oma & Harata 2011](#)). However, a number of F-actin-binding proteins are found in nuclei. Although their exact function(s) remain unclear, proposed roles include regulation of mRNA processing and transport, chromatin structure, and transcription ([Ozanne *et al.* 2000](#), [Loy *et al.* 2003](#), [Archer *et al.* 2005](#), [Prante *et al.* 2008](#), [Zheng *et al.* 2009](#)).

The Palladin (PALLD) gene encodes a highly conserved F-actin-bundling protein that is both widely expressed and essential for development (Parast & Otey 2000, Luo *et al.* 2005, Otey *et al.* 2009). We previously showed that PALLD relocates from the cytoplasm to nuclei of Sertoli cells as they undergo terminal differentiation (Niedenberger *et al.* 2013). This predominant nuclear localization was also indicated in a report published at the same time (see Fig. 1D in Qian *et al.* (2013)). In that study, the role of PALLD in Sertoli cells was assessed by siRNA-mediated knockdown (KD) *in vitro*. The most obvious phenotype following PALLD KD *in vitro* was a loss of organization of the F-actin cytoskeleton, with multiple short fragments scattered throughout the cytosol (Qian *et al.* 2013). *In vivo* treatment with adjuvin, a specific disrupter of anchoring junctions in the apical ES causing infertility (Cheng *et al.* 2001, Mok *et al.* 2011), resulted in loss of PALLD signal at the apical ES by 48 h (Qian *et al.* 2013).

In this study, we utilized primary Sertoli cells in culture as a model to investigate the mechanisms regulating PALLD nuclear localization that occurs as Sertoli cells mature during puberty. In serum-free culture, PALLD remained nuclear, but the addition of serum resulted in a dramatic relocation to the cytoplasm. We found that PALLD nuclear localization is regulated by increased cAMP levels through Rho signaling and responds to the modulation of the G:F-actin ratios. We also found that localization is modified *in vivo* by perturbation of gonadotropin signaling. Taken together, these results provide a functional link between hormonal signaling and localization of a critical actin-bundling protein that regulates cytoskeletal dynamics in a wide variety of tissues and cell types. In addition, this provides the first example we are aware of where localization of an F-actin binding protein is determined by changes in polymerization of the actin cytoskeleton.

Materials and methods

Primary Sertoli cell culture

The Institutional Animal Care and Use Committee at East Carolina University approved all procedures involving mice (AUP# A178). CD1 mice were killed at 10 and 18 days *postpartum* (dpp) and Sertoli cells isolated essentially as previously described (Anway *et al.* 2003), without hypotonic shock treatment. After 2 days in culture, Sertoli cells represented $\geq 95\%$ of adherent junctions of cells. The cells were cultured up to 4 days on coverslips in 50:50 DMEM:Ham's F-12 (Cellgro-Mediatech, Manassas, VA, USA) with or without 10% FBS (ATCC, Manassas, VA, USA)-containing $1 \times$ penicillin-streptomycin at 33 °C in 95% air with 5% CO₂. The primary cell cultures were treated with the following compounds at least twice with the following (final concentration): 10 μ M cytochalasin D (A2618, Sigma-Aldrich), 2.5 μ M latrunculin B (L5288, Sigma-Aldrich), 100 μ M 8-bromo-cAMP (1140, Tocris Bioscience, Bristol, UK), 10 μ M forskolin (BML-CN100, Enzo Biochem, New York, NY,

USA), 1 μ g/ml C3 transferase (CT104, Cytoskeleton, Inc., Denver, CO, USA), 50 nM leptomycin B (L2913, Sigma-Aldrich), and 0.1 U/ml FSH (F2293, Sigma-Aldrich).

Indirect immunofluorescence on coverslips

The cells grown on coverslips were fixed for 1 h in 4% PFA. Blocking and antibody dilutions were carried out in PBS+0.1% Triton X-100-containing 3% BSA, pH 7.3. C-terminal anti-PALLD (1:200, ProteinTech Group, Inc., Chicago, IL, USA) or N-terminal PALLD (1:1000, Rachlin & Otey 2006) was added and incubated overnight at 4 °C. Alexa Fluor-488 anti-rabbit IgG (1:500, Life Technologies) was added with Alexa Fluor-594 phalloidin (1:1000, Life Technologies) for 1 h at room temperature. The coverslips were mounted with Vectastain-containing DAPI (Vector Laboratories, Burlingame, CA, USA) and images acquired using a Nikon E600 fluorescence microscope with an Orca II CCD camera (Hamamatsu Corp., Middlesex, NJ, USA) or a Fluoview FV1000 confocal laser scanning microscope (Olympus America). DAPI staining was used to visually determine the position of nuclei and PALLD localization. If PALLD staining was of greater intensity within the area stained by DAPI than outside, PALLD localization was determined to be nuclear. If PALLD staining was of lesser or similar intensity within the area stained by DAPI than outside, PALLD localization was determined to be cytoplasmic. For each treatment, when PALLD nuclear localization was determined, it was observed in $> 85\%$ of Sertoli cells.

Indirect immunofluorescence on frozen sections

The frozen sections were prepared as described previously (Niedenberger *et al.* 2013) from mouse testes. PBS+0.1% Triton X-100 containing 3% BSA of pH 7.3 was used for both blocking and antibody dilution. After blocking for 1 h, N-terminal PALLD (1:1000, Rachlin & Otey 2006) was applied to sections. Alexa Fluor-488 anti-rabbit IgG (1:500, Life Technologies) was added with Alexa Fluor-594 phalloidin (1:1000, Life Technologies) for 1 h at room temperature. The images were acquired using a Fluoview FV1000 confocal laser scanning microscope (Olympus America).

Immunohistochemistry

The testes were fixed in 4% PFA overnight at 4 °C and then processed using standard methods. Immunohistochemistry (IHC) was carried out on 5 μ m sections as described previously (Niedenberger *et al.* 2013). Blocking and antibody dilutions were done in 0.05 M Tris-HCl, 0.15 M NaCl, 0.02% Triton-X-100+1% BSA, pH 7.6. The antibodies were against PALLD C-terminus (1:500, ProteinTech Group, Inc.) and AR (#sc-816, 1:500, Santa Cruz Biotechnology, Inc.). The sections were imaged using a Zeiss Axio Observer A1 (Carl Zeiss Microscopy LLC, Thornwood, NY, USA) and images captured using a Dage XL16C digital camera and Dage Exponent software (Dage-MTI, Michigan City, IN, USA).

RNA isolation and quantitative (q)RT-PCR

Total RNA was isolated from the tissues using Trizol (Life Technologies), according to the manufacturer's instructions.

Table 1 Primer sequences.

Gene	Accession	Forward (5' → 3')	Reverse (5' → 3')
<i>Palld</i>	JX477684	TACAGAGCGTATTTGGTGCCAGT	TCAAAGGCCTCAGCAATGACCAAG
<i>Trf</i>	NM_133977	TAGACAGAACCCTGCTTGGAAACA	TTGAGTGGGGCCAATACACAGGTCA
<i>Fos</i>	NM_010234	AGAGAAACGGAGAATCCGAAGGGA	ATTGGCAATCTCAGTCTGCAACGC
<i>Vcl</i>	NM_009502	ACAGTGGCAGAGGTAGTGGAAACT	TCACCAACATCACACGGGTGTTCT
<i>B2m</i>	NM_009735	CCGTGATCTTTCTGGTGCTT	CGTAGCAGTTCAGTATGTTTCG
<i>Actb</i>	NM_007393	TCCGATGCCCTGAGGCTCTTTTC	CTTGCTGATCCACATCTGCTGGAA

Briefly, 100 ng of RNA from cultured cells were reverse transcribed and amplified in triplicate using the iScript One-Step RT-PCR Kit with SYBR green on an iQ5 real-time PCR detection system (Bio-Rad Laboratories). The primer pairs were designed to amplify across an intron/exon junction to avoid amplification of genomic DNA. Primer sequences are listed in Table 1.

Steady-state mRNA levels for commonly used housekeeping genes such as glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and beta actin (*Actb*) increased in response to serum (Schmittgen & Zakrajsek 2000); therefore Ct values were normalized to beta 2 microglobulin (*B2m*), which changed the least in response to serum. Relative mRNA levels were calculated with the delta–delta Ct (ddCt) method. Student's *t*-test was used for all statistical analyses, and significant differences are indicated with an asterisk at $P < 0.05$.

Immunoblot analysis

Fifteen microgram of protein were separated on a 10% SDS–polyacrylamide gel before immunoblot analysis using standard methods as described previously (Niedenberger *et al.* 2013). The gels were blotted onto PVDF membranes and blocked in 5% nonfat milk in PBS-T. The antibodies used were against C-terminal PALLD (1:1000, ProteinTech Group, Inc.), N-terminal PALLD, (1:1000, Rachlin & Otey 2006), and ACTB (1:1000, A2066, Sigma).

Results

PALLD localization is dynamic in primary Sertoli cells

We first isolated primary Sertoli cells from the testes at 10 dpp (when PALLD is cytoplasmic) and 18 dpp (when PALLD is nuclear) and maintained them in serum-free culture for up to 4 days to determine whether PALLD retained proper *in vivo* localization (Fig. 1A). We carried out indirect immunofluorescence (IIF) and found that PALLD localization was predominantly cytoplasmic at 10 dpp (Fig. 1B, Supplementary Figure S2A, see section on supplementary data given at the end of this article), but was mostly nuclear in Sertoli cells isolated from 18 dpp mice (Fig. 1C, Supplementary Figure S2B), as was seen *in vivo* (Niedenberger *et al.* 2013). It was previously shown that Sertoli cell morphology and cytoskeletal organization were modified *in vitro* by the addition of serum to culture media (Hutson *et al.* 1980). We tested whether the addition of serum would affect PALLD localization in 18 dpp Sertoli cells and found that

PALLD relocated to the cytoplasm in the presence of 10% serum (Fig. 1D, Supplementary Figure S2C). Conversely, in 10 dpp Sertoli cells, PALLD localization was not significantly affected by the addition of 10% serum. The addition of as little as 1% serum was sufficient to direct PALLD cytoplasmic localization (data not shown). To determine whether removal or addition of serum would change PALLD localization once it was established, we maintained 18 dpp Sertoli cells in serum-free medium for 3 days, then serum-stimulated for 24 h, and found that PALLD relocated from the nucleus to the cytoplasm. We did not, however, observe

Figure 1 PALLD localization in cultured primary Sertoli cells. Treatments with various inhibitors are described in the Materials and Methods section. (A) Timeline for primary Sertoli cell culture experiments. (B, C, and D) IIF to detect PALLD (in green) in primary Sertoli cells from mice aged 10 dpp (B) and 18 dpp (D and C) in the absence (B and C) or presence (D) of serum. Phalloidin-labeled F-actin cytoskeleton (in red). (E and F) Levels of *Palld* mRNA (E) and protein (F) were slightly elevated in Sertoli cells grown in the presence of serum ($P < 0.0271$). Scale bars = 25 μ m.

PALLD nuclear localization in cells that were maintained with serum for 3 days and then serum-starved for 24 h (data not shown). Therefore, in the remainder of this report, we employed primary Sertoli cells isolated from 18 dpp mice to investigate the mechanisms regulating the nuclear and cytoplasmic localization of PALLD and gross changes in the F-actin cytoskeleton were monitored by phalloidin staining (Supplementary Figures S3, S4 and S5, see section on supplementary data given at the end of this article).

The addition of serum elevates *Palld* mRNA and protein levels

We investigated whether increased expression of PALLD might underlie its dramatic nuclear relocalization. We found that the addition of serum caused a small but statistically significant increase ($P=0.027$) in mRNA levels for the 140 kDa *Palld* isoform (Fig. 1E), which is the predominant isoform in Sertoli cells (Niedenberger *et al.* 2013). This was accompanied by a small increase in the level of PALLD protein (Fig. 1F). The addition of serum to cultured cells has been reported to increase the abundance of most housekeeping mRNAs, including *Actb* and *Gapdh* (Schmittgen & Zakrajsek 2000). In accordance with this, we saw increased levels for beta actin (*Actb*) = +1.8-fold ($P=0.004$). In contrast, mRNAs encoding markers of differentiated Sertoli cells showed no change (*Vcl*, $P=0.273$) or decreased in response to serum, including FBJ osteosarcoma oncogene (*Fos*) = -3.4-fold ($P=0.0003$), and transferrin (*Trf*) = -3.8-fold ($P=0.000001$) (Chaudhary *et al.* 1996, Gineitis & Treisman 2001; Fig. 2).

Figure 2 Gene expression changes in primary Sertoli cells in response to serum. Message levels were quantitated by qRT-PCR for the indicated genes (*Vcl*, vinculin; *Actb*, beta actin; *Fos*, c-FBJ osteosarcoma oncogene; *Trf*, transferrin). Statistical significance is indicated by an asterisk, with associated P values.

PALLD localization is dependent upon XPO1

A leucine-rich (LxLxxxL) putative nuclear export signal (NES) resides at the extreme C-terminus of PALLD (la Cour *et al.* 2004). The NES of target proteins is bound by exportin 1 (XPO1, previously CRM1), which directs their exit from the nucleus (Kutay & Guttinger 2005). We previously found that deletion of C-terminal sequence containing the NES resulted in nuclear localization of GFP-PALLD expression constructs in the TM4 Sertoli cell line (Niedenberger *et al.* 2013). We tested whether shuttling of endogenous PALLD occurred in primary Sertoli cells by treating serum-stimulated cells with leptomycin B, a specific inhibitor of XPO1. As early as 1 h after treatment with leptomycin B, PALLD became predominantly nuclear (Fig. 3B), and this was enhanced by 7 h (Fig. 3C).

Depolymerization of the F-actin cytoskeleton results in PALLD nuclear localization

Addition of serum to cells in culture stimulates polymerization of the F-actin cytoskeleton, including in primary Sertoli cells (Hutson *et al.* 1980). Accordingly, the phalloidin-labeled Sertoli F-actin cytoskeleton appeared less robust in serum-free culture. We treated primary Sertoli cells with cytochalasin D and latrunculin B, two drugs which cause rapid depolymerization of the F-actin cytoskeleton (Brenner & Korn 1979, Brown & Spudich 1979, Lin & Lin 1979, Flanagan & Lin 1980, MacLean-Fletcher & Pollard 1980, Spector *et al.* 1983). In Sertoli cells maintained in serum-containing medium, PALLD rapidly relocalized to nuclei following the addition of cytochalasin D (Fig. 4B) or latrunculin B (Fig. 4C), coinciding with breakdown of the F-actin cytoskeleton as assessed by phalloidin staining.

cAMP and Rho signaling regulate PALLD nuclear localization in vitro

The Sertoli cell cytoskeleton undergoes substantial changes during the prepubertal period, with the creation of unique and highly specialized actin-rich basal and apical ES structures (Mruk & Cheng 2010). Addition of cAMP to Sertoli cells in culture profoundly affected cellular morphology and the cytoskeleton (Tung & Fritz 1975, Tung *et al.* 1975, Hutson 1978, Hutson *et al.* 1980), and the addition of serum blocked these effects (Tung *et al.* 1975). Therefore, we tested whether localization of PALLD, as a dominant modulator of the F-actin cytoskeleton, was affected in response to elevated cAMP levels using two approaches. We treated serum-stimulated Sertoli cells with 8-bromo-cAMP, a stable analog resistant to phosphodiesterase degradation, and forskolin, which stimulate adenylyl cyclase activity. PALLD relocates to the nucleus within 24 h in serum-stimulated Sertoli cells in response to both

Figure 3 PALLD accumulates in nuclei after inhibition of nuclear export. (A, B, and C) IIF to detect PALLD (in green) in serum-stimulated Sertoli cells grown without (A) or with leptomycin B treatment for 1 h (B) and 7 h (C). Phalloidin-labeled F-actin cytoskeleton (in red). Scale bars = 25 μ m.

8-bromo-cAMP (Fig. 4D) and forskolin (Fig. 4E). Serum stimulation drives actin polymerization by activating Rho signaling (Van Aelst & D'Souza-Schorey 1997, Bishop & Hall 2000, Hall 2012), and Rho-induced actin polymerization results in relocalization of MKL1 to the nucleus (Miralles *et al.* 2003). We therefore tested whether inactivation of Rho signaling directed PALLD nuclear localization. We treated serum-stimulated primary Sertoli cells with the specific Rho inhibitor C3-transferase, and PALLD relocated to the nucleus within 24 h (Fig. 4F).

Gonadotropin signaling regulates PALLD nuclear localization *in vivo*

Several studies uncovered the roles of hormone signaling in remodeling the Sertoli cell cytoskeleton *in vivo* (Muffly *et al.* 1993, 1994, Allan & Handelsman 2005). We tested whether PALLD nuclear localization depended upon gonadotropin signaling *in vivo* in *Hpg*-mutant mice, which have a spontaneous mutation in the gonadotropin-releasing hormone (*Gnrh1*) gene. Male *Hpg* mice are infertile, with dramatically reduced levels of FSH and luteinizing hormone (LH) and small testes (Cattanach *et al.* 1977, Mason *et al.* 1986). In contrast to the robust nuclear PALLD localization that we found in adult WT testes (Fig. 5A, Niedenberger *et al.* 2013), only a subset of immature-appearing Sertoli cells in *Hpg* mutants displayed faint nuclear PALLD staining, with many having no detectable PALLD (Fig. 5B and C). As GNRH1 stimulates the anterior pituitary to release LH and FSH, this suggests that impaired signaling through LH or FSH decreased nuclear localization *in vivo*.

PALLD nuclear localization is not dependent upon AR, but is reduced in *Fshb* KO testes

We tested the hypothesis that nuclear translocation of AR (downstream of testosterone production by LH) was required for PALLD localization within Sertoli cell nuclei using testes from Sertoli cell *Ar* knockout (SCARKO) mice (De Gendt *et al.* 2004, Zhou *et al.* 2011). As expected, AR was not detectable, although it was present in interstitial Leydig cells (Fig. 5D). In contrast, PALLD was present in all Sertoli cell nuclei in adult SCARKO mice (Fig. 5E), indicating that the loss of testosterone signaling through AR did not affect nuclear localization of PALLD. However, it is possible that translocation of AR depends on PALLD, rather than the other way around as we originally predicted.

cAMP is one of the most well-studied molecules induced by FSH during pubertal Sertoli cell differentiation *in vivo* (Walker & Cheng 2005), which is the time period during which PALLD localization changes from cytoplasmic to nuclear (Niedenberger *et al.* 2013). Therefore, we determined whether nuclear localization was affected in the absence of FSH signaling. We carried out IHC with

Figure 4 PALLD accumulates in nuclei in response to increased cAMP and after disruption of the F-actin cytoskeleton. (A, B, and C) IIF to detect PALLD (in green) in serum-stimulated Sertoli cells grown without (A) or with cytochalasin D (B), latrunculin (C), 8-bromo-cAMP (D), forskolin (E), or C3 transferase (F). Phalloidin-labeled F-actin cytoskeleton (in red). Scale bar = 25 μ m.

Figure 5 PALLD nuclear localization is disrupted in response to aberrant gonadotropin signaling *in vivo*. IHC was used to detect PALLD (brown), and sections were counterstained with hematoxylin (blue). PALLD in adult testis sections from WT (A), *Hpg* mutant (B and C), SCARKO (D and E), or *Fshb* KO (F) mice. Arrows indicate unstained Sertoli cell nuclei in panels C–D. Scale bars = 25 μ m.

anti-PALLD on sections from *Fshb* KO mice (Kumar *et al.* 1997). Nuclear PALLD was reduced, with localization around the periphery of the nucleus (Fig. 5E and F). We tested whether FSH would direct PALLD nuclear localization in primary Sertoli cells from 10 dpp mice (when it is cytoplasmic). We found that the addition of 0.1 U/ml FSH for 24 h did not affect PALLD nuclear localization, suggesting that other mechanisms are preventing PALLD nuclear localization in immature Sertoli cells.

Discussion

In this study, we demonstrate that nuclear localization of the essential F-actin bundling protein PALLD is regulated by changes in the polymerization status of the actin cytoskeleton. Our results suggest that FSH signaling modulates the nuclear localization of the actin binding protein PALLD in Sertoli cells, and this relationship provides a functional link between hormone signaling, intracellular signaling downstream of cAMP, and Sertoli cell cytoskeletal changes during puberty.

It was shown many years ago that addition of serum to primary Sertoli cells resulted in dramatic changes in morphology and cytoskeletal organization (Hutson *et al.*

1980), and we found that it also caused a dramatic relocalization of PALLD from the nucleus to the cytoplasm. Serum contains a complex but poorly defined mixture of macromolecules, nutrients, hormones, and growth factors. Therefore, instead of trying to identify responsible component(s), we focused on the fact that serum induces polymerization of the F-actin cytoskeleton. We tested the hypothesis that PALLD relocalization was regulated by the polymerization status of the actin cytoskeleton and showed that depolymerization by latrunculin B and cytochalasin D induced rapid PALLD nuclear localization. Therefore, our results support the model that PALLD is in the cytoplasm in cells with abundant F-actin, and in the nucleus with low F-actin levels. Three positively charged amino acid residues in the third immunoglobulin domain of PALLD were found to direct its F-actin binding and bundling capabilities (Dixon *et al.* 2008, Beck *et al.* 2013). Although WT recombinant PALLD was detected in the cytoplasm, mutation of these residues (K to A) resulted in dramatic nuclear localization of mutant recombinant PALLD (Beck *et al.* 2013). Taken together, these data indicate that, when not bound to F-actin, PALLD migrates into the nucleus. This localization paradigm provides a counterexample to the actin polymerization-directed nuclear localization of the G-actin binding-transcription factors MKL1 (previously MAL and MRTF-A) and MKL2 (previously MRTFB). As the actin cytoskeleton polymerizes, there is reduced G-actin to retain MKL1 and MKL2 in the cytoplasm, and therefore they shuttle into the nucleus (Wang *et al.* 2002, Miralles *et al.* 2003, Olson & Nordheim 2010), where they activate transcription in association with serum response factor (Wang *et al.* 2002, Miralles *et al.* 2003).

These elegant studies involving MKL1 and MKL2 demonstrate that changes in cytoplasmic G:F-actin result in their nuclear localization to ultimately regulate transcription. This provides a mechanism by which the cell can sense and respond to cytoplasmic changes at the level of gene expression. We envision a similar scenario for PALLD, whose localization is regulated in a reciprocal manner. There is precedence for a role of PALLD in transcription, as other studies have shown that PALLD also partially localizes to the nucleus in certain cell lines (Endlich *et al.* 2009, Goicoechea *et al.* 2010, Jin *et al.* 2010), where it can bind to transcriptional regulators that influence the patterns of gene expression.

However, others and we have shown that PALLD is present in both Sertoli cell nuclei and the cytoplasmic F-actin-rich structures that are alternately formed and disassembled at precise points during the cycle of the seminiferous epithelium (Niedenberger *et al.* 2013, Qian *et al.* 2013). The change in the intracellular localization of PALLD temporally accompanies the development of the ES structures described earlier, suggesting an important role for PALLD in F-actin cytoskeletal rearrangements during Sertoli cell development. It is likely that the appearance of specialized F-actin cytoskeletal structures

(BTB at puberty, and apical ES later) shifts cytoplasmic F-actin ratios, resulting in the redirection of PALLD into the nucleus. Alternatively, this shuttling mechanism may function to reduce cytoplasmic levels of PALLD, a dominant regulator of the cytoskeleton, as the F-actin cytoskeleton changes during Sertoli cell development. It has been shown that transfection of excess PALLD into cells results in dramatic changes in the cytoskeleton, with the formation of robust cable-like and stellate actin arrays (Rachlin & Otey 2006). Therefore, PALLD may play multiple roles in Sertoli cells by regulating transcription and ES formation, and may also provide a signal to the nucleus as to the status of the F-actin cytoskeleton.

We found that PALLD nuclear localization was impaired in Sertoli cells in *Hpg* mice, which indicates that it was at least indirectly affected by either LH or FSH signaling. The major role of LH in the testis is to direct the production of testosterone by interstitial Leydig cells. In Sertoli cells, testosterone binding to the androgen receptor (AR) mediates its nuclear translocation (Bremner *et al.* 1994), which occurs in a similar developmental pattern to PALLD (Niedenberger *et al.* 2013). As mentioned previously, this nuclear translocation was facilitated through an interaction with another F-actin-binding protein, filamin A (Ozanne *et al.* 2000, Loy *et al.* 2003). Therefore, this association seemed to be a likely mechanism for directing PALLD nuclear localization. However, we did not see any difference in SCARKO testes, indicating that PALLD localization does not depend upon AR, although the converse may be true. Our results do, however, suggest a role for FSH signaling in the nuclear localization of PALLD, and there have been a number of reports supporting a link between FSH signaling and cytoskeletal regulation. Cultured primary Sertoli cells from 20 dpp rats undergo significant morphological changes in response to FSH, but not other hormones such as insulin, LH, growth hormone, testosterone, or estradiol (Hutson 1978). FSH signaling is required for qualitatively and quantitatively normal fertility (Allan *et al.* 2004, Walker & Cheng 2005), and it stimulates adenylate cyclase in Sertoli cells to increase intracellular cAMP levels (Murad *et al.* 1969, Kuehl *et al.* 1970, Dorrington *et al.* 1972, Means & Vaitukaitis 1972, Bhalla & Reichert 1974, Fritz *et al.* 1975, Tung *et al.* 1975). The addition of FSH to rats receiving GNRH immunogen (reducing testosterone and FSH) increased actin-containing ES and adherens junctions (Sluka *et al.* 2006). FSH also induced elongated cytoplasmic extensions resembling the arbor-like cytoplasmic processes of differentiated Sertoli cells *in vivo* (Tung & Fritz 1975, Tung *et al.* 1975, Hutson 1978, Hutson & Stocco 1978), and it regulated proper localization of actin, espin, and vimentin (Sluka *et al.* 2006). We also found that increased cAMP resulted in robust nuclear localization of PALLD, and this effect was likely mediated by Rho activation.

In conclusion, the differentiating Sertoli cell population provides an excellent model system to

investigate the functions of nuclear-localized actin-binding proteins such as PALLD in a relevant developmental context. We have used a combination of primary cell culture and tissues from mutant mice to explore the mechanisms regulating nuclear localization of PALLD. Our current model is that FSH-induced organization of the Sertoli cell cytoskeleton resulting in the formation of discrete F-actin-containing structures results in a reduction in the overall F-actin cytoskeleton, which in turn directs the nuclear localization of PALLD. Future experiments will test these complex interactions and investigate the specific role of nuclear PALLD.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0147>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by startup funds from the Department of Anatomy and Cell Biology and Division of Research and Graduate Studies at ECU (to C B Geyer).

Acknowledgements

The authors thank Joani Zari-Oswald for technical assistance, and David Tulis and Myles Cabot (East Carolina University) for helpful advice. SCARKO testis sections were provided by Marvin Meistrich (MD Anderson), and *Fshb* KO testis sections were provided by Rajendra Kumar (UKMC).

References

- Allan CM & Handelsman DJ 2005 Transgenic models for exploring gonadotropin biology in the male. *Endocrine* **26** 235–239. (doi:10.1385/ENDO:26:3:235)
- Allan CM, Garcia A, Spaliviero J, Zhang FP, Jimenez M, Huhtaniemi I & Handelsman DJ 2004 Complete Sertoli cell proliferation induced by follicle-stimulating hormone (FSH) independently of luteinizing hormone activity: evidence from genetic models of isolated FSH action. *Endocrinology* **145** 1587–1593. (doi:10.1210/en.2003-1164)
- Anway MD, Folmer J, Wright WW & Zirkin BR 2003 Isolation of Sertoli cells from adult rat testes: an approach to *ex vivo* studies of Sertoli cell function. *Biology of Reproduction* **68** 996–1002. (doi:10.1095/biolreprod.102.008045)
- Archer SK, Claudianos C & Campbell HD 2005 Evolution of the gelsolin family of actin-binding proteins as novel transcriptional coactivators. *Bioessays* **27** 388–396. (doi:10.1002/bies.20200)
- Beck MR, Dixon RD, Goicoechea SM, Murphy GS, Brungardt JG, Beam MT, Srinath P, Patel J, Mohiuddin J, Otey CA *et al.* 2013 Structure and function of Palladin's actin binding domain. *Journal of Molecular Biology* **425** 3325–3337. (doi:10.1016/j.jmb.2013.06.016)

- Bhalla VK & Reichert LE Jr 1974 Properties of follicle-stimulating hormone–receptor interactions. Specific binding of human follicle-stimulating hormone to rat testes. *Journal of Biological Chemistry* **249** 43–51.
- Bishop AL & Hall A 2000 Rho GTPases and their effector proteins. *Biochemical Journal* **348** 241–255. (doi:10.1042/0264-6021:3480241)
- Bremner WJ, Millar MR, Sharpe RM & Saunders PT 1994 Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology* **135** 1227–1234. (doi:10.1210/endo.135.3.8070367)
- Brenner SL & Korn ED 1979 Substoichiometric concentrations of cytochalasin D inhibit actin polymerization. Additional evidence for an F-actin treadmill. *Journal of Biological Chemistry* **254** 9982–9985.
- Brown SS & Spudich JA 1979 Cytochalasin inhibits the rate of elongation of actin filament fragments. *Journal of Cell Biology* **83** 657–662. (doi:10.1083/jcb.83.3.657)
- Cattanach BM, Iddon CA, Charlton HM, Chiappa SA & Fink G 1977 Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature* **269** 338–340. (doi:10.1038/269338a0)
- Chaudhary J, Whaley PD, Cupp A & Skinner MK 1996 Transcriptional regulation of Sertoli cell differentiation by follicle-stimulating hormone at the level of the c-fos and transferrin promoters. *Biology of Reproduction* **54** 692–699. (doi:10.1095/biolreprod54.3.692)
- Cheng CY, Silvestrini B, Grima J, Mo MY, Zhu LJ, Johansson E, Saso L, Leone MG, Palmery M & Mruk D 2001 Two new male contraceptives exert their effects by depleting germ cells prematurely from the testis. *Biology of Reproduction* **65** 449–461. (doi:10.1095/biolreprod65.2.449)
- la Cour T, Kierner L, Molgaard A, Gupta R, Skriver K & Brunak S 2004 Analysis and prediction of leucine-rich nuclear export signals. *Protein Engineering, Design & Selection* **17** 527–536. (doi:10.1093/protein/gzh062)
- De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C *et al.* 2004 A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *PNAS* **101** 1327–1332. (doi:10.1073/pnas.0308114100)
- Dixon RD, Arneman DK, Rachlin AS, Sundaresan NR, Costello MJ, Campbell SL & Otey CA 2008 Palladin is an actin cross-linking protein that uses immunoglobulin-like domains to bind filamentous actin. *Journal of Biological Chemistry* **283** 6222–6231. (doi:10.1074/jbc.M707694200)
- Dorrington JH, Vernon RG & Fritz IB 1972 The effect of gonadotrophins on the 3',5'-AMP levels of seminiferous tubules. *Biochemical and Biophysical Research Communications* **46** 1523–1528. (doi:10.1016/0006-291X(72)90780-2)
- Endlich N, Schordan E, Cohen CD, Kretzler M, Lewko B, Welsch T, Kriz W, Otey CA & Endlich K 2009 Palladin is a dynamic actin-associated protein in podocytes. *Kidney International* **75** 214–226. (doi:10.1038/ki.2008.486)
- Flanagan MD & Lin S 1980 Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *Journal of Biological Chemistry* **255** 835–838.
- Fritz IB, Louis BG, Tung PS, Griswold M, Rommerts FG & Dorrington JH 1975 Biochemical responses of cultured Sertoli cell-enriched preparations to follicle stimulating hormone and dibutyryl cyclic AMP. *Current Topics in Molecular Endocrinology* **2** 367–382. (doi:10.1007/978-1-4613-4440-7_26)
- Gettemans J, Van Impe K, Delanote V, Hubert T, Vandekerckhove J & De Corte V 2005 Nuclear actin-binding proteins as modulators of gene transcription. *Traffic* **6** 847–857. (doi:10.1111/j.1600-0854.2005.00326.x)
- Gineitis D & Treisman R 2001 Differential usage of signal transduction pathways defines two types of serum response factor target gene. *Journal of Biological Chemistry* **276** 24531–24539. (doi:10.1074/jbc.M102678200)
- Goicoechea SM, Bednarski B, Stack C, Cowan DW, Volmar K, Thorne L, Cukierman E, Rustgi AK, Brentnall T, Hwang RF *et al.* 2010 Isoform-specific upregulation of Palladin in human and murine pancreas tumors. *PLoS ONE* **5** e10347. (doi:10.1371/journal.pone.0010347)
- Hall A 2012 Rho family GTPases. *Biochemical Society Transactions* **40** 1378–1382. (doi:10.1042/BST20120103)
- Hutson JC 1978 The effects of various hormones on the surface morphology of testicular cells in culture. *American Journal of Anatomy* **151** 55–69. (doi:10.1002/aja.1001510106)
- Hutson JC & Stocco DM 1978 Specificity of hormone-induced responses of testicular cells in culture. *Biology of Reproduction* **19** 768–772. (doi:10.1095/biolreprod19.4.768)
- Hutson JC, Garner CW & Stocco DM 1980 Effects of serum components on the morphology of Sertoli cells in culture. *Anatomical Record* **197** 205–211. (doi:10.1002/ar.1091970209)
- Jin L, Gan Q, Zieba BJ, Goicoechea SM, Owens GK, Otey CA & Somlyo AV 2010 The actin associated protein Palladin is important for the early smooth muscle cell differentiation. *PLoS ONE* **5** e12823. (doi:10.1371/journal.pone.0012823)
- Kuehl FA Jr, Patanelli DJ, Tarnoff J & Humes JL 1970 Testicular adenyl cyclase: stimulation by the pituitary gonadotrophins. *Biology of Reproduction* **2** 154–163. (doi:10.1095/biolreprod2.1.154)
- Kumar TR, Wang Y, Lu N & Matzuk MM 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nature Genetics* **15** 201–204. (doi:10.1038/ng0297-201)
- Kutay U & Guttinger S 2005 Leucine-rich nuclear-export signals: born to be weak. *Trends in Cell Biology* **15** 121–124. (doi:10.1016/j.tcb.2005.01.005)
- de Lanerolle P & Serebryanny L 2011 Nuclear actin and myosins: life without filaments. *Nature Cell Biology* **13** 1282–1288. (doi:10.1038/ncb2364)
- Lie PP, Mruk DD, Lee WM & Cheng CY 2010 Cytoskeletal dynamics and spermatogenesis. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **365** 1581–1592. (doi:10.1098/rstb.2009.0261)
- Lin DC & Lin S 1979 Actin polymerization induced by a motility-related high-affinity cytochalasin binding complex from human erythrocyte membrane. *PNAS* **76** 2345–2349. (doi:10.1073/pnas.76.5.2345)
- Loy CJ, Sim KS & Yong EL 2003 Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. *PNAS* **100** 4562–4567. (doi:10.1073/pnas.0736237100)
- Luo H, Liu X, Wang F, Huang Q, Shen S, Wang L, Xu G, Sun X, Kong H, Gu M *et al.* 2005 Disruption of Palladin results in neural tube closure defects in mice. *Molecular and Cellular Neurosciences* **29** 507–515. (doi:10.1016/j.mcn.2004.12.002)
- MacLean-Fletcher S & Pollard TD 1980 Mechanism of action of cytochalasin B on actin. *Cell* **20** 329–341. (doi:10.1016/0092-8674(80)90619-4)
- Mason AJ, Hayflick JS, Zoeller RT, Young WS III, Phillips HS, Nikolics K & Seeburg PH 1986 A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. *Science* **234** 1366–1371. (doi:10.1126/science.3024317)
- Means AR & Vaitukaitis J 1972 Peptide hormone "receptors": specific binding of 3 H-FSH to testis. *Endocrinology* **90** 39–46. (doi:10.1210/endo-90-1-39)
- Miralles F, Posern G, Zaromytidou AI & Treisman R 2003 Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113** 329–342. (doi:10.1016/S0092-8674(03)00278-2)
- Mok KW, Mruk DD, Lie PP, Lui WY & Cheng CY 2011 Adjudin, a potential male contraceptive, exerts its effects locally in the seminiferous epithelium of mammalian testes. *Reproduction* **141** 571–580. (doi:10.1530/REP-10-0464)
- Mruk DD & Cheng CY 2010 Tight junctions in the testis: new perspectives. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **365** 1621–1635. (doi:10.1098/rstb.2010.0010)
- Muffly KE, Nazian SJ & Cameron DF 1993 Junction-related Sertoli cell cytoskeleton in testosterone-treated hypophysectomized rats. *Biology of Reproduction* **49** 1122–1132. (doi:10.1095/biolreprod49.5.1122)
- Muffly KE, Nazian SJ & Cameron DF 1994 Effects of follicle-stimulating hormone on the junction-related Sertoli cell cytoskeleton and daily sperm production in testosterone-treated hypophysectomized rats. *Biology of Reproduction* **51** 158–166. (doi:10.1095/biolreprod51.1.158)
- Murad F, Strauch BS & Vaughan M 1969 The effect of gonadotropins on testicular adenyl cyclase. *Biochimica et Biophysica Acta* **177** 591–598. (doi:10.1016/0304-4165(69)90324-9)
- Niedenberger BA, Chappell VK, Kaye EP, Renegar RH & Geyer CB 2013 Nuclear localization of the actin regulatory protein Palladin in Sertoli cells. *Molecular Reproduction and Development* **80** 403–413. (doi:10.1002/mrd.22174)

- Olson EN & Nordheim A 2010 Linking actin dynamics and gene transcription to drive cellular motile functions. *Nature Reviews. Molecular Cell Biology* **11** 353–365. (doi:10.1038/nrm2890)
- Oma Y & Harata M 2011 Actin-related proteins localized in the nucleus: from discovery to novel roles in nuclear organization. *Nucleus* **2** 38–46. (doi:10.4161/nucl.2.1.14510)
- Otey CA, Dixon R, Stack C & Goicoechea SM 2009 Cytoplasmic Ig-domain proteins: cytoskeletal regulators with a role in human disease. *Cell Motility and the Cytoskeleton* **66** 618–634. (doi:10.1002/cm.20385)
- Ozanne DM, Brady ME, Cook S, Gaughan L, Neal DE & Robson CN 2000 Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. *Molecular Endocrinology* **14** 1618–1626. (doi:10.1210/mend.14.10.0541)
- Parast MM & Otey CA 2000 Characterization of Palladin, a novel protein localized to stress fibers and cell adhesions. *Journal of Cell Biology* **150** 643–656. (doi:10.1083/jcb.150.3.643)
- Pederson T 2008 As functional nuclear actin comes into view, is it globular, filamentous, or both? *Journal of Cell Biology* **180** 1061–1064. (doi:10.1083/jcb.200709082)
- Prante BC, Garman KL, Sims BN & Lindsey JS 2008 Matrix-coated transwell-cultured TM4 Sertoli cell testosterone-regulated gene expression mimics *in vivo* expression. *In Vitro Cellular & Developmental Biology. Animal* **44** 434–443. (doi:10.1007/s11626-008-9135-8)
- Qian X, Mruk DD, Wong EW, Lie PP & Cheng CY 2013 Palladin is a regulator of actin filament bundles at the ectoplasmic specialization in adult rat testes. *Endocrinology* **154** 1907–1920. (doi:10.1210/en.2012-2269)
- Rachlin AS & Otey CA 2006 Identification of Palladin isoforms and characterization of an isoform-specific interaction between Lasp-1 and Palladin. *Journal of Cell Science* **119** 995–1004. (doi:10.1242/jcs.02825)
- Romrell LJ & Ross MH 1979 Characterization of Sertoli cell–germ cell junctional specializations in dissociated testicular cells. *Anatomical Record* **193** 23–41. (doi:10.1002/ar.1091930103)
- Ross MH 1976 The Sertoli cell junctional specialization during spermiogenesis and at spermiation. *Anatomical Record* **186** 79–104. (doi:10.1002/ar.1091860107)
- Russell L 1977 Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. *American Journal of Anatomy* **148** 313–328. (doi:10.1002/aja.1001480303)
- Schmittgen TD & Zakrajsek BA 2000 Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *Journal of Biochemical and Biophysical Methods* **46** 69–81. (doi:10.1016/S0165-022X(00)00129-9)
- Sluka P, O'Donnell L, Bartles JR & Stanton PG 2006 FSH regulates the formation of adherens junctions and ectoplasmic specialisations between rat Sertoli cells *in vitro* and *in vivo*. *Journal of Endocrinology* **189** 381–395. (doi:10.1677/joe.1.06634)
- Sotiropoulos A, Gineitis D, Copeland J & Treisman R 1999 Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* **98** 159–169. (doi:10.1016/S0092-8674(00)81011-9)
- Spector I, Shochet NR, Kashman Y & Groweiss A 1983 Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. *Science* **219** 493–495. (doi:10.1126/science.6681676)
- Tung PS & Fritz IB 1975 Follicle stimulating hormone and cyclic AMP directed changes in ultrastructure and properties of cultured Sertoli cell-enriched preparations: comparison with cultured testicular peritubular cells. *Current Topics in Molecular Endocrinology* **2** 459–508. (doi:10.1007/978-1-4613-4440-7_34)
- Tung PS, Dorrington JH & Fritz IB 1975 Structural changes induced by follicle-stimulating hormone or dibutyl cyclic AMP on presumptive Sertoli cells in culture. *PNAS* **72** 1838–1842. (doi:10.1073/pnas.72.5.1838)
- Van Aelst L & D'Souza-Schorey C 1997 Rho GTPases and signaling networks. *Genes and Development* **11** 2295–2322. (doi:10.1101/gad.11.18.2295)
- Visa N & Percipalle P 2010 Nuclear functions of actin. *Cold Spring Harbor Perspectives in Biology* **2** a000620. (doi:10.1101/cshperspect.a000620)
- Vogl AW, Vaid KS & Guttman JA 2008 The Sertoli cell cytoskeleton. *Advances in Experimental Medicine and Biology* **636** 186–211. (doi:10.1007/978-0-387-09597-4_11)
- Walker WH 2003 Molecular mechanisms controlling Sertoli cell proliferation and differentiation. *Endocrinology* **144** 3719–3721. (doi:10.1210/en.2003-0765)
- Walker WH & Cheng J 2005 FSH and testosterone signaling in Sertoli cells. *Reproduction* **130** 15–28. (doi:10.1530/rep.1.00358)
- Wang DZ, Li S, Hockemeyer D, Sutherland L, Wang Z, Schratt G, Richardson JA, Nordheim A & Olson EN 2002 Potentiation of serum response factor activity by a family of myocardin-related transcription factors. *PNAS* **99** 14855–14860. (doi:10.1073/pnas.222561499)
- Zheng B, Han M, Bernier M & Wen JK 2009 Nuclear actin and actin-binding proteins in the regulation of transcription and gene expression. *FEBS Journal* **276** 2669–2685. (doi:10.1111/j.1742-4658.2009.06986.x)
- Zhou W, Wang G, Small CL, Liu Z, Weng CC, Yang L, Griswold MD & Meistrich ML 2011 Gene expression alterations by conditional knockout of androgen receptor in adult Sertoli cells of Utp14b jsd/jsd (jsd) mice. *Biology of Reproduction* **84** 400–408. (doi:10.1095/biolreprod.110.090530)

Received 13 March 2014

First decision 7 April 2014

Revised manuscript received 18 June 2014

Accepted 2 July 2014