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Characterization of Profilin and Actin Depolymerizing Factors Expression and Function in The Testis

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Summary

The actin cytoskeleton is a structure found in all eukaryotes, known to be essential for a wide range of cellular processes. Cytoskeletal dynamics is regulated by a large number of proteins, commonly known as actin binding proteins.

Although the role of the actin cytoskeleton has been well studied in somatic cells, its function in germ cells remains unclear. The goal of this thesis was to shed some light on the function of the actin cytoskeleton in sperm development, by investigating the possible function of the actin monomer binding protein profilin, and the actin depolymerising factors.

Profilins are small actin binding proteins found in all eukaryotes. 4 profilin genes have been identified in mice so far. Although all profilins are expressed in the testis, the distinct expression patterns I observed for each profilin during both postnatal testicular development, and at a subcellular level suggest these proteins have distinct cellular functions in spermatogenesis. Profilin 1 is highly expressed in meiotic cells, where it is believed to play a role in cell division. Profilin 2 is highly expressed during the stages of germ cell maturation in which the acrosome forms, and might be involved in vesicle trafficking from the golgi to this structure. Profilin 3 is exclusively expressed in haploid cells, in an insoluble, actin rich structure known as the perinuclear theca. The role of profilin 4, which is expressed highly in meiotic and haploid cells, is not clear yet.

Knockout mice for profilin 2 have a defect in spermatogenesis leading to an increase in apoptotic germ cells and decrease in sperm number. To study the role of profilin 3 in the testis, I generated a profilin 3 knockout mouse, which is currently being analyzed.

The three main actin depolymerizing factors in mice, ADF, gelsolin and cofilin in the male reproductive system have distinct roles in spermatogenesis. Gelsolin is expressed as a secreted and non-secreted form by somatic cells in the testis, whereas in the epididymis, only the secreted form of gelsolin is expressed, by secretory cells of the epididymal epithelium. Gelsolin seems to play a role in sperm maturation, as spermatozoa from gelsolin knockout mice have morphological defects which impede their swimming, such as angular tails and residual cytoplasm at the neck.

Cofilin is the only actin depolymerising factor expressed in germ cells, and a germ-cell specific knockout for this gene was generated. Mice which do not express cofilin in the haploid stages of spermatogenesis are viable and show no severe defects in the morphology of the testis or in germ cell maturation.

The presented studies on the various actin-binding proteins will provide a better understanding of the role of the actin cytoskeleton dynamics in germ cell maturation and function, as well as in clinical applications such as infertility.

Zusammenfassung

Das Aktinzytoskelett ist eine Struktur, die in allen eukaryotischen Zellen gefunden wurde und die für verschiedenste zelluläre Prozesse essenziell ist. Die Dynamik des Aktinzytoskeletts wird durch eine Vielzahl von Proteinen reguliert, die als Aktin-bindende Proteine bezeichnet werden.

Die Funktion des Aktinzytoskelets wurde intensiv in somatischen Zellen untersucht, seine Funktion in Keimzellen ist dagegen weitestgehend unklar. Ziel dieser Arbeit ist es, zum Verständnis der Bedeutung des Aktinzytoskeletts für die Spermienentwicklung beizutragen, indem mögliche Funktionen des Aktinmonomerbindenden Proteins Profilin und von Aktin-Depolymerisationsfaktoren untersucht wurden.

Profiline sind kleine, Aktin-bindende Proteine, die in allen eukaryotischen Zellen gefunden wurden. Bislang konnten in der Maus vier Gene, die für Profiline kodieren, identifiziert werden. Obwohl alle vier Gene im Hoden exprimiert werden, deuten einige Ergebnisse meiner Arbeit wie unterschiedliche Expressionsmuster während der postnatalen Hodenentwicklung und Unterschiede in der subzellulären Lokalisation auf distinkte Funktionen während der Spermatogenese hin. Profilin 1 wird besonders stark in meiotischen Zellen exprimiert. Es wird angenommen, dass es dort eine wichtige Rolle bei der Zellteilung spielt. Profilin 2 dagegen wird während der Keimzellreifung während der Ausbildung des Akrosomens exprimiert. Es ist möglicherweise am Vesikeltransport vom Golgi zum Akrosom beteiligt. Profilin 3 wird ausschließlich in haploiden Zellen exprimiert und ist dort in der perinukleären Theca, einer unlöslichen, Aktin-reichen Struktur lokalisiert. Die Funktion von Profilin 4, das sowohl in haploiden als auch in meiotischen Zellen stark exprimiert wird, ist bislang unklar.

Um die Funktion von Profilin in vivo zu charakterisieren habe ich Profilin 2-defiziente Mäuse analysiert. Diese Mäuse zeigen einen Defekt in der Spermatogenese, der zu einem Anstieg apoptotischer Keimzellen und einer Abnahme in der Spermienzahl führt. Außerdem habe ich ein Knock-out Mausmodell für Profilin 3 generiert, um auch die Funktion dieses Proteins im Hoden aufklären zu können. Die Profilin 3 Mausmutanten werden zurzeit von mir analysiert.

Die drei wichtigsten Aktin-Depolymerisationsfaktoren des männlichen Reproduktionssystems in der Maus, ADF, Gelsolin und Cofilin, besitzen unterschiedliche Funktionen während der Spermatogenese. Gelsolin wird in der sekretorischen und in der nicht-sekretorischen Form von somatischen Zellen im Testis exprimiert, wohingegen in der Epididymis nur die sekretorische Form exprimiert wird. Gelsolin scheint von besonderer Bedeutung für die Spermienreifung zu sein, da die Spermatozoen dieser Knock-out Mäuse morphologische Defekte wie einen geknickten Spermienschwanz und Restzytoplasma im Halsbereich der Spermien aufweisen, welche die Fortbewegung beeinträchtigen.

Da Cofilin als einziger Aktin-Depolymerisationsfaktor in Keimzellen exprimiert wird, habe ich einen Keimzell-spezifischen Knock-out generiert. Mäuse die kein Cofilin während der haploiden Phase der Spermatogenese exprimieren, sind lebensfähig und zeigen keinen schweren Defekt in der Hodenmorphologie und der Keimzellreifung.

Die hier dargestellten Untersuchungen zur Funktion verschiedener Aktin-bindender Proteine tragen zum besseren Verständnis der Bedeutung der Aktinzytoskelettdynamik für die Reifung und Funktion von Keimzellen bei und eröffnen neue Aspekte in Bezug auf Unfruchtbarkeit beim Mann.

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Introduction

1. Spermatogenesis

Spermatogenesis is a paradigm of development that continues throughout adult life in most mammals (Eddy 2002). During spermatogenesis, diploid progenitor germ cells known as spermatogonia divide and undergo extensive genetic, biochemical and morphological transformation to become highly specialized, haploid spermatozoa (Russell 1990).

1.1 Overview of The Mammalian Testis

Despite a variation in position, the overall anatomy of the testis is very similar in most mammals. In the 17th century, it was believed that the testis was filled with a 'glandulous', 'pultaceous' or 'porridge-like' substance, until de Graaf showed convincingly that the testis was densely packed with a series of convoluted tubules known as the seminiferous tubules (De Graaf 1668).

Seminiferous tubules, which take up about 95% of the space in the testis, are populated by two kinds of cells: germ cells that will mature into spermatozoa, and sertoli cells, which provide the necessary physiological environment for germ cell development. Several functions have in fact been attributed to the sertoli cells, including phagocytosis to remove residual cytoplasm from the germ cells, secretion of critical growth factors and nutrients, and creating a blood-testis barrier (Russell 1993)

Between the Sertoli cells and the basal lamina of the seminiferous tubules lie the stem cells of the male germ line, the spermatogonia. As these proliferate and mature, their progeny are translocated by the sertoli cells in rings around the circumference of the tubule toward the lumen, where they will be released as spermatozoa. In a cross-section of the mouse testis, the annular migration pattern of the germ cells can be seen clearly, as circumferential zones of more mature cells lie inside zones of less advanced cells (see Fig.1). Although germ

cells must be free to migrate through the seminiferous epithelium, intimate association with the sertoli cells is constantly maintained by actin rich junctions unique to the testis (Grove and Vogl 1989; Guttman et al. 2004) .

The interstitial space surrounding the seminiferous tubules contains blood vessels, lymph vessels, nerves, and, intimately associated with all 3 of these, leydig cells, which produce the majority of the steroid hormones formed *de novo* in the testis from cholesterol (Benton et al. 1995; Mendis-Handagama and Ariyaratne 2001).
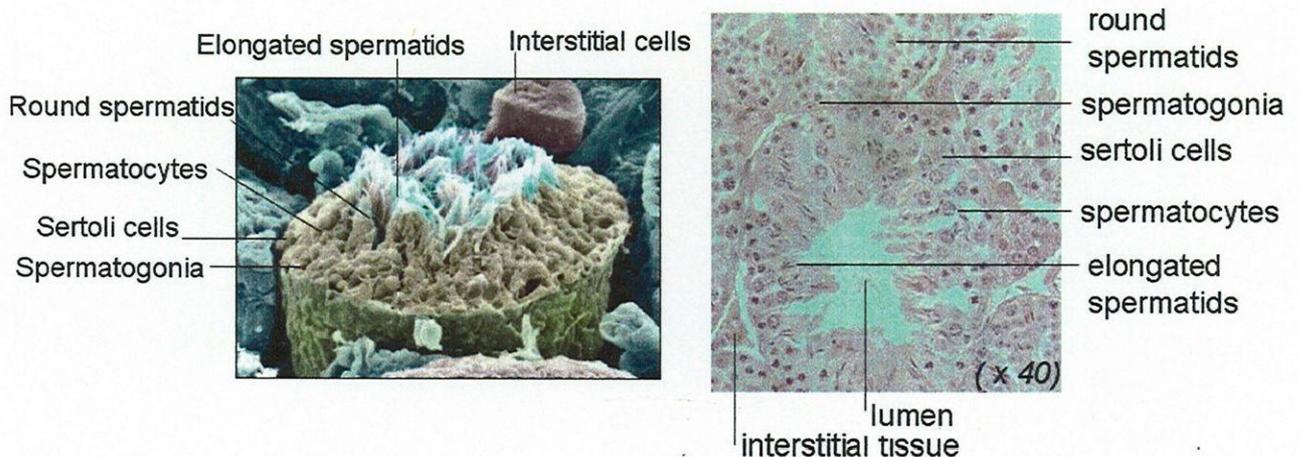
Figure 1 a) Scanning electron micrograph of seminiferous tubule **b)** Light microscopy histological section of a seminiferous tubule stained with haematoxylin and eosin. The different steps of germ cell development can be distinguished on the basis of nuclear morphology.

Considering the complexity and lengthiness of spermatogenesis, it is not surprising that 10,000 out of the 30,000 mouse genes are expressed in the mouse testis (Shima et al. 2004). The process, which takes 35 days in the mouse (see Fig. 2), and 72 days in the human (Clermont 1972), is dependent upon the capacity of **spermatogonial stem cells** to self-renew and at the same time to give rise to a progeny committed to differentiate. As spermatogonia undergo 10-12 divisions, 4096 haploid spermatids can theoretically be formed by a single

stem cell, so that few stem cells are sufficient to maintain the huge sperm production observed in mammals.

The process of spermatogenesis can be divided into 3 stages, spermatogonial proliferation (mitosis), meiosis and spermiogenesis:
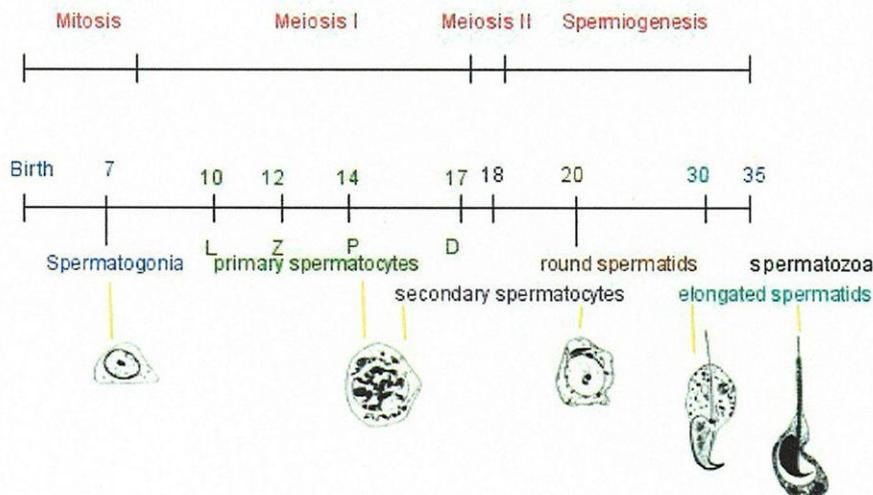
Figure 2 Overview of mouse spermatogenesis: time scale for a new-born mouse to go through the first wave of spermatogenesis, indicating the day at which the main steps of germ cell development first appear in the testis.

1. *Mitosis* - each spermatogonium goes through 8 mitotic divisions, generating type A spermatogonia, which repopulate the stem cell pool, and type B spermatogonia, which will proceed to the next stage of germ cell maturation, meiosis. The mitotic divisions are characterized by incomplete cytokinesis, so that clones of germ cells joined by intercellular bridges are formed and proceede synchronously throughout spermatogenesis (Oakberg 1971)

2. *Meiosis* - prior to meiosis, type B spermatogonia mature into primary spermatocytes, which proceed through the lengthy prophase of the first meiotic division and are indicated as **pre-leptotene, leptotene, zygotene,** and **pachytene spermatocytes,** which then divide to become secondary spermatocytes. After the second meiotic division, which is far more rapid, the

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secondary spermatocytes form haploid, round spermatids (Oakberg 1956). The rapidity of the second division means that secondary spermatocytes are very rarely found in the testis.

3. *Spermiogenesis* - during this step, in which spherical spermatids mature into elongated spermatozoa, dramatic changes occur to the morphology of the cells. The spermatid nucleus condenses and histones are replaced by protamines, a flagellar tail develops and vesicles from the golgi bud off to form a vesicular structure called the acrosome. The spermatid will also shed most of its excess cytoplasm as a residual body, which is phagocytosed by the Sertoli cell, and develop new cytoskeletal elements in the head region. Once the spermatid is mature, it is released from the testis, in a process known as spermiation (Huckins 1971; Oakberg 1971).

As each batch of germ cells progresses through the spermatogenic process, further rounds of spermatogenesis initiate at the basal lamina. As a result, in the seminiferous epithelium of an adult rodent several layers of germ cells in different stages of development are associated. Because the various layers of germ cells develop in a coordinated manner, these associations are not random but only a limited number of combinations exist. In the mouse twelve different germ cell associations, designed as **stages** I to XII, can be recognized cytologically (Oakberg 1956), and any given cross section of a seminiferous tubule will only contain the cell types present in one of these 12 combinations (see Fig.3).
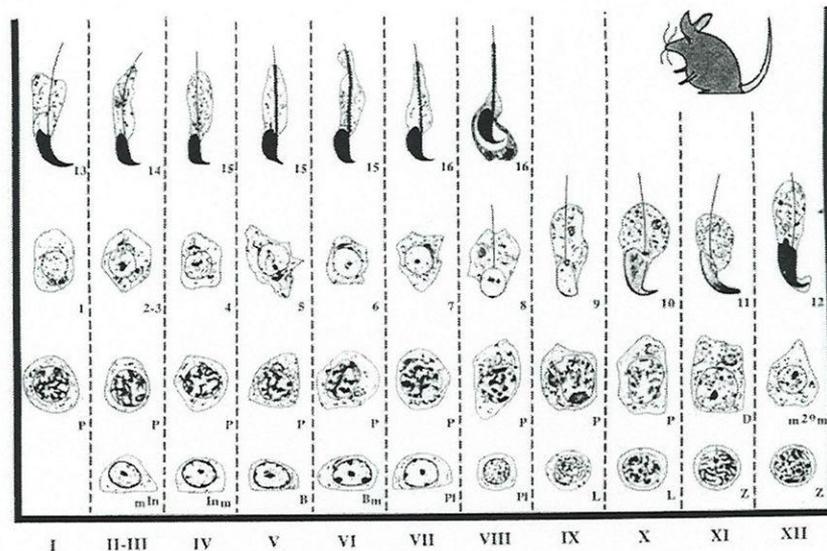
Figure 3 The 12 stages of the spermatogenic cycle in mice. The vertical columns, designated by roman numerals, illustrate any of the 12 cell associations that will be observed in a cross-section of a seminiferous tubule.

1.3 Post-Testicular Sperm Maturation

When spermatozoa leave the testis, they are neither motile, nor capable of recognizing or fertilizing an egg. They must therefore be released into the lumen of the seminiferous tubules, and flow through the rete testis into the epididymis, where they will, among other things, acquire these abilities (Burkin et al. 2004).

The epididymis is a single convoluted duct divided anatomically into caput (head), corpus (body), and cauda (tail). Different maturation events occur at distinct locations in the epididymis, for example, it is believed that motility is acquired in the caput, ability to fertilize is acquired at the corpus, and that the caudal region is used as a reservoir for spermatozoa, protecting them for example from toxic substances. Throughout the epididymis, proteins that will

form part of the surface architecture of spermatozoa are also secreted (Cooper 1998).

The final stages of spermatozoon maturation, however, do not occur in the male reproductive system, but in the female's reproductive tract, by a poorly understood, calcium-dependant process known as capacitation (Austin 1951; Chang 1951).

1.4 Morphology of the Mature Spermatozoon
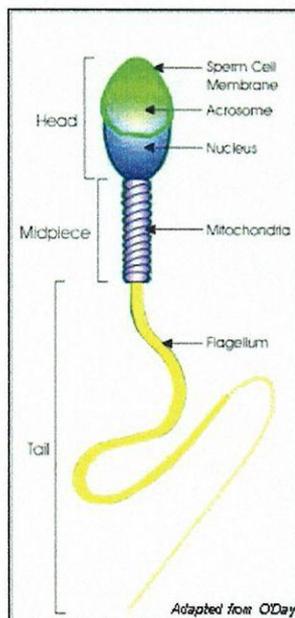
Figure 4 Basic morphology of the mammalian spermatozoon

By the time the spermatozoon is ready for ejaculation, it has become a highly specialized cell (Fig. 4). In the head region, the nucleus is capped by the acrosome, a vesicle containing lytic enzymes that will digest the zona pellucida and allow the spermatozoa to pass into the perivitelline space of the egg. An actin cytoskeleton structure called the acroplaxome has formed in the subacrosomal space to dock the acrosome to the nucleus. By replacing the nuclear histones by arginine–rich protamines, the nucleus has become extremely condensed, so that the DNA takes up only 5% of the space it occupied when bound by histones (Ward and Coffey 1991). Behind the head region, the midpiece is filled with mitochondria to drive the lashing motion of the attached flagellar tail.

Nothing is superfluous in the spermatozoon; in these highly specialized cells, almost all the cytoplasm has been shed, and only a few hundred proteins are present in these cells as transcription is almost entirely stopped after meiosis. Until recently this "minimalism" led us to believe that spermatozoa contributed nothing more than their DNA to the fertilized egg. However, recent discovery has revealed the error of this view. In a recent study

on fertile men, 3,000 mRNAs were detected in spermatozoa, some of these encoding for genes known to be important for embryonic development (Ostermeier et al. 2004; Ostermeier et al. 2005), and some of them coding for previously unknown genes, which do not appear to be transcribed in the egg (Ostermeier et al. 2002). Considering spermatozoa like to travel lightly, a question springs to mind: are proteins and mRNAs expressed in the late stages of spermatogenesis remnants of their past lives in the testis, or will they have post ejaculatory functions, for example in embryonic development?

2. The Actin Cytoskeleton

The actin cytoskeleton is a structure common to all eukaryotes involved in a plethora of basic cellular activities, including cytokinesis (Sanger et al. 1989) endo- and exocytosis (Perrin et al. 1992), cell motility (Stossel 1993), cell polarity and intracellular trafficking (Witke 2004). Whereas the function of the cytoskeleton and localization of actin in somatic cells has been well characterized, its function in germ cells is not well understood (Howes et al. 2001).

The principal goal of the present thesis is therefore to begin to understand how the actin cytoskeleton is regulated in the testis and how it contributes to the development of male germ cells.

2.1 Actin: A Versatile Building Block

Actin is a 45 kD protein, which in the cell exists in two forms, monomeric (G-actin) and polymeric (F-actin). Each actin molecule can bind one molecule of ATP, which is hydrolyzed irreversibly to ADP + Pi after incorporation of the actin monomer into the filament. Although energy is not required for incorporation of actin monomers into the filaments, it can increase the rate of polymerization, as it was shown that ATP-bound actin is added on faster to growing filaments than ADP-bound actin (Pollard and Cooper 1986). This difference in binding affinity leads to a polarization of the growing filament - the fast-growing end (+end) capped with ATP-actin, and the slow-growing end (- end) capped with ADP-actin.

Since ADP-actin has lower affinity that ATP-actin for the filament, ATP-actin monomers add on to the fast growing end of the filament while ADP-actin monomers are lost from the slow-growing end, in a process known as treadmilling (Fig.5). Actin forms the cytoskeletal filaments, however, it is not

capable of regulating its filament length and three-dimensional structure independently.
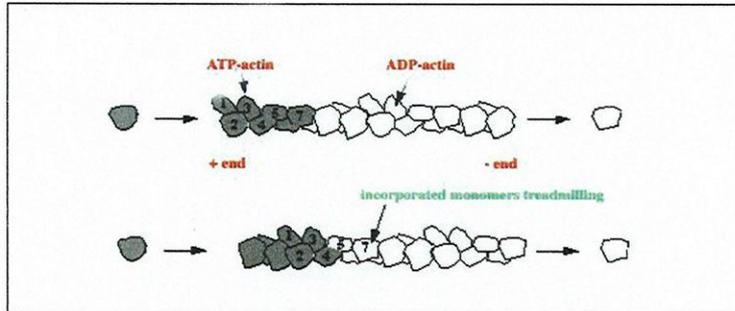
Figure 5 Actin filament treadmilling

Many cellular processes depend on a precise temporal and spatial reorganization of the actin cytoskeleton, which is co-coordinately regulated by a large number of proteins collectively known as actin binding proteins (Fig.6). These proteins function in diverse ways, and can be classified according to the action they perform on actin: proteins that sever (e.g. gelsolin, cofilin, ADF) nucleate (e.g. ARP 2/3, (Pollard et al. 2000)) cross link (e.g. filamin) or cap (e.g. CapG) actin filaments or proteins that sequester monomeric actin (e.g. profilin, thymosin).
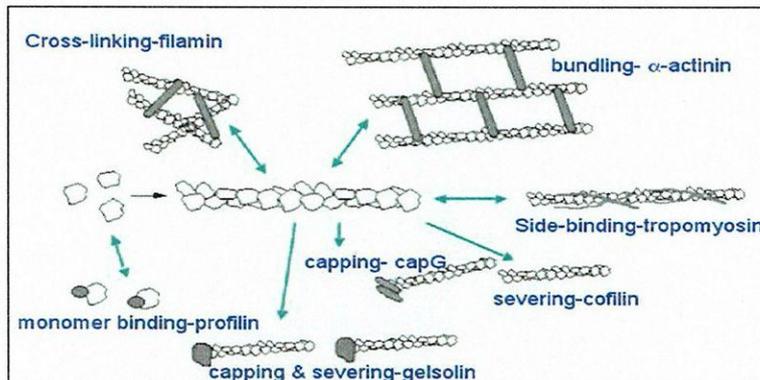
Figure 6 Functions of actin binding proteins on actin filaments and monomers.

The actin cytoskeleton is therefore a complex network of actin, and many actin-binding proteins, which control the filaments length, and spatial organization in response to internal or external cues.

2.2 The Actin Cytoskeleton in Spermatogenesis

The rapidly changing levels of filamentous and monomeric actin indicate that the actin cytoskeleton in germ cells is very dynamic during spermatogenesis and fertilization.

In developing spermatids, actin exists mainly in its filamentous form (Vogl 1989), but as development proceeds it depolymerizes so that in the latest stages of spermiogenesis and in spermatozoa only G-actin is present (Howes et al. 2001). *In vitro* capacitation of sperm from several mammalian species was shown to be accompanied by a second increase in actin polymerization, but prior to the occurrence of the acrosome reaction, F-actin depolymerizes again, allowing the sperm's outer acrosomal membrane and overlying plasma membrane to come into close proximity and fuse. *In vivo* this occurs when the sperm binds to the egg's zona pellucida, causing a fast increase in sperm intracellular calcium, and activation of actin severing proteins which break down the actin fibers, thus allowing the acrosome reaction to take place.

As well as studying the levels of polymerized/depolymerized actin during germ cell maturation, several studies on the subcellular localization of actin in the testis have also been carried out (Vogl et al. 1985; Vogl et al. 1986; Russell et al. 1987; Russell et al. 1987; Vogl 1989; Oko et al. 1991; Kurohmaru et al. 1992). From these studies it was shown that in spermatogenic and sertoli cells, actin filaments are concentrated in specific regions, forming structures that are unique to the testis.

2.3 Localization of Actin in Sertoli Cells

In sertoli cells, actin is enriched in specialized junctions that form between sertoli cells and germ cells, the ectoplasmic specialization, and the tubulobulbar complexes (Grove and Vogl 1989) (Fig.7). Ectoplasmic specializations are modified adherens junctions consisting of the plasma membrane at the attachment site, a layer of actin filaments (hexagonally packed in bundles), and a cistern of endoplasmic reticulum. Apart from being responsible for the mechanical adhesion of germ cells to sertoli cells, ectoplasmic specializations are crucial for cell morphogenesis (Ozaki-Kuroda et al. 2002), correct positioning of the germ cells in the seminiferous epithelium, and preventing premature release of elongating spermatids. Abnormal or absent Sertoli ectoplasmic specializations have been associated with spermatid sloughing and subsequent oligozoospermia (Meyer et al. 1992; Toyama et al. 1999; Wolski et al. 2005).

To allow the germ cells to move towards the lumen, ectoplasmic specializations must break down, by depolymerizing the actin filaments. Several actin binding proteins such as gelsolin (Guttman et al. 2002) espin (Bartles et al. 1996) and vinculin (Pfeiffer and Vogl 1991) have been identified at these junction sites, indicating that rapid polymerization and depolymerization of the actin filaments is required for the dismantling and rebuilding of these junctions at the moment of germ cell release.

Ectoplasmic specializations disassemble at two specific times: 1) prior to when maturing spermatocytes are moved from the basal compartment of the seminiferous epithelium toward the lumen, and 2) prior to spermatid release into the lumen. As the ectoplasmic specialization is broken down between sertoli cells and elongated spermatids prior to sperm release, transient junctions known as tubulobulbar complexes are formed in the concave part of the spermatid head (Russell 1979).

These long finger-like structures consist of tubular structures protruding from the germ cell, that are "cuffed" by actin filaments in the corresponding

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invagination of the sertoli cell. About 4-24 of these tubular structures form per spermatid, suggesting the importance of these structures during cell movement. Tubulobulbar complexes are the final structures that link the mature spermatids to the sertoli cells (Guttman et al. 2004), and are thought to be involved in the removal of excess cytoplasm from the spermatids, as well as preventing spermatid release in the final stages of spermatogenesis and internalizing other junctions between the sertoli cell and the germ cell that must be removed prior to spermiation (the release of into the lumen) (Guttman et al. 2004). Not much is known about the proteins involved in regulating tubulobulbar complexes, however one of the actin binding proteins localized in these complexes so far is the actin severing protein cofilin.
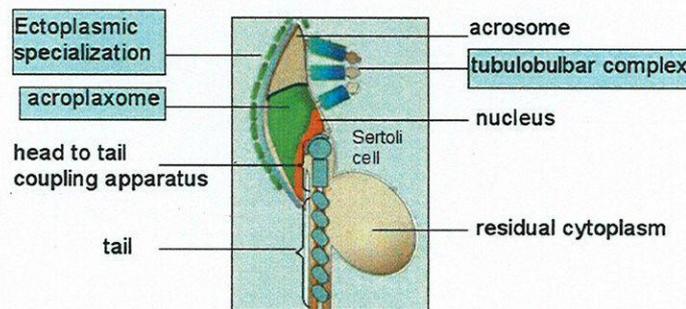
Figure 7 Actin rich regions of germ cells and sertoli cells. The spermatid head is engilfed by the sertoli cell (light green). Tubulobulbar complexes and ectoplasmic specializations are responsible for the association of germ cells and sertoli cells throughout spermatogenesis.

2.4 Localization of Actin in Germ Cells

In mammalian germ cells, actin is enriched in the perinuclear theca (PT) (Korley et al. 1997), a "cytoskeletal coat" encasing the mammalian sperm nucleus entirely, except at the base, which is removed from the sperm head at fertilization (Fig.7). The PT is implicated in cellular processes such as acrosome formation during spermiogenesis, egg activation and pronuclear formation during fertilization, and is also known to store the oocyte-activating factor (SOAF) (Sanchez-Gutierrez et al. 2002), a yet-to-be-characterized substance responsible

for triggering the signaling cascade of oocyte activation (Kimura et al. 1998; Perry et al. 1999; Perry et al. 2000). When this insoluble cytoskeletal structure is removed form the sperm, it retains the shape of the nucleus, rather like an empty shell. The PT must be removed by the egg, to allow the sperm pronuclear DNA to form. Structurally, it is divided into 2 components: the subacrosomal layer (designated by Kierzenbaum as the acroplaxome (Kierszenbaum et al. 2003)) and the postacrosomal calyx (Heid et al. 2002). The acroplaxome is an F-actin and keratin-containing cytoskeletal plate sandwiched between the acrosome and nucleus of spermatids (see Fig. 7). It is important for nuclear shaping during spermiogenesis, as it tethers the acrosomal granule to the nucleus in the early stages of acrosome biogenesis, and provides a scaffold for anchoring the nucleus to the acrosome as the nucleus elongates (Kierszenbaum et al. 2003; Kierszenbaum et al. 2003), but is also known to associate with manchette microtubules and golgi-derived vesicles (Kierszenbaum et al. 2003; Kierszenbaum et al. 2004). The calyx, so called for it's funnel-like shape, is a structure which displays remarkable morphological differences between species (Heid et al. 2002). No function specific to the calyx is known so far.

Apart from the head region of spermatozoa, actin has also been observed in the tail and manchette of many mammalian species, although its function in these regions also remains unclear (Brener et al. 2003).

2.5 Evidence for the Importance of the Actin Cytoskeleton in Germ Cells

The importance of controlling the levels of actin polymerization and depolymerization in germ cells has been shown in vitro in several studies on various mammalian and invertebrate species. Inhibition of F-actin formation by cytochalasin D (Castellani-Ceresa et al. 1993) or with actin antibodies (Liu et al. 2002) has been shown to block sperm capacitation and reduce the *in vitro* fertilization rate of mouse and human spermatozoa, by preventing the sperm

from incorporating into the egg cytoplasm. Inhibition of actin polymerization by latrunculin A or B was also shown to inhibit post-testicular sperm maturation by blocking the initiation of sperm motility in epididymal spermatozoa of tammar wallabies (Lin et al. 2002), which also suggests a role for actin in the development of the sperm tail. In mice, sperm DNA decondensation, which occurs at fertilization, is also blocked when sperm are incubated with cytochalasin B (Kumakiri et al. 2003). Finally, in echinoderm sperm, incubation with cytochalasin B inhibits the acrosome reaction by preventing the elongation of the acrosomal process (Tilney et al. 1983).

In vitro studies have also demonstrated the importance of actin depolymerization in precise events leading to fertilization. In mice, inhibition of actin depolymerization in spermatozoa, upon incubation with phalloidin, was shown to inhibit calcium ionophore induced exocytosis, indicating that the dispersion of actin is necessary for the acrosome reaction to occur (Spungin et al. 1995). Although these *in vitro* studies on actin strongly suggest that actin polymerization may represent an important regulatory pathway in fertilization, the information we can obtain from these studies is limited for several reasons. Firstly, they can only provide us with information on the role of actin during fertilization and capacitation (i.e. in mature spermatozoa) but not spermatogenesis, although we know the actin cytoskeleton to be extremely dynamic during this process. This is because it is not possible to maintain testicular germ cells for more than a few hours in culture, but actin-rich structures such as the perinuclear theca develop over a period of days (Kierszenbaum et al. 2003). Secondly, the actin cytoskeleton in germ cells cannot be disrupted with such assays (using disruptors of the cytoskeleton) *in vivo*, without affecting other cell types, such as sertoli cells, which nourish the germ cells. Finally, complete disruption of actin polymerization in spermatozoa, may cause defects in multiple cellular functions, which may indirectly cause a failure in fertilization ability.

Given the presence of many actin binding proteins in the testis such as gelsolin (Rousseaux-Prevost et al. 1997), cofilin (Ono et al. 1994), calicin (Lecuyer et al. 2000). scinderin (Pelletier et al. 1999), and the existence of a

number of testis-specific isoforms of actin binding proteins such as profilin (Hu et al. 2001; Obermann et al. 2005), actin-capping protein (von Bulow et al. 1997), and fascin (Tubb et al. 2002), studies on the expression and function of these proteins in the testis may give us a deeper understanding of the underlying mechanisms controlling the dynamics of actin and its function in spermatogenesis, by causing more subtle or specific defects in the actin cytoskeleton. This approach may unmask mechanisms of actin regulation in particular cell types or structures that would be impossible to distinguish by complete disruption of the actin cytoskeleton.

For these reasons, the following thesis presents a study on the testicular actin monomer-binding protein profilin, and the most common actin severing proteins, gelsolin, cofilin and ADF in the testis.

3. Profilins: A Function in the Mammalian Testis?

Profilin was the first actin-monomer binding protein to be discovered and isolated from inactivated echinoderm sperm (Carlsson et al. 1977). This 15 kDa protein, one of the most abundant proteins in the cytoplasm, is ubiquitously expressed in eukaryotes, and even the vaccinia virus genome contains a potential profilin gene (Blasco et al. 1991). In spite of the considerable degree of sequence variation between profilins from different species, profilins' biochemical properties have been extremely well conserved throughout evolution (Lambrechts et al. 1997). It has, for example, been shown that bovine profilin is able to rescue the defects in cell shape, cytokinesis, and development that were observed in Dictyostelium profilin null mutant (Karakesisoglou et al. 1996; Schluter et al. 1998).

3.1 Profilin Ligands

It has been shown that by sequestering G-actin in a 1:1 complex, profilin can both enhance and delay the rate of actin polymerization. In the absence of actin filaments, or when the filaments are capped, profilin sequesters G-actin, inhibiting actin polymerization by preventing spontaneous nucleation of actin filaments, (Pollard and Cooper, 1984). However, when uncapped filaments are already present, profilin enhances actin polymerization by acting as an ATP-nucleotide exchange factor, recharging ADP-actin with ATP and thus delivering ATP-charged actin to the uncapped barbed filament ends (Pring et al. 1992).

In addition to regulating actin assembly, profilin can bind to other molecules, such as the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (Lassing and Lindberg 1985). The binding of profilin to this ligand has in fact been suggested to be the primary role of the protein in cells, since

has in fact been suggested to be the primary role of the protein in cells, since phosphoinositides provide a very important cell signaling cascade, transducing messages from outside to inside the cell. Profilins also bind to proline–rich domains of over 40 proteins, such as vasodilator-stimulated phosphoprotein (VASP) (Watanabe et al. 1997), mammalian Ena (Mena) (Gertler et al. 1996), and Wiskott-Aldrich Syndrome protein (WASP) (Suetsugu et al. 1998) known to be involved in a wide range of cellular activities.

These binding properties map to different domains of the protein as shown in figure 8. The binding of profilin to PtdIns(4,5)P$_2$ however, inhibits the binding of profilin to actin as well as proline-rich domains of the ligands, whereas actin and poly-L-proline binding can occur concomitantly, as the binding sites lie on opposite sides of profilin.
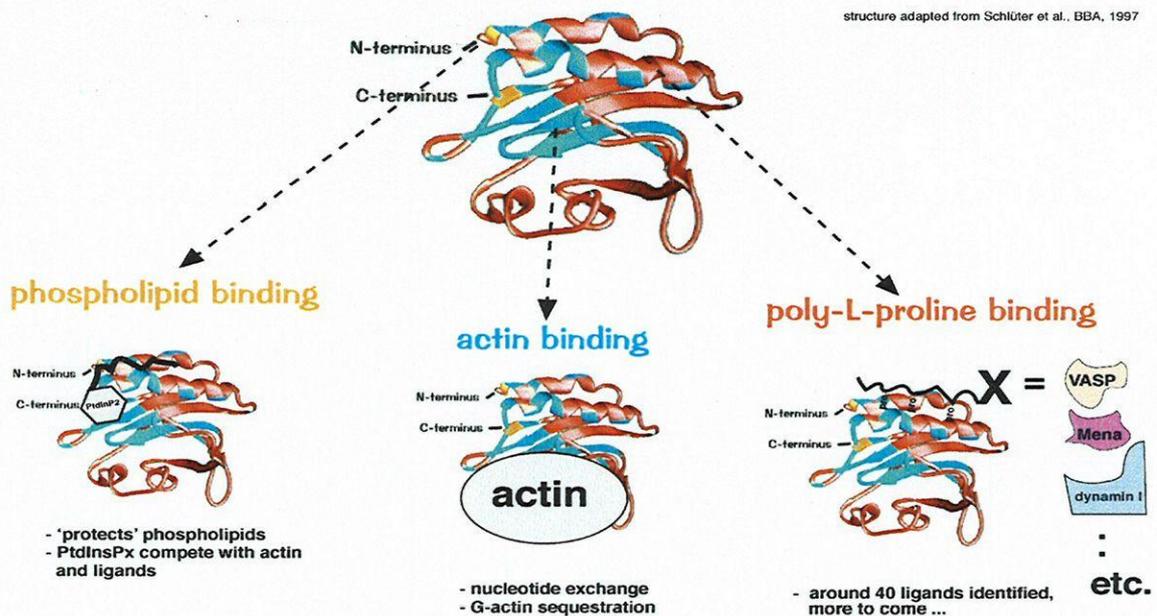
Figure 8 The 3 main ligands of profilins, PtdIns(4,5)P$_2$, actin and poly-L-proline rich regions

3.2 Profilin Genes in Mice

Until 1993, only one profilin gene was known to be present in mammalian cells. To date, 4 profilin genes have been identified so far in mice and humans, (Kwiatkowski and Bruns 1988; Honore et al. 1993; Braun et al. 2002; Obermann et al. 2005).

In mice, profilin 1 is highly expressed in most tissues except skeletal muscle, and knockout mice for this gene do not develop beyond the 2- cell stage due to a defect in cytokinesis and failure of the embryo to implant in the uterus (Witke et al. 2001).

Profilin 2, for which two splice variants (profilin 2A and profilin 2B) have been identified, is instead expressed predominantly in neuronal tissues, and at lower levels in uterus and kidney (Witke et al. 1998; Di Nardo et al. 2000). The deletion of profilin 1 or 2 in mice causes very different phenotypes, as profilin 2 -/- mice are viable, but show neurological and behavioral defects, whereas profilin 1 mice die at the 2-cell stage due to a defect in cytokinesis. This observation leads us to believe that profilins have different as opposed to redundant functions in the organism, profilin 1 acting as a housekeeping gene, and profilin 2 having a more specialized function in the nervous system.

Profilin 3 was first isolated from a cDNA library of rat kidney (Hu et al. 2001). As the gene is made up of 1 exon, which lies on the DNA strand opposite the transcribed region of the sodium renal transporter gene slc34a1, it was initially thought to be expressed in the kidney as well as the testis. A second study however proved this was this to be a testis-specific profilin, which at the RNA level was shown to be expressed in elongated spermatids (Braun et al. 2002).

In 2005, a fourth profilin was discovered in rats (Obermann et al. 2005), and shown to be expressed at the RNA level in post-meiotic germ cells. Although profilin 3 is also expressed in these cells, it is known that in rats the onset of their expression during post-natal development is distinct (Obermann et al. 2005).

3.3 The Role of Profilin in the Testis

In mice, little is known about the role or localization of profilin in the testis. It has been shown by immunoblotting that profilin I is expressed in the testis (Lambrechts et al. 2000), although nothing is known about it's localization or function in germ cell development. As for profilin 2, it is not known whether either isoform is expressed in mouse testis at all. Testis-specific profilins 3 and 4 have instead been localized by *in situ* and by northern blotting to elongated spermatids and round spermatids respectively.

It is interesting to note that profilin was first isolated from echinoderm spermatozoa (Tilney et al. 1983) where it was shown to sequester monomeric actin thereby preventing actin polymerization until fertilization of the egg (Fig.9).
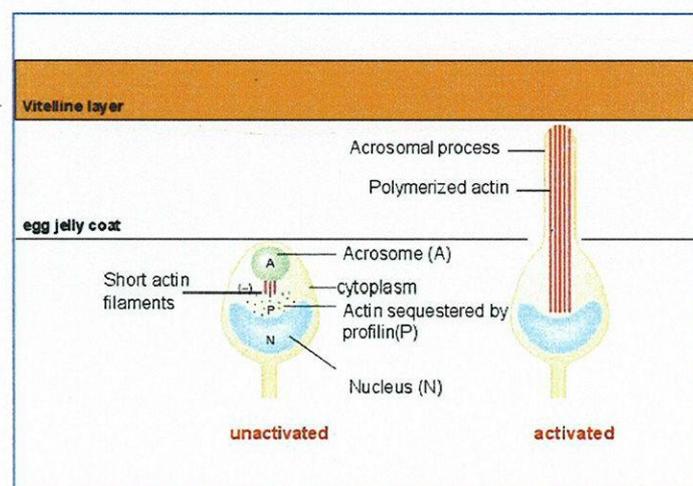
Figure 9 The acrosome reaction in echinoderm. Actin is sequestered by profilin in unactivated eggs. During the acrosome reaction, profilin releases the actin, which rapidly polymerizes to form the acrosomal process.

Prior to fertilization, most of the actin in the sperm is unpolymerized and sequestered by profilin so that only short actin filaments are present in the sperm head, hidden under the acrosomal vesicle. Upon contact with the thick jelly surrounding the egg, the acrosomal vesicle moves, and in doing so exposes the barbed ends of the actin filaments. This acts as a signal for profilin to release the

actin so that the filaments rapidly polymerize. The elongation of the filaments pushes the sperm membrane, along with the acrosomal vesicle just behind it, through the jelly coat and the vitelline layer of the egg, until it reaches and fuses with the egg membrane, allowing the sperm nucleus to enter the egg.

A central role of profilin in spermatogenesis was also observed in lower eukaryotes such as drosophila, where mutants lacking the profilin homologue *chickadee* are sterile (Verheyen and Cooley 1994).

As profilin appears to play an important role in spermatogenesis in lower eukaryotes, a more detailed analysis of the expression of profilins in mice, and an analysis of the effects of profilin loss in mammalian testis may contribute significantly to our understanding of sperm development and the function of the actin cytoskeleton in mammalian germ cells. As lower eukaryotes only contain one profilin gene, in mice, profilins may have redundant roles in spermatogenesis, or may have evolved specialized functions in germ cell development.

4. Actin Filament-Severing Proteins

Different types of actin binding proteins are capable of severing actin filaments, including proteins classified as monomer binding or actin capping proteins. Among the most abundant actin severing proteins in higher vertebrates are gelsolin, Actin Depolymerizing Factor (ADF) and cofilin.

4.1 Actin Depolymerizing Factors

Actin depolymerizing factors are small (15-20 kDa) ubiquitous, essential eukaryotic proteins that can bind to both monomeric and filamentous actin. These proteins contribute to actin dynamics by depolymerizing filaments from their pointed ends, but are also capable of severing actin filaments, by binding to the side of ADP-actin filaments, and twisting the filament more tightly(McGough et al. 1997), which causes it to destabilize and break. As ADF/cofilin bind to ADP-actin filaments and ADP-actin monomers with higher affinity than ATP-actin monomers (Carlier and Pantaloni 1997; Blanchoin and Pollard 1999), they can also prevent nucleotide exchange, thereby slowing down actin polymerization because as previously mentioned, ADP-actin is incorporated slower into growing filaments than ATP-actin.

Whereas lower eukaryotes express only one actin depolymerizing factor, mammals have three highly conserved genes coding for actin depolymerizing factors known as n-cofilin for non-muscle cofilin, m-cofilin for muscle cofilin and ADF, which stands for 'actin depolymerizing factor' (Bamburg 1999; Paavilainen et al. 2004). It is not yet known whether ADF is expressed in the testis, while n-cofilin and m-cofilin have been observed in the adult mouse tissue by northern blotting (Ono et al. 1994), and in rats, where the levels of n-cofilin compared to m-cofilin were estimated to be 8 times higher, n-cofilin was shown to be

expressed in tubulobulbar complexes of elongated spermatids (Guttman et al. 2004).

One way in which cofilin is thought to be regulated *in vivo,* is through phosphorylation at Ser-3, which abolishes its ability to bind to and depolymerize actin (Toshima et al. 2001). At least 4 kinases are known to phosphorylate cofilin at this site: LIM kinase 1 (LIMK1) Lim kinase 2 (LIMK2) (Takahashi et al. 2003), testicular protein kinase 1 (TESK1), and testicular protein kinase 2 (TESK2).

The importance of cofilin in murine spermatogenesis has already been suggested by studies on the LIMK2 knockout mouse. Not only were the testes in these mice smaller in size, but also a partial degeneration of spermatogenic cells in the seminiferous tubules in association with increased apoptosis was apparent (Fig.10) (Takahashi et al. 2003).
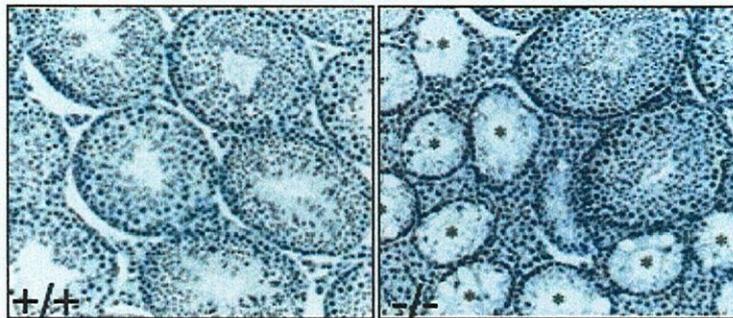
Figure 10 Spermatogenesis in wild type and LIMK2 KO testes after germ cell depletion induced by heat stress. Many seminiferous tubules in the knockout do not repopulate with germ cells due to an impairment in spermatogenesis

As the homozygous knockout mouse for n-cofilin dies during embryogenesis due to a failure in closure of the neural tube (Gurniak et al. 2005), it is not possible to study the effect of deleting n-cofilin in the adult testis in the complete cofilin knockout. To circumvent this problem, a germ cell-specific deletion of cofilin using a conditional mutant mouse would be extremely useful.

4.2 Gelsolin, an Actin-capping and Severing Protein

Gelsolin is able to instantaneously sever all the filaments into a network of short fragments, and remains tightly bound to the barbed end of one piece, to prevent the addition of any more actin subunits. The capping and severing activities of gelsolin are calcium dependant, so that in low calcium concentrations, the protein is inactive. However, when a cell's calcium concentration increases, for example when the sperm comes into contact with the fluids in the female reproductive tract, the gelsolin binds calcium, and this in turn activates its severing and capping activities.

Gelsolin is highly expressed in many tissues, but its expression pattern in the mammalian testis appears to have some degree of species-specific variation. In the rat testis it has been shown to be present in the ectoplasmic specializations, where it may be involved with the rapid actin depolymerization during detachment of the germ cells during their translocation through the seminiferous epithelium (Guttman et al. 2002). Whereas gelsolin is absent in human germ cells (Rousseaux-Prevost et al. 1997), it is present in germ cells of other mammalian species. In the hamster for example, gelsolin is present in the spermatozoa head, where it is thought to be involved in the depolymerization of F-actin prior to acrosome reaction (Cabello-Agueros et al. 2003). It therefore appears that gelsolin is present in germ cells from some mammalian species, and is involved in processes such as fertilization, and is completely absent in others. So far gelsolin studies have not been carried out on mice, so that neither its function nor localization is known.

5. Aim of Thesis

The aim of the present thesis is to provide a detailed analysis of the profilins and the actin depolymerizing factors in the mouse testis and begin to understand the function of these genes in the testis, using a genetic approach, by constitutively or conditionally inactivating gene expression.

Results

1. Characterization of Profilin in the Testis: Identification of a Novel, Testis-Specific Profilin

1.1 Sequence Comparison of Mouse Profilins

To date, 4 profilin genes have been identified in mice. As profilin 1 and profilin 2 were the first profilins to be identified in mice, they have been most extensively characterized so far. Profilin 1 and 2 have very different expression patterns in mouse tissues (see Fig.11) and the knockout mice for these genes have very different phenotypes, profilin 1 being an embryonic lethal mutation (Witke et al. 2001), and profilin 2 mice being viable but with neurological defects (Di Nardo 2001). Such differences suggest profilin 1 and 2 have distinct cellular roles, profilin 1 functioning more as a housekeeping gene, and profilin 2 having a more tissue-specific function in the brain.
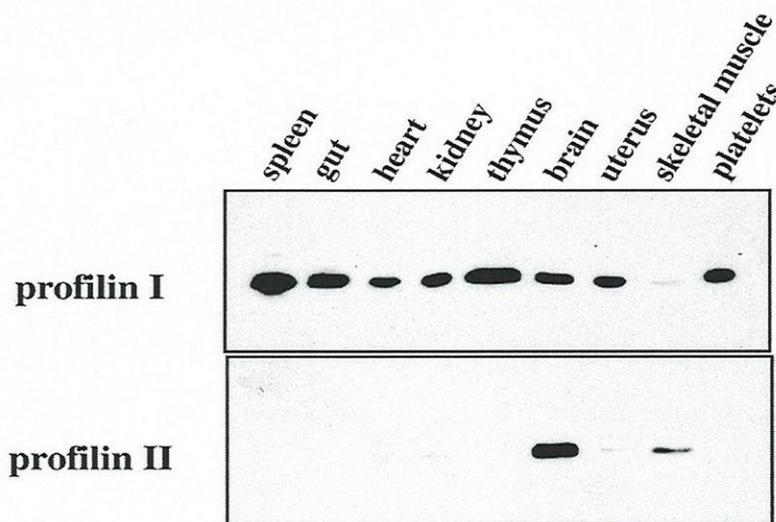
Figure 11. Tissue western blot for profilin 1 and profilin 2 expression in mouse. Profilin 1 is ubiquitously expressed with the exception of skeletal muscle, while profilin 2 is mainly restricted to brain (taken from Witke et al. 1998)

Despite their differences in expression and probably function, the proteins' amino acid sequence, biochemical properties, and binding affinity for their 3 major ligands, actin, PtdIns(4,5)P_2, and poly-L-proline (Gieselmann et al. 1995; Lambrechts et al. 1995) are very similar. At the amino acid level, mouse profilin 1 and 2 show 63% identity(Gieselmann et al. 1995), and their protein

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crystal structure is virtually identical (Nodelman et al. 1999). Profilin 1 and 2 are both encoded on 3 exons seperated by relatively short introns, and the exon-intron boundaries are conserved. Such similarity in the organization of their genomic loci suggests they may have arisen from a duplication event (Di Nardo 2001).

In contrast to the detailed characterization of profilin 1 and 2 that has been carried out so far, little is known about the biochemical properties, structure or function of profilin 3 and 4. This is due to the fact that these profilins were identified more recently, and so far, no tools such as antibodies and recombinant proteins have been available for the study of these proteins. So far, the expression patterns for profilin 3 and 4 suggest they are both testis-specific proteins. In a first attempt to characterize profilin 3, I compared mouse profilin 3 to profilin 1, 2 and 4. The first striking difference that emerged at a genomic level is that profilin 3 is encoded on a single exon, whereas profilin 4, like profilin 1 and 2 is encoded on 3 exons. Furthermore, the peptide sequence of profilin 3 is three amino acids shorter than profilin 1 and 2, but longer than profilin 4, which in turn is eleven amino acids shorter than profilin 1 and 2.

Table 1 summarizes the percentage identity for the profilins. Overall profilin 3 has quite a low degree of conservation with the other profilins. It is most closely related to profilin 1 (40% identity) and profilin 2 (36% percentage identity). The identity between profilin 3 and profilin 4 protein sequence is very low (12,5%). The profilin 4 sequence is in fact, very different to all mouse profilins, as it's percentage identity with these is between 12-15%.

	mPfn1	mPfn2	mPfn3	mPfn4
mPfn1	100%			
mPfn2	62.9%	100%		
mPfn3	40.1%	35.8%	100%	
mPfn4	12.4%	14.7%	12.4%	100%

Table 1. Percentage identity between mouse profilins.

The percentage identity of the protein sequences shown in Table 1 correlates with the evolutionary distances I traced to determine the evolutionary positions of mouse profilins 1-4 (Fig.12).
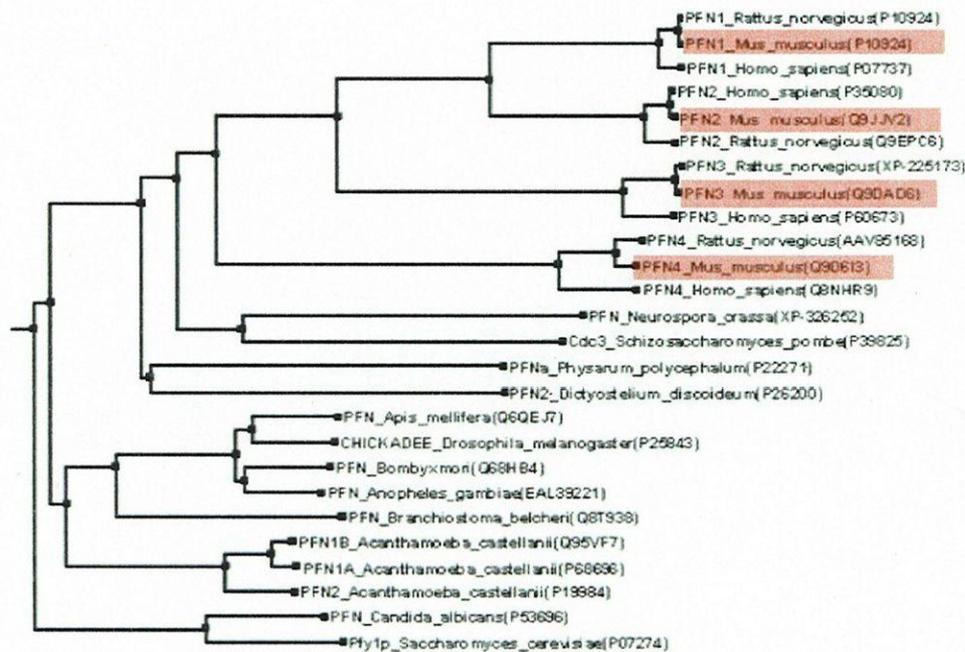
Figure 12. Phylogenetic tree for profilins from several species (using Clustal method) to evaluate the evolutionary relationship between different profilins from different species. Mouse Profilin 1-4 are more closely related to their homologues in other species than between themselves. Profilin 4 is more closely related to profilins from lower eukaryotes.

Profilin 1 and 2 are most closely related, and profilin 3 diverged from them before they became two distinct genes. Profilin 4 diverged from all mouse profilins much earlier in evolution, and is more closely related to profilins from

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lower eukaryotes such as *Neurospora crassa*, which explains why the peptide sequence differs so extensively from other mouse profilins. The phylogenetic tree also shows that mouse profilins are more closely related to their homologues in rat and human, than between themselves, which suggests the 4 profilin genes were present in a common ancestor before the species diverged.

Invariant residues

The amino acid residues **Trp3, Tyr6, Trp31, His133, Leu134 and Tyr139** are strictly invariant across profilins from all species studied so far, except in the putative profilin from vaccinia virus (Schutt et al. 1993), and the majority of these residues have been shown to be essential for profilin ligand binding, suggesting that this evolutionarily conserved activity must be an important function of profilin. In mouse profilins, the invariant residues are also conserved among profilin 1-3, except **His133** and **Tyr139**, which have non-conservative substitutions in profilin 2 and 3. In contrast, in profilin 4, only one of the invariant amino acids is conserved, and the rest have non conservative substitutions (see Fig.13).

The crystalline structure for profilin alone, or complexed to actin or poly-L-proline peptides has been solved for profilins from several species (Archer et al. 1993; Schutt et al. 1993; Vinson et al. 1993; Cedergren-Zeppezauer et al. 1994; Fedorov et al. 1994; Mahoney et al. 1997; Eads et al. 1998; Liu et al. 1998; Nodelman et al. 1999). These structures, along with studies on site-directed mutagenesis of profilins from a wide range of species, have identified some of the most important residues involved in actin-binding and PLP-binding of profilin.
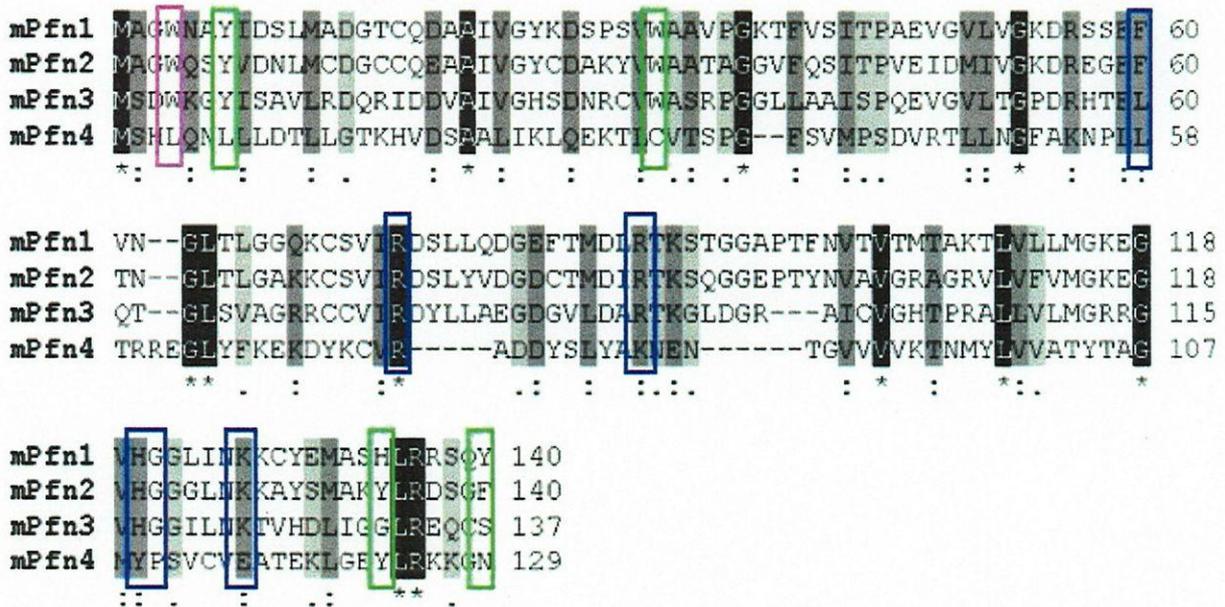
Figure 13 Sequence alignent of mouse profilins on the amino acid level. The alignent was performed using GeneDoc software. Symbols denote the degree of conservation observed in each column. Conservation of residue properties in all profilins is highlighted in black and by an asterisk below the alignent. Conserved substitutions are highlighted in dark grey and by ": " . Semi-conserved substitutions are highlighted in light grey and denoted with "." Based on results form site-directed mutagenesis studies, Blue boxes indicate amino acids required in actin binding, green boxes indicate amino acids required in poly-L-proline binding, Red boxes indicate amino acids required both ligand binding.

Actin Binding

Structural studies have shown that profilin residues that interact with actin molecules are: **Iso73, Asn124, His119, Gly121** (Schutt et al. 1993) Using site-directed mutagenesis, amino acids shown to be essential for actin binding are **Phe59** (Schluter et al. 1998), **His119** (Suetsugu et al. 1998), **Lys125** (Schluter et al. 1998), **Trp3** (Bjorkegren et al. 1993; Bjorkegren-Sjogren et al. 1997), **Arg88** (Sohn et al. 1995), **Gly120** (Schluter et al. 1998), as it has been shown in several species, that when these amino acids are mutated, actin binding is highly reduced. Other profilin mutations have been shown to reduce actin binding, but in a species-specific manner. For example, the deletion of **Pro96** and **Thr97** is known to reduce actin binding by 50% in *S. cerevisiae* (Hajkova et al. 1997).

Analysis of the peptide sequences for all 4 mouse profilins, showed that all the amino acids identified from the protein structure and from site directed mutagenesis as being involved in actin binding are conserved among profilin 1-3, except that in profilin 3 there is a conserved substitution of **Phe59** to **Leu59**, and the residues **Pro96** and **Thr97** are completely missing from profilin 3, whose peptide sequence is three amino acids shorter than profilin 1 and 2. In contrast, none of the amino acids known to be involved in actin binding have remained completely invariant in the profilin 4 peptide sequence, as 5/8 residues have a conservative, 1/8 a semi-conservative, and 2/8 a non-conservative substitution. **Pro96** and **Thr97** are also completely missing in profilin 4, which is eleven amino acids shorter than profilin 1 and 2.

Poly-L-Proline Binding

Profilin residues involved with poly-L-proline binding are the most conserved across all species (Lu and Pollard 2001) and it has been shown in several species that some of the main profilin residues interacting with poly-L-proline rich regions are **Trp3** (Bjorkegren et al. 1993; Bjorkegren-Sjogren et al. 1997; Ostrander et al. 1999), **Tyr6, Trp31, His 133** (Bjorkegren et al. 1993; Bjorkegren-Sjogren et al. 1997; Yang et al. 2000), and **Tyr 139**. Four of these residues are among the amino acids shown to be invariant across all profilins so far. Whereas all of these residues are conserved in profilin 1-3, except **Tyr139** which has mutated to a **Phe** in profilin 2 and **Ser** in profilin 3, profilin 4 does not conserve a single residue of the amino acids known to be involved in poly-L-proline binding.

PtdIns(4,5)P$_2$ Binding

Little is known about the amino acid residues involved in PtdIns(4,5)P$_2$, binding (Sohn et al. 1995; Chaudhary et al. 1998) as no site-directed mutagenesis studies have succeeded in generating a profilin mutant with markedly reduced binding affinity for PtdIns(4,5)P$_2$, It is likely that the binding site contains positively charged amino acids (Fedorov et al. 1994) and among the putative residues thought to be involved are 4 residues from helix 4 : **Lys125, Lys126, Arg135** and **Arg136**. The degree of conservation of these

residues is variable among the mouse profilins, as **Arg135** is conserved in all 4 mouse profilins, **Lys125** is identical in profilin 1-3 but has a conservative substitution in profilin 4, and non-conservative substitutions are present in profilin 4 for **lys126**, and in profilin 2 and 3 for **Arg136**.

Summary

From the analysis we carried out on the profilin peptide sequences, we would expect profilin 3 to share the actin and poly-L-proline binding ability of profilin 1 and 2, although its affinity to bind actin may be lower due to the deletion of 2 potentially key residues for this ligand (**Pro96** and **Thr97**). The ability of profilin 4 to interact with these ligands, particularly actin is questionable. Finally, it is not possible to predict from the peptide sequence whether profilin 3 and profilin 4 can bind to PtdIns(4,5)P$_2$, as the binding site for this ligand on the profilin molecule is still poorly defined. Based on the residues we believe could be involved in binding to this ligand, the binding affinity of profilin 3 for PtdIns(4,5)P$_2$ could equal that of profilin 2.

1.2 Biochemical Properties of Profilin 3

In order to study the biochemical properties of profilin 3, I wanted to express it as a recombinant protein in *E.coli*. To do this, I amplified the profilin 3 from mouse genomic DNA, and cloned it into the pMW-72 plasmid vector, which would express profilin 3 as an untagged protein. The untagged protein could then be purified from bacterial lysates by incubation with poly-L-proline beads, to which the profilin would bind. This vector and purification method had been successfully used in the laboratory for the generation of recombinant profilin 1 and 2, which express very readily in bacteria, and recombinant untagged profilin 1 and profilin 2 have been used extensively for the biochemical characterization of these proteins. Untagged profilin 3 was also highly expressed in *E.coli,* however, it remained insoluble, forming inclusion bodies in the bacteria. To increase the solubility of the protein, we solubilized the inclusion bodies with 8M urea, and attempted to renature the protein by dialysing it against gradually decreasing concentrations of urea to

allow it to refold correctly. However, this did not increase the amount of soluble protein extracted from the bacteria, as the protein would not refold correctly, and therefore would precipitate.

Profilin 3 cDNA was subsequently cloned into the pGEX vector, to express profilin 3 as a GST- (Glutathione-S-transferase) fusion protein, but similar solubility problems were encountered also with this expression system. To test whether the solubility could be increased using a different tagging protein, GST tag was also exchanged by cloning the profilin 3 cDNA into pMAL and pQE vectors, to express recombinant profilin 3 tagged with maltose binding protein (MBP), and 6 x histidine chain (His$_6$) respectively. However, neither of these fusion proteins was more soluble than the GST-profilin 3. A similar problem in protein solubility has been encountered during expression and purification profilin 4 (Katerina Salimova, personal communication). We suspect insolubility may be an innate property of these profilins, as they may localize to an insoluble structure in the germ cells known as the perinuclear theca (see chapter 2.6 "Subcellular Localization of Profilins")

Although I was not able to produce sufficient recombinant profilin 3 to carry out biochemical assays such as actin binding and actin polymerization, enough recombinant profilin 3 was obtained from the soluble fraction after bacterial lysis to inject as an antigen to generate polyclonal antibodies, and for a preliminary study on the ability of profilin 3 to bind to poly-L-proline stretches.

1.3 Profilin 3 can Bind to Poly-L-Proline Stretches

One common feature of profilins is the ability to bind to poly-L-proline (PLP) stretches and many proteins containing PLP stretches have been shown to interact with profilins via profilins' PLP-binding site. To determine whether profilin 3 has an affinity for proline-rich sequences, recombinant GST-profilin 3 was expressed and the soluble fraction purified from *E.coli*. To avoid any sterical hindrance, the GST moiety was cleaved off with factor X prior to incubation with PLP coated beads. After incubating the protein with the coated beads, the fraction that bound to the beads as well as the supernatant were

collected. The bound and unbound fractions were then subjected to SDS-PAGE, and visualized by coomassie staining.
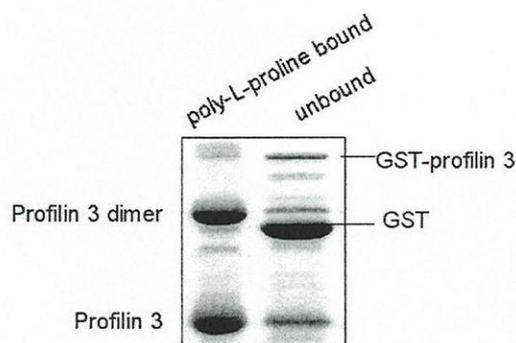
Figure 14 Poly-L-proline binding of the bacterial expressed profilin 3. Cleaved profilin 3 bound to the beads, whereas GST and uncleaved profilin 3 were found in the unbound fraction.

As shown in figure 14, the cleaved profilin 3 bound to the proline repeats, whereas the GST tag remained in the unbound fraction. Interestingly, the small amounts of uncleaved GST-profilin 3 did not bind to the PLP beads, suggesting GST tagging at the N terminus obstructs the PLP binding domain.

My results show that profilin 3 has poly-L-proline binding ability similar to profilin 1 and 2. As profilins have different binding affinities for poly-L-proline stretches, for example mouse profilin 2 binds with greater affinity to poly-L-proline than profilin 1, it will be interesting to compare the poly-L-proline binding affinity of profilin 3 to that of the other mouse profilins.

As mentioned earlier, actin-binding and PtdIns(4,5)P$_2$ studies were not carried out so far, due to the small amount of recombinant protein available.

1.4 Generation of Profilin 3-Specific Antibodies

No commercial antibodies for profilin 3 are available. In order to study the protein expression pattern, I generated a polyclonal antibody in rabbits by immunizing the rabbits with full-length recombinant GST-profilin 3. To attenuate the immune reaction of the rabbit towards the GST-tag, and increase the specificity of its antibodies towards profilin 3, full-length

recombinant profilin 3 fused to a different tag (MBP) was used to boost the immune response.
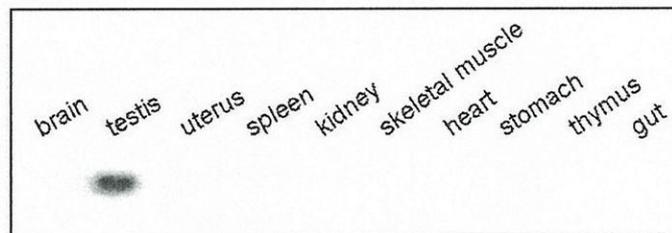
Figure 15. Western blot analysis for testing profilin 3 specific sera (BEG6) on tissue lysates. A signal was detected in the testis lysates, confirming the antibody is specific for profilin 3. BEH3 also gave a testis-specific signal, indicating a good specificity for profilin 3 (Data not shown). For comparison of western blotting with profilin 1 and 2 antibodies in tissues (See Fig.11.)

By western blotting, two of the sera obtained, BEH3 and BEG6, showed a good signal when tested against recombinant profilin 3. When tested in tissues, the antibodies showed a signal in testis and no signal in other tissues in which profilin 1 and 2 are highly expressed, indicating a high specificity of the antibody for profilin 3 (see Fig.15). Both antibodies also worked well in immunohistochemistry and immunofluorescence (see chapter 1.5 "Tissue Expression of Profilin 3 in the Mouse", Fig.17 & 18, chapter 2.6 "Subcellular localization of Profilins in Germ Cells" Fig. 29).

1.5 Tissue Expression of Profilin 3 in the Mouse

Hu et al. first isolated profilin 3 from a rat kidney cDNA library, and showed the RNA to be also expressed in testis (Hu et al. 2001). Later it was shown by Braun et al. that the signal in kidney was in fact due to the presence of a slc34a1 transcript. The gene for this protein is located on the DNA strand opposite profilin 3 and contains the entire profilin 3 open reading frame in its 3'-untranslated region (Braun et al. 2002). It was therefore concluded that profilin 3 was a testis-specific profilin gene (Braun et al. 2002).

To test whether profilin 3 expression was indeed restricted to the testis, I screened a number of mouse tissues by RT-PCR, using profilin 1 as a positive control (Fig.16).
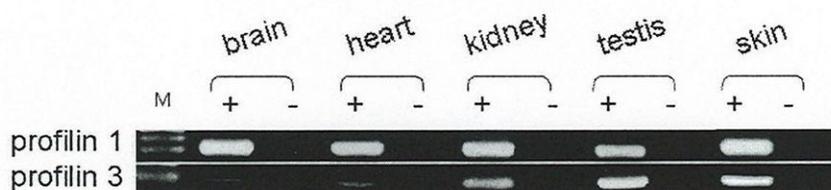
Figure 16. RT-PCR on tissues to detect profilin 3 (below). Profilin 1 (above) was used as a control. Note the expression of profilin 3 in skin

The expression of profilin 3 in the testis was confirmed by RT-PCR. As profilin 3 is a single exon gene located opposite the 3'UTR region of an alternative splice form of the slc34a1 gene, it is not possible to selectively amplify profilin 3 and not the slc34a1 mRNA. The signal observed in the kidney was probably due to the slc34a1 mRNA present in this tissue as we did not detect profilin 3 expression in the kidney at the protein level (see Fig. 15) nor at the RNA level by northern blotting (see Fig.21).

RT-PCR screening revealed a signal for profilin 3 in skin. To confirm that this signal was due to the expression of profilin 3 and not slc34a1 in this tissue, I carried out histological analysis on adult skin sections.

Immunohistochemical staining using the profilin 3 specific antibody BEG6 on skin sections demonstrated profilin 3 was expressed in the epidermis and in the outer sheath of hair follicles, specifically in the distal region of the follicle, which elongates to allow the hair to push through to the skin surface (Fig. 17).
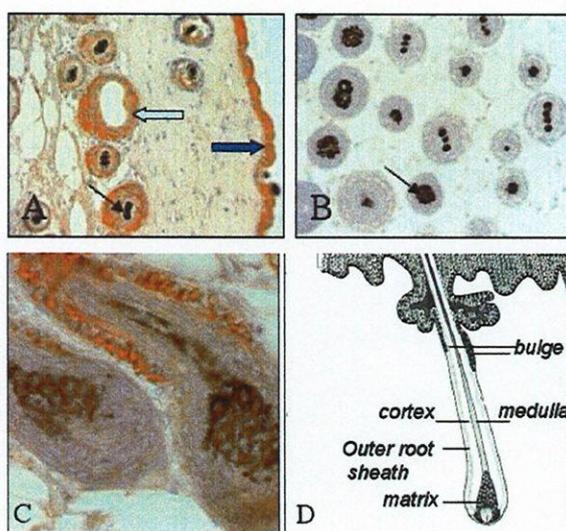
Figure 17 DAB immunohistochemical stainings for profilin in skin sections. Blue arrows indicate profilin 3 expression in hair follicle (pale blue) and epidermis (dark blue). Black arrows indicate growing hair
A) immunohistochemical staining for profilin 3.
B) (negative control (no incubation with primary antibody)
C) longitudinal section of a hair follicle stained with profilin 3,showing expression in the outer sheath of the hair follicle
D) diagram of a hair follicle.

Immunohistochemical staining for profilin 1 was also carried out, to determine whether profilin 1 and 3 colocalized in this tissue. Profilin 1 was not expressed in the same region as profilin 3 but near the root of the hair, suggesting these proteins have non-redundant functions in hair follicles (data not shown).

This result shows that although the expression of profilin 3 is highest in testis, its expression at lower levels in selected cell types of other tissues cannot be excluded per se.

1.6 Expression of Profilin 3 During Postnatal Development of The Testis

Although profilin 3 has been shown to be expressed in the adult mouse testis, nothing was known about its expression during postnatal testicular development.

The first month of postnatal development in the male mouse is the period that marks the first round of spermatogenesis. If transcription of a particular gene is turned on at a specific stage of spermatogenesis, mRNA for that gene will appear after a certain number of days from birth. The spermatogenic cell

type in which the transcript is first expressed can therefore be deduced from the age of the mouse at which the transcript is first detected (Braun et al. 1989) Days 8, 15, 20, 30 are particularly informative, as they coincide with the appearance of the major cellular steps of spermatogenesis (see introduction, Fig.2). Total RNA and proteins from testis of these ages were used to determine at what developmental stage profilin 3 expression started, and at what age its expression was highest.

By northern blotting, profilin 3 is not expressed in the testis during the first 20 days of postnatal development (Fig.18) and starts to be expressed between day 20 and day 30. This period marks the spermatogenic stage during which elongated spermatids first appear in the seminiferous tubules.
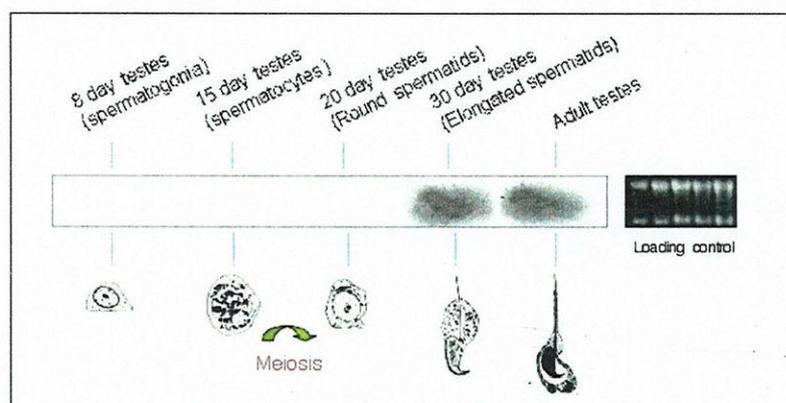
Figure 18 Northern blotting for profilin 3 during postnatal development. Profilin 3 appears to be expressed only after meiosis, by day 30. This corresponds to the period in which elongated spermatids first appear in the testis.

By performing western blotting on testis from mice of the same ages as those used for northern blotting, a small amount of profilin 3 was already visible at day 20, the stage in which round spermatids first appear in the testis. However, the peak of protein expression was found to be in adult testis (Fig.19). Again, these results strongly point towards a specifically post-meiotic expression of profilin 3 during germ cell development.
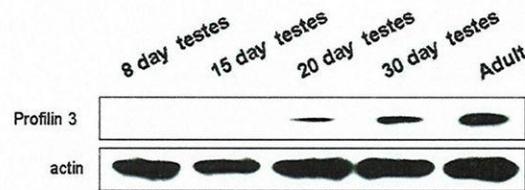
Figure 19 Western blotting of profilin 3 on testis lysates from 8, 15, 20, 30 day and adult mice. Protein expression is limited to post-meiotic stages of germ cell development. Actin was used as a loading control.

1.7 Post-Testicular Expression of Profilin 3

The late appearance of profilin 3 in the developing testis suggests profilin 3 is expressed only in the final stages of spermatogenesis, when the sperm leave the testis, and move into the epididymis. This was confirmed by performing immunohistochemical staining of testis sections (Fig. 20a).
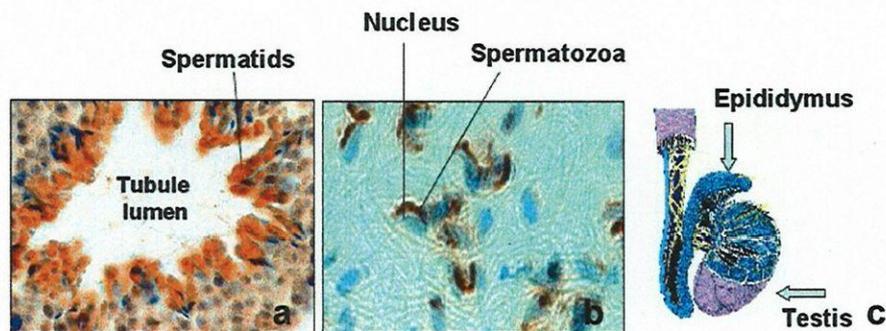
Figure 20 Immunohistochemistry showing late expression of profilin 3 in the the testis (a) and continuation of protein expression in sperm during epididymal transit (b). DNA is stained in blue. Diagram of the epididymis and testis (c).

The most mature germ cells, which lie closest to the lumen of the tubule, ready to be released into the epididymis, strongly express profilin 3. I therefore wondered whether profilin 3 expression arrested at the end of spermatogenesis, with the release of the spermatozoa into the lumen of the seminiferous tubule, or continued during epididymal maturation.

By performing immunohistochemistry on epididymal tissue sections (see Fig.20b) I observed that profilin 3 expression was still highly expressed by spermatozoa during epididymal transit, where it localizes to the head region.

2. Comparison of Profilins Expression in the Testis

The expression pattern for profilin 3 suggests this protein may have a specialized function in haploid germ cells. However, if profilin 3 is only expressed after such an important cellular activity as meiosis, other profilins should be expressed earlier in spermatogenesis, as the actin cytoskeleton needs to be regulated during this cellular process. I therefore determined which profilins were expressed in the adult testis, and subsequently compared their expression patterns both in the developing testis, and at a subcellular level in germ cells.

2.1 All Four Profilins are Expressed in the Testis

Although in lower species, such as drosophila and echinoderma, profilin is known to be important in spermatogenesis, little is known about the expression of the profilins in the mouse testis.

To determine whether all the profilins are expressed in the testis and how strongly they are expressed in this tissue relative to other organs, northern blotting analysis was performed on a wide range of adult mouse tissues (Fig. 21) using full-length cDNAs for each probe.
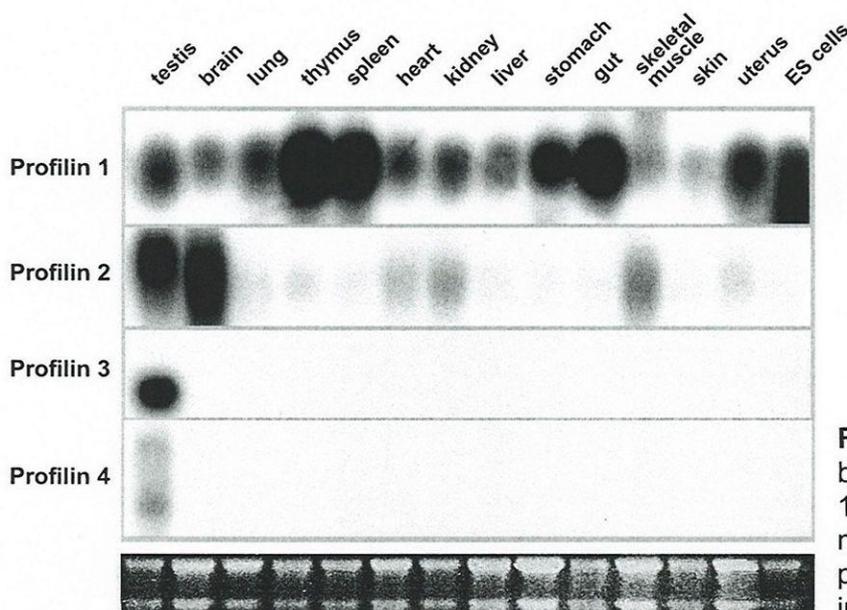
Figure 21 Northern blot analysis of profilin 1-4 expression in mouse tissues. All four profilins are expressed in the testis.

Profilin 1 is the most abundant of the profilins, as it is almost ubiquitously expressed in mouse tissues, with highest expression levels in thymus, spleen, and gut. This is not surprising, as profilin 1 seems to be involved in a wide range of basic cellular functions, such as cytokinesis, and cell migration.

Although profilin 2 is traditionally regarded as a brain-specific profilin, partly due to the neurological defects of the profilin 2 knockout mice, its expression is almost equally high in the testis. By northern blotting, the expression of profilin 3 and profilin 4 appears to be specific to the testis. In conclusion, my results demonstrate that the testis is the first and perhaps only mouse tissue known to express all four profilins.

2.2 Expression of Profilins During Testicular Postnatal Development

Having shown that all profilins are expressed in the adult testis, the next question to answer was whether their expression overlapped during testicular development. I therefore compared the expression pattern of all four profilins during postnatal development both at the RNA and protein level (Fig. 22). By northern and western blotting, good correlation between the expression patterns at the protein and mRNA level was found.
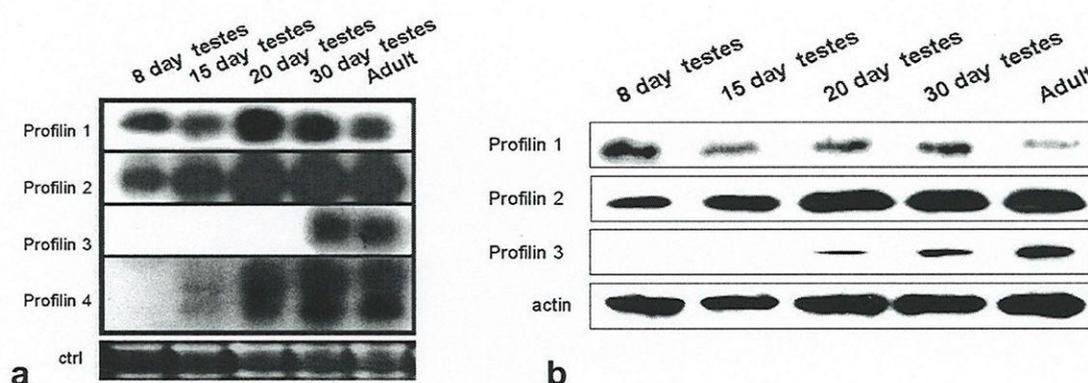
Figure 22 Comparison of profilin expression at the mRNA (a) and protein level (b) during postnatal testis development by northern and western blotting respectively. For northern blotting, ethidium bromide stained ribosomal RNA was used as a loading control.

At day 8, when the only germ cells present in the testis are spermatogonia, profilin 1 is already abundant and reaches a peak by day 20, after which its expression diminishes significantly. Profilin 2 is also expressed at low levels by day 8, but expression increases significantly between day 15 and 20, with the appearance of the first haploid spermatids.

Profilin 4 showed yet another expression pattern, as it starts to be expressed at very low levels during meiosis at day 15, and increases postmeiotically by day 20. Protein levels for profilin 4 could not be determined as no antibodies are currently available. In conclusion, profilin 3 is the last profilin to be expressed during spermatogenesis, and is the only profilin to be exclusively found in haploid cells. Although all profilins are expressed in the testis, each profilin has a unique developmental expression pattern, which was further confirmed by *in situ* hybridizations for all 4 profilins on adult testis sections.Full-length sense and anti-sense probes were used, and the cytoplasm was counterstained with Carmallume Mayer (see Fig. 23).
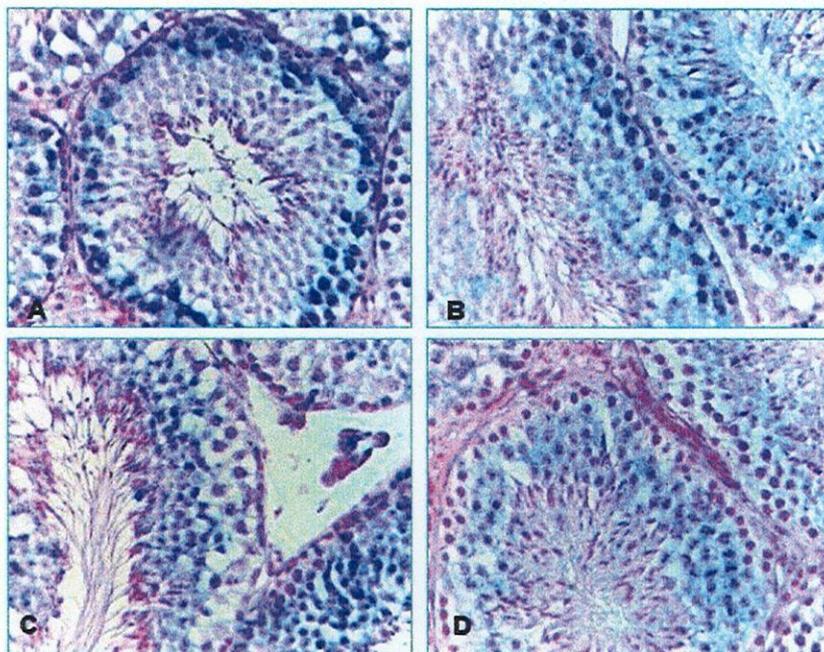
Figure 23 *In situ* hybridization for profilins on adult mouse testis. Cytoplasm is stained with Carmallume Mayer. A) Profilin 1, B) Profilin 2, C) Profilin 3, D) Profilin 4

Profilin 1 is expressed at the earlier stages of spermatogenesis, as the cells showing highest transcription levels are the germ cells lying close to the basal membrane of the seminiferous tubule (Fig.23 A). It is difficult to determine by *in situ* whether spermatogonia express profilin 1 or not, as these cells are very small and sparse in the tubules, however, the expression of profilin 1 is strongest in pachytene spermatocytes, cells which are about to go through meiotic division and is absent or significantly reduced in spermatocytes at earlier stages of development (e.g. lepoptene, zygotene) and in postmeiotic, round and elongated spermatids, which lie inside the spermatocyte layer, closer to the lumen.

Profilin 2 (Fig. 23B) and 4 (Fig. 23D) are expressed more broadly throughout several stages of spermatogenesis. Their expression is significantly reduced if not absent in the earliest stages of spermatogenesis, after which, profilin 4 expression seems to reach its highest expression in spermatocytes, and decrease slightly in postmeiotic cells, whereas profilin 2 remains highly expressed constantly from the spermatocyte stage through to the elongated spermatids. In contrast, profilin 3 is only expressed in the final stages of spermatogenesis, in round and elongated spermatids, which lie close to the lumen of the tubule (Fig. 23C).

The results obtained by *in situ* hybdridization confirmed and summarized the differences in expression patterns observed so far by northern and westernblotting of profilins in the developing testis.

2.3 Somatic Versus Germ Cell Expression of Profilins

Profilins may also be expressed in cell types other than germ cells in the developing testis, making it difficult to estimate how much of the profilin RNA and protein observed by northern or western blotting on extracts from the developing testes is contributed by somatic cells and how much by germ cells. This consideration is particularly important if the gene is highly expressed in the first days of postnatal development, when fewer germ cells are present in the testis, and the somatic cells contribute a higher proportion of RNA/protein compared to germ cells.

One way to estimate the extent of germ cell and somatic cell contribution of profilin is to compare the amount of protein present in a normal testis, and a germ cell free testis. The adult testis can be completely depleted of germ cells by treating the mouse with Busulfan, a chemical routinely used in humans to treat blood cancers, and known to be toxic to proliferating cells. Busulfan therefore causes endogenous spermatogenesis to arrest, so that 30 days after busulfan treatment all the mature spermatozoa have left the testis, but have not been replaced by new ones, leaving the testis completely void of germ cells, except for a few spermatogonial stem cells, which do not divide frequently. The depletion of germ cells in the testis is temporary, as after busulfan treatment, the spermatogonial stem cells will eventually repopulate the testis. We therefore treated adult wild type mice with a single i.p. injection of busulfan to eliminate the germ cells from the testis, sacrificed them 4-8 weeks after the treatment, and compared the levels of profilin expression in normal and germ cell depleted testis, via western blotting (Fig. 24).

As shown in Fig. 24, no reduction in profilin 1 busulfan-treated testes compared to the untreated adult tissue was evident. This suggests that most of the profilin 1 present in the testis is expressed either by somatic cells, or by the spermatogonial stem cells, the only germ cells that are not affected by busulfan treatment. It would be interesting to determine whether profilin 1 is expressed in the spermatogonial stem cells, as it is the only profilin known to be highly expressed in embryonic stem cells (Witke et al. 2001).
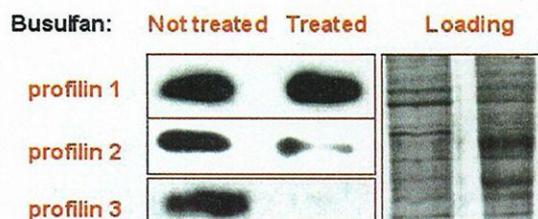
Figure 24 Profilin 1-3 expression in adult testis lysate from an untreated mouse compared to germ-cell depleted adult testis lysate from a busulfan-treated mouse.

In contrast to profilin 1, profilin 3 seems to be almost exclusively expressed by germ cells. Profilin 2 is expressed by both somatic and germ cells, although the levels of profilin 2 are very reduced in busulfan-treated testes, indicating that most profilin 2 in the testis is contributed by germ cells.

2.4 Comparison of Profilin Levels in Purified Germ Cell Stages.

After having shown that profilins in the whole testis lysates showed distinct expression patterns during postnatal development, I wanted to extend my expression analysis and compare the expression levels of profilins at different stages of germ cell development.

It is possible to purify germ cells from the testis (see materials and methods). What is obtained is a mixed population of germ cells at all stages of germ cell maturation, which I will refer to as the "whole population". From the whole population, the germ cells can be further fractionated according to their maturation stage by Staput sedimentation (Boitani et al. 1980) (see materials and methods). These purification techniques allow a more precise comparison of the protein levels directly in the germ cells during the major steps of spermatogenesis, because they remove the somatic cell compartment and enrich each developmental population. Spermatocytes, round spermatids and elongate spermatids were purified from whole germ cell populations, and the purity of each fraction was determined by light microscopy, based on cell morphology (see materials and methods). On average, the fractions were shown to have > 85% purity.

To compare the profilins' expression levels in specific germ cell populations, western blotting on protein extracts from the purified germ cell fractions was performed (see Fig. 25).
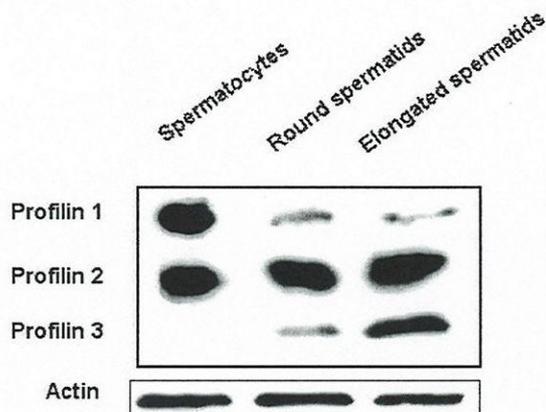
Figure 25 Western blotting for profilin 1-3 on purified germ cell extracts. Profilin 1 expression decreases significantly after meiosis, profilin 2 expression remains fairly constant throughout spermatogenesis, and profilin 3 is expressed only in haploid spermatids.

The expression patterns seen in the analysis of the developing testis for profilin 1, 2 and 3 is in agreement with the expression patterns I obtained with the germ cells fractions, but the differences in expression levels are more pronounced in the purified germ cells. For example, the large decrease in profilin 1 expression we observe after meiosis (i.e. in round spermatids) was also visible in the northern and western blotting on the whole testis lysates (i.e. after day 15), but the decrease in the strength of the signal was not as dramatic in the whole testis lysates due to the high contribution of somatic cell expression of profilin 1.

Profilin 2 expression levels are constant and high in the different stages of spermatogenesis represented. The increase of profilin 2 during testicular development (compare Fig. 22) could therefore be explained by the overall increase in the total number of germ cells in the testis expressing profilin 2, as testis development proceeds.

Finally, although profilin 3 showed a post-meiotic expression, western blotting on purified germ cell extracts allowed us to see that its expression is stronger in elongated than round spermatids.

2.5 Subcellular Localization of Profilins

I have shown that during spermatogenesis, most stages of spermatogenesis express more than one profilin. To determine whether the overlap in expression is possibly linked to an overlap in function, I analysed the subcellular localization of profilin 1-3. Germ cell populations were purified by velocity sedimentation using the Staput method (see materials and methods), and subsequently cytocentrifuged onto slides in order to perform immunofluorescent staining.

Profilin 1 appears to be broadly expressed throughout the cytoplasm of spermatocytes and round spermatids, with no specific localization in germ cell structures present during these stages of spermatogenesis although a perinuclear staining was observed in round spermatids. Profilin 1 appears to be absent in the head region of elongated spermatids (Fig. 26).

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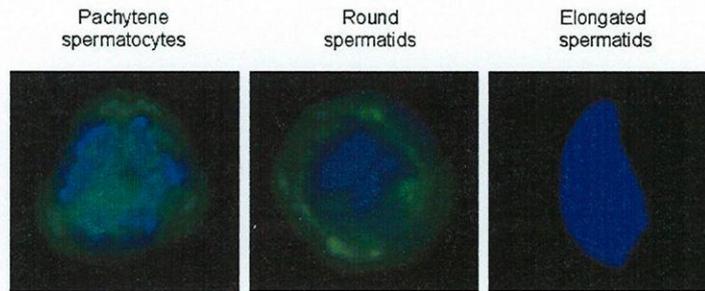
Figure 26 Profilin 1 staining in purified germ cells. Profilin 1 is broadly expressed throughout the cytoplasm of pachytene spermatocytes and round spermatids, but is not expressed in later stages.

Profilin 2 is also broadly expressed throughout the cytoplasm in pachytene spermatocytes, although it's expression is not as strong as profilin 1 in these cells (Fig. 27). After meiosis, profilin 2 expression increases, and an asymmetric distribution in the cytoplasm of round spermatids develops and becomes evident in a small, rounded region on one side of the nucleus (Ramalho-Santos et al. 2001). In elongated spermatids, the profilin 2 signal is very punctuated, and spreads along the nucleus, being strongest along the anterior half of the spermatid head, known as the acrosomal region (Toshimori and Ito 2003).
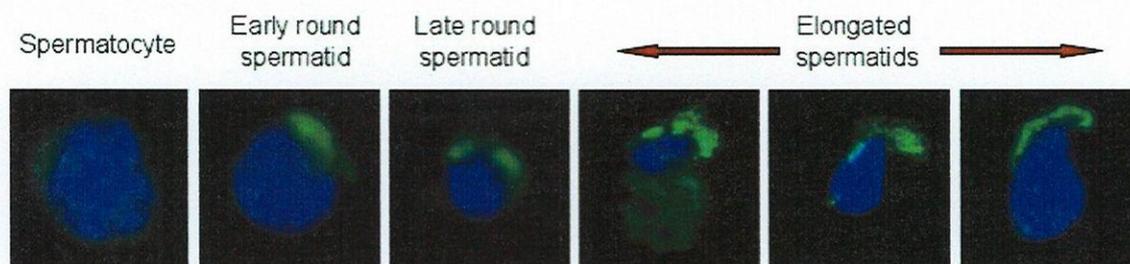
Figure 27 Immunofluorescent staining of profilin 2 in germ cells. The punctuated staining pattern and asymmetrical distribution in round spermatids of profilin 2 suggests it may be involved in acrosome biogenesis

The pattern closely resembles the expression pattern of proteins involved in the formation of the acrosome, which starts to develop in round spermatids, when vesicles bud off from the golgi body and fuse to form the acrosomal vesicle on one side of the cell. Like profilin 2, such proteins show a punctuated pattern in the cytoplasm of spermatocytes and early round

spermatids, and develop an asymmetrical distribution pattern as the acrosome forms in late round spermatids (Moreno et al. 2000) intensifying in a small region on one side of the cell nucleus, and finally spreading across the anterior half of the elongated spermatid head. The similarity in the subcellular staining between these proteins and profilin 2 therefore raises the possibility that in germ cells, profilin 2 is involved in vesicle trafficking from the golgi to the developing acrosome.

Profilin 3 is not expressed in pachytene spermatocytes, and its expression in round spermatids is more evenly distributed throughout the cytoplasm than profilin 2 (Fig. 28). As round spermatids develop into elongating spermatids, profilin 3 is not expressed in a punctuated manner around the acrosomal region like profilin 2, and its expression in elongated spermatid heads extends along the entire exterior edge of the nucleus, almost until the neck of the spermatozoa.

Pachytene spermatocytes   Round spermatids   Elongated spermatids
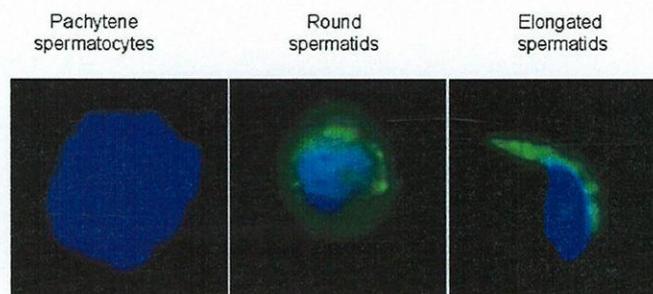
Figure 28 Profilin 3 staining in purified germ cells. Profilin 3 is not expressed in spermatocytes, and is expressed broadly throughout the cytoplasm of round spermatids. In elongated spermatids, it is expressed along the entire exterior edge of the nucleus.

Like profilin 2 therefore, profilin 3 is also expressed in the acrosomal region, but extends further, into the region known as the postacrosomal region (Toshimori and Ito 2003). To determine whether a protein is present in the acrosome or in the cytoplasm or cytoskeletal elements of the sperm head, one can empty the acrosomal contents by inducing the acrosome reaction *in vitro*. To do this, the sperm must first be capacitated in capacitating media, and subsequently incubated with

calcium ionophore, which induces this exocytotic process, thereby causing the release of the acrosomal contents (see materials and methods). After induction of the acrosome reaction in spermatozoa, the cells were spotted on glass slides, and immunofluorescent staining for profilin 3 was performed (see Fig. 29). As not all the spermatozoa will have undergone the acrosome reaction, double staining with FITC-PNA (peanut agglutinin), which labels intact acrosomes, was carried out to identify which spermatozoa were acrosome reacted and which were not.
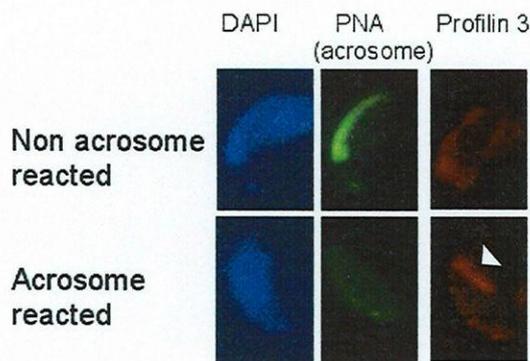
Figure 29. Profilin 3 expression in acrosome reacted and non-reacted spermatozoa. Nuclei were stained with Hoechst, acrosomes with FITC-PNA. Presence of FITC-PNA indicates the acrosome is intact, loss indicates the sperm underwent the acrosome reaction. In non-reacted sperm, profilin 3 (red) is present in the acrosomal region. Staining remains in the perinuclear theca (white arrow) after the acrosome is removed.

In non acrosome-reacted spermatozoa, profilin 3 is expressed along the exterior edge of the nucleus. In acrosome reacted spermatozoa, profilin 3 was still strongly expressed in the head region, where it appeared to localize to the perinuclear theca, an insoluble actin rich structure encasing the nucleus. The staining of this structure becomes more evident after the removal of the acrosome.

3. Studies on the *in vivo* Function of Profilins in the Testis

The generation of knockout mice has proved to be a powerful technique to study the *in vivo* functions of a protein. Since cell culture systems are very limited for the study of spermatogenesis, due to the sensitivity of germ cells, which cannot be maintained in culture for extended periods of time, the possibility to use *in vivo* techniques is essential to study this developmental process.

In addition to comparative studies on the expression of profilins in the testis, we also wanted to carry out *in vivo* analysis of profilin function in this tissue. Profilin 1 and 2 knockouts have already been generated in our laboratory. Profilin 1 mutants, however, are embryonic lethal, and it would have been difficult to generate a profilin 1 conditional knockout in germ cells, as no cre-lines are known so far to express cre at a very early stage in development, when profilin 1 is already highly expressed. We therefore decided to proceed with analysis of profilin 2 knockouts, which are viable. Of the two profilins for which knockouts have not yet been generated, profilin 3 and 4, we chose to generate a profilin 3 knockout, which is more closely related to mouse profilin 1 and 2. In this section I will therefore present my results on the *in vivo* studies for profilins in the mouse testis, carried out so far for profilin 2 and 3.

3.1 Generation of a Profilin 3 Knockout Mouse

In order to generate a knockout mouse for profilin 3 (see material and methods) a targeting construct carrying the profilin 3 gene flanked by loxP sites was generated as shown in Fig. 30. The neomycin resistance cassette (neo) was flanked by FRT sites, which allowed its removal it upon FLP recombination.

The first targeting construct contained a 1.5 kb 5' homology arm and a 4.8 kb 3' homology arm for the recombination in the genomic locus of mouse profilin 3, as shown in Fig. 30 (construct 1).
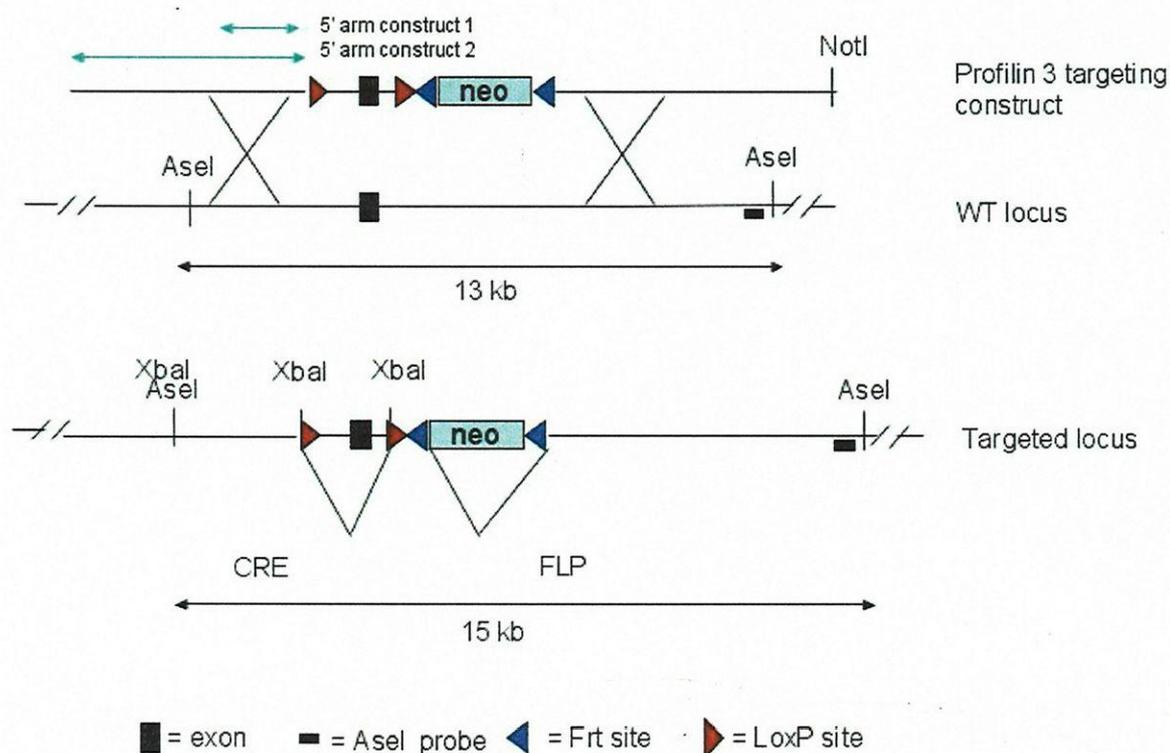
Figure 30. Schematic drawing representing the profilin 3 wild type region, the second targeting construct and the targeted locus where profilin3 is flanked by loxP sites and carries a neomycin resistance flanked by FRT sites, downstream of its 3' UTR. The 5' arm for the first targeting construct is also marked.

ES cells were electoporated as described with the linearized targeting vector 300 neomycin resistant clones were picked, and analyzed by Southern blot, using AseI restriction digest and the external probe, as shown in Fig.30. A band of 13 kb was expected from the wild type allele and a band of 15 kb in case of an homologous recombination event due to the presence of the 2 kb neomycin cassette inserted downstream of the gene (see Fig. 31). Single

copy integration was subsequently confirmed using a neomycin probe (data not shown).
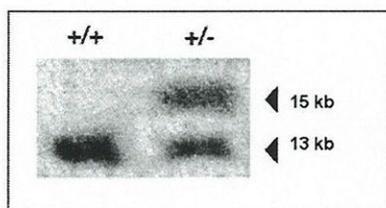
Figure 31 Southern blot analysis of genomic DNA from homologous recombinant ES cell and wild type clones digested with AseI.

Although the AseI digest identified the clones in which homologous recombination had occurred upstream of the neo resistance gene, it could not distinguish whether recombination had occurred in the 5' homology arm or between the loxP sites (in which case the 5' loxP site would have been lost).
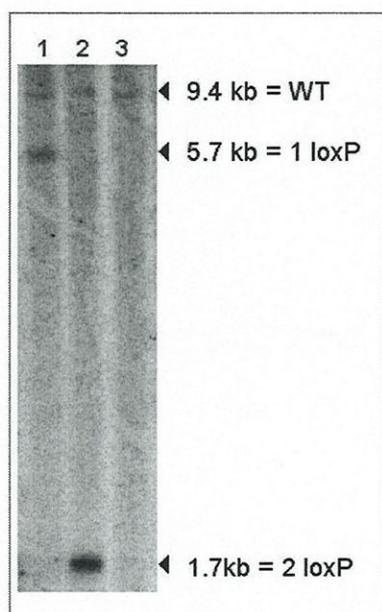
Figure 32 Southern blot of XbaI digested genomic DNA probed with an internal probe, showing a targeted ES clone in which recombination occurred between the loxP sites (lane 1) a clone in which recombination occurred in the homologous arms, (lane 2,) and a WT clone (lane 3).

Southern blotting after restriction digest using XbaI, allowed us to determine unequivocally whether both loxP sites were present in the recombinant clones, as the targeting construct brought in a new XbaI site immediately upstream of the 5' loxP site. From this Southern blot, for which an internal probe (profilin 3 cDNA) had to be used, it was possible to see that none of the clones contained both loxP sites (Fig. 32 lane 2).

One of the reasons recombination occurred between the loxP sites and not in the 5' homologous arm may have been that the 5' arm was too short. To increase the probability that recombination would occur in the homologous arms and not between the loxP sites, I modified the targeting construct by generating a 5 kb 5' arm (see Fig. 30, construct 2). With this targeting vector I obtained 20 clones out of a total of 300 in which homologous recombination had occurred. Three clones out of these 20

contained both loxP sites (Fig. 32, lane 2). All three correctly targeted ES cell clones (#9, #11 and #20) were subsequently expanded, and injected into C57Bl/6 blastocysts. Six male and two female chimeras were obtained and have been crossed with wild type C57 for germline transmission. I recently obtained germline and we are currently breeding these mice to cross them to cre-deleter mice to obtain the complete knockout for profilin 3.

3.2 Analysis of Profilin 2 Knockout Mice

Although profilin 2 is highly expressed in the testis, its function in this organ has not been studied so far. I therefore analyzed profilin 2 null mutants to determine whether the absence of profilin 2 affected fertility, and if so, whether this was a testis-related phenotype, or a consequence of their altered social behaviour.
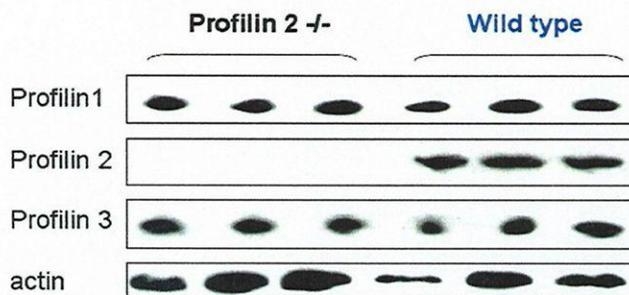
Figure 33 Western blotting for profilins in wild type and profilin 2 knockout mice. No upregulation is detectable in whole testis lysates for profilin 1 and 3 at the protein level. Profilin 2 is absent in profilin 2-/- testes.

In order to determine whether there was any upregulation of profilin 1 and 3 in profilin 2 mutant mouse testis and to confirm the absence of profilin 2 at the protein level in the knockouts, I carried out western blots on protein extracts from three wild type and three mutant mouse testes. As expected, profilin 2 was absent from the knockout testes. The levels of profilin 1 and 3 in whole testis lysates are comparable in profilin 2 knockouts and wild type mice (Fig. 33).

3.2.1 Morphology of the Testis of Profilin 2 -/- Mice is Normal

To compare the overall morphology of profilin 2 knockout and wild type testes, testis sections were stained with haematoxilin and eosin, which stain the cytoplasm and DNA respectively (Fig. 34 (a, c)). With such general staining, no morphological differences were evident, as spermatogenesis and spermiogenesis appeared normal in the mice lacking profilin 2.
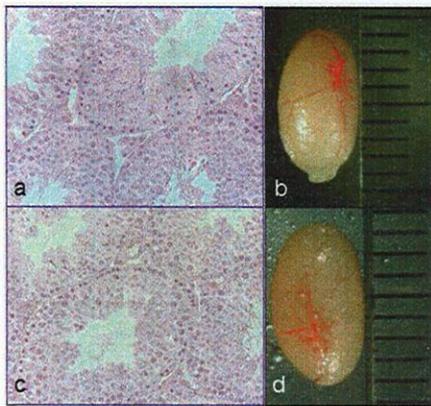
Figure 34. Analysis of profilin 2 knockout testes. Deletion of the profilin 2 gene does not affect spermatogenesis or spermiogenesis in adult testis. Shown are representative sections of the testis stained with hematoxylin-eosin and whole testis extracted from wild type (a, b) and profilin 2 knockout (c, d) mice.

Overall size of the profilin 2 knockout testis was comparable to wild type testes (Fig.34 b,d). The epididymes weight relative to the total body weight was also comparable in wild type and mutant mice, however, profilin 2 knockout testes displayed a tendency to be lighter compared to wild type mice testis with respect to the total body weight (Fig.35, P= 0.07, Student t-test, n=6 for KO, n=9 for WT). A lighter testis or epididymis can be an indication that less germ cells or spermatozoa are present in the respective tissues.
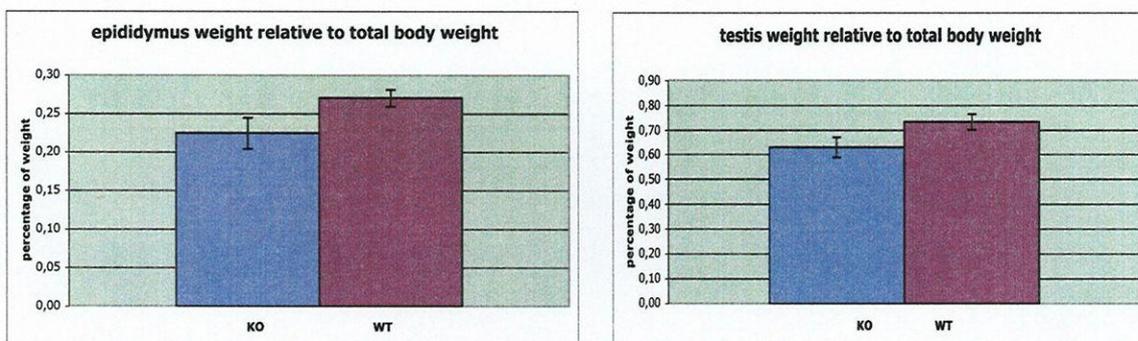
Figure 35 Epididymis and testicular mass relative to total body mass. A tendency towards lighter testis relative to the total body mass was observed in profilin 2 mutant mice (P= 0.07, Student's test, n=6 for KO, n=9 for WT).

3.2.2 Reduced Sperm Counts in Profilin 2 Mutant Mice

Typically, a C57 mouse stores roughly 25 million spermatozoa per epididymis. To determine whether the reduction in the testis weight was due to a lower number of spermatozoa being produced by the testis, I performed sperm counts on mutant and control mice. Whole epididymes were dounced to release the sperm and sonicated (see materials and methods for protocol) to lyse all cells except sperm heads, which are resistant to such treatments. The sperm heads were counted using a hemocytometer.
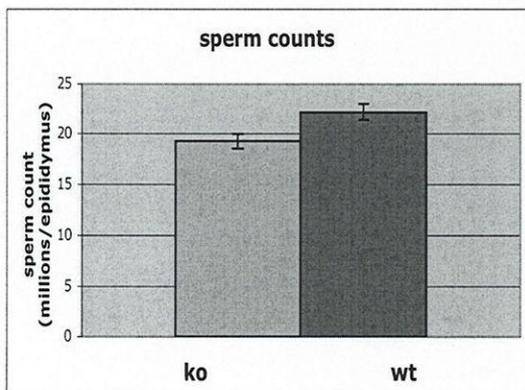
Figure 36. Sperm counts in wild type and mutant mice. Profilin 2 -/- mice show a 15% reduction in sperm number, with an average of 18,9 million sperm compared to 22 million in wildtype (n=7 for KO n= 9 for WT p< 0.05).

The sperm count showed a significant, reproducible 15% reduction in sperm number (n=7 for KO n= 9 for WT p< 0.05 Student's t-test) in mutant mice, which had on average 18,9 million spermatozoa, compared to 22,1 million in wild type. (see Fig 36).

3.2.3 Increase in Cellular Apoptosis in Profilin 2 -/- Mice

A possible cause for sperm number reduction in the profilin 2 mutants could be an increase in cellular apoptosis during spermatogenesis. Apoptosis is a naturally occurring phenomenon in the mammalian testis (Rodriguez et al. 1997; Jahnukainen et al. 2004), where a proper balance of germ cell and Sertoli cell numbers is crucial, and high levels of apoptosis are seen both during development and in adult testes. However, a defect in germ cell development can sometimes be seen by an increased number of apoptotic cells present in the adult testis. To visualize the number of apoptotic cells in

the seminiferous tubules, I used the TUNEL assay, which fluorescently stains apoptotic cells (Fig.37).
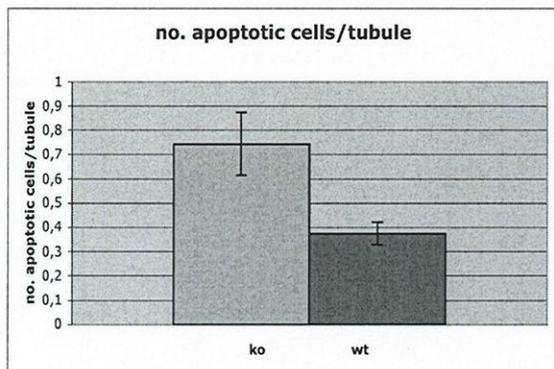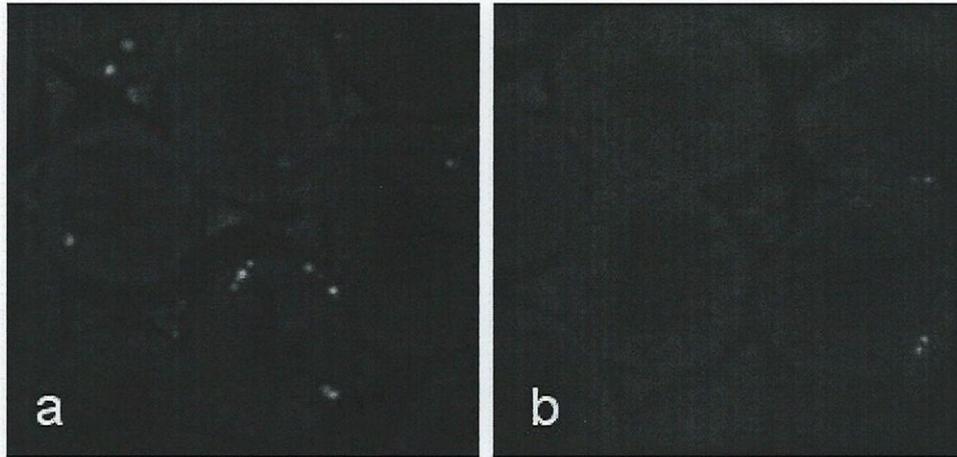
Figure 37 TUNEL staining on profilin 2 -/- (a) and wild type (b) testis. DNA was counterstained with Hoechst. A higher incidence of apoptotic germ cells in profilin 2 -/- seminiferous tubules was observed (C) (n= 5 for KO, n= 5 for wild type, p >0.05 Student's t-test)

The number of apoptotic cells was significantly higher in the profilin 2 knockout mice than in wild type mice. I determined that knockouts had 0.74 apoptotic cells/ tubule compared to the wild type mice in which there were only 0.37 apoptotic cells/ tubule (n= 5 for wild type and KO p >0.05 Student's t-test). Therefore, it is likely that the increased apoptosis of germ cells in the profilin 2 –/– testis contributes to the decrease in epididymal sperm number found in these mutants.

3.2.4 Mating Behaviour and Fertility of Profilin 2 Knockouts

To determine whether the lack of profilin 2 affected mouse fertility, I carried out fertility assays on age matched knockout and wild type mice. Fertility was measured by the number of pups/litter, and the number of plugs.

Each male was housed with three virgin females at a time, and when plugged, the female was transferred to a separate cage. The pregnant females were kept in separate cages to give birth, and the litter sizes counted on day P1.
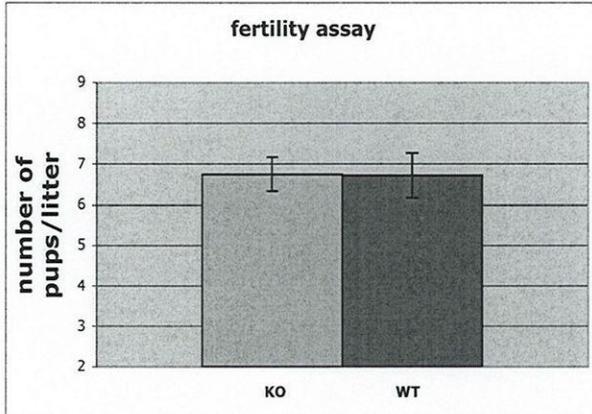
Figure 38. Fertility assay in profilin 2 -/- and wild type. The average litter sizes were comparable.

The average days taken for a male mouse to plug a female was comparable in knockout and wild type mice, suggesting a normal mating behaviour in these mice. The litter size was comparable in wild types and mutants (see Fig. 38) as an average of 6.7 mice/litter were born from both wild type and mutant males, and the number of plugs that produced litters also showed no significant difference, as I obtained litters from 11/15 plugs by profilin 2 -/- and 12/16 plugs by wild type mice.

Although profilin 2 mice have a defect in spermatogenesis that causes a higher incidence of germ cell death and reduction in sperm production, it does not seem to compromise their fertility.

Summary

Although the testis is the first and only tissue in mice known to express high levels of all 4 profilins, these proteins have distinct expression patterns during testicular development, which have been summarized in Fig. 39.
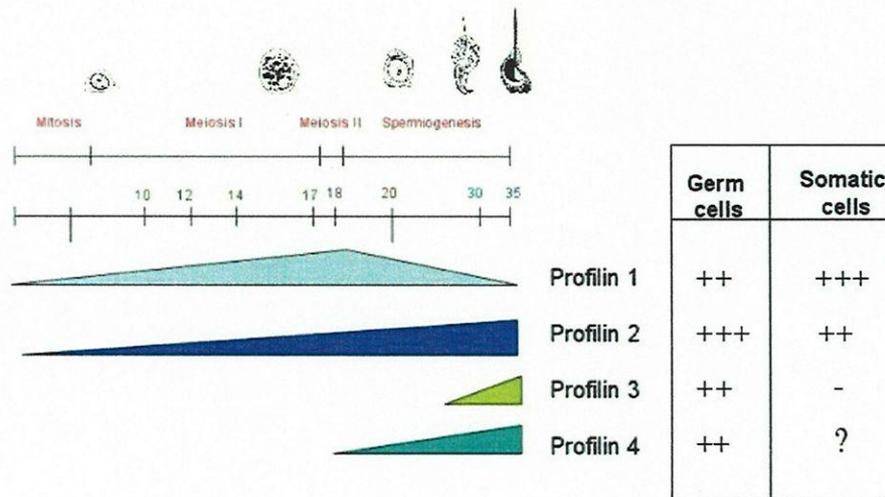
Figure 39 Summary of mouse profilin expression pattern in the testis. A distinct expression pattern can be observed for each profilin during testicular development, with expression levels peaking at different ages. The protein contribution by germ cell and somatic cells for each profilin is also summarized (+++=high, ++=medium, +=low expression -= not expressed).

Expression studies carried out so far for profilin 1-3 in purified germ cells, also suggest that in stages of germ cell maturation where more than one profilin is expressed, the proteins have distinct subcellular localizations as summarized in Fig.40.
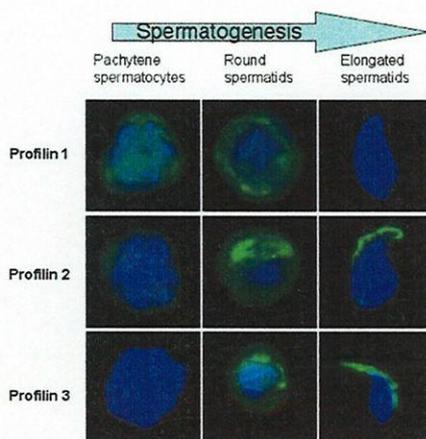
Figure 40 Summary of the subcelluar localization of profilin 1-3 in various developmental stages of sperm maturation. Progressive stages of germ cell maturation are shown from left to right.

Taken together, these results suggest that profilins have distinct functions in germ cell development. Although these functions are yet to be elucidated, we speculate that profilin 1 may be involved in meiotic division, profilin 2 in vesicle trafficking and profilin 3 in the formation of the acroplaxome and perinuclear theca. The absence of profilin 2 in the testis causes an increase in testicular germ cell apoptosis, leading to a 15% reduction in the number of spermatozoa produced by the profilin 2-/- knockout mice. This does not affect their fertility that is comparable to that of wild type mice. No upregulation or downregulation of profilin 1 and 3 in profilin 2 knockouts is detectable at the protein level in whole testis lysates. We are currently generating the profilin 3 knockout mouse, for which we have obtained germ line transmission of the floxed profilin 3 allele.

4. Actin Depolymerizing Factors in The Testis

During my studies of the role of actin binding proteins in the testis, I wanted to characterize and compare a second kind of actin binding protein, the actin depolymerising factors. The 3 main actin depolymerizing factors in mammals are gelsolin, ADF, and cofilin. Although all three proteins have been shown to be expressed in the mammalian testis, so far no detailed studies within this tissue have been carried out in mice.

Some conflicting results regarding the localization of these proteins in the mammalian testis, in particular gelsolin, are found in the literature, and this may in part be due to the fact that different mammalian species, including humans (Rousseaux-Prevost et al. 1997), guinea pigs (Cabello-Agueros et al. 2003), and rats (Guttman et al. 2002) have been used to study these proteins. I decided study the actin depolymerising factors in the testis, with particular focus on gelsolin and cofilin.

4.1 Expression of Gelsolin in the During Testicular Development

Gelsolin is already present in the testis at a very early postnatal age, and its expression levels remain constant throughout the first month of development and in the adult (Fig. 41). The constant protein levels during the development of the testis suggests that gelsolin is not expressed in any particular stage of germ cell maturation, but that its expression in the testis must largely be contributed by somatic cells.
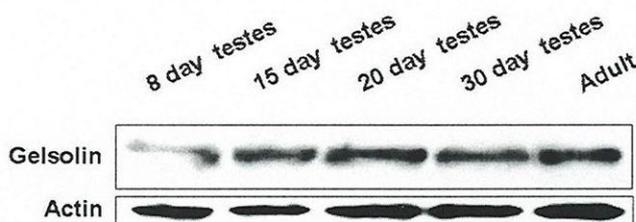
Figure 41. Expression of gelsolin in the developing testis. Expression remains constant during development

In some mammalian species, gelsolin is expressed in spermatozoa, where it is involved in the rapid depolymerization of actin occurring prior to

fertilization (Cabello-Agueros et al. 2003). To determine whether in mice, gelsolin was also expressed in germ cells, western blotting on purified germ cell lysates was carried out. No expression in the testicular germ cells could be seen (see Fig. 42) indicating that in mouse testis, gelsolin is only expressed by somatic cells.
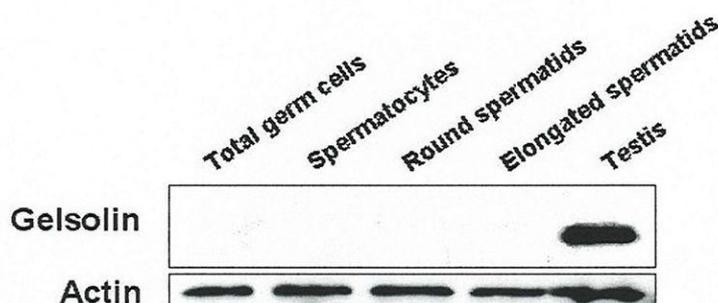
Figure 42 Gelsolin is not expressed by germ cells at any stage during development. Expression in the testis is therefore appears to be restricted to somatic cells

To characterize the expression of gelsolin in the testis, I carried out whole mount double staining and confocal microscopy on seminiferous tubules I isolated from the testis, for gelsolin and F-actin. The whole mount immunofluorescence allows us to maintain the structure of the seminiferous tubules and layering of the germ cell epithelium intact. Gelsolin colocalized with actin around the heads of elongated spermatid, (Fig. 43). As germ cells do not express gelsolin, we can conclude that the protein is present in the Sertoli cells, which surround the spermatid heads. The area of the Sertoli cell encircling the spermatid's head contains the ectoplasmic specialization, an actin rich structure, responsible for maintaining the contact between germ cells and the nurturing sertoli cell, as well as the correct positioning of the spermatids in the germ cell epithelium. Gelsolin has previously been found to be expressed in ectoplasmic specializations in rats (Guttman et al. 2002).

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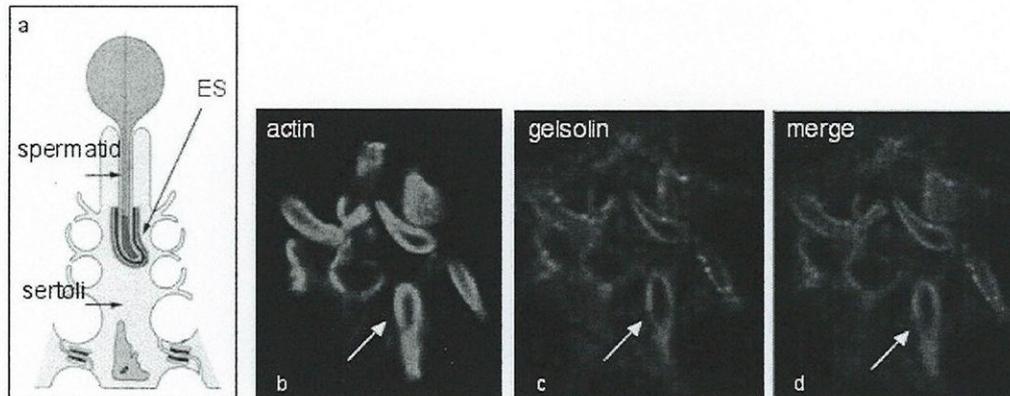
Figure 43 In mice, as in rats, gelsolin is expressed in ectoplasmic specializations, structures surrounding the spermatid heads, which are important for the correct positioning of the germ cells in the seminiferous epithelium.

4.2 Expression of Gelsolin in the Epididymis

In the epididymis, I found that cells in the pseudostratified columnar epithelium of the epididymal ducts strongly express gelsolin (see Fig. 44).

Figure 44. Gelsolin is expressed in principal cells, secretory cells in the lining of the epididymal duct. a) epididymis stained for gelsolin b) magnification of a) showing the principal cells in more detail. Arrowhead points to a principal cell expressing gelsolin c) Negative control (secondary antibody only)

These cells, which span the lining of the duct, are known as principal cells, and are known to have a secretory activity, producing and secreting, for example, glycerophosphorylcholine (Brooks et al. 1974), which is responsible for inhibiting capacitation of the sperm, until they are ready for fertilization,

and carnitine (Brooks 1979), believed to be essential for mitochondrial metabolism in sperm once they have acquired motility (Jeulin and Lewin 1996).

As secretion is the main activity of principal cells, I wondered whether the gelsolin produced by these cells was secreted into the epididymal fluid. It has been shown that a secreted form of gelsolin exists, that is slightly larger than the non-secreted form (83 kDa as opposed to 80 kDa) and that is mainly expressed in muscle (Kwiatkowski et al. 1988). As our gelsolin antibody recognises both isoforms, I performed western blotting on epididymis and testis lysates, using as controls blood serum (known to contain only secreted gelsolin) and thymus (known to express only non-secreted gelsolin). By running the lysates on a low percentage gel, I was able to separate the two forms of gelsolin, and observed that whereas the testis expresses both the secreted and non-secreted forms of gelsolin, the epididymis only expresses the secreted form (Fig. 45).
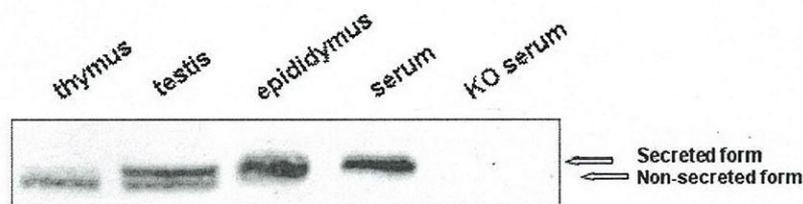
Figure 45 In the testis, gelsolin is expressed as secreted (83 kDa) and non-secreted (80 kDa). In the epididymis, only the secreted form seems to be present.

This result suggests that gelsolin is expressed by the principal cells, and released into the seminal fluid surrounding the spermatozoa. Although gelsolin knockout mice are fertile, no detailed studies have been carried out on the testes of the mice so far. As gelsolin is secreted by the epididymis into the seminal fluid, I wanted to determine whether loss of gelsolin affects epididymal sperm, as this fluid contains many proteins and nutrients that are important for the final steps of sperm maturation.

Preliminary analysis of epididymal sperm morphology suggests that in mice gelsolin has a role in the epididymal maturation of spermatozoa, as gelsolin -/- spermatozoa extracted from the epididymis show a high incidence

of angular flagellation (see Fig. 46). The tail of these spermatozoa presents a kink at the end of the neck region, where frequently some residual cytoplasm that should have been shed prior to sperm release is still present. Although this morphology is also seen in wild type, it normally occurs in immature spermatozoa residing in the caput epididymis, where the spermatozoa first enter the epididymis. In the gelsolin knockout mice, this morphology was more common throughout the epididymis, which may suggest that the gelsolin secreted by the principal cells into the epididymal fluid plays a role in sperm maturation, in a way that sperm maturation is delayed in gelsolin knockout mice.
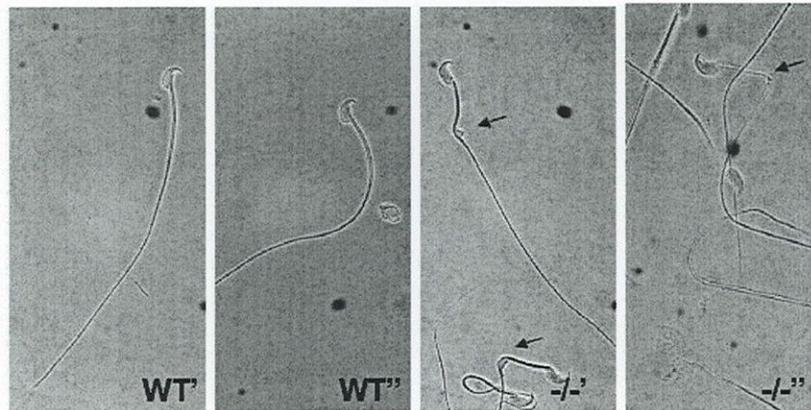
Figure 46. Wild type and gelsolin knockout spermatozoa. Arrows indicate residual cytoplasm and angular tails of the spermatozoa, which prevent the correct beating of the tail.

4.3 Cofilin is the Only Actin Depolymerising Factor in Germ Cells

Interestingly, the actin depolymerizing factors ADF and gelsolin, are not expressed in germ cells, and are both highly expressed in the epididymis (Fig.47). It has in fact been previously shown that ADF is present in the epididymal epithelia (Vartiainen et al. 2002). In contrast, cofilin is highly expressed in all stages of sperm maturation, and is the only depolymerizing factor to be expressed by germ cells.
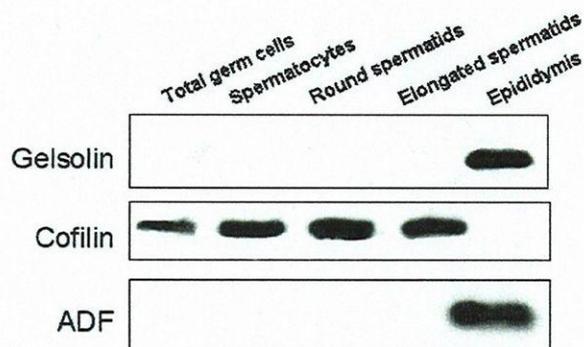
Figure 47 Western blotting carried out on purified germ cells for actin depolymerising factors, showing that only cofilin is expressed in germ cells, Gelsolin and ADF are both highly expressed in the epididymis.

To observe the expression pattern for n-cofilin in the developing mouse testis, northern blotting was performed on RNA extracts from testis of 8, 15, 20, 30 day and adult mice, using a full-length cDNA probe. At the mRNA level n-cofilin is already present in the young testis, but expression is highest during and after meiosis, from day 20 until adulthood (Fig.48 A).
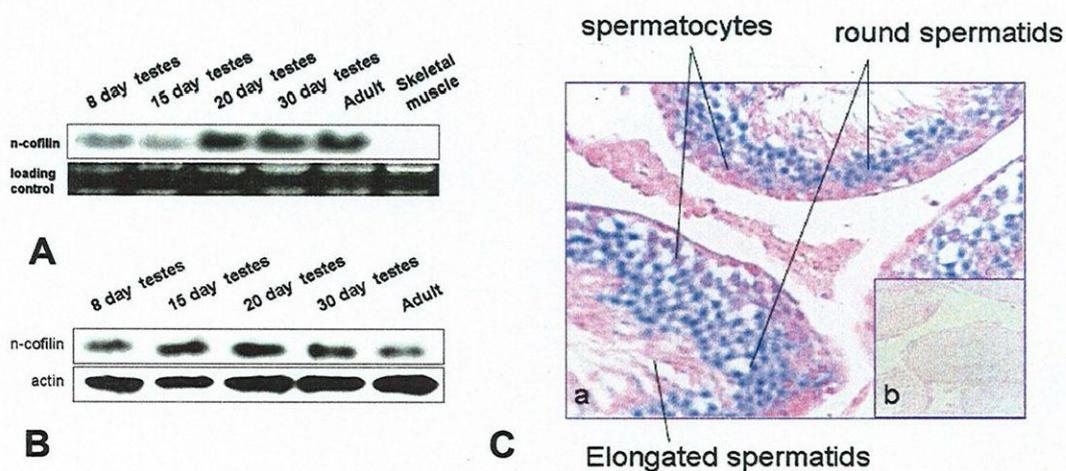
Figure 48 Expression of n-cofilin during postnatal development of the testis by northern blotting (A) and western blotting (B). Skeletal muscle mRNA was used as a negative control. The expression pattern observed at the protein level correlates with the mRNA expression pattern. C) N-cofilin *in situ*. Testis sections were probed with sense (a) and anti-sense (b) probes. Cofilin transcription appears to increase significantly in haploid round spermatids, which would be consistent with the increase in mRNA and protein expression observed around day 20 by northern and western blotting.

The developmental pattern observed at the mRNA level for n-cofilin by northern as well as by *in situ* correlated with the protein expression pattern observed by western blotting on whole testis lysates, using an n-cofilin specific antibody, as protein levels were highest around day 20 (Fig. 48B). By *in situ* hybridization, where the high expression of n-cofilin was observed in haploid round spermatids (Fig. 48C).

4.4 Deletion of N-Cofilin Using a Germ-Cell Specific Cre-Line Mouse

In spermatogenesis a role for cofilin, shown to be the only actin depolymerizing factor present in mouse germ cells, has been suggested by the phenotype of the knockout mice for LIMK2, a cofilin-specific kinase, (see introduction section 4.1), in which an increase in germ cell apoptosis is observed (Takahashi et al. 2002) and in *twinstar* mutants, the cofilin homologue in *D.melanogaster*, in which primary spermatocytes develop abnormal cytoskeletal structures and form large actin aggregates in association with centrosomes (Gunsalus et al. 1995).

Since in mice, the complete knockout of n-cofilin results is an embryonic lethal phenotype, it is not possible to study spermatogenesis in these knockout animals. To circumvent this problem, I wanted to create a germ-cell specific knockout for n-cofilin, by crossing a cofilin conditional mouse model with a mouse expressing cre recombinase under a germ–cell specific promoter. The conditional (floxed) cofilin mouse (cof $^{flx/flx}$) was generated in our laboratory and generously provided by Christine Gurniak. The cre-expressing mouse I chose for the testis-specific deletion the protamine 1-cre (prm-cre) knock-in mouse, generated by O'Gorman et. al (O'Gorman et al. 1997). Protamine-1 is known to be transcribed premeiotically, but its translation is suppressed until after meiosis (Cho et al. 2001). As it is not known whether the translation of cre-recombinase is also repressed until after meiosis, it is not known at precisely what stage in spermatogenesis recombination between the loxP sites occurs. So far, the exact cell stage at which recombination occurs has not been important, as the protamine-cre

mice have never previously been used to study spermatogenesis, but were instead used simply as a tool to obtain recombination in the germ line.

To be sure that the protamine-cre deleted the n-cofilin gene efficiently in germ cells, I crossed homozygous cofilin conditional male mice also carrying one protamine-cre allele (cof $^{flx/flx}$/prm-cre$^{+/-}$), with wild type females. If deletion of the n-cofilin gene was 100% efficient, all the pups born should have been heterozygous for the deleted cofilin allele. By genotyping the pups, we determined that deletion of the cofilin gene by protamine-cre occurred in approximately 90% of spermatozoa of the conditional cofilin knockout mice, as 14/16 pups born from these matings inherited a deleted allele, and 2/16 maintained a floxed allele from their father.

4.5 Analysis of Protamine-Cre Cofilin Mice.

The mice used to analyze the *in vivo* function of cofilin were cof $^{flx/-}$/prm-cre $^{+/-}$. In these mice, the floxed allele would express cofilin as a wild type allele until recombination in the germ cells by cre. The other cofilin allele was a null, so that only half the wildtype amount of cofilin was present in this mouse testis to begin with. We therefore expected to see a further reduction of the cofilin levels only in the germ cells after the deletion of the floxed cofilin by protamine- cre.

4.5.1 N-Cofilin Expression is Reduced in the Mutant Testis

To determine whether the deletion of n-cofilin by protamine-cre caused a decrease in the levels of n-cofilin protein in the testis, western blotting was performed on protein extracts from whole testis lysates, using an n-cofilin specific antibody. Various genotypes for the cofilin and protamine-cre loci were used as controls for the expression levels.

With respect to the actin loading, we observed a reduction in n-cofilin levels in whole testis lysates in cof $^{flx/+}$/prm-cre $^{+/-}$ mice, comparable to the

levels observed in cof $^{+/-}$/ prm-cre $^{+/+}$ mouse. Also with respect to the actin loading, the n-cofilin level in cof$^{flx/-}$ / prm-cre $^{+/-}$ mice was even further reduced (Fig.49). A small amount of n-cofilin was still present however, probably due to the contribution of n-cofilin by somatic cells or early germ cells expressing n-cofilin from the floxed allele, as protamine-cre is not expressed in these cells.
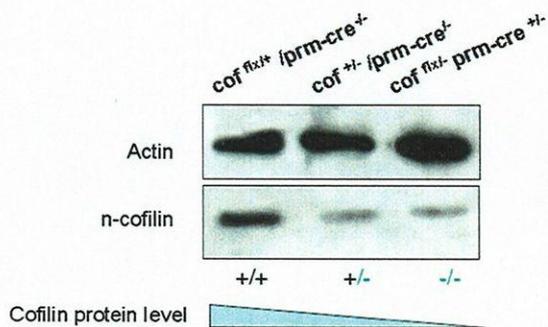
Figure 49 Expression levels of n-cofilin in protamine-cre cofilin conditional mice. In proportion to the actin loading, the deletion of cofilin by protamine-cre appears to decrease the cofilin levels in the testis. +/- under the lanes indicates the expected levels of cofilin for each genotype.

4.5.2 No Apparent Phenotype in Prm-Cre N-Cofilin Mutant Mice

As well as determining the expression levels of n-cofilin in the germ cells of the cofilin conditional mice, we analysed the mutant testis to determine whether there were any obvious defects in spermatogenesis or in testicular morphology.
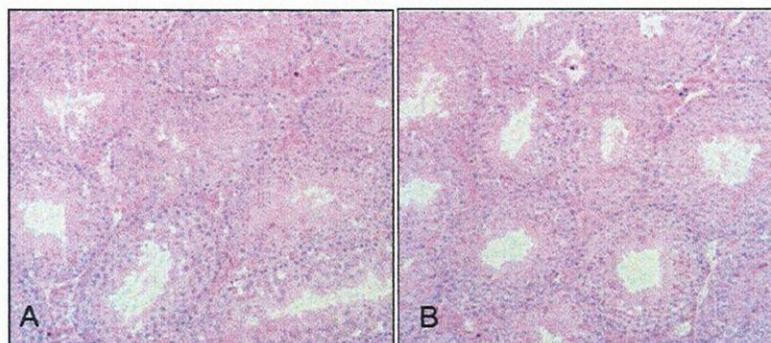
Figure 50 Analysis of prm-cre n-cofilin conditional knockout testis. Conditional deletion of cofilin in germ cells does not affect spermatogenesis in adult testes. Shown are representative sections of the testis stained with hematoxylin-eosin from wild type (A) and cof $^{flx/-}$ / prm-cre $^{+/-}$ (B) mice.

Overall, the morphology of the testis appeared normal (Fig. 50) as was the weight of the testis and epididymis relative to the total body weight (Fig. 51 A, B). To determine whether the deletion of cofilin could affect the fertility of these mice, we carried out fertility assays on cof $^{flx/-}$ / prm-cre $^{+/-}$ mice, using wild type and prm-cre $^{+/-}$ as control mice, to exclude that any phenotype we may have seen was due to the expression of cre under the protamine promoter, and not due to the deletion of cofilin in the germ cells.
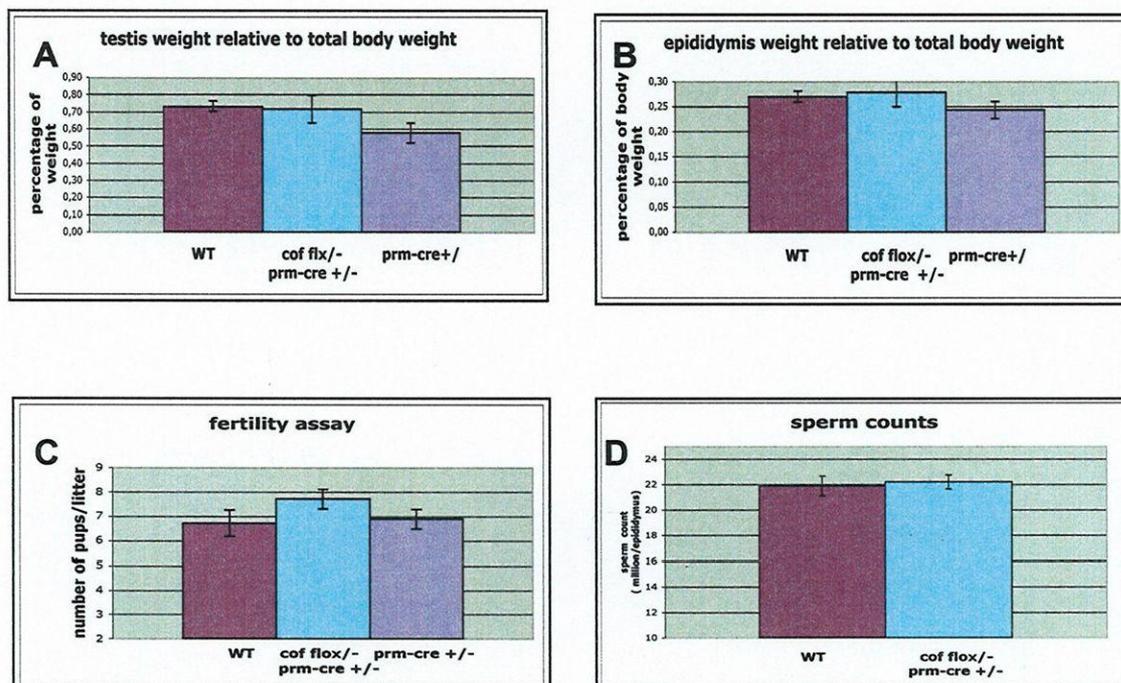
Figure 51 Analysis of protamine-cre cofilin conditional knockout mice. The weight of the testis (A) and epididymis (B) relative to the total body weight was comparable to wild type mice and to mice containing the protamine-cre allele, as was the fertility, judged by litter size (C) and the number of sperm stored in the epididymis (D).

The fertility of these mice also was not affected, as the litter sizes were comparable to wildtype and prm-cre $^{+/-}$ litter sizes (Fig.51C). Finally, epididymal sperm counts from cof $^{flx/-}$ /prm-cre $^{+/-}$ were also comparable to those of wildtype mice (Fig.52D). In summary, although the levels of n-cofilin are reduced in the testis of cof $^{flx/-}$ /prm cre $^{+/-}$ mice, so far no phenotypic effect has been observed in these mice.

Summary

Mammals express three main actin depolymerising factors - gelsolin, ADF and cofilin. Although all three proteins are known to be expressed in the adult testis, little is known about the functions of the actin depolymerising factors in this tissue.

I hereby show that in mice, gelsolin is not expressed in germ cells at any stage of germ cell development, although it is present in the testis from a very young age. Instead, it is expressed in sertoli cells, where it localizes to an actin rich structure known as the ectoplasmic specialization. Gelsolin is also secreted by the epididymal epithelium, from secretory cells known as principal cells that are located in the lining of the epididymal ducts.

Little is known about ADF in the testis. Like gelsolin, it is present in the epididymal epithelium, and in the testis, it is expressed by somatic cells only, as we did not find it to be expressed at any stage in purified germ cells.

Cofilin is the only actin depolymerizing factor expressed in gem cells. To study the *in vivo* function of cofilin I generated a germ-cell specific conditional n-cofilin knockout by crossing the n-cofilin conditional (floxed) mouse to a mouse expressing cre recombinase under the protamine 1 promoter.

Although the levels of n-cofilin are reduced in the testis, n-cofilin is dispensable for spermatogenesis, as so far no phenotypic defect has been observed in these mice. The spermatogenic cycle in these mutants is comparable to wild type, as judged from histological analysis of the adult testis, as well as their testicular weight, fertility, and sperm count.

Discussion

Elucidating the Role of the Actin Cytoskeleton in the Testis

The actin cytoskeleton has long been known to be essential for a wide range of cellular activities, such as motility, exo/endocytosis, and cell division, and its disruption causes dramatic defects in many cell types, which have in turn linked it to a wide range of human pathologies such as neurodegenerative diseases, muscular dystrophy, haemolytic anemias, and cardiomyopathies. Although the function of actin has been analyzed in many cell types, tissues, and cellular activities, its role in male germ cells and its involvement in testicular pathologies, which often disrupt spermatogenesis and frequently result in infertility, have not been closely examined.

In vitro and cell culture techniques have been extremely useful to study the role of the actin cytoskeleton in somatic cells, however they cannot be applied to the study of actin in germ cells, because of the difficulty to maintain germ cells in culture over extended periods of time. Furthermore, the study of male germ cells is complicated by the extremely structured organization of the seminiferous epithelium, which contains multiple layers of germ cells at different stages of germ cell maturation, all connected by cytoplasmic bridges, due to multiple rounds of incomplete cytokinesis. Although a limited number of *in vitro* studies on spermatozoa have provided evidence for the importance of the actin cytoskeleton in fertilization, *in vivo* studies will be essential to understand the role of actin in spermatogenesis.

The complete disruption of the actin cytoskeleton *in vivo* cannot be carried out to study the function of actin due to its lethality. However, expression studies and targeted deletions of genes coding for actin binding proteins have provided a wealth of understanding on the function of the actin cytoskeleton and its localization in many tissues, cell-types, and cellular activities. The absence of actin binding proteins often causes subtle phenotypic defects in the cytoskeleton or in a cellular function, which are not lethal to the organism, and thereby can shed some light on a specific function of the cytoskeleton.

The interest in actin-binding proteins during germ cell development stems from the fact that the actin cytoskeleton has been shown to be highly dynamic throughout spermatogenesis and fertilization, and that it forms cytoskeletal structures in germ cells and sertoli cells that are unique to these cell types. Furthermore, it has recently been discovered that testes-specific isoforms of actin (T-actin) exist (Tanaka et al. 2003), which may in turn be regulated by testes-specific isoforms identified for many actin binding proteins, e.g. destrin (Howes et al. 2001), actin capping protein (Hurst et al. 1998) thymosin β10 (Lin and Morrison-Bogorad 1991),and profilin (Braun et al. 2002; Obermann et al. 2005).

Using the mouse as a model for mammalian spermatogenesis, the goal of this thesis was to characterize the expression and possibly function of the monomer actin binding protein profilin, and the three actin depolymerising factors known to be expressed in the mammalian testis, gelsolin, cofilin and ADF.

Profilins in the Testis: How Far Have we Come?

Since its discovery as an actin monomer-binding protein, profilin has been known to be involved in sperm function. Profilin was in fact first isolated in echinoderma sperm, and in several invertebrate species has been shown to be centrally involved in the acrosome reaction, by causing the rapid actin filament elongation required for the acrosomal process to cross the jelly coat and reach the egg's plasma membrane (Tilney et al. 1983). Also in *D. melanogaster*, profilin is essential in the testis, as the *chickadee* mutant, the profilin homologue in drosophila, causes severe disruptions in spermatogenesis (Verheyen and Cooley 1994).

The importance of this protein in spermatogenesis in lower eukaryotes, along with the recent discovery of two testis-specific profilins in mice and humans, were the premises for our belief that a detailed study of profilins in the mouse testis may shed some light on the regulation of the actin cytoskeleton during

mammalian spermatogenesis. Could one of these testis-specific mouse profilins have maintained an ancestral function in actin polymerization during fertilization, or would profilin 1 be a more likely candidate, with its more basic function and ubiquitous expression?

The mouse testis is a "common ground" for profilin expression, being the only tissue known so far in which all profilins are highly expressed. I asked myself whether this was purely coincidental, as so many proteins (1 in 3 in mice) are expressed in the mouse testis, or whether profilins had overlapping functions in germ cells. The distinct expression patterns for profilins observed during testicular development, and in purified germ cells, strengthened the hypothesis that profilins have distinct functions in spermatogenesis. Furthermore, the subcellular localization of profilin 1-3 in germ cells at different stages of maturation and the precise temporal expression observed in specific germ cell stages in adult tissue, allowed us to speculate on the possible roles of the profilins in the spermatogenic process. Could the role we believe profilin 1 and 2 play in germ cells be correlated to a somatic cell function already attributed to each of these proteins?

For profilin 1, for example, we have shown it to be involved in somatic cell division, because knockout mice for this gene do not develop beyond the 8-cell stage, due to a defect in cytokinesis (Witke et al. 2001). My observation that profilin 1 is expressed at its highest levels in spermatocytes, cells that are undergoing meiotic division, supports the hypothesis that profilin 1 plays an important role in cell division also in germ cells. Further evidence that its key function in germ cells could be cell division, is that after a very high expression of profilin 1 in meiotic cells, it is completely absent in haploid germ cells, where most germ-cell specific structures start to develop.

Spermatocytes are not the only germ cells undergoing cell division, as spermatogonial stem cells must divide frequently in order to generate a constant pool of type B spermatogonia, which will differentiate into spermatozoa, and type A spermatogonia, which will remain as undifferentiated stem cells. If profilin 1 was indeed involved in cell division, and considering its high expression in

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embryonic stem cells, it will be interesting to determine whether it is also present in spermatogenic stem cells.

Studies in the brain have linked profilin 2 to vesicle trafficking in neuronal synapses (Pietro Pilo-Boyl, personal communication). Many proteins involved in membrane trafficking in somatic cells are also involved in the trafficking of vesicles from the golgi to the acrosome formation (Ramalho-Santos et al. 2001) and in developing round spermatids they are seen to localize to a rounded region on one side of the nucleus, and develop a punctuated pattern in elongated spermatid heads. The pattern closely resembles the expression pattern for profilin 2 in these stages of germ cell maturation, which supports my hypothesis that, like in somatic cells, in germ cells profilin 2 is involved in vesicle trafficking, in this case, from the golgi to the developing acrosome. Profilin functions are therefore not only related to their actin-binding properties.

Profilin 3 expression on the other hand is clearly restricted to haploid cells, where it appears to be expressed in the actin-rich structure known as the perinuclear theca. Although the function of profilin 3 in the perinuclear theca is not known, the localization of the protein in this structure may explain the insolubility of the recombinant profilin 3 protein, which we were surprised to discover when trying to produce it in bacteria. The perinuclear theca, is in fact an extremely insoluble, actin rich structure, resistant even to sonication. This also explains why not only was profilin 3 difficult to obtain in a soluble form from bacteria, but also the extraction from germ cells for western blotting required harsher tissue-lysis conditions than the other profilins.

From the expression patterns determined for profilin 3 both during germ cell development and at the subcellular level we can confidently classify profilin 3 as a "chauvinist gene". Chauvinist genes are so called because male germ cells "favor their expression with such strong prejudice" (Eddy 2002), and are believed to be key players in spermatogenesis.

A gene is considered a chauvinist gene on the basis of 3 main characteristics:

1. it encodes a protein with essential roles for structures or functions specific to spermatogenic cells;
2. it is expressed in a developmentally regulated pattern
3. it is transcribed only in spermatogenic cells or produces mRNAs unique to spermatogenic cells.

According to these characteristics, profilin 3 can be classified as chauvinist as it

1. localizes to the perinuclear theca, a structure specific to elongated spermatids.
2. is expressed in a developmentally regulated manner,
3. is specifically expressed in the testis.

Like profilin 3, 20% of genes expressed in germ cells are testis-specific, and again like profilin 3, 99% of testis-specific genes are expressed during or after meiosis (Schultz et al. 2003). It has been postulated that germ cell-specific genes play important roles in germ cell maturation, and that furthermore, transcripts displaying a markedly increased expression in haploid germ cells have an important function in fertilization. Based on these assumptions, and what I have demonstrated for the expression of profilin 3, we believe that a knockout model for this gene will have an interesting testicular phenotype.

Not much can be concluded on profilin 4, mainly due to the lack of tools such as antibodies and recombinant profilin 4 proteins. Studies at the RNA level have shown its expression is also exclusive to the testis. Although this gene may also be considered a chauvinist gene, we have no information on the subcellular localization yet.

In vivo Analysis of Profilins

Although knockout mice are extremely useful for studies on spermatogenesis, as the study of germ cells in their natural environment is of fundamental importance, the mouse model has some limitations. For example, it has been demonstrated that rodents exhibit male hyper fertility, presumably to allow for continued reproduction even during non-ideal conditions. Male rats, for example, will inject 10- to 100-fold excess sperm than what is required for them to be fertile (Pearse et al. 1997). Due to this massive excess of sperm production in rodents, a decrease in fertility, will not be easily obtained unless sperm counts are dramatically reduced (Cooke and Saunders 2002).

The profilin 2-/- mice are a good example for the robustness of rodent fertility, as the 15% reduction of sperm number observed in these mice was not sufficient to decrease their fertility. Several knockout mice have been described whose reduced sperm count had no effect on fertility, and also in these cases, this was due to the hyper fertility of mice (Schurmann et al. 2002; Santti et al. 2005). From the expression pattern of profilin 2, we may have expected to see a reduction in fertility in the male knockout mice, due to a malformation of the acrosome. Although we have not yet followed the process of acrosome formation closely in these mice, the fertility levels of the knockouts did not appear to be reduced compared to wild type mice.

When analysing the profilin 2 -/- testicular phenotype, a couple of experimental conditions may have influenced our results. It has been shown that the genetic background of the mouse has been shown to have an impact on the fertility levels of a mouse. In Sprm -/- mice, for example, the subfertility phenotype is specific to the 129/Sv background as the *Sprm-1* mutants backcrossed into the C57BL/6 background have relatively normal fertility (Pearse et al. 1997). This is probably due to the fact that male C57BL/6 have a higher fecundity, and are therefore capable of overcoming the defect in the laboratory

environment. Studies on the profilin 2 deletion were carried out on mice with a C57BL/6 background, which might explain their fertility.

The females used for the fertility assays were C57BL/6, which normally produce 6/7 pups/litter. The use of CD1 females, which on average generate 11pups/litter, might make the fertility assay more sensitive. The mutant mice analysed were 3-5 months old, and therefore in their most fertile age. It might be worth testing the fertility in older (e.g. 6-12 months) knockout mice to determine whether the fertility level declines more rapidly in these mice.

A larger difference in sperm number between wild type and mutant mice may have been observed in the testis than in the epididymis of profilin 2 knockouts. Whereas the epididymis can be considered a place of storage for spermatozoa, which can remain in the epididymis for extended periods, gradually accumulating in this tissue, the testis is the site of production and therefore can be seen as the "rate-limiting step". We can therefore consider the number of sperm in the epididymis as close to the maximum number of spermatozoa the mouse will have at any one time, whereas the number of sperm produced by the testis might show a more dramatic difference between mutant and wild type sperm numbers.

The decrease in sperm number in profilin 2 mutant mice is due to the increase in apoptosis observed in testicular germ cells. Apoptosis is a naturally occurring phenomenon in the developing and adult testis, which is carried out by Sertoli cells on germ cells. The increased apoptosis of spermatogenic cells we observed could have been due to an abnormality of Sertoli cells, which do not phagocytose efficiently. However, because profilin 2 expression in testis was mainly localized in spermatogenic cells, and expression was low if not absent in Sertoli cells, I consider this possibility highly unlikely. In addition, if profilin 2 were to have a role in phagoyctotic activities in sertoli cells, findings from other cell types, suggest these cells would exhibit more efficient phagocytosis as compared to wildtype cells (Temesvari et al. 2000) as profilin 2 is believed to control membrane uptake as a negative regulator.

In the profilin 2 -/- mice, apoptosis might be occurring at a very precise stage of spermatogenesis, as, although we calculated an average of apoptotic

cells/tubule, I most frequently found that the tubules contained either several or no apoptotic cells. As discussed in the introduction (see ch. 1.2 "The Spermatogenic Cycle") the various layers of germ cells in a tubule develop in a coordinated manner, so that associations between spermatogenic cells at particular maturation steps are not random, and only a limited number of combinations exist. In the mouse, twelve different germ cell associations, designed as stages I to XII, can be recognized cytologically (Oakberg 1956), and any given cross section of a seminiferous tubule will only contain the cell types present in one of these 12 combinations. In retrospect, I wondered whether the tubules containing many apoptotic cells could all have been in a particular stage. If this were the case, I would be able to identify at precisely what point during spermatogenesis the profilin 2 -/- germ cells become apoptotic. For now, all we can confidently say is that profilin 2 -/- germ cells become apopototic in the pre-meiotic stages.

Although the levels of profilins 1,3 and 4 were not altered in profilin 2 -/- mice, they may however compensate the lack of profilin 2, by covering its functions in germ cell development. To determine whether this could be the case, double and triple knockouts should be generated, to see whether any combination of profilin deletions causes more severe defects.

The crossing between profilin 1 and profilin 2 could only be made by generating profilin 2 -/- profilin 1 +/- mice, as profilin 1 homozygous mice are embryonic lethal. It should be kept in mind, however, that because mammalian spermatogenesis occurs in a syncytium, often a heterozygous knockout mouse phenotype will be no different from the wild type phenotype, due to the cytoplasmic bridges that maintain a flow of cytoplasm from the early spermatogonial stages, until the very end of spermatogenesis, thereby possibly rescuing even haploid mutant cells.

Alternatively, a germ cell specific knockout for profilin 1 could be created. However, as profilin 1 is expressed very early on in spermatogenesis, a cre line must be found that deletes in spermatogonial stem cells. It will also be interesting to observe the effect of deleting profilin 2 and the testis-specific forms of profilins.

We are currently crossing profilin 3 conditional mice to obtain a line that can also be used to combine the profilin 2 and 3 deficiencies.

ADF and Gelsolin: Regulating Actin Dynamics in the Epididymal Sperm?

Although actin depolymerising factors are expressed in the testis, they also appear to have an interesting role in the mouse epididymis. Spermatozoa undergo important maturational changes during epididymal transit, acquiring for example motility and the ability to fertilize an egg. The maturation events taking place in the epididymis depend on the local environment provided by the epididymal fluid (Jungsu Oh et al. 2005), which is composed primarily of proteins secreted by the epididymal epithelium.

Although a number of epididymal secretory proteins have been identified, our knowledge of their roles is limited to a few of them. Transgenic mice with disruptions in the secretory function of the epididymal epithelia (Yeung et al. 1999; Sipila et al. 2002) are known to have severe defects in sperm morphology, such as angular flagellation, and retention of cytoplasm in the cytoplasmic droplet, suggesting that secreted proteins in the epididymis are involved in sperm volume regulation and in motility. Even though the number of spermatozoa produced is comparable to the numbers produced by wild type animals, these mutants are infertile, because the angular spermatozoa are unable to swim through the female reproductive tract to reach the egg.

Interestingly, although ADF and gelsolin are expressed in the murine testis, they are not expressed in germ cells. In the testis, two forms of gelsolin, secreted, and non-secreted are expressed. The secretion of gelsolin probably occurs from sertoli cells, which are known to express gelsolin, and have a secretory function. The Sertoli cells also express the non-secreted form of

gelsolin, which localizes to ectoplasmic specializations, structures that allow the Sertoli cell to maintain the germ cells in their correct position within the seminiferous epithelium. The role of gelsolin in this structure however, is still unknown. On the other hand, in the epididymis, gelsolin is only expressed as the secreted form, by principal cells, which are the main secretory cells of the epididymal epithelium.

Preliminary data on analysis of the gelsolin knockout mice has been collected, and from the testis, no gross morphological defect was observed. However, in the epididymis, the spermatozoa appeared to have a defect in the tail morphology, possibly due to a small droplet of residual cytoplasm in the sperm tail, which causes it to kink. This morphology is not completely absent in wild type mice, however, it is normally characteristic of an immature spermatozoon. If the number of angular sperm in gelsolin knockout is indeed higher, this would suggest that secreted plasma gelsolin has an important role in epididymal sperm maturation. This would be extremely interesting, as it may elucidate a novel function of gelsolin that is unrelated to actin depolymerisation.

The defect in sperm morphology in gelsolin -/- mice could arise either in the testis or in the epididymis. Normally, the cytoplasmic droplet is removed in the testis, by tubulobulbar complexes (TBC), modified ectoplasmic specializations (Guttman et al. 2004). Whereas ectoplasmic specializations are found in the convex part of the elongated spermatid head, the TBC are located on the concave side. So far, gelsolin has been observed in the ectoplasmic specialization, but it cannot be excluded that it is also expressed in the neighbouring TBC, and thus be involved in the removal of residual cytoplasm by this structure.

Another aspect to consider is the possible functional redundancy between the actin depolymerising factors. The similarity in expression patterns of gelsolin and ADF raises the possibility that ADF can compensate the lack of gelsolin in the testis, where it is also only expressed in the somatic compartment, the epididymis, and the epididymal epithelia. To determine whether there is

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functional redundancy, double mutants could be generated for gelsolin and ADF, for which we also have the knockout.

Is Cofilin Dispensable for Spermatogenesis in Mouse?

The actin depolymerizing factor cofilin is known to be expressed in germ cells of several species, and its importance in spermatogenesis has been suggested by studies in the drosophila *twinstar* mutant, (cofilin homologue), where spermatocytes show an abnormal actin cytoskeleton, and form large actin aggregates in association with centrosomes (Gunsalus et al. 1995). In the mouse, studies on LIMK2, whose only role known so far is to phosphorylate cofilin, indirectly suggested the importance of cofilin in murine spermatogenesis. Not only were the testes of LIMK2 -/- mice smaller in size but a partial degeneration of spermatogenic cells in the seminiferous tubules in association with increased apoptosis was apparent (Takahashi et al. 2003)

Out of the three main actin depolymerizing factors expressed in mice, cofilin is the only one to be expressed in germ cells. This was an advantage in the generation of a cofilin germ-cell specific knockout, as we could exclude any other actin depolymerizing factors could compensate for the lack of cofilin, thus potentially masking an otherwise severe phenotype.

As the complete deletion of n-cofilin in the mouse causes embryos to die due to a failure in closure of the neural tube (Gurniak et al. 2005), we needed to generate a tissue-specific deletion of cofilin in germ cells. We chose to cross the conditional cofilin mouse to the cre line expressing cre under the protamine 1 promoter because it was one of the few germ–cell specific cre-lines available at the time, that was known to have a good deletion efficiency in the germ line. When generating a protamine-cre/cofilin conditional mouse, one potential problem could have been the toxicity of cre-recombinase when constitutively expressed in germ cells. Schmidt et al. observed that the constituent expression

of cre in germ cells caused severe defects in spermatozoa, as chromosomal rearrangements in the sperm nuclei caused these mice to be infertile (Schmidt et al. 2000). However, in our hands, mice expressing protamine cre had fertility levels and sperm numbers that were comparable to wildtype animals, so I was able to exclude this possibility.

Although every single cell type in which cofilin has been deleted by crossing with cell-type specific cre-lines showed a pronounced phenotype (Christine Gurniak, personal communication) the absence of cofilin did not appear to affect spermatogenesis or fertility of these mice severely. We cannot exclude yet however that there might be a subtler phenotype we have not yet identified in these mice. The somewhat unexpected lack of a strong phenotype in these mice could not be explained by a compensatory effect by other actin depolymerizing factors, because, as previously mentioned, ADF and gelsolin are absent from germ cells. One possibility we cannot yet exclude however, is an up-regulation of muscle (m-cofilin).

Although both m-cofilin and n-cofilin are present in germ cells (Vartiainen et al. 2002), n-cofilin is considered to be the predominant isoform, as at the mRNA level it was shown to be 8.5 fold more abundant in the rat sertoli cells than m-cofilin and at the protein level, only n-cofilin is observed by immunoblotting on testis tissue lysates (Guttman et al. 2004). Furthermore, the expression or up-regulation of m-cofilin has never been shown to rescue a phenotype caused by deletion of n-cofilin in a tissue-specific manner or in the complete knockout, so we believe it to be unlikely to be the case in germ cells.

A second problem we may have encountered in the conditional knockout is an earlier expression of cofilin, prior to recombination of the loxP sites, in spermatocytes. The onset of expression of cre in the protamine cre-line has never been established, and protamine 1, like many testis-specific genes, is expressed as a protein in elongated spermatids, but is transcribed at least 7 days earlier, in round spermatids. The repression of translation is caused by an RNA-binding protein, which binds to a sequence element in the 3'UTR also present in the 3' UTR of protamine 2 (Braun et al. 1989). Whether the translation of cre is

also repressed, is still unknown. If translation of cre is also repressed, some cofilin will be expressed prior to meiosis, in spermatocytes, before the gene is deleted in haploid germ cells. In this scenario, the haploid germ cells will not be void of cofilin, due to the cytoplasmic bridges joining them to the germ cells they develop with in a syncitium.

One solution to this problem would be to cross the conditional mouse with a cre line expressing cre earlier in spermatogenesis, and a possible candidate line has been identified for this. The PrP-Cre-ER (T) cre-expressing mouse expresses tamoxifen inducible cre under the prion protein promoter that is expressed in spermatogonia and spermatocytes but not sertoli or leydig cells (Weber et al. 2003). By deleting n-cofilin earlier, we may therefore avoid the sharing of cofilin by earlier germ cells and germ cells who have deleted the cofilin gene.

An alternative approach to target cofilin in germ cells, which we have recently started to develop, is to use *in vitro* targeted germ cells for germ-cell transplantation. Germ-cell transplantation is a relatively new technique, which has so far been developed to treat clinical problems such as infertility, but which may become an established method for the study of spermatogenesis. To induce *In vitro* recombination in germ cells between two loxP sites, cre recombinase can be by transduced into cultured cells, using HTNC, a histidine-tagged cre fused to TAT, an 11 amino acid peptide derived from HIV. The HIS-TAT-NLS-CRE (HTNC) recombinase has been shown to be the most efficient for transduction of cre into cells, with approximately 90% of deletion efficiency (Peitz et al. 2002).

So far, the *in vitro* deletion of a gene using recombinant cre recombinase has not been tested on germ cells. However, if recombination in these cells is efficient, the treated germ cells could then be transplanted into a germ-cell free (busulfan-treated) adult testis so that, as they repopulate the testis, the spermatogenic cycle of the knockout germ cells can be studied *in vivo.* The establishment of such a technique would be interesting in itself, as it could become an extremely useful tool for studying spermatogenesis *in vivo.*

The efficiency of deletion in the germ cells we have obtained so far is still low (approximately 30%) and an optimal balance between the concentration of HTNC and the incubation time required for an efficient deletion rate is currently being investigated.

Actin Binding Proteins in the Testis: What next?

While these studies on actin binding proteins will lead to a better understanding of the role of actin in spermatogenesis, they may prove to be useful in other fields of research as well. For example, one use, based on the expression pattern for profilin 4 has already been postulated. It was demonstrated that the pattern of profilin 4 expression in the human testis is similar to that observed in mice, and it has been suggested that by carrying out RT–PCR analysis of human testicular biopsy specimens the presence or absence of profilin 4 expression could be used as a molecular diagnostic parameter suitable for supplementing conventional histopathological diagnostics in the assessment of testicular biopsies (Obermann et al. 2005).

Human infertility affects 10–15% of couples, with approximately equal contributions from both partners. Although environmental, behavioural, and genetic factors can affect male fertility, it is estimated that 60% of male infertility problems are due to genetic factors. However, very few mutations that cause infertility have been identified so far.

At present, *in vitro* fertilization techniques are becoming an increasingly common solution to infertility issues, but they cannot be considered a long-term solution, as they circumvent rather than treat male infertility problems. Unfortunately, until the underlying basis of fertilization continues to be poorly understood, partly due to the lack of information in the number and nature of molecules involved in this process, *in vivo* treatments for infertility, such as germ cell transplantation, cannot be efficiently developed. Although in humans, several

mutations affecting male fertility have been localized to regions of the Y chromosome, the possibility that also perturbations of the actin cytoskeleton due to the absence of a regulating actin binding protein can lead to defective spermatozoa merits attention.

If we were to relate our studies on profilin 2 to humans, a 15 % decrease of sperm would not be sufficient to cause a fertility problem in humans, as fertility problems in humans containing morphologically normal spermatozoa arise when the sperm counts decrease by at least 60% from 66 million, to below 20 million sperm/ml (Cooke and Saunders 2002). However, while the absence of profilin 2 does not affect murine fertility, we cannot exclude that the effect of deleting profilin 2 in human would be more severe, as previous studies have shown this to be the case with other genes (O'Neal et al. 1993).

The second field of research that relies on a deeper understanding of spermatogenesis, and in which knowledge on the function of actin binding proteins in the testis may prove to be useful, is research on alternative methods of male contraception. Two relatively new strategies for contraception are the disruption of the actin rich sertoli-germ cell junctions, known as ectoplasmic specializations, and the targeting of spermatozoa in the epididymis.

Because spermatozoa undergo a lengthy maturation process in the epididymis, where they are then stored prior to ejaculation, they can be targeted by contraceptive agents while in the epididymis. The possibility that male contraception could be based on action in the epididymis is suggested by several cases of infertility in mice that involve epididymal dysfunction. Despite normal testicular maturation, these animals ejaculate spermatozoa, which are either coiled or bent, which prevents their transit through the oviduct. Genetic mutations causing such morphological defects therefore identify attractive targets for developing contraceptive drugs that act on epididymal spermatozoa – could gelsolin be one of them?

Ectoplasmic specializations are also attractive targets for disruption of spermatogenesis, as like the epididymis, they can be targeted without perturbing the endocrine system. Knockout mice for proteins present in these complexes,

such as nectin 2, have been shown to be infertile due to a malformation of the ectoplasmic specialization, making this a promising strategy for contraception. Because ectoplasmic specializations must break down during spermatogenesis to allow germ cells to move through the seminiferous epithelium, many actin binding proteins, like gelsolin, are present in these structures, as the actin filaments must rapidly depolymerize prior to germ cell release, and reform once the germ cells have moved to the next layer of the seminiferous epithelium. Again, these proteins may become candidate targets for drugs that prevent spermatogenesis from taking place.

Materials and Methods

1. Molecular biology

All DNA and RNA manipulations, unless otherwise specified, were carried out according to Sambrook (Sambrook 1989)

1.1 RNA Preparation for RT-PCR and Northern Blot Analysis

Total RNA was prepared from wildtype testes of mice between ages 8-30 days and from different adult tissues by using TRIzol reagent (GIBCO/BRL) according to the manufacturer's instructions.

For northern blotting, RNA samples (10µg) were denatured at 65 °C in RNA sample buffer (25% formamide, 3%formaldehyde, 5µg/ml ethidium bromide, 20mM MOPS/NaOH, 5mM Na-acetate, 1mM EDTA) and after denaturation, 1/10 volume of loading buffer (50% saccharose, bromophenolblue) was added. RNA samples were separated overnight by gel electrophoresis (1.2% gel, 30V) and transferred overnight by capillary blotting to Hybond-N (Amersham Pharmacia) membranes. Probes were generated from cDNA fragments labelled by random priming with ^{32}Pα-dGTP (Feinberg and Vogelstein 1983) After capillary transfer, the membrane was baked at 80°C for 1 hour, wet in 2xSSC, and pre-incubated in 10ml hybridization buffer (1% BSA, 1mM EDTA, 0,5 M Na-phosphate buffer pH 7.2, 7% SDS) at 62°C for 30 minutes. The labelled probes were added to 10ml fresh hybridization buffer and allowed to hybridize to the membrane overnight at 62°C. The next day the membranes were washed in wash buffer (1mM EDTA, 40mM Na-phoshate buffer pH 7.2, 1% SDS) at 62°C and exposed to autoradiographic film over night at -80°C.

For RT-PCR, total tissue RNA was treated with DnaseI (Promega) and reversed transcribed, using random hexamers, with SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. 1 µl cDNA obtained from this first strand synthesis, was subsequently

used for 20μl PCR reactions. Negative controls were performed using 1 μl DNAse I treated RNA, that had not been subjected to first strand synthesis. PCR was performed using *Pfu* polymerase (Stratagene) using the following primers:

Profilin 1:

Oligo 362: 5'-AAC GCC TAC ATC GAC AGC CTT-3'
Oligo 363: 5'-GAC TAG CGT CTT GGC AGT CAT-3'

Profilin 3:

Oligo 365: 5'-ATC AGT GCA GTG CTG CGG GAT-3'
Oligo 363: 5'-CCT TGG TGC GCG CAT CCA GTA -3'

PCR program

Step 1) 98°C   2:00
Step 2) 96°C   0:30
Step 3) 55°C   0:40
Step 4) 72°C   0:30
Step 5) back to step 2) 35 x
Step 6) 72°C   5:00

1.2 Cloning Strategy used for Targeting of Profilin 3 Locus in Mice

The pFLRT-AscI plasmid was used as a base for generating the profilin 3 targeting construct (plasmid 271, Witke archive).

Step 1

Subcloning of the fragment to insert between the loxP sites was performed by PCR amplification from a phage containing a genomic fragment on which included the profilin 3 gene. The phage was isolated from a mouse genomic

phage library by screening the library with profilin 3 cDNA (Sambrook 1989). The following PCR program and primers were used:

<div align="center">BamHI</div>

Oligo 382: 5'- GCG**GGATCC**TGTGTCTGAAGACAGCTCCAGTG-3'

<div align="center">BamHI</div>

Oligo 383:5'-GCG**GGATCC**GTAGAGTTTGAGAATTGGCCACC -3'

<u>PCR Program</u>
Step 1) 98°C   2:00
Step 2) 96°C   0:30
Step 3) 60°C   1:00
Step 4) 72°C   0:30
Step 5) back to step 2) 30 x
Step 6) 72°C   5:00

= Frt site   = LoxP site   =Neo
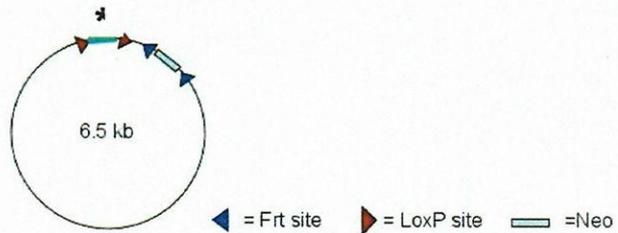
Expected fragment length: 1.5 kb

Step 2

Subcloning of the 5' arm was performed by PCR amplification of the genomic fragment from ES cell C57 mouse genomic DNA, The arm was amplified using using Pfu (Stratagene) (for targeting construct 1) and Expand Fidelity PCR™ (Roche) (targeting construct 2). In both cases, the reaction was carried out in 50µl total volume and the PCR fragment subcloned directly into the pCRII-TOPO-TA vector (Invitrogen) The following oligos and PCR programs were used for targeting construct 1 and 2:

Targeting Construct 1

<div align="center">NotI</div>

Oligo 380: 5': - AATA**GCGGCCGC**GGATCCAGAAGCATATTGCTTCATAAC

<div align="center">SalI</div>

Oligo 381: 5'- GACT**GTCGAC**CCAGAGGGCATCAGATCTC-3'

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PCR Program
Step 1) 98°C   2:00
Step 2) 96°C   0:30
Step 3) 60°C   1:00
Step 4) 72°C   0:30
Step 5) back to step 2) 30x
Step 6) 72°C   5:00

Expected fragment length: 1.7 kb

Targeting Construct 2

                 NotI

Oligo 375:   5' – TAT**GCGGCCGC**AGACTCGACCGATCA-3'

                 SalI

Oligo 451: 5'- CGC**GTCGAC**ATGCGCCACCAC-3'

PCR Program
Step 1) 94°C   2:00
Step 2) 94°C   0:15
Step 3) 63°C   0:30
Step 4) 68°C   4:00
Step 5) back to step 2) 9x
Step 6) 94°C   0:15
Step 7) 63°C   0:30
Step 8) 68°C   4:00 +20secs/cycle
Step 9) back to step 6) 23 x
Step10) 72°C   5:00
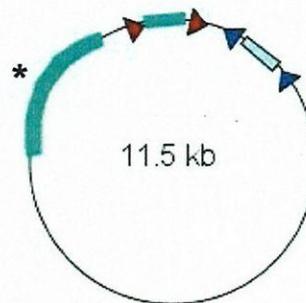
Expected fragment length: 5 kb

11.5 kb

Step 3

Subcloning of the 3' arm was also performed by PCR amplification on phage isolated from a mouse genomic phage library by probing with profilin 3 cDNAThe PCR fragment was cloned directly into the pCRII-TOPO-TA vector (Invitrogen)

The following oligos and PCR programs were used TA vector. The following PCR program and primers were used:

BstBI

Oligo 384: 5': -CGA**TTCGAA**CTCAGGATTCAGCAATCTTTCTCA-3'

AscI

Oligo 385: 5'-GC**GGGCGCGCCC**TGGAATTCACTTTGTAGACCCTG-3'

PCR Program
Step 1) 98°C   2:00
Step 2) 96°C   0:30
Step 3) 60°C   1:00
Step 4) 72°C   0:30
Step 5) back to step 2) 30x
Step 6) 72°C   5:00
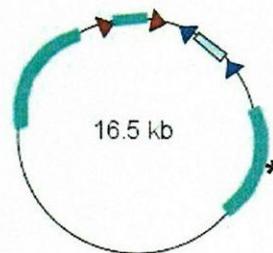
Expected fragment length: 5 kb

1.3 Genomic DNA Isolation from Mouse-Tail Biopsy and PCR Analysis of Mutant Mice.

Genomic DNA was prepared from a 5mm tail biopsy, taken from 3-weeks old pups, after over night digestion in 500µl of DNA lysis buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 5mM EDTA, 1% SDS) with 100µg/ml of proteinase K at 55°C. The following day, the digested tails were vortexed for 20 secs and centrifuged at 13,000 r.p.m. for 10 minutes. The supernatant was transferred to a fresh tube, and genomic DNA was extracted by mixing with an equal volume of isopropanol and allowing it to precipitate. The DNA was then washed in 70% ethanol, dehydrated in 100% Ethanol, and resuspended in 200µl Tris-EDTA (TE). 1 µl of resuspended DNA was used per PCR reaction, or 20µl per digestion for southern blotting.

2. Cell Biology

2.1 ES Cell Cultures

IB10 embryonic stem cells derived from 129Sv blastocyst were grown on embryonic fibroblast feeder layer (EF-feeders) plated on 0.2% gelatin coated dishes in D-MEM (with 4.5 g/l pyruvate) containing 15% fetal bovine serum, 2mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM ß-mercaptoethanol (Sigma), 100 u/ml penicillin/streptomycin mixture, 1000U/ml leukemia inhibitory factor (LIF) at 37°C and 5% CO_2.

2.2 ES Cell Transfection

Before transfection, cells were washed twice in PBS buffer, harvested by trypsinization and after washing in electroporation buffer (10mM HEPES buffer in DMEM without FCS) were resuspended at 1x 10^7 cells/ml in a final volume 0.7ml. Cell suspension was electroporated using BioRad Gene Pµlser set at 250 V/cm and 500µF for one pulse at RT. 30µg of linearized plasmid DNA was used for each transfection. After pulsing, cell suspension was diluted in 10ml of complete medium and plated on 10 cm dishes. Neomycin selection started 1 day after transfection with 0.25mg/ml G418.

2.3 ES Cell Selection

After having reached a visible size (approx.7 days) single ES cells clones were picked and expanded in 96-well plates grown on EF-layer in selection medium. Once reached a confluence of 70-80%, the clones were washed twice with 100µl PBS and trypsinized for 5 mins with 50 ul trypsin at 37°C. 100µl ES cell culture medium was added to the trypsinized clones, which were resuspended by

pipetting vigorously, and split into 3 96-well plates. 2 of the 96-well plates were previously plated with an EF-layer, and were used to freeze down and store the clones 2 and 3 days after splitting. The third plate was further expanded on 0.2% gelatin coated dishes for genomic DNA. Screening of the ES cells clones for homology recombination event was performed by Southern blot.

2.4 Genomic DNA Isolation from ES Cells and Southern Blot Analysis

To prepare genomic DNA from ES cells, cells were grown to confluency without feeder layer in 96- well dishes , washed with 100µl PBS, and lysed with 50µl lysis buffer (10mM Tris-HCl, pH 7,5, 10mM EDTA, 0.5% Sarcosyl, 10mM NaCl) with 100µg/ml of proteinase K overnight at 56°C in a humidified chamber. The next day the box was cooled for one hour and genomic DNA was precipitated by adding 100µl ethanol into each plate, and allowed to stand for 1-2 hours. Once the filamentous DNA could be observed, the plate was inverted gently to remove the ethanol, and the wells washed with 70% ETOH, air dried for 15 mins and resuspended in 100µl of 1xTE buffer.

For Southern blot assay, the genomic DNA was digested overnight directly in the wells, in a final volume of 50 ul with the respective restriction enzyme. The DNA fragments were then separated on a 0,7% agarose gel, and transferred overnight by capillary flow onto a surface of a charged nylon membrane (Gene Screen Plus NEF 976). The following day, the membrane was baked at 80°C for 1 hour and pre-incubated in hybridization buffer (1% BSA, 1mM EDTA, 0,5 M Na-phosphate buffer pH 7.2, 7% SDS) at 65°C for 30 minutes. DNA probes labelled by random priming with ^{32}Pα-dGTP (Feinberg and Vogelstein 1983) were added to the fresh hybridization buffer and allowed to hybridize to the membrane overnight at 65°C. The next day the membranes were washed in wash buffer (1mM EDTA, 40mM Na - phoshate buffer pH 7.2, 1% SDS) at 65°C and exposed to autoradiographic film over night at -80°C.

3. Biochemistry

3.1 Purification of GST-tagged Profilin 3 Expressed in *E.coli*

1L of 37°C LB-medium with 50 μg/ml ampicillin was inoculated 1:10-1:20 with fresh overnight cultures of bacteria harbouring the profilin 3 cDNA in a pGEX-vector (Clontech Inc, USA). When the cells had grown to an OD_{600} of 0.8, protein-expression was induced by adding 0.5mM IPTG and growing the bacteria further at 37°C for 4-6 hrs. The bacteria were harvested by centrifugation at 4000 rpm in a JLA 10.500-rotor (Beckman) for 10 min at 4°C, after which the pellet was resuspended in lysis buffer (PBS, 0.1 mM EDTA, 0.1 % Triton X-100 + EDTA-free protease inhibitors (Roche)) and subjected to 2 rounds of sonication (2x30 s, MS73-probe in a Bandelin Sonopuls GM 200 sonicator) and homogenization in a Dounce-homogenizer (Kontes). After clearing the lysate by a 30 min spin at 4°C and 40.000 rpm in a TLA 45-rotor (Beckman), the supernatant was passed over an appropriate amount of glutathione-4B-sepharose beads (Amersham Pharmacia) which had been washed and equilibrated in lysis-buffer. After extensive washing with cold lysis-buffer and briefly with PBS, proteins were eluted with 20 mM glutathione in PBS. After determining the protein concentration using the Bradford assay, aliquots were snap-frozen in liquid nitrogen and stored at -80°C.

3.2 Preparation of Protein Lysates from Mouse Tissues and Germ cells for Western Blot Analysis

Dissected mouse organs or germ cells were placed in lysis buffer (20mM Tris-HCl, pH8.0, 100mMNaCl, 5mM EGTA, 2mM EDTA, 0.5% Triton-X), and dounced

until the tissue was completely dissociated. After centrifugation of the lysate for 45 min at 65.000 x g at 4 °C, the supernatant was transferred to fresh tubes and protein concentration determined using the Bradford assay. Proteins were then denatured in SDS sample buffer.

3.3 Western Blot Analysis

For SDS-PAGE, proteins were diluted in 1x SDS sample buffer, denatured by heating 10 min at 95°C and subjected to discontinuous gel electrophoresis. Proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) using a semi-dry apparatus by applying 20V for 1h. Gel and membrane were equilibrated in transfer buffer (25mM Tris, 190mM Glycine, Methanol pH8.3) before transfer. After the transfer membrane was incubated in blocking solution (5% non-fat powder milk dissolved in NCP buffer (0.15M NaCl, 20 mM Tris, 0.05% Tween-20) at 4°C overnight. Primary and secondary antibodies were diluted in blocking solution to the appropriate concentrations. The enhanced chemiluminescence system (Amersham) was used followed by the exposure to x-ray films (X-Omat AR, Eastman Kodak Co.) Between the antibodies incubations, the membranes were washed 5 times in NCP buffer. For detection horseradish peroxidase (HRP) conjugated secondary goat anti-rabbit, goat anti-mouse antibodies (Pierce).

Detailed information regarding the antibodies used in the assays:

Antibody	Dilution western	Dilution immunofluorescence	Secondary used
anti-profilin1 (1T)	1:1000	1:100	Rabbit
anti-profilin 2A (3003)	1:1000	1:100	Rabbit
anti-profilin 3 (BEH3,BEG6)	1:1000	1:100	Rabbit
anti n- cofilin (KG60)	1:500	-	Rabbit
anti-gelsolin (3825)	1:5000	-	Rabbit
anti-actin(224-236-1) (hybridoma supernatant)	1:5	-	Mouse

3.4 Preparation of Poly-L-Proline Beads

Poly-L-proline beads were made by dissolving 1 g poly-L-proline, MW 40.000 (Sigma) in 50 ml 1 x coupling buffer (0.1 M NaCO3, 0.5 M NaCl, pH 8.5) for several days at 4°C and deblocking 15 g of dry CNBr-activated sepharose 4B (Amersham Pharmacia) with 1 mM HCl according to supplier's instructions. After deblocking, the beads were washed briefly with cold water and coupling-buffer. The beads were added immediately to the dissolved poly-L-proline and incubated overnight and over at 4°C. The next day, the beads were washed three times with 200 ml couplingbuffer and blocked with 0.1 M Tris pH 8.0/0.5 M NaCl for 2 hrs at RT. Then the resin was washed 4 times alternatingly with 0.1 M sodium acetate pH 4.0/0.5 M NaCl and 0.1 M Tris pH 8.0/0.5 M NaCl. Afterwards, the beads were washed and stored in 20 mM Tris pH 8.0, 5 mM EDTA/0.1 M NaCl/0.1 % NaN3 at 4°C.

3.5 Generation of Polyclonal Antibodies against Recombinant Profilin 3

0.3-0.5 mg antigen was diluted in 0.5 ml of normal saline, mixed with 0.5 ml of Titermax adjuvant (Sigma), chilled on ice, sonicated 3 times for 10 s with the MS72 probe in a Bandelin Sonopuls GM200-sonicator and injected into rabbits subcutaneously. The rabbits were boosted after 3 weeks with another injection of antigen, bleeds were checked by western-blotting of tissue-lysates and recombinant antigens. Boosts were repeated every 3-4 weeks until a satisfactory immune reaction was seen.

3.6 *In situ* Hybridisation

Full-length cDNA fragments for profilins and cofilin were cloned into pBluescript SK-. *In vitro* transcription was performed according to Jostarndt et al. (Jostarndt et al. 1994) , using digoxigenin labeled nucleotides (Boehringer Mannheim), from the T3 or from the T7 promoter, for transcription of sense and antisense-probes, respectively. The probes were used for *in situ* hybridization on paraffin sections of adult testis. Hybridisation solution contained the digoxygenin-11-UTP-labeled riboprobe (1ng/ml) in 40% formamide, 5x SSC, 1x Denardt's solution, 100 ug/ml each of the denatured salmon testis DNA and tRNA. Hybridisation was performed overnight at 65°C. Slides with sections were floated off in 5x SSC, followed by a 40 min wash in 0.5x SSC, 20% formamide at 60°C and a 15 min wash in NTE (0.5M NaCl, 10 mM Tris/HCl, pH 7.0, 5 mM EDTA). The sections were treated with 20µg/ml RNase A in NTE for 30 min at 37°C and washed in NTE for 15 min, in 0.5x SSC, 20% formamide for 20 min at 60°C, and then in 2x SSC for 25 min. Antibody incubation and detection were performed according to the manufacturer's description (Boehringer Mannheim) and signal was developed using BCIP/NBT (Vector Labs).

3.7 Depletion of Germ-Cells in Wild Type Testis

To obtain adult germ-cell depleted testis, mice were treated with a single i.p. injection of busulfan (Sigma: 40mg/Kg) performed at 5-6 weeks of age to destroy endogenous spermatogenesis. Animals were then sacrificed 4-8 weeks after busulfan treatment.

3.8 Purification and Fractionation of Whole Germ Cell Populations from Adult Testis

To obtain highly purified germ cell fractions, testes from 30-day-old mice were removed, decapsulated and digested in D-MEM (Gibco, Invitrogen, Italy) + 1mM pyruvate, 0.45 U/ml collagenase (Serva, Italy) and 0.08 mg/ml DNase (Gibco) whilst vigorously shaking the tube for 5-8 minutes. Once the tubules had dispersed, they were allowed to settle for 2 minutes, and the medium changed. A second digestion was performed by adding fresh enzymes to the seminiferous tubules for 45 min under shaking at 32°C. At the end of digestion, the suspension was pipetted up and down with a 5ml Pasteur pipette for several minutes to release the germ cells from the tubules. The suspension was allowed to settle for 5 mins, and the supernatant removed to a fresh 15ml falcon tube. The cells were washed twice by spinning at 1000 rpm for 5 minutes, and resuspended in fresh D-MEM. Germ cell fractions were isolated by Staput method of sedimentation velocity at unit gravity.(Boitani et al. 1980). Purity of cell fractions was estimated by light microscopy. Pachytene spermatocytes and round spermatids fractions were > 85 % average purity.

3.9 Germ Cell Immunostaining

Purified germ cells were attached to microscope slides by cytocentrifugation using the cytospin apparatus (Cytopsin[3], (Shandon)). The cells were fixed in 4% paraformaldehyde (PFA)/PBS for 10 min and rinsed once in PBS. The cells were then blocked in 1M Glycine for 10 min at RT, and in PBS+1%BSA,5% serum,0.1%Triton X-100 for 45 min at RT in a humidity chamber. Primary antibodies were diluted in PBS + 5% BSA+0,1% Triton X-100 for 1hr at RT. The slides were then washed with PBS+1%BSA, (3 x 1ml rapidly with a pipette, 2x 5 mins, 1x 10 mins) and fluorescence-conjugated secondary antibodies were allowed to bind for 30 min at room temperature diluted in PBS + 1% BSA+0,1% Triton X-100. Nuclei were stained by including a 1:30.000 dilution of Hoechst 33342 (10 mg/ml, Molecular Probes) in PBS in one of the final washes. After briefly dipping in water, the slides were mounted with glass coverslips using gelvatol.

3.10 Spermatozoa staining

Spermatozoa were resuspended in PBS, spun down 2 mins at $600xg$, and resuspended to a final concentration of 2000 sperm/µl. 30,000 spermatozoa/spot were air-dried on SuperFrost® Plus glass coated slides (Menzel-Glaser) and stored at –20 until required. Prior to staining, slides were warmed to room temp, and sperm were fixed for 10 mins in acetone at -20°C.

After drying and washing once in PBS, unspecific binding was inhibited by blocking in PBS 1%BSA for 30 min. Primary antibodies were diluted in blocking-buffer and incubated on the cells for 45 min, After washing in PBS 1% BSA (3x1ml rapidly with a pipette, 2x 5 mins, 1x 10 mins) fluorescence-conjugated secondary antibodies were allowed to bind for 30 min at room temperature diluted in blocking-buffer. Nuclei were stained by including a dilution of Hoechst

33342 (10 mg/ml, (Molecular Probes)) 1:30.000 in PBS in one of the final washes. Acrosomes were stained by incorporating a 1:500 dilution in PBS of FITC-PNA (Sigma-Aldrich, 1mg/ml). After briefly dipping in water, the slides were mounted with glass coverslips using gelvatol.

3.11 Induction of the Acrosome Reaction

Mature sperm from the cauda epididymis and vas deferens were incubated in M2 medium (119 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.0 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.6 mM glucose, 0.5 mM sodium pyruvate, and 4 mg/ml bovine serum albumin) (23) at 37 °C in a humidified incubator with 5% CO_2, 95% air to allow capacitation. After 1.5 hours, calcium ionophore A23187 (Sigma) was added at a final concentration of 10 µM to induce the acrosome reaction. After a 15-min incubation, sperm were spotted onto microscope glass slides, air dried, and examined using immunofluorescence techniques.

4. Analysis of Knockout Mice

Mice with the following genotypes were used in this work:
- C57 wild type (Charles River)
- Prm-cre [+/-] C57 background (O'Gorman et al. 1997)
- Cof [floxed/-] C57/129Sv mixed background (C. Gurniak)
- Profilin 2 -/- , C57 background (P. Pilo Boyl, A. DiNardo)
- Gelsolin -/- ,C57 background (W. Witke)

4.1 Genotyping of Mice by PCR

Profilin 2-PCR

Oligo229: 5'-GTC TTG GTC TTT ATG GGA AAA G-3'

Oligo 268:5'-GGA GTA CAC AAG GAA AC-3'

PCR Program
Step 1) 98°C   2:00
Step 2) 96°C   0:30
Step 3) 60°C   1:00
Step 4) 72°C   0:30
Step 5) back to step 2) 34 x
Step 6) 72°C   5:00

Expected PCR products: 250 for the wildtype band, 450 for the mutant band

Prm1 CRE-recombinase transgene

Oligo 357: 5'-ATG GTA CAG GTC CTC ACT–3'

Oligo 358: 5'-TTT TGC TGC GGC AGC ATC–3'

PCR Program
Step 1) 98°C   2:00
Step 2) 96°C   0:30
Step 3) 60°C   1:00
Step 4) 72°C   0:30
Step 5) back to step 2) 30x
Step 6) 72°C   5:00

Expected PCR product: 360bp CRE-recombinase band, 235bp wild type band

Cofilin PCR

Oligo 347: 5'-CGC TGG ACC AGA GCA CGC GGC ATC-3'

Oligo 348: 5'- CTG GAA GGG TTG TTA CAA CCC TGG-3'

Oligo 349 : 5'-CGC TGG ACC AGA GCA CGC GGC ATC-3'

PCR Program

Step 1) 94°C   2:00
Step 2) 94°C   0:30
Step 3) 58°C   0:30
Step 4) 68°C   0:40
Step 5) back to step 2) 42 x
Step 6) 68°C   5:00

Expected PCR product: 380bp wildtype band, 170bp deleted band, 420 floxed band

4.2 Tunel Assay

Apoptotic cells were detected using the DeadEnd™ Fluorometric TUNEL System kit (Promega) according to the manufacturer's instruction. Testes were fixed in 4% parafolmaldehyde and embedded in paraffin wax. 6µm sections were deparaffinized, rehydrated, and permeabilized with 20 µg/ml of proteinase K. The tissue sections were then incubated with TdT enzyme at 37C for 60 min and the nuclei counterstained with Hoechst. The number of TUNEL-positive cells and tubules were counted from at least 4 sections for each mouse, and the number of apoptotic cells expressed in terms of cells per tubules. A total of 8 mutant and 8 wildtype mice were used.

4.3 Epididymal Sperm Counts

Whole epididymes from mutant and wild type littermates were homogenized in 0.15M NaCl2, 0.05% Triton X-100, and sonicated for 1 minute to remove sonication-sensitive cells. The spermatozoa heads were counted using a hemotcytometer, and an average from both epididymes was calculated.

4.4 Fertility Assays

Fertility assays on mutant and wild type control mice were performed using age-matched mice, and when possible, littermates. Wild-type/control and mutant male mice were mated with 6-8 week old C57 females. Once plugged, the females were moved, and the litter sizes scored upon delivery. For each genotype, at least 4 males were mated, and 16 litters were counted.

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