Essential role of citron kinase in cytokinesis of spermatogenic precursors

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Summary

During spermatogenesis, the first morphological indication of spermatogonia differentiation is incomplete cytokinesis, followed by the assembly of stable intercellular cytoplasmic communications. This distinctive feature of differentiating male germ cells has been highly conserved during evolution, suggesting that regulation of the cytokinesis endgame is a crucial aspect of spermatogenesis. However, the molecular mechanisms underlying testis-specific regulation of cytokinesis are still largely unknown. Citron kinase is a myotonin-related protein acting downstream of the GTPase Rho in cytokinesis control. We previously reported that Citron kinase knockout mice are affected by a complex neurological syndrome caused by cytokinesis block and apoptosis of specific neuronal precursors. In this report we show that, in addition, these mice display a dramatic testicular impairment, with embryonic and postnatal loss of undifferentiated germ cells and complete absence of mature spermatocytes. By contrast, the ovaries of mutant females appear essentially normal. Developmental analysis revealed that the cellular depletion observed in mutant testes is caused by increased apoptosis of undifferentiated and differentiating precursors. The same cells display a severe cytokinesis defect, resulting in the production of multinucleated cells and apoptosis. Our data indicate that Citron kinase is specifically required for cytokinesis of the male germ line.

Key words: Spermatogenesis, Cytokinesis, Rho GTPases, Cytoskeleton, Apoptosis

Introduction

The division cycle of most eukaryotic cells is concluded by cytokinesis, during which the daughter nuclei acquire separate plasma membrane and cytoplasm. In metazoans, cytokinesis is driven by a cortical acto-myosin ring, which contracts, furrowing the overlying plasma membrane (Glotzer, 2001). When the cleavage furrow reaches the remnants of the mitotic spindle, an electron-dense structure is formed, known as the midbody, followed by abscission of the two daughter cells (Glotzer, 2001). A remarkable variation of this standard sequence occurs in the male germline of many organisms, including insects and mammals. Indeed, the committed spermatogenic precursors of these species do not complete cytokinesis and remain connected by intercellular communications, named ring canals in insects (Mahowald, 1971) and intercellular cytoplasmic bridges (ICBs) in mammals (de Rooij, 1998; de Rooij and Grootegoed, 1998). Mammalian spermatogenic stem cells originate from the primordial germ cells (PGCs), which are derived during early embryogenesis from a small population of epiblast cells (Ginsburg et al., 1990). After migrating to the genital ridges, PGCs become enclosed by Sertoli cells, forming the seminiferous cords and change their morphology, giving rise to the gonocytes (Huckins and Clermont, 1968). Gonocytes proliferate for a few days and then arrest in the G0/G1 phase of the cell cycle (Huckins and Clermont, 1968; Vergouwen et al., 1991). In mice and rats, they resume proliferation a few days after birth to become A_{single} (A_s) spermatogonia, marking the beginning of spermatogenesis (de Rooij and Grootegoed, 1998; Vergouwen et al., 1991). Upon division, A_s spermatogonia can undergo either complete or incomplete cytokinesis generating, respectively, two A_s spermatogonia or two morphologically similar cells connected by an ICB, which are named A-paired (A_{pr}) spermatogonia (de Rooij, 1998). Although probably A_{pr} spermatogonia maintain stem cell properties, their generation is the first sign of commitment to terminal differentiation. This type of incomplete cytokinesis occurs in all the subsequent mitotic and meiotic divisions, leading to long chains of haploid spermatids (de Rooij and Grootegoed, 1998). The above observations strongly suggest that tissue-specific regulation of cytokinesis is a critical aspect of the spermatogenic differentiation program. However, the molecular mechanisms underlying this specificity are still largely obscure.

In the past few years, remarkable progress has been made in understanding how cytokinesis is controlled in animal cells (Glotzer, 2001; Hales et al., 1999). In particular, small GTPases of the Rho family have been recognized as playing a crucial role in this process (Glotzer, 2001; Hales et al., 1999). Rho GTPases are molecular switches that control cytoskeletal dynamics, cycling between an active GTP-bound, and an inactive, GDP-bound state (Hall, 1998; Van Aelst and Souza-

Schorey, 1997). This conformational transition is tightly regulated by guanine-nucleotide exchange factors (GEFs), which stimulate the binding to GTP, and GTPase-activating proteins (GAPs), which increase their basal GTPase activity (Van Aelst and Souza-Schorey, 1997). Signal transduction by Rho GTPases is mediated by a complex network of effector molecules, which specifically bind to the active conformation and change their biological properties upon binding (Bishop and Hall, 2000). Cytokinesis is blocked in different model organisms by inhibition of Rho signaling (Drechsel et al., 1997; Kishi et al., 1993; Nakano et al., 1997). Moreover, Rho regulators and effectors specifically involved in cytokinesis have been recently isolated (Hirose et al., 2001; Jantsch-Plunger et al., 2000; Kosako et al., 1999; Lehner, 1992; Madaule et al., 1998; Tatsumoto et al., 1999). Citron kinase (CIT-K) is a myotonin-related protein highly enriched in the cleavage furrow and in the midbody of dividing cells (Madaule et al., 1998). The first in vitro studies on this molecule suggested that it could be one of the principal ubiquitous downstream effectors of Rho during cytokinesis (Madaule et al., 1998). However, the subsequent production of knockout ($^{-/-}$) mice showed that CIT-K is functionally required for cytokinesis only by specialized cell populations, like proliferating neuronal precursors (Di Cunto et al., 2000).

Here we report that CIT-K is required for cytokinesis by mouse spermatogenic precursors, which become multinucleated and display increased apoptosis in CIT-K$^{-/-}$ animals. As a consequence, the seminiferous tubules of these mice are characterized by progressive loss of undifferentiated germ cells and complete absence of spermatocyte differentiation. Despite this severe testicular phenotype, the ovogenesis of CIT-K mutant females is apparently preserved. Our results strongly suggest that CIT-K is a key regulator of the cytokinesis endgame in developing and differentiating testicular germ cells.

Materials and Methods

Western blotting

Western blotting was performed essentially as described previously (Di Cunto et al., 1998). Briefly, tissues were homogenized in 2×SDS sample buffer (65 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol) and incubated for 4 minutes at 95°C. 30 μg of total cell extracts were separated by 6% SDS-PAGE and transferred onto PVDF membranes (Millipore). The anti-Citron-K polyclonal antiserum was used at 1:2000 dilution. Equal loading of the samples was verified by probing the same lysates with anti-GAPDH monoclonal antibodies (Sigma).

Histology

The genotype of mice was determined by PCR as previously described (Di Cunto et al., 2000). The sex of 12.5-day-old (E12.5) embryos was determined by PCR with SRY-specific oligonucleotides (Kunieda et al., 1992). For light microscopy, embryos were fixed by immersion in fixative solution (4% paraformaldehyde in PBS), whereas postnatal mice were deeply anaesthetized and transcardially perfused with fixative solution. Testes and ovaries were removed, postfixed for at least 12 hours, embedded in paraffin, and sectioned at 5 μm. Sections were stained with hematoxylin-eosin. For electron microscopy, testes were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH. 7.4, postfixed in osmium tetroxide, dehydrated in ethanol and embedded in epoxy resin. Semi-thin sections were stained with toluidine blue. Ultra-thin sections were viewed using an Hitachi 7000 transmission electron microscope.

Alkaline phosphatase staining

Detection of endogenous alkaline phosphatase (AP) activity was carried out as described previously (Zackson and Steinberg, 1988), with minor modifications. Briefly, embryos were fixed by immersion in fixative solution, embedded in paraffin and sectioned at 5 μm. After rehydration sections were permeabilized for 10 minutes with 0.1% Triton in TBS and incubated for 40 minutes in AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris pH 9.5) at room temperature. Two chromogenic substrate systems were employed to visualize the activity: 165 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and 330 μg/ml nitro blue tetrazolium (NBT, Sigma) were added to AP buffer. The reaction was performed in the dark at room temperature and stopped in PBS.

Immunohistochemistry

The TUNEL assay was performed as previously described (Migheli et al., 1999). For BrdU immunostaining, mice were labeled with a single injection of BrdU (Sigma) at 100 mg/kg and sacrificed after 1 hour. Tissues were fixed in Carnoy, embedded in paraffin and sectioned at 5 μm. After rehydration, sections were treated for 20 minutes with 1N HCl. Anti BrdU antibodies (Chemicon) were used at a 1:100 dilution. For detection of CIT-K, sections were treated for 30 minutes with trypsin 0.1% in PBS, rinsed in HCl 0.5N at 4°C and then incubated with affinity-purified rabbit polyclonal anti-Citron antibodies at 1:200. The other immunostainings were performed as described previously (Migheli et al., 1999) using the following antibodies: anti-activated Caspase-3 (1:500, New England Biolabs); anti-Cyclin D1 (1:1000, Santa Cruz). In every case, sections were counterstained with hematoxylin.

Quantitative analysis

All the quantitative analyses were performed on inbred 129Sv CIT-K$^{+/+}$ and $^{-/-}$ mice. Numbers represent the average of at least three independent animals per genotype. For the embryonic gonads, the total number of germ cells was determined by counting all the alkaline-phosphatase-positive cells on serial sections. For post-natal testes, the volume of gonads was estimated on serial sections using the Image Pro Plus program (Olympus). The average density of gonocytes and undifferentiated spermatogonia per surface area was determined on at least six sections and then referred to the estimated volume. The proliferative and apoptotic indices were determined at E14.5 and P4 as the ratio between the number of tubular immunohistochemistry-positive cells and the sum of gonocytes and spermatogonia, counted on at least six sections.

The percentage of polyploid germ cells was determined at P4 on semi-thin sections of seminiferous tubules; the total number of analyzed tubules was 274 for $^{+/+}$ and 296 for $^{-/-}$ testes.

For determination of the percentage of binucleated apoptotic cells, caspase-3-positive intratubular elements at E14.5 and P4 were scored at high power as early or late apoptotic cells on the basis of the absence/presence of nuclear condensation and/or fragmentation. Early apoptotic cells were then scored as mononucleate or binucleate.

In every case, the statistical significance of differences between CIT-K and control samples was assessed by the Student *t*-test.

Reverse transcription (RT)-PCR

Total RNA was isolated from 8- and 14-day-old (P8 and P14) testes of CIT-K$^{+/+}$ and $^{-/-}$ 129Sv inbred mice (three animals per age per genotype) using the Rneasy kit (Qiagen). Single-strand cDNAs were prepared by reverse transcription of 2 μg of testis total RNA with

MMLV reverse transcriptase (GIBCO/BRL). Each PCR amplification was performed in a 50 μl reaction volume using as a 5 μl template of the room temperature reaction. Amplification primers were synthesized as previously described (Tanaka et al., 2000). The linear amplification range was determined for each primer pair, and control for equal loading was performed using the 18S primers of the QuantumRNA kit (Ambion), according to the manufacturer's specifications.

Results

Expression of CIT-K during testicular development

It has previously been shown that CIT-K is very abundant in the adult mouse testis (Di Cunto et al., 1998), but its cellular localization and developmental expression pattern were not characterized. To clarify these points, CIT-K protein levels and localization were analyzed at different developmental stages using a specific polyclonal antibody (Di Cunto et al., 2000). Total cell extracts prepared from postnatal day (P) 4 and adult mouse testis displayed robust expression of CIT-K, comparable with the levels observed in the developing cerebellum (Fig. 1A). Interestingly, no expression was detectable in adult ovaries (Fig. 1A). Immunohistochemistry performed with the same antibody revealed that, during embryogenesis and in the first postnatal days, CIT-K immunoreactivity was concentrated in the gonocytes (Fig. 1B). In the adult tubules, CIT-K expression was localized to the germ cells of the basal compartment (Fig. 1C) and was particularly pronounced in differentiating type-A spermatogonia (Fig. 1C). By contrast, the expression of CIT-K in cells of the suprabasal layers was very low or undetectable (Fig. 1C).

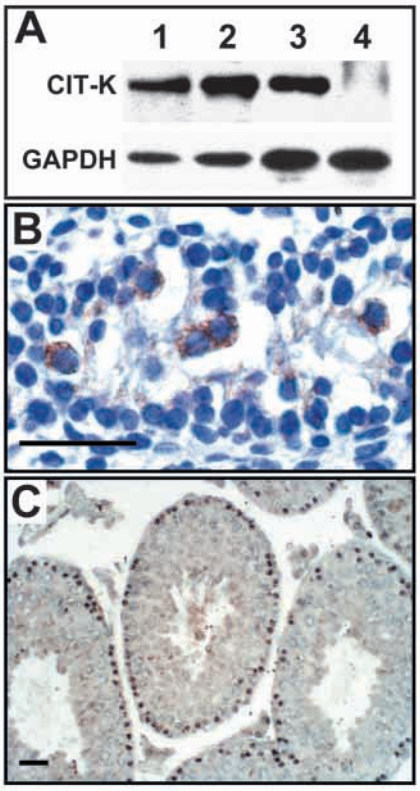

Fig. 1. Expression of CIT-K during testicular development. (A) Protein extracts from mouse wild-type tissues were probed by western blotting using anti-CIT-K (upper panel) or anti GAPDH (lower panel) antibodies. The samples were cerebellum at P4 (1), testis at P4 (2) and P40 (3), ovary at P4 (4). **(**B-C**)** Sections from mouse wild-type testes at P4 (B) and P40 (C) were analyzed by immunohistochemistry with affinity-purified anti-Citron antibodies. Bars, 50 μm.

Inactivation of CIT-K results in specific depletion of testicular germ cells

Mice heterozygous for a null mutation of the *CIT-K* gene display normal life span and fertility. By contrast, CIT-K$^{-/-}$ mice are characterized by reduced growth and die during the first three postnatal weeks due to lethal epilepsy (Di Cunto et al., 2000). At P14, if compared with $^{+/+}$ and $^{+/-}$ controls, the body weight of $^{-/-}$ mice is reduced by approximately 25% (Di Cunto et al., 2000). Autopsy performed at the same age revealed that most somatic organs, with the only exception being the brain, display a similar size reduction but maintain a normal histological structure (Di Cunto et al., 2000). However, the volume of CIT-K$^{-/-}$ testes was reduced by approximately 70% (Fig. 2A), thus suggesting a specific impairment. Using

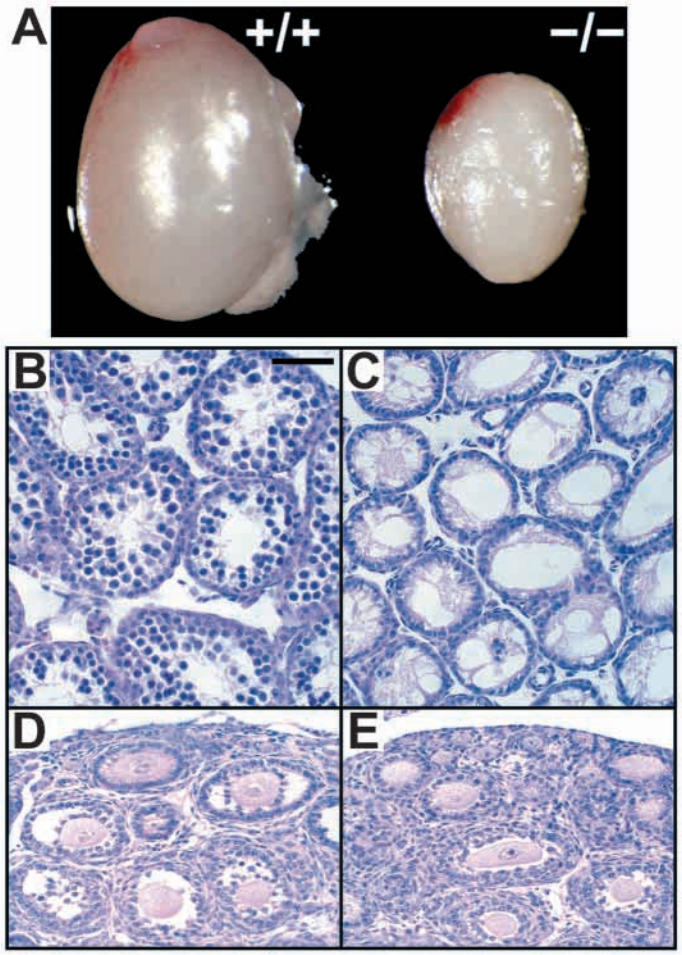

Fig. 2. Specific testicular impairment in CIT-K$^{-/-}$ mice. (A) Frontal view of P14 CIT-K$^{+/+}$ and $^{-/-}$ testes. (B-E) Histological sections of P14 testes (B,C) and ovaries (D,E) from P14 CIT-K$^{+/+}$ (B,D) and $^{-/-}$ (C,E) mice. Staining hematoxylin and eosin, bar, 50 μm.

histological examination in control animals, mature spermatocytes appeared to differentiate in the innermost layer of the seminiferous tubules (Fig. 2B). By contrast, testes of the knockout mice were severely depleted of proliferating and differentiating spermatogenic precursors (Fig. 2C). The testicular epithelium was dramatically simplified and appeared to consist mostly of a single row of Sertoli cells (Fig. 2C). A comparable number of Leydig cells was detected in the interstitial stroma of $^{-/-}$ and control animals. Other reproductive organs such the vas deferens and epididymus appeared to be normal in the knockouts (data not shown). Strikingly, histological analysis of P14 ovaries from knockout and control females did not show gross morphological differences among genotypes (Fig. 2D,E). In particular, several resting and growing follicles were detected throughout the ovarian cortex of $^{-/-}$ animals (Fig. 2D,E). Since no significant differences were observed between $^{+/+}$ and $^{+/-}$ controls, all the subsequent analysis were performed only on $^{+/+}$ and $^{-/-}$ animals.

Analysis of the spermatogenic block in CIT-K$^{-/-}$ testes

In order to elucidate whether postnatal testes of CIT-K knockout mice still contain spermatogenic cells and to establish at which stage of differentiation the observed depletion occurs, we carried out semi-quantitative RT-PCR of different spermatogenic markers (Tanaka et al., 2000). At P8, analysis of transcripts that appear in early spermatogenic cells before they reach the spermatocyte stage, such as the mouse Vasa homologue (Mvh) and A-Myb (Mettus et al., 1994; Tanaka et al., 2000), showed no differences among genotypes (Fig. 3A). By contrast, the mRNAs of Dmc1 and Mlh1, which are expressed in spermatocytes before they reach the pachytene stage (Baker et al., 1996; Habu et al., 1996), were dramatically reduced in the knockout samples (Fig. 3A). Strikingly, the expression of Calmegin, which normally starts in pachytene spermatocytes (Watanabe et al., 1994), was not detectable in the knockouts (Fig. 3A). At P14 the relative levels of spermatocyte marker expression was comparable to P8 samples (data not shown), and Mvh and A-Myb mRNAs were still detectable in the knockout testes (Fig. 3B). However, if compared with controls, their expression levels were clearly reduced in the $^{-/-}$ samples (Fig. 3B). Taken together, these observations indicate that the simplified testicular epithelium of CIT-K$^{-/-}$ mice still contains germinal cells, which are completely unable to differentiate as meiotic spermatocytes and undergo progressive depletion.

In order to address whether male germ cells of CIT-K knockout mice are already compromised during embryonic development, serial sections of $^{+/+}$ and $^{-/-}$ male embryos were analyzed at different developmental stages by histology and alkaline phosphatase histochemistry. As shown in Fig. 3C,D PGCs were easily detected in the genital ridges of both control and knockout embryos, and no ectopic alkaline-phosphatase-positive cells were observed, thus indicating that the specification and migration of spermatogenic precursors are not severely affected by the absence of CIT-K. However, their number was significantly reduced in the knockout mice as early as E12.5 (Fig. 3C,D). Accordingly, at E14.5 the number of gonocytes in the lumen of CIT-K$^{-/-}$ seminiferous cords was reduced to approximately 60% of the controls (Fig. 3G). After birth, cells displaying the morphological features of gonocytes, and type A spermatogonia were easily detectable on histological sections of knockout testes (data not shown), in good agreement with the RT-PCR analysis. To further confirm the presence of these cells, we performed on the same samples immunohistochemical detection of cyclinD1, which has been recently recognized as a specific marker (Beumer et al., 2000). As shown in Fig. 3F, cyclinD1-positive cells were detected in the tubular lumen and on the basal membrane of the knockout tubules, further indicating that both gonocytes and type A spermatogonia were present. However, if compared with the embryonic testes, the relative number of germinal cells was further decreased, being only 30% of the control at P4 (Fig. 3E,G). These data indicate that, besides the differentiating spermatogonia, even the PGCs and the gonocytes are significantly compromised by the absence of CIT-K.

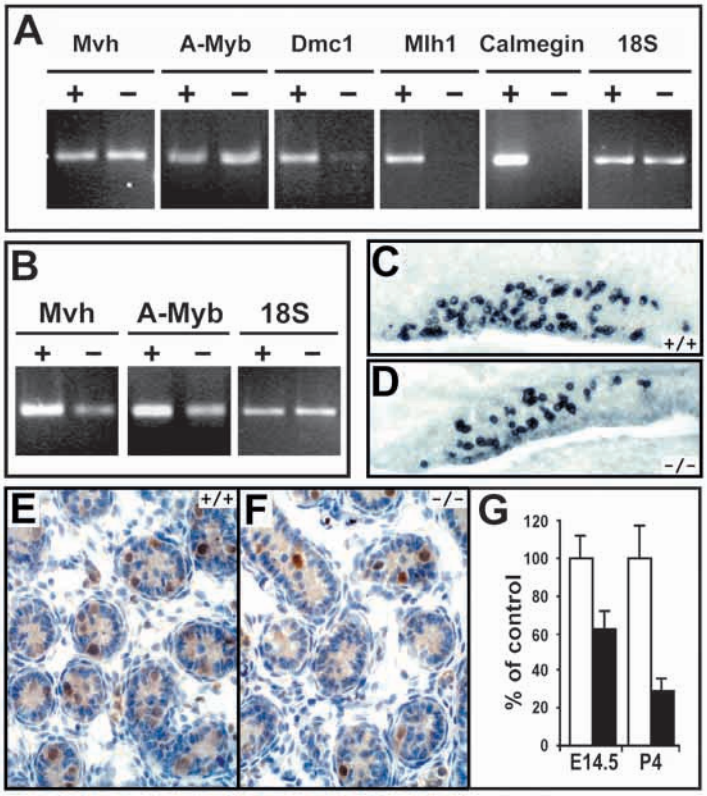

Fig. 3. Analysis of the spermatogenic block in CIT-K$^{-/-}$ mice. (A-B) Expression of the indicated spermatogenic markers was analyzed by RT-PCR under semi-quantitative conditions. The assay was performed on total RNA extracted from CIT-K knockout (–) and littermate control (+) mice at P8 (A) and P14 (B). (C,D) Primordial germ cells were identified by alkaline phosphatase staining of comparable longitudinal sections of E12.5 CIT-K$^{+/+}$ (C) and $^{-/-}$ (D) embryonic testes. (E,F) Gonocytes and undifferentiated spermatogonia were identified on sections of P4 CIT-K$^{+/+}$ (E) and $^{-/-}$ (F) testes by anti-cyclinD1 immunostaining. (G) The total number of gonocytes and undifferentiated spermatogonia was determined on hematoxylin- and eosin-stained histological sections of testes from CIT-K$^{+/+}$ (open bars) and $^{-/-}$ (closed bars) mice at the indicated times. Values are expressed as a percentage of the control. Error bars are standard deviation, $P<0.01$.

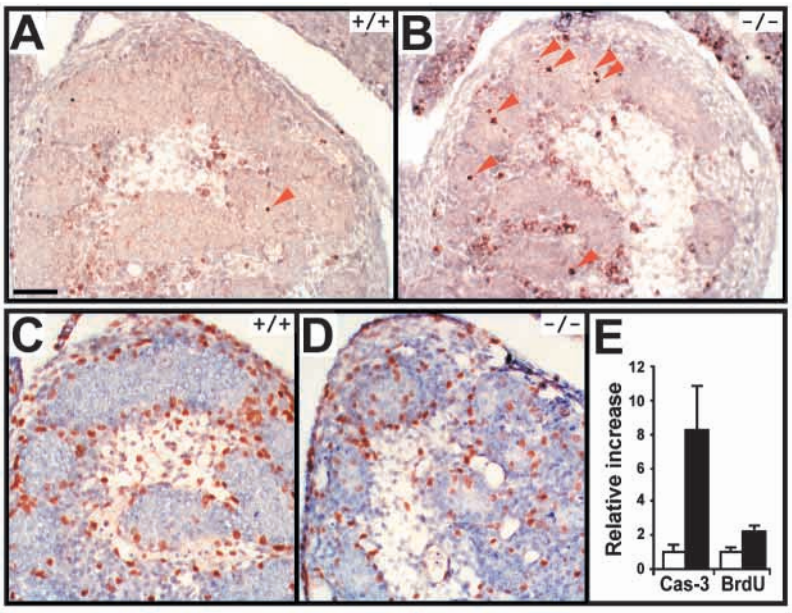

Fig. 4. Apoptotic loss of spermatogenic precursors in CIT-K$^{-/-}$ mice. E14.5 embryos from CIT-K heterozygous intercrossing were labeled for 1 hour in utero with BrdU and then processed for immunohistochemistry. (A,B) Apoptotic gonocytes were identified by immunohistochemistry for activated Caspase-3, as intratubular-positive cells (red arrowheads). The assay was performed on comparable sections from CIT-K$^{+/+}$ (A) and $^{-/-}$ (B) testes. Interstitial cells displayed a background cytoplasmic positivity in both control and knockout samples. (C,D) The tubular proliferative index was determined on sections adjacent to those used for the apoptosis assay, labeled by anti-BrdU immunostaining. (E) Graphic representation of the apoptotic (Cas-3) and proliferative (BrdU) indexes at E14.5 in testes of CIT-K$^{+/+}$ (open bars) and $^{-/-}$ (closed bars). Values are expressed as the relative increase compared with the control. Error bars showstandard deviation, $P<0.01$.

Apoptotic loss of CIT-K$^{-/-}$ spermatogenic precursors

To investigate if the observed depletion of testicular germ cell precursors was caused by reduced proliferation or increased cell death, the apoptotic and proliferative indices of testicular tubular cells were determined in both embryonic and postnatal testes of wild-type and knockout animals by anti-activated Caspase-3 immunostaining and BrdU labeling, respectively.

As shown in Fig. 4A,B, the number of apoptotic cells detected in the lumen of seminiferous cords at E14.5 was clearly increased in sections of $^{-/-}$ versus $^{+/+}$ embryos. The relative increase was even more significant when the absolute counts were normalized for the reduced number of gonocytes observed in the knockouts (Fig. 4E).

Strikingly, despite the reduced numbers of gonocytes, the BrdU labeling index of knockout seminiferous cords was increased by approximately 2.5 fold (Fig. 4C,E). Similar results were obtained at P4 (data not shown).

Taken together, the above observations indicate that the striking depletion of testicular germ cells occurring in CIT-K knockout mice is caused by progressive apoptotic loss of embryonic and post-natal precursors.

Defective cytokinesis in CIT-K$^{-/-}$ spermatogenic precursors

CIT-K has been previously shown to be required for cytokinesis in specific populations of the developing central nervous system (CNS) but not in the other somatic tissues that express it at significant levels (Di Cunto et al., 2000). In order to address whether the increased cell death of CIT-K$^{-/-}$ testicular precursors is correlated with a specific impairment of this process, we asked whether an increase in germinal cells displaying a binucleate or multinucleate morphology could be detected in the knockouts. Examination of semi-thin sections obtained from postnatal testes revealed that the number of gonocytes and spermatogonia with at least two nuclei was increased by approximately four-fold in the $^{-/-}$ samples (Fig. 5A,C). Electron microscopy of ultra-thin sections, obtained from the same samples, demonstrated that these cells were not adjacent mononuclear elements but real syncitia (Fig. 5D), thus indicating a cytokinesis block. Strikingly, many cells with more than two nuclei were observed in the CIT-K$^{-/-}$ tubules, but never in the control samples (Fig. 5A,B,D), indicating that cytokinesis-deficient cells are able to re-enter the cell cycle and to undergo multiple rounds of DNA synthesis.

Interestingly, in cyclinD1 immunohistochemistry we noticed that many of the binucleate and multinucleate gonocytes and spermatogonia display high immunoreactivity for this protein (Fig. 5E).

To establish a direct link between the cytokinesis block and the induction of apoptosis in the knockout testicular germ cells, we first attempted to quantify the percentage of polyploid cells undergoing apoptosis by FACS analysis after double labeling with propidium iodide and anti-Caspase-3 antibodies, as previously reported for the brain (Di Cunto et al., 2000). However, probably because of the low ratio between germinal and support cells at the analyzed stages (P4 and P8), no significant results were obtained (data not shown). Therefore, to address the same point, we tried to establish the percentage of activated Caspase-3-positive cells clearly displaying mononucleate versus binucleate or multinucleate morphology. One potential complication for this kind of analysis is that cells at late stages of the apoptotic process often undergo nuclear condensation and fragmentation (Walker et al., 1988), making it impossible to assess the original number of nuclei. Therefore, cells displaying condensed and fragmented nuclei were excluded from the counts. Using these criteria, we were not able to identify early apoptotic cells with more than one nucleus in wild-type sections (total number of scored cells=92 on 50 sections). By contrast, approximately 10% of the Caspase-3-positive cells displayed a binucleate or multinucleate morphology in the knockouts (Fig. 5F, total number of scored cells=250 on 25 sections).

Taken together, these observations indicate that the absence

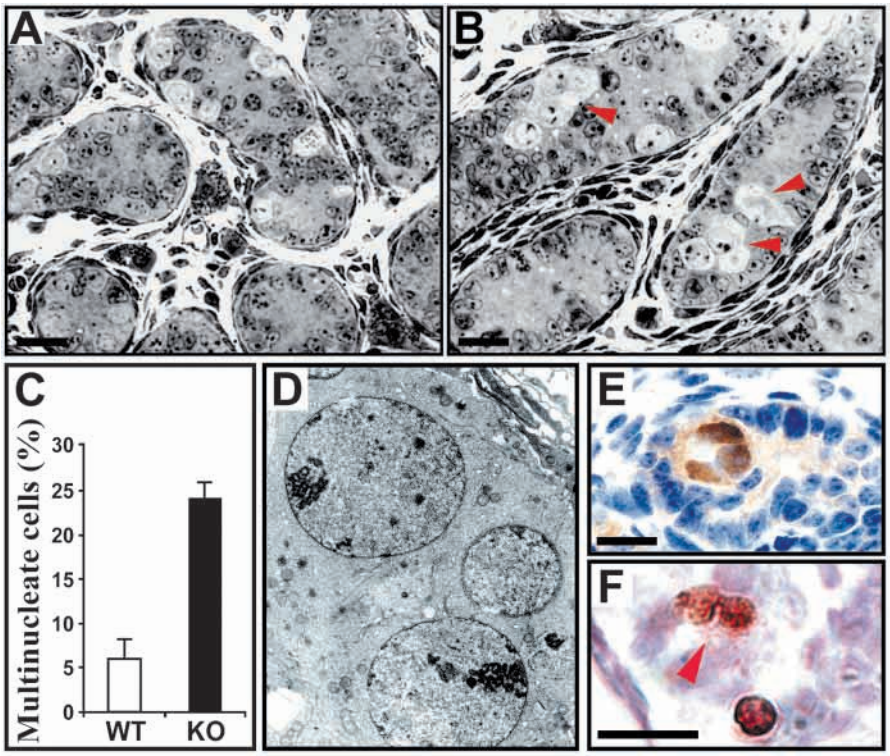

Fig. 5. Defective cytokinesis in CIT-K$^{-/-}$ undifferentiated male germ cells. (A,B) Semi-thin sections of P4 CIT-K$^{+/+}$ (A) and $^{-/-}$ (B) testes were analyzed by optical microscopy. Red arrowheads point to type A spermatogonia displaying more than one nucleus. (C) The percentage of undifferentiated germ cells displaying a binucleate/multinucleate morphology was determined on semi-thin sections by optical microscopy. Error bars show standard deviation, $P<0.005$. (D) Electron microscopy picture of a P4 CIT-K$^{-/-}$ type A spermatogonium, displaying three nuclei surrounded by the same cytoplasm. (E) Anti-cyclinD1 immunohistochemistry performed on P4 CIT-K$^{-/-}$ testis, showing a multinucleated spermatogonium expressing high levels of this protein. (F) High-power field of anti-activated Caspase-3 immunostaining, performed on P4 CIT-K$^{-/-}$ testis. Red arrowhead indicates an early apoptotic, binucleate cell. Bars, 20 μm.

of CIT-K results in abnormal cytokinesis and polyploidization of spermatogenic precursors, which can be followed by re-entering in the cell cycle or by induction of programmed cell death.

Discussion

Spermatogenesis is characterized by a fine-tuned sequence of mitotic and meiotic cellular divisions, which lead from type A_S spermatogonia to haploid spermatids (de Rooij, 1998). Previous studies, performed in evolutionarily distant species, such as insects and mammals, strongly suggest that regulation of the cytokinesis endgame is a crucial aspect of the spermatogenic differentiation program. For instance, mutation of several *Drosophila* genes involved in cytokinesis results in male sterility (Brill et al., 2000; Castrillon and Wasserman, 1994; Hime et al., 1996). Moreover, it is well established that in mammalian spermatogenesis the first morphological indication of spermatogonia differentiation is incomplete cytokinesis, followed by the formation of a stable intercellular bridge between the daughter cells (de Rooij and Grootegoed, 1998). Nevertheless, the molecular mechanisms responsible for the testis-specific aspects of cytokinesis are still largely obscure, especially in mammals.

The Rho effector CIT-K is the first protein that has been shown to be required in vivo for cytokinesis of specialized mammalian cell types (Di Cunto et al., 2000; Glotzer, 2001). Indeed, inactivation of this molecule by gene targeting resulted in a dramatic malformative syndrome of the CNS, caused by cytokinesis failure and apoptosis of specific neuronal precursors, whereas no abnormalities were observed in the other somatic tissues (Di Cunto et al., 2000). In this report we have shown that, in addition, CIT-K$^{-/-}$ mice display a dramatic testicular phenotype, characterized by progressive loss of germ cells during development.

The number of spermatogenic precursors of CIT-K$^{-/-}$ mice was significantly reduced as early as E12.5, even though the specification and migration of PGCs appeared to be conserved. In the first postnatal days, gonocytes and type A spermatogonia were still present in CIT-K$^{-/-}$ testes, although their number was severely reduced. At later developmental stages, the presence of undifferentiated germ cells was confirmed by the expression of the spermatogenic markers Mvh and A-Myb, even though they were not easily identified by morphological analysis. Interestingly, the expression levels of the two markers were higher than expected from the morphological data because no difference between $^{+/+}$ and $^{-/-}$ samples was detected at P8, and only a partial reduction was observed at P14. Since the density of the other testicular elements was not dramatically changed, a possible explanation for this observation could be that the Mvh and A-Myb are upregulated in the residual spermatogenic precursors of knockout animals. By contrast, both morphological and molecular analysis consistently showed that production of mature spermatocytes is completely blocked in CIT-K$^{-/-}$ testes.

Taken together, these observations indicate that CIT-K is necessary for the normal expansion of undifferentiated male germ cell precursors and is absolutely required to ensure the transition from spermatogonia to spermatocytes. The most likely cause of this phenotype is increased apoptosis rather than reduced proliferation.

The complex brain malformations of CIT-K$^{-/-}$ mice, which lead them to death in the first postnatal weeks (Di Cunto et al., 2000), raised the possibility that this testicular phenotype could be a secondary consequence of neuro-endocrine defects. In particular, among their distinctive features, CIT-K knockout

mice display a dramatic cellular depletion in the olfactory subventricular stream (Di Cunto et al., 2000) involved in the migration of GnRH-producing cells (Kim et al., 1999). However, the requirement for CIT-K in gametogenesis appears to be exquisitely sex specific, as the ovaries of knockout females were histologically comparable to their littermate controls. This finding strongly argues against the hormonal mechanism, since GnRH deficiency results in both male and female hypogonadism (Halpin et al., 1986; Hargreave, 2000; Kendall et al., 1991). Moreover, the good correlation between the expression pattern and the defects observed in the knockouts strongly suggests that CIT-K acts in these cells through a cell-autonomous mechanism.

Histological and EM analysis clearly showed that gonocytes and spermatogonia with multiple nuclei are produced in the seminiferous tubules of CIT-K$^{-/-}$ mice, thus indicating a cytokinesis block. To our knowledge, CIT-K is the first molecule that has been shown to be specifically required for cytokinesis of mammalian testicular stem cells.

It is remarkable that, although CIT-K is expressed in many proliferating tissues and was initially identified as a crucial, generic player of cytokinesis (Madaule et al., 1998), the in vivo requirement for its function is restricted to male germ cells and specific neuronal precursors. One explanation for this specificity could be that the affected cell types are physiologically devoid of the molecule(s) that is able to compensate for the absence of CIT-K in the non-affected cell types of the knockouts. Extensive homology search of the mouse and human genome sequence databases performed using the BLAST programs (Altschul et al., 1997) revealed that CIT-K is encoded by a unique gene, as no closely related paralogs could be identified (data not shown). Among the other members of the myotonin protein kinase subfamily, Rho-kinases (ROCKs) and myotonin-related-CDC42-binding kinases (MRCKs) displayed the highest similarity to CIT-K, as previously reported (Leung et al., 1998). However these molecules are very unlikely to compensate for CIT-K loss. Indeed, although the ROCKs have been involved as Rho effectors in cytokinesis (Kosako et al., 1999), they are ubiquitous proteins and are very well expressed in the testis (Nakagawa et al., 1996) and in the proliferating neuroblasts affected by the absence of CIT-K (Di Cunto et al., 2000). On the other hand, the MRCKs display a relatively tissue-specific expression pattern but have been reported to bind only to CDC42 and have not been localized to the cleavage furrow and the midbody of dividing cells (Leung et al., 1998).

An attractive alternative possibility could be that the cytokinesis machinery of the affected cell types shares important and tissue-specific molecular details, which require CIT-K in order to act properly.

The characterization of molecules capable of physically and functionally interacting with CIT-K in the affected versus spared cell types will be very important to clarify these points.

Despite the impossibility of rigorously establishing a direct link between abnormal cytokinesis and increased apoptosis, our results strongly suggest that, as in the developing brain, in the testes of CIT-K knockout mice the induction of programmed cell death is secondary to the cytokinesis block. The mechanisms responsible for activation of the apoptotic pathway in these cells are still unknown. Interestingly, we found that multinucleate cells in post-natal testes of CIT-K knockout mice display elevated levels of cyclinD1. This observation could be simply explained by the ability of the cytokinesis-deficient gonocytes and spermatogonia to re-enter the G1 phase of the cell cycle. However, the available evidence suggests that the expression of cyclinD1 is directly linked to activation of the apoptotic pathway. Indeed, it has been previously shown that overexpression of cyclinD1 is necessary and sufficient for apoptotic induction in neuronal cells (Kranenburg et al., 1996). Moreover, elevated levels of this protein were previously detected in the apoptotic brain regions of CIT-K$^{-/-}$ mice (Di Cunto et al., 2000). Finally, activation of the cyclinD-dependent CDKs by double inactivation of the proteins p18(Ink4c) and p19(Ink4d) resulted in increased apoptosis of testicular germ cells (Zindy et al., 2001).

The results of this study are consistent with the idea that proper temporal regulation of cytokinesis is essential for normal differentiation of the testicular epithelium. Indeed, the incomplete cytokinesis of gonocytes and undifferentiated spermatogonia occurring in CIT-K$^{-/-}$ testes does not result in the anticipated formation of ICBs but produces multinucleated cells, which are unable to further differentiate and are eventually lost by programmed cell death. The mechanisms by which committed spermatogenic cells arrest their cytokinesis at the midbody stage and then assemble stable ICBs (Kojima, 1992) remain obscure. In particular, it is not understood whether the incomplete cell division of differentiating male germ cells is caused by downregulation of molecules required for completion of cytokinesis, by upregulation of specific cytokinesis inhibitors or from a combination of both. Since CIT-K is necessary for cytokinesis of the basal spermatogonia and its expression is downmodulated at later stages of differentiation, it is tempting to speculate that its inactivation could play a role in this process. Although no direct evidence in support of this model has been so far obtained, a testable prediction would be that the ectopic overexpression of CIT-K in differentiating spermatogenic precursors should be able to interfere with the establishment of their ICBs.

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References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

Baker, S. M., Plug, A. W., Prolla, T. A., Bronner, C. E., Harris, A. C., Yao, X., Christie, D. M., Monell, C., Arnheim, N., Bradley, A. et al. (1996). Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over. *Nat. Genet.* **13**, 336-342.

Beumer, T. L., Roepers-Gajadien, H. L., Gademan, I. S., Kal, H. B. and de Rooij, D. G. (2000). Involvement of the D-type cyclins in germ cell proliferation and differentiation in the mouse. *Biol. Reprod.* **63**, 1893-1898.

Bishop, A. L. and Hall, A. (2000). Rho GTPases and their effector proteins. *Biochem. J.* **348**, 241-255.

Brill, J. A., Hime, G. R., Scharer-Schuksz, M. and Fuller, M. T. (2000). A phospholipid kinase regulates actin organization and intercellular bridge formation during germline cytokinesis. *Development* **127**, 3855-3864.

Castrillon, D. H. and Wasserman, S. A. (1994). Diaphanous is required for cytokinesis in *Drosophila* and shares domains of similarity with the products of the limb deformity gene. *Development* **120**, 3367-3377.

de Rooij, D. G. (1998). Stem cells in the testis. *Int. J. Exp. Pathol.* **79**, 67-80.
de Rooij, D. G. and Grootegoed, J. A. (1998). Spermatogonial stem cells. *Curr. Opin. Cell Biol.* **10**, 694-701.
Di Cunto, F., Calautti, E., Hsiao, J., Ong, L., Topley, G., Turco, E. and Dotto, G. P. (1998). Citron Rho-interacting kinase, a novel tissue-specific ser/thr kinase encompassing the Rho-Rac-binding protein Citron. *J. Biol. Chem.* **273**, 29706-29711.
Di Cunto, F., Imarisio, S., Hirsch, E., Broccoli, V., Bulfone, A., Migheli, A., Atzori, C., Turco, E., Triolo, R., Dotto, G. P. et al. (2000). Defective neurogenesis in citron kinase knockout mice by altered cytokinesis and massive apoptosis. *Neuron* **28**, 115-127.
Drechsel, D. N., Hyman, A. A., Hall, A. and Glotzer, M. (1997). A requirement for Rho and Cdc42 during cytokinesis in *Xenopus* embryos. *Curr. Biol.* **7**, 12-23.
Ginsburg, M., Snow, M. H. and McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development* **110**, 521-528.
Glotzer, M. (2001). Animal cell cytokinesis. *Annu. Rev. Cell Dev. Biol.* **17**, 351-386.
Habu, T., Taki, T., West, A., Nishimune, Y. and Morita, T. (1996). The mouse and human homologs of DMC1, the yeast meiosis-specific homologous recombination gene, have a common unique form of exon-skipped transcript in meiosis. *Nucleic Acids Res.* **24**, 470-477.
Hales, K. G., Bi, E., Wu, J. Q., Adam, J. C., Yu, I. C. and Pringle, J. R. (1999). Cytokinesis: an emerging unified theory for eukaryotes? *Curr. Opin. Cell Biol.* **11**, 717-725.
Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.
Halpin, D. M., Charlton, H. M. and Faddy, M. J. (1986). Effects of gonadotrophin deficiency on follicular development in hypogonadal (hpg) mice. *J. Reprod. Fertil.* **78**, 119-125.
Hargreave, T. B. (2000). Genetics and male infertility. *Curr. Opin. Obstet. Gynecol.* **12**, 207-219.
Hime, G. R., Brill, J. A. and Fuller, M. T. (1996). Assembly of ring canals in the male germ line from structural components of the contractile ring. *J. Cell Sci.* **109**, 2779-2788.
Hirose, K., Kawashima, T., Iwamoto, I., Nosaka, T. and Kitamura, T. (2001). MgcRacGAP is involved in cytokinesis through associating with mitotic spindle and midbody. *J. Biol. Chem.* **276**, 5821-5828.
Huckins, C. and Clermont, Y. (1968). Evolution of gonocytes in the rat testis during late embryonic and early post-natal life. *Arch. Anat. Histol. Embryol.* **51**, 341-354.
Jantsch-Plunger, V., Gonczy, P., Romano, A., Schnabel, H., Hamill, D., Schnabel, R., Hyman, A. A. and Glotzer, M. (2000). CYK-4: A Rho family gtpase activating protein (GAP) required for central spindle formation and cytokinesis. *J. Cell Biol.* **149**, 1391-1404.
Kendall, S. K., Saunders, T. L., Jin, L., Lloyd, R. V., Glode, L. M., Nett, T. M., Keri, R. A., Nilson, J. H. and Camper, S. A. (1991). Targeted ablation of pituitary gonadotropes in transgenic mice. *Mol. Endocrinol.* **5**, 2025-2036.
Kim, K. H., Patel, L., Tobet, S. A., King, J. C., Rubin, B. S. and Stopa, E. G. (1999). Gonadotropin-releasing hormone immunoreactivity in the adult and fetal human olfactory system. *Brain Res.* **826**, 220-229.
Kishi, K., Sasaki, T., Kuroda, S., Itoh, T. and Takai, Y. (1993). Regulation of cytoplasmic division of *Xenopus* embryo by Rho p21 and its inhibitory GDP/GTP exchange protein (Rho GDI). *J. Cell Biol.* **120**, 1187-1195.
Kojima, Y. (1992). Ultrastructure of goat testes: intercellular bridge between germ cells. *J. Vet. Med. Sci.* **54**, 213-219.
Kosako, H., Goto, H., Yanagida, M., Matsuzawa, K., Fujita, M., Tomono, Y., Okigaki, T., Odai, H., Kaibuchi, K. and Inagaki, M. (1999). Specific accumulation of Rho-associated kinase at the cleavage furrow during cytokinesis: cleavage furrow-specific phosphorylation of intermediate filaments. *Oncogene* **18**, 2783-2788.
Kranenburg, O., van der Eb, A. J. and Zantema, A. (1996). Cyclin D1 is an essential mediator of apoptotic neuronal cell death. *EMBO J.* **15**, 46-54.
Kunieda, T., Xian, M., Kobayashi, E., Imamichi, T., Moriwaki, K. and Toyoda, Y. (1992). Sexing of mouse preimplantation embryos by detection of Y chromosome-specific sequences using polymerase chain reaction. *Biol. Reprod.* **46**, 692-697.
Lehner, C. F. (1992). The pebble gene is required for cytokinesis in *Drosophila*. *J. Cell Sci.* **103**, 1021-1030.
Leung, T., Chen, X. Q., Tan, I., Manser, E. and Lim, L. (1998). Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization. *Mol. Cell Biol.* **18**, 130-140.
Madaule, P., Eda, M., Watanabe, N., Fujisawa, K., Matsuoka, T., Bito, H., Ishizaki, T. and Narumiya, S. (1998). Role of citron kinase as a target of the small GTPase Rho in cytokinesis. *Nature* **394**, 491-494.
Mahowald, A. P. (1971). The formation of ring canals by cell furrows in *Drosophila*. *Z Zellforsch Mikrosk. Anat.* **118**, 162-167.
Mettus, R. V., Litvin, J., Wali, A., Toscani, A., Latham, K., Hatton, K. and Reddy, E. P. (1994). Murine A-myb: evidence for differential splicing and tissue-specific expression. *Oncogene* **9**, 3077-3086.
Migheli, A., Piva, R., Casolino, S., Atzori, C., Dlouhy, S. R. and Ghetti, B. (1999). A cell cycle alteration precedes apoptosis of granule cell precursors in the weaver mouse cerebellum. *Am. J. Pathol.* **155**, 365-373.
Nakagawa, O., Fujisawa, K., Ishizaki, T., Saito, Y., Nakao, K. and Narumiya, S. (1996). ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett.* **392**, 189-193.
Nakano, K., Arai, R. and Mabuchi, I. (1997). The small GTP-binding protein Rho1 is a multifunctional protein that regulates actin localization, cell polarity, and septum formation in the fission yeast *Schizosaccharomyces pombe*. *Genes Cells* **2**, 679-694.
Tanaka, S. S., Toyooka, Y., Akasu, R., Katoh-Fukui, Y., Nakahara, Y., Suzuki, R., Yokoyama, M. and Noce, T. (2000). The mouse homolog of *Drosophila* Vasa is required for the development of male germ cells. *Genes Dev.* **14**, 841-853.
Tatsumoto, T., Xie, X., Blumenthal, R., Okamoto, I. and Miki, T. (1999). Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. *J. Cell Biol.* **147**, 921-928.
Van Aelst, L. and Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes Dev.* **11**, 2295-2322.
Vergouwen, R. P., Jacobs, S. G., Huiskamp, R., Davids, J. A. and de Rooij, D. G. (1991). Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. *J. Reprod. Fertil.* **93**, 233-243.
Walker, N. I., Harmon, B. V., Gobe, G. C. and Kerr, J. F. (1988). Patterns of cell death. *Methods Achiev. Exp. Pathol.* **13**, 18-54.
Watanabe, D., Yamada, K., Nishina, Y., Tajima, Y., Koshimizu, U., Nagata, A. and Nishimune, Y. (1994). Molecular cloning of a novel Ca(2+)-binding protein (calmegin) specifically expressed during male meiotic germ cell development. *J. Biol. Chem.* **269**, 7744-7749.
Zackson, S. L. and Steinberg, M. S. (1988). A molecular marker for cell guidance information in the axolotl embryo. *Dev. Biol.* **127**, 435-442.
Zindy, F., den Besten, W., Chen, B., Rehg, J. E., Latres, E., Barbacid, M., Pollard, J. W., Sherr, C. J., Cohen, P. E. and Roussel, M. F. (2001). Control of spermatogenesis in mice by the cyclin D-dependent kinase inhibitors p18(Ink4c) and p19(Ink4d). *Mol. Cell Biol.* **21**, 3244-3255.