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FK506, a Calcineurin Inhibitor, Prevents Cadmium-Induced Testicular Toxicity in Mice

Lisa Joy Martin,*,† Haiyan Chen,*,[1] Xiaoyan Liao,* Hooman Allayee,†,[2] Diana Mouhan Shih,† Grace Sangeun Lee,*,[3] David Norman Hovland, Jr,*,[4] Wendie Anne Robbins,‡ Kay Carnes,§ Rex Allen Hess,§ Aldons Jake Lusis,† and Michael David Collins*,[5]

*Interdepartmental Program in Molecular Toxicology, University of California, Los Angeles School of Public Health, 10833 LeConte Avenue, Los Angeles, California 90095; †Department of Human Genetics, David Geffen School of Medicine at University of California, Los Angeles, 695 Charles E. Young Drive South, Los Angeles, California 90095; ‡Center for Occupational and Environmental Health, Factor Building, University of California, Los Angeles, Los Angeles, California 90095; and §Department of Veterinary Biosciences, University of Illinois, 2001 S. Lincoln Avenue, Urbana, Illinois 61802

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Cadmium, a ubiquitous environmental contaminant, damages several major organs in humans and other mammals. The molecular mechanisms for damage are not known. At high doses (5 mg/kg cadmium chloride or higher), testicular damage in mice, rats, and other rodents includes interstitial edema, hemorrhage, and changes in the seminiferous tubules affecting spermatogenesis. Necrosis is evident by 48 h. The goal of this study was to fine map and identify the *cdm* gene, a gene that when mutated prevents cadmium-induced testicular toxicity in mouse strains with a mutation in this gene. A serine-threonine phosphatase, calcineurin (CN), subunit A, α isoform (*Ppp3ca*), was one of the seven candidates in the *cdm* region that was narrowed from 5.6 to 2.0 Mb on mouse chromosome 3. An inhibitor of CN, the immunosuppressant, FK506, prevented cadmium-induced testicular damage in five pathological categories, including vascular endothelial and seminiferous epithelial endpoints. Inductively coupled plasma-mass spectrometry revealed that FK506 protected without lowering the amount of cadmium in the testes. *Ppp3ca*$^{-/-}$ mice were investigated but were found to exhibit endogenous testicular abnormalities, making them an inappropriate model for determining whether the inactivation of the *Ppp3ca* gene would afford protection from cadmium-induced testicular toxicity. The protection afforded by FK506, found by the current study, indicated that CN is likely to be important in the mechanism of cadmium toxicity in the testis and possibly other organs.

Key Words: **cadmium; testes; *cdm*; calcineurin; Ppp3ca; FK506; multinucleated germ cells; SSeCKS.**

Cadmium is a highly reactive metal, adversely affecting many mammalian organ systems, including kidney, liver, lung, pancreas, testis, prostate, ovary, and placenta (Bridges and Zalups, 2005). In humans, chronic environmental and occupational exposure causes severe bone and kidney damage and acute occupational exposure from cadmium fumes causes pulmonary edema (Sittig, 1991). Cadmium-induced testicular damage in mice, rats, and other rodents includes interstitial edema and hemorrhage, accompanied by damage to seminiferous tubules affecting sperm cells and their precursors (Parizek, 1957).

Testicular endothelial tissue is a primary target of systemically administered cadmium, with edema detected 1 h after an injection of a very high dose of $CdCl_2$ (10 mg/kg, sc) in rats (Gabbiani *et al*., 1974). At lower doses, however, peritubular cells and the seminiferous epithelium have been shown to be primary targets (Hew *et al*., 1993b; Setchell and Waites, 1970). For example, a single low dose of $CdCl_2$ (1 mg/kg, ip) in rats caused failure of spermiation and fragmentation of basal Sertoli cell junction microfilament bundles in the absence of edema or necrosis (Hew *et al*., 1993a). Similarly, a study investigating Sertoli cell junctional dynamics in rats using a moderate dose of $CdCl_2$ (3 mg/kg, ip) found that damage to Sertoli cell barriers occurred prior to damage to vascular endothelial barriers (Wong *et al*., 2004). Therefore, seminiferous epithelial toxicity is not necessarily a consequence of vascular damage. A disruption of cell-to-cell endothelial and epithelial junctions, possibly due to disruption of the internal cytoskeleton, has been proposed as a mechanism for cadmium toxicity in the testis (Janecki *et al*., 1992; Wong *et al*., 2004) and other organs (Prozialeck *et al*., 2006).

A single homozygous recessive gene, named *cdm*, was found to confer complete resistance to cadmium-induced testicular

[1] Institute of Oral Health Research, School of Dentistry, University of Alabama at Birmingham, 1530 3rd Avenue South Birmingham, Alabama 35294

[2] Department of Preventive Medicine, Keck School of Medicine USC, 1441 Eastlake Avenue, Los Angeles, California 90089

[3] Schering-Plough Research Institute, 556 Morris Avenue, Summit, New Jersey 07901

[4] Amgen Inc., One Amgen Center Drive, Thousand Oaks, California 91320

[5] To whom correspondence should be addressed at Department of Environmental Health Sciences, Interdepartmental Program in Molecular Toxicology, University of California, Los Angeles School of Public Health CHS 71-297, 10833 LeConte Avenue, Los Angeles, CA 90095-1772. Fax: (310) 206-9903. E-mail: mdc@ucla.edu.

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toxicity in one-third of 45 inbred mouse strains tested (Taylor, 1973). *Cdm* was determined to be either a polymorphism or a mutated version of an unidentified gene in a 2.1-cM region on mouse chromosome 3 (MGD, 2006). Because of the compelling biological story of the *cdm* gene, that of a complex toxicological phenotype controlled by a single unidentified gene, many laboratories over the years have pursued investigations into the identity of the *cdm* gene. In fact, after the current study was underway, a different laboratory identified the metal ion transporter *Slc39a8* as the *cdm* gene (Dalton *et al*., 2005; Wang *et al*., 2007). We continued our study because our genotyping results for the recombinant inbred strain, RI*Ty14*, which was the key for establishing the distal boundary of the *cdm* region, differed from previous findings (Dalton *et al*., 2000), and because we had determined that FK506 antagonized cadmium-induced testicular toxicity prior to the identification of the *cdm* gene (Dalton *et al*., 2005). Therefore, the current study used F_2 mice from a BXD F_1 cross to narrow the *cdm* region to 1.97 Mb and seven genes (one of which was *Slc39a8*). The catalytic subunit, α isoform of the calcium/calmodulin-dependent serine-threonine phosphatase, calcineurin (CN), *Ppp3ca*, was pursued as a *cdm* gene candidate in the current study. CN is a heterodimer composed of the catalytic subunit CN A (Ppp3c) and the regulatory subunit CN B (Ppp3r), each with chromosome-specific isoforms (Klee *et al*., 1998).

The importance of several common elements in cadmium toxicity and CN regulatory pathways, including zinc, iron, calcium/calmodulin, and oxidative stress, made *Ppp3ca* an intriguing candidate for the *cdm* gene. Zinc is very important in spermatogenesis (Merker and Gunther, 1997), and coadministration of zinc ameliorated cadmium-induced testicular toxicity (Parizek, 1957). Low nutritional iron status increases intestinal absorption of cadmium, indicating a role that iron may play in cadmium transport (Zalups and Ahmad, 2003). Both metal ions, Zn^{2+} and Fe^{2+}, are located in the catalytic core of Ppp3c (Wang *et al*., 1996) and could possibly be targeted directly by Cd^{2+} replacement (Stohs *et al*., 2001) or, in the case of Fe^{2+}, indirectly targeted through oxidation to the Fe^{3+} state. Cadmium toxicity is associated with oxidative stress (Valko *et al*., 2005), and CN activity is decreased when the iron ion in the catalytic core is oxidized (Wang *et al*., 1996). Finally, the phosphatase activity of CN is calcium/calmodulin dependent, and cadmium has been shown to interfere with calcium signaling pathways (Barbier *et al*., 2004). Calmodulin inhibitors prevented cadmium-induced testicular damage in mice (Niewenhuis and Prozialeck, 1987), as well as cytoskeletal cadmium-induced damage in cells (Perrino and Chou, 1986).

We administered an inhibitor of CN, FK506 (Dumont, 2000), to mice before cadmium chloride as an initial test of the hypothesis that *Ppp3ca* was the *cdm* gene. FK506 conferred protection from cadmium-induced testicular edema, hemorrhage, Sertoli cell toxicity, and germ cell damage. Therefore, in the process of searching for the *cdm* gene, we found that FK506 antagonized cadmium-induced testicular toxicity, which suggested that CN plays a role in the cadmium toxicity pathway in the testis and possibly other organs.

MATERIALS AND METHODS

Mouse models. The mice used in this study included the inbred C57BL/6J, SWV, and DBA/2J strains. The colony of SWV mice was established from mice originally provided by Dr Richard Finnell (Texas A&M University, College Station, TX). C57BL/6J, DBA/2J mice, and DNA from RI*14Ty* were obtained from Jackson Laboratories (Bar Harbor, ME). Congenic mice (one segment of genomic DBA on a C57BL/6 background) were originally from Parke Davis (Iakoubova *et al*., 2001). *Ppp3ca*$^{-/-}$ mice were a gift from Dr Jonathan Seidman (Howard Hughes Medical Institute, Harvard Medical School, Boston, MA). Animals were housed in transparent plastic cages with stainless steel lids and microisolator filter covers (University of California, Los Angeles) with wood shavings for bedding. Water and a commercial diet (Purina Formulab 5008, St Louis, MO) were available *ad libitum*. Animals were maintained in climate-controlled rooms 22°C under an alternating 12-h light/dark cycle.

Genotyping and fine mapping. DNA from C57BL/6J × DBA/2J F2 mouse tail clips was isolated via phenol:chloroform extraction (Couse *et al*., 1994). Primers D3Nds2, D3Mit291, D3Mit255, D3Mit125, and D3Mit349 (Invitrogen, Carlsbad, CA) and new custom-made primers (supplementary data) were used to detect recombinations in the F_2 mice. National Center for Biotechnology Information Build 36, accessed through the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu), was used to map commercial primers inside the *cdm* region (Hinrichs *et al*., 2006; Karolchik *et al*., 2003); the BLAT (Kent, 2002) was used to map custom-made primers. Congenic mice BDChr3D and BDChr3M were genotyped with D3Mit259, D3Mit163, D3Mit97, and D3Mit45 (Invitrogen). *Ppp3ca*$^{+/-}$ breeding pair offspring were genotyped as previously reported (Gooch *et al*., 2006).

Scoring system for cadmium-induced damage in the testis. Damage due to cadmium and protection by FK506 were assessed by a scoring system using five categories, each scored 0–3, with 3 describing the most severe damage (Table 1). For photographs of cadmium damage in the mouse testis, see supplementary data. (1) Hemorrhage: presence of erythrocytes in the interstitium; (2) Periodic Acid Schiff positive (PAS+) material and edema or increased interstitial space. Increased PAS+ material in the interstitium correlated with edema, and although possibly distinct effects, these endpoints were assigned one score to avoid redundancy. (3) Sertoli cell barrier breakdown: indicated by germ cell disorganization within the seminiferous tubule; (4) Loss of Sertoli cells: detachment from the basement membrane leading to sloughing into the lumen; and (5) Multinucleated cells comprised of either spermatocytes or spermatids: indicated a failure of Sertoli cell cytoplasmic bridge management. These five categories were chosen because they produced nonredundant scores for each category in individual animals. The total damage score was the sum of the five individual category scores.

FK506, rapamycin, and cadmium chloride administration. Male mice, 8–12 weeks of age, were administered single doses of $CdCl_2$ at 2.0, 2.5, or 6.0 mg/kg body weight by sc injection. The 2.0 and 2.5 mg/kg doses and the 24-h time point were used in the drug protection experiments. The 6.0 mg/kg dose and the 48-h time point were used to phenotype F_2 recombinant mice because hemorrhage in sensitive mice at this dose and time point was obvious to the naked eye, yet completely absent in resistant mice. The lower doses and 24-h time point required histopathology to determine the phenotypes. Injections were administered at 10 ml/kg body weight. FK506, a gift from Fujisawa, Inc. or purchased from LC Laboratories (Woburn, MA), was administered from a 1 mg/ml solution in 95% soybean oil (Sigma Aldrich, St Louis, MO) and 5% ethanol (Fisher Scientific, Pittsburg, PA). Mice were administered FK506 at two time points prior to injection of $CdCl_2$. The first FK506 injection was

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TABLE 1
Testicular Histopathological Damage Due to Cadmium Exposure[1]

	Categories[2]				
	A	B	C	D	E
Score	Hemorrhage	PAS+ edema	Sertoli cell barrier breakdown	Loss of Sertoli cells	Multinucleated germ cells
0	None	Normal	None	None	None
1	1–10%	1–20%	1–20%	1–20%	1–2
2	10–20%	20–50%	20–50%	20–50%	3–10
3	> 20%	> 50%	> 50%	> 50%	> 10

[1]Damage was assessed 24 h after $CdCl_2$ administration. One entire histological section was examined to determine the score in each category. Sections that had damage at the lower or upper ends of the range were assigned +/− 0.5.

[2]A, % of interstitial space occupied by red blood cells; B, % of interstitial space occupied by PAS+ material; C, % of tubules in which this occurs; D, % of tubules in which this occurs; E, number of multinucleated spermatocytes and spermatids per section.

sc, administered 4 h prior to $CdCl_2$, and the second was ip, administered 1 h prior to $CdCl_2$ injection. Two different injection sites were used due to irritation at the injection site. Rapamycin (LC Laboratories) was administered from a sterile 1 mg/ml aqueous suspension containing 0.2% carboxymethyl cellulose and 0.25% polysorbate-80 (Sigma Aldrich). Mice were sacrificed 24 h after injection of $CdCl_2$ by exposure to isoflurane and subsequent cervical dislocation. One testis was taken for histopathology analysis and the other for protein, RNA, or inductively coupled plasma-mass spectrometry (ICP-MS) analysis. For histology, testes were preserved in modified Davidson's: 30% of a 37–40% solution of formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled H_2O. After 24 h, the tissues were transferred to 70% ethanol and stored at 4°C until they were embedded in paraffin. Sections were stained with hematoxylin and counterstained with eosin or PAS solution.

Tissue preparation. For protein assays and ICP-MS, the testes of treated and untreated mice were extracted, immersed in liquid nitrogen, and stored at −80°C. Proteins were isolated by first disrupting one whole testis in a 1.5-ml sterile microtube with sterile 3-in. disposable pestle (Kontes Glass Company, Vineland, NJ) on ice using 200 µl lysis buffer per testis. Lysis buffer contained 0.1M Tris-HCl (pH 7.5), 2mM ethylenediaminetetraacetic acid, 2mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA), 2mM dithiothreitol (Fisher Scientific), and protease inhibitors (per milliliters of lysis buffer solution): 50 µg Trypsin inhibitor 1-S from soybean, 10 µg aprotinin, 10 µg leupeptin, and 50 µg phenylmethylsulfonyl fluoride (Sigma Aldrich). The homogenate was centrifuged at 40,000 × g at 4°C for 45 min, after which the supernatant was removed and measured by the BioRad (Hercules, CA) protein assay (Bradford method) with Lyophilized Bovine Plasma Gamma Globulin as the standard.

Western blots. Western blots were run on 10% and 4–12% Tris-Glycine PAGEr gold precast gels (Cambrex, Rockland, ME). Primary antibodies to Ppp3ca for Western blots were (1) N-terminal, epitope CDPKLSTTDRVV (1:1000) (Chemicon, Temecula, CA) and (2) C-terminal (1:1000), epitope MPSDANLNSINKALASETNGTDSNG (Claude Klee, National Cancer Institute, National Institutes of Health, Bethesda, MD). Anti-rabbit horseradish peroxidase conjugated secondary antibody with the Enhanced chemiluminescent (ECL) Western Blotting Analysis System (Amersham Biosciences, Buckinghamshire, England).

CN activity assay. CN enzymatic activity was measured with the BioMol CN activity kit (BioMol, Plymouth Meeting, PA). The Protein Kinase A Regulatory Subunit II-phosphopeptide (BioMol) was used as a highly specific substrate for CN, and the detection of free phosphate released from RII by CN was measured by the malachite green dye reaction. CN activity was determined as phosphate released from RII in the presence of okadaic acid minus phosphate released in the presence of okadaic acid plus EGTA. Reactions were terminated after 30 min, and absorption was read on a microtiter plate reader at 620 nm.

Immunohistochemistry. Paraffin sections were deparaffinized by xylene and ethanol solutions (100, 95, and 70% ethanol and deionized water), with citrate buffer (pH 6.0) used for antigen retrieval. After rinsing by phosphate-buffered saline (PBS) and PBS with 0.1% Tween 20, the slides were immersed in a blocking solution of 5% normal goat serum in PBS. The slides were incubated overnight at 4°C with the primary polyclonal C-terminal antibody Ppp3ca (1:200) (Claude Klee) or the primary polyclonal antibody for Src suppressed C kinase substrate (SSeCKS) (1:200) (Irwin Gelman, Roswell Park Cancer Institute, Buffalo, NY), and after rinsing, exposed to the secondary Cy3-conjugated polyclonal antibody for 1 h. Photographs were taken using the digital camera (SPOT, Diagnostic Instruments, Sterling Heights, MI) on a fluorescence microscope (Nikon Labophot-2, Melville, NY).

Inductively coupled plasma-mass spectrometry. Testicular extracts were assessed for cadmium accumulation via ICP-MS (Agilent 7500c series; Agilent Technologies, Santa Clara, CA). Fourteen of the 24 testes that were assessed for cadmium damage were also assayed for cadmium content (one testis was used per assay). Testes were taken from −80°C storage and homogenized with an acid-washed pestle in 2.5 ml of 3% HNO_3. The homogenate was heated at 120°C in a dry heat block for 2.5 days until completely dry. Dry weight was obtained by subtracting the weight of the preweighed tube from the weight of the tube with dry homogenate. In total, 2.5 ml of 3% HNO_3 was added to each tube, tubes were reweighed, and placed on a heat block for 5 min at 120°C. The solution was decanted to 14 ml polypropylene tubes, and cadmium content was measured by ICP-MS. Selectivity and sensitivity parameters for the equipment may be found in supplementary data.

Graphs and figures. Figures were made with Adobe Photoshop Creative Suite 2 (San Jose, CA) or with GraphPad Prizm 4 software (San Diego, CA). Statistical significance parameters were determined with Prizm 4 software (Motulsky, 2003) using the Mann-Whitney or Kruskal-Wallis statistical tests, with a p value ≤ 0.05 considered to be significant.

RESULTS

Cdm Gene Fine Mapping Experiments

Our fine mapping experiments, initiated in 1998, narrowed the *cdm* region to 1.97 Mb and seven *cdm* gene candidates between Intv6 and D3Mit349 (Fig. 1A). This was accomplished by identifying recombinations in a 2.1-cM region between proximal flanking marker D3Nds2 (64.1 cM) and distal marker D3Mit291 (66.2 cM; MGD, 2006) from a total of 1830 F_2 mice from a BXD F_1 cross (Fig. 1A). Seventeen informative recombinant F_2 mice were generated: since the gene is recessive, only mice that were homozygous C57BL/6 (*cdm*/*cdm*, resistant) at one marker and heterozygous (+/*cdm*, sensitive) at the other were informative. We designed new polymorphic microsatellite markers for fine mapping between D3Nds2 and D3Mit291 (supplementary data), which enabled us to eliminate 3.6 Mb from the *cdm* region. Regions could be eliminated because a locus with a homozygous C57BL/6 genotype on a mouse with a sensitive phenotype or heterozygous genotype with a resistant phenotype was incompatible with the presence of the *cdm* gene (Fig. 1B).

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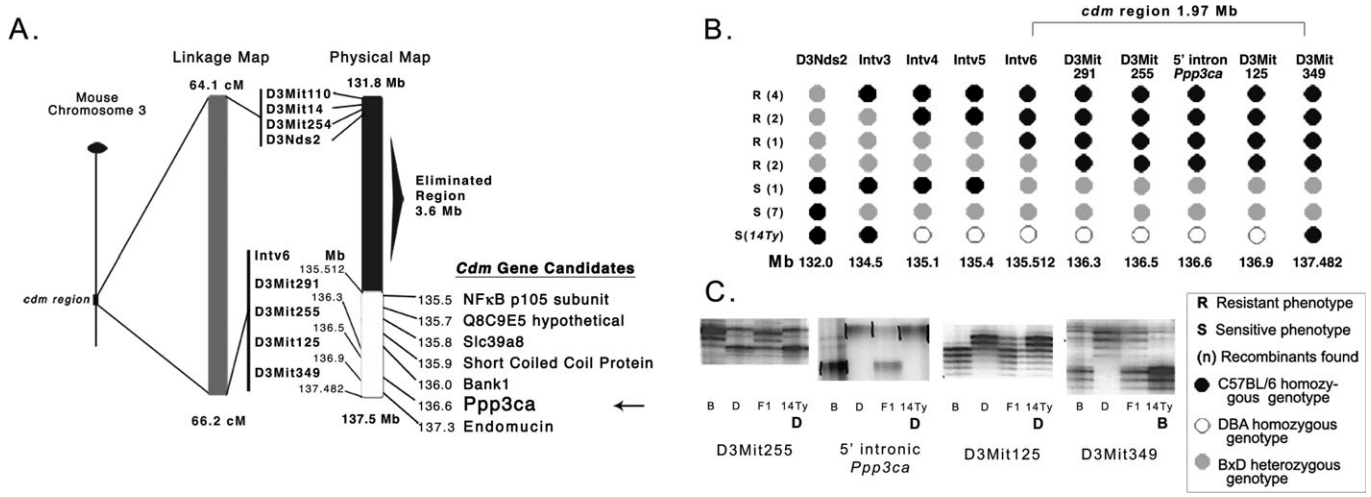

FIG. 1. (A) Linkage and physical maps used to fine map the *cdm* gene. The region was narrowed to 1.97 Mb and seven candidate genes. *Ppp3ca* is the *cdm* candidate that was investigated in the current study. (B) Haplotypes of informative recombinant F_2 (BXD) mice and RI strain BxD*14Ty* using polymorphic microsatellite markers. (C) PCR products of RI BxD*14Ty* genotyping showing D3Mit349 as the *cdm* gene region distal boundary.

A previous study by Dalton *et al*. (2000) found that RI BXD strain *14Ty* had a double recombination between D3Mit110 and D3Mit255 (Fig. 1B), which established the proximal and distal boundaries, respectively, for the *cdm* region (Dalton *et al*., 2000). To confirm the *cdm* distal boundary, we genotyped RI*14Ty* with a custom-made polymorphic microsatellite marker for *Ppp3ca*. We discovered that the DNA of RI*14Ty* was actually DBA at this locus (Fig. 1C). RI*14Ty* also genotyped as DBA at the D3Mit255 and D3Mit125 loci and did not genotype as C57BL/6 until D3Mit349, adding 1 Mb to the region containing the *cdm* gene (Fig. 1C).

Protection from Cadmium-Induced Testicular Toxicity by FK506

As an initial test of the hypothesis that *Ppp3ca* was the *cdm* gene, we administered an inhibitor of CN, FK506, before cadmium chloride injection. FK506 conferred complete or nearly complete protection from cadmium-induced testicular toxicity (Figs. 2A–D). Low- to mid-range cadmium-induced testicular damage is shown in Figure 2B. Prior administration of FK506 led to protection (Fig. 2C) of both vascular endothelial and seminiferous epithelial (e.g., Sertoli cell) toxic endpoints. Two doses of cadmium chloride were utilized to determine the efficacy of FK506 for antagonizing cadmium-induced testicular damage. The 2 mg/kg $CdCl_2$ dose was close to a threshold dose for many pathological parameters, with several animals at this dose sustaining no damage in any category (Fig. 2D); therefore, the demonstration of protection from 2 mg/kg $CdCl_2$ of cadmium at the 24-h time point was difficult ($p = 0.4819$; Fig. 2D). A 2.5 mg/kg $CdCl_2$ dose was a more robust toxic dose (Fig. 2D), with cadmium damage increasing to levels that allowed detection of antagonism by FK506 ($p < 0.0001$).

Increasing the dose from 2.0 to 2.5 mg/kg $CdCl_2$ resulted in higher damage in every category, with hemorrhage and loss of Sertoli cells displaying the most striking differences (Fig. 3). The 2.0 mg/kg $CdCl_2$ produced hemorrhage in only two of nine animals (22%), compared with 12 of 12 (100%) for animals in the 2.5 mg/kg treatment group. Likewise, the percentage of animals that sustained loss of Sertoli cells increased from 11% at the lower dose to 83% at the higher dose (Fig. 3). FK506 significantly reduced the vascular damage indicated by hemorrhage and edema ($p < 0.0001$), as well as epithelial Sertoli cell barrier breakdown and loss of Sertoli-basement membrane connections (loss of Sertoli cells) ($p < 0.0001$; Fig. 3B). The significant reduction ($p < 0.002$) of multinucleated cells by FK506 using 2.5 mg/kg $CdCl_2$ indicated protection of the Sertoli cell actin-based bridges that form between developing germ cells (Fig. 3B). Except for one mouse that sustained substantial cadmium damage in spite of FK506 pretreatment, the FK506-treated animals scored 0 or 0.5 in all categories (Fig. 3B). Of the four FK506-only-treated animals, one had testicular damage (Fig. 3D), with a relatively high score of 2 for loss of Sertoli cells and 0 in all other categories.

ICP-MS Verified Cadmium in Testis

To determine whether the antagonism of cadmium-induced testicular toxicity by FK506 was due to changes in cadmium distribution, we measured testicular cadmium levels at the 24-h time point in both cadmium-only-treated animals and FK506-pretreated animals. An equal amount of cadmium was detected in the testes of both groups of animals, as measured by ICP-MS (Fig. 4). The cadmium concentration in the testis of cadmium-only-treated animals versus cadmium with FK506 pretreatment was (mean ± SE) 1.16 ± 0.083 versus 1.29 ± 0.047 ng of cadmium/mg dry weight testis, respectively, which was

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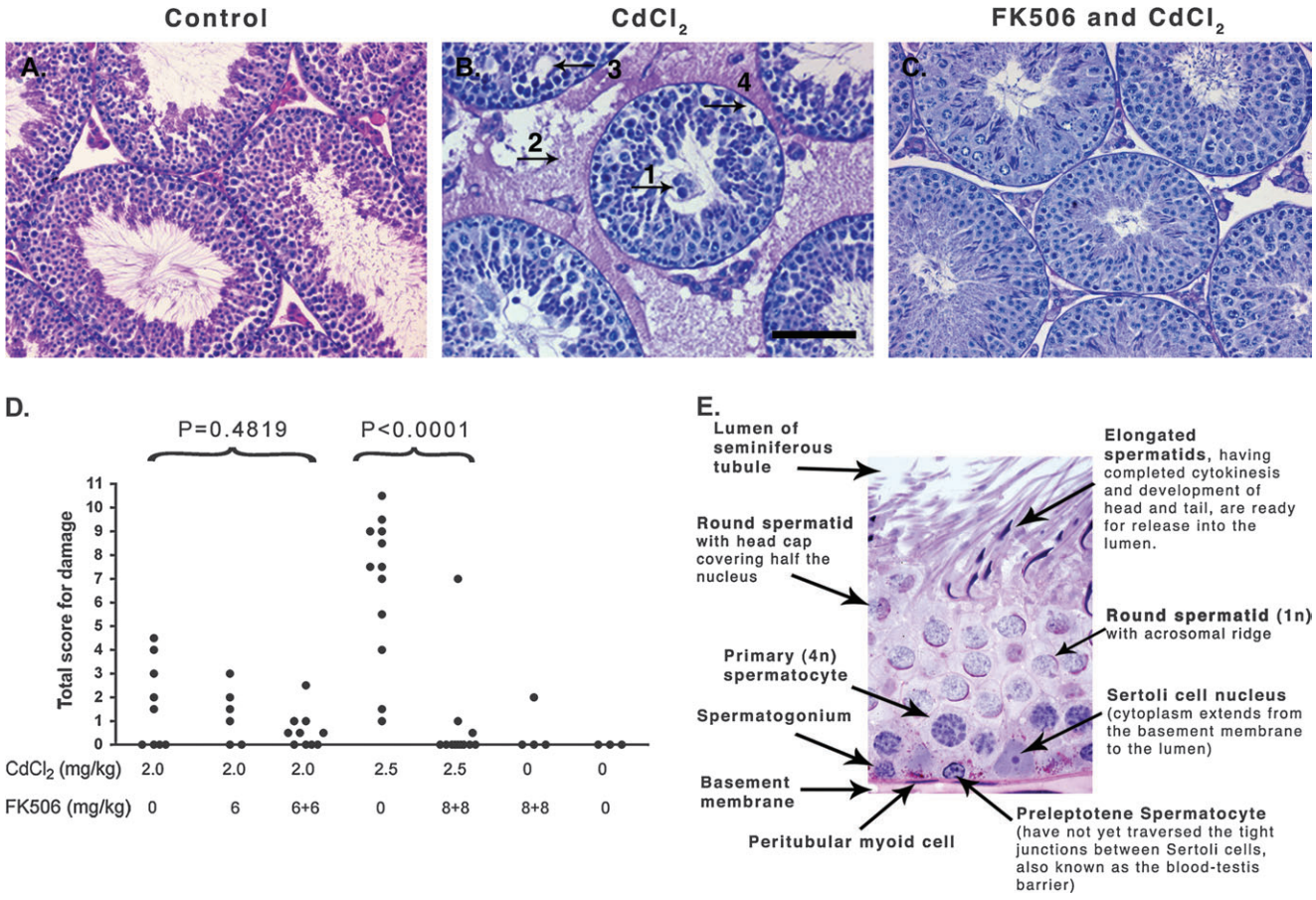

FIG. 2. Comparisons of testis sections at 20× magnification from 8-week-old SWV mice (A–C). (A) Untreated control (B) 24 h after 2.5 mg/kg $CdCl_2$ sc injection. Line is 100 microns. Damage includes (1) premature release of immature germ cells into the lumen, (2) PAS+ material in the interstitium, (3) Sertoli cell vacuolization, and (4) detachment of Sertoli cells from the basement membrane (C) 24 h after 2.5 mg/kg $CdCl_2$ sc injection, with prior injection of 8 mg/kg (×2) FK506, demonstrating protection by FK506. (D) Quantification of damage due to $CdCl_2$ and protection by FK506 in SWV mice. Each data point represents the sum of the scores of the five categories (see "Materials and Methods" section) describing damage due to $CdCl_2$ for an individual mouse testis. (E) Cross section of rat testis seminiferous tubule in stage VII of the seminiferous epithelial cycle containing several generations of germ cells in different stages of development.

a nonsignificant difference ($p = 0.2055$). The testes that were analyzed for cadmium content were from a subset of those that had been previously scored histologically for damage. The one FK506-pretreated animal that was not protected by FK506 did not have more cadmium in the testis.

Rapamycin Control Experiment

To determine if the antagonism of cadmium-induced testicular toxicity by FK506 was due to a general immunosuppression or a CN-specific pathway, we administered rapamycin prior to $CdCl_2$. Rapamycin, an immunosuppressant that blocks the T-cell response through mammalian target of rapamycin inhibition (Sehgal, 2003), did not provide protection with any treatment regimen, including single doses of 3, 5, 10, or 12 mg/kg administered 1 h before 2.5 mg/kg $CdCl_2$ or double doses of 5 (×2), 6 (×2), 8 (×2) mg/kg, 4 h and 1 h prior to 2.5 mg/kg $CdCl_2$. The effective immunosuppressant dosage of rapamycin is approximately equal to that of FK506 (Vu *et al*., 1997).

Knockout Mouse Had Endogenous Defects in Spermatogenesis

Because chemical inhibition of CN antagonized cadmium-induced testicular toxicity, we obtained a mouse model with genetic inactivation of *Ppp3ca* (the *cdm* candidate gene) to determine whether cadmium-induced testicular toxicity would also be antagonized under these conditions. However, the $Ppp3ca^{-/-}$ mice had many testicular problems, possibly due to the loss of Ppp3ca during development, as well as during adulthood (Fig. 5). Therefore, while not being a good model for testing the effect of cadmium, these mice certainly showed the importance of *Ppp3ca* in the testis, which has not been previously reported.

Many $Ppp3ca^{-/-}$ mice died before sexual maturity, which occurred by 7 weeks of age, making it difficult to obtain mice at an appropriate age for testing the effect of cadmium. A total of four $Ppp3ca^{-/-}$ mice were examined histologically: two no-cadmium controls, one with a 3 mg/kg $CdCl_2$ injection, and

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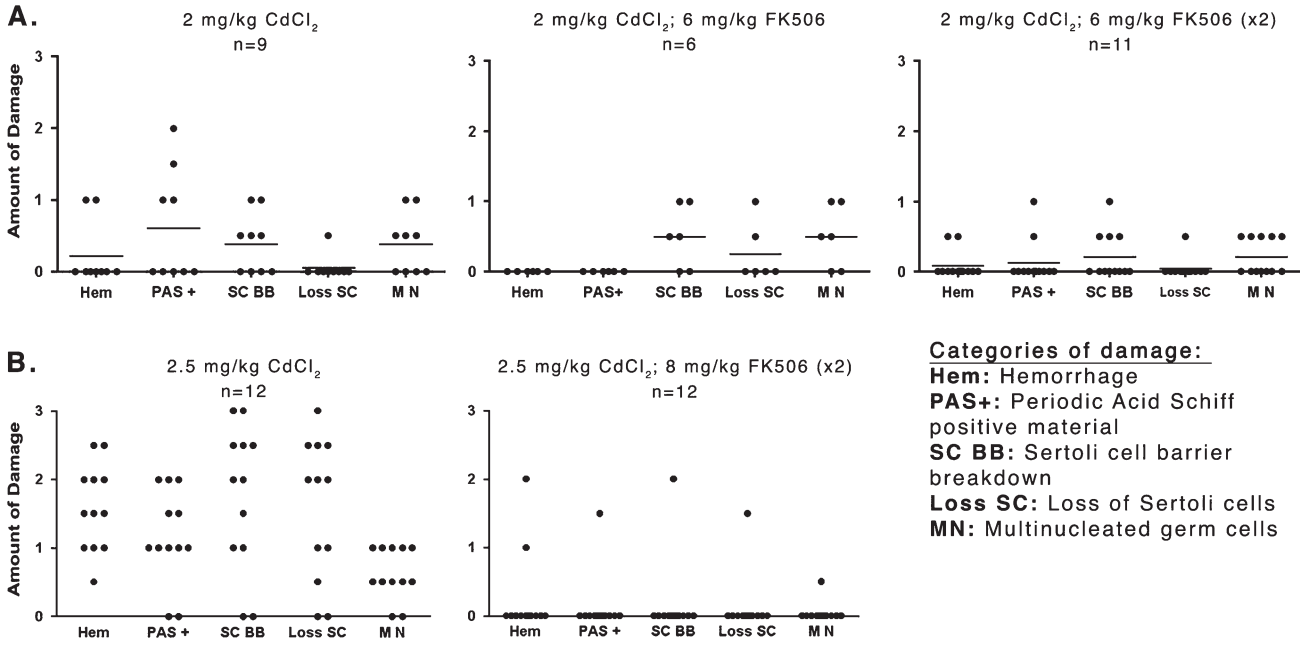

FIG. 3. Individual category scores for testis sections. (A) Details of damage with 2 mg/kg $CdCl_2$ and two different prior doses of FK506. (B) Details of damage with 2.5 mg/kg $CdCl_2$ and a prior dose of FK506.

one with 6 mg/kg FK506 (×2) prior to a 2 mg/kg $CdCl_2$ injection. In untreated *Ppp3ca*$^{-/-}$ mice, germ cells either did not complete meiosis or, if completed, developed into misshapen later-stage spermatids (Lisa J. Martin, Rex A. Hess, Michael D. Collins). As in the cadmium-treated wild-type animals, several multinucleated germ cells were present, indicating a failure of Sertoli cell cytoplasmic bridge management (Fig. 5C). The smaller seminiferous tubule diameter in *Ppp3ca* $^{-/-}$ mouse testis (Fig. 5B), compared with wild type (Fig. 5A), suggested that the increase in interstitial space was due to shrinkage of seminiferous tubules. Cadmium administration in the *Ppp3ca* $^{-/-}$ animal produced hemorrhage, PAS+ material, Sertoli cell barrier breakdown, and loss of Sertoli cells. Administration of FK506 did not protect from cadmium toxicity in this mouse.

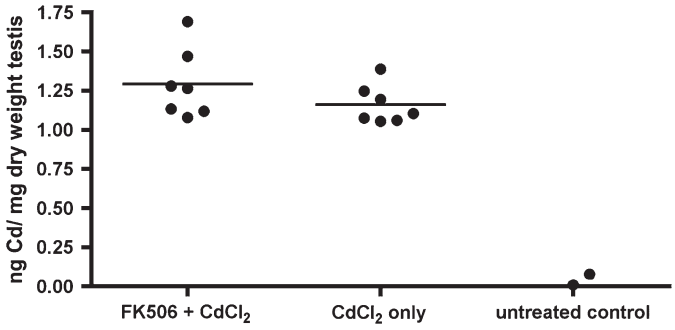

FIG. 4. ICP-MS measurements of cadmium metal in mouse testis of cadmium-only-treated mice (2.5 mg/kg) compared with those pretreated, and protected, by FK506. The differences between the two treated groups were not significant ($p = 0.2055$).

Western Blotting, CN Activity, and Immunohistochemistry

Testicular protein levels, phosphatase activity, and localization of Ppp3ca in the testis were assayed in order to determine whether strain differences in these parameters in response to cadmium administration might contribute to the *cdm* phenotype. Western blots revealed that the levels of Ppp3ca protein expression did not change appreciably in the testes of resistant C57BL/6 mice or in sensitive SWV mice in response to cadmium or FK506 at 24 h after injection (Figs. 6A and 6B). In brain protein lysates, both N- and C-terminal antibodies detected proteins of 60 kDa, which is the expected size of the Ppp3ca subunit (Figs. 6A and 6B; lane 9). In the testis lysate, however, the N-terminal antibody detected one band of approximately 35 kDa (Fig. 6A); the C-terminal antibody detected several bands, none the same size as that found in the brain, likely indicating splice variations of Ppp3ca in the testes. The cadmium-treated SWV (sensitive mice) had a slightly

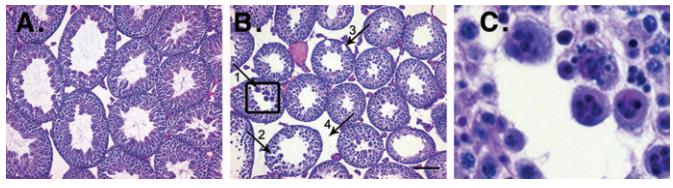

FIG. 5. Comparison of (A) wild-type testis with (B) *Ppp3ca*$^{-/-}$ testis (both 10× magnification). The *Ppp3ca*$^{-/-}$ testis has (1) giant multinucleated cells; (2) gaps between some Sertoli cells; (3) vacuoles inside Sertoli cells; and (4) an increase in intertubular space and a severe reduction of mature spermatids. Line in lower right corner is 100 microns. (C) Enlarged area of the box in (B) showing multinucleated germ cells in the lumen of the seminiferous tubule.

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reduced Ppp3ca expression (Fig. 6B; lane 6) detected by the C-terminal antibody; however, the actin control band was also lower (Fig. 6C). Because the dilution factor for cadmium-only-treated lysates (which were very red due to hemorrhage) was higher than for other treatment groups, it was not known whether reduced Ppp3ca and actin protein levels were real or due to an artificially high absorbance reading.

We measured CN phosphatase activity in order to detect possible strain differences in protein function with and without cadmium exposure (Fig. 6D). CN phosphatase activity in control mouse testis lysates was found to be slightly higher, although not significantly, for the resistant C57BL/6 mice. At 24 h after 2.0 mg/kg $CdCl_2$ injection, CN activity was decreased compared with nontreated controls in all strains but the decrease was significant only in SWV mice; DBA phosphatase activity had a nearly significant decrease due to cadmium ($p = 0.054$). Other experiments using testis tissue taken 2 and 4 h after 6 mg/kg $CdCl_2$ sc injection displayed the same trend of higher activity in the untreated C57BL/6 mice compared to the untreated SWV mice and decreasing activity due to cadmium in both strains (data not shown).

Ppp3ca was shown via immunohistochemistry (IHC) to be highly expressed in the testis in vascular endothelial cells and at the acrosomal ridge in round spermatids (1*n*) (Figs. 7A and 7B). Ppp3ca protein expression may be germ cell–specific at the acrosome or may also be expressed in the surrounding Sertoli cell. The expression of Ppp3ca could not be detected above background in pachytene primary spermatocytes (4*n*) (Fig. 1E for histology). However, because the abnormalities in the *Ppp3ca*$^{-/-}$ mice originated at the pachytene spermatocyte stage of development (data not shown), the protein must be expressed at low yet critical levels in either these developing germ cells or the Sertoli cells. Ppp3ca was highly expressed in endothelial tissue (Figs. 7A and 7B). In the round spermatids of cadmium-treated animals, Ppp3ca was shown to be delocalized compared with control at 24 h after injection of 2.5 mg/kg (Figs. 7E and 7F). The delocalization was prevented in the FK506-treated animals (Fig. 7G).

The A Kinase anchoring protein (AKAP), Src Suppressed C Kinase Substrate (SSeCKS), was assayed via IHC to ascertain whether it might be considered as a CN scaffolding protein. SSeCKS colocalized with Ppp3ca in round spermatids and in vascular endothelial tissue (Fig. 7E), indicating that it may serve as a scaffold for CN and other AKAP-associated proteins in the testis. SSeCKS also delocalized from the membrane of round spermatids due to cadmium treatment (Figs. 7H and 7I), and the delocalization was prevented by FK506 (Fig. 7J). SSeCKS was much more widely distributed than Ppp3ca with clear expression at Sertoli cell junctions (Fig. 7C) and in the apical part of Sertoli cells in tubules with late-stage spermatids (not shown).

Congenic Mice

To determine the relative contribution to the cadmium-induced toxicity phenotype of the chromosome 3 region containing the *cdm* gene versus the genetic background of the mouse, congenic mice with chromosome 3 regions from a sensitive strain on the resistant strain background were tested. Two congenic mouse lines, one with a small region of chromosomal DBA/2J (sensitive) spanning the *cdm* region (BDChr3M) and the other with DBA/2J chromosomal DNA distal to the *cdm* region (BDChr3D) on a C57BL/6J (resistant) background displayed distinctly different responses to cadmium (Fig. 8). The congenic line, BDChr3M, with 60 Mb of DBA chromosomal DNA spanning the *cdm* region sustained severe damage from 6 mg/kg $CdCl_2$. This was compared with the other

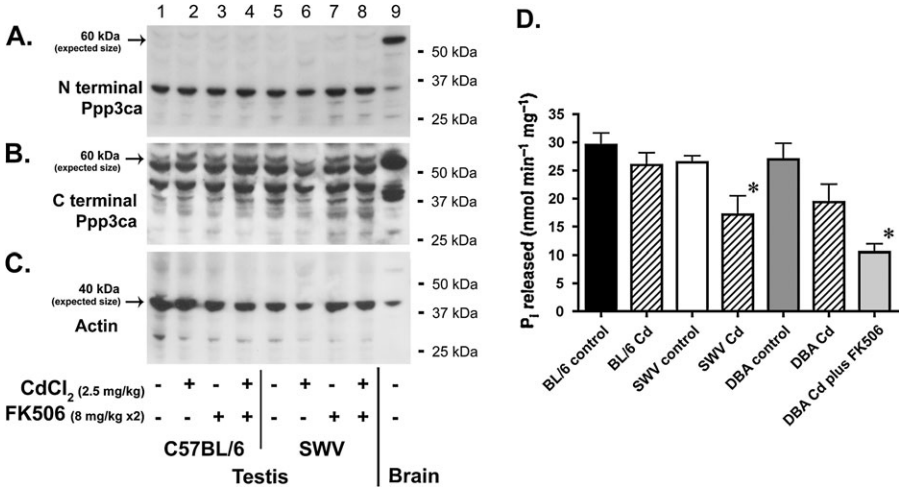

FIG. 6 Western blots of Ppp3ca and CN phosphatase activity from protein lysates of tissue taken 24 h after treatment. Lanes 1–8: 25 μg total protein from testis; Lane 9: 7.5 μg total protein from brain of C57BL/6 male. (A) N-terminal antibody showing 35-kDa band from testis tissue and the expected 60-kDa band from brain tissue. (B) C-terminal antibody showing multiple bands of different sizes. (C) Actin at 40 kDa. Blot for actin was on the same membrane as the C-terminal antibody (stripped). (D) CN activity assay, $n = 6$ for each group, with a significant decrease between control and cadmium treatment for SWV mice ($p < 0.05$). Between DBA control and DBA plus cadmium, $p = 0.054$. The protein lysates were from testes taken 24 h after 2 mg/kg $CdCl_2$ sc injection.

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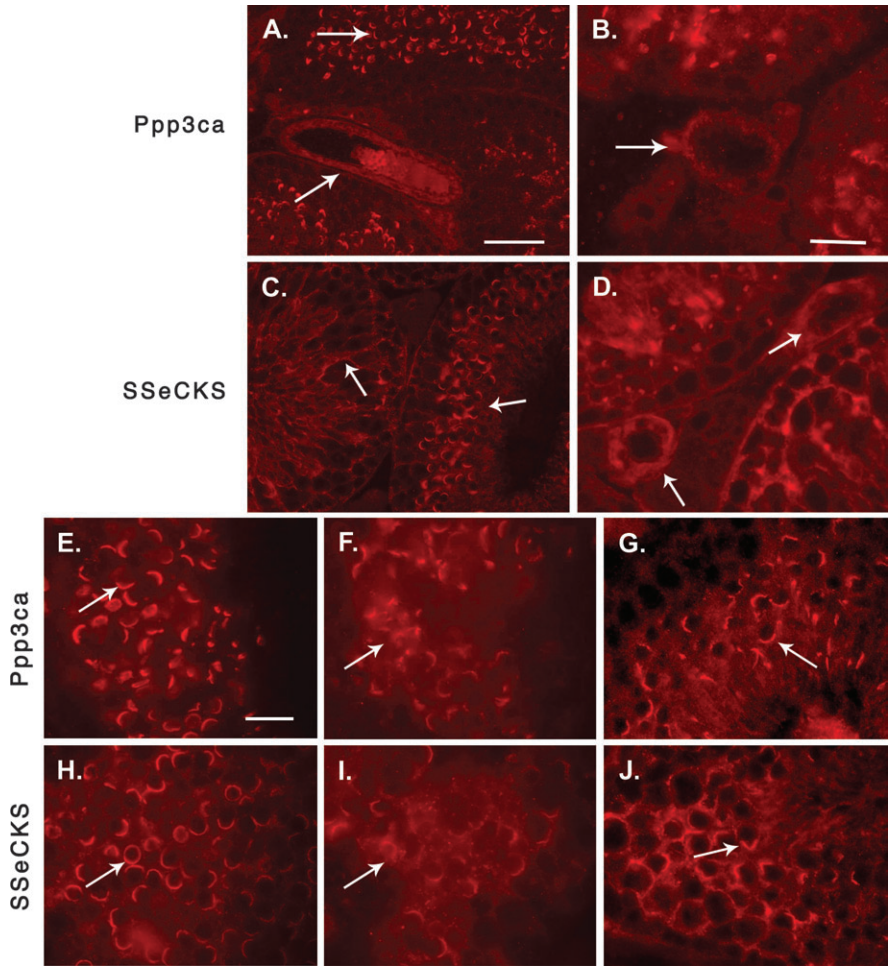

FIG. 7. Immunohistochemistry with SWV mouse testis sections showing colocalization of Ppp3ca with the AKAP SSeCKS. (A) 40× magnification; Ppp3ca prominently expressed in round spermatids (upper arrow) vascular endothelial cells (lower arrow). Line represents 50 microns for (A) and (C). (B) 100× of Ppp3ca in capillary (arrow). Line represents 10 microns. (C) 40× of two seminiferous tubules with SSeCKS expression. Left arrow indicates expression at Sertoli cell junctions, and right arrow indicates expression in round spermatids. (D) 100×; arrows indicating SSeCKS expression in capillaries (both arrows). (E and H) 100× of Ppp3ca and SSeCKS, respectively, in acrosomal ridge of round spermatids in control testis. Line represents 15 microns for (E–J). (F and I) delocalization due to 2.5 mg/kg sc $CdCl_2$ injection, 24 h after injection. (G and J) Rescue of delocalization by FK506, 24 h after 2.5 mg/kg $CdCl_2$ sc injection.

congenic line, BDChr3D, essentially a C57BL/6 mouse with 10 Mb of DBA DNA on mouse chromosome 3, which was completely resistant to this high dose (Fig. 8). The reversal of the cadmium-induced testicular damage phenotype was therefore achieved by exchanging less than 60 Mb of genomic DNA on mouse chromosome 3 between the two strains.

Real-Time PCR and Sequencing Experiments

We conducted real-time PCR experiments in order to detect possible strain differences at the expression level and did not detect significant expression differences between strains for any of the seven genes in the 1.97 Mb region, including *Ppp3ca* (data not shown). Once it was discovered that FK506 prevented the damage in SWV mice and in Black Swiss mice, the background for the *Ppp3ca*$^{-/-}$ mice (data not shown), a concerted effort was undertaken to find a strain difference that would support the claim that *Ppp3ca* was the *cdm* gene. This included sequencing the 1580 bases of *Ppp3ca* cDNA for C57BL/6, SWV, and DBA mice, with six different sets of primers covering the entire cDNA sequence. Additionally, at least 90 bases of 5′ and 3′ intronic DNA from all 14 exons were sequenced, with no differences between strains (data not shown). The only region that was not successfully sequenced was the 5′ intronic region before exon 1 that included the promoter. Due to the 80% GC content in that region, a reliable PCR product could not be generated, even though several different primer combinations and conditions were tested.

DISCUSSION

In the process of searching for the *cdm* gene, a gene that confers resistance to cadmium-induced testicular toxicity in

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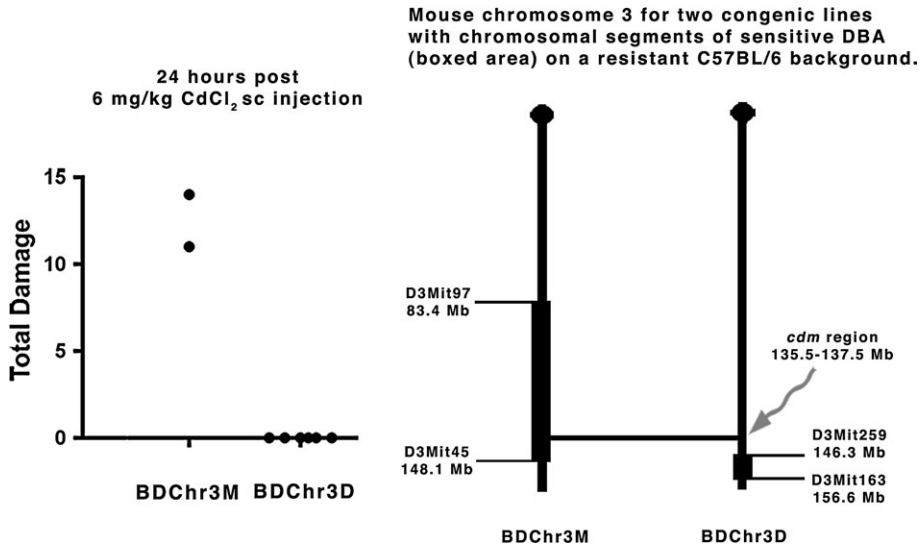

FIG. 8. Comparison of damage in the testis due to 6 mg/kg $CdCl_2$ in congenic mice. The *cdm* gene was located within the DBA chromosomal region on strain BDChr3M and slightly proximal to the DBA chromosomal region on BDChr3D. Mice were homozygous for the DBA regions; all other chromosomes were homozygous C57BL/6.

mice, we discovered that FK506, an inhibitor of CN, prevented cadmium-induced damage in mouse testes. Prior injections of FK506 prevented testicular vascular endothelial, epithelial, and germ cell toxicity due to cadmium chloride administration. A different immunosuppressant, rapamycin, did not prevent damage, indicating that FK506 was working through a CN-specific pathway. Most importantly, FK506 protected testicular tissue without changing the amount of cadmium that reached the testes. An attempt to reproduce the chemical antagonism of cadmium-induced testicular toxicity with a genetic model led us to the finding that *Ppp3ca*$^{-/-}$ mice had endogenous defects in spermatogenesis. The formation of multinucleated germ cells in the *Ppp3ca*$^{-/-}$ mice, as well as in cadmium-treated mice, was likely due to disruption of the Sertoli cell actin cytoskeleton.

FK506 antagonized cadmium-induced endothelial, as well as seminiferous epithelial, toxicity. Cadmium has long been known to target the vascular endothelium in the testes (Gunn and Gould, 1970), and it has been suggested that all other damage is secondary to the edema-related constriction of the vasculature, causing ischemia and subsequent reperfusion (Chiquoine, 1964). Typically, studies drawing those conclusions have used either very high doses of cadmium chloride (6–10 mg/kg) (Chiquoine, 1964; Gunn and Gould, 1970) or low doses administered for a longer duration (1 mg/kg daily for 4 weeks) (Haffor and Abou-Tarboush, 2004). In contrast, the single dose 2.5 mg/kg $CdCl_2$, used in the current study to demonstrate protection by FK506, was a low- to mid-range dose, and the 24-h time point detected relatively rapidly-induced damage.

The effects of cadmium on the seminiferous epithelium appeared to be mediated through the Sertoli cell. Cadmium administration increased Sertoli cell barrier breakdown and loss of Sertoli cells, both of which were prevented by FK506. Detachment of Sertoli cells from the basement membrane of seminiferous tubules was significantly elevated only with the higher dose of 2.5 mg/kg $CdCl_2$ treatment, indicating more severe toxicity, whereas Sertoli-Sertoli junctional damage was apparent at both 2.0 and 2.5 mg/kg doses. In several mice at the 2.0 mg/kg dose, Sertoli-Sertoli junctional damage appeared without any accompanying damage to the vasculature. Loss of connections between cells arises through perturbations of transcellular proteins that are anchored by the internal cell cytoskeleton (Pelletier and Byers, 1992; Wong *et al*., 2005). Consistent with evidence that cadmium disrupts intercellular junctions in the kidney by targeting the internal cytoskeleton (Gennari *et al*., 2003), the junctional perturbations in the testes could also originate with perturbations of the cytoskeleton. The protection of intercellular junctions afforded by FK506, while not shown by the current study to be directly related to protection of the cytoskeleton, suggested that CN plays a key role in junctional dynamics in the testis, including both the seminiferous epithelium and the vasculature.

On the other hand, the formation of multinucleated germ cells by cadmium treatment and prevention by FK506 provided more direct evidence that cadmium disrupted the actin cytoskeleton of the Sertoli cell and that CN was likely involved. Multinucleated germ cells are ''symplasts'' of germ cells and are formed by the degradation of normal Sertoli cell cytoplasmic indentations that play a role in germ cell division (Russell *et al*., 1987; Weber *et al*., 1988). The indentations are made structurally sound by Sertoli cell actin filaments that form narrow channels, or bridges, between developing germ cells of a clonal unit. TEX14, a germ cell–specific protein, is also

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involved in the formation of these intercellular bridges, but the *Tex14*$^{-/-}$ mouse testis does not show symplast formation (Greenbaum *et al*., 2006); therefore, Sertoli cell cytoplasm appears to be responsible for maintenance of the bridges after their formation. The importance of actin in the maintenance of intercellular bridges was highlighted by the finding that administration of the actin disruptor, cytochalasin D, resulted in the formation of multinucleated germ cells in rat testes (Russell *et al*., 1987). The current study found that short-term inhibition of CN by FK506 prevented the opening of these bridges by cadmium. This discovery, along with the finding of multinucleated germ cells in the *Ppp3ca*$^{-/-}$ mouse, strongly suggested that CN is involved in the maintenance of the Sertoli cell actin cytoskeleton.

In looking for possible mechanisms by which inhibition of CN might lead to protection of cellular junctions, protein complexes involved in cytoskeletal maintenance were considered. AKAPs are large scaffolding proteins that tether kinases and phosphatases at subcellular locations and are associated with the cytoskeleton in the brain, kidney, and pancreas (Dell'Acqua *et al*., 2006; Jo *et al*., 2001; Lester *et al*., 2001). An important AKAP in the testis is SSeCKS, which is a 280-kDa scaffolding protein with binding sites for CN, Protein Kinase A, and Protein Kinase C (Erlichman *et al*., 1999). SSeCKS colocalized in the current study with Ppp3ca in testicular endothelial tissue and at the Sertoli cell-round spermatid (1*n*) interface. Unlike Ppp3ca, SSeCKS was also prominent at Sertoli-Sertoli junctions. However, it cannot be said with certainty that Ppp3ca is not expressed at Sertoli-Sertoli junctions. The fact that meiosis was severely perturbed in the *Ppp3ca*$^{-/-}$ mouse as early as the pachytene spermatocyte (4*n*) stage (Lisa J. Martin, Rex A. Hess, Michael D. Collins) indicated that Ppp3ca must also be expressed at a level not detected in the current study in Sertoli cells, early stage germ cells, or at Sertoli-germ cell junctions. The colocalization of Ppp3ca and SSeCKS allows for speculation about how FK506 might protect from cadmium toxicity. If cadmium does interfere with cytoskeletal architecture through an SSeCKS/CN pathway, then a short-term inhibition of CN by FK506, preventing downstream signaling that leads to cytoskeletal disruption, might explain the protection of testicular endothelial and epithelial cellular junctions by FK506.

A transgenic mouse showed that the identified *cdm* gene, S*lc39a8*, is critical for the induction of cadmium-induced toxicity in the testes (Wang *et al*., 2007). However, certain aspects of the *cdm* phenotype cannot be explained by increased transport of cadmium into testicular endothelial cells, leaving open the possibility that *Ppp3ca* may also be involved in some aspects of toxicity, namely, seminiferous epithelial toxicity with lower doses of cadmium. For instance, if damage to testicular endothelial cells due to increased transport in sensitive strains is responsible for Sertoli and germ cell damage, how does damage to Sertoli and germ cells occur in the absence of endothelial damage? In addition, why are Sertoli cells of resistant strains not affected by cadmium at any dose? High doses of $CdCl_2$ result in nearly equivalent levels of cadmium in the testis of resistant strains compared with sensitive strains (Liu *et al*., 2001), and therefore lack of exposure to cadmium in resistant strains cannot be the explanation for Sertoli cell resistance. Cadmium likely has multiple entryways into cells (Bridges and Zalups, 2005), including active transport by the DMT-1 transporter (Leazer *et al*., 2002) and possibly other metal transporters (Augustine *et al*., 2005). If cadmium uses *Slc39a8* to enter and cause toxicity in endothelial cells, then it also likely uses *Slc39a8* to enter and cause toxicity in Sertoli cells. *Slc39a8* is highly expressed in Sertoli cells of both resistant and sensitive strains (Dalton *et al*., 2005), and cadmium was found to be transported at the same rate into Sertoli cells of sensitive and resistant strains of mice (King *et al*., 1999), leaving unanswered the question as to why Sertoli cells in resistant strains are unaffected by cadmium at any dose.

The proximity of *Ppp3ca* and *Slc39a8*, genes that are separated by less than 1 Mb, and the findings that (1) an inhibitor of CN antagonized cadmium-induced testicular toxicity, (2) Ppp3ca was critically important in spermatogenesis, and (3) Ppp3ca was highly expressed during the stages of the seminiferous epithelium most sensitive to a low dose of $CdCl_2$ suggested that *Ppp3ca* warrants further study before discounting its possible role in the *cdm* phenotype. It might be possible, for example, that the toxicity arising from lower doses of cadmium, specific to the seminiferous epithelium, is mediated by Ppp3ca.

Independent of the *cdm* gene question, the current study provides important contributions to reproductive toxicology. First, it contributes to the understanding of mechanisms for cadmium-induced testicular toxicity by demonstrating that inhibition of CN antagonized cadmium toxicity in mouse testes. Of equal importance is the contribution to the evolving body of knowledge about intercellular junctional dynamics in the testes. The current study showed that CN is likely to participate in Sertoli-Sertoli junctional dynamics, but also, by virtue of FK506 protection of endothelial cellular barriers, that CN is likely to participate in endothelial junctional dynamics, as well. Finally, the defects in cytoskeletal architecture leading to multinucleated germ cell formation, induced by the absence of functional Ppp3ca and by cadmium administration, but prevented by FK506, indicated that CN likely plays a role in the maintenance of the Sertoli cell actin cytoskeleton.

SUPPLEMENTARY DATA

Supplementary Data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Augustine, L. M., Markelewicz, R. J., Jr, Boekelheide, K., and Cherrington, N. J. (2005). Xenobiotic and endobiotic transporter mRNA expression in the blood-testis barrier. *Drug Metab. Dispos.* **33,** 182–189.

Barbier, O., Jacquillet, G., Tauc, M., Poujeol, P., and Cougnon, M. (2004). Acute study of interaction among cadmium, calcium, and zinc transport along the rat nephron in vivo. *Am. J. Physiol. Renal. Physiol.* **287,** F1067–F1075.

Bridges, C. C., and Zalups, R. K. (2005). Molecular and ionic mimicry and the transport of toxic metals. *Toxicol. Appl. Pharmacol.* **204,** 274–308.

Chiquoine, A. D. (1964). Observations on the early events of cadmium necrosis of the testis. *Anat. Rec.* **149,** 23–35.

Couse, J. F., Davis, V. L., Tally, W. C., and Korach, K. S. (1994). An improved method of genomic DNA extraction for screening transgenic mice. *Biotechniques* **17,** 1030–1032.

Dalton, T. P., He, L., Wang, B., Miller, M. L., Jin, L., Stringer, K. F., Chang, X., Baxter, C. S., and Nebert, D. W. (2005). Identification of mouse SLC39A8 as the transporter responsible for cadmium-induced toxicity in the testis. *Proc. Natl. Acad. Sci. USA* **102,** 3401–3406.

Dalton, T. P., Miller, M. L., Wu, X., Menon, A., Cianciolo, E., McKinnon, R. A., Smith, P. W., Robinson, L. J., and Nebert, D. W. (2000). Refining the mouse chromosomal location of Cdm, the major gene associated with susceptibility to cadmium-induced testicular necrosis. *Pharmacogenetics* **10,** 141–151.

Dell'Acqua, M. L., Smith, K. E., Gorski, J. A., Horne, E. A., Gibson, E. S., and Gomez, L. L. (2006). Regulation of neuronal PKA signaling through AKAP targeting dynamics. *Eur. J. Cell Biol.* **85,** 627–633.

Dumont, F. J. (2000). FK506, an immunosuppressant targeting calcineurin function. *Curr. Med. Chem.* **7,** 731–748.

Erlichman, J., Gutierrez-Juarez, R., Zucker, S., Mei, X., and Orr, G. A. (1999). Developmental expression of the protein kinase C substrate/binding protein (clone 72/SSeCKS) in rat testis identification as a scaffolding protein containing an A-kinase-anchoring domain which is expressed during late-stage spermatogenesis. *Eur. J. Biochem.* **263,** 797–805.

Gabbiani, G., Badonnel, M. C., Mathewson, S. M., and Ryan, G. B. (1974). Acute cadmium intoxication. Early selective lesions of endothelial clefts. *Lab. Invest.* **30,** 686–695.

Gennari, A., Cortese, E., Boveri, M., Casado, J., and Prieto, P. (2003). Sensitive endpoints for evaluating cadmium-induced acute toxicity in LLC-PK1 cells. *Toxicology* **183,** 211–220.

Gooch, J. L., Guler, R. L., Barnes, J. L., and Toro, J. J. (2006). Loss of calcineurin Aalpha results in altered trafficking of AQP2 and in nephrogenic diabetes insipidus. *J. Cell Sci.* **119,** 2468–2476.

Greenbaum, M. P., Yan, W., Wu, M. H., Lin, Y. N., Agno, J. E., Sharma, M., Braun, R. E., Rajkovic, A., and Matzuk, M. M. (2006). TEX14 is essential for intercellular bridges and fertility in male mice. *Proc. Natl. Acad. Sci. USA* **103,** 4982–4987.

Gunn, S. A., and Gould, T. C. (1970). Specificity of the vascular system of the male reproductive tract. *J. Reprod. Fertil.* **10**(Suppl), 75–95.

Haffor, A. S., and Abou-Tarboush, F. M. (2004). Testicular cellular toxicity of cadmium: Transmission electron microscopy examination. *J. Environ. Biol.* **25,** 251–258.

Hew, K. W., Ericson, W. A., and Welsh, M. J. (1993a). A single low cadmium dose causes failure of spermiation in the rat. *Toxicol. Appl. Pharmacol.* **121,** 15–21.

Hew, K. W., Heath, G. L., Jiwa, A. H., and Welsh, M. J. (1993b). Cadmium in vivo causes disruption of tight junction-associated microfilaments in rat Sertoli cells. *Biol. Reprod.* **49,** 840–849.

Hinrichs, A. S., Karolchik, D., Baertsch, R., Barber, G. P., Bejerano, G., Clawson, H., Diekhans, M., Furey, T. S., Harte, R. A., Hsu, F., *et al.* (2006). The UCSC Genome Browser Database: Update 2006. *Nucleic Acids Res.* **34,** D590–D598.

Iakoubova, O. A., Olsson, C. L., Dains, K. M., Ross, D. A., Andalibi, A., Lau, K., Choi, J., Kalcheva, I., Cunanan, M., Louie, J., *et al.* (2001). Genome-tagged mice (GTM): Two sets of genome-wide congenic strains. *Genomics* **74,** 89–104.

Janecki, A., Jakubowiak, A., and Steinberger, A. (1992). Effect of cadmium chloride on transepithelial electrical resistance of Sertoli cell monolayers in two-compartment cultures–a new model for toxicological investigations of the "blood-testis" barrier in vitro. *Toxicol. Appl. Pharmacol.* **112,** 51–57.

Jo, I., Ward, D. T., Baum, M. A., Scott, J. D., Coghlan, V. M., Hammond, T. G., and Harris, H. W. (2001). AQP2 is a substrate for endogenous PP2B activity within an inner medullary AKAP-signaling complex. *Am. J. Physiol. Renal. Physiol.* **281,** F958–F965.

Karolchik, D., Baertsch, R., Diekhans, M., Furey, T. S., Hinrichs, A., Lu, Y. T., Roskin, K. M., Schwartz, M., Sugnet, C. W., Thomas, D. J., *et al.* (2003). The UCSC Genome Browser Database. *Nucleic Acids Res.* **31,** 51–54.

Kent, W. J. (2002). BLAT–the BLAST-like alignment tool. *Genome Res.* **12,** 656–664.

King, L. M., Banks, W. A., and George, W. J. (1999). Differences in cadmium transport to the testis, epididymis, and brain in cadmium-sensitive and -resistant murine strains 129/J and A/J. *J. Pharmacol. Exp. Ther.* **289,** 825–830.

Klee, C. B., Ren, H., and Wang, X. (1998). Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J. Biol. Chem.* **273,** 13367–13370.

Leazer, T. M., Liu, Y., and Klaassen, C. D. (2002). Cadmium absorption and its relationship to divalent metal transporter-1 in the pregnant rat. *Toxicol. Appl. Pharmacol.* **185,** 18–24.

Lester, L. B., Faux, M. C., Nauert, J. B., and Scott, J. D. (2001). Targeted protein kinase A and PP-2B regulate insulin secretion through reversible phosphorylation. *Endocrinology* **142,** 1218–1227.

Liu, J., Corton, C., Dix, D. J., Liu, Y., Waalkes, M. P., and Klaassen, C. D. (2001). Genetic background but not metallothionein phenotype dictates sensitivity to cadmium-induced testicular injury in mice. *Toxicol. Appl. Pharmacol.* **176,** 1–9.

Merker, H. J., and Gunther, T. (1997). Testis damage induced by zinc deficiency in rats. *J. Trace Elem. Med. Biol.* **11,** 19–22.

MGD. (2006). *Mouse Genome Database (MGD). In Mouse Genome Informatics Web Site.* The Jackson Laboratory, Bar Harbor, ME.

Motulsky, H. (2003). *Prizm 4 Statistics Guide-Statistical Analyses for Laboratory and Clinical Researchers.* GraphPad Software, Inc, San Diego, CA.

Niewenhuis, R. J., and Prozialeck, W. C. (1987). Calmodulin inhibitors protect against cadmium-induced testicular damage in mice. *Biol. Reprod.* **37,** 127–133.

Parizek, J. (1957). The destructive effect of cadmium ion on testicular tissue and its prevention by zinc. *J. Endocrinol.* **15,** 56–63.

Pelletier, R. M., and Byers, S. W. (1992). The blood-testis barrier and Sertoli cell junctions: Structural considerations. *Microsc. Res. Tech.* **20,** 3–33.

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Perrino, B. A., and Chou, I. N. (1986). Role of calmodulin in cadmium-induced microtubule disassembly. *Cell Biol. Int. Rep.* **10,** 565–573.

Prozialeck, W. C., Edwards, J. R., and Woods, J. M. (2006). The vascular endothelium as a target of cadmium toxicity. *Life Sci.* **79,** 1493–1506.

Russell, L. D., Vogl, A. W., and Weber, J. E. (1987). Actin localization in male germ cell intercellular bridges in the rat and ground squirrel and disruption of bridges by cytochalasin D. *Am. J. Anat.* **180,** 25–40.

Sehgal, S. N. (2003). Sirolimus: Its discovery, biological properties, and mechanism of action. *Transplant. Proc.* **35,** 7S–14S.

Setchell, B. P., and Waites, G. M. (1970). Changes in the permeability of the testicular capillaries and of the 'blood-testis barrier' after injection of cadmium chloride in the rat. *J. Endocrinol.* **47,** 81–86.

Sittig, M. (1991). *Handbook of Toxic and Hazardous Chemical and Carcinogens*. Noyes Publications, Park Ridge, NJ.

Stohs, S. J., Bagchi, D., Hassoun, E., and Bagchi, M. (2001). Oxidative mechanisms in the toxicity of chromium and cadmium ions. *J. Environ. Pathol. Toxicol. Oncol.* **20,** 77–88.

Taylor, B. A., Heininger, H. J., and Meier, H. (1973). Genetic analysis of resistance to cadmium-induced testicular damage in mice. *Proc. Soc. Exp. Biol. Med.* **143,** 6.

Valko, M., Morris, H., and Cronin, M. T. (2005). Metals, toxicity and oxidative stress. *Curr. Med. Chem.* **12,** 1161–1208.

Vu, M. D., Qi, S., Xu, D., Wu, J., Fitzsimmons, W. E., Sehgal, S. N., Dumont, L., Busque, S., Daloze, P., and Chen, H. (2003). Tacrolimus (FK506) and sirolimus (rapamycin) in combination are not antagonistic but produce extended graft survival in cardiac transplantation in the rat. *Transplantation* **64,** 1853–1856.

Wang, B., Schneider, S. N., Dragin, N., Girijashanker, K., Dalton, T. P., He, L., Miller, M. L., Stringer, K. F., Soleimani, M., Richardson, D. D., *et al.* (2007). Enhanced cadmium-induced testicular necrosis and renal proximal tubule damage caused by gene-dose increase in a Slc39a8-transgenic mouse line. *Am. J. Physiol. Cell Physiol.* **292,** C1523–C1535.

Wang, X., Culotta, V. C., and Klee, C. B. (1996). Superoxide dismutase protects calcineurin from inactivation. *Nature* **383,** 434–437.

Weber, J. E., Turner, T. T., Tung, K. S., and Russell, L. D. (1988). Effects of cytochalasin D on the integrity of the Sertoli cell (blood-testis) barrier. *Am. J. Anat.* **182,** 130–147.

Wong, C. H., Mruk, D. D., Lui, W. Y., and Cheng, C. Y. (2004). Regulation of blood-testis barrier dynamics: An in vivo study. *J. Cell Sci.* **117,** 783–798.

Wong, C. H., Mruk, D. D., Siu, M. K., and Cheng, C. Y. (2005). Blood-testis barrier dynamics are regulated by {alpha}2-macroglobulin via the c-Jun N-terminal protein kinase pathway. *Endocrinology* **146,** 1893–1908.

Zalups, R. K., and Ahmad, S. (2003). Molecular handling of cadmium in transporting epithelia. *Toxicol. Appl. Pharmacol.* **186,** 163–188.

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