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Zinc Transport Differs in Rat Spermatogenic Cell Types and Is Affected by Treatment with Cyclophosphamide[1]

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ABSTRACT

Adequate zinc levels are required for proper cellular functions and for male germ cell development. Zinc transport is accomplished by two families of zinc transporters, the ZIPs and the ZnTs, that increase and decrease cytosolic zinc levels, respectively. However, very little is known about zinc transport in the testis. Furthermore, whether cytotoxic agents such as cyclophosphamide (CPA), a known male germ cell toxicant, can affect zinc transport and homeostasis is unknown. We examined zinc transporter expression and zinc transport in pachytene spermatocytes (PS) and round spermatids (RS) in a normal state and after exposure to CPA. We observed differences in the expression of members of the ZnT and ZIP families in purified populations of PS and RS. We also observed that RS accumulate more zinc over time than PS. The expression of many zinc binding genes was altered after CPA treatment. Interestingly, we found that the expression levels of ZIP5 and ZIP14 were increased in PS from animals treated daily with 6 mg/kg CPA for 4 wk but not in RS. This up-regulation led to an increase in zinc uptake in PS but not in RS from treated animals compared to controls. These data suggest that CPA treatment may alter zinc homeostasis in male germ cells leading to an increased need for zinc. Altered zinc homeostasis may disrupt proper germ cell development and contribute to infertility and effects on progeny.

chemotherapy, gene expression, immunosuppressant, male germ cells, pachytene spermatocytes, round spermatids, spermatogenesis, testis, toxicology, zinc, zinc transport

INTRODUCTION

Zinc is an essential trace element that is important for growth and development, metabolism, brain and immune system function, and reproduction [1]. It is present in varying amounts in different tissues and organs [2]. At the cell level, zinc is necessary for proper cellular function because it binds more than 10% of all proteins [3], is a cofactor for over 300 enzymes, and is required for more than 2000 transcription factors [4]. Zinc has an important function in modulating oxidative stress through its roles in antioxidant enzymes, the electron transport chain [5, 6], prevention of Fe/Cu Fenton reactions [7, 8], endoplasmic reticulum protein misfolding [9, 10], and inhibition of NADPH oxidase activity [11, 12]. Moreover, apart from being important in DNA–protein binding through zinc finger proteins, DNA repair enzyme activity is influenced by zinc levels, indicating its importance in DNA damage repair [13–15].

Because zinc is an essential trace element, its levels need to be tightly controlled. This is accomplished by two families of zinc transporters: the ZIP family and the ZnT family of zinc transporters; these transporters work in an opposite fashion to regulate cytosolic zinc. The ZIP family is composed of 14 members and increases cytosolic zinc by importing zinc from extracellular spaces and releasing zinc from intracellular spaces [16]. The ZnT family consists of 10 members and decreases cytosolic zinc by exporting zinc into the extracellular space and sequestering zinc in intracellular spaces [17].

The importance of zinc in reproduction is very apparent in the male reproductive system. Tissue zinc concentration is greatest in the prostate, and semen contains very high concentrations of zinc [18]. Zinc is proposed to play an important role in sperm chromatin condensation by stabilizing chromatin structure [19]. Zinc is also abundant in the testis, where it is present in all stages of germ cells [20]. Zinc deficiency studies have been convincing in demonstrating the importance of zinc in male reproductive function, as zinc deficiency leads to increased oxidative stress, DNA damage, and apoptosis in the testis and an arrest in spermatogenesis [21–25]. On the other hand, zinc overload also has the same detrimental effects on spermatogenesis [26].

It is clear that proper control of zinc levels is essential in the testis; however, very little is known about zinc transport in male germ cells. Only two studies have described the presence of select members of both the ZIP and ZnT families in human and mouse testes [27, 28], while a third study measured the kinetics of zinc transport in spermatids [29]. No studies have investigated whether exposure to drugs or chemicals toxic to germ cells alters zinc transport.

Cyclophosphamide (CPA) is an alkylating agent commonly used in cancer and immunosuppression therapies and is a known male germ cell toxicant [30–38]. Men treated chronically with CPA have an increased incidence of azoospermia and oligozoospermia [30]. Studies in animal models have shown that CPA causes increased DNA damage [31] and oxidative stress [32] in male germ cells, as well as a decrease in sperm chromatin quality [33–36]. The detrimental effects on male germ cells in turn lead to adverse progeny outcomes that are time-specific and dose-dependent [37]. Postimplantation loss is greatest at 4 wk after the initiation of treatment, whereas preimplantation loss is greatest 5 to 6 wk after the initiation of treatment [37], respectively corresponding

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to targeting spermatids and spermatocytes [38]. The underlying molecular mechanisms of CPA toxicity and how male germ cells respond to such an insult remain to be elucidated.

Given the important role of zinc in spermatogenesis and in mediating oxidative stress and DNA damage and the lack of knowledge of zinc transporters in germ cells, we examined the expression of ZIP and ZnT family members and zinc transport in purified populations of male germ cells in a normal state and after treatment with CPA.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (350–400 g; Charles River Canada, St-Constant, Qc, Canada) were maintained on a 12L:12D cycle and had access to food and water ad libitum. After 1 wk of acclimatization, rats were randomly assigned to 1 of 2 treatment groups and gavaged with saline (vehicle) or CPA (CAS 6055-19-2; Sigma Chemical Co., St. Louis, MO), 6 mg/kg body weight, 6 days per week for 4 wk. All animal care and handling were done in accordance with the guidelines outlined by the Canadian Council on Animal Care (McGill Animal Resources Centre protocol 2144).

Germ Cell Isolation and Separation

After 4 wk of treatment, rats were euthanized by CO_2 asphyxiation. Spermatogenic germ cells were isolated and separated using a velocity sedimentation method (STA-PUT; Proscience, Don Mills, ON, Canada) as described by Bellve et al. [39] and modified by Aguilar-Mahecha et al. [40]. Briefly, testes were removed, decapsulated, and digested by incubation with collagenase (Sigma Chemical). After a brief washing, seminiferous tubules were further digested by incubation with trypsin (type 1; T8003; Sigma Chemical) and DNase I (product DN-25; Sigma Chemical). Seminiferous tubules were subsequently dissociated in the presence of DNase I and filtered through 70-μm nylon mesh, followed by washing with RPMI 1640 medium (Life Technologies, Grand Island, NJ) containing 0.5% bovine serum albumin (BSA; Sigma Chemical) and filtering once more through 55-μm nylon mesh. A total of 5.6×10^8 cells suspended in 25 ml of RPMI medium containing 0.5% BSA was loaded into a velocity sedimentation cell separator apparatus and separated by unit gravity sedimentation with a 2%–4% BSA/RPMI gradient. Pachytene spermatocyte- and round spermatid-containing fractions were identified by phase-contrast microscopy. Fractions with more than 80% (pachytene spermatocyte) and 85% (round spermatid) purity were pooled and either pelleted and frozen at −80°C for further processing or cultured overnight (see below).

RNA Extraction and Microarray

Total RNA was extracted from frozen pachytene spermatocyte and round spermatid fractions using TRIzol reagent (Life Technologies) and cleaned using RNeasy mini kit columns (Qiagen, Mississauga, ON, Canada). Total RNA concentration was determined by spectrophotometry (Nanodrop 2000; Nanodrop Technologies, Wilmington, DE), and quality was determined by electrophoresis (BioAnalyzer 2100 Expert; Agilent Technologies, Santa Clara, CA). Only RNA samples with an RNA integrity number higher than 8 were used for microarray and quantitative reverse transcriptase-PCR (qRT-PCR) experiments. Total RNA was reverse transcribed, labeled, and hybridized to rat gene expression arrays (SurePrint G3 rat GE 8 × 60K microarrays; Agilent Technologies) following the manufacturer's instructions (one-color microarray-based gene expression analysis protocol; Agilent). Raw data were quantile-normalized and further analyzed using GeneSpring version 12.0 software (Agilent Technologies). Student *t*-test was used to determine statistically significant differences in gene expression between treated and control samples. Genes that were significantly altered were further filtered using a 1.5-fold cutoff. Gene Ontology analysis (GeneSpring software) and Ingenuity Pathway Analysis software (Qiagen) were used to further characterize altered transcripts. All data were placed in Gene Expression Omnibus database (under accession number GSE79471; NCBI).

Real-Time qRT-PCR

Real-time qRT-PCR validation of microarray results was done by two-step qRT-PCR. Reverse transcription was done with 50 ng of total RNA input using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. Complementary DNA (cDNA) was diluted 1:2, and qRT-PCR was carried out using TaqMan gene expression assays and reagents (Applied Biosystems), following the manufacturer's instructions (OneStepPlus real-time PCR system; Applied Biosystems). The list of accession numbers for the primers is available in Table 1. The expression levels of all genes of interest were determined using the cycle threshold ($\Delta\Delta C_t$) method and normalized to the expression of 18S rRNA [41]. All samples were run in triplicate for each primer.

TABLE 1. Primers used in qRT-PCR.

Gene name	Accession no.	TaqMan assay no.
Slc39a5	NM_001108728.1	Rn01527167_m1
Slc39a6	NM_001024745.1	Rn01405813_m1
Slc39a13	NM_001039196.1	Rn01485759_m1
Slc39a14	NM_001107275.1	Rn01468336_m1
18S rRNA	NM_213557.1	Rn01428913_gH

Protein Extraction and Western Blotting

Total protein was extracted from both the pachytene spermatocytes and round spermatids by using transmembrane protein extraction reagent (Fivephoton Biochemicals, San Diego, CA), following the manufacturer's instructions; total protein concentrations were determined by Bradford assay using a protein assay reagent (Bio-Rad, Saint-Laurent, Qc, Canada). Samples were resolved on 10% (w/v) polyacrylamide gels and then transferred onto polyvinylidene fluoride membranes. Membranes were blocked in 10% non-fat cow's milk in TBS 0.1%-Tween-20. Proteins were detected using antibodies specific for ZIP5 (1:1000 dilution; product ARP44143_P050; Aviva Systems Biology, San Diego, CA) and β-actin (1:5000 dilution; code sc-1616; Santa Cruz Biotechnology, Dallas, TX) diluted in 5% non-fat milk/TBS-0.1% Tween and incubated overnight at 4°C. Primary antibodies were followed by horseradish peroxidase-linked secondary antibodies (donkey anti-rabbit immunoglobulin G [product NA93V]; 1:25 000 dilution; GE LifeSciences, Mississauga, ON, Canada; donkey anti-goat immunoglobulin G; 1:10 000 dilution; product sc-2056; Santa Cruz Biotechnology) diluted in 5% non-fat milk/TBS-0.1% Tween-20, incubated for 2 h at room temperature. Protein bands were detected by electrochemiluminescence prime Western blotting detection reagent (GE LifeSciences).

Germ Cell Culture

After germ cell separation, pachytene spermatocytes and round spermatids were seeded (pachytene spermatocytes at 100×10^5 cells/well; round spermatids at 300×10^5 cells/well) into 96-well cell culture plates (Costar 3595; Corning Life Sciences, Tewksbury, MA) in phenol red-free Dulbecco modified Eagle medium/F12 medium; Life Technologies) with streptomycin and penicillin G added and supplemented with HEPES, lactic acid, and fetal bovine serum, as adapted from the method of LaSalle et al. [42]. Cells were cultured overnight for approximately 11 h at 32°C in 5% CO_2.

Zinc Uptake Assay

After overnight culture, cells were removed from culture plates and transferred to 1.5-ml microcentrifuge tubes. Cells were incubated with 1 μM Fluozin3-AM (Molecular Probes, Eugene, OR) in Live Cell Imaging solution (Molecular Probes) for 1 h at 32°C. Following incubation and washing, cells were resuspended in imaging solution containing Hoechst nuclear stain (2,5′-bi-1H-benzimidazole, 2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]; Invitrogen, Burlington, ON, Canada) and incubated for 5, 15, 30, or 45 min at 32°C with 100 μM $ZnSO_4$. After 45 min, TPEN (500 μM *N*,*N*,*N*′,*N*′-tetrakis[2-pyridylmethyl] ethane-1,2-diamine; Sigma Chemical) was added for 30 min to chelate zinc. Propidium iodide (PI; Invitrogen) was used to evaluate cell viability. After a second wash in imaging solution, cells were transferred to a 96-well cell carrier (PerkinElmer, Woodbridge, ON, Canada) plate with an optically clear bottom. The plate was spun down at 1000 rpm at 4°C for 5 min and immediately scanned (Operetta HTS imaging system; PerkinElmer) at 20× magnification, with 15 fields of view per well. Image analysis software (Columbus version 2.2; PerkinElmer) was used to quantify the mean fluorescent signals from individual cells in each well.

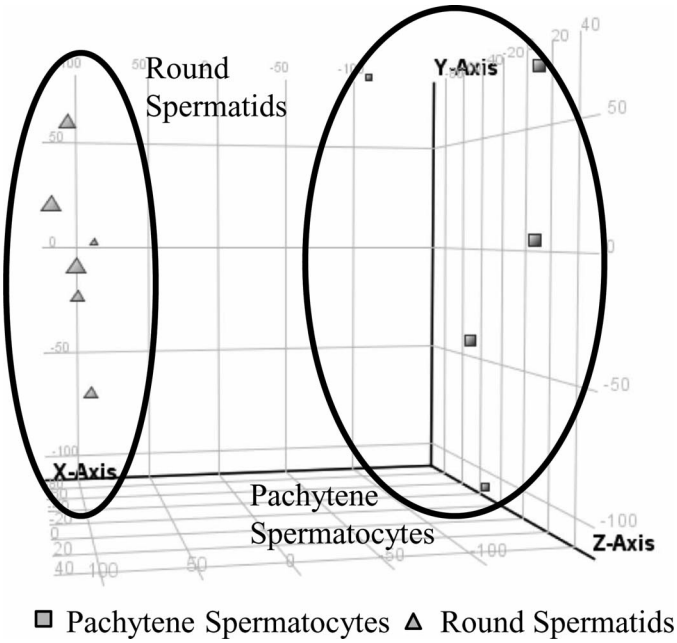

FIG. 1. PCA showing distribution of pachytene spermatocytes and round spermatids in a 3D plot. Pachytene spermatocyte (n = 5) and round spermatids (n = 6) occupy distinct spaces.

Statistical Analyses

Results are expressed as mean ± standard error of the mean (SEM) and were analyzed using Student *t*-test in Prism version 6 software (GraphPad Software, Inc., LaJolla, CA).

RESULTS

Pachytene Spermatocytes and Round Spermatids Show Differences in Gene Expression Profiles

We assessed the differences in gene expression in purified pachytene spermatocytes and round spermatids, two germ cell populations at different stages of differentiation. Principle component analysis (PCA) was used to reduce the dimensionality of the gene expression data sets and allow for easier discernment of the general relationships of groups (Fig. 1). As expected, PCA indicated clear differences, accounting for approximately 78% of the variance in gene expression profiles between pachytene spermatocytes and round spermatids.

Expression of the ZIP Family Zinc Transporters in Pachytene Spermatocytes and Round Spermatids

The expression of members of the ZIP family of zinc transporters, responsible for increasing cytosolic zinc, in pachytene spermatocytes was evaluated and compared with that in round spermatids. All members of this family of zinc transporters were expressed to various degrees in both of the germ cell types (Fig. 2a). The most abundantly expressed member in both of the cell types was ZIP3, followed by ZIP4; both members were present at levels up to 2 orders of magnitude higher than the lowest expressed member, ZIP2. These differences in expression of ZIP transporters are even more evident when visualized on the linear scale (Supplemental Fig. S1a; all Supplemental Data are available online at www.biolreprod.org). Additionally, the expression levels of the different ZIP family members vary between the two cell types (Fig. 2b). The largest difference in expression was seen with ZIP12, which was greater than 5 times more abundant in round spermatids than in pachytene spermatocytes, followed by ZIP11, which was almost 4 times more abundant in round spermatids. In contrast, ZIP7 was almost 3.5 times more abundant in pachytene spermatocytes than in round spermatids. ZIP4, the second most abundant ZIP member in the two cell types, was present in pachytene spermatocytes at levels more than 2 times those found in round spermatids.

Expression of the ZnT Family Zinc Transporters in Pachytene Spermatocytes and Round Spermatids

The expression of members of the ZnT family of zinc transporters, responsible for decreasing cytosolic zinc, in pachytene spermatocytes was evaluated and compared with that in round spermatids (Fig. 2c). ZnT family members were expressed to various degrees in both of these cell types. The most abundantly expressed ZnT family members in both cell types were ZnT3 and ZnT1, which were expressed at levels more than 2 orders of magnitude higher than the lowest expressed members, ZnT2, ZnT4, and ZnT5. These differences in the expression of ZnT transporters were even more evident when visualized on a linear scale (Supplemental Fig. S1b). The expression of ZnT members differed significantly between the two cell types, with the exception of ZnT3, ZnT9, and ZnT10 (Fig. 2d). The largest difference was seen with ZnT6, which was greater than 5 times more abundant in pachytene spermatocytes than in round spermatids. In addition, ZnT1, the second most abundant ZnT member in both cell types, was expressed at a level almost 5 times higher in round spermatids than in pachytene spermatocytes.

Zinc Transport in Pachytene Spermatocytes Compared to Round Spermatids

Considering the differences in expression of zinc transporters, we next evaluated zinc transport over time in both pachytene spermatocytes and round spermatids by live cell imaging using a fluorescent zinc probe. Within 5 min after the addition of zinc, the mean fluorescent signal in both cell types increased by 10% above baseline levels and continued to increase steadily (Fig. 3, a, b, and c). There were statistically significant differences in zinc uptake between the two cell types at 45 min after the addition of zinc; at this time the mean fluorescent signal in round spermatids was increased above that of pachytene spermatocytes by 18% above baseline (Fig. 3c). The addition of TPEN, an intracellular zinc chelator, decreased the fluorescence signal to levels well below baseline, indicating that the observed fluorescence signal was indeed from zinc (Fig. 3d). If alterations in membrane integrity were to account for the differences seen in zinc accumulation, round spermatids would be expected to have decreased membrane integrity. However, membrane integrity, as assessed by PI staining, showed a decreased number of cells with PI signal in round spermatids compared to that in pachytene spermatocytes (5% vs. 16% of cells, respectively) (Supplemental Fig. S2a), supporting the results obtained in the zinc uptake assay.

Chronic Low-Dose CPA Treatment Alters Gene Expression in Pachytene Spermatocytes and Round Spermatids

We assessed the impact of chronic low-dose CPA treatment on gene expression in pachytene spermatocytes and round spermatids by using whole-rat genome microarrays. Principal component analysis was used to examine relationships between samples (Fig. 4, a and b). Samples from CPA-treated animals

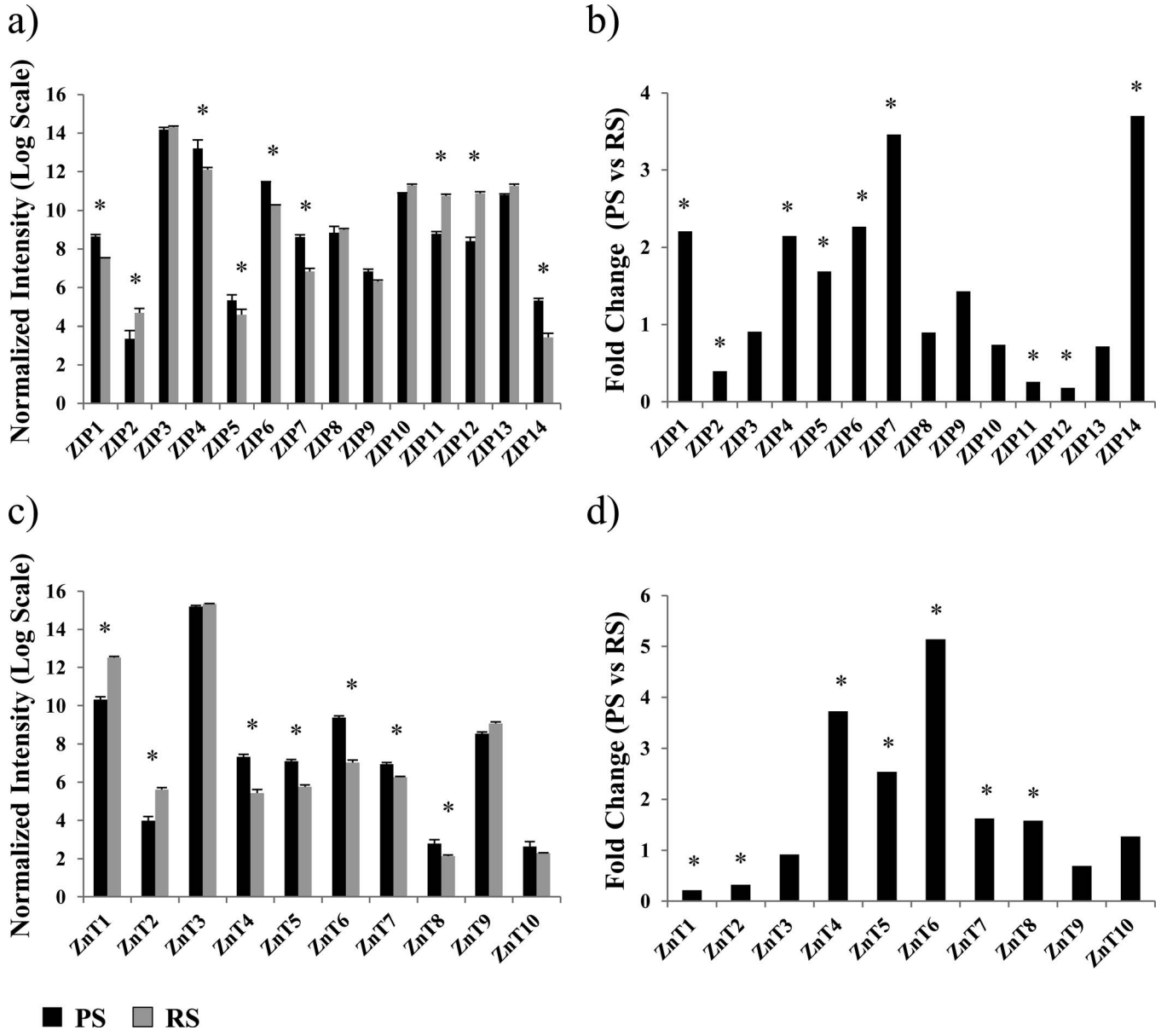

FIG. 2. Expression of zinc transporters in male germ cells. Log scale expression levels of ZIP family (**a**) and ZnT family (**c**) of zinc transporters in pachytene spermatocytes and round spermatids. Fold change of expression levels of ZIP members (**b**) and ZnT members (**d**) in pachytene spermatocytes versus round spermatids. n = 5 pachytene spermatocytes; n = 6 round spermatids. *$P < 0.05$.

and control animals separated along the *y* and *z* axes. The analysis also revealed differences in gene expression profiles after CPA treatment in both of the cell types.

Of the 30 507 probe sets present on the arrays, 20 449 (67%), corresponding to 13 524 known genes, were considered expressed in both CPA-treated and control pachytene spermatocyte samples (Fig. 5a). A number of these known genes were significantly altered and had a 1.5 or greater fold change with respect to treatment (Fig. 5c). Nearly twice as many genes were down-regulated than were up-regulated after treatment. Additionally, more than 10 times more genes were reduced to undetectable levels after CPA treatment than were induced (Fig. 5a). These results suggest that CPA treatment may have a repressive effect on gene expression in pachytene spermatocytes.

In round spermatids, 20 972 probe sets (68.8%), corresponding to 13 939 known genes, were present in both treated and control samples (Fig. 5b). The number of known genes significantly altered over 1.5-fold after treatment in round spermatids and was similar to the number in pachytene spermatocytes (Fig. 5d). However the numbers of known genes that were down- and up-regulated after CPA treatment were approximately equal. Similarly, although the number of genes that was induced after treatment in round spermatids was greater than the number of genes repressed, the differences were not as striking as in the pachytene spermatocytes (Fig. 5d). The comparable numbers of transcripts or known genes that were induced or up-regulated and repressed or down-regulated suggest that CPA treatment has a greater effect on gene expression in round spermatids.

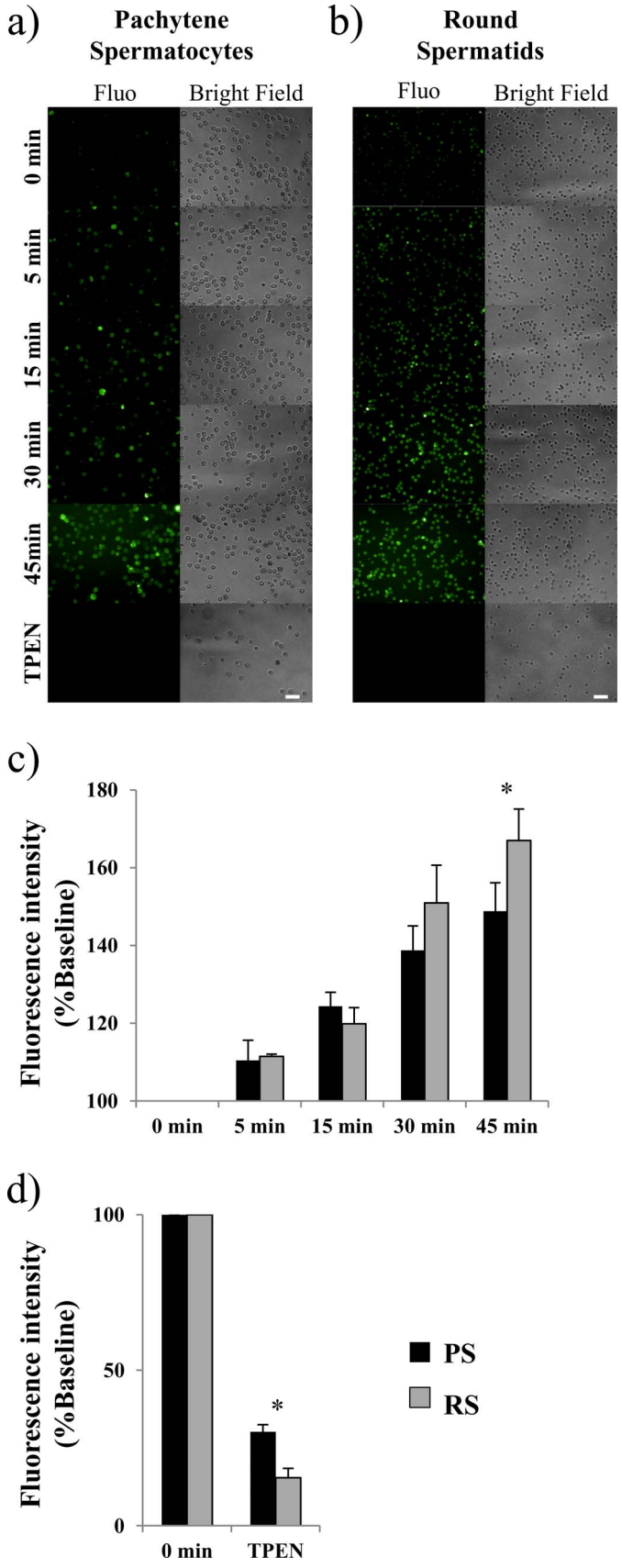

FIG. 3. Zinc transport in pachytene spermatocytes and round spermatids. High-throughput imaging of fluorescent zinc probe Fluozin-3AM and brightfield view in pachytene spermatocytes (n = 8 [**a**]) and round spermatids (n = 5 [**b**]) incubated with 100 nM $ZnSO_4$ for 0, 5, 15, 30, or 45 min and after the addition of the zinc chelator TPEN. Bar = 50 μm. Quantification of fluorescence intensity over time (**c**) and after addition of TPEN (**d**), as a percentage of baseline values. *$P < 0.05$.

Expression of Members of the ZIP Family of Zinc Transporters Is Increased in Pachytene Spermatocytes After CPA Treatment

Bioinformatic analysis using ingenuity pathway analysis was used to further characterize transcripts that were significantly altered by CPA treatment. As expected, genes involved in the response to stress, response to DNA damage, DNA repair, regulation of cell death, and spermatogenesis were altered after CPA treatment in pachytene spermatocytes and round spermatids (Fig. 6, a and b). Unexpectedly, many genes involved in ion transport and zinc binding were altered in both of the cell types after CPA treatment. Supplemental Tables S1 and S2 list the genes involved in zinc binding and transport. In both of the cell types, treatment altered the expression of many genes involved in transcription, including multiple zinc finger proteins (Fig. 6, c and d). Both estrogen-related receptor alpha (*Essra*) and PR domain containing 13 (*Prdm13*) were altered by CPA treatment in both cell type, although in opposite directions. Of particular interest were four members of the ZIP family of zinc transporters, *Zip5*, *Zip6*, *Zip13*, and *Zip14*, which were all significantly up-regulated in pachytene spermatocytes after CPA treatment (Figs. 6a and 7, a–d). The expression of these zinc transporters, or any others, was not altered in round spermatids (Supplemental Fig. S3).

Real-time qRT-PCR assay was used to validate the microarray results in pachytene spermatocytes and confirmed the significantly increased expression of Zip5 and Zip14 (Fig. 7, e and h). However, Zip6 and Zip13 were not significantly up-regulated (Fig. 7, f and g). The results from the qRT-PCR experiment were remarkably similar to those from the microarray results, reflecting the sensitivity of the microarray experiment.

Western blots were analyzed to determine whether the changes in expression of *Zip5* and *Zip14* transcripts translated to proteins. The results revealed that ZIP5 levels were 2.26-fold up-regulated in pachytene spermatocytes (Fig. 8a). Protein expression levels were also verified in the round spermatids to determine whether altered transcript levels in pachytene spermatocytes could carry over into the more differentiated germ cells; ZIP5 levels in round spermatids were not significantly affected by CPA treatment (Fig. 8b). The protein levels of ZIP14 were not assessed due to the lack of a specific antibody.

Chronic Low-Dose CPA Treatment Results in an Increase in Zinc Uptake in Pachytene Spermatocytes

Whether the increased expression of ZIP5 and ZIP14 resulted in an increase in zinc accumulation was assessed by live cell imaging using a fluorescent zinc probe and monitoring the fluorescence signal over time after the addition of zinc (Fig. 9, a and c). The mean fluorescence signal in pachytene spermatocytes from CPA-treated animals was significantly increased above that of control cells by 24% above baseline levels at 45 min after addition of zinc. Addition of the intracellular zinc chelator TPEN decreased the fluorescence signal to levels well below baseline, indicating the specificity of the fluorescent zinc probe (Fig. 9e). The number of cells with a positive PI signal was not significantly different between the two treatment groups (Supplemental Fig. S2b), indicating

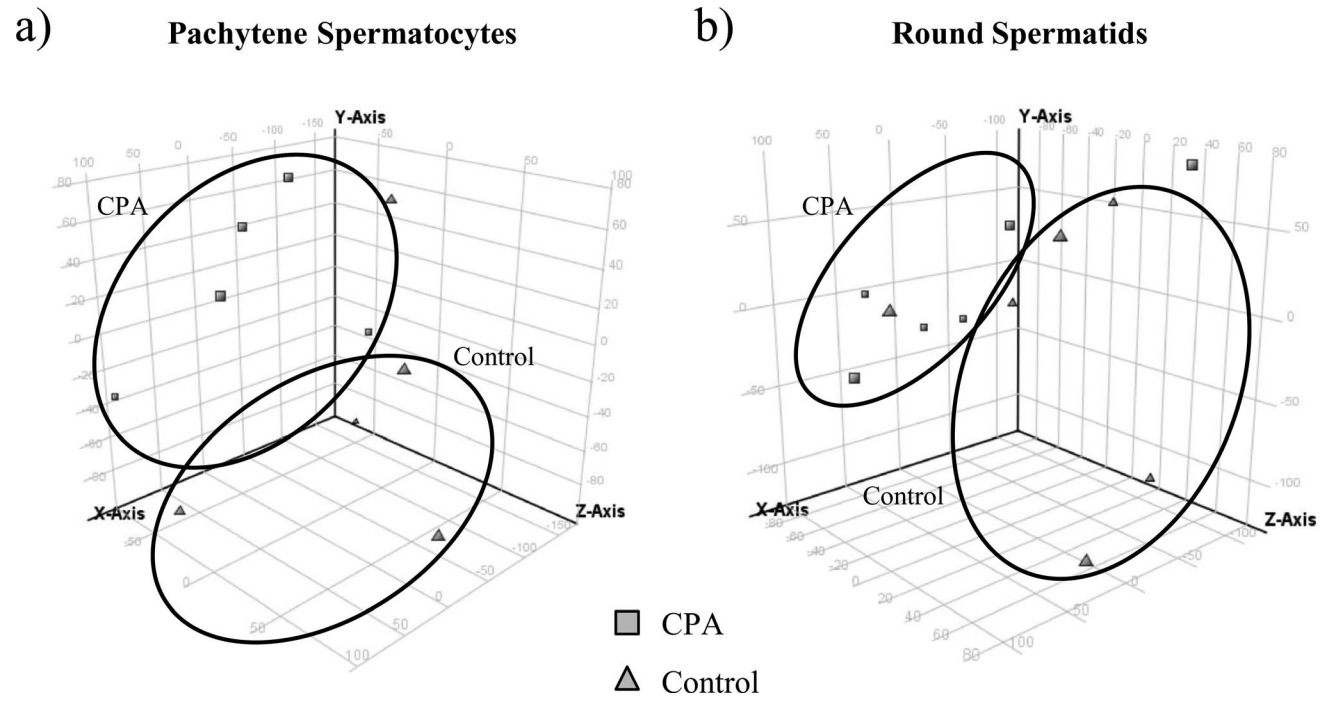

FIG. 4. PCA showing the distribution of CPA-treated and control pachytene spermatocytes (n = 5 [**a**]) and round spermatids (n = 6 [**b**]).

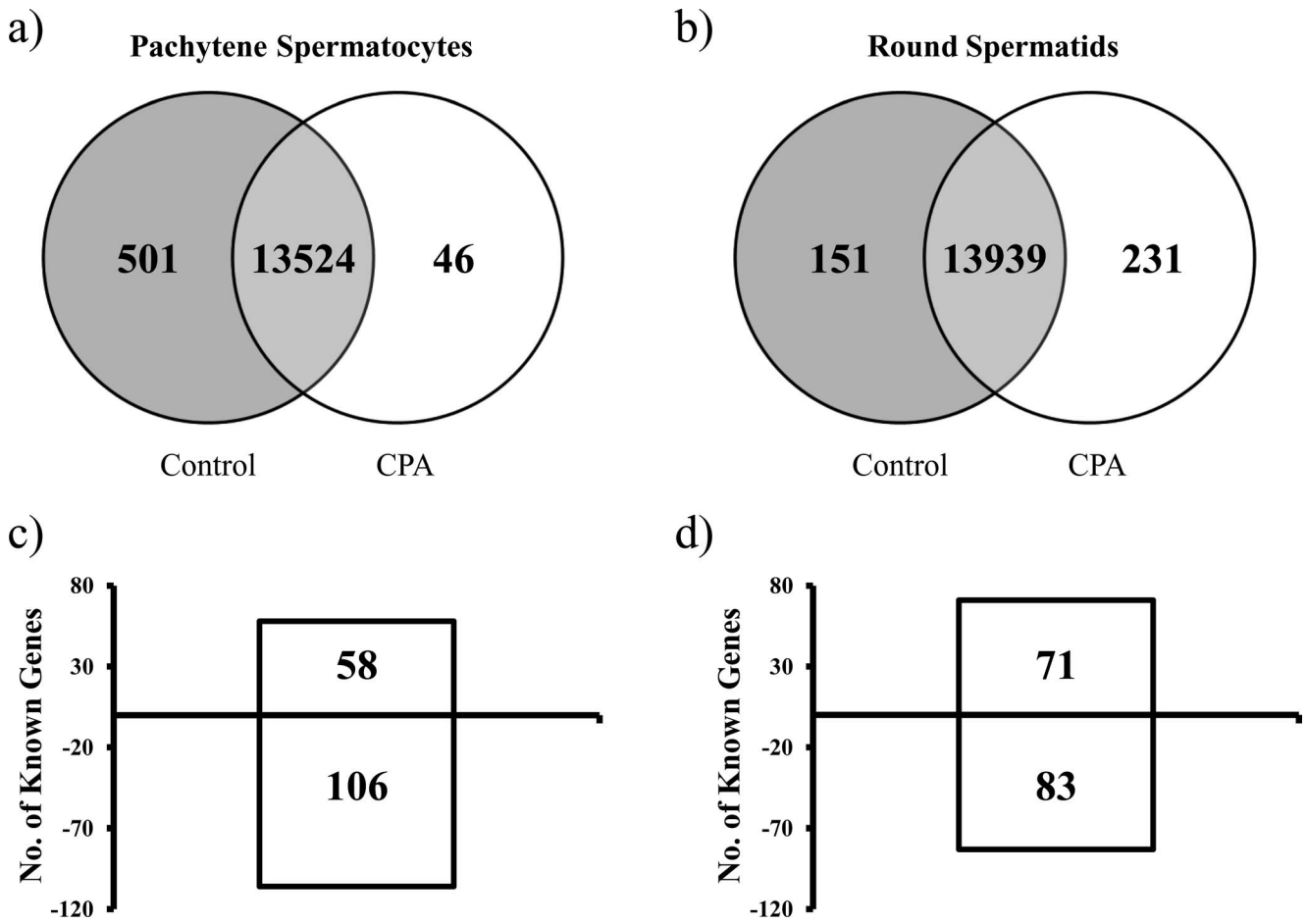

FIG. 5. Changes in expression of known genes after CPA treatment. Venn diagram of known genes expressed in both the control and the CPA-treated samples and those that are exclusively expressed in one treatment group in pachytene spermatocytes (n = 5 [**a**]) and round spermatids (n = 6 [**b**]). Numbers of known genes that are significantly 1.5-fold altered after CPA treatment in pachytene spermatocytes (**c**) and round spermatids (**d**). $P > 0.05$.

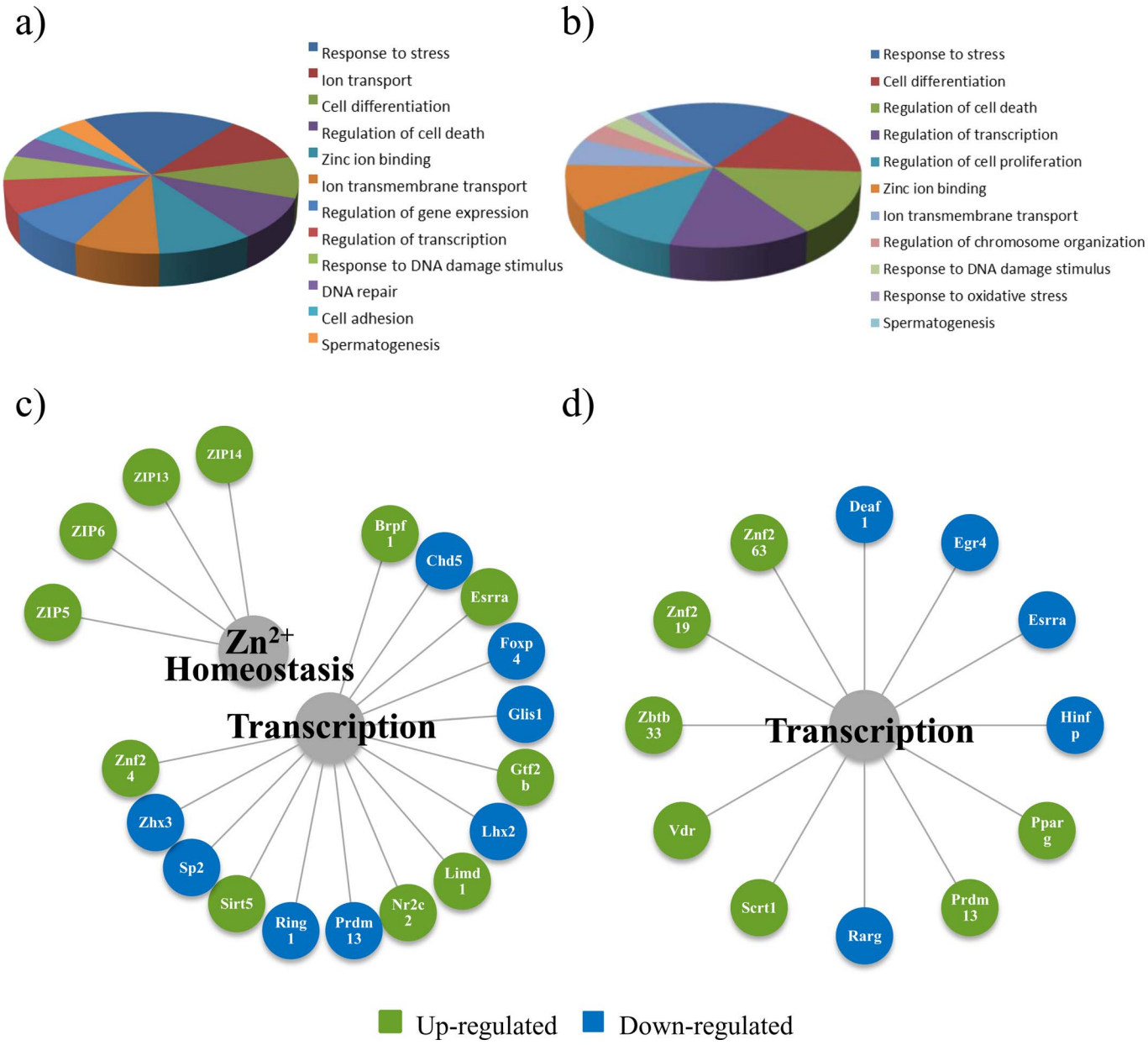

FIG. 6. Functional characterization of genes significantly altered by CPA treatment. Pie charts show the prevalence of different GO terms associated with genes altered by CPA treatment in pachytene spermatocytes (**a**) and round spermatids (**b**). Functional analysis of altered genes involved in zinc ion binding in pachytene spermatocytes (**c**) and round spermatids (**d**).

that the differences in zinc accumulation between cells from CPA treated animals and control cannot be explained by a detrimental effect of CPA on membrane integrity.

Chronic Low-Dose CPA Treatment Does Not Increase Zinc Uptake in Round Spermatids

The effect of CPA treatment on zinc uptake was also determined in round spermatids which did not have altered expression of zinc transporters (Fig. 9, b and d). After the addition of zinc the fluorescent zinc signal increased over time in round spermatids from both treated and control animals; however, there was no significant difference in fluorescent signal between the two groups at any time point. Again, consistent with the pachytene spermatocytes, there were no significant differences in the number of cells with PI signal between round spermatids from CPA-treated and control animals (Supplemental Fig. S2c). These results indicate that CPA treatment does not affect zinc transport in round spermatids.

DISCUSSION

Despite clear evidence of the importance of zinc in male germ cell development and fertility, there is little understanding of zinc transport in the testis. Here we examined the expression of ZIP and ZnT family members and zinc transport in purified populations of male germ cells in a normal state and after treatment with CPA. We found that both pachytene spermatocytes and round spermatids expressed many members of both families of zinc transporters and that the level of expression of some transporters differed between the two cell types. Additionally, we found that round spermatids accumulated more zinc over time than pachytene spermatocytes. Treatment

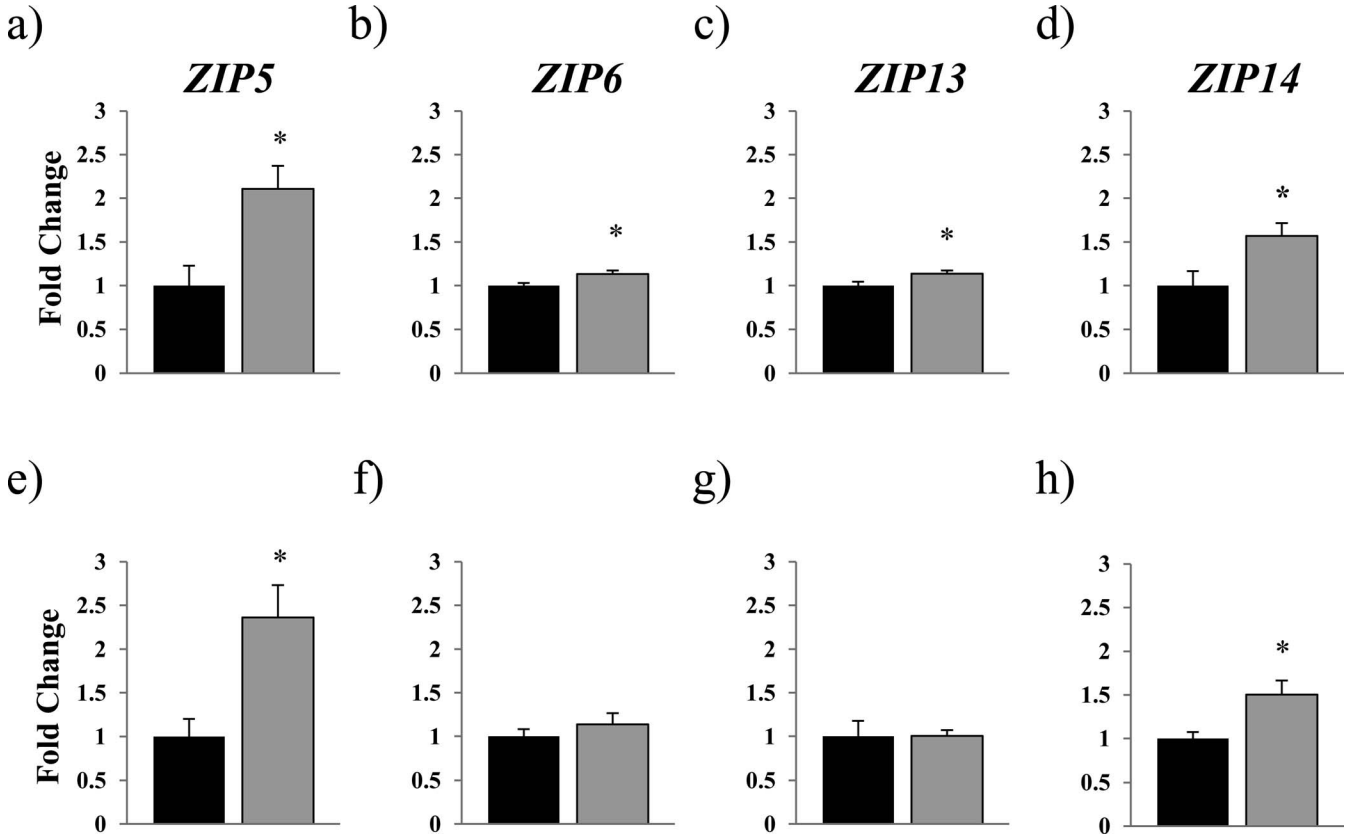

FIG. 7. CPA increases the expression of members of the ZIP family of zinc transporters. The expression of ZIP family members is significantly increased after CPA treatment in pachytene spermatocytes as shown by microarray results (**a**–**d**) and qRT-PCR validation (**e**–**h**). n = 5; *$P < 0.05$.

with the alkylating agent CPA led to an increase in the expression of ZIP5 and ZIP14 in pachytene spermatocytes but not in round spermatids. Consistent with our expression data, CPA treatment resulted in an increase in zinc uptake in pachytene spermatocytes but not in round spermatids.

Many studies have investigated differences in gene expression between different germ cell types. However, no studies have investigated zinc transporters in particular. Furthermore, only a few studies have addressed the expression of zinc transporters in the testis. To our knowledge, our study is the first to evaluate the expression of all members of the ZnT and ZIP families in purified populations of pachytene spermatocytes and round spermatids. Other studies have investigated the expression of select transporters in whole-testis homogenates or immunohistochemically [27, 28, 43–47].

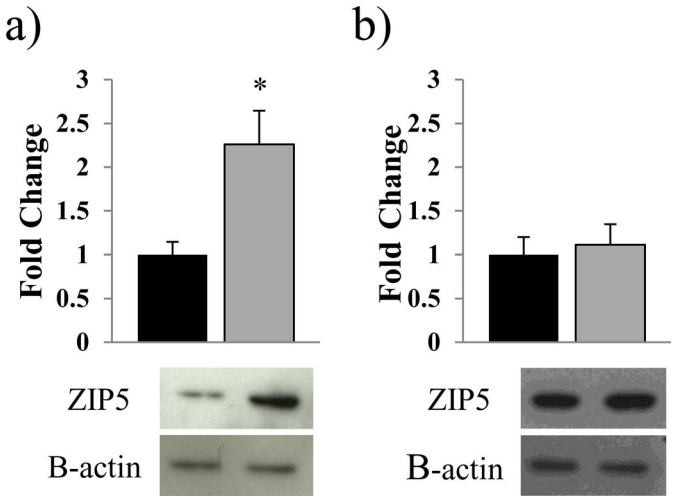

FIG. 8. CPA treatment increases ZIP5 expression at the protein level. The protein expression of ZIP5 is increased in pachytene spermatocytes (**a**) but not in round spermatids (**b**) after CPA treatment. n = 3; *$P < 0.05$.

In previous studies, ZnT1 was detected immunohistochemically in Sertoli cells but not in germ cells in mouse and human testes [27, 43], whereas ZnT7 showed strong immunoreactivity in mouse spermatocytes and spermatids [44]. At the mRNA level, ZnT1, ZnT2, and ZnT3 have been found in mouse testis homogenates and purified Sertoli cells [45, 46]. We found that both ZnT1 and ZnT3 are highly expressed in both pachytene spermatocytes and round spermatids. All other ZnT family members were also expressed in both cell types with the exception of ZnT2 in pachytene spermatocytes and ZnT8 and ZnT10 in round spermatids.

ZIP family members 5, 6, and 8 have been detected in spermatogonia, spermatocytes, spermatids, and spermatozoa in human testis biopsies [27]. ZIP1 was detected only in spermatids and spermatozoa, and ZIP14 was not detected in any cell of the human testis [27]. In mice, ZIP14 has been localized to spermatogonia; ZIP5, ZIP8, and ZIP10 have been localized to spermatocytes; ZIP6, ZIP8, and ZIP10 have been localized in round spermatids; ZIP1 and ZIP6 have been localized in elongating spermatids; and ZIP5 has been localized in Sertoli cells [28]. At the transcript level, the testis has shown the highest level of expression of ZIP3 [47]. Similarly, we found ZIP3 was the most abundant ZIP transporter in both pachytene spermatocytes and round spermatids. In our study we also found the expression of ZIP5, ZIP6, ZIP8, and ZIP10 in pachytene spermatocytes and ZIP6, ZIP8, and ZIP10 in

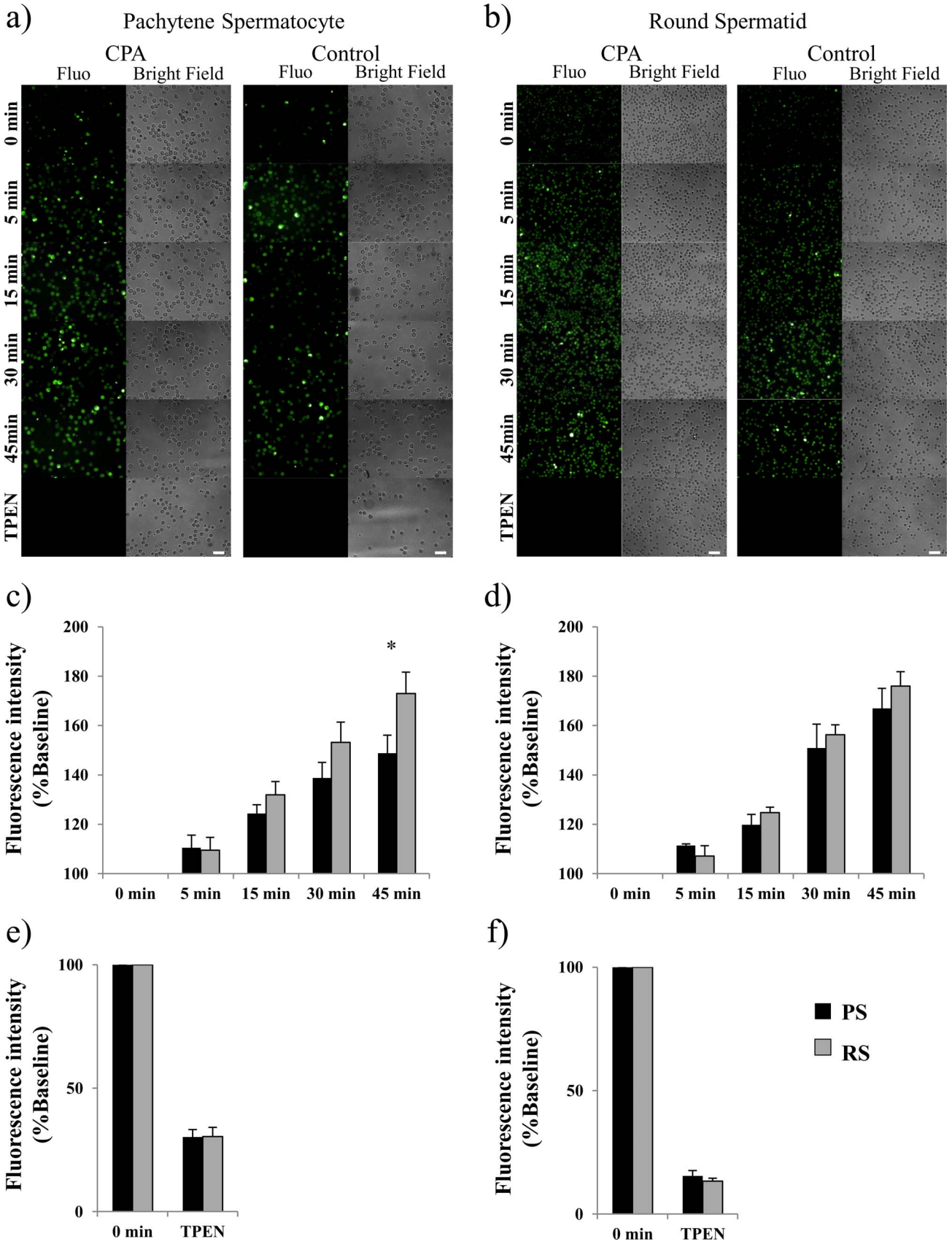

FIG. 9. Zinc transport in pachytene spermatocytes and round spermatids after CPA treatment. High-throughput imaging of fluorescent zinc probe Fluozin-3am and brightfield of pachytene spermatocytes (**a**) and round spermatids (**b**) from treated and control animals, incubated with 100 nM ZnSO4 for 0, 5, 15, 30, or 45 min and after the addition of the zinc chelator TPEN. Bar = 50 μm. Quantification of fluorescence intensity over time in pachytene spermatocytes (**c**) and round spermatids (**d**) as a percentage of baseline values. Quantification of fluorescence after addition of TPEN, as a percentage of baseline values in pachytene spermatocytes (**e**) and round spermatids (**f**). Pachytene spermatocytes n = 9 CPA, n = 8 control; round spermatids n = 5. *$P < 0.05$.

round spermatids at the mRNA level. Transcripts for all other members of the ZIP family were also found.

The expression of most of the different ZnT and ZIP family members in germ cells may serve a compensatory role as ZnT or ZIP knockout models have not shown any obvious reproductive phenotype [16, 17]. In fact, testis zinc levels are not altered in either ZnT3 or ZIP3 knockout mice [48, 49]. However, fertility was not specifically evaluated in these models.

The expression of many of the zinc transporters differed between pachytene spermatocytes and round spermatids. Considering this, we next examined whether there were differences in zinc accumulation in these cells. We found that round spermatids took up more zinc over time than pachytene spermatocytes. To our knowledge, this is the first study to compare zinc transport in these germ cell types. A previous study investigated the kinetics of zinc transport in spermatids (round and elongating) using Zn-65 labeling and found a temperature-independent fast kinetic component which corresponded to extracellular zinc binding, followed by a temperature-dependent slower kinetic component which corresponded to an active transport of zinc into the cell [29]. Inside the cell, zinc was primarily bound to organelles or large cytoskeletal elements. Zinc efflux was slower than influx, presumably due to a slow release of zinc from intracellular organelles and binding sites. Our study was not designed to assess the kinetics of zinc influx and efflux in the germ cells. However, from our results we can postulate that the zinc kinetics of pachytene spermatocytes are different from those of round spermatids.

The observed differences between zinc transporter expression and zinc uptake in pachytene spermatocytes and those in round spermatids likely reflect cell- and stage-specific zinc requirements. Because zinc plays an important role in sperm chromatin condensation, it is possible that round spermatids may accumulate more zinc for use in later stages of spermiogenesis than pachytene spermatocytes do. The significance of the differences in zinc transporter expression and zinc transport, as well as the specific roles played by zinc in these two germ cell types, warrant further investigation.

We also evaluated the effect of CPA on global gene expression in pachytene spermatocytes and round spermatids. We found that CPA treatment repressed and downregulated many genes in pachytene spermatocytes, suggesting a global repression of transcription. The dysregulation of transcripts in round spermatids was more evenly distributed. As expected, CPA treatment altered the expression of genes involved in the response to stress. The results from this study are consistent with those of a previous study from our laboratory, where we showed that chronic CPA treatment resulted in a decrease in the expression of transcripts involved in the stress response in pachytene spermatocytes and round spermatids [50]. Interestingly, acute CPA treatment resulted in an increase in gene expression, particularly in round spermatids [51]. Other cancer treatment regimens, such as the combination of bleomycin, etoposide, and *cis*-platinum, have been shown to alter gene expression in male germ cells [52]. Unexpectedly, we found that many transcripts involved with zinc were altered after CPA treatment, including members of the ZIP family of zinc transporters.

Changes in zinc transporter expression have been associated with neurodegenerative diseases, immunological impairment, and cancer progression and metastasis [53]. The potential for a xenobiotic to modulate the expression of zinc transporters, to our knowledge, has not been tested. Here we show that chronic treatment with CPA upregulated the expression of two zinc transporters, ZIP5 and ZIP14, in pachytene spermatocytes. ZIP5 was highly upregulated after CPA treatment. ZIP5 has been localized to the basolateral membranes of intestinal enterocytes and pancreatic acinar cells [54, 55]. In periods of dietary zinc deficiency, ZIP5 is internalized and degraded [55]. These data and results from ZIP5 knockout mice suggest that ZIP5 plays a role in intestinal zinc excretion and zinc accumulation/retention in the pancreas [55, 56].

ZIP14 expression was also increased after CPA treatment. ZIP14 is most abundantly expressed in the liver where it has been localized to the plasma membrane of hepatocytes and is upregulated in response to inflammation through interleukin-6 (IL-6), IL-1β, and nitric oxide, leading to increased hepatic zinc uptake and contributing to hypozincemia [57, 58]. ZIP14 has high expression in the duodenum, at the apical membrane of polarized cells, where it is thought to play a role in zinc absorption [59]. Additionally, ZIP14 may have a function in chondrocyte and adipocyte differentiation via signaling pathways involving zinc [60–62].

What roles these zinc transporters play in male germ cells are unknown. Neither the expression of ZIP5 nor of ZIP14 in the testis was affected by moderate zinc deficiency [28]. However, the expression of zinc transporters was assessed only in whole testis. Whether zinc deficiency affects the expression of different zinc transporters in germ cells at different stages of development is unknown.

We next asked whether the increased expression of ZIP5 and ZIP14 would lead to greater zinc uptake. Using a fluorescent zinc probe, we measured an accumulation of zinc that was greater in pachytene spermatocytes from CPA-treated animals than in controls. Consistent with the expression data, no increase in zinc uptake was observed over time in round spermatids. The fact that ZIP transporters and zinc transport were increased in pachytene spermatocytes but not round spermatids may be due to inherent differences in the two cell types. Perhaps pachytene spermatocytes are better suited to adapt and respond to CPA treatment. Indeed, although proliferating premeiotic and meiotic germ cells respond to alkylating agents, postmeiotic germ cells and spermatozoa are more susceptible [63, 64].

Previously we and others have shown that CPA treatment causes oxidative stress and DNA damage in male germ cells [31]. We propose that this stress leads to an increased zinc requirement in these cells. Pachytene spermatocytes may respond to a CPA-induced zinc deficiency by increasing the expression of ZIP5 and ZIP14, which leads to greater zinc uptake. A previous study showed that, in the testis, ZIP6 and ZIP10 expression levels were decreased in response to moderate zinc deficiency [28]. However, it is possible that germ cells, in particular pachytene spermatocytes, respond differently to different stressors, in this case dietary zinc deficiency versus cellular stress caused by CPA, by altering the expression of different zinc transporters. ZIP5 and ZIP14 are not the most abundant zinc transporters expressed in the pachytene spermatocytes; however, they may be the ZIP transporters most involved in responding to cellular insult by a toxic agent. Whether chronic low-dose CPA treatment causes zinc deficiency in male rats was not assessed in this study. However, the known importance of zinc in transcription, along with our observations of an apparent global repression in gene expression, and the altered expression of many transcription factors that we observed after CPA treatment may also indicate a state of zinc deficiency in pachytene spermatocytes. Additionally, high doses of CPA administered to male rats have been shown to result in reduced serum and testis zinc levels [65].

Sperm chromatin from CPA-treated rats has decreased protamination and sulfhydryl groups [34]. Because zinc plays an important role in sperm chromatin structure [19], we propose that a decrease in zinc levels in the germ cells may be responsible for the reduced sperm chromatin quality observed after CPA treatment.

Treatment with an acute high dose but not a chronic low dose of CPA impairs meiotic progression in pachytene spermatocytes [66]. It is possible that altered zinc homeostasis in pachytene spermatocytes is involved in this impairment. Zinc is important for meiotic progression in oocytes [67–69], but it is not known whether it plays an important role in meiosis in male germ cells. The lack of impairment after chronic CPA treatment may in fact be due to the cells adapting by increasing zinc uptake.

In conclusion, the expression of ZIP and ZnT family members as well as zinc transport differ in purified populations of pachytene spermatocytes and round spermatids. Importantly, we have shown that an alkylating agent such as CPA is capable of modulating the expression of zinc transporters in male germ cells, leading to changes in zinc uptake. Considering the importance of zinc in proper cellular function and spermatogenesis, any changes in the regulation of zinc levels have the potential to alter normal spermatogenesis. How these changes affect germ cell survival, development, and quality will need to be examined further. These novel data have important implications for understanding damage to male germ cells from toxic agents as they suggest that zinc homeostasis is altered after CPA treatment. Zinc supplementation could potentially be an interesting strategy to protect male germ cells from damage induced by toxic agents such as CPA.

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