Loss of the Atp2c1 Secretory Pathway Ca\(^{2+}\)-ATPase (SPCA1) in Mice Causes Golgi Stress, Apoptosis, and Midgestational Death in Homozygous Embryos and Squamous Cell Tumors in Adult Heterozygotes*  

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Loss of one copy of the human ATP2CI gene, encoding SPCA1 (secretory pathway Ca\(^{2+}\)-ATPase isoform 1), causes Hailey-Hailey disease, a skin disorder. We performed targeted mutagenesis of the Atp2c1 gene in mice to analyze the functions of this Golgi membrane Ca\(^{2+}\) pump. Breeding of heterozygous mutants yielded a normal Mendelian ratio among embryos on gestation day 9.5; however, null mutant (Spca1\(^{−/−}\)) embryos exhibited growth retardation and did not survive beyond gestation day 10.5. Spca1\(^{−/−}\) embryos had an open rostral neural tube, but hematopoiesis and cardiovascular development were ostensibly normal. Golgi membranes of Spca1\(^{−/−}\) embryos were dilated, had fewer stacked leaflets, and were expanded in amount, consistent with increased Golgi biogenesis. The number of Golgi-associated vesicles was also increased, and rough endoplasmic reticulum had fewer ribosomes. Coated pits, junctional complexes, desmosomes, and basement membranes appeared normal in mutant embryos, indicating that processing and trafficking of proteins in the secretory pathway was not massively impaired. However, apoptosis was increased, possibly the result of secretory pathway stress, and a large increase in cytoplasmic lipid was observed in mutant embryos, consistent with impaired handling of lipid by the Golgi. Adult heterozygous mice appeared normal and exhibited no evidence of Hailey-Hailey disease; however, aged heterozygotes had an increased incidence of squamous cell tumors of keratinized epithelial cells of the skin and esophagus. These data show that loss of the Golgi Ca\(^{2+}\) pump causes Golgi stress, expansion of the Golgi, increased apoptosis, and embryonic lethality and demonstrate that SPCA1 haploinsufficiency causes a genetic predisposition to cancer.

The mammalian Ca\(^{2+}\)-transporting ATPases include two secretory pathway Ca\(^{2+}\)-ATPases (SPCAs)\(^{2}\) (1–3), three sarco-(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (4), and four plasma membrane Ca\(^{2+}\)-ATPases (5, 6). The biochemical, cell biological, and physiological functions of SERCA and plasma membrane Ca\(^{2+}\)-ATPases have been intensively studied (4, 7). Only limited information is available for the SPCAs (8), which are closely related to PMR1, a P-type Ca\(^{2+}\)-ATPase in yeast that is expressed in Golgi membranes (9) and transports both Ca\(^{2+}\) and Mn\(^{2+}\) (10). Loss of PMR1 affects outer chain glycosylation, proteolytic processing, and trafficking of proteins in the secretory pathway (9). In mammals, SPCA1 is expressed in all tissues (1), whereas SPCA2 is expressed in only a limited set of tissues (3). Like PMR1, both SPCA1 and SPCA2 are localized to the Golgi and transport Ca\(^{2+}\) and Mn\(^{2+}\) (3, 11). There is evidence that the cell biological functions of SPCA1 are also similar to those of PMR1 (12).

Loss of one copy of the human ATP2CI gene, encoding SPCA1, causes Hailey-Hailey disease (HHD), an autosomal dominant skin disorder (13, 14). SPCA1 protein levels in HHD keratinocytes are reduced to about half of normal levels, and Golgi Ca\(^{2+}\) handling is impaired (15). HHD is similar to Darier disease, which is caused by null mutations in one copy of the human ATP2A2 gene, encoding SERCA2 (16). Both diseases are characterized by acantholysis (a disruption of cell-cell contacts) in the suprabasal layers of the skin. As the major ER Ca\(^{2+}\) pump in most tissues, including keratinocytes, the function of SERCA2 is similar to that of SPCA1 in that it maintains luminal Ca\(^{2+}\) concentrations in a major compartment of the secretory pathway. In mice, SERCA2 haploinsufficiency does not cause Darier disease but does lead to squamous cell tumors of keratinized epithelial cells (17, 18), the same cell type affected in Darier disease. In humans, a low incidence of squamous cell tumors has been reported in both Darier disease (19) and HHD (20, 21), but it is unclear whether this is a chance association or is caused by the reduction in Ca\(^{2+}\) pump levels and activity.

In the current study, we developed a gene-targeted mouse model for SPCA1 and analyzed the phenotype resulting from heterozygous and homozygous null mutations. The results show that SPCA1 null embryos undergo a substantial degree of...
structural development and survive until gestation day 10.5. However, embryonic tissues exhibited a high incidence of apoptosis and ultrastructural evidence of severe Golgi stress, thus establishing SPCA1 deficiency as the first clear example of a condition that causes Golgi stress. Heterozygous mutants exhibited no evidence of HHD but did develop squamous cell tumors, as observed in SERCA2 heterozygous mice. The parallels between the effects of SPCA1 deficiency and SERCA2 deficiency are consistent with a model in which species differences in the balance between prosurvival and proapoptotic responses of keratinocytes to secretory pathway stress favor development of cancer in mice and acantholytic skin disease in humans.

**EXPERIMENTAL PROCEDURES**

**Generation of Mutant Mice**—A 2.1-kb BamHI-HindIII fragment beginning in intron 7 and ending in intron 9 and a 2.4-kb BamHI-PstI fragment beginning in intron 13 and extending to intron 15 were inserted into the pMJKO vector (22). The vector contained the neomycin resistance and HSV-thymidine kinase genes for positive and negative selection. Electroporation of embryonic stem cells and blastocyst-mediated transgenesis were performed as described previously (22), and male chimeric mice were bred with Black Swiss females. Southern blot analysis was performed using a 3′ outside probe corresponding to a 1.25-kb PstI-BamHI site from intron 15 and 5′ inside probe corresponding to codons 182–228 from exon 8. The mice used in the aging study were from early generations and of the original mixed 129/SvJ and Black Swiss background; mice used for analysis of the embryo lethality phenotype had been backcrossed onto the Black Swiss background. The morning on which a vaginal plug was observed was regarded as embryonic day (ED) 0.5.

**Genotype Analysis**—Genotyping was performed by Southern blot analysis of tail or spleen DNA or by PCR analysis of tail DNA, whole embryos, or embryonic yolk sac using a set of three primers. A 276-bp product from the wild-type allele was amplified using forward (5′-GCTTCGATGGAATGTGTCATCC-TGTA-3′) and reverse (5′-ACTGAAACTATGCATCCCA-CTGAG-3′) primers corresponding to nucleotides 475–501 and 725–750 of intron 9, respectively. This fragment contained the HindIII site used to prepare the 5′ arm. A 188-bp product from the mutant allele was amplified in the same reaction using the forward primer described above and a reverse primer (5′-GCATGCTCCAGACTGCCTTG-3′) based on a sequence in the promoter of the neomycin resistance gene.

**Northern Blot and PCR Analysis of Mutant and Wild-type SPCA1 mRNAs**—Total RNA was isolated from tissues of adult wild-type and heterozygous mice and from embryos at different stages of development. Northern blot analysis of RNA from adult tissues was performed as described previously (22) using an SPCA1 cDNA probe and an L32 ribosomal protein cDNA probe as a loading control. For PCR analysis, first strand cDNA was prepared using oligo(dT) primers and reverse transcriptase. For adult tissues, PCR primers corresponded to codons 182–190 in exon 8 and the reverse complement of codons 428–435 in exon 15. For embryos, PCR primers corresponded to codons 182–190 and the reverse complements of either codons 366–373 in exon 13 or codons 660–667 in exon 21. PCR products were analyzed by agarose gel electrophoresis and DNA sequencing.

**Immunoblot Analysis of SPCA1 Protein**—Newborn pups (day 1) were euthanized, and back-skins were collected and incubated in 1× Dispase II (Roche Applied Science) overnight at 4°C with gentle agitation. After incubation, the epithelial layer, consisting of keratinocytes, was peeled off the dermal layer, frozen in liquid nitrogen, and stored at −80°C until further use. Epithelial samples were manually homogenized as described before (17). Adult mouse hearts were manually pulverized in liquid nitrogen and homogenized in buffer H (10 mM Tris, 1 mM EDTA, 0.25% Nonidet P-40, 2 mM dithiothreitol, containing a mixture of protease and phosphatase inhibitors) using a Polytron 3000 mechanical homogenizer. Protein concentrations were determined using the Coomassie protein assay reagent (Pierce). Proteins were resolved on a discontinuous, reducing 9% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and probed using an anti-SPCA1 antibody (23) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (KPL Inc., Gaithersburg, MD). Chemiluminescence was developed using the LumiGlo chemiluminescent substrate kit (KPL), and autoradiograms were developed using BioMax Films ML and MR (Eastman Kodak Co.).

**Tissue Preparation**—Tissues were fixed for light and electron microscopy and morphometry, as described previously (22).

**Histology and Light Microscopic Morphometry**—Paraffin sections of embryos were stained with H&E (93 pups). Placenta was analyzed, and morphometry of yolk sac vessels was performed using H&E-stained sections of embryos in utero. At ×40 magnification, the area (μm²) of vessel lumen (blood islands) was determined, and the number of visceral endodermal yolk sac and mesenchymal cell nuclei/μm of yolk sac was determined. In addition, the mitotic index and the number of visceral yolk sac cells containing more than three lipid droplets was counted. The thickness of visceral yolk sac endoderm was digitized (μm), as was the nuclear area (μm²).

Plastic blocks containing embryos were sectioned at 2 μm thick and stained with toluidine blue for light microscopy and light microscopic morphometry. Other embryo blocks were first trimmed to the level of the heart, where 2-μm-thick sections were made. All null embryos were grossly smaller and less well developed than age-matched wild-type embryos at ED 9.5–10.5. Tissue sites were identified according to an atlas of mouse development (24). Data were obtained from anatomical sites as follows: dorsal and ventral rostral neural tube, dorsal and ventral head mesenchyme, dorsal and ventral distal neural tube and mesenchyme, limb bud mesenchyme, surface ectoderm, bulbus cordis and outflow tract, branchial arch mesenchyme, primitive gut, otic and optic placodes, blood vessels, and red blood cells. Within these sites, the percentage of each of the following was determined: 1) mitosis (late prophase through late telophase), 2) apoptotic cells and apoptotic debris, and 3) cells containing more than three fat droplets.

**Electron Microscopic Morphometry**—The cell ultrastructure of eight Spca1+/+ and 11 Spca1−/− embryos was examined from selected sites (rostral neural tube, heart, and yolk sac). Thin sections were placed on naked copper grids, stained with uranyl acetate and lead citrate, and photographed at magnifica-
FIGURE 1. SPCA1 gene targeting, genotyping, and mRNA analysis. A, targeting strategy. Top, structure of the wild-type allele. Middle, targeting construct, with the neomycin resistance gene (Neo) replacing sequences from exons 10–13 and the herpes simplex virus thymidine kinase gene (TK) included for negative selection. Bottom, structure of the targeted allele. The locations of the wild-type 9.7- and 5-kb fragments and the mutant 8.5-kb fragment are shown above each allele, and the diagnostic probes are indicated above the wild-type allele. B, Southern blot analysis of wild-type (+/+) and targeted (+/-) embryonic stem (ES) cell DNA and spleen DNA of wild-type and heterozygous mice using the indicated enzymes and probes. C, PCR genotyping revealed wild-type and heterozygous offspring but no homozygous mutant offspring. D, RNA (20 μg/lane) from the indicated tissues (Stom, stomach; Mamm, mammary gland) was hybridized with cDNA probes for SPCA1 and the L32 ribosomal subunit (as a loading control). E, reverse transcription (RT)-PCR analysis of SPCA1 mRNA from SPCA1+/+ and Spca1−/− mice using primers from exons 8 and 15 and agarose gel electrophoresis. DNA sequence analysis revealed a 366-bp difference in size between the wild-type (WT) and knock-out (KO) PCR products, which corresponded to a deletion of exons 10–13.

**RESULTS**

**Generation of Mice with a Null Mutation in the Spca1 Gene**—Embryonic stem cells carrying a null mutation in the Spca1 gene were prepared using a construct in which exons 10–13 were replaced with the neomycin resistance gene (Fig. 1A). Because these exons encode both the catalytic phospho-rylation site and transmembrane domains 3 and 4, their excision ensured that the mutation would be a functional null. Blastocyst-mediated transgenesis yielded chimeric male mice that transmitted the mutant allele through the germ line (Fig. 1B). PCR genotyping of tail DNA of live offspring (Fig. 1C) of heterozygous breeding pairs revealed that only wild-type and heterozygous pups, in a 1:2 ratio (228:425), were present at birth, indicating that the homozygous null mutation caused embryonic lethality. Spca1−/− mice exhibited no evidence of skin lesions indicative of Hailey-Hailey disease, and there were no histologic abnormalities, except in aged mice, where there was an increased incidence of squamous cell tumors (see below).

Northern blot analysis of RNA from Spca1−/− and wild-type tissues identified SPCA1 mRNAs of 4.6–4.8 kb in all tissues examined (Fig. 1D). For some tissues, such as stomach, liver, and colon, heterozygotes exhibited a reduction in signal intensity, consistent with loss of the mutant mRNA, whereas in others there was a broadening of the mRNA size in Spca1−/− samples, consistent with the expression of both wild-type and mutant mRNAs. PCR and DNA sequence analysis confirmed that Spca1−/− tissues contained a mutant mRNA in which the donor site of exon 9 was spliced to the acceptor site of exon 14, thereby producing an mRNA that lacked codons 252–373 (Fig. 1E).
Embryonic Lethality of Null Mutants Occurs at Gestation Day 10.5—Heterozygous mice were mated, and embryos were examined at various stages of gestation. PCR genotyping revealed the presence of ostensibly live null mutants as late as ED 10.5 (Fig. 2A), and reverse transcriptase–PCR analysis of RNA from null embryos at day 8.5 confirmed the absence of wild-type SPCA1 mRNA and the presence of mutant SPCA1 mRNA (Fig. 2B). On EDs 7.5 and 8.5, there was no evidence of increased death among null mutants (12+/+, 38−/−, and 22−/−). A normal Mendelian ratio for Spca1−/− embryos was also observed on ED 9.5 (56+/+, 116+/−, and 54−/−). On ED 10.5, however, the number of null mutants was reduced (38+/+, 86+/−, and 20−/−), and by ED 11.5 and later, live null mutants were not observed.

Reduction in SPCA1 Protein Levels in Heterozygous Tissues—As discussed below, keratinocytes are the only cell type or tissue exhibiting a clear phenotype in adult tissues, and the heart exhibited no apparent structural or functional abnormalities even in homozygous null embryos. Immunoblot analysis of both the epithelial (keratinocyte) layer of newborn mouse skin (Fig. 3A) and adult heart (Fig. 3B) showed that the loss of one allele caused a significant reduction in SPCA1 protein levels in both tissues.

Incomplete Closure of Neural Tube in Null Mutant Embryos—The most striking gross defect in Spca1−/− embryos was failure of neural tube closure (Fig. 4). During the early stages of neural tube development on ED 8.5, null mutants were morphologically indistinguishable from Spca1+/+ and Spca1+/− embryos, and there was no apparent growth retardation. Primary neurulation appeared to have proceeded normally in Spca1−/− embryos, with development of the notochord, neural plate, neural crests, and epidermis; the caudal and ventral aspects of the neural tube were similar among all genotypes, and successful closure of the caudal portion of the neural tube
Normal spontaneous cardiac contractions in Spca1+/+ and Spca1−/− embryos. Spca1+/+ and Spca1−/− embryos were removed from the yolk sac, and if no spontaneous contractions were apparent, the left ventricle was mechanically stimulated. The frequency of the resultant rhythm contractions (which does not correspond to the normal embryonic heart rate in utero) was essentially the same for both genotypes at ED 9.5 (n = 13+/+ and 19−/− embryos) and ED 10.5 (n = 2+/+ and 3−/− embryos).

On ED 9.5, yolk sac blood islands (Fig. 5A), red blood cells (Fig. 5B), and blood vessels, including the dorsal aorta (Fig. 5C) and yolk sac vessels, appeared to be similar in Spca1+/+ and Spca1−/− embryos, indicating that hematopoiesis and vascular development were relatively normal at this stage. Specific indicators of yolk sac health were measured histologically at ED 9.5 in an attempt to identify and quantify any subtle impairments of development that might be a forerunner of embryonic death on ED 10.5. The mitotic index and percentage of yolk sac cells with more than three lipid droplets (see below) were not significantly different between Spca1+/+ and Spca1−/− embryos, and apoptosis was not elevated. Visceral endodermal thickness was similar in Spca1+/+ and Spca1−/− embryos (21.8 ± 3.1 and 19.31 ± 1.94 μm, respectively), and the numbers of both visceral endodermal cells/μm (0.11 ± 0.02 in Spca1+/+; 0.09 ± 0.01 in Spca1−/−) and mesodermal cells/μm (0.05 ± 0.01 in Spca1+/+; 0.04 ± 0.01 in Spca1−/−) were essentially the same. Finally, there was no significant difference between the two genotypes in the nuclear area of visceral endodermal cells (52.3 ± 3.15 μm² in Spca1+/+; 54.6 ± 3.06 μm² in Spca1−/−), as would be expected if yolk sac cells were blocked in either S phase or G2, or exhibited severe aneuploidy.

Cardiac function and development appeared to be grossly normal in null mutants. The hearts of ED 9.5 and 10.5 embryos stopped beating when the embryos were removed from the uterus; however, spontaneous beating could be elicited by gentle mechanical stimulation, although it was at a much lower rate relative to normal heart rates. There was no significant difference in the rates of this mechanically induced spontaneous heartbeat in Spca1+/+ and Spca1−/− embryos (Fig. 6). Hearts of both genotypes had sufficiently developed myofilaments (see below) and cell-cell contacts to produce coordinated cardiac contractions, which progressed in waves from the primitive

FIGURE 6. Normal spontaneous cardiac contractions in Spca1+/+ and Spca1−/− embryos. Spca1+/+ and Spca1−/− embryos were removed from the yolk sac, and if no spontaneous contractions were apparent, the left ventricle was mechanically stimulated. The frequency of the resultant rhythm contractions (which does not correspond to the normal embryonic heart rate in utero) was essentially the same for both genotypes at ED 9.5 (n = 13+/+ and 19−/− embryos) and ED 10.5 (n = 2+/+ and 3−/− embryos).

FIGURE 5. Yolk sac, hematopoiesis, and vascular development in Spca1+/+ and Spca1−/− embryos are ostensibly normal. A, H&E-stained sections of yolk sac from Spca1+/+ and Spca1−/− embryos revealed no differences in mesothelium (white arrows) or visceral endoderm (black arrows), and nucleated red blood cells were abundant in blood islands of both genotypes (white arrowheads). B, nucleated red blood cells examined by electron microscopy appeared normal in both wild-type (left) and null mutant (right) embryos. N, nucleus. C, H&E-stained sections of wild-type (left) and null mutant (right) embryos revealed apparently normal dorsal aorta (black arrows) in both genotypes. The notochord (white arrow), mesenchyme (M), and oropharynx (P) are indicated.

occurred by ED 8.5. By ED 9.5, however, Spca1−/− embryos exhibited a failure of neural tube closure from the hind brain rostrally. Around ED 9.5, the presumptive nerve cells in the rostral neural tube had developed neural processes containing neurotubules. Subjectively, these appeared to be less well developed in mutants than in wild-type embryos, but they occurred at the appropriate interface between the menenchyme and the open rostral neural tube. Spca1−/− embryos that survived to ED 10.5 were growth-retarded and continued to have an open rostral neural tube, and ruffling of the edges of the open neural tube was observed in some of the mutants (red arrows in Fig. 4, A and B). The medial hinge point of the neural tube often appeared appropriate in Spca1−/− embryos, but dorsolateral hinging was incomplete from the hind brain forward. Despite growth retardation and nonclosure of the rostral neural tube in null embryos, some growth and development continued between EDs 8.5 and 10.5, and, very infrequently in older Spca1−/− embryos, partial closure of the neural tube occurred at the maxilla and face.

Hematopoiesis and Cardiovascular Development Appear Normal in Null Mutant Embryos—Despite their dramatic appearance, neural tube defects cannot account for the observed embryonic death at ED 10.5. However, because impaired hematopoiesis or cardiovascular development can be a major cause of death during this period, we examined these processes.
ventricle through the outflow tract, propelling blood into the dorsal aortae. Red blood cells within the embryos were present in sufficient numbers to be visible as a flow within both the heart tube and dorsal aortae, demonstrating that the circulatory system was intact.

Presumptive myocardial and endocardial cells, trabeculae, and division of the heart tube into appropriate segments occurred in both genotypes (Fig. 7). Endocardial cushions formed in the absence of SPCAl, since similar outflow tract morphology was seen in both Spca1+/+ and Spca1−/− embryos (Fig. 7A). Heart development progressed with apparent normality through ED 10.5; the looped heart, paired dorsal aortae, bulbous cordis, primitive ventricle, and common atrial chamber were all distinguishable. At higher resolution, the endocardium and trabeculae appeared similar in both wild-type and Spca1−/− hearts (Fig. 7B). Cardiac myocytes contained glycogen deposits by ED 9.5. Ultrastructurally, ED 10.5 myocytes of Spca1+/+ and Spca1−/− hearts contained actin and myosin filaments and exhibited clear evidence of banding, and both genotypes showed typical thin and thick filament organization in longitudinal sections and cross-sections (Fig. 7C).

TABLE 1

| Morphometric analyses of the effects of SPCAl ablation on Golgi membranes, Golgi-associated vesicles, and rough endoplasmic reticulum |
| --- |
| 8.5–10.5 ED | +/+ | −/− | p |
| Golgi bodies/cell profile | 0.08 ± 0.02 | 0.40 ± 0.15 | 0.012 |
| Golgi bodies (Vd) | 0.18 ± 0.08 | 0.78 ± 0.4 | 0.075 |
| Flat lamellae (number per Golgi body) | 4.7 ± 1.8 | 1.36 ± 0.68 | 0.017 |
| Dilated Golgi (percentage of total Golgi) | 44.4 ± 20.5 | 89.1 ± 4.2 | 0.010 |
| Golgi-associated vesicles (Vd) | 0.51 ± 0.16 | 1.57 ± 0.64 | 0.007 |
| Golgi-associated dense vesicles/cell profile | 0.30 ± 0.20 | 1.17 ± 0.3 | 0.051 |
| Dense vesicles on cis side (%) | 3.2 ± 2.8 | 42.7 ± 11.5 | 0.016 |
| Rough ER (Vd) | 2.52 ± 0.30 | 2.62 ± 0.49 | 0.83 |
| Dilated RER (percentage of total) | 31.7 ± 7.6 | 48.6 ± 14.9 | 0.069 |
| Ribosomes/μm of RER | 27.08 ± 2.11 | 19.71 ± 1.45 | 0.0001 |

*Vd, volume density.

* This Vd measurement largely excluded the small dense vesicles.
Membrane specializations for cell adhesion appeared similar in cells from Spca1−/− embryos. The incidence of coated pits, desmosomes, junctional complexes, and basement membranes appears normal in most, if not all, tissues. The number of Golgi-associated lamellae typical of Golgi bodies had been lost or diminished in plasmic reticulum were altered (Fig. 8). The flattened, stacked copy indicated that both Golgi membranes and rough endoplasmic reticulum were increased in the mutant, and dense vesicles associated with the Golgi appeared to have been sharply increased, particularly on the cis-side. Profiles of RER were often dilated, and the RER seemed to have diminished numbers of ribosomes.

To quantify these changes, the numbers of these organelles per cell were counted, and/or their volume densities were determined (Table 1). Major alterations in Spca1−/− cells included at least a 4-fold increase in Golgi bodies, dilation of Golgi membranes, and significant changes in the number and distribution of Golgi-associated vesicles. The amount of RER was not altered, and the increase in dilated RER did not achieve a high level of significance; however, the number of ribosomes per μm of RER was significantly reduced.

Given the changes in Golgi and RER membranes, one might anticipate that structures dependent on processing and trafficking of proteins in the secretory pathway would be perturbed. However, ultrastructural analysis of the neural tube, which was the only embryonic structure that was clearly altered, revealed normal-appearing coated pits, junctional complexes, desmosomes, and basement membranes (Fig. 9). Ultrastructural analyses of other tissues, such as visceral endodermal cells, yielded similar results (data not shown).

Morphometric analyses (Table 2) revealed no significant differences in the numbers of coated pits, desmosomes, or junctional complexes, and similar numbers of mitochondria were present in both genotypes.

Apoptosis and Intracellular Lipid Droplets Are Increased in Null Mutant Embryos—The apparent growth retardation raised the possibility that Spca1−/− embryos on EDs 9.5 and 10.5 might have a reduced rate of cell division and/or an increased rate of apoptosis. While determining mitotic and apoptotic indices, a frequent observation in Spca1−/− cells was the apparent accumulation of lipid droplets within the cytoplasm, which was confirmed by ultrastructural studies (Fig. 10). Therefore, a count of cytoplasmic lipid droplets was also conducted.

Analysis of neural tube, mesenchyme, and the developing cardiovascular system of wild-type and null embryos revealed no significant differences in mitotic index (Table 3). The apoptotic index, however, was significantly increased in both neu-


SPCA1 Gene Targeting

TABLE 3

Morphometric analyses of the effects of SPCA1 ablation on mitotic index, apoptotic index, and the number of lipid inclusions in embryonic cells

Data were collected from H&E-stained sections from ED 8.5–10.5 embryos. Values are means ± S.E. and represent the percentage of affected cells in that tissue. n = 8–12 for each genotype.

|                  | Neural tube | Mesenchyme | Heart and vessels |
|------------------|-------------|------------|------------------|
|                  | +/+         | −/−        | +/+              | −/−            |
| Mitotic index    | 1.62 ± 0.37 | 1.97 ± 0.31| 0.94 ± 0.31      | 1.13 ± 0.35    |
| Apoptotic index  | 0.42 ± 0.18 | 2.91 ± 1.38| 0.85 ± 0.36      | 3.72 ± 1.11    |
| >3 lipid droplets/cell | 0.08 ± 0.06 | 3.27 ± 1.36 | 1.72 ± 1.5       | 18.7 ± 5.8     |

*p < 0.02.

TABLE 4

Squamous cell carcinomas and papillomas in Spca1+/−/− mice

Spca1+/− and Spca1+/-/+ mice were aged for up to 2 years. Tumors of nonepithelial origin did not differ significantly between the genotypes and were not included.

| Skin                | Carcinoma | Papilloma | Esophagus (papilloma) |
|---------------------|-----------|-----------|-----------------------|
| +/− (n = 26)        | 3         | 1         | 2                     |
| +/+ (n = 23)        | 0         | 0         | 0                     |

FIGURE 11. Squamous cell papillomas and carcinomas in SP5A1 heterozygous mutant adults. A, papilloma of the esophagus isolated from a 23-month-old Spca1+/+ male; note the extensive keratinization (top). An additional focus of hyperplasia was located elsewhere in this esophagus. B, skin carcinoma from a 20-month-old Spca1+/−/− female located on the back of the right hind limb. Bar, 100 μm.

DISCUSSION

Midgestational Death and Exencephaly in Spca1−/− Embryos—The normal appearance of null embryos on ED 8.5 showed that the loss of SPCA1 did not impair blastocyst development, implantation, formation of the three germ layers, or gastrulation. By ED 9.5, however, null embryos exhibited growth retardation and exencephaly, and death occurred between ED 10 and ED 11. The most common causes of embryonic lethality during this period are failure of hematopoiesis and impaired development of the cardiovascular system (25). However, defects in yolk sac development or yolk sac-based hematopoiesis (26, 27) were not responsible for the observed embryonic lethality. Similarly, none of the hallmarks of impaired cardiovascular development (28–31) occurred in Spca1−/− embryos. The only apparent structural defect was incomplete closure of the neural tube, although there was some bending at the dorsolateral and medial hinge points, suggesting that signaling via factors secreted from the notochord are required for neural plate bending (32) was not seriously impaired. Neural tube defects have a diversity of causes (33), including apoptosis (34), which is the likely cause of these defects in Spca1−/− embryos.

Embryolethality Correlates with Apoptosis and Secretory Pathway Stress—The increased apoptosis suggests that embryonic death was due to widespread failure of cell viability rather than the failure of any one cell type or organ system. Because the primary defect was the loss of SPCA1, it is clear that the apoptosis is due, directly or indirectly, to a deficiency in Golgi Ca2+ and/or Mn2+ handling. In yeast, PMR1 null mutants are toxic, suggesting that one function of the Mn2+ transport activity of the pump is to expel Mn2+ from the cell via the exocytic pathway (35). Mn2+ toxicity can occur in humans and has been shown to cause apoptosis in mammalian cells (36); however, it is unclear that Mn2+ detoxification would be a critical function in a rapidly growing embryo. Because Mn2+ is an important cofactor for glycosyltransferases in the Golgi (37), impaired glycosylation of...
proteins could contribute to secretory pathway stress and apoptosis; however, Mn$^{2+}$ can enter mammalian secretory membranes via inositol 1,4,5-trisphosphate receptors (38), which are present in both the ER and Golgi (39). Thus, the more likely cause of the apoptosis phenotype is the defect in Golgi Ca$^{2+}$ handling.

A reduction in ER Ca$^{2+}$ stores or inhibition of SERCA2 activity by thapsigargin is a well-established mechanism for inducing ER stress (40, 41), which leads to induction of the UPR and ER-associated degradation (42, 43), with activation of both apoptotic and survival pathways. The Golgi also plays a role in stress sensing and apoptotic signaling (44); however, well-documented conditions producing Golgi-specific stress have not been identified (44). The current study provides strong evidence that impaired Ca$^{2+}$ sequestration by SPCA1 can cause Golgi-specific stress, which seems analogous to ER-specific stress resulting from SERCA2 deficiency.

Major ultrastructural manifestations of Golgi stress included dilation of the Golgi and a reduction in the number of stacked leaflets. An increase in Golgi membranes and Golgi-associated vesicles was also observed and, as discussed below, may be a response to Golgi stress that allows cell survival. Dilation of Golgi membranes and expansion of the Golgi have been observed both in human Tangier disease and in mice lacking ATP-binding cassette transporter 1 (45, 46), which is expressed in both Golgi and plasma membranes (47). The cytoplasmic lipid droplets in Spca1$^{-/-}$ cells may also be due to Golgi stress, since the Golgi plays a critical role in lipid metabolism and trafficking (48).

There was some dilation of the RER and a significant decrease in the number of membrane-bound ribosomes, consistent with increased accumulation of protein and reduced protein synthesis that occurs during ER stress (41, 49). The sharp increase in the number of dense vesicles on the cis-side of the Golgi suggests that trafficking of vesicles between the ER and Golgi was affected. The effects of SPCA1 null mutations on the ER are consistent with observations that a reduction in SPCA1 expression in cultured cells causes hypersensitivity to ER stress and impairs ER-associated protein degradation (12).

**Expansion of the Golgi as a Possible Survival Response to Golgi Stress**—Despite increased apoptosis and Golgi stress, the viability of Spca1$^{-/-}$ embryos during earlier stages of embryogenesis and the normal development of coated pits, desmosomes, junctional complexes, and basement membranes, which would require a functional secretory pathway, shows that the loss of SPCA1 does not cause a massive failure of Golgi function. That a substantial degree of Golgi function is retained suggests that at least partial compensation occurs for any functional deficits caused by the loss of SPCA1. It is unlikely that SPCA2 is involved, since it is expressed in only a limited set of tissues (5, 6), and reverse transcription-PCR analysis showed that its mRNA is not expressed in ED 8.5 embryos (data not shown). Other means by which delivery of Ca$^{2+}$ to the Golgi might occur is by the activity of a thapsigargin-sensitive Ca$^{2+}$ pump (presumably SERCA2) that mediates Ca$^{2+}$ uptake into an agonist-sensitive compartment of the Golgi (8) and by trafficking of Ca$^{2+}$-containing vesicles from the ER to the Golgi.

The ~4-fold expansion of the Golgi in Spca1$^{-/-}$ embryos would appear to be an important compensatory mechanism, since it should improve the overall capacity for processing and trafficking of proteins in the secretory pathway. We are aware of only one other documented example of Golgi expansion in a disease state (45, 46); however, expansion of the ER occurs during differentiation and in response to ER stress resulting from a variety of conditions (reviewed in Ref. 50). The transcription factor XB1, which is activated in response to ER stress and is an effector of the survival pathways of the UPR (42), induces ER biogenesis (51, 52). Overexpression of XB1 leads to induction of genes encoding proteins of multiple compartments of the secretory pathway, including the Golgi (53), and to expansion of the Golgi itself (54). Thus, it is possible that expansion of the Golgi in Spca1$^{-/-}$ embryos is elicited by survival pathways activated in response to Golgi stress, which are directly analogous to (and may interact or overlap with) survival pathways activated in response to ER stress.

**Squamous Cell Tumors in SPCA1 Heterozygous Mice**—Spca1$^{+/+}$ mice exhibited no manifestations of HHD but did develop squamous cell carcinomas and papillomas. The phenotype is similar to that of SERCA2 heterozygous mice, which developed squamous cell tumors but not Darier disease (17, 18). Thus, the Spca1$^{+/+}$ mouse is the second genetic model of cancer involving a deficiency in Ca$^{2+}$ transport. Because SPCA1, like SERCA2, functions in the secretory pathway but plays much less of a role in Ca$^{2+}$ signaling than SERCA2, it is likely that impairments of the secretory pathway itself is part of the mechanism of tumorigenesis in both models.

The incidence of squamous cell tumors was ~25% in Spca1$^{+/+}$ mice, compared with ~90% among SERCA2 heterozygous mutants (17, 18). The normal incidence is very low; e.g. among 4900 control mice in the National Toxicology Program studies (55), only one esophageal and 11 skin squamous cell tumors were observed, six of which were carcinomas. Tumors in SERCA2 heterozygous mice did not exhibit loss of the wild-type allele (18), thereby ruling out the possibility that SERCA2 serves as a classical tumor suppressor in mice. The late age of onset makes it unlikely that loss of heterozygosity is part of the genetic mechanism of tumorigenesis in Spca1$^{+/+}$ mice.

In humans, there is at least one case of type 2 segmental HHD in which loss of heterozygosity occurred at the ATP2C1 (SPCA1) locus during embryonic development, leading to mosaicism and severe HHD in Spca1 null cells (56). The lack of skin tumors in this patient shows that SPCAI does not serve as a classical tumor suppressor in humans.

**Similarities between Phenotypic Effects of SPCA1 and SERCA2 Deficiency Suggest a Common Mechanism**—Despite major species differences in disease phenotypes, there are clear parallels between the effects of SPCA1 and SERCA2 heterozygosity in humans and mice. For each Ca$^{2+}$ pump and for both species, the affected cell type is keratinocytes, and the site of the primary deficit in Ca$^{2+}$ handling is a compartment of the secretory pathway. In humans, loss of one copy of either gene causes acantholytic skin disease (13, 14, 16), whereas squamous cell tumors, with no evidence of skin disease, are the major phenotype in mice (17, 18) (this study). These similarities suggest that the mechanisms of each disease are similar. However, why
would reduced activity of either SPCA1 or SERCA2 cause squamous cell tumors in mice and acantholytic skin disease in humans? The answer is undoubtedly complex but may involve differential responses of mouse and human keratinocytes to secretory pathway stress, with prosurvival pathways that favor tumorigenesis predominating in mice and proapoptotic pathways that favor acantholysis predominating in humans. In considering this possibility, which is admittedly speculative, some uncertainties should be noted. Although a great deal is known about ER stress responses, almost nothing is known about Golgi stress responses other than the observations that severe Golgi stress can affect ER function, induce Golgi expansion (an apparent prosurvival response), and induce apoptosis.

Conditions causing deficiencies in protein processing and trafficking in the secretory pathway, including nutrient deprivation, hypoxia, exposure to certain anti-tumor agents, or a reduction in luminal Ca²⁺ concentrations, can cause ER stress (57). This leads to induction of the UPR, a set of powerful stress response pathways that activate both prosurvival and proapoptotic mechanisms. Severe ER stress, to which the cell cannot adapt, leads to apoptosis; however, activation of the UPR by chronic low level ER stress leads to adaptation, since prosurvival stimuli remain active and proapoptotic stimuli diminish (58). The major effectors of the UPR are the transcription factors XBP1 and ATF6, and the kinase PERK, which affects translation (41, 59). Thus, the UPR causes major alterations in mRNA and protein expression patterns that either enable the cell to survive or, alternatively, lead to death.

Although the details are not well understood, the role of the UPR in tumor survival and progression is well established (60), with both XBP1 and PERK playing prominent roles (61, 62). Activation of the UPR by exposure to conditions causing ER stress can lead to protection against ER stress caused by the same or different conditions (58). That mild SERCA2 deficiency has the potential to cause ER stress and induce adaptive components of the UPR is supported by the observation that treatment of cultured cells with very low concentrations of the SERCA inhibitor thapsigargin leads to sustained activation of prosurvival pathways of the UPR with only transient activation of proapoptotic pathways (58). Thus, it is possible that sustained activation of prosurvival pathways of cellular responses to ER or Golgi stress might play a mechanistic role in tumor development in SERCA2 and SPCA1 heterozygous mice.

In humans, it has been suggested that HHD and Darier disease may result from defective processing and trafficking of desmosomal components (63). In the case of HHD, this mechanism seems unlikely, since desmosomes and other cell surface structures appeared normal in null mutant mice. Also, in a patient with type 2 segmental HHD, in which both copies of the ATP2C1 (SPCA1) gene were defective (56), some of the genetically affected skin appeared normal. One might expect that if heterozygosity caused a sustained defect in trafficking and processing of desmosomal proteins, then homozygous null mutations would cause a massive failure. Type 2 segmental HHD was more severe and affected deeper layers of the skin but was still recognized as HHD, suggesting that the disease process was fundamentally the same as in heterozygous HHD. During differentiation, keratinocytes undergo a highly controlled process of “incomplete” apoptosis (64–67) in which they lose their nuclei and die but retain the intercellular connections and intracellular keratin intermediate filaments that provide structural integrity to the skin. Perhaps the loss of one copy of either gene causes ER or Golgi stress, which in turn elicits responses that activate proapoptotic pathways that interfere with the regulation of incomplete apoptosis needed for terminal differentiation. Ultrastructural changes in cells from Darier disease lesions (68) are consistent with aberrant apoptosis as part of the disease mechanism. Desmosomal proteins are targeted during apoptosis (69, 70), so if the incomplete apoptosis that occurs during normal keratinocyte differentiation in humans were accentuated by additional apoptotic stimuli, breakdown of desmosomal contacts between cells and their attachments to intermediate filaments, one of the hallmarks of HHD and Darier disease, could occur.

Conclusions—Our findings establish SPCA1 deficiency as the first documented example of a Golgi-specific stress condition and identify Sca1⁺/− mice as the second genetic model in which a deficiency in Ca²⁺ handling causes cancer. When considered in the light of previous studies, a number of interesting parallels between the phenotypic effects of SPCA1 and SERCA2 haploinsufficiency are apparent. A reduction in the activity of either Ca²⁺ pump, each of which serves in the secretory pathway, caused squamous cell tumors in mice and acantholytic skin disease in humans, and, in each case, keratinocytes were the affected cell type. This suggests a common disease mechanism in which 1) chronic secretory pathway stress, originating in either the ER or Golgi, leads to 2) activation of cellular stress responses, such as the UPR or some comparable Golgi-specific stress response (about which almost nothing is known), and 3) differential activities of prosurvival or proapoptotic arms of the stress response in mice or humans, respectively, leads to the observed disease manifestations in each species. If this view is correct, then the prosurvival and proapoptotic stress responses themselves serve as major components of the disease mechanism.

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