

Development of pheochromocytoma in ceramide synthase 2 null mice

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Abstract

Pheochromocytoma (PCC) and paraganglioma are rare neuroendocrine tumors of the adrenal medulla and sympathetic and parasympathetic paraganglia, for which mutations in ~15 disease-associated genes have been identified. We now document the role of an additional gene in mice, the ceramide synthase 2 (CerS2) gene. CerS2, one of six mammalian CerS, synthesizes ceramides with very-long (C22–C24) chains. The CerS2 null mouse has been well characterized and displays lesions in several organs including the liver, lung and the brain. We now demonstrate that changes in the sphingolipid acyl chain profile of the adrenal gland lead to the generation of adrenal medullary tumors. Histological analyses revealed that about half of the CerS2 null mice developed PCC by ~13 months, and the rest showed signs of medullary hyperplasia. Norepinephrine and normetanephrine levels in the urine were elevated at 7 months of age consistent with the morphological abnormalities found at later ages. Accumulation of ceroid in the X-zone was observed as early as 2 months of age and as a consequence, older mice displayed elevated levels of lysosomal cathepsins, reduced proteasome activity and reduced activity of mitochondrial complex IV by 6 months of age. Together, these findings implicate an additional pathway that can lead to PCC formation, which involves alterations in the sphingolipid acyl chain length. Analysis of the role of sphingolipids in PCC may lead to further understanding of the mechanism by which PCC develops, and might implicate the sphingolipid pathway as a possible novel therapeutic target for this rare tumor.

Key Words

- ▶ sphingolipid
- ▶ ceramide
- ▶ acyl chain length
- ▶ oxidative stress
- ▶ ceroid
- ▶ lipofuscin
- ▶ pheochromocytoma

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Introduction

The adrenal gland contains two distinct hormone-producing zones, the outer adrenal cortex, which produces glucocorticoids and mineralocorticoids, and the adrenal medulla, which is directly innervated from sympathetic nerves and secretes norepinephrine (NE) and epinephrine

(E), and which participate in the fight-or-flight response (Rosol *et al.* 2001). In mice, particularly in females, a region defined as the X-zone is located between the cortex and the medulla. However, there are significant differences between the X-zone in mice and humans, with the

X-zone apparent long after birth in mice (Sucheston & Cannon 1968). Moreover, in aged mice, accumulation of lipofuscin, also known as aging pigment or ceroid, is detected in the X-zone (Rosol *et al.* 2001, Seehafer & Pearce 2006) and may extend into the medulla (Greaves 2007). Ceroid, a generic name given to lipopigments that accumulate in association with lipid degeneration or lipid excess, accumulates in lysosomes and consists of advanced lipoxidation end-products (ALEs), which harbor biochemical modifications leading to cross-linking of proteins, lipids and carbohydrates. Ceroid accumulation is associated with vitamin E deficiency, and with neurodegenerative diseases such as the neuronal ceroid lipofuscinoses (NCLs), possibly reflecting chronic oxidative stress (Weglicki *et al.* 1968, Seehafer & Pearce 2006, Hayashi 2009). Chronic oxidative stress and ceroid formation have been associated with tumor formation in the adrenal gland, and in particular with pheochromocytoma (PCC) (Pacak 2011). In humans, PCC is a rare tumor of which ~25% of cases are associated with germline mutations, commonly in subunits of succinate dehydrogenase (SDH), which is part of mitochondrial complex II (Kantorovich & Pacak 2010).

We recently generated a ceramide synthase 2 (*CerS2*) null mouse (Pewzner-Jung *et al.* 2010a) and now document a high incidence of bilateral medullary hyperplasia and PCC in mice older than 13 months of age. *CerS2* is one of six mammalian *CerS* (Levy & Futerman 2010, Park *et al.* 2014), each of which synthesizes ceramide, a key intermediate in the pathway of sphingolipid (SL) biosynthesis (Levy & Futerman 2010, Park *et al.* 2014) and an important signaling lipid (Hannun & Obeid 2008), particularly with acyl chains of different length. The *CerS2* null mouse is unable to synthesize very-long chain (VLC) ceramides (C22–C24-ceramides). As a result, the ceramide and SL profile is altered such that the mice contain no VLC-SLs but rather contain elevated levels of long chain (LC) SLs, and in some cases, elevated levels of the *CerS* substrate, sphinganine (Pewzner-Jung *et al.* 2010a, Ben-David *et al.* 2011). These mice develop lesions in several organs including the liver (progressive hepatopathy), lung (emphysema and chronic inflammation) and brain (bilateral and symmetrical demyelination) (Pewzner-Jung *et al.* 2010b, Ben-David *et al.* 2011, Petrache *et al.* 2013), and as we now report, in the adrenal gland. The mechanism of PCC formation in *CerS2* null mice may be related to mitochondrial dysfunction due to complex IV deficiency, as has been observed in hepatocytes from these mice (Zigdon *et al.* 2013).

Materials and methods

Materials

N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (suc-LLVY-MCA) was from Sigma–Aldrich. The antibodies used in this study were anti-*CerS2* (Sigma–Aldrich), anti-cathepsin D, anti-cathepsin L and anti-cathepsin S (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HNE-Michael adducts (Calbiochem, San Diego, CA, USA), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Millipore, Temecula, CA, USA) and anti-F4/80 (Biolegend, San Diego, CA, USA).

Generation and maintenance of *CerS2* null mice

CerS2 null mice were generated as described (Pewzner-Jung *et al.* 2010a). All mice were treated according to the Animal Care Guidelines of the Weizmann Institute of Science Animal Care Committee and the National Institutes of Health's Guidelines for Animal Care.

Real time PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was generated using the Verso cDNA Synthesis Kit (Thermo Scientific, Hudson, NH, USA). Real time PCR was performed using a SYBR Green PCR master mix (Finnzyme, Espoo, Finland) and an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Primers for cathepsin were from Vitner *et al.* (2010).

Macroscopic and microscopic analyses

Mice were removed for necropsy at various ages. The pathological study was based on 49 mice (30 *CerS2* null (15 males and 15 females) and 19 WT mice (five males and 14 females)) aged 2–19.5 months. This group includes 21 mice older than 13.5 months (the youngest age at which PCC was observed), 11 *CerS2* null mice (six females and five males) and ten WT mice (seven females and three males). The adrenal gland was freshly excised, weighed, photographed and fixed. Samples for histology were fixed in 10% neutral buffered formalin, processed routinely and stained with hematoxylin and eosin (H&E), Nissl, Periodic acid-Schiff (PAS) or Ziehl-Nielsen and Pearl's iron stains. Sections for immunohistochemistry were fixed in 3% paraformaldehyde for 2 days. Paraffin sections (4 μm) were deparaffinized and antigen retrieval was performed using

10 mM citric acid (pH 6.0) prior to blocking in 20% horse serum in PBS containing 0.2% Triton X-100 for 2 h. Antibodies were added overnight at 4 °C and a biotinylated secondary antibody was added for 2 h. Sections were processed with an avidin-biotin HRP kit (ABC kit, Vector Laboratories, Burlingame, CA, USA) for 2 h and developed using diaminobenzidine (DAB) (Sigma–Aldrich).

Electrospray ionization-tandem mass spectrometry

Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was performed using a PE-Sciex API 3000 triple quadrupole mass spectrometer and an ABI 4000 quadrupole-linear ion trap mass spectrometer (Shaner *et al.* 2009, Sullards *et al.* 2011).

Western blotting

The adrenal gland was homogenized with radioimmuno-precipitation assay (RIPA) buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors) and incubated at 4 °C for 30 min. Lysates were centrifuged (10 min, 10 000 g, 4 °C) and protein in the supernatant measured using a BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL, USA). Fifty micrograms was loaded on 10%

SDS–PAGE gels and transferred to a nitrocellulose membrane. Membranes were blocked with 5% BSA in PBST (PBS, 0.1% Tween-20) and primary and secondary antibodies were applied. Chemiluminescence was performed using a SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Inc., Bremen, Germany).

Stress-induced corticosterone secretion

CerS2 null mice were restrained for 30 min using a 50 ml conical tube with the bottom removed (Chen *et al.* 2006). Blood was collected before, 30 and 60 min after restraint and serum was isolated. Corticosterone levels were measured using a corticosterone ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). Epinephrine, metanephrine (MN), NE and normetanephrine (NMN) levels in urine were analyzed by the Bio-Rad HPLC method (Peaston *et al.* 1996).

Biochemical assays

Cathepsin D activity was measured using a Cathepsin D activity assay kit (Biovision, Palo Alto, CA, USA). Activities of mitochondrial respiratory complexes were measured as described (Zigdon *et al.* 2013). For activity of the 20S proteasome (Sitte *et al.* 2000), the adrenal gland was

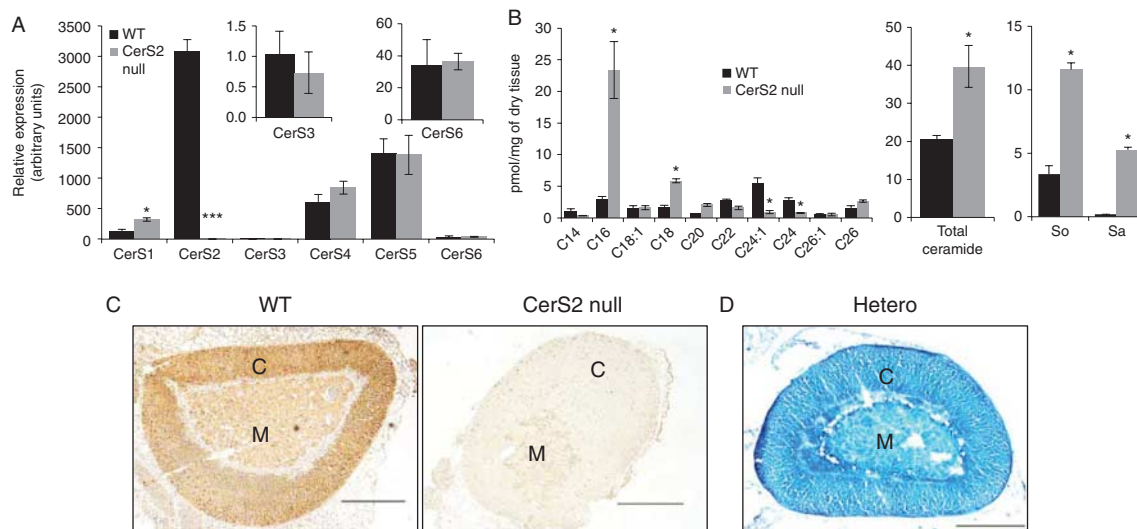


Figure 1

CerS2 is expressed at high levels in the adrenal gland. (A) Relative *CerS* mRNA levels in 2-month-old WT and *CerS2* null mouse adrenal gland. Data are mean \pm s.e.m., $n=3$. * $P<0.05$, *** $P<0.001$. (B) Ceramide and long chain base composition. The left-hand panel shows the acyl chain composition of ceramide, the middle panel levels of total ceramide and the right-hand panel levels of sphingosine (So) and sphinganine (Sa) in 4-month-old mice.

Data are means \pm s.e.m., $n=2$. * $P<0.05$. (C) Immunostaining using an anti-*CerS2* antibody in 2-month-old female mouse adrenal gland. Scale bar, 500 μ m. C, cortex; M, medulla. (D) X-gal staining on cryo-sections of adrenal gland isolated from 3-month-old female *CerS2* heterozygous mice. C, cortex; M, medulla. Scale bar, 500 μ m.

homogenized in lysis buffer (0.25 M sucrose, 25 mM HEPES (pH 7.8), 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol) and centrifuged (10 min, 14 000 g, 4 °C). Five micrograms of protein from the supernatant was diluted in 100 µl proteolysis buffer (50 mM Tris (pH 7.8), 20 mM KCl, 5 mM MgOAc, 0.5 mM dithiothreitol) and incubated with 200 µM Suc-LLVY-MCA at 37 °C for 1 h. The reaction was terminated by adding 1 volume of cold ethanol and measured at 380 nm excitation and 440 nm emission after addition of 0.125 M sodium borate (pH 9.0). Free methylcoumarin (MCA) was used for quantification.

Results

CerS2 is the major *CerS* transcribed in the adrenal gland (Fig. 1A) and as expected, no *CerS2* mRNA was detected in the adrenal gland of *CerS2* null mice, in which a small elevation of *CerS1* mRNA was detected (Fig. 1A). VLC-ceramides were the major ceramide species in the adrenal gland of WT mice and were significantly reduced in *CerS2* null mice, while levels of C16:0 and C18:0-ceramides were elevated (Fig. 1B). As a result, total ceramide levels were elevated approximately two fold, similar to that observed in some other tissues (Ben-David *et al.* 2011, Pewzner-Jung *et al.* 2014). Sphinganine and sphingosine levels were also elevated (Fig. 1B). *CerS2* was detected both in the cortex and medulla of WT adrenal glands, although at higher levels in the cortex, but was not detected in *CerS2* null mice (Fig. 1C). A similar expression pattern was observed

by x-gal staining (which detects the LacZ gene inserted in the first intron of the *CerS2* gene in the *CerS2* null allele) (Fig. 1D). *CerS2* was not detected in the X-zone (Fig. 1C and D).

Ceroid accumulation in *CerS2* null mouse adrenal gland

Since *CerS2* was the major *CerS* in the adrenal gland, we examined the effect of ablating *CerS2* on adrenal gland pathology. Mild to marked ceroid accumulation was observed in the X-zone of *CerS2* null mice as early as 2 months of age, and was often more severe in older mice (Fig. 2A), although no differences were observed between male and female mice. The material accumulated in, and distended the cytoplasm of cells arranged into variably-sized clusters. The clusters were most-commonly concentrated in the X-zone but in some cases were also found in the cortex and medulla (Fig. 2A). The ceroid was finely granular, light brown by the HE stain, PAS-positive, acid fast negative for iron (Fig. 2B), and autofluorescent (Fig. 2C), as is typical of ceroid (Greaves 2007). Ceroid-laden cells were F4/80-positive (Fig. 2D) and sometimes multinucleated, consistent with macrophages.

We also examined levels of cathepsins since ceroid is known to affect lysosome function (Keller *et al.* 2004). mRNA levels of a number of cathepsins were significantly elevated (Fig. 3A), as were protein levels of cathepsin D, L and S (Fig. 3B) and activity of cathepsin D (Fig. 3C). Cathepsins D, L and S were localized in ceroid aggregates

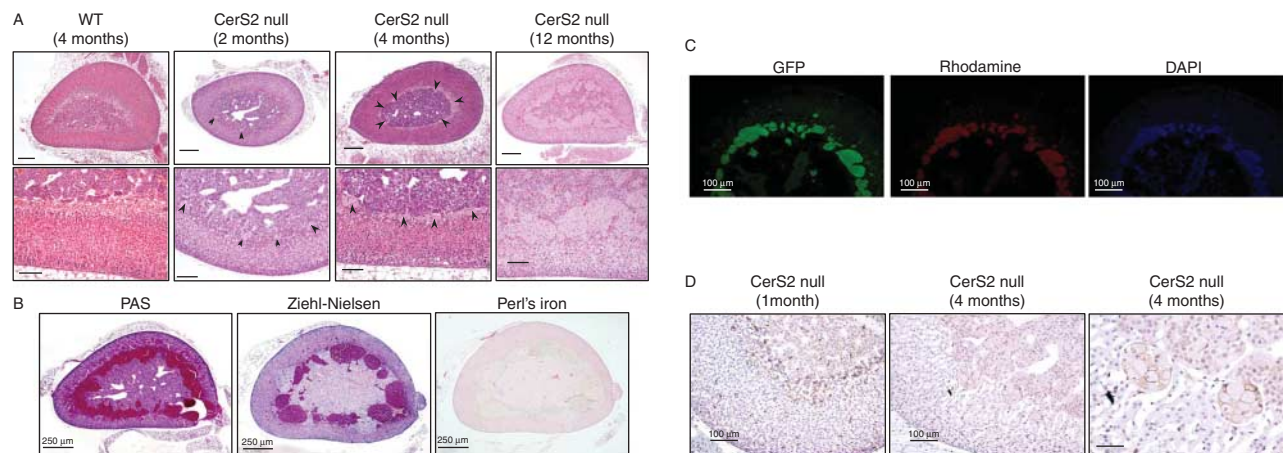
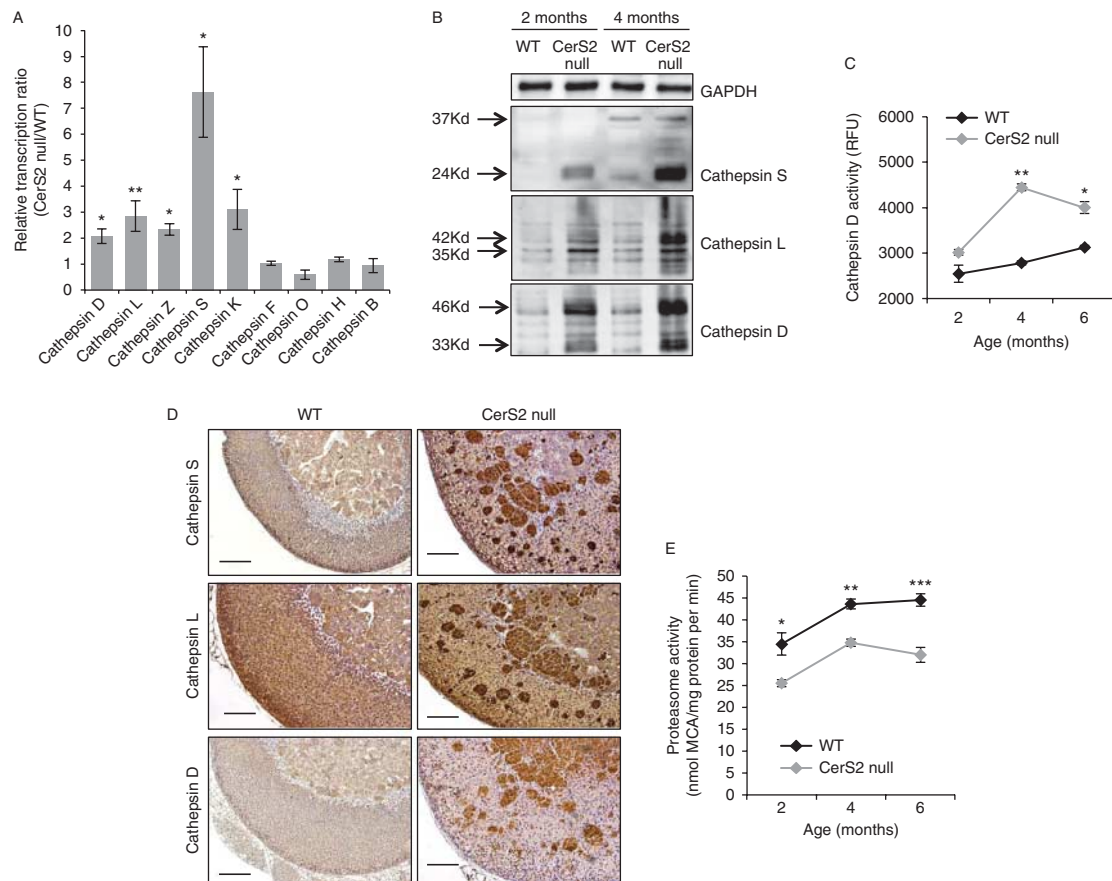


Figure 2

Ceroid accumulation in *CerS2* null mouse adrenal gland. (A) Hematoxylin and eosin staining. Arrowheads indicate ceroid accumulation in the X-zone. Scale bar, 250 µm (upper panels), 100 µm (lower panels). (B and C) Ceroid accumulation in the X-zone of the adrenal gland of 2–4 month-old male and female *CerS2* null mice. Ceroid is PAS-positive, acid fast (Ziehl-Nielsen stain) and negative for iron (Perl's iron stain) scale bars, 250 µm (B),

100 µm (C). (D) F4/80 staining of macrophages in adrenal gland of *CerS2* null mice at 1 and 4 months of age. Arrowheads are aggregates of macrophages which are found in the X-zone between the medulla and the cortex. The right-hand panel is an enlargement of the middle panel. Scale bar 25 µm.

**Figure 3**

Lysosomal enzyme overexpression and decreased proteasome activity. (A) Relative mRNA levels of cathepsins in the adrenal gland of *CerS2* null mice. $n=3$; data are means \pm s.e.m. * $P<0.05$, ** $P<0.01$. (B) Representative western blot of cathepsins L, S and D in 2–4 month-old WT and *CerS2* null mouse adrenal gland. GAPDH was used as loading control. (C) Activity of cathepsin D in 2, 4, and 6 month-old adrenal gland homogenates. $n=3$;

data are means \pm s.e.m. * $P<0.05$, ** $P<0.01$. (D) Immunohistochemical staining of 4 month-old WT and *CerS2* null adrenal gland using anti-cathepsin S, L and D antibodies. Scale bar, 50 μ m. (E) 20S proteasome activity of WT and *CerS2* null adrenal gland in 2, 4 and 6 month-old adrenal gland homogenates. $n=3$. Data are means \pm s.e.m. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

(Fig. 3D). Proteasome activity, which can be reduced by lipofuscin or ceroid (Sitte *et al.* 2000), was decreased between 2 and 6 months of age (Fig. 3E).

Mitochondrial dysfunction in *CerS2* null mouse adrenal gland

Since ceroid can be formed as a result of oxidative stress (Kurz *et al.* 2007), we analyzed oxidative stress in the adrenal glands of *CerS2* null mice. Lipid peroxidation products (identified using the anti-4-hydroxy-2,3-nonenal (HNE)-Michael adducts antibody) co-localized with ceroid aggregates (Fig. 4A), suggesting that chronic oxidative stress may lead to ceroid formation, or vice versa. No difference in the activity of the mitochondrial electron transport chain was detected at 2 months of age

(not shown), but a significant reduction in the activities of mitochondrial complexes III and IV was detected in adrenal glands from 6 month-old *CerS2* null mice (Fig. 4B), consistent with the notion that mitochondrial dysfunction is secondary to ceroid formation (Terman *et al.* 2006).

CerS2 null mice develop PCC and secrete elevated levels of epinephrine and NE

Bilateral PCC was found in six out of 11 *CerS2* null mice (three males and three females) between the ages of 13–19 months (Fig. 5A). In the remaining five *CerS2* null mice, medullary hyperplasia (unilateral or bilateral) was observed (Fig. 5B). Microscopic examination of mice with PCC showed marked enlargement of the adrenal gland in

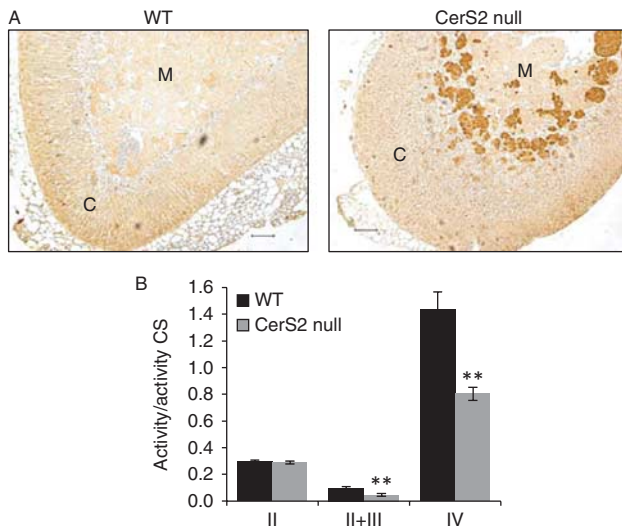


Figure 4
Oxidative stress in *CerS2* null mouse adrenal gland. (A) HNE staining in 4 month-old mice. C, cortex, M, medulla. Scale bar, 100 μ m. (B) Activity of mitochondrial respiratory complexes (II, II+III, and IV), normalized to citrate synthase (CS) in 6 month-old adrenal glands ($n=3$). Data are means \pm s.e.m. ** $P < 0.01$.

medullary tissue of heterogeneous appearance, which at times compressed the cortex into a thin peripheral band (Fig. 6A). The neoplastic cells were of variable cellular and nuclear size, variable cytoplasmic staining (dark blue to clear), variable nuclear to cytoplasmic ratio, and were arranged in irregular nodules (Fig. 6B). The mitotic rate was high (Fig. 6B, arrows). Clusters of ceroid-laden cells were common throughout the tumor (Fig. 6C, asterisks), as were irregular areas of fibrillar eosinophilic material, consistent with neuronal processes.

Medullary hyperplasia (Mohr 2001) was seen at an earlier age than PCC in the *CerS2* null mouse (Fig. 6D). It was present in three out of six of the 10.5–11 month-old

males and in four out of five of the 12 month-old females. In this group, hyperplasia appeared to be in its early stage and the histologic findings were subtle and in some cases equivocal. By definition, in all cases with hyperplasia, the architecture of the adrenal gland was well-preserved and without significant cortical atrophy (Fig. 6D and E). Paragangliomas were not identified. None of the histologic changes (i.e. PCC or hyperplasia) were observed in WT mice (Fig. 6F).

No difference in stress-induced corticosterone secretion was detected between WT and *CerS2* null mice (not shown). In contrast, evaluation of medullary function, ascertained by measuring urinary levels of epinephrine (E), NE, MN and NMN demonstrated that NE and NMN were elevated in 3 month-old *CerS2* null mouse urine, while MN and E were elevated at 7 and 10 months of age (Fig. 7). This finding correlates with, and may precede, the morphological abnormalities observed in the medulla of *CerS2* null mouse adrenal gland at a later age.

Discussion

The main finding of the current study is that *CerS2* null mice display a high incidence of PCC, which is preceded by medullary hyperplasia. A number of genes have been associated with the development of PCC in humans, such as the SDH gene (mitochondrial complex II subunits), RET proto-oncogene, von Hippel-Lindau (VHL) tumor suppressor gene or neurofibromin 1 (NF1) gene (Fishbein & Nathanson 2012, Shah *et al.* 2012). Mutations in VHL and SDH result in accumulation of succinate which directly inhibits prolyl hydroxylase (PHD), thereby inducing Hif-1 α accumulation (Favier & Gimenez-Roqueplo 2010). Hif-1 α causes PCC tumorigenesis by increasing angiogenesis, proliferation, invasion and metastasis (Favier &

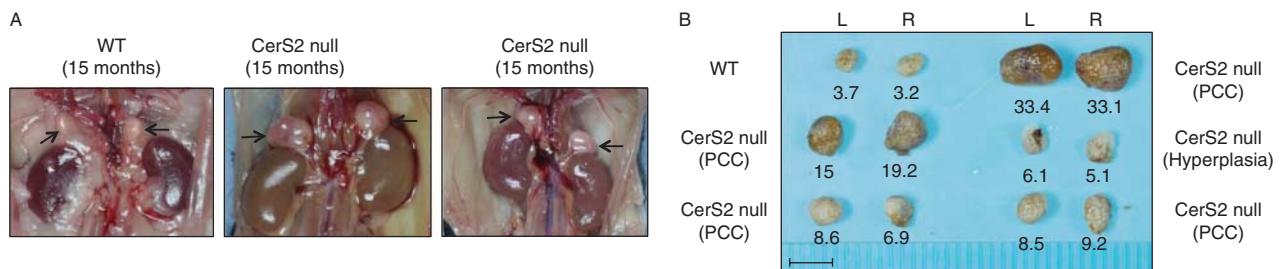
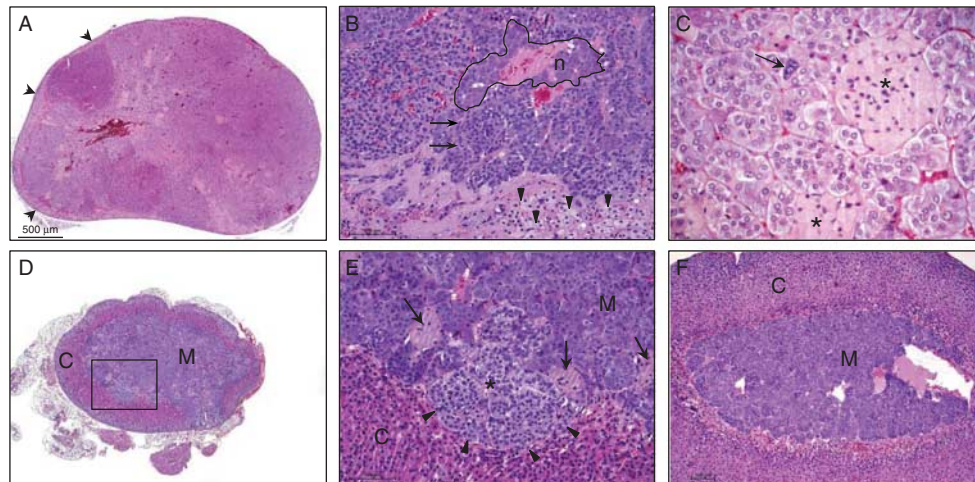


Figure 5
Macroscopic examination of PCC in *CerS2* null mice. (A) Enlarged adrenal glands (black arrows) are observed in 15-month-old *CerS2* null mice. (B) *CerS2* null adrenal glands. The weight of the left (L) and right (R) adrenal gland (mg) of each mouse is given beneath each image. Scale bar, 5 mm.

**Figure 6**

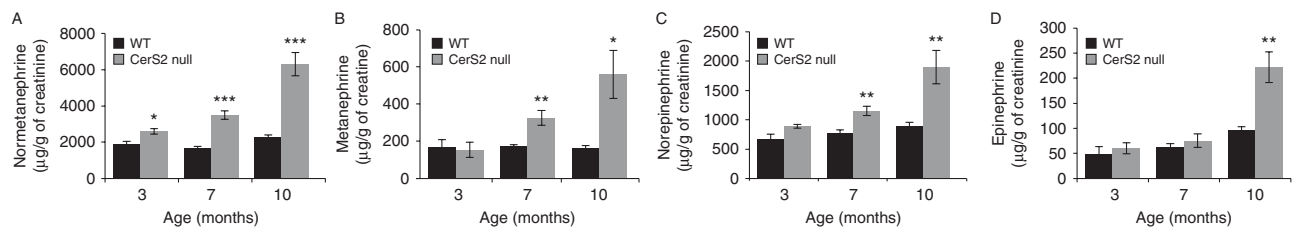
Microscopic analysis of PCC in *CerS2* null mice. (A) Low magnification (4 \times) of a PCC in the adrenal gland of a 15 month-old *CerS2* null mouse. (B) Medium magnification (20 \times) of a typical area at the edge of a complex pheochromocytoma. Neoplastic medullary cells occupy the top 3/4 of the field. Note the higher nuclear:cytoplasmic ratio of medullary (chromaffin) cells on the left vs the right. Arrows point to two mitotic figures in the transformed population. A group of neurons is delimited by a black line. Residual cortical cells are preserved at the bottom of the field (arrowheads). (C) High magnification (40 \times) of PCC. The neoplastic cells show a range of cellular and nuclear sizes (anisocytosis and anisokaryosis). A cell with a large nucleus is identified (arrow). There are clusters of ceroid-laden cells in the tumor (asterisks). (D) Low magnification (4 \times) of an adrenal

gland from a case with medullary hyperplasia. The medulla (M) is somewhat expanded but the overall corticomedullary architecture and ratio are relatively preserved. The boxed area is shown in E. (E) Medium magnification (20 \times) of the insert in panel D. A small nodule of hyperplastic medullary tissue is identified with an asterisk. Note the different appearance of chromaffin cells in the nodule. The cells have a higher nuclear:cytoplasmic ratio and their cytoplasm stains more lightly. The nodule causes compression of adjacent cortical cells (arrowheads). Several clusters of ceroid-laden cells are marked (arrows). (F) Medium magnification (10 \times) of an adrenal gland from a WT mouse. The cortex (C) and medulla (M) are defined.

Gimenez-Roqueplo 2010). Mutations in the RET proto-oncogene and in NF1 activate RAS/RAF/MAPK and PI3K/Akt/mTOR signaling pathways, which play critical roles in tumorigenesis (Shah *et al.* 2012).

The mechanism responsible for PCC in *CerS2* null mice is not known, but is clearly related to the changes in the SL profile. Three major changes in SL levels occur in *CerS2* null mice; reduction in levels of VLC SLs, elevation of long-chain SLs and elevation of sphinganine (and sphingosine), although the pathways by which the latter two are altered is not known. Transcriptional

up-regulation of either *CerS1* or *CerS5/6* cannot account for increased C18- and C16-ceramide. Rather, it is likely that changes in levels of these lipids are related to other changes in their metabolism (such as altered rates of synthesis vs degradation), although little information is currently available to assess this possibility. However, some of the pathology can be explained by changes in levels of specific lipids. For instance, C16-ceramide and sphinganine both directly inhibit complex IV activity in the liver (Zigdon *et al.* 2013), which results in oxidative stress. Altered levels of sphingosine, which is known to

**Figure 7**

Catecholamine levels are increased in the urine of *CerS2* null mice. Levels of (A) normetanephrine, (B) metanephrine, (C) norepinephrine and (D) epinephrine in 3, 7 and 10 month-old WT and *CerS2* null mouse urine ($n=4-7$). Data are means \pm S.E.M. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

permeabilize lysosomes and cause relocation of lysosomal enzymes to the cytosol (Kågedal *et al.* 2001), may be related to the altered cathepsin levels, which together with oxidative stress might reduce lysosome function and thus accelerate lipofuscin accumulation.

Lipofuscin accumulates in CerS2 null mice as young as 2 months of age. This is somewhat similar to the events that occur in ABCD2-deficient mice (Lu *et al.* 2007) in which spontaneous and premature deposition of ceroid, an end-product of oxidative damage, occurs predominantly in adrenal medullary cells. Lipofuscin is normally found in senescent post-mitotic cells such as cardiac myocytes, neurons, and in the area between the zona reticularis and the medulla in adrenal glands in old age (Kurz *et al.* 2007). Thus, the CerS2 null mouse provides additional indications that oxidative stress is a key player in PCC formation, although the mechanism of oxidative stress is somewhat different inasmuch as SDH function was normal, the Hif-1 α protein was not up-regulated, and Akt and mTOR phosphorylation was not detected in the PCC (data not shown). Moreover, ceroid accumulation is not observed in PCC caused by SDH mutations, suggesting that the mechanism of PCC formation differs between SDH mutations and that observed in the CerS null mouse. We conclude that defects in cytochrome c oxidase (mitochondrial complex IV) (Lee & Wei 2007) may be the main mechanism by which oxidative stress is generated in the CerS2 null mouse.

The relevance of our findings on altered SL levels to human PCC is currently unclear, with only one study relating to this issue, in which SMPD1, the gene which encodes for acid sphingomyelinase, was elevated in benign compared to malignant PCC (Thouënon *et al.* 2007). There is one report of a human patient with a mutation in CerS2, in which a 27 kb *de novo* deletion in one allele of chromosome 1q21 was detected (Mosbech *et al.* 2014), although there is no evidence that this patient displays either mitochondrial dysfunction or PCC. There is, however, a relationship between altered CerS expression and cancer. Thus, increased expression of CerS2 occurs in breast cancer (Erez-Roman *et al.* 2010, Hartmann *et al.* 2012, Fan *et al.* 2013) and CerS2 may play a role in prostate cancer (Wang *et al.* 2012, Xu *et al.* 2012). Moreover, ~15% of CerS2 null mice develop hepatocellular carcinoma (HCC) and most mice develop proliferative nodules (Pewzner-Jung *et al.* 2010b).

The enhanced levels of secretion of NE and MNE into the urine is consistent with data showing that these two hormones increase in PCC (Pacak 2011). Several factors are known to enhance catecholamine secretion from the

medulla of the adrenal gland, including chronic hypoglycemia and increased leptin levels (Takekoshi *et al.* 2001, Shibuya *et al.* 2002), both of which are observed in CerS2 null mice (Park *et al.* 2012). Leptin stimulates the hypothalamus and increases sympathetic nerve tone (Rosol *et al.* 2001). Whether these factors are also involved in PCC formation in the CerS2 null mouse is unknown, but irrespective of the precise details of the molecular mechanism, interrogating the SL pathway might pave the way for understanding the etiology of PCC formation and for development of novel potential therapeutic approaches for the treatment of PCC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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