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Palmoplantar keratoderma along with neuromuscular and metabolic phenotypes in Slurp1-deficient mice

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Abstract

Mutations in SLURP1 cause mal de Meleda, a rare palmoplantar keratoderma (PPK). SLURP1 is a secreted protein that is expressed highly in keratinocytes but has also been identified elsewhere (e.g., spinal cord neurons). Here, we examined Slurp1-deficient mice (Slurp1−/−) created by replacing exon 2 with β-gal and neo cassettes. Slurp1−/− mice developed severe PPK characterized by increased keratinocyte proliferation, an accumulation of lipid droplets in the stratum corneum, and a water barrier defect. In addition, Slurp1−/− mice exhibited reduced adiposity, protection from obesity on a high-fat diet, low plasma lipid levels, and a neuromuscular abnormality (hind limb clasping). Initially, it was unclear whether the metabolic and neuromuscular phenotypes were due to Slurp1 deficiency because we found that the targeted Slurp1 mutation reduced the expression of several neighboring genes (e.g., Slurp2, Lypd2). We therefore created a new line of knockout mice (Slurp1X−/− mice) with a simple nonsense mutation in exon 2. The Slurp1X mutation did not reduce the expression of adjacent genes, but Slurp1X−/− mice exhibited all of the phenotypes observed in the original line of knockout mice. Thus, Slurp1 deficiency in mice elicits metabolic and neuromuscular abnormalities in addition to PPK.

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Conflict of interest

The authors have declared that no conflict of interest exists.
Introduction

Mal de Meleda, a severe form of palmoplantar keratoderma (PPK), is caused by mutations in SLURP1 (Eckl et al., 2003; Fischer et al., 2001; Marrakchi et al., 2003). The disease is very rare and there are relatively few thorough descriptions of disease phenotypes (Charfeddine et al., 2003; Eckl et al., 2003; Fischer et al., 2001; Marrakchi et al., 2003; Oh et al., 2011). Affected patients have thickening of the skin on the palms and soles, occasionally with pseudoainhum formation and autoamputation of digits. The skin of the palms and soles is often malodorous. Mal de Meleda patients have two defective SLURP1 alleles; heterozygous carriers are normal.

The protein that is defective in Mal de Meleda, SLURP1, is a member of the “Lymphocyte Antigen 6” (Ly6) protein family. The hallmark of Ly6 proteins is an ~80–amino acid domain containing 10 cysteines, all disulfide-bonded and arranged in a characteristic spacing pattern (Galat et al., 2008; Kieffer et al., 1994). The same motif is found in cobra and viper toxins, many of which target the neuromuscular acetylcholine receptor (Fry et al., 2003; Galat et al., 2008). The majority of mammalian Ly6 proteins are glycosylphosphatidylinositol (GPI)-anchored proteins, but SLURP1 is a secreted protein. SLURP1 is expressed at high levels in keratinocytes, with particularly high levels in the palms and soles (Favre et al., 2007). Because SLURP1 is secreted, it can be found in plasma and urine (Andermann et al., 1999; Mastrandeli et al., 2003). SLURP1 has also been found in rat spinal cord neurons (Moriwaki et al., 2009).

There are many causes of PPK, and nearly all involve defects in structural proteins of keratinocytes (Bowden, 2010; Brooke et al., 2012; Heathcote et al., 2000; Liu et al., 2009; Maestrini et al., 1999; Petrof et al., 2012; Richardson et al., 2006; Xu and Nicholson, 2013). Mal de Meleda is unusual in being caused by a secreted protein. Neither SLURP1’s interactions with other proteins nor its role in epidermal differentiation are fully understood, but SLURP1 was reported to potentiate acetylcholine signaling through the α7-nicotinic acetylcholine receptor (α7nAChR) (Chimienti et al., 2003). Subsequent studies suggested that SLURP1 “ligation” to α7nAChR leads to increased acetylcholine signaling, which in turn leads to increased calcium signaling in keratinocytes and effects on kinase expression (Arredondo et al., 2005; Chernyavsky et al., 2010). However, no one has yet tested whether SLURP1 directly interacts with α7nAChR on the surface of cells.

SLURP1 is one of nine Ly6 genes within a ~550-kb segment of human chromosome 8 (chromosome 15 in the mouse). Only one of these genes, Gpihbp1, has been studied in depth (Beigneux et al., 2007; Beigneux et al., 2011; Davies et al., 2010; Weinstein et al., 2008). We showed that GPIHBP1, a GPI-anchored protein of endothelial cells, is crucial for plasma triglyceride metabolism; it binds lipoprotein lipase and transports it to the capillary lumen (Beigneux et al., 2007; Davies et al., 2010). Several other genes in this cluster (e.g., Slurp1, Slurp2, Ly6d) are expressed highly in keratinocytes.

Over the past few years, we have begun to investigate the functional relevance of “Ly6 genes” near Gpihbp1. We had dual motivations. One was to investigate the in vivo relevance of Ly6 proteins in keratinocytes, and a second was to determine if other Ly6 genes, aside
from Gpihbp1, were relevant to metabolism. We began our studies with Slurp1 because there were no published reports of Slurp1 knockout mice and we were intrigued by reports that SLURP1 is a small secreted protein that can be found in the plasma. Our studies yielded fresh insights into SLURP1 function in the skin and uncovered completely unexpected consequences of Slurp1 deficiency in mice.

Results

Slurp1 knockout mice, created by replacing exon 2 with neo and lacZ cassettes (Fig. S1), appeared normal at birth but developed PPK by 6 weeks of age (Fig. 1 A–C). By H&E staining, the epidermis in Slurp1−/− paw skin was thickened and the stratum granulosum was poorly demarcated (Fig. 1 B). No inflammatory infiltrates were present. The stratum corneum contained abundant lipid droplets, as judged by H&E staining, BODIPY staining, and electron microscopy (Fig. 1 C–E). Lamellar bodies were present in stratum granulosum keratinocytes in both wild-type and Slurp1−/− mice, as determined by electron microscopy (Fig. S 2), but whether this technique is capable of picking up subtle differences in the efficiency of lamellar body secretion is uncertain. BrdU incorporation into keratinocytes of paw skin in Slurp1−/− mice was markedly increased (Fig. 1 F). Aside from the paw, the skin and hair of Slurp1−/− mice were normal, both grossly and by routine histology (Fig. S3). The gastrointestinal tract, bronchial epithelium, and lungs of Slurp1−/− mice were histologically normal. Humans with mal de Meleda have been reported to have perivascular lymphocytic infiltrates and perioral involvement (Bouadjar et al., 2000; Nath et al., 2012), but these features were not present in Slurp1−/− mice. Slurp1+/− mice were indistinguishable from wild-type mice.

SLURP1 could be documented in the paw skin of wild-type mice but was absent in Slurp1−/− mice (Fig. 2 A). Our mouse SLURP1 antibodies were not useful for immunohistochemistry (and the lacZ cassette was not expressed), making it impossible to visualize SLURP1 expression in mouse skin. However, a human SLURP1-specific antibody detected SLURP1 in the suprabasal layers (including the spinous and granular layers) of human skin (Fig. 2 C), confirming earlier reports (Favre et al., 2007; Mastrangeli et al., 2003).

Because SLURP1 is expressed in the stratum granulosum of human skin (the layer that creates the water barrier) and also because of the accumulation of lipids in the stratum corneum of Slurp1−/− mice, we assessed transepidermal water loss in Slurp1−/− and wild-type mice. Water loss from the paw skin of Slurp1−/− mice was significantly greater than in wild-type mice, but water loss from the skin on the ear and back was similar in the two groups of mice (Fig. 3).

Slurp1−/− mice exhibited a neuromuscular phenotype: they clasped their hind limbs when picked up by the tail (a phenotype often observed in the setting of central nervous system disease, myopathy, or peripheral neuropathy) (Dequen et al., 2010; Hayward et al., 2008; Lalonde and Strazielle, 2011) (Fig. 4 A–B). The sciatic nerve of Slurp1−/− mice was normal by routine histology and by electron microscopy (Fig. S4), and no pathology was observed.
in the cerebral cortex or cerebellum. Serum chemistries in Slurp1−/− mice, including creatine phosphokinase levels, were within normal limits.

Slurp1−/− mice also exhibited metabolic phenotypes; they had reduced body weight and adiposity on a chow diet, despite consuming more food (Fig. 4 C–E). They were also protected from obesity on a high-fat diet (Fig. 4 F–G). The plasma levels of triglycerides, cholesterol, insulin, and leptin were lower in Slurp1−/− mice than in wild-type littermate controls (Fig. 5 A–C). Metabolic cage studies showed that mean O₂ consumption and CO₂ production were higher in Slurp1−/− mice; the respiratory quotient was higher in Slurp1−/− mice during the dark phase (Fig. 5 D–F and Fig. S5 A–C). Surprisingly, physical activity in Slurp1−/− mice was lower, as judged by laser beam breaks in metabolic cages (Fig. 5 G and Fig. S5 D). We considered the possibility that hyperventilation might contribute to increased oxygen consumption, but this appeared unlikely because the serum bicarbonate levels were within normal limits (13.6 ± 0.56 mEq/L in wild-type mice and 11.55 ± 2.30 mEq/L in Slurp1−/− mice). It is conceivable that increased grooming in Slurp1−/− mice (a form of activity that might not lead to laser beams breaks) could account for the increased oxygen consumption, but studies of grooming behavior in Slurp1−/− mice with continuous video monitoring provided no evidence that this was the case.

We suspected that the metabolic abnormalities would be accompanied by gene-expression perturbations in peripheral tissues. Hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) were increased in brown and white adipose tissue (Fig. S6). Also, PGC-1α expression was higher in the soleus muscle, liver, and heart of Slurp1−/− mice (Fig. S6), and the expression of the fatty acid transport protein (FATP-1), which plays an important role in facilitating mitochondrial fatty acid oxidation (Sebastian et al., 2009; Wiczer and Bernlohr, 2009), was increased in the quadriceps, soleus, brown adipose tissue, and white adipose tissue (Fig. S6). UCP1 expression in brown adipose tissue was similar in Slurp1−/− and wild-type mice (Fig. S6).

Because the neuromuscular and metabolic phenotypes were unexpected, we worried that the targeted Slurp1 mutation, which involved insertions of β-gal and neo cassettes, might have influenced the expression of neighboring genes (Olson et al., 1996). Indeed, the expression of two Ly6 genes adjacent to Slurp1 (Slurp2 and Lypd2) was reduced by 60–75% in Slurp1−/− mice, while the expression of a more distant Ly6 family member, Ly6d, was unaffected (Fig. S7 A). Perturbed expression of Slurp2 and Lypd2 was also present in Slurp1+/− mice, where the paw skin is normal, implying that the effects of the targeted Slurp1 mutation on neighboring genes were due to effects on chromatin structure and not an indirect consequence of PPK.

The fact that the Slurp1 mutation lowered the expression of nearby genes suggested that the “non-PPK” phenotypes in Slurp1−/− mice might be due to altered expression of neighboring genes rather than to SLURP1 deficiency. To address that possibility, we created new Slurp1 knockout mice harboring only a simple nonsense mutation (Fig. S7 B–D). This allele, designated Slurp1X, did not lower the expression of adjacent genes (Fig. S7 E). Nevertheless, homozygous knockout mice (Slurp1X−/−) exhibited all of the phenotypes found in the original Slurp1−/− mice (PPK, reduced body weight, hind limb clasping) (Fig. S7 F–G).
6). In fact, the neuromuscular abnormalities were more striking in \textit{Slurp1X}^{−/−} mice; aside from hind limb clasping, they dragged their hind limbs and tail by 4 months of age (Supplementary Movies 1 and 2).

\textbf{Discussion}

In the current study, we investigated \textit{Slurp1} knockout mice, fully expecting to encounter PPK. That expectation was fulfilled, but we also found neuromuscular and metabolic/body weight abnormalities. Initially, whether SLURP1 deficiency caused the “non-PPK” phenotypes seemed uncertain because the targeted mutation, which involved insertions of β-gal and neo cassettes, lowered the expression of nearby genes. However, a new line of \textit{Slurp1} knockout mice harboring a simple nonsense mutation—and where the expression of surrounding genes was normal—displayed the same neuromuscular and metabolic phenotypes, leaving no doubt that the “non-PPK” phenotypes were \textit{bona fide} features of SLURP1 deficiency. We considered the possibility that the unexpected phenotypes could be due to “passenger genes” segregating with \textit{Slurp1} (Smithies and Maeda, 1995), but this explanation is highly unlikely because neuromuscular and body weight phenotypes are absent in chow-fed knockout mice for nearby Ly6 genes (\textit{Lynx1} and \textit{Gpihbp1}) (Beigneux et al., 2007; Ibanez-Tallon et al., 2002; Miwa et al., 2006; Weinstein et al., 2012; Weinstein et al., 2010a; Weinstein et al., 2010b).

Why a deficiency of SLURP1, a secreted Ly6 protein, causes PPK is uncertain, but one possibility, suggested by Fischer et al. (Fischer et al., 2001), is that SLURP1 is a ligand for a cell-surface receptor on keratinocytes. Chimienti et al. (Chimienti et al., 2003) reported that SLURP1 potentiates calcium flow mediated by the α7 nicotinic acetylcholine receptor (α7nAChR). Also, Arredondo et al. (Arredondo et al., 2005) reported that SLURP1 competitively inhibits binding of small-molecule ligands to that receptor. The involvement of acetylcholine receptors in the pathogenesis of \textit{mal de Meleda} is potentially attractive because acetylcholine signaling is thought to be important in multiple aspects of keratinocyte biology and because the three-fingered structure of SLURP1 resembles cobra toxins that bind to acetylcholine receptors at the neuromuscular junction (Andermann et al., 1999; Fischer et al., 2001; Kini, 2002). Also, a “SLURP1–acetylcholine connection” could potentially be relevant to both the skin and neuromuscular phenotypes of \textit{Slurp1}^{−/−} mice. At this point, what is needed is firm evidence for specific protein–protein interactions between SLURP1 and acetylcholine receptors. Thus far, we have not been successful in documenting SLURP1 binding to α7nAChR on the surface of CHO-K1 cells using the same cell-based binding assays that have been used extensively to document the binding of a protein ligand to GPIHBP1 (an Ly6 protein that is structurally related to SLURP1) (Beigneux et al., 2011; Beigneux et al., 2009; Franssen et al., 2010; Olafsen et al., 2010; Voss et al., 2011). However, it is possible that our cell-based binding assays were not sufficiently sensitive to detect SLURP1–α7nAChR interactions. In addition, we have not yet tested the possibility that SLURP1 might bind to other acetylcholine receptors. In any case, we believe that a “SLURP1–acetylcholine receptor connection” is an attractive concept and deserves further study. We also believe that it is important to be open to other possibilities for SLURP1 function. One is that SLURP1 binds to and modifies the function of structural proteins already implicated in the pathogenesis of PPK, for example demosomal proteins (Bowden,
2010; Brooke et al., 2012; Liu et al., 2009; Petrof et al., 2012; Richardson et al., 2006) or connexin 26, a gap junction protein (Heathcote et al., 2000; Maestrini et al., 1999; Xu and Nicholson, 2013). The idea that SLURP1 might affect cell–cell junctions has been raised previously (Fischer et al., 2001) and is not farfetched because another Ly6 protein, LY6D, is present in desmosomes (Brakenhoff et al., 1995; Fischer et al., 2001). Also, Drosophila Ly6 proteins are crucial for the septate junctions that cement epithelial cells together (Hijazi et al., 2011; Hijazi et al., 2009; Kim and Marques, 2012; Nilton et al., 2010).

The mechanism by which SLURP1 deficiency elicits the neuromuscular findings is unknown. One possibility is that SLURP1 plays a role in peripheral nerves, perhaps (as suggested by Moriwaki et al. (Moriwaki et al., 2009)) by acting as a neurotransmitter. Improved antibody reagents will be required to investigate this possibility. A more speculative idea relates to the possible involvement of SLURP1 in cell–cell junctions in peripheral nerves (Banerjee et al., 2006). Genetic manipulations of axo-glial septate junction proteins in mice alter nerve conduction and elicit the same hind limb clamping phenotype found in Slurp1−/− mice (Cifuentes-Diaz et al., 2011; Lee et al., 2013). No one has yet reported neuropathy in mal de Meleda patients, but we would not be surprised if sophisticated testing were to uncover subtle abnormalities.

SLURP1 deficiency reduced body weight and adiposity, increased oxygen consumption, and lowered plasma lipid and insulin levels. The same phenotypes have been observed in mice lacking stearoyl-CoA desaturase-1 (SCD1), which is required for lipid synthesis and the formation of the epidermal water barrier (Binczek et al., 2007; Sampath et al., 2009). Scd1 knockout mice display increased evaporative water loss, leading to loss of body heat and increased energy expenditure (Binczek et al., 2007). We doubt that the same mechanism applies in Slurp1−/− mice. First, the water barrier defect in Slurp1−/− mice is confined to the paw, and it seems unlikely that water loss from this tiny area of skin would explain the striking metabolic abnormalities. Second, Slurp1 deficiency did not increase expression of thermogenic genes in brown adipose tissue. Third, reduced body weight is not a general feature of PPK; grainy head-like 1 knockout mice have severe PPK but no alterations in body weight (Wilanowski et al., 2008).

While body weight and adiposity abnormalities in SLURP1-deficient mice are profound, the underlying mechanisms are not clear. It is tempting to speculate that SLURP1, a secreted peptide found in the plasma, may act directly or indirectly to increase fuel utilization in metabolically active tissues. We did observe increased expression of HSL and ATGL (genes involved in intracellular lipolysis) in adipose tissue of Slurp1−/− mice; we also found increased Pgc1α and Fatp1 expression in multiple tissues of Slurp1−/− mice.

We found an accumulation of neutral lipid droplets in the stratum corneum of paw skin of Slurp1−/− mice. In earlier studies, lipid droplets were observed in the fingernails of mal de Meleda patients (Salamon, 1986; Salamon et al., 1984), and higher triglyceride levels were also found in the epidermis of several mal de Meleda patients (Kuster et al., 2003). The increased numbers of lipid droplets in the stratum corneum are likely due to impaired hydrolysis of triglycerides, a process that is essential for the formation of acylceramides and the epidermal water barrier (Elias et al., 2008). A similar accumulation of neutral lipid
droplets in the stratum corneum is found in neutral lipid storage disease due to CGI-58 deficiency (Demerjian et al., 2006), where triglyceride hydrolysis is clearly impaired (Radner et al., 2011; Radner et al., 2010; Ujihara et al., 2010). We did not find reduced transcript levels for CGI-58, ATGL, triglyceride hydrolase K, or triglyceride hydrolase N in paw skin of Slurp1−/− mice (not shown). Nevertheless, it seems likely that SLURP1 deficiency influences the efficiency of triglyceride hydrolysis in keratinocytes, perhaps indirectly as a consequence of altered keratinocyte proliferation or differentiation.

Our findings are relevant to clinical dermatology. The discovery of a defective water barrier in SLURP1-deficient skin, along with increased neutral lipids in the stratum corneum, could help to explain malodorous skin in mal de Meleda (Oh et al., 2011). Leakage of interstitial fluids into the stratum corneum along with the accumulation of triglyceride droplets would favor the growth of microorganisms, and bacterial lipases are known to release malodorous aldehydes, alcohols, and ketones (Chung and Seok, 2012). Also, our studies reinforce the fact that genetic defects causing PPK can be accompanied by “non-skin” disease phenotypes. Other genetic defects causing PPK are associated with deafness, right ventricular dysplasia, and central nervous system disease (Brooke et al., 2012; Heathcote et al., 2000; Petrof et al., 2012; Xu and Nicholson, 2013). In the current studies, we found that SLURP1 deficiency in the mouse leads to neuromuscular and body weight abnormalities.

Materials and Methods

Slurp1-deficient mice

Slurp1−/− mice were obtained from Lexicon. The Slurp1 knockout allele was detected by PCR genotyping as described in the Supplementary Methods. Slurp1X mice were created by introducing a premature stop codon into exon 2 (N35X), as described in the Supplementary Methods and Supplementary Figure 6. All mice had a mixed genetic background (<10% 129/Sv and >90% C57BL/6). Unless otherwise specified, the mice were fed a chow diet (LabDiet No. 5001, Purina) and housed in a barrier facility with a 12-h light/dark cycle. All studies were approved by UCLA’s Animal Research Committee.

Histology and immunofluorescence microscopy

Skin biopsies were fixed overnight in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For immunohistochemistry, cultured cells and frozen sections of human skin were fixed in methanol. The cells and tissues were blocked with 10% donkey serum and incubated with primary antibodies at 4°C overnight. Antibodies included a goat polyclonal antibody against the S-protein tag (Abcam; 1:500), a mouse antiserum against human SLURP1 (Novus Biologicals; 1:100), a rat monoclonal antibody against BrdU (Abcam; 1:200), or an Alexa 568-labeled mouse monoclonal antibody against cytokeratin 14 (Abcam; 1:500). Secondary antibodies included an Alexa 568–conjugated donkey anti-goat IgG (Invitrogen; 1:800), an Alexa 488–conjugated donkey anti-mouse IgG (Invitrogen; 1:800), and an Alexa 488–conjugated donkey anti-rat IgG (Invitrogen; 1:200). DNA was visualized with DAPI. Microscopy was performed with an Axiovert 200M microscope.
**Western Blotting**

Proteins from tissue or cell culture extracts (25 μg) were size-fractionated on a 12% Bis-Tris SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. Antibodies included a mouse monoclonal antibody against β-actin (1:500) (Abcam, Cambridge, MA); a rabbit antiserum against a SLURP1 peptide (CKTVLETVEAAFPFNHSPMVTRS) (5 μg/ml; an IRdye800-conjugated donkey anti-rabbit and donkey anti-mouse IgG (1:2,000) (Li-Cor, Lincoln, NE). Antibody binding was detected with an Odyssey infrared scanner (Li-Cor).

**Quantification of Transepidermal Water Loss**

Transepidermal water loss (TEWL) measurements on the skin of the back, rear paws, and ear were recorded at room temperature on age- and sex-matched wild-type and Slurp1−/− mice (n = 6/group) with an RG1 evaporimeter (cyberDERM) equipped with a 0.3-mm circular adaptor. The hair on a small portion of the back was shaved with a clipper before TEWL measurements were performed.

**Body Weight/Metabolic Phenotypes**

Body weights of male and female mice were recorded weekly from 4 to 15 weeks of age, and measurements of adiposity were made by NMR (Weinstein et al., 2010a). Measurements of oxygen consumption and respiratory quotient were performed using sealed metabolic cages (Oxymax, Columbus Instruments) (Weinstein et al., 2012). Sensors attached to the sealed chamber measured oxygen and carbon dioxide concentrations, and physical activity was assessed by the number of laser beam breaks. Data was collected and analyzed with Oxymax/CLAMS software. All metabolic cage studies were performed using male mice.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| PPK          | palmoplantar keratoderma |
| Ly6          | lymphocyte antigen 6 |
| GPIHB1       | glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 |
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Figure 1. Palmoplantar keratoderma and increased stratum corneum lipid droplets in Slurp1^−/− mice

(A) Paws from wild-type and Slurp1^−/− mice, revealing markedly thickened skin in Slurp1^−/− mice. (B–C) Hematoxylin and eosin–stained sections revealing a thickened epidermis in the paw skin of a Slurp1^−/− mouse, along with many tiny lipid droplets in the stratum corneum (arrowheads). Scale bars = 50 µm and 10 µm for B and C respectively. (D) BODIPY 493/503 (green) staining showing lipid droplets in the stratum corneum (SC) of the paw skin of a Slurp1^−/− mouse. DNA is stained with DAPI (blue). Scale bar = 50 µm. (E)
Electron micrograph showing lipid droplets in the stratum corneum of Slurp1<sup>−/−</sup> paw skin (arrowheads). Scale bar = 1 µm. (F) Increased BrdU incorporation into DNA of paw keratinocytes of Slurp1<sup>−/−</sup> mice (green). DNA is stained with DAPI (red). Scale bar = 50 µm.
Figure 2. Immunochemical detection of SLURP1

(A) Western blot of extracts of paw skin from wild-type (Slurp1+/+) and Slurp1−/− mice with an antibody against a mouse SLURP1 peptide. CHO cells transfected with a flag-tagged mouse SLURP1 expression vector were used as a control. SLURP1, like nearly all other Ly6 proteins, has an N-linked glycosylation site, and the two SLURP1 bands likely reflect glycosylated and nonglycosylated versions of the protein (Beigneux et al., 2008). The SLURP1 antibody did not cross-react with SLURP2. (B) The specificity of the human SLURP1 antibody (green) in these studies was assessed by immunocytochemistry. CHO-K1
cells were transiently transfected with an empty vector or an S-protein–tagged human SLURP1 expression vector. Cells that had been transiently transfected with the S-protein–tagged SLURP1 vector were identified with an S-protein–specific antibody (red). (C) Detection of SLURP1 (green) in human skin by immunohistochemistry with a human SLURP1 antibody. Keratin 14 (red) was used as a marker of basal keratinocytes; DNA was stained with DAPI (blue). Scale bars = 20 μm.
Figure 3. Evaporative water loss from the paw, ear, and back skin of $Slurp1^{+/+}$ and $Slurp1^{-/-}$ mice

Measurements of evaporative water loss were performed with an RG1 evaporimeter ($n = 6$ group). TEWL, Transepidermal water loss. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. 

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Figure 4. “Non-skin” phenotypes in Slurp1<sup>−/−</sup> mice

(A) Hind limb clasping in male Slurp1<sup>−/−</sup> mice when picked up by the tail (similar results were observed with female Slurp1<sup>−/−</sup> mice). (B) Quantification of the hind limb clasping phenotype (0 for no hind-limb retraction, 1 for unilateral retraction, 2 for bilateral retraction) (n = 16 for Slurp1<sup>+/+</sup>; n = 19 for Slurp1<sup>+/−</sup>; and n = 17 for Slurp1<sup>−/−</sup> mice). ***P < 0.001 (Slurp1<sup>+/+</sup> vs. Slurp1<sup>−/−</sup>). (C) Weight gain in chow-fed female Slurp1<sup>+/+</sup> and Slurp1<sup>−/−</sup> mice (4–15 weeks of age; n = 11/group). Similar results were observed with male mice (D) Reduced adiposity in 7-month-old chow-fed female Slurp1<sup>−/−</sup> mice, as judged by NMR (n =
Food consumption in male Slurp1+/+ and Slurp1−/− mice over 24 h. Similar results were observed in two independent experiments (n = 3 mice/group/experiment). (F) Slurp1−/− mice are resistant to diet-induced obesity. Female Slurp1−/− (n = 4) and Slurp1+/+ mice (n = 3) were fed a high-fat diet for 11 weeks and weight gain was analyzed. Similar results were observed with male mice. *P < 0.05; **P < 0.01; ***P < 0.001. (G) Consumption of the high-fat diet (HFD) by Slurp1+/+ and Slurp1−/− mice over 24 h (n = 3/group).
Figure 5. Altered metabolic parameters in Slurp1^-/- mice
Plasma levels of cholesterol and triglycerides (males, n = 10/group) (A), insulin (males, n = 9 wild-type mice; n = 10 Slurp1^-/- mice) (B), and leptin (males, n = 6 wild-type mice; n = 4 Slurp1^-/- mice) (C) in wild-type and Slurp1^-/- mice. *P < 0.05; **P < 0.01; ***P < 0.001.

(D) Metabolic cage studies revealing increased oxygen consumption (VO2) in male Slurp1^-/- mice.

(E) CO2 production.

(F) Respiratory quotient measurements during two dark and light cycles.

(G) Decreased activity in male Slurp1^-/- mice. Bar graph showing total activity (the sum of X Total, X Ambulatory, and Z Total). X Total, total counts of beam interference in the X dimension, including stationary positions; X Ambulatory, multiple beam breaks in the X dimension; Z Total, line breaks in the Z dimension (rearing up) (n = 3/
group). Similar results were observed in two independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. 
Figure 6. Slurp1<sup>X<sup>−/−</sup></sup> mice exhibit PPK and the same “non-skin” disease phenotypes observed in the original line of Slurp1 knockout mice.

(A–B) Paws of male Slurp1<sup>X<sup>+</sup></sup> and Slurp1<sup>X<sup>−/−</sup></sup> mice at 12 weeks of age. (C–D) Hematoxylin and eosin–stained sections revealing a thickened epidermis in the paw skin of a Slurp1<sup>X<sup>−/−</sup></sup> mouse. Higher-powered images revealed many small lipid droplets in the stratum corneum (arrowhead). Scale bar = 50 µm. (E–F) Hind limb clasping in a male Slurp1<sup>X<sup>−/−</sup></sup> mouse. Similar results were observed with female mice. (G) Reduced body weight in Slurp1<sup>X<sup>−/−</sup></sup> mice. Serial measurements of body weight revealed a 15–20%
reduction in body weight in 9-week-old Slurp1X−/− female mice on chow diet, very similar to findings in the original line of Slurp1 knockout mice. *P < 0.05; ***P < 0.001.