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Authors
Allan, Christopher M
Procaccia, Shiri
Tran, Deanna
et al.

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Palmoplantar keratoderma in *Slurp2*-deficient mice

Christopher M. Allan[1,*], **Shiri Procaccia**[1,*], **Deanna Tran**[1], **Yiping Tu**[1], **Richard H. Barnes II**[1], **Mikael Larsson**[1], **Bernard B. Allan**[4], **Lorraine C. Young**[1], **Cynthia Hong**[3,5], **Peter Tontonoz**[3,5], **Loren G. Fong**[1,†], **Stephen G. Young**[1,2,†], and **Anne P. Beigneux**[1,†]

[1]Department of Medicine (Divisions of Cardiology and Dermatology), David Geffen School of Medicine, University of California, Los Angeles, CA 90095

[2]Department of Human Genetics, David Geffen School of Medicine, University of California, Los Angeles, CA 90095

[3]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095

[4]Department of Molecular Biology, Genentech, South San Francisco, CA 94080

[5]Howard Hughes Medical Institute

Abstract

SLURP1, a member of the Ly6 protein family, is secreted by suprabasal keratinocytes. Mutations in *SLURP1* cause a palmoplantar keratoderma (PPK) known as *mal de Meleda.* Another secreted Ly6 protein, SLURP2, is encoded by a gene located ~20 kb downstream from *SLURP1*. SLURP2 is produced by suprabasal keratinocytes. To investigate the importance of SLURP2, we first examined *Slurp2* knockout mice in which exon 2–3 sequences had been replaced with *lacZ* and *neo* cassettes. *Slurp2*$^{-/-}$ mice exhibited hyperkeratosis on the volar surface of the paws (*i.e.*, PPK), increased keratinocyte proliferation, and an accumulation of lipid droplets in the stratum corneum. They also exhibited reduced body weight and hind limb clasping. These phenotypes are very similar to those of *Slurp1*$^{-/-}$ mice. To solidify a link between *Slurp2* deficiency and PPK and to be confident that the disease phenotypes in *Slurp2*$^{-/-}$ mice were not secondary to the effects of the *lacZ* and *neo* cassettes on *Slurp1* expression, we created a new line of *Slurp2* knockout mice (*Slurp2X*$^{-/-}$) in which *Slurp2* was inactivated with a simple nonsense mutation. *Slurp2X*$^{-/-}$ mice exhibited the same disease phenotypes. Thus, *Slurp2* deficiency and Slurp1 deficiencies cause the same disease phenotypes.

†Correspondence: Anne P. Beigneux, Stephen G. Young, Loren G. Fong, 650 Charles E. Young Dr. South, Los Angeles, CA 90095. Tel: (310) 825-4934; Fax: (310) 206-0865; ; Email: abeigneux@mednet.ucla.edu, ; Email: sgyoung@mednet.ucla.edu, ; Email: lfong@mednet.ucla.edu

*To be considered co-first authors.

Conflict of interest

The authors have declared that no conflict of interest exists.

Introduction

Every clinical dermatologist knows that *SLURP1* mutations cause a palmoplantar keratoderma (PPK) known as *mal de Meleda* (Eckl *et al.,* 2003; Fischer *et al.,* 2001a; Marrakchi *et al.,* 2003). *Mal de Meleda* patients have a thickened epidermis on the palms and soles, occasionally with pseudoainhum formation, but the skin elsewhere is normal or minimally affected. *Mal de Meleda* is a recessive syndrome; every patient carries two mutant *SLURP1* alleles. Heterozygous carriers are free of disease.

SLURP1 is an 8.9-kDa protein of the “Lymphocyte Antigen 6” (Ly6) family. The hallmark of this family is an “Ly6 domain” with 8–10 cysteines, all arranged in a characteristic spacing pattern and all disulfide-linked so as to create a three-fingered motif (Galat *et al.,* 2008; Kieffer *et al.,* 1994). The same structure is found in many secreted toxins in viper and cobra venom (Fry *et al.,* 2003; Kini, 2002). Most Ly6 proteins in mammals are tethered to the plasma membrane by a glycosylphosphatidylinositol (GPI)-anchor, but SLURP1 is an exception. SLURP1 is synthesized and secreted by keratinocytes (Favre *et al.,* 2007), enters the plasma, and can be found in the urine (Andermann *et al.,* 1999; Mastrangeli *et al.,* 2003).

The function of SLURP1 in the skin is not well defined, although several studies have reported that it modulates acetylcholine signaling (Arredondo *et al.,* 2005; Chernyavsky *et al.,* 2010; Chimienti *et al.,* 2003). SLURP1 is often presumed to bind to a cell-surface receptor on keratinocytes (Fischer *et al.,* 2001a), but thus far no one has documented binding of SLURP1 to a specific keratinocyte protein.

Inactivating *Slurp1* in mice (either by replacing exon 2 with *neo* and *lacZ* cassettes or by introducing a premature stop codon into exon 2) causes PPK (Adeyo *et al.,* 2014). The PPK is obvious by ~6–8 weeks of age. *Slurp1* knockout mice also exhibit increased energy consumption and reduced body weight (Adeyo *et al.,* 2014). The mechanism for those phenotypes is not clear, but they might be secondary to increased grooming (as a result of the PPK). *Slurp1*-deficient mice also exhibit hind limb clasping, a nonspecific neuromuscular phenotype (Dequen *et al.,* 2010; Hayward *et al.,* 2008; Lalonde and Strazielle, 2011). Again, the mechanism is unclear.

In mammals, SLURP1 is not the only secreted Ly6 protein. SLURP2, an ~8-kDa Ly6 protein, is synthesized and secreted by keratinocytes. Studies of human skin with a human SLURP2–specific monoclonal antibody revealed that SLURP2, like SLURP1, is made by suprabasal keratinocytes (Arredondo *et al.,* 2006). SLURP2 was initially identified as a cDNA that is upregulated in psoriasis vulgaris (Tsuji *et al.,* 2003). The gene for SLURP2 is immediately upstream from *LYNX1* and 21.9 kb downstream from *SLURP1; SLURP2* is ~443 kb upstream from the gene for GPIHBP1, a GPI-anchored Ly6 protein that shuttles lipoprotein lipase to the capillary lumen (Beigneux *et al.,* 2007; Davies *et al.,* 2010; Goulbourne *et al.,* 2014). The function of SLURP2 is unclear, but one paper proposed that SLURP2 modulates acetylcholine signaling (Arredondo *et al.,* 2006). Similar to the situation with SLURP1, no one has yet identified specific interactions between SLURP2 and any keratinocyte protein.

There have been no insights into possible consequences of *SLURP2* deficiency. One could easily imagine that the consequences of SLURP2 and SLURP1 deficiency might be similar, given that both are members of the Ly6 family and both are secreted by keratinocytes. On the other hand, one could be skeptical about that possibility, given that different Ly6 family members can play very diverse functions in mammalian biology (Galat *et al.*, 2008). Moreover, the level of amino acid sequence identity between SLURP1 and SLURP2 is extremely low—only 17% after the 10 cysteines of the Ly6 domain are excluded from consideration (Fig. S1). The level of sequence identity between the Ly6 domains of SLURP2 and GPIHBP1 (the LPL transporter) is 21%.

To define the *in vivo* functional relevance of SLURP2 in mammals and to determine whether SLURP2 might be relevant to skin disease, we characterized two independent lines of *Slurp2* knockout mice.

Results

We first examined *Slurp2* knockout mice (*Slurp2*$^{-/-}$) that were created by replacing an exon 2–3 fragment with *neo* and *lacZ* cassettes (Fig. S2). As expected, *Slurp2* transcripts were half-normal in heterozygotes and absent in homozygotes (see Fig. 3 below). We attempted to visualize mouse SLURP2 in the skin of wild-type mice by western blotting and immunohistochemistry with our rabbit antiserum against a mouse SLURP2 peptide, but we were unable to detect a specific signal.

Slurp2$^{-/-}$ mice appeared normal at birth and at weaning, but hyperkeratosis on the volar surface of the paws (*i.e.*, PPK) was invariably present by 6–8 weeks of age (Fig. 1a). Grossly, the PPK in *Slurp2*$^{-/-}$ mice was indistinguishable from that in *Slurp1*$^{-/-}$ mice (Adeyo *et al.*, 2014). On H&E–stained sections, the epidermis of the paw in *Slurp2*$^{-/-}$ mice exhibited hyperkeratosis, and the stratum granulosum was poorly demarcated (Fig. 1b). The stratum corneum contained many tiny lipid droplets, as judged by H&E and BODIPY staining (Fig. 1c–d). There was no inflammation in the dermis or epidermis (confirmed by a UCLA dermatopathologist, Dr. Peter G. Sarantopoulos). Also, we did not observe consistently higher cytokine transcripts in the paw skin of *Slurp2X*$^{-/-}$ mice, whereas the expression of each of the cytokines was increased in the skin of *Apoe*$^{-/-}$*Lxra*$^{-/-}$ mice (where cholesterol accumulation in the skin is accompanied by histologic evidence of inflammation) (Fig. S3) (Bradley *et al.*, 2007).

BrdU incorporation into basal keratinocytes was increased in the paws of *Slurp2*$^{-/-}$ mice (Fig. 1e). These findings are similar to those in *Slurp1*$^{-/-}$ mice (Adeyo *et al.*, 2014). Apart from the paw, the skin in *Slurp2*$^{-/-}$ mice was normal, both by gross appearance and by routine histology (Fig. S4). Heterozygous knockout mice (*Slurp2*$^{+/-}$) were free of disease and indistinguishable from wild-type mice.

Like *Slurp1*$^{-/-}$ mice (Adeyo *et al.*, 2014), *Slurp2*$^{-/-}$ mice clasped their hind limbs when picked up by the tail (a phenotype often observed in mice with cerebellar disease, myopathy, or peripheral neuropathy) (Dequen *et al.*, 2010; Hayward *et al.*, 2008; Lalonde and Strazielle, 2011) (Fig. 2a–b). The onset of the hind limb clasping in *Slurp2*$^{-/-}$ mice

coincided with the development of obvious PPK (~6–8 weeks of age). Also, like *Slurp1*$^{-/-}$ mice (Adeyo *et al.*, 2014), *Slurp2*$^{-/-}$ mice had lower body weights than littermate wild-type mice (Fig. 2c–d) despite consuming similar amounts of food (Fig. 2e). The lower body weight in *Slurp2*$^{-/-}$ mice was primarily due to reduced adiposity (Fig. 2f). Metabolic cage studies (n = 3 mice/group) revealed increased oxygen consumption but reduced numbers of laser beam breaks in *Slurp2*$^{-/-}$ mice (Fig. 2g–h). The plasma cholesterol levels in *Slurp2*$^{-/-}$ mice were lower than in wild-type mice (p = 0.002); the plasma glucose levels were similar (Fig. S5).

Slurp2 transcripts were absent in the paw skin of *Slurp2*$^{-/-}$ mice. We previously showed that replacing exon 2 of *Slurp1* with *lacZ* and *neo* cassettes resulted in reduced expression of several nearby genes, including *Slurp2* (Adeyo *et al.*, 2014). Given those results, we examined the expression of *Slurp1* and two "nearby Ly6 genes" (*Lypd2, Ly6d*) in the paw skin of *Slurp2*$^{-/-}$ mice. *Slurp1* transcripts were reduced by ~60% in *Slurp2*$^{-/-}$ mice and ~50% in *Slurp2*$^{+/-}$ mice (Fig. 3a). The expression of *Lypd2* (located ~11.8 kb upstream from *Slurp2*) was reduced by ~35% in the paw skin of *Slurp2*$^{-/-}$ mice. The expression of *Ly6d* (~15 kb downstream from *Slurp2*) was not reduced and actually appeared to be increased in *Slurp2*$^{-/-}$ mice (Fig. 3a).

The fact that the *Slurp2* knockout allele reduced *Slurp1* transcripts in paw skin suggested the possibility that the disease phenotypes in *Slurp2*$^{-/-}$ mice might be caused by reduced *Slurp1* expression. To examine this issue, we measured *Slurp1* transcript levels in the paw skin of an independent group of *Slurp2*$^{-/-}$ mice as well as an age-matched group of *Slurp1*$^{+/-}$ mice (where the paw skin was normal). Again, *Slurp1* expression in the paw skin of *Slurp2*$^{-/-}$ mice was reduced by ~60%. Not surprisingly, *Slurp1* expression in *Slurp1*$^{+/-}$ mice was reduced by ~50% (Fig. 3b).

It seems unlikely that a ~60% reduction in *Slurp1* transcripts in *Slurp2*$^{-/-}$ mice would lead to PPK, reduced body weight, and hind limb clasping, while a 50% reduction in *Slurp1* in *Slurp1*$^{+/-}$ mice would yield no disease phenotypes at all. Nevertheless, to further examine the link between SLURP2 deficiency and PPK, we created a new *Slurp2* knockout allele (designated *Slurp2X*) by inserting a simple nonsense mutation into exon 2 (*i.e.*, no *lacZ* or *neo* insertions) (Fig. S6). Heterozygous *Slurp2X* knockout mice (*Slurp2X*$^{+/-}$) were normal, indistinguishable from wild-type littermates. Homozygous knockout mice (*Slurp2X*$^{-/-}$) exhibited PPK (Fig. 4a–b) that was similar to that in the original *Slurp2*$^{-/-}$ mice (Fig. 1). The *Slurp2X*$^{-/-}$ mice also exhibited increased trans-epidermal water loss from the skin of the paw (Fig. 4c). Like the original line of *Slurp2*$^{-/-}$ mice, *Slurp2X*$^{-/-}$ mice exhibited hind limb clasping and reduced body weight (Fig. 5). Given that the nonsense mutation in *Slurp2X*$^{-/-}$ mice was located ~20 kb downstream from *Slurp1*, it is difficult to avoid the conclusion that *Slurp2* is a "PPK gene" in mice.

Given the severity of the PPK in *Slurp2X*$^{-/-}$ mice, we predicted that the expression of many "keratinocyte genes" would be perturbed in paw skin. Indeed, this was the case, as judged by qRT-PCR studies on selected keratinocyte genes (Table S1). For example, keratin 6b and keratin 16 expression levels were markedly upregulated in *Slurp2X*$^{-/-}$ mice. Similar gene-expression changes were observed in the paw skin of *Slurp1X*$^{-/-}$ mice (Table S1), although

we caution against comparing levels of transcripts in *Slurp2X*$^{-/-}$ and *Slurp1X*$^{-/-}$ mice because the two groups of mice were not perfectly matched for age. Several transcripts that we found to be increased in the setting of SLURP2 deficiency, for example transcripts for keratin 6b, keratin 16, late cornified envelope protein 3a, and defensin 4B were also increased in the setting of the PPK associated with pachyonychia congenital (Cao *et al.,* 2015). *Slurp1* transcripts in *Slurp2X*$^{-/-}$ mice were reduced by 50%, regardless of whether the *Slurp1* transcript levels were normalized to cyclophilin A (expressed in all cells) or LYPD5 (expressed in suprabasal keratinocytes). Given the striking epidermal pathology and massive changes in the expression of many keratinocyte genes (Table S1), the relatively small change in *Slurp1* transcripts is probably not surprising. Again, we would contend that a ~50% reduction in *Slurp1* transcripts in the *Slurp2X*$^{-/-}$ mice is unlikely to be relevant to PPK, given that a 50% reduction in *Slurp1* transcripts in heterozygous *Slurp1* knockout mice does not elicit PPK or any other disease phenotype.

Heterozygosity for the *Slurp1X* or *Slurp2X* alleles lowered transcript levels by one-half but caused no disease (Fig. 3), whereas homozygosity for either allele caused severe PPK. A formal possibility is that SLURP1 and SLURP2 play redundant functions and that a threshold level of "SLURP protein" (*i.e.*, SLURP1 plus SLURP2) is necessary to ward off PPK. If that were the case, we reasoned that mice heterozygous for both *Slurp1X* and *Slurp2X* alleles (*Slurp1X*$^{+/-}$*Slurp2X*$^{+/-}$ mice) might exhibit PPK. This was not the case. *Slurp1X*$^{+/-}$*Slurp2X*$^{+/-}$ mice did not develop PPK (Fig. S7), whereas PPK was invariably evident in *Slurp1X*$^{-/-}$ and *Slurp2X*$^{-/-}$ mice by 6–8 weeks of age. *Slurp1X*$^{+/-}$*Slurp2X*$^{+/-}$ and wild-type mice had normal digits, whereas *Slurp1X*$^{-/-}$ and *Slurp2X*$^{-/-}$ mice exhibited PPK (evident by the bulbous appearance of the distal phalanges).

Discussion

The link between SLURP1 deficiency and PPK is well documented. SLURP1 is produced by keratinocytes, and many different *SLURP1* mutations have been uncovered in *mal de Meleda* patients (Adeyo *et al.,* 2015b; Bakija-Konsuo *et al.,* 2002; Chimienti *et al.,* 2003; Fischer *et al.,* 2001b; Marrakchi *et al.,* 2003; Nellen *et al.,* 2013). Also, two independent lines of *Slurp1* knockout mice (*Slurp1*$^{-/-}$ and *Slurp1X*$^{-/-}$ mice) developed PPK (Adeyo *et al.,* 2014). In contrast, the functional relevance of SLURP2, another secreted Ly6 protein, has been unclear. In the current studies, we initially investigated *Slurp2* knockout mice (*Slurp2*$^{-/-}$ mice) in which exon 2–3 sequences were replaced with *neo* and *lacZ* cassettes. The *Slurp2*$^{-/-}$ mice developed PPK, similar to that in *Slurp1*$^{-/-}$ mice (Adeyo *et al.,* 2014). The PPK in *Slurp2*$^{-/-}$ mice was accompanied by increased keratinocyte proliferation, a poorly demarcated stratum granulosum, and an accumulation of small lipid droplets in the stratum corneum. Like *Slurp1*-deficient mice (Adeyo et al., 2014), *Slurp2*$^{-/-}$ mice exhibited hind limb clasping and reduced body weight.

The fact that the phenotypes of *Slurp2*$^{-/-}$ mice closely resembled *Slurp1*$^{-/-}$ mice was somewhat surprising because SLURP1 and SLURP2 have negligible levels of sequence identity (apart from the 10 cysteines of the Ly6 domain). We were initially concerned by the fact that *Slurp1* transcripts were reduced in *Slurp2*$^{-/-}$ mice and that the disease phenotypes in *Slurp2*$^{-/-}$ mice might result from reduced *Slurp1* expression (conceivably reflecting the

effects of the *neo* and *lacZ* insertions on the expression of nearby genes) (Adeyo *et al.*, 2014). That worry was mitigated by the finding that *Slurp1* transcripts in the paw skin of *Slurp2*$^{-/-}$ mice (which manifest severe PPK) were in the same range as those in *Slurp1*$^{+/-}$ mice (which had no disease). The worry was further mitigated by the discovery that *Slurp2X*$^{-/-}$ mice (where *Slurp2* was inactivated with a nonsense mutation) manifested the same disease phenotypes. Finding disease in mice harboring a simple *Slurp2* nonsense mutation strongly supports the idea that *Slurp2* is a *bona fide* "PPK gene" in mice.

We do not fully understand why the PPK in *Slurp1*- and *Slurp2*-deficient mice is accompanied by hind limb clasping and metabolic phenotypes. Those phenotypes could be due to the effects of SLURP1 and SLURP2 deficiencies on other tissues, but a more parsimonious explanation would be that these phenotypes are consequences of the PPK. The metabolic phenotypes might relate to increased grooming of paw skin (a form of locomotor activity that often goes undetected in metabolic cages) or to effects of water loss (*i.e.*, increased loss of saliva) during grooming (Gordon, 1990). Hind limb clasping was present at 6–8 weeks of age, coinciding perfectly with visible evidence of PPK. It seems possible that hind limb clasping could relate to impaired nociception/proprioception as a result of the thickened epidermis on the paws. Alternatively, the hind limb clasping could relate to the presence of a passenger gene that segregates with *Slurp2* (Ji *et al.*, 2010; Smithies and Maeda, 1995; Westrick *et al.*, 2010).

Thus far, no one has identified *SLURP2* mutations in humans. It is possible that SLURP2 is simply dispensable in humans. However, we suspect that, sooner or later, dermatologists will uncover a *SLURP2* mutation in a human subject with PPK. It is noteworthy that some patients with PPK resembling *mal de Meleda* do not have *SLURP1* mutations (Charfeddine *et al.*, 2003; Lestringant *et al.*, 2001; van Steensel *et al.*, 2002). In some cases, the linkage data seem inconsistent with *SLURP2* but in none of the cases was *SLURP2* sequenced.

The functions of SLURP1 and SLURP2 proteins require more study. Most of the earlier research focused on the possibility that these proteins (like secreted snake toxins) affect acetylcholine signaling (Chimienti *et al.*, 2003) (Arredondo *et al.*, 2006; Arredondo *et al.*, 2005; Chernyavsky *et al.*, 2010). Several studies suggested that SLURP1 affects signaling through the α7 subtype of nicotinic acetylcholine receptors (Chimienti *et al.*, 2003) (Arredondo *et al.*, 2005; Chernyavsky *et al.*, 2010), and one study found that SLURP2 competed with radiolabeled nicotinic ligands for binding to keratinocytes (Arredondo *et al.*, 2006). However, no study has yet demonstrated a *direct* protein–protein interaction between SLURP1 or SLURP2 and another keratinocyte protein. Adeyo and coworkers were not able to detect SLURP1 binding to CHO cells that overexpressed the α7-nicotinic acetylcholine receptor (Adeyo *et al.*, 2014; Adeyo *et al.*, 2015a), but the possibility that SLURP1 binds to other acetylcholine receptors was not tested.

We considered the possibility that *Slurp1* and *Slurp2* play purely redundant functions and that heterozygosity for *both* knockout alleles would elicit the same disease phenotypes found in *Slurp1*$^{-/-}$ and *Slurp2*$^{-/-}$ mice. This was not the case; double heterozygous mice (*Slurp1*$^{+/-}$ *Slurp2*$^{+/-}$) were free of disease. This finding points against functional redundancy of *Slurp1* and *Slurp2*, but finding precise roles for SLURP1 and SLURP2 will require more

studies. For example, it would be desirable to determine if a complete deficiency of both SLURP1 and SLURP2 (*Slurp1*$^{-/-}$ *Slurp2*$^{-/-}$) would result in particularly severe disease—or whether the double-knockout mice would be indistinguishable from single-knockout mice. If the latter were the case, it would suggest that SLURP1 and SLURP2 are involved in the same pathway or that they were components in the same multi-protein complex. Still another possibility would be that SLURP2 is required for the expression of "the SLURP1 receptor." Such a scenario could explain why the phenotypes of SLURP1 and SLURP2 deficiencies are so similar.

Inflammation and infection are prominent features of the PPK in *mal de Meleda* patients, but we found no histologic evidence of inflammation in the paw skin of *Slurp1* and *Slurp2* knockout mice. The absence of inflammatory infiltrates in the epidermis or dermis of the mouse PPK models suggests that the inflammation in humans with *mal de Meleda* may be secondary to infections in the markedly thickened palms and soles (as opposed to being a *direct* consequence of the absence of a functional SLURP protein).

The knockout mice described in the current study represent an important step forward in defining the function of SLURP2. However, more work is needed. In the case of SLURP1 and SLURP2, digging deeper into their precise function and their link to PPK will require improved reagents, for example monospecific antibodies for *mouse* SLURP1 and SLURP2 and recombinant proteins. Once improved reagents are in hand, it ought to be possible to identify proteins that interact with SLURP1 and SLURP2 and pursue mechanisms for disease. The availability of recombinant proteins could also open the door to testing a therapy for *mal de Meleda*.

Materials and Methods

Slurp2-deficient mice. *Slurp2*$^{-/-}$ mice were obtained from the UC Davis Mutant Mouse Regional Resource Center (MMRRC). The Slurp2 knockout allele was originally created by Lexicon Genetics for Genentech (Tang *et al.*, 2010). Genotyping was performed by PCR. The mutant allele was detected with oligonucleotides 5′-GCAGCGCATCGCCTTCTATC-3′ and 5′-CATTGGACAACTATGTGACCCAGGTA-3′ (amplifying a 396-bp fragment from the *neo* to sequences downstream from *Slurp2*). The wild-type allele was detected with oligonucleotides 5′-TGGCTCCAATGATTACTG-3′ and 5′-CTAGACGGGTGAGA-3′ (amplifying a 287-bp fragment from intron 1 to exon 2). We created a second line of *Slurp2* knockout mice ("*Slurp2X* mice") by gene targeting with a sequence-replacement vector designed to change Leu-27 in exon 2 of *Slurp2* to a premature stop codon. A description of the *Slurp2X* knock-in vector is included in Fig. S6. Targeted clones were identified by long-range PCR (Fig. S6). Two targeted clones were injected into C57BL/6 blastocysts to produce chimeric mice, which were then bred to create "*Slurp2X* knockout mice." The *neo* was removed by breeding *Slurp2X*$^{+/-}$ mice with a deleter *Cre* transgenic line (Ella-*Cre* transgenic mice from The Jackson Laboratory). Genotyping was performed by PCR with oligonucleotides 5′-CTGGGCTGGATGCAAGACCT-3′ and 5′-ACACTCACGGGTGGCAATGA-3′ (amplifying a 663-bp fragment spanning the targeted point mutation). The 663-bp product from the mutant alleles could be cleaved by *Spe*I into 575- and 88-bp fragments.

Slurp1 knockout mice (one in which exon 2 coding sequences were replaced with *neo* and *lacZ* cassettes, and a second in which a nonsense mutation was inserted into exon 2) have been described previously (Adeyo *et al.,* 2014). All mice had a mixed genetic background (129/OlaHsd and C57BL/6). 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 in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Images were obtained with a Nikon Eclipse E600 microscope (Plan Fluor 20×/0.50 NA or 40×/0.75 NA objectives, air) with a DS-Fi2 camera (Nikon). To detect lipid droplets, skin biopsies were placed in Optimal Cutting Temperature (OCT) compound, frozen, and cryosectioned (10 μm). Sections were fixed in 4% formalin, washed three times in phosphate-buffered saline, and stained with BODIPY 493/503 (Adeyo *et al.,* 2014). To assess BrdU uptake, 4-week-old mice were given an intraperitoneal injection of BrdU (40 mg/kg of body weight) and euthanized 1 h later. Cryosections were fixed with acetone for 10 min, treated with 1 N HCl for 10 min on ice, 2 N HCl for 10 min at room temperature and 10 min at 37°C, and neutralized with 0.1 M sodium borate pH 8.5 for 12 min (Adeyo *et al.,* 2014). Sections were then blocked with 10% donkey serum, and incubated overnight at 4°C with a rat monoclonal antibody against BrdU (Abcam, 1:200), followed by a 1-h incubation with an Alexa Fluor 488–conjugated donkey anti-rat IgG (Life Technologies, 1:200) (Adeyo *et al.,* 2014). DNA was stained with DAPI. Images were obtained with a Zeiss LSM700 laser-scanning microscope [Plan Apochromat 20×/0.80 NA (air) or 63×/1.4 NA (oil) objectives]. We attempted to detect SLURP2 in skin sections from wild-type mice with a rabbit antiserum against a mouse SLURP2 peptide (CVIIATRSPISFTDLPLVTKM), but no signal was detected.

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 *Slurp2X*$^{-/-}$ mice (n = 5/group) with an RG1 evaporimeter (cyberDERM) (Adeyo *et al.,* 2014).

Body Weight/Metabolic Phenotypes

Measurements of oxygen consumption were performed using sealed metabolic cages (Oxymax, Columbus Instruments), and physical activity was assessed by numbers of laser beam breaks (Weinstein *et al.,* 2012). Data was collected and analyzed with Oxymax/CLAMS software. Body weights of male and female mice were recorded weekly. Adiposity was assessed by NMR (Weinstein *et al.,* 2010).

Gene Expression

RNA was isolated with TRI reagent (Molecular Research), and qRT-PCR measurements were performed in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems) (Jung *et al.,* 2013; Weinstein *et al.,* 2012; Yang *et al.,* 2011). Gene-expression was calculated with the comparative C_T method and normalized to cyclophilin A. Primers

for *Slurp1* were 5′-CACGGCCATTAACTCATGC-3′ and 5′-CCATGGGACTGTGGTTGAA-3′ (exons 2 and 3, respectively). Primers for *Slurp2* were 5′-TGGTCTTGAGCATGGAGCTA-3′ and 5′-TCCATGGGCAGCTAGACG-3′ (exons 1 and 2, respectively). Primers for *Lypd2* and *Ly6d* were described previously (Adeyo *et al.*, 2014). We also used qRT-PCR to assess levels of *Krt6b, Krt16, Krt24, Lce1m, Lce31, Lce3f, Areg,* and *Defb4* expression the paw skin of *Slurp2X*$^{-/-}$, *Slurp1X*$^{-/-}$, and littermate wild-type mice (primers listed in Table S2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PPK	palmoplantar keratoderma
Ly6	lymphocyte antigen 6
GPIHBP1	glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1

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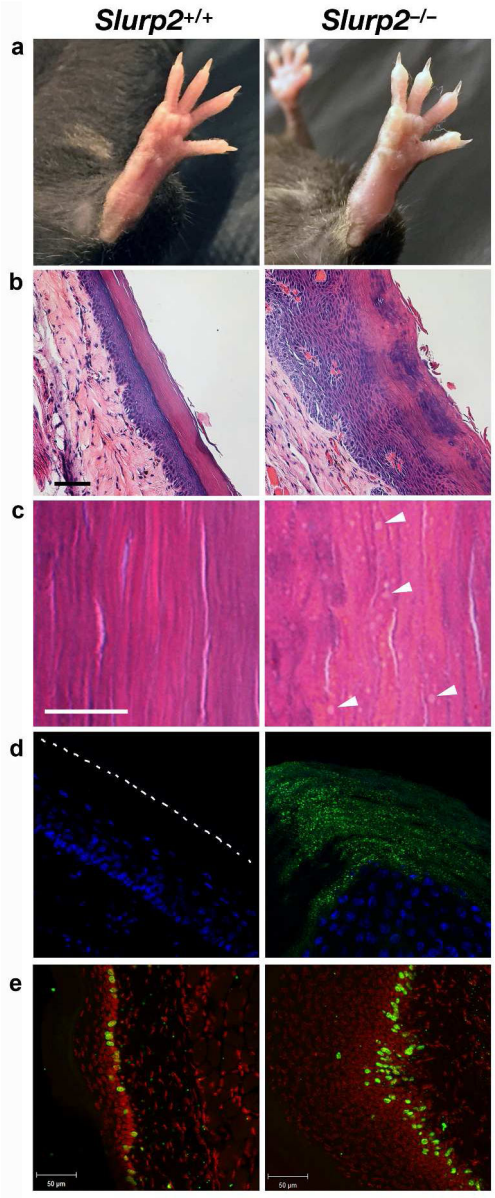

Figure 1. Palmoplantar keratoderma in *Slurp2*$^{-/-}$ mice

(a) Paws from wild-type and *Slurp2*$^{-/-}$ mice. The epidermis of the entire paw was thick but the PPK was quite evident at 6–8 weeks of age by the bulbous appearance of the tips of the digits. **(b)** H&E–stained sections showing hyperkeratosis in *Slurp2*$^{-/-}$ paw skin. Scale bar, 50 μm. **(c)** Numerous small lipid droplets in the stratum corneum of *Slurp2*$^{-/-}$ mice (arrowheads). Scale bar, 10 μm. **(d)** BODIPY 493/503 staining showing tiny lipid droplets (green) in the stratum corneum of *Slurp2*$^{-/-}$ paw skin. DNA was stained with DAPI (blue). In the left-hand panel (wild-type mouse), the stratum corneum is above the white line. **(e)** Increased BrdU incorporation (green) into the paw skin of *Slurp2*$^{-/-}$ mice. DNA was stained with DAPI (red). Scale bar, 50 μm.

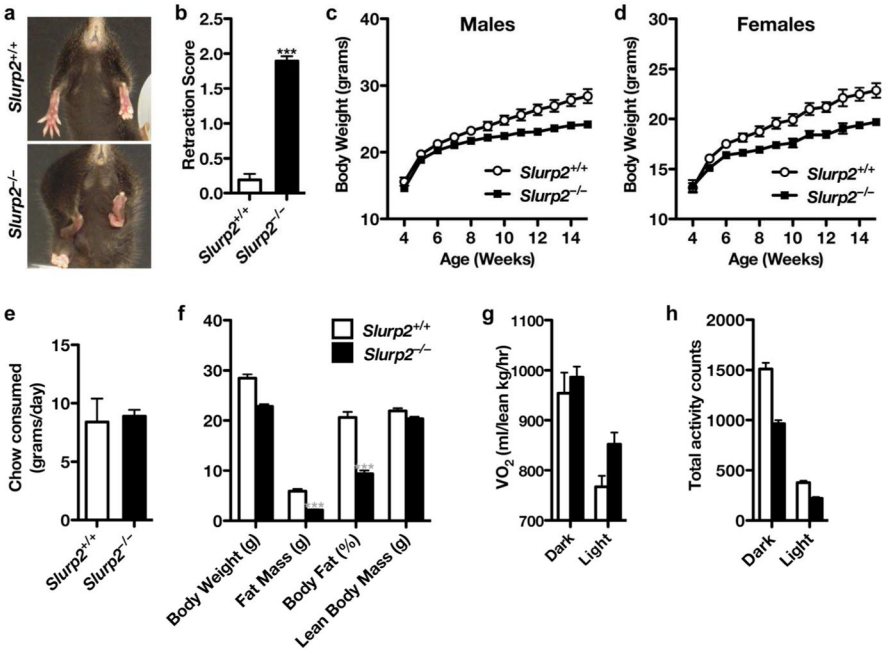

Figure 2. "Non-skin" phenotypes in *Slurp2*$^{-/-}$ mice

(a) Hind limb clasping in *Slurp2*$^{-/-}$ mice. **(b)** Quantification of hind limb clasping (0 for none; 1 for unilateral retraction; 2 for bilateral retraction). Shown are means ± SEM; $n = 12$ for *Slurp2*$^{+/+}$ mice; $n = 13$ for *Slurp2*$^{-/-}$ mice; ***$p < 0.0001$). **(c–d)** Weight gain in chow-fed male and female *Slurp2*$^{+/+}$ and *Slurp2*$^{-/-}$ mice, beginning at ~4 weeks of age. Males: $n = 10$ *Slurp2*$^{+/+}$ and $n = 9$ *Slurp2*$^{-/-}$. Females: $n = 8$ *Slurp2*$^{+/+}$ and $n = 5$ *Slurp2*$^{-/-}$. Means ± SEM. Males: differences were significant at weeks 8–15 at p values ranging from 0.046 to 0.002. Females, p values for weeks 9–15 were <0.05 except for week 13 (0.066). **(e)** Chow consumption in *Slurp2*$^{-/-}$ and wild-type mice. **(f)** Adiposity in 7-month-old chow-fed male *Slurp2*$^{-/-}$ mice ($n = 7$/group; $p < 0.001$ for fat mass and % body fat). **(g–h)** Increased O_2 consumption but reduced activity (reduced numbers of laser beam breaks) in *Slurp2*$^{-/-}$ mice. The differences in O_2 consumption and activity (two light-dark cycles for each of the 3 mice/group) were consistent and significant during the light cycle at $p < 0.001$ and $p < 0.05$, respectively.

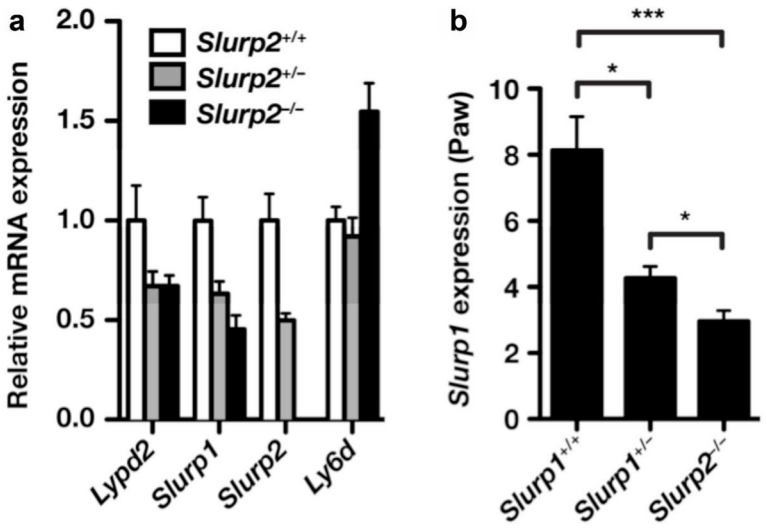

Figure 3. Expression of *Slurp2* and nearby genes in *Slurp2*$^{-/-}$ mice

(a) Expression of *Slurp2*, *Slurp1*, and genes encoding two other Ly6 proteins (*Lypd2, Ly6d*) in the paw skin of *Slurp2*$^{-/-}$ mice, as judged by qRT-PCR, means ± SEM. *Slurp2*$^{+/+}$ ($n = 6$), *Slurp2*$^{+/-}$ ($n = 5$), and *Slurp2*$^{-/-}$ mice ($n = 4$/group). The levels of *Slurp1* and *Slurp2* transcripts in *Slurp2*$^{-/-}$ mice were lower than in *Slurp2*$^{+/+}$ mice ($p < 0.01$) **(b)** Expression of *Slurp1* in the paw skin of wild-type ($n = 9$), *Slurp1*$^{+/-}$ ($n = 5$), and *Slurp2*$^{-/-}$ mice ($n = 7$). . *$p < 0.05$; ***$p < 0.001$.

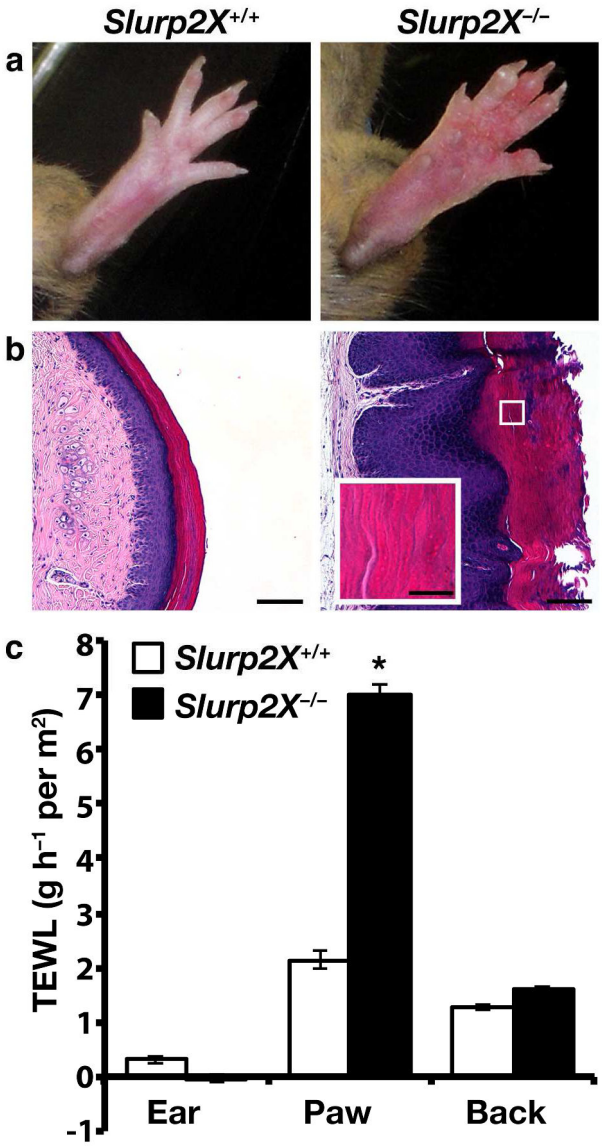

Figure 4. *Slurp2X*$^{-/-}$ mice develop PPK

(a) Paws of *Slurp2X*$^{+/+}$ and *Slurp2X*$^{-/-}$ mice at 12 weeks of age. **(b)** H&E–stained sections of paw skin in *Slurp2X*$^{+/+}$ and *Slurp2X*$^{-/-}$ mice, revealing a thickened epidermis in the paw skin of a *Slurp2X*$^{-/-}$ mouse. Scale bar, 100 μm. Insert in the right-hand panel shows tiny lipid droplets in the stratum corneum of *Slurp2X*$^{-/-}$ paw skin; scale bar, 10 μm. **(c)** Increased transepidermal water loss (TEWL) from the paw skin of age-matched *Slurp2X*$^{+/+}$ and *Slurp2X*$^{-/-}$ male mice (10–15 weeks old) as measured with an RG1 evaporimeter (n = 5/group). Means ± SEM; *$p < 0.05$).

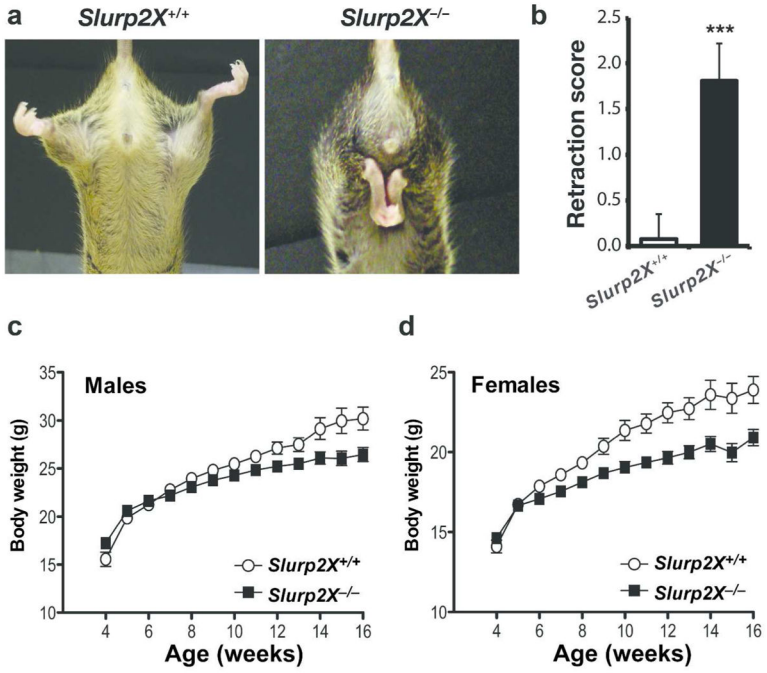

Figure 5. *Slurp2X*$^{-/-}$ mice exhibit hind limb clasping and reduced body weight

(a) Hind limb clasping in male *Slurp2X*$^{-/-}$ mice when picked up by the tail (similar results were observed with female *Slurp2X*$^{-/-}$ mice). **(b)** Quantification of the hind limb clasping phenotype (0 for no hind limb retraction; 1 for unilateral retraction; 2 for bilateral retraction) (n = 26 for *Slurp2X*$^{+/+}$ mice and n = 16 for *Slurp2X*$^{-/-}$ mice). Means ± SEM; ***$p < 0.001$. **(c–d)** Weight gain in chow-fed male and female *Slurp2X*$^{+/+}$ (wild-type) and *Slurp2X*$^{-/-}$ mice (4 to 16 weeks of age; n = 9/group). Males: n = 8/group; differences were significant at weeks 12–16 ($p < 0.05$). Females: n = 13 *Slurp2X*$^{+/+}$ and n = 14 *Slurp2X*$^{-/-}$; p <0.03 for weeks 7–16. Means ± SEM.