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Functional roles for *Piezo1* and *Piezo2* in urothelial mechanotransduction and lower urinary tract interoception

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Running title: *Piezo* function in bladder urothelium

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ABSTRACT

The mechanisms that link visceral mechanosensation to the perception of internal organ status (i.e., interoception) remain elusive. In response to bladder filling, the urothelium releases ATP, which is hypothesized to stimulate voiding function by communicating the degree of bladder fullness to subjacent tissues including afferent nerve fibers. To determine if PIEZO channels function as mechanosensors in these events, we generated conditional urothelial Piezo1-, Piezo2-, and dual Piezo1/2-knockout (KO) mice. While functional PIEZO1 channels were expressed in all urothelial cell layers, Piezo1-KO mice had a limited phenotype. Piezo2 expression was limited to a small subset of superficial umbrella cells, yet male Piezo2-KO mice exhibited incontinence (i.e., leakage) when their voiding behavior was monitored during their active dark phase. Dual Piezo1/2-KO mice had the most significant phenotype, characterized by decreased urothelial responses to mechanical stimulation, diminished ATP release, bladder hypoactivity in anesthetized Piezo1/2-KO females, but not male ones, and urinary incontinence in both male and female Piezo1/2-KO mice during their dark phase, but not inactive light one. Our studies reveal that the urothelium functions in a sex and circadian manner to link urothelial PIEZO1/2 channel-driven mechanotransduction to normal voiding function and behavior, and in the absence of these signals, bladder dysfunction ensues.
INTRODUCTION

In addition to well-known senses that allow us to perceive external stimuli (e.g., taste, hearing, sight, touch, smell), body movement (vestibular senses), and body position (proprioception), is the “hidden” sense of interoception (1-3). It links the sensation of internal bodily functions such as heartbeat, breathing, digestion, and bladder filling to higher-order functions including conscious awareness (e.g., my bladder is full), behavior (e.g., urination), and emotions (e.g., relief) (4). Integral to interoception are “interceptors,” which are generally classified as sensory nerve endings (4, 5); however, other than astrocytes (6), a functional role for other non-neuronal tissues in interoception is an open question. In the case of the lower urinary tract (LUT), the stratified urothelium that lines most of these organs is thought to be a site of visceral sensation, mechanotransduction in particular (7). For example, in response to bladder filling, the urothelium releases ATP, a mediator that is hypothesized to stimulate bladder activity and voiding function by communicating the degree of bladder fullness to subjacent tissues including sensory afferents (7-9). Testing this “current model” in an in vivo setting has proven difficult because of a lack of fundamental insights into how tension in the plasma membrane of urothelial cells is sensed, how or if the associated mechanotransduction pathways contribute to mediator release from the serosal surfaces of the tissue, and whether these events are conducive to normal voiding function and behavior.

A diversity of proteins instrumental to cellular mechanotransduction have been identified including junction-associated proteins, G-protein coupled receptors, and stretch-activated channels (10-13). Nominally stretch-sensitive channels expressed by the urothelium include TRPV4, K⁺ channels, and the PIEZO channels (7). However, of this group, only PIEZO channels belong to the small class of channels (other members include TRAK1/2, KCNK2, OSCA/TMEM63) that have been classified as bona fide mechanosensors, i.e., molecular force transducers that change their conformation in direct response to mechanical stimuli (12). Discovered by Dr. Ardem Patapoutian and colleagues using an siRNA screen (14), PIEZO1 (2521...
amino acids in humans) and PIEZO2 (2752 amino acids in humans), form a small family of non-selective cation channels that are activated by various mechanical stimuli including cell probing, changes in hydrostatic pressure, stretch, or laminar flow (14-17). Functional PIEZO channels can be reconstituted in vitro, their structures are known, and they regulate a diversity of stretch-sensitive processes in the body (18, 19).

Despite the rapid increase in knowledge of PIEZO channels over the past few years, our current understanding of PIEZO1 and PIEZO2 expression, distribution, and function in the urothelium is limited. The urothelium expresses *Piezo1* message, which reportedly exhibits circadian rhythmicity (20, 21). Using *Piezo1*^{tdT/tdT} reporter mice, Dalghi et al. showed that the urothelium lining the renal pelvis, ureters, bladder, and urethra express PIEZO1, as do underlying interstitial fibroblasts, smooth muscle cells, endothelial cells, and mesothelial cells (22). In isolated urothelial cells, PIEZO1 along with TRPV4 are required for stretch-induced release of ATP (21), but if release of mediators from native urothelium is also *Piezo1* dependent has not been reported. Currently, there are no insights into whether PIEZO1-dependent urothelial mechanotransduction pathways promote normal voiding function or behavior. While *Piezo2* expression is also reported in the urothelium (23), the expression and localization of PIEZO2 protein in the urinary tract is unknown. Intriguingly, patients deficient in PIEZO2 expression exhibit voiding dysfunction (23), although this may not be the case for all patients (24). In addition, mouse LUT function is also reportedly dependent on *Piezo2* expression in sensory neurons and possibly the urothelium (23); however, it remains unknown if *Piezo2* expression is critical for urothelial mechanotransduction, if urothelial-expressed PIEZO channels regulate voiding behavior, or whether PIEZO1 and PIEZO2 act in a coordinate fashion to regulate these events.

To better understand roles for the urothelium in mechanotransduction and LUT interoception, we generated conditional urothelial *Piezo1*-knockout (KO), *Piezo2*-KO, and *Piezo1/2*-KO mice. Our studies reveal that the urothelium acts as a functional interoceptor,
transducing mechanical stimuli in a PIEZO-dependent fashion, ultimately contributing to normal voiding function and behavior.

RESULTS

Piezo1 and Piezo2 are expressed in mouse bladder urothelium

Expression of Piezo1 in mouse urothelial cells was initially assessed by fluorescent in situ hybridization (FISH), which confirmed Piezo1 expression in all three layers of the urothelium, as well as subjacent cell types including fibroblasts (Figure 1A). We used Piezo1<sup>tdT/tdT</sup> reporter mice (25), coupled with Western blot analysis, to show that PIEZO1-tandem-dimer Tomato (tdT) expression was enriched in a urothelial fraction (Figure 1B; urothelium to detrusor ratio of 1.00 ± 0.01 to 0.53 ± 0.10; normalized to urothelium, mean ± SEM, n=3), which is consistent with the tissue distribution of PIEZO1-tdT assessed by immunofluorescence (Figure 1C). Furthermore, and like our previous report (7), we observed that PIEZO1-tdT was localized to the basal and lateral surfaces of umbrella cells, as well as at the plasma membranes of underlying intermediate and basal cells (Figure 1D-E). PIEZO1 is reported to be enriched at the tight junction of intestinal adenocarcinoma-derived Caco2 cells (26); however, in native umbrella cells, PIEZO1-tdT was found along the lateral membrane, but did not extend into the region of the CLDN8-labeled tight junction (Figure 1E).

Using FISH, we explored the expression of Piezo2, which was recently reported to be highly expressed in ~13% of umbrella cells, but with up to 75% of umbrella cells expressing smaller amounts of message (23). To selectively label the urothelium, but not other bladder tissues, we used probes against Upk3a, which exhibits highest expression in umbrella cells and lowest expression in basal cells (27-29). Piezo2 message was mostly limited to a small population of apparently scattered umbrella cells, which also expressed relatively high amounts of Upk3a message (Figure 1F). Only, small and variable amounts of Piezo2 message were detected in the
adjacent urothelial cells. *Piezo2* message was also observed in suburothelial fibroblasts and blood vessels. We attempted to immunolocalize PIEZO2 in the urothelium using commercial antibodies, or a previously published one (30), but were unable to convincingly confirm PIEZO2 expression in the bladder. We also employed a *Piezo2* mouse reporter line (*Piezo2*GFP-IRES-Cre), which expresses GFP fused to PIEZO2 as well as the Cre recombinase under the direction of the *Piezo2* promoter (30). However, we were unable to detect PIEZO2-GFP in any LUT tissue, even after amplifying the signal by use of anti-GFP antibodies.

We also mated *Piezo2*GFP-IRES-Cre mice with Ai9 reporter mice, which express tdT upon Cre-mediated recombination. This technique identifies sites of *Piezo2*-driven gene expression in adult tissues, but also serves as a form of lineage tracing, allowing for the identification of the adult progeny of *Piezo2*-expressing progenitor cells. The pattern of tdT expression was similar to that observed for FISH analysis: scattered tdT-positive umbrella cells, as well as tdT-positive interstitial fibroblasts (and blood vessels) (Figure 1G). The lack of expression in either basal or intermediate cells (both of which are precursors for umbrella cells) (31, 32), indicates that umbrella cells likely express *Piezo2* sometime after undergoing terminal differentiation. While the tdT-positive umbrella cells appeared to be randomly distributed, they formed a gradient along the dome-to-neck axis of the bladder, with increasing numbers near the neck region (Figure 1G). In sum, *Piezo1* message and PIEZO1 protein are expressed throughout the urothelium, whereas *Piezo2* message is mostly restricted to a scattered population of umbrella cells.

**Urothelial cells express Yoda1-responsive Piezo1 channels, but Piezo1-KO urothelium remains responsive to mechanical stimulation**

To understand whether PIEZO1 was integral to urothelial mechanotransduction, function, and interoception, we initially attempted to generate conditional urothelial *Piezo1*-KO mice by mating *Piezo1*fl/fl mice with those expressing inducible *Upk2-iCreERT2* (33); however, this approach was
ineffective at decreasing Piezo1 expression by more than ~30%. We subsequently used a mouse strain with constitutive Upk2-driven Cre expression (Upk2\(^{Cre-/-}\))(34), and confirmed that Cre was expressed in all three layers of the urothelium lining the bladder, ureter, and proximal urethra, but not in non-urothelial tissues (Figure S1). Using qRT-PCR, we observed a significant ~75% decrease in Piezo1 expression in urothelial lysates prepared from Piezo1-control (Piezo1\(^{fl/fl}\); Upk2\(^{Cre-/-}\)) versus Piezo1-KO mice (Piezo1\(^{fl/fl}\); Upk2\(^{Cre-/-}\)) (Figure S2A). Because of the potential for contribution of Piezo1 message from non-urothelial tissues in these lysates (e.g., from Piezo1-expressing fibroblasts), we also quantified Piezo1 expression using FISH. In this case, we observed a significant ~85% reduction in urothelial Piezo1 expression in Piezo1-KO mice versus controls (Figure S2B-C). Loss of Piezo1 expression had no obvious effects on urothelial tissue morphology, expression of urothelial cell-type specific markers, or ultrastructure (Figure S2D-E).

The lack of access to the basolateral surfaces of native umbrella cells prevented us from measuring PIEZO channel activity using techniques like patch clamp. Instead, we measured changes in [Ca\(^{2+}\)], a commonly used surrogate that increases downstream of PIEZO channel opening (14). We transduced the urothelium in situ with an adenovirus encoding the Ca\(^{2+}\) sensor GCAMP5G (35), made a sheet preparation of urothelium, and perfused it with the nominally PIEZO1-selective activator Yoda1 (Figure 2A) (36). This treatment stimulated a substantial increase in [Ca\(^{2+}\)] (e.g., see Figure 2B), which was significantly reduced in urothelial sheets prepared from conditional urothelial Piezo1-KO mice, but not in urothelium derived from Piezo2-KO mice (Figure 2C). Unfortunately, there are no known agonists of PIEZO2 channels, and thus we could not use a similar strategy to confirm the presence of functional PIEZO2 channels in the urothelium.

To assess the sensitivity of urothelial PIEZO1 channels to mechanical stimuli, we used fire-polished and sealed patch pipettes to gently “poke” random umbrella cells (Figure 2D). This stimulus elicits large changes in PIEZO channel activity, and in the case of PIEZO1, poking is ~20-fold better than increasing membrane tension by use of suction (14, 30). Poking resulted in an
instantaneous rise in umbrella cell $[Ca^{2+}]$, followed by a rapid fall (Figure 2E; Video 1). The reversibility of the effect, combined with the ability to trigger repeated $[Ca^{2+}]$, spikes from the same cell, confirms that the cells were not damaged by this treatment. A careful examination of the videos revealed that adjacent cells, including those several cell lengths from the target cell, exhibited $[Ca^{2+}]$ responses, but after a several second delay (Video 1). The nature of these secondary events was not explored further. Surprisingly, we did not detect a significant difference in poking-induced $[Ca^{2+}]$ responses when comparing Piezo1-control umbrella cells to those from Piezo1-KO tissue (Figure 2F and Figure S3). While it is feasible that mechanical poking is ineffective at stimulating urothelial-expressed PIEZO1 channels, an alternative possibility is that the loss of Piezo1 is compensated by expression of Piezo2, or other gene products. This possibility is explored in more detail below.

**Serosal release of ATP from the native urothelium is not impaired in Piezo1-KO mice**

The urothelium is proposed to regulate LUT function by releasing mediators, ATP in particular, in response to mechanical stimuli such as bladder filling (7, 37). To test the Piezo1-dependence of ATP release from native urothelium, we used a “peeled” bladder preparation (Figure 2G and S4), which allowed us measure release of ATP from the serosal surfaces of the urothelium, and in response to filling (albeit from the urethral side). We previously showed, using dissected rabbit urothelium, that it is the urothelium and not the underlying fibroblasts that are the primary source of ATP (38). The urothelium in these preparations was intact and maintained tight junctions, while the majority of lamina propria, muscularis, and serosa was removed (Figure S4). We observed that ATP was released from the serosal surfaces of both Piezo1-control and Piezo1-KO mice in peeled bladders (Figure 2H). No significant differences were observed between these two groups. Thus, unlike the previous report that ATP release from isolated urothelial cells is (partially) Piezo1-
dependent (21), serosal release of ATP from the native urothelium was not significantly affected by loss of *Piezo1* expression.

**Piezo1-KO mice exhibit near-normal voiding function and voiding behavior**

To assess LUT function, we performed continuous cystometry in urethane-anesthetized *Piezo1*-control and *Piezo1*-KO mice. In female mice, none of the measured cystometric parameters were significantly affected by loss of urothelial *Piezo1* expression (Figure 3A-B). This included voiding efficiency (1.26 ± 0.08 in *Piezo1*-control mice versus 1.17 ± 0.15 in *Piezo1*-KO mice; no significant difference, t-test), a measure of the ability of the bladder to completely release its contents due to efficient detrusor contractions coupled with coordinate “opening” of the bladder sphincter. Relative to *Piezo1*-control mice, male *Piezo1*-KO mice also exhibited no significant differences in their cystometric parameters (Figure 3B). As it is difficult to accurately collect fluid from male mice, we were not able to measure their bladder efficiency. To further assess detrusor function in *Piezo1*-KO mice, we measured contractions of isolated, full-thickness bladder strips. However, there were no significant effects, even after triggering strong detrusor contractions using carbachol treatment or electrical field stimulation (Figure 3C).

Finally, we performed video-monitored void-spot assays on *Piezo1*-control and *Piezo1*-KO mice. Unlike cystometry, these assays were performed on freely moving, non-anesthetized mice, allowing us to assess conscious voiding behavior (Figure 4A). Because *Piezo1* expression is reported to exhibit circadian rhythmicity, with four-fold greater expression during the mouse’s active dark phase (20, 39), we measured voiding behavior in six-hour time window during either the light or dark phase. We also assessed whether there were any sex-related differences. Like previous studies, we sorted urine output into two classes: primary void spots (PVS; which are typically > 20 µl), or secondary void spots (SVS; those < 20 µl) (40-42). While the latter are uncommon in control mice (except in the case of aggressive α-males), they increase in animals
exhibiting urinary incontinence (i.e., leakage), including mice with experimental cystitis, outlet obstruction, or those exposed to stress (43-46).

Female Piezo1-control mice had ~1 PVS per hour during their active dark phase, but ~1/3 that number was observed during the light phase, when the mice were most often inactive (i.e., resting or sleeping; Figure 4B). The relatively low numbers of PVS during the light phase, the lack of SVS, and the increase in PVS during the dark phase, confirms that the mice retained their circadian patterns of urination and were not overtly stressed by being monitored. When comparing female Piezo1-control mice to Piezo1-KO ones, we noted no significant differences in the PVS or SVS parameters (Figure 4B). Technically, the average volume/PVS is indeterminate when the number of PVS falls to zero, which we observed in a few animals. However, if we performed a secondary analysis in which we excluded those animals with no voids, the values for this and other parameters remained non-significant. We also calculated a urinary continence score; however, we noted no significant effects on continence (Figure 4B). Again, when the PVS is zero, the continence score becomes indeterminate. In this case, the values were excluded, as a value of zero would indicate complete incontinence, which is the opposite of not voiding. Like females, Piezo1-control male mice had larger numbers of PVS during their dark phase compared to their light phase, and few SVS were observed. During the dark phase, none of the measured parameters were significantly different between Piezo1-control and Piezo1-KO male mice (Figure 4C). However, we observed a significant increase in number of PVS during the inactive light phase in male Piezo1-KO mice (independent of including or excluding animals that did not void; Figure 4C).

In summary, poking-induced changes in $[\text{Ca}^{2+}]_i$, serosal ATP release, cystometry, and voiding function were largely unaffected in Piezo1-KO mice. However, and opposite of the predictions of the current model, Piezo1-KO male mice exhibited a form of “nocturia”, in which they voided more frequently during their less active, light phase.
Conditional urothelial Piezo2-KO mice display normal mechanotransduction and LUT function, but male mice exhibit defects in voiding behavior

It was recently reported that conditional urothelial Piezo2-KO mice have abnormalities in bladder function (assessed using cystometry) and sphincter function (23). This study did not, however, assess whether urothelial mechanotransduction or voiding behavior are impacted in Piezo2-KO animals. To explore these possibilities, we generated conditional urothelial Piezo2-control and Piezo2-KO mice. We were unable to quantify any decrease in Piezo2 expression using qPCR as the amount of Piezo2 expression in the urothelium of Piezo2-control mice was too low to accurately measure by this technique. However, by using FISH to estimate the number of Piezo2-expressing umbrella cells per length of luminal membrane, we confirmed that Piezo2 expression was reduced to almost zero in Piezo2-KO mice (Figure S5A). There was no significant difference between Piezo2-control and Piezo2-KO responses to poking-induced changes in [Ca^{2+}]. (Figure 2C). Moreover, their robust response to Yoda1 indicated that umbrella cells lacking Piezo2 expression still expressed functional PIEZO1 channels (Figure 2C). We could not demonstrate any reliance of serosal ATP release on Piezo2 (Figure 2H), and in cystometry, we could not detect significant differences in bladder function between Piezo2-control versus Piezo2-KO male or female mice (Figure S6). There was no significant effect on female Piezo2-KO voiding efficiency (1.03 ± 0.10 in Piezo2-control mice versus 1.12 ± 0.03 in Piezo2-KO mice; t-test)

Finally, we assessed voiding behavior in Piezo2-control vs Piezo2-KO mice (Figure 5A). In the case of female mice, we noted a significant decrease in the number of PVS during the light phase, but this did not affect PVS total volume. When a secondary statistical analysis was performed, in which we excluded the animal that did not void, the differences became not significant. The most striking change we observed was in male mice during their active dark phase. Here, we noted a decrease in continence, which apparently resulted from a significant decrease in total PVS volume (Figure 5B). No significant differences were observed for the other
measured parameters, or during the male light phase. In sum, conditional urothelial male Piezo2-KO mice had a reduced total PVS volume in their dark phase, likely contributing to the overall decrease in continence. Interestingly, this occurred in the absence of any Piezo2-KO-dependent change in ATP release or in sensitivity to mechanical stimulation.

**Role for urothelial mechanotransduction is revealed in Piezo1/2-KO mice**

Up to this point, it was possible to conclude that urothelial expressed PIEZO channels have limited (or no) roles in urothelial mechanotransduction or voiding function. However, the relatedness of PIEZO1 and PIEZO2 led us to determine if loss of urothelial Piezo1 expression was compensated for by upregulated expression of Piezo2. However, we could not detect a significant difference in urothelial Piezo2 expression in Piezo1-control versus Piezo1-KO mice (1.00 ± 0.12 and 0.95 ± 0.14, respectively; data normalized to Piezo1-control values, mean ± SEM, n=3; t-test). To determine whether expression of both channels was necessary, we generated Piezo1/2-control or Piezo1/2-KO mice. FISH was used to confirm that Piezo1/2-KO mice had significantly lower amounts of Piezo1 and Piezo2 expression compared to Piezo1/2-control mice (Figure S7). Furthermore, Piezo1/2-KO umbrella cells were also significantly less responsive to treatment with Yoda1 (Figure 2C), confirming a significant reduction in their expression of PIEZO1. The urothelium of Piezo1/2-KO mice exhibited no obvious abnormalities, maintaining a wild-type-like organization and expression of urothelial marker proteins (Figure S8).

We first determined if the Piezo1/2-KO mice would reveal the existence of PIEZO1/2-dependent mechanotransduction pathways in the urothelium. Consistent with this possibility, Piezo1/2-KO umbrella cells exhibited a significant reduction in poking-induced changes in \([\text{Ca}^{2+}]\), (Figure 2F). The mechanotransduction pathway that contributes to the remaining poking-induced rise in \([\text{Ca}^{2+}]\), is unknown, and was not explored further. Next, we measured ATP release from the urothelium of Piezo1/2-control and Piezo1/2-KO peeled bladders. Strikingly, we observed that serosal ATP release was blocked in peeled Piezo1/2-KO bladders (Figure 2G), revealing an
almost complete dependence of serosal ATP release on PIEZO1/2 channel-dependent mechanotransduction.

The dramatic loss of serosal ATP release in Piezo1/2-KO mice urothelium allowed us to assess whether urothelial ATP release (and possibly other mediators) is integral to voiding function. In the case of female Piezo1/2-KO mice, we observed no significant effects on resting pressure, pressure threshold, maximum contraction, or amplitude in animals undergoing cystometry (Figure 6A). Nor was there any significant difference in voiding efficiency (1.09 ± 0.02 in Piezo1/2-control and 1.02 ± 0.05 in Piezo1/2-KO mice; data normalized to Piezo1/2-control, mean ± SEM, n=6, data not significantly different using Mann-Whitney test). Instead, we noted a significant increase in intervoid interval, and an associated increase in bladder compliance (Figure 6A). This would indicate that anesthetized female Piezo1/2-KO mice were less sensitive to bladder filling, the bladder required more volume to initiate a contraction, and the detrusor of Piezo1/2-KO mice was likely more relaxed. However, in the case of male Piezo1/2-KO mice, there was no effect on intervoid interval or compliance. Instead, their threshold pressure was significantly decreased relative to Piezo1/2-control mice (Figure 6B), indicating that the pressure needed to trigger voiding was reduced in these mice. None of the other cystometric parameters were significantly affected.

Finally, we assessed voiding behavior in the Piezo1/2-KO mice. Despite the cystometry results described above, freely mobile unanesthetized Piezo1/2-KO female mice exhibited no signs of PVS or SVS defects during their inactive light phase (Figure 7A). However, if we performed a secondary analysis where we excluded non-voiders, the average volume/PVS was significantly different between Piezo1/2-control versus Piezo1/2-KO mice. Somewhat like the Piezo2-KO male mice described above (Figure 5B), we observed a significant decrease in bladder continence in Piezo1/2-KO female mice; however, the latter exhibited no PVS defects, but instead presented with an increase in SVS number and total SVS volume (Figure 7A). We note that SVS were individual events, not obviously tied in time to PVS, and were voluntary in the sense that
animals moved to the corners, urinated, and then left. In the case of male Piezo1/2-KO mice, we observed no significant differences in PVS number, average volume per PVS, or total PVS volume during their dark phase, but like female mice they did present with a significant increase in SVS number and total SVS volume (Figure 7B). In turn, this led to a significant decrease in continence. No significant differences in voiding activity were observed during the light phase of male Piezo1/2-KO mice versus Piezo1/2-control ones (Figure 7B). Taken together, our studies reveal an important role for PIEZO1/2 channels in urothelial mechanotransduction, including functions in mechanosensing, serosal release of ATP, and modulation of voiding function and behavior.
DISCUSSION

Visceral organs experience mechanical forces that include rhythmic contractions (e.g., heartbeat and peristalsis) and in most cases gases, fluids, and solids enter, exit, or flow through them. While PIEZO channels are important to visceral organ functions that include blood pressure sensing (47), lung inflation (48), and release of hormones (49), few studies have made direct links between Piezo-channel dependent mechanotransduction and behavior, a critical link that underpins interoception. Perhaps the clearest evidence to date are recent studies that couple Piezo expression to neurons that innervate the Drosophila melanogaster crop and anterior midgut and are important for regulating fly feeding behavior (50, 51). Other potential examples include the voiding anomalies reported by Marshall et al. in patients with PIEZO2 mutations or in Hoxb8-cre;Piezo2<sup>fl/fl</sup> mice (23), but which also suffer from severe defects in motility and proprioception (52). However, the reported expression of Hoxb8 in all mouse bladder tissue types (28), combined with the reported expression of Piezo2 in blood vessels, fibroblasts, neurons, and urothelium, makes it difficult to parse which tissue(s) is acting as the interoceptor (this manuscript; 23, 25, 52). Marshall et al. also reported that Piezo2 deletion in afferent nerves or the urothelium alters LUT function (23); however, they did not assess if urothelial mechanotransduction was affected in these Piezo2-KO mice, nor did they measure voiding behavior in these mice. Using conditional urothelial Piezo KO mice, our studies reveal the following insights: (i) urothelial mechanotransduction depends in part on expression of Piezo1 or Piezo2; (ii) the urothelium acts as a mechanotransducer, and ATP release from the serosal surfaces of the native urothelium is Piezo1/2 dependent; and (iii) the urothelium functions as a non-neuronal interoceptor, linking PIEZO1/2 mechanotransduction to serosal mediator release (i.e., ATP) and to normal voiding function and behavior.

Our initial insight is that expression of Piezo1 or Piezo2 is integral to umbrella cell responses to mechanical stimulation. Interestingly, gentle poking of randomly chosen umbrella cells increases [Ca<sup>2+</sup>], but only in Piezo1/2-KO mice did we observe an inhibition of these responses.
This dual PIEZO channel requirement is surprising given that PIEZO1 is apparently expressed in all urothelial cells (and could be stimulated by Yoda1), whereas Piezo2 expression is seemingly limited to a small population of scattered umbrella cells, but whose function remains unknown. Unfortunately, we still have no insights into the expression, distribution, and rate of turnover of the PIEZO2 protein due to lack of suitable reagents. The most parsimonious solution to our conundrum would be that loss of Piezo1 would be compensated for by an increase in Piezo2 expression; however, we found no evidence that this was occurring. An additional possibility is that the PIEZO1/2-expressing umbrella cells produce a diffusible signaling molecule that is critical for mechanical responses in adjacent urothelial cells. This hypothesized signaling molecule could function by way of a paracrine signaling pathway, or by way of connexin channels that are expressed by the urothelium (20, 53). In this model, our results could be explained, for example, if PIEZO1 and PIEZO2 form heteromeric channels, but in the absence of its binding partner, the PIEZO channels form homomeric ones that still retain some degree of functionality. An alternative possibility relates to our use of mouse strains that constitutively express Upk2-driven Cre, a necessity to effectively reduce Piezo1/2 expression. As a result, loss of Piezo1 alone or Piezo2 alone could be compensated for during development (and/or postnatally) by altered expression of other genes integral to the mechanotransduction pathway. In this scenario, loss of both Piezo channel genes becomes too much to overcome. Finally, we also observed a large fraction of poking-induced [Ca\textsuperscript{2+}] that was not attenuated in Piezo1/2-dKO mice, indicating that other mechanotransduction pathways are likely operating in the urothelium. The nature of these pathways remains mysterious, but the urothelium potentially expresses multiple stretch-activated channel activities (reviewed in 7). For example, we have described a non-selective, stretch-activated channel activity at the apical surfaces of umbrella cells (54), and the basolaterally distributed TRPV4 channel was previously implicated in regulating bladder function and in stretch-induced [Ca\textsuperscript{2+}] changes in isolated urothelial cells (21, 55).
Our second insight relates to the PIEZO channel-dependence of serosal mediator release from the native urothelium, and its hypothesized role in communication with subjacent tissues (37). In the case of ATP, there are a plethora of studies that have explored the mechanisms and pathways of urothelial ATP release, including reports that filling-induced ATP release is decreased in P2rx3, Trpv1, Trpv4, Slc17a9 (VNUT), Cnx43 KO mice (53, 55-59), or increased in urothelial conditional Itgb1 (β1-integrin) KO mice (60). However, none of these studies unambiguously identified the mechanosensor, and in all cases, only mucosal ATP release was measured. While mucosal ATP can trigger events via a transmural pathway (7, 61), it is most likely that serosally released mediators are integral to urothelial interoception. Miyamoto et al. reported that stretch-induced ATP release from isolated mouse urothelial cells is (partially) Piezo1 dependent (21), and subsequent studies showed the Piezo1-dependence of endothelial cell ATP release (62). Using a peeled bladder preparation, we demonstrate that filling-induced release of ATP from the serosal surface of the native urothelium is wholly dependent on a PIEZO1/2 mechanotransduction pathway. While this preparation is arguably more physiologically relevant than isolated cells, by necessity it requires the removal of blood vessels, fibroblasts, nerves, and smooth muscle cells, whose contributions may include effector functions (e.g., release of mediators), modulation of urothelial signaling, and roles in defining the mechanical properties of the mucosa. Despite these limitations, the tools we have employed make it possible to begin exploring whether the serosal (and mucosal) release of other urothelial mediators (e.g., acetylcholine, adenosine, prostaglandins, NO)(7) is similarly PIEZO1/2 dependent.

Our final insight relates to the hypothesized role that the urothelium plays in regulating LUT function and voiding behavior, and in particular its role as an interoceptor. In the current model, bladder filling causes the urothelium to release ATP, which is hypothesized to act in a stimulatory manner to promote voiding function downstream of a local urothelial:afferent reflex (or via effects on other subepithelial tissues)(63). Notwithstanding the possibility that other mediators are also affected, the almost complete loss of serosal ATP release in Piezo1/2-KO mice afforded us an
ideal opportunity to test this model. Even so, the phenotype of the Piezo1/2-KO mice varied depending on whether we measured voluntary voiding behavior (i.e., void-spot assays) versus involuntary reflex micturition (i.e., cystometry), the animal sex, and the light phase during which measurements were made. The extended intercontraction interval and increased compliance we observed in female Piezo1/2-KO undergoing cystometry is consistent with the current model, as is the increased average volume per PVS during the light phase of conscious female mice (when we exclude non-voiding events). However, the decreased threshold pressure that we measured when performing cystometry in Piezo1/2-KO male mice would not be. Nor is the observation that voiding behavior during the light phase of male Piezo1/2-KO mice was not significantly different than control mice. The greatest divergence from the current model occurred when we assessed the voiding behavior of Piezo1/2-KO mice during their active dark phase. In both sexes, we observed a marked increase in SVS (but no significant effect on PVS), indicating urinary incontinence.

The proximal cause of this incontinence is unknown, but one possibility is that during the dark phase, urothelial-released ATP functions in an inhibitory fashion to dampen pathways that give rise to urinary incontinence. For example, the urethral sphincter is under control of a guarding reflex that strengthens urethral tone as the bladder becomes full (64). If urothelial ATP regulates these pathways, then loss of this mediator could allow urine leakage. Alternatively, incontinence may result from increased (or decreased) production of other mediators that are also under the control of the urothelial PIEZO channel-dependent mechanotransduction pathway. This could explain why Piezo1-KO and Piezo2-KO mice, males in particular, exhibited alterations in voiding behavior (nocturia and incontinence, respectively), even though serosal ATP release was apparently unaffected in these animals. In any case, both the mediators involved, and the target tissues (e.g., afferent nerve endings, detrusor, or urethral outlet) affected, will need to be defined by future studies. A final alternative is related to original identification of PIEZO1 (known as FAM38A at the time) as a regulator of ITGB1 activity and cell motility (65, 66). Intriguingly,
conditional urothelial Itgb1-KO mice are reported to exhibit incontinence, producing increased numbers of small void spots (0.8-4.0 µl range) (60). While Itgb1-KO mouse phenotype does not completely phenocopy that of Piezo1/2-KO mice (e.g., Itgb1-KO mice have large numbers of non-voiding contractions), integrins are known to play important roles in mechanotransduction (67), and thus some aspects of the incontinence we observe may involve the interplay between PIEZO channel-dependent signaling and that associated with ITGB1.

Finally, while urination can bring a feeling of relief in a normal setting, in pathological settings, LUT symptoms (including urgency, frequency, hesitancy, and nocturia) and incontinence give rise to emotional distress. As noted above, a subset of patients with PIEZO2 mutations exhibit LUT dysfunction (23). Their symptoms include bladder underactivity, or urgency in some cases, and stress-urinary incontinence. However, and unlike our urothelial-focused studies, patients with PIEZO2 mutations are deficient in PIEZO2 expression in all tissues including the urothelium and nervous system, and are more closely mimicked by the aforementioned Hoxb8-cre:Piezo2fl/fl mouse, which are also described as being incontinent (23). Other mouse studies have proposed (although not directly tested) roles for Piezo1 in partial bladder outlet obstruction, cystitis, and conditions that may arise from altered circadian expression of genes (including Piezo1), which are hypothesized to contribute to nocturia and enuresis (20, 39, 68, 69). While our studies did not directly address the clock gene-regulated aspects of Piezo1 expression, we did observe that loss of Piezo1 expression alone results in nocturia in male mice, and that disruption of voiding behavior in Piezo1/2-KO (and Piezo2-KO) mice is primarily affected during their active, dark phase. Our insights, combined with the tools and techniques we have developed, will not only enhance our understanding of the roles of urothelial mechanotransduction in LUT interoception, but should also guide future investigations into the roles of PIEZO1/2 in normal and abnormal LUT function, and the roles of sex and circadian rhythmicity in these events.
METHODS

Reagents including antibodies

Unless otherwise specified, all chemicals were reagent grade or better and obtained from Sigma Aldrich (St. Louis, MO). Primary antibodies were as follows: rat monoclonal anti-CDH1 (E-cadherin, clone DECMA-1; catalog number MABT26, Millipore-Sigma, Burlington, MA); goat polyclonal anti-m-Cherry (catalog number MBS448057; MyBioSource, San Diego, USA); chicken polyclonal anti-KRT5 (catalog number 905901, Biolegend, San Diego, CA); rabbit polyclonal anti-KRT5 (catalog number 905501, Biolegend); rabbit polyclonal anti-KRT20 (catalog number ab53120, Abcam, Cambridge, MA); rabbit polyclonal anti-RFP (catalog number 600-401-379; Rockland Immunochemicals, Limerick, PA); rabbit polyclonal anti-TJP1 (ZO-1; catalog number 61-7300, Thermo Fisher Scientific, Waltham, MA); goat polyclonal anti-TP63 (p63/TP73L; catalog number AF1916, R&D Systems, Minneapolis, MN); rabbit polyclonal pan-uroplakin was a kind gift from Dr. T.-T. Sun (New York University, NY, NY)(70). Minimal cross reactivity goat and donkey secondary antibodies conjugated to Alexa488, Alexa548, Cy5, or HRP were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Animals

Both female and male mice were used in our studies and purpose bred from the strains described below. Mice were housed in standard caging, with automatic water dispenser and dry mouse chow ad libitum. All experimental mice, female and male, were virgins and group housed after weaning (up to five females/cage or up to four males/cage). Breeding mice were never used for experiments. Non-pregnant does were housed up to five per cage. Male breeders were always housed individually when not breeding. Animals were harem bred. The bottom of the cage was covered in standard bedding, and included a plastic igloo or running wheel, and a small square of paper for shredding. The animals were held under a 12-hour day/night cycle. The weights and
ages of animals used in our studies are presented in Table S1. Euthanization of mice was accomplished by inhalation of CO₂ gas, followed by thoracotomy or cervical dislocation.

To define PIEZO1 expression and distribution, Piezo1<sup>tdT/+</sup> reporter mice (strain number 029214, Jackson Laboratory, Bar Harbor, ME) were mated to generate homozygous Piezo1<sup>tdT/tdT</sup> mice or wild-type Piezo1<sup>+/+</sup> mice (25). To assess Piezo2 expression, a Piezo2 mouse reporter line (Piezo2<sup>GFP-ires-Cre</sup>, strain number 027719, Jackson Laboratory) was employed (30). It expresses GFP fused to PIEZO2 and the Cre recombinase under the direction of the Piezo2 promoter. Piezo2<sup>GFP-ires-Cre</sup> mice were mated with Ai9 mice (strain number 007909, Jackson Laboratory), which express CAG promoter-driven TdT expression upon Cre-mediated removal of a stop codon upstream of the TdT sequence. To generate urothelial-specific conditional KO mice, we used constitutive Upk2-driven Cre expression (Upk2<sup>Cre+</sup>; strain number 029281, Jackson Laboratory)(34). By mating Piezo1<sup>fl/fl</sup> mice (strain number 029213, Jackson Laboratory)(71) with Upk2<sup>Cre+</sup>;Piezo1<sup>fl/fl</sup> mice, we generated Piezo1-control (Piezo1<sup>fl/fl</sup>;Upk2<sup>Cre+</sup>) and Piezo1-KO mice (Piezo1<sup>fl/fl</sup>;Upk2<sup>Cre+</sup>). Similarly, by mating Piezo2<sup>fl/fl</sup> mice (strain number 027720, Jackson Laboratory)(30) with Upk2<sup>Cre+</sup>;Piezo2<sup>fl/fl</sup> mice we generated Piezo2-control (Piezo2<sup>fl/fl</sup>;Upk2<sup>Cre+</sup>) and Piezo2-KO mice (Piezo2<sup>fl/fl</sup>;Upk2<sup>Cre+</sup>). To produce urothelial-specific conditional double KO mice, Piezo1<sup>fl/fl</sup> mice were crossed with Piezo2<sup>fl/fl</sup> mice to create Piezo1<sup>fl/fl</sup>;Piezo2<sup>fl/fl</sup> mice. In turn, these were mated with Upk2<sup>Cre+</sup>;Piezo1<sup>fl/fl</sup>;Piezo2<sup>fl/fl</sup> mice to generate conditional urothelial Piezo1/2-control (Piezo1<sup>fl/fl</sup>;Piezo2<sup>fl/fl</sup>;Upk2<sup>Cre+</sup>) or Piezo1/2-KO mice (Piezo1<sup>fl/fl</sup>;Piezo2<sup>fl/fl</sup>;Upk2<sup>Cre+</sup>). In all experiments, non-Cre-expressing, sex-matched littermates were used as controls. Age-matched animals were used when littermates were not available. Genotyping was performed on tail snips collected from 21-d to 25-d old pups. Tail DNA was extracted using the QuickExtract DNA Extraction Solution (cat number QE09050; Lucigen, Middleton, WI) and analyzed using the primer sequences and PCR protocols described by Jackson Laboratory to confirm the animals’ genotype.
Detection and quantitation of Piezo channel expression using fluorescent in situ hybridization. To analyze gene expression of Piezo1, Piezo2, and Upk3a in the urothelium, we employed the RNAscope™ multiplex fluorescent kit (ACD; Newark, CA) and the following probes: Mm-Piezo1 in channel 1 (catalog number 500511), Mm-Piezo2 in channel 3 (catalog number 400191), and Mm-Upk3a in channel 2 (catalog number 505891). Details about controls, the staining protocols, and quantitation are provided in the Supplementary Methods.

Western Blot analysis of tdT-PIEZO1 expression in bladder fractions. Methods for Western blotting are described in the Supplementary Methods.

Immunofluorescence labeling and tissue imaging. Details of tissue recovery, fixation, freezing, sectioning, immunolabeling and data acquisition are provided in the Supplementary Methods.

RT-qPCR analysis. Preparation of samples, primers, and details of the methods are provided in the Supplementary Methods. Relative gene expression was assessed using the 2-ddCt method (72).

Peeled bladder and urothelial sheet preparations. A peeled bladder preparation, similar to that described by Durnin et al., was made (73). A description of the methods to prepare peeled bladders and urothelial sheets is provided in the Supplementary Methods.

[Ca^{2+}], measurements, including those in response to cell poking. An adenovirus encoding the Ca^{2+} sensor GCAMP5G was prepared and mouse bladders transduced using our established protocols (74, 75). As this technique involves transurethral catheterization, only female mice were used in these experiments. After 48 h, the mice were euthanized and a urothelial sheet...
preparation made. The Krebs buffer was replaced with recording solution (135 mM NaCl, 5.0 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4, gassed with 100% O₂), and the culture dish containing the sheet preparation was mounted in a DH-35iL culture dish incubator (Warner Instruments, Holliston, MA). The tissue was continuously perfused with recording solution using an in-line heater (model SH-27B; Warner Instruments). The temperature of the chamber and solutions was maintained at ~37°C with a dual channel bipolar temperature controller (model TC-344B; Warner Instruments). Images of GCAMP5G-transduced umbrella cells were acquired using a Hamamatsu ORCA fusion digital CMOS camera (Hamamatsu Corporation, Bridgewater Township, NJ), coupled to an upright BX51W Olympus microscope (Center Valley, PA), equipped with a Lambda XL light source (Sutter Instrument, Novato, CA). To measure poking-induced changes in [Ca²⁺], individual umbrella cells expressing GCAMP5G were mechanically stimulated using a glass micropipette. The micropipettes were pulled from Corning 7056 glass (Warner Instruments) and the tip was fire polished and sealed to a diameter of ~1-3 µm using a Narishige MF-830 Micro Forge (Narishige, Amityville, NY). The micropipettes were attached to a pipette holder, which was coupled to a Thorlabs piezoelectric actuator (model PAS005, Thorlabs Inc., Newton, NJ), which was itself mounted on a MP-285 micromanipulator (Sutter Instruments). To poke umbrella cells, the micropipette, positioned next to the cell’s apical surface, was programmed to move 20 µm, hold for a 1-sec duration, and then return to its starting position. Poking was triggered by a signal from an open-loop piezo controller (model MDT694B, Thorlabs), remotely operated through a Digidata 1440A controller (Molecular Devices). Images were acquired every 185 ms and analyzed using cell-Sens software (Olympus) running on a Precision 5820 tower computer (Dell, Round Rock, TX) outfitted with and Intel Xeon W-2145 3.7 GHz processor. The change in fluorescence intensity is denoted as ∆F/F, where F is the basal intensity of GCAMP5G at t=0 and ∆F is the difference between the evoked fluorescence intensity and the basal intensity. In some cases, 30 µM Yoda-1 (catalog number 5580/10; Tocris Biotechne,
Minneapolis, MN), prepared as a 30 mM stock in DMSO, was added to the perfusate bathing the bladder sheet preparation and images were acquired every 5 s.

**Measurement of serosal ATP release.** A peeled bladder preparation was made, residual fluid was removed from the bladder lumen using a catheter attached to a 5-ml syringe. Subsequently, a 1-ml syringe, filled with Krebs-HEPES buffer (Krebs buffer supplemented with 10 mM HEPES, pH 7.4, and oxygenated by bubbling pure O₂ gas through the solution), was attached to the catheter hub of the bladder preparation. The peeled bladder, attached to a micromanipulator, was submerged in 1ml of Krebs-HEPES buffer that was contained within a custom, 3D-printed epoxy cylindrical chamber (I.D.= 1.0 cm; height = 2.5 cm) with integral rectangular base (1.8 x 4.0 cm). The base was designed to fit in the slot of a PM-5 heated platform with magnetic clip (Warner Instruments). The PM-5 was heated by a TC344B dual automatic temperature controller (Warner Instruments). The chamber was perfused through an inlet with Krebs-HEPES buffer at a flow rate of 1.5 ml/min using a Harvard Apparatus PHD-Ultra syringe pump. The buffer entering the chamber was heated to 37˚ C using a model SH-27B (Warner Instruments) inline heater connected to the TC-344B unit. The cylindrical chamber also had a side port, which was connected to a Gilson Minipulse 3 pump (flow rate = 1.5 ml/min; Madison, WI), which released fluid from the chamber as the volume approached ~1 ml. The effluent from the pump was collected into 5-ml sterile screw-cap tubes (DNase-, RNAse, apyrase-free, and cytoxin free; catalog number C2540, MTC Bio, Woburn, MA). During experiments, the peeled bladders, mounted in the setup, were flushed with Krebs-HEPES buffer for 20 min, and then for 10 min with Krebs-HEPES buffer supplemented with 30 µM Ebselen. Three 1.5-ml aliquots were collected in the 5-ml tubes, and then the bladder was filled over a 10 s period-of-time with Krebs-HEPES-Ebselen buffer. The volume used to fill the bladders varied with the age of the animals, as bladder size varies: 120 µl in female or 150 µl in males that were 7-11-weeks old. However, this amount was increased to 200 µl in all animals > 12-weeks old. Subsequently, a sample was collected every
minute (~1.5 ml), for 12 minutes. Immediately after each sample was collected it was heated at 98˚ C for 2 min and then frozen in liquid nitrogen. The samples were recovered from the liquid nitrogen and stored at -20˚ C until ATP measurements were made. Details of the ATP assays can be found in the Supplementary Methods.

**Muscle strip analysis.** These analyses were performed as described previously (76), using the modifications described in the Supplementary Methods.

**Cystometry.** Mouse voiding function was assessed by cystometry using our previously described methods (77), with modifications described in the Supplementary Methods.

**Video-monitored void-spot assay.** To evaluate voiding behavior in awake, freely moving mice, we modified the standard void-spot assay to incorporate video monitoring, akin to the setup described by Keller et al. (78) (Figure 2). By incorporating real-time video monitoring into these assays, we could follow mouse activity over extended periods of time while overcoming the difficulties of distinguishing overlapping voiding spots (a result of mice urinating multiple times in the same location), an oft-cited shortfall of these assays (40). Moreover, we could readily detect small voiding events, confirming that they were not a result of non-specific carryover from the mouse’s fur or trailing associated with larger void spots. The system consisted of a custom-made frame with two compartments, constructed of T-slot aluminum framing material, connectors, and panels (80/20 Inc., Columbia City, IN). The upper compartment (dimensions of 37 x 25 x 20 cm) was divided in half to house two mice in a side-by-side configuration. The top, side walls, and bottom of the upper chamber were made of clear UV-transmitting acrylic plastic. The side wall between adjacent animals was covered with opaque paper, and the bottom of the upper compartment was covered in its entirety with blotting paper (see below) which was illuminated from below by two 24”-long UV tube lights (model T8-F20BLB24; ADJ Products LLC, Los Angeles,
CA) mounted to the floor of the lower compartment, but with light directed upwards. Reflecting differences in our ability to visualize spots, we used two different papers: Cosmo blotting paper (catalog number 10422-1005; Blick Art Materials, Galesburg, IL) was used to monitor voiding behavior during the dark phase, while chromatography paper (cat #057144, ThermoFisher) was used during the light phase. The lower compartment of the chamber, made of mirrored plexiglass panels (80/20 Incorporated), allowed the UV light to reflect light upwards. Mouse activity and voiding behavior were monitored using wide-angle “webcam” cameras (model C930e, Logitech, Newark, CA): one positioned above the cage and another mounted at the base of the lower compartment. The upper camera was most useful to monitor events in the dark phase, while the lower camera was most useful during the light phase. Each mouse compartment was furnished with the following: an igloo-shaped sleeping chamber, an Eppendorf tube as enrichment, and a dish with standard mouse chow and water in the form of Hydrogel (ClearH2O, Westbrook, ME). The mice were routinely housed in a facility with 12-hour light-dark cycles, with 7:00AM being zeitgeber time (ZT) =0 (start of light cycle). To analyze their voiding behavior in the dark phase, the mice were placed in the upper chamber between ZT=10-11, and analysis of void spots was performed from ZT=17 to ZT=23. The extended period of acclimatization reflected access limitations to the facility after 7:00 PM. For analysis during the light phase, the mice were placed in the chamber between ZT=3-4, allowed to acclimatize for one hour, and analysis performed during the subsequent 6-h time window (ZT=4-5 to ZT=10-11). Video was captured at 1 frame per second with a 1920 × 1080 pixel resolution using an Apple iMac computer running SecuritySpy software (BenSoftware.com). The movies were saved in .m4v format and viewed on an Apple iMac computer using Quicktime (Apple) software.

Voiding events were identified by visual inspection of the movies. Males had a tendency to “walk” while voiding, giving the impression of multiple spots/smear for a single voiding event. If confirmed to be a single voiding event by video, it was recorded as such. To demonstrate that voiding behavior was voluntary in nature, we confirmed that the mice moved to the area of interest,
urinated, and then left. When a voiding event was observed, the video was played frame-by-frame to capture the exact time of voiding and the frame in which the urine spot had diffused maximally. A screen shot of the entire video frame was made and the resulting TIF file opened in NIH ImageJ. The Freehand tool was used to select the boundary of the spot. The same tool was used to select the perimeter of paper. The ratio of spot area to paper area was recorded. Calibration curves, made by spotting mouse urine (2 µl – 750 µl) on the appropriate paper type, were similarly analyzed, and used to calculate the volume/void spot. Consistent with previous reports, spots were categorized as primary void spots (PVS; those ≥ 20 µl) or secondary void spots (SVS; < 20 µl) (43). The parameters measured in our analysis were number of PVS, average volume per void, total PVS volume, number of SVS, and total SVS volume. Urinary continence scores were calculated as described by:

\[ CS = \frac{\sum V_{PVS}}{\sum V_{PVS} + \sum V_{SVS}} \]

where \( \sum V_{PVS} \) is the sum of all PVS volumes and \( \sum V_{SVS} \) is the sum of all SVS volumes. The following animals were excluded from analysis: aggressive, dominant α-male mice, those with > 100 voids during the dark phase or > 50 voids during the light phase; those animals that chewed or damaged the paper prior to or during the time window of analysis.

**Statistical analysis.** Data are reported as mean ± SEM (n), where n equals the number of independent experiments or individual animal. In experiments where multiple, replicate values were measured (e.g., during cystometry), the values for an individual mouse were averaged prior to assessing statistics for the sample population. Parametric or nonparametric tests were employed based on the results of the Shapiro-Wilk and Kolmogorov-Smirnov normality tests. If data were normally distributed, then t-tests were performed, using Welch’s correction if the variance between groups was unequal. If data were not normally distributed, then a Mann-
Whitney test was employed. A $p$ value of $\leq 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 8/9 (GraphPad Software, San Diego, CA).

**Study approval.** All animal studies were performed in accordance with relevant guidelines/regulations of the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Animal Welfare Act, and under the approval of the University of Pittsburgh Institutional Animal Care and Use Committee.
AUTHOR CONTRIBUTIONS

M.D., G.A., and M.D.C conceived the idea of studying the role of PIEZO channels in urothelial mechanotransduction. W.G.R. established the peeled bladder preparation in our labs and also developed the bladder sheet preparation. M.D.C. designed the ATP assay and created the assay chamber to measure ATP release. M.D., N.M., and W.G.R. carried out experiments using the ATP assay chamber. M.D.C. developed and performed experiments measuring poking-induced changes in intracellular calcium. M.D.C. and N.M. conceived and oversaw the construction of the void-spot chambers. M.D. and W.G.R. designed and carried out experiments using the void-spot chambers. D.C., W.G.R., M.D., and G.A. conceived and executed experiments requiring image analysis. D.C. performed image capture, image processing, and quantitation. W.G.R. performed the EM analysis. J.B. and S.L.D. designed and helped execute along with M.D. the muscle strip analysis. G.A. and M.D. wrote the initial drafts of the manuscript, with subsequent contributions from all of the other participants.

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Figure 1. Expression and distribution of *Piezo1* and *Piezo2* in mouse bladder urothelium. (A) Distribution of *Piezo1* (signal dots in red, surrounded by yellow circles) and *Upk3a* (green) assessed using FISH. Urothelial boundary is outlined with white dashed lines (lumen indicated by red arrowheads), scale bars are 50 μm. (B) Western blot analysis of PIEZO1-tdT expression in urothelium (Ut) or detrusor (D) fractions taken from *Piezo1*<sup>−/−</sup> (lanes 1 and 3) or *Piezo1<sup>+/−</sup>tdT<sup>−/−</sup>* (lanes 2 and 4) mouse bladders. (C) Distribution of PIEZO1-tdT in bladder wall, scale bar is 100 μm. (D-E) Localization of PIEZO1-tdT with respect to the actin cytoskeleton or CLDN8. Apical surface of umbrella cells marked with white dashed lines and arrows point to the location of the junctional complex, closed white circles indicate umbrella cell nuclei, and the region of tissue in the yellow dashed box is magnified in the insets. Scale bars are 20 μm. (F) *Piezo2* (red) and *Upk3a* (green) expression in mouse bladder urothelium defined using FISH. Arrows mark the position of Piezo2-expressing umbrella cells. The boxed region, indicated by a dashed yellow line, is magnified in the insets. The larger panel is a photomerge of 11 images, collected using a wide-field microscope. The area bound by the rectangular box includes a stitching error when the samples were merged. Scale bar is 200 μm. (G) Expression of tdT in *Piezo2<sup>Cre-ires-GFP</sup>* mice mated with Ai9 reporter mice. In the confocal images at the left and at the center, a single tdT-positive umbrella cell is located at the tip of a bladder rougae (also note tdT+ fibroblasts in LP). The region of the yellow dashed line is magnified in the images below. Confocal images to the right show tdT-positive umbrella cells, viewed en face in whole-mounted bladder tissue. Examples of the regional expression of tdT-positive umbrella cells (and the tight-junction protein TJP1) from the dome, equator, and neck region of the bladder are shown. All confocal images are 3D reconstructions of 32-48 optical sections. Legend: DIC, differential interference contrast; LP, lamina propria; L, lumen; Ut, urothelium. Scale bars are 100 μm.
Figure 2. Evidence of PIEZO channel-dependent mechanotransduction in bladder urothelium. (A-C) Yoda1-stimulated PIEZO1 activation in GCAMP5G-transduced urothelium. (A) Diagram of experimental approach. (B) Example of Yoda1-induced [Ca\(^{2+}\)] increases in Piezo1/2-control or Piezo1/2-KO urothelium. Scale bar is 100 μm. (C) Yoda1-induced changes in [Ca\(^{2+}\)], normalized to control responses. Data are mean ± SEM (n = 3). Data were analyzed using t-tests, and significant differences indicated with an asterisk (p ≤ 0.05). (D-F) Piezo channel-dependence of poking-induced changes in [Ca\(^{2+}\)]. (D) Diagram depicting experimental approach. (E) Example of poking-induced increase in [Ca\(^{2+}\)] in urothelium transduced with adenovirus encoding GCAMP5G. In the three images to the right, the indicated cell (yellow arrow) was poked at 10.0 s and the changes in [Ca\(^{2+}\)] recorded over the next several seconds. Scale bar is 50 μm. (F) Poking-induced [Ca\(^{2+}\)] changes in randomly selected umbrella cells. Data, normalized to matched controls, are mean ± SEM (n=3 animals for each group; the value of each animal is the average from 11-14 cells). Data were analyzed using t-tests, and significant differences indicated with an asterisk (p ≤ 0.05). (G-H) Dependence of serosal ATP release on Piezo expression. (G) Schematic of the experimental setup. (H) Upper panels: ATP release from peeled bladders of the indicated strain of mouse. Both males and females were used in this analysis. The peeled bladders were filled after fraction 3. Bottom panels: The total filling-induced ATP release from the serosal surfaces of peeled bladders was calculated. Data are mean ± SEM (Piezo1-control, n=5; Piezo1-KO, n=6; Piezo2-control and Piezo2-KO, n=3; Piezo1/2-control and Piezo1/2-KO, n=5). Data were analyzed using t-tests, and significant differences indicated with a double asterisk (p ≤ 0.01).
Figure 3. Bladder function in Piezo1-control and Piezo1-KO mice. (A) Example cystometrogram. Key: A, amplitude (PP-TP); IVI, intervoid interval (time between voiding events); PP, peak pressure associated with voiding event; RP, resting pressure; TP, threshold pressure; ΔV/ΔP (compliance), change in pressure in response to an incremental change in volume. (B) Comparison of cystometric parameters for male and female, urethane-anesthetized Piezo1-control and Piezo1-KO mice. Voiding events are marked with red arrowheads (line below arrowhead indicates a single voiding event with multiple pressure spikes). Data, analyzed using Mann-Whitney tests, are mean ± SEM (Piezo1-control, males and females, n=6; Piezo1-KO female, n=6; Piezo1-KO male, n=5). (C) Contraction of muscle strips in response to carbachol and electric field stimulation. Data are mean ± SEM, (n=3).
Figure 4. Voiding behavior in freely mobile Piezo1-control and Piezo1-KO mice. (A) Left panel: Diagram of void-spot chamber. Right panel: Representative video still of a mouse in a void-spot chamber, illuminated from below with UV light, and recorded using the top camera. Primary void spots (PVS) are outlined with dashed yellow lines. A small void spot (SVS), on top of a previous PVS, is indicated with a red arrowhead. (B-C) Void spot parameters in male (B) and female (C) mice analyzed over a six-hour time window during the dark phase or light phase. Data are mean ± SEM (Piezo1-control and Piezo1-KO females, n=8; Piezo1-control and Piezo1-KO males, n=6). Data were analyzed using a Mann-Whitney test, and significant differences indicated with a single asterisk (p ≤ 0.05).
Figure 5. Voiding behavior in freely mobile Piezo2-control and Piezo2-KO mice. (A-B) Void-spot parameters in female (A) and male (B) mice were analyzed over a six-hour time window during the dark phase or light phase. Data are mean ± SEM (dark-phase females, Piezo2-control and Piezo2-KO, n=6; light-phase females, Piezo2-control and Piezo2-KO, n=9; dark-phase males, Piezo2-control and Piezo2-KO, n=8; light-phase males, Piezo2-control, n=6, Piezo2-KO, n=7). Data were analyzed using a Mann-Whitney test, and significant differences indicated with a single asterisk (p ≤ 0.05) or a double asterisk (p ≤ 0.01).
Figure 6. Voiding function of anesthetized Piezo1/2-control and Piezo1/2-KO mice as assessed by cystometry. (A-B) Urethane-anesthetized female (A) or male (B) mice were subjected to cystometry. Representative cystometrograms are shown to the right of the figure. Data are mean ± SEM (female Piezo1/2-control and Piezo1/2-KO, n=6; male Piezo1/2-control, n=5; male Piezo1/2-KO, n=6). Data were analyzed using Mann-Whitney tests, and significant differences indicated with a single asterisk (p ≤ 0.05) or a double one (p ≤ 0.01).
Figure 7. Voiding behavior in freely mobile Piezo1/2-control or Piezo1/2-KO mice. (A-B) Void-spot parameters in female (A) and male (B) mice were analyzed in a six-hour time window during the dark phase or light phase. Data are mean ± SEM (dark-phase females, Piezo1/2-control and Piezo1/2-KO, n=7; light-phase females, Piezo1/2-control and Piezo1/2-KO, n=11; dark-phase males, Piezo1/2-control and Piezo1/2-KO, n=6; light-phase males, Piezo1/2-control and Piezo1/2-KO, n=6). Data were analyzed using Mann-Whitney tests, and significant differences indicated with a single asterisk (p ≤ 0.05) or with a double asterisk (p ≤ 0.01).