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Impaired contractility and detrusor hypertrophy in cavin-1-deficient mice

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Abstract

Caveolae are membrane invaginations present in a variety of cell types. Formation of caveolae depends on caveolins and on the more recently discovered family of proteins known as the cavins. Genetic ablation of caveolin-1 was previously shown to give rise to a number of urogenital alterations, but the effects of cavin-1 deletion on urogenital function remain unknown. Here we characterized detrusor contractility and structure in cavin-1-deficient mice. Electron microscopy demonstrated essentially complete lack of caveolae in the knock-out detrusor, and immunoblotting disclosed reduced levels of cavin-3 and of all caveolin proteins. Bladder weight was increased in male knock-out mice, and length-tension relationships demonstrated a reduction in depolarisation-induced contraction. Contractility in response to muscarinic receptor activation was similarly reduced. Despite these functional changes, micturition patterns were similar in conscious and freely moving animals and diuresis was unchanged. Our breeding additionally disclosed that the number of knock-out mice generated in heterozygous crosses was lower than expected, suggesting embryonic/perinatal lethality. In conclusion, this is the first study to show that cavin-1 is critical for detrusor caveolae and for the overall contractility and structure of the urinary bladder.

Keywords: Polymerase transcript release factor (PTRF), Serum deprivation protein response (SDPR), sdr-related gene product that binds to c kinase (SRBC), pacsin2, caveolae, carbachol, purinergic.

1. Introduction

Caveolae are highly ordered 50-100 nm flask-shaped membrane invaginations present in a variety of cell types including smooth and skeletal muscle, endothelial cells, and adipocytes (Hnasko and Lisanti, 2003). The functional roles of caveolae are diverse and tissue-specific and include cellular processes such as lipid and glucose homeostasis and endocytosis (Capozza et al., 2005; Razani et al., 2002). Caveolae are moreover considered to play an important but complex role in cellular signalling through their ability to sequester signalling molecules (Bastiani and Parton, 2010).

Biogenesis of caveolae depends on the caveolin proteins. Three caveolin proteins have been recognized: caveolin-1, caveolin-2 and caveolin-3. The latter is primarily expressed in striated muscle (Tang et al., 1996), whereas caveolin-1 and -2 dominate elsewhere. Moreover, two different groups have proposed that pacsin2 sculpts the membrane of caveolae (Hansen et al., 2011; Senju et al., 2011). Recent studies have identified a novel family of proteins named the cavin proteins. These proteins appear to be crucial for formation, stability and dynamics of caveolae. Four cavin proteins exist and they are named cavin-1 (a.k.a. polymerase I and transcript release factor, PTRF) (Hill et al., 2008; Vinten et al., 2005), cavin-2 (serum deprivation protein response, SDPR), cavin-3 (sdr-related gene product that binds to c kinase, SRBC) and cavin-4 (muscle restricted coiled-coiled protein, MURC). The cavin proteins form tissue and cell-specific complexes in the cytosol. These complexes then associate with caveolins at the cytoplasmic leaflet of the plasma membrane (Bastiani et al., 2009; Briand et al., 2011; McMahon et al., 2009). It has been demonstrated that cavin-1 is critical for formation of caveolae in cultured cells (Liu et al., 2008), and a cavin-1 knock-out mouse model has confirmed this to be the case in some tissues also *in vivo*. With the exception for a

detailed metabolic characterization (Liu et al., 2008), the phenotypic properties of cavin-1 knock-out mice remain unexplored.

Caveolae are abundant in the urogenital tract. In detrusor myocytes they are arranged in parallel longitudinal membrane stripes and they reach a density of close to 2 caveolae per μm membrane (Murakumo et al., 1993; Sadegh et al., 2011). All caveolin isoforms are represented here, and it has been speculated that the different caveolins may play unique roles. Work from several different laboratories including ours has demonstrated urogenital changes in caveolin-1 knock-out mice. These include impaired detrusor contractility and cholinergic neuroeffector transmission, bladder and prostate hypertrophy, enlargement of the seminal vesicles and disturbed erectile mechanisms (Lai et al., 2007; Lai et al., 2004; Sadegh et al., 2011; Shakirova et al., 2009; Woodman et al., 2004). Here we have used cavin-1 knock-out mice to explore the consequences of cavin-1 loss for detrusor structure, function and protein expression. Our findings confirm the critical role of cavin-1 for caveola formation and for the stability of caveolin and cavin proteins, and additionally unveil bladder hypertrophy and impairment of contractility in the absence of this protein.

2. Material and methods

2.1. Animals and breeding

Heterozygous (cavin-1^{+/-}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred to obtain cavin-1^{-/-} animals. Genotyping was done using the PCR protocol supplied by Jackson. 4-5 month-old cavin-1^{-/-} (knock-out, KO) and cavin-1^{+/+} (wild type, WT) littermates of both sexes were used. Caveolin-1 knock-out mice were from our breeding colony. Experiments were approved by the local animal ethics committee.

2.2. Exploiting β -galactosidase to follow cavin-1-expression *in situ*

The gene targeting vector used to create the cavin-1-KO mice (Liu et al., 2008) contained prokaryotic β -galactosidase (β -gal). The artificial substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) is cleaved by β -gal. Oxidation of the cleavage product yields an intensely blue and insoluble pigment. This was exploited to examine cavin-1 expression in tissues of adult heterozygous mice. Briefly, micro-dissected organs from the urogenital tract of both male and female heterozygous mice were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.0) for 10 min at 4 °C. Organs were then incubated with continuous shaking using β -gal staining solution (150 mM NaCl, 2 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 40 mM citric acid, 12 mM sodium phosphate, pH 6.0, containing 1 mg/ml X-gal (Sigma, St. Louis, MO) at 37 °C for either 1h or overnight. Photographs were acquired with an Infinity 1 camera mounted on an Olympus (SZ61) microscope.

2.3. Electron microscopy

Anaesthesia was induced with 4% isoflurane (Abbot Scandinavia, Solna, Sweden) using an anaesthesia unit (Univentor, Zejtan, Malta). Tracheotomy was performed and anaesthesia was maintained with 2% isoflurane (mouse ventilator 28025, Ugo Basile, Comerio, Italy). The animals were perfusion-fixed with 2.5 % glutaraldehyde in 150 mM cacodylate buffer (pH 7.4) through a carotid cannula. The bladders were dissected and transferred to new fixative, and then to cacodylate buffer and further prepared as described (Sadegh et al., 2011). Semithin sections were stained with toluidine blue, and used for measurements of the relative amount of detrusor muscle. Thin sections were cut from areas with cross-sectioned muscle bundles and examined in an electron microscope. Digital photos were analysed using ImageJ (NIH, Bethesda, MD, USA).

2.4. Western blotting

Preparations were frozen in liquid nitrogen and pulverized in a mortar. In some experiments detrusor strips from WT and cavin-1 KO mice were treated with or without 1 μ M carbachol for 5 min and snap frozen in liquid nitrogen prior to protein extraction. Protein preparations were generated as described (Sadegh et al., 2011). 20 μ g of protein was loaded on TGX Criterion gels (BioRad). Protein was transferred to nitrocellulose membranes using the Trans-Blot Turbo system (BioRad). Membranes were incubated with primary antibodies: cavin-1 (ab78553, Abcam, 1:500), cavin-2 (AF5759, R&D systems, 1:500), cavin-3 (16250-1-AP, Protein Tech Group, 1:500), caveolin-1 (D46G3, Cell signalling, 1:2000), caveolin-2 (C57820-050, BD Transduction laboratories, 1:500), caveolin-3 (610421, BD Transduction laboratories, 1:10000), pacsin2 (GTX104204, Gene Tex, 1:500), Glyceraldehyde-phosphate-dehydrogenase (GAPDH, MAB374, Millipore, 1:1000), Desmin (#4024, Cell Signaling, 1:1000), Calponin (ab46794, Abcam, 1:1000) and SM22 (ab14106, Abcam, 1:2000), HSP90 (#610418,

BD Transduction labs., 1:1000), Myosin heavy chain, MHC (BT-562, Biomed technologies, 1:1000), LIM Kinase, LIMK (#3842, #3841S cellsignaling, 1:500), Cofilin (#07-300, #07-326, Millipore, 1:500) . Protein bands were visualized using horseradish peroxidase-conjugated secondary antibodies (#7074, 7076, Cell signalling, 1:5000) and West Femto chemiluminescence reagent (Pierce, Rockford, IL). Images were acquired using a LI-COR Odyssey Fc instrument (LI-COR Biosciences).

2.5. *In vitro* contractility

Ring preparations were prepared under a dissection microscope by removing the uppermost part of the dome as well as the trigone. The urothelium was then removed. Three preparations were demarcated by 6-0 silk sutures. The length of the equatorial strip and the individual preparations was measured, preparations were cut, and loops were tied using the attached silk sutures. Length-tension relationships were obtained as described (Sadegh et al., 2011). Concentration-response curves were generated at the optimal circumference for force development (L_0) by cumulative addition of carbachol. After termination of the experiments, preparations were measured and weighed after gentle blotting on filter paper. With exception for electrical field stimulations, where peak responses were analysed, all contractions were averaged over the 7 min stimulation period. We then multiplied average force (mN) with length (mm) and density (assumed to be 1.06 mg/mm^3), and divided the product by weight (mg) to obtain stress in mN/mm^2 .

2.6. Electrical field stimulation

Bladder smooth muscle preparations were prepared and stimulated as described (Sadegh et al., 2011). After removing the urothelium two detrusor strips from the midportion of the bladder were prepared from each animal. Electrical field stimulation was performed in the

absence and presence of scopolamine (1 μ M) followed by α , β -methylene-ATP (10 μ M) as described (Sadegh et al., 2011). Each experiment was started and ended by activating the smooth muscle with K⁺-high solution (KCl: 124 mM, NaHCO₃: 15 mM, NaH₂PO₄: 1.2 mM, MgCl₂: 1.2 mM, CaCl₂: 1.5 mM and glucose: 5.5 mM).

2.7. *In vivo* micturition

Micturition was assessed according to (Gevaert et al., 2007) with some simplification. Mice were housed individually with filter papers placed in the bottom of their cages. After 15h the filter paper was collected and photographed under UV illumination. The total number of urine spots on each filter paper was then counted. Water consumption was measured by weighing water bottles every day (with 24 h interval) as an estimate of diuresis.

2.8. Statistics

Values are presented as means \pm S.E.M. n-values refer to the number of mice. Student's unpaired t-test (one or two-tailed as appropriate) or ANOVA was used to test for significance. Deviation from Mendelian distribution was tested using Chi-square statistics. P<0.05 was considered significant. Significance is indicated by *P<0.05, **P<0.01, and ***P<0.001.

3. Results

3.1. Increased bladder weight in knock-out mice

When offspring from heterozygous crosses was genotyped at 4 weeks 9% (19/217) were knock-outs (-/- or KO), to be compared with 26% (51/217) wild-types (+/+ or WT). This distribution differs from that expected for Mendelian inheritance (P<0.001). At ~18 weeks,

body weights were similar (25 ± 1 g for wild-type vs. 23 ± 1 g for knock-out, $n=18$). Bladder weights were increased in knock-outs (32 ± 1 vs. 38 ± 2 mg, $P < 0.05$, $n=18$) as was the bladder to body weight ratio (1.3 ± 0.1 vs. 1.6 ± 0.1 mg/g, $P < 0.01$, $n=17$). Subgroup analysis based on sex showed a significant difference of bladder weight in males ($P < 0.001$ for the six males, $P = 0.2$ for the 11 females). However, the bladder weight of male KO and female KO bladders was not significantly different. On toluidine blue stained sections the proportion of muscle relative to total wall thickness was unchanged (76 ± 2 vs. $75 \pm 2\%$), suggesting a proportional increase in all bladder wall layers.

3.2. Cavin-1 reporter expression in urogenital organs

The bladder, the distal ureters and the proximal urethra were intensely stained after overnight incubation with x-gal substrate (Fig. 1a). Shorter incubations provided better contrast and revealed staining of smooth muscle cells, oriented as swirls, in the wall of the detrusor (Fig. 1b). Detrusor arteries were also intensely stained, but no staining of bladder nerves or intramural ganglia, which are present in the bladder wall of some species but not in mice (Gabella, 1990), were seen. Except for feeding arterioles and some haphazardly oriented smooth muscle cells, the seminal vesicles were negative (1c, detail in e). The prostate was however strongly positive and a conspicuous regional heterogeneity was observed with exceptionally high reporter expression in the ventral and lateral lobes (1d) and lower expression in the anterior lobes (1c, e). The finger-like ductal projections of the anterior prostate were more intensely stained at their tips than at their bases (1e). A faint blue outline of the seminiferous tubules was seen in the testicles (1f), and the head of the epididymis was strongly positive (1g), contrasting with its body which was almost negative; staining then increased again towards the tail. The uterine horns in female mice were weakly

stained, whereas the arterial supply was strongly positive (1h). Finally, the oviduct (1i) was more strongly stained than the uterus.

3.3. Lack of caveolae in detrusor smooth muscle and reduced contents of caveolin and cavin proteins

We focused henceforth on the urinary bladder. Electron microscopy (Fig. 2a-d) did not disclose any conspicuous changes in the KO detrusor smooth muscle layer except for the essentially complete lack of caveolae (wild-type: 1.8 ± 0.1 caveolae per μm membrane; knock-out: 0.1 ± 0.04 caveolae per μm membrane, $P < 0.001$, $n=3$). The overall domain organisation of the membrane was similar (proportion occupied by dense bands was 59 ± 5 vs. $62 \pm 3\%$, $n=3$), and the extracellular matrix appeared unchanged; collagen fibre diameters for example were identical (44 ± 3 vs. 45 ± 1 nm). In view of the possible role of caveolae in neuro-muscular transmission, neural varicosities were studied. Varicosities were dispersed at similar distances in the muscle layer in wild-type and knock-out (7.5 ± 0.7 vs. 8.2 ± 0.3 μm) and their density of neurotransmitter vesicles was not different (69 ± 4 vs. 88 ± 12 vesicles per μm^2).

Caveolin and cavin protein levels were next examined using immunoblotting. Caveolin-1-deficient bladders were included for comparison. Caveolins-1 and -2 and cavin-3 were markedly reduced in cavin-1-deficient detrusor (Fig. 2e). A slight reduction of caveolin-3 and Pacsin2 was also seen. Expression of caveolin-2 and cavin-3 appeared to be somewhat better maintained in caveolin-1 knock-out detrusors than in cavin-1 knock-out detrusor. The relative expression levels for caveolins, cavins and pacsin2 are summarized in Table I.

3.4. Impaired neurogenic detrusor activation and decreased response to carbachol

Length-tension relationships were generated in myographs to assess the optimum circumference for force development of bladder strips (L_0 , Fig. 3a-d). L_0 was unchanged (Fig. 3c), but active stress (force per cross-sectional area) in response to 60 mM K^+ (high K^+ solution) at L_0 was reduced (Fig. 3a and d). Passive force was unchanged at circumferences ranging between 14 and 26 mm (Fig. 3b). Separate analyses of male and female data indicated similar and significant differences in active force in both sexes (not shown). Taken together, this suggests preserved passive elastic properties, but reduced active force in response to depolarisation.

We next performed electrical field stimulation to assess detrusor activation by intrinsic nerves. The cholinergic component of activation was reduced at intermediate stimulation frequencies (Fig. 4a shows data from female mice). The purinergic component of activation was reduced at higher stimulation frequencies (Fig. 4b). Pooled data from males and females showed largely similar and significant differences (not shown), but no statistically significant differences were seen when males were considered alone ($n=3$). Taken together with the unchanged density of detrusor nerve terminals and the reduced high K^+ responses, this suggests impaired contractility due either to a deficiency in signalling or a change in the contractile machinery of the smooth muscle cells.

Concentration response relationships for the muscarinic receptor agonist carbachol were generated at L_0 . Carbachol-induced force was reduced in cavin-1 knock-out mice at concentrations ranging between 0.1 μ M to 10 μ M (Fig. 5a). Stress was similarly reduced (Fig. 5b, $n=7$). When the carbachol response was normalised to high K^+ contraction, a modest but significant reduction was seen (Fig. 5c). Subgroup analyses based on sex, again showed

significant reductions in force and stress, respectively, for both male and female KO mice (for example at 1 μ M carbachol: $P < 0.05$, $n = 4$, males and $P < 0.05$, $n = 3$, females).

3.5. Smooth muscle differentiation and cellular signalling in cavin-1-deficient detrusor

Caveolae have been proposed to influence smooth muscle cell phenotype expression. We therefore assessed the contents of several contractile proteins, including myosin heavy chain, desmin, calponin and SM22 α which are markers of the contractile phenotype of smooth muscle cells. However, no difference in the content of any of these proteins was observed (Fig. 6a).

Contraction of smooth muscle depends in part on influx of Ca^{2+} and activation of myosin light chain kinase, and in part on RhoA/ROCK -dependent Ca^{2+} sensitization pathways. A read-out of RhoA/ROCK activation is phosphorylation of LIM kinase and of cofilin. We therefore measured phosphorylation of these proteins in control and carbachol-stimulated (1 μ M carbachol for 5 min) detrusor muscle from wild-type and knock-out mice (Fig. 6b). No apparent differences were noted.

3.6. No change in micturition pattern in freely moving cavin-1 knock-out mice

Impaired activation of detrusor muscle by intrinsic nerves may affect micturition. This was assessed *in vivo* by placing filter papers in cages overnight and measuring the number and distribution of urine spots under UV light (Fig. 6c). Caveolin-1 knock-out mice were included for comparison. Except for a difference between cavin-1 knock-out and caveolin-1 knock-out mice in the total number of micturitions, no differences between genotypes were observed (Fig. 6d, $n = 7$). Water consumption was also measured and related to body weight as an

estimation of diuresis. No difference was observed (wild-type: $0.149 \pm 0.023 \text{ g} \cdot \text{g}^{-1} / 24 \text{ h}$; cavin-1- knock-out: $0.146 \text{ g} \pm 0.012 \text{ g} \cdot \text{g}^{-1} / 24 \text{ h}$, n=5).

4. Discussion

The current study, which represents the first characterization of bladder function in cavin-1 knock-out mice, shows that ablation of cavin-1 is associated with alterations in detrusor contractility and structure. Cavin-1 was found to be expressed throughout the urogenital tract of both male and female mice, and lack of this protein led to the absence of caveolae in detrusor smooth muscle, confirming the importance of cavin-1 for stability and biogenesis of caveolae. Genetic ablation of cavin-1 also led to a reduced content of cavin-3 and of all caveolins. Except for the maintained level of cavin-2 in the bladder, a finding that needs to be confirmed using additional antibodies, this is largely in keeping with recent work with other cavin-1-deficient tissues (Bastiani et al., 2009; Liu and Pilch, 2008). Pacsin2 was recently shown to be associated with caveolae and to play a role in their formation (Hansen et al., 2011). We find here that this protein is modestly reduced in cavin-1-deficient but not in caveolin-1-deficient detrusors. The effect was not as remarkable as that seen for cavin-3 and caveolin-2, but in the same range as seen for caveolin-3 (Table 1).

Bladder weights were increased in the absence of cavin-1. This is consistent with the observation that 1-year-old male caveolin-1-deficient mice have bladder hypertrophy (Woodman et al., 2004). However, caveolin-1-deficient male mice of the same age do not display bladder hypertrophy, suggesting a more rapidly progressing phenotype in the cavin-1-deficient mouse strain. A difference between males and females with regard to structural changes in the bladder has previously been reported for mice that lack the muscarinic M_3

receptor. In those mice, the response to carbachol was eliminated in both males and females ($3 \times 10^{-8} \rightarrow 3 \times 10^{-3}$ M); yet only male mice had enlarged bladders and mild hydronephrosis (Matsui et al., 2000). Such differences may relate to the higher bladder outlet resistance in males. In caveolin-1-deficient mice it is possible that outflow obstruction caused by a hypertrophic prostate (Woodman et al., 2004) leads to urine retention and bladder growth with age. Here we report a remarkable cavin-1 reporter expression in the prostate, and studies are therefore warranted to determine if and why the prostate undergoes changes in cavin-1-deficient mice. In this respect it is interesting to note that caveolin-1 has long been considered a candidate gene for hormone-resistant prostate cancer in man (Gumulec et al., 2012). Intriguingly, a polymorphism near Pcsin2 was recently identified as a prostate cancer susceptibility locus (Eeles et al., 2009).

Even if the changes in the bladder largely recapitulate those in caveolin-1-deficient mice (Lai et al., 2007; Lai et al., 2004; Sadegh et al., 2011; Woodman et al., 2004), these strains do not have identical phenotypes. A speaking example of this is the lower than expected numbers of cavin-1 knock-out animals generated in heterozygous crosses. To the best of our knowledge no similar observations have been made in any of the caveolin knock-outs or in double knock-outs for caveolin-1 and -3. We sporadically found dead and rejected mice shortly after birth, but timed breeding needs to be undertaken to determine when the mice succumb and for what reason. The other published studies using this strain did not report increased embryonic/perinatal mortality (Bastiani et al., 2009; Liu et al., 2008). If the increased mortality depends on background or breeding environment is not known.

Detrusor contractility in cavin-1 knock-out mice was impaired in both males and females.

This again resembles the scenario in caveolin-1-deficient mice, where clear-cut changes in

detrusor contractility are seen in both male and female mice (Sadegh et al., 2011). We cannot rule out that the changes observed here in cavin-1 knock-out mice are due to reduced caveolin-1 expression (reduced by ~80%). However, subtle differences between our present findings and those previously reported for caveolin-1-deficient mice are worth noting. These include a reduced purinergic component of activation during electrical field stimulation, and a selective impairment carbachol-induced contractility relative to high-K⁺ contraction in the cavin-1 knock-out bladder. In contrast, both KO strains have a reduction of the cholinergic component of neurogenic bladder activation (shown here for female mice) and a reduced post-junctional response to the muscarinic receptor agonist carbachol (this study, Lai et al., 2004; Sadegh et al., 2011; Woodman et al., 2004). The reduced neurogenic activation, at least in the caveolin-1-KO mouse, may involve a reduced release of acetylcholine (Lai 2004). As shown here in cavin-1-KO bladder, the density of neural varicosities and their content of vesicles were similar, but this does not rule out a reduced neurotransmitter content or reduced vesicle exocytosis. Species differences exist for the relative contribution of cholinergic and purinergic mechanisms in the bladder. Because the cholinergic component of neurogenic detrusor activation is smaller, and the purinergic component is larger, in mouse than in human bladder (Wust et al., 2002), it is possible that muscarinic activation may be more severely disturbed in humans that lack caveolae than in the corresponding mouse strains. Indeed, pharmacological disruption of caveolae in human detrusor has profound effects on muscarinic activation (Ekman et al., 2012).

The biochemical basis of the reduced detrusor contractility in the absence of caveolae remains obscure. We did not find evidence for a reduced level of smooth muscle differentiation, as indicated by maintained expression of myosin heavy chain, desmin, calponin or SM22 α , and by the preserved ultrastructure. We did also not find evidence for

altered phosphorylation of either LIM-kinase or cofilin, both of which represent read-outs of ROCK activation, but detailed time-course studies should be undertaken.

Micturition *in vivo* is expected to be affected by a multitude of compensatory mechanisms; increased efferent signalling, altered sensory mechanisms, detrusor hypertrophy and a prolongation of micturition may all contribute. According to La Place's law, pressure generation by the cavin-1-deficient detrusor would be normalized by a reduction of radius by as little 15%. Given that diuresis (drinking) was similar, this change should be reflected in an increase in micturition frequency. Such a trend was seen but this was only significant when comparison was made with caveolin-1-deficient mice which may have reduced diuresis (Sadegh et al., 2011). A difference between our *in vitro* and our *in vivo* findings is that the former experiments were done using preparations without mucosa. We chose this approach to simplify the interpretation of any changes, and we have previously shown that staining for caveolin-1 is very low in the urothelial layer (Sadegh et al., 2011). Compensatory changes in urothelial signalling mechanisms can however not be ruled out as a mechanism for the largely normal micturition pattern in mice that lack cavin-1.

In conclusion, we show that cavin-1 is critical for of caveolae in the urinary bladder and demonstrate that lack of cavin-1 leads to changes in detrusor contractility and increased bladder mass. Our findings also suggest embryonic or perinatal lethality in cavin-1-deficient mice.

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Figure legends

Figure 1. Cavin-1 reporter staining in urogenital organs from male and female mice.

Overnight (**a**) and 1 h (**b-i**) incubation with β -galactosidase substrate results in blue staining of several urogenital organs from heterozygous (*cavin-1*^{+/-}) mice. The bladder and urethra are shown in panels **a** and **b**. Panel **c** shows the seminal vesicles and the prostate. Intense blue staining was seen in ventral and lateral lobes of the prostate (**d**). Panel **e** shows a detail of the anterior prostate and one seminal vesicle. Panel **f** shows a testicle with a prominent central artery. Panel **g** shows the epididymis, **h** a uterine horn with its vascular supply, and **i** the oviduct.

Figure 2. Cavin-1-deficient detrusor lacks caveolae and have reduced caveolin protein

expression. Electron micrographs (**a-d**) of detrusor smooth muscle cells from wild-type (WT; **a, c**) and cavin-1 knock-out (**b, d**) mice. Representative caveolae are indicated with black arrowheads (**a, c**). Dense bands are highlighted with white arrowheads (**c, d**). A nerve terminal is seen in **b** (denoted by the letter n). Panel **e** shows western blots for proteins involved in biogenesis of caveolae. Caveolin-1-deficient detrusor was included for comparison.

Figure 3. Impaired depolarization-induced contractility but no remodelling in the cavin-1

KO urinary bladder. Circumference-tension relationships were generated to investigate the optimum circumference for force development, L_0 . Panel **a** shows active force in response to 60 mM K^+ as function of bladder circumference. Panel **b** shows passive force in the absence of Ca^{2+} as a function of circumference. Panel **c** shows the circumference at L_0 , and panel **d** shows depolarisation-induced stress (force per cross-sectional area) at L_0 . Pooled data from male and female mice throughout, $n=7$.

Figure 4. Reduced cholinergic and purinergic components of neurogenic activation in female cavin-1 KO mice. Bladders strips without mucosa were stimulated at increasing frequencies (1-50 Hz) in absence and presence of scopolamine (muscarinic M₃ receptor antagonist) and after desensitization of purinergic receptors using α , β -methylene-ATP. The cholinergic component (**a**) was obtained by subtracting force in the presence of scopolamine from total force in control conditions. The purinergic component (**b**) was obtained by subtracting the scopolamine and α , β -methylene-ATP-resistant component from force in the presence of scopolamine. Contractility was expressed as stress (mN/mm², see Methods, n=4 for both panels).

Figure 5. Impaired muscarinic activation of the cavin-1 deficient detrusor. Panel **a** shows original records of force in response to 60 mM K⁺ (HK) followed by cumulative addition of carbachol. Summarized concentration-response data is presented as stress in panel **b**. Carbachol-induced contraction was averaged over the stimulation period (7 min, 1 μ M carbachol) and expressed in relation to the average high K⁺ response (7 min) in the same strip in panel **c** (n=7 throughout).

Figure 6. No effect on the expression of contractile differentiation markers in the isolated detrusor or *in vivo* micturition. Assessment of smooth muscle differentiation markers and phosphorylation of proteins involved in contractile activation were explored with immunoblotting. **a.** No change in expression of myosin heavy chain (MHC), desmin, calponin or SM22 α , relative to either of the housekeeping proteins glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or heats shock protein 90 (HSP90), were noted (n=2-3). **b.** Phosphorylated (P) or total (T) protein expression of LIM kinase (LIMK) and cofilin (Cof) following stimulation with carbachol (Cch, 1 μ M) for 5 min (n=2-3). Micturition was assessed

in wild type (WT), cavin-1 knock-out (KO) mice and caveolin-1 KO mice by placing filter papers in cages for 15 h and visualizing urine spots under UV light **(c)**. **d.** Summary of the number of micturitions (n=7 in each group).

Table I. Relative expression levels for caveolin and cavin proteins in cavin-1 and caveolin-1 knock-out (-/-) mice.

	cavin-1^{-/-} (%)	caveolin-1^{-/-} (%)
cavin-1	0 ^a	44±2 ^{a, b}
cavin-2	70±11	59±14 ^a
cavin-3	2±1 ^a	7±0.5 ^{a, b}
caveolin-1	21±3 ^a	0 ^{a, b}
caveolin-2	9±4 ^a	14±3 ^{a, b}
caveolin-3	52±6 ^a	66±3 ^a
pacsin2	59±8 ^a	78±31

Expression was normalized to GAPDH and shown as a percentage of the level in wild type bladders. Values are means±SEM, a: P<0.05 vs. wild type and b: P<0.05 vs. cavin-1 knock-out.

Figure 1

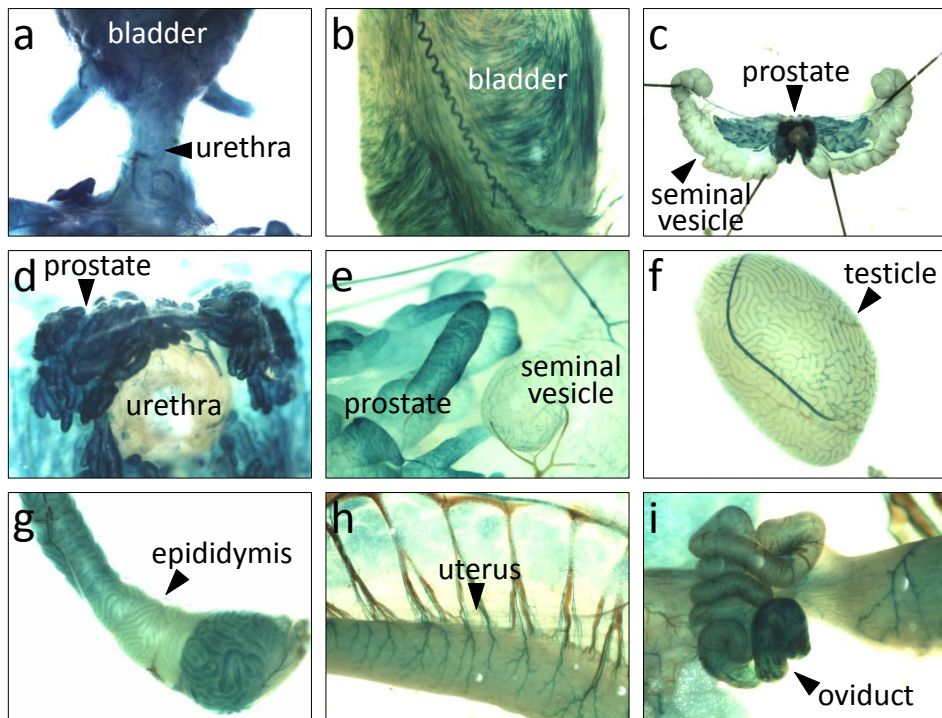


Figure 2

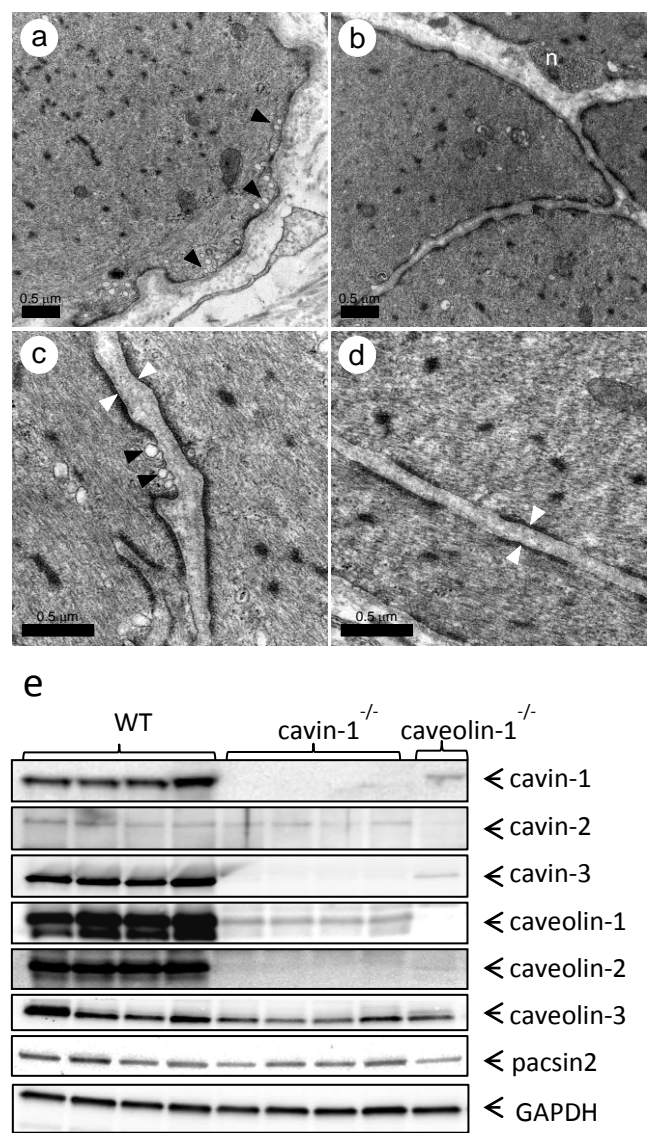


Figure 3

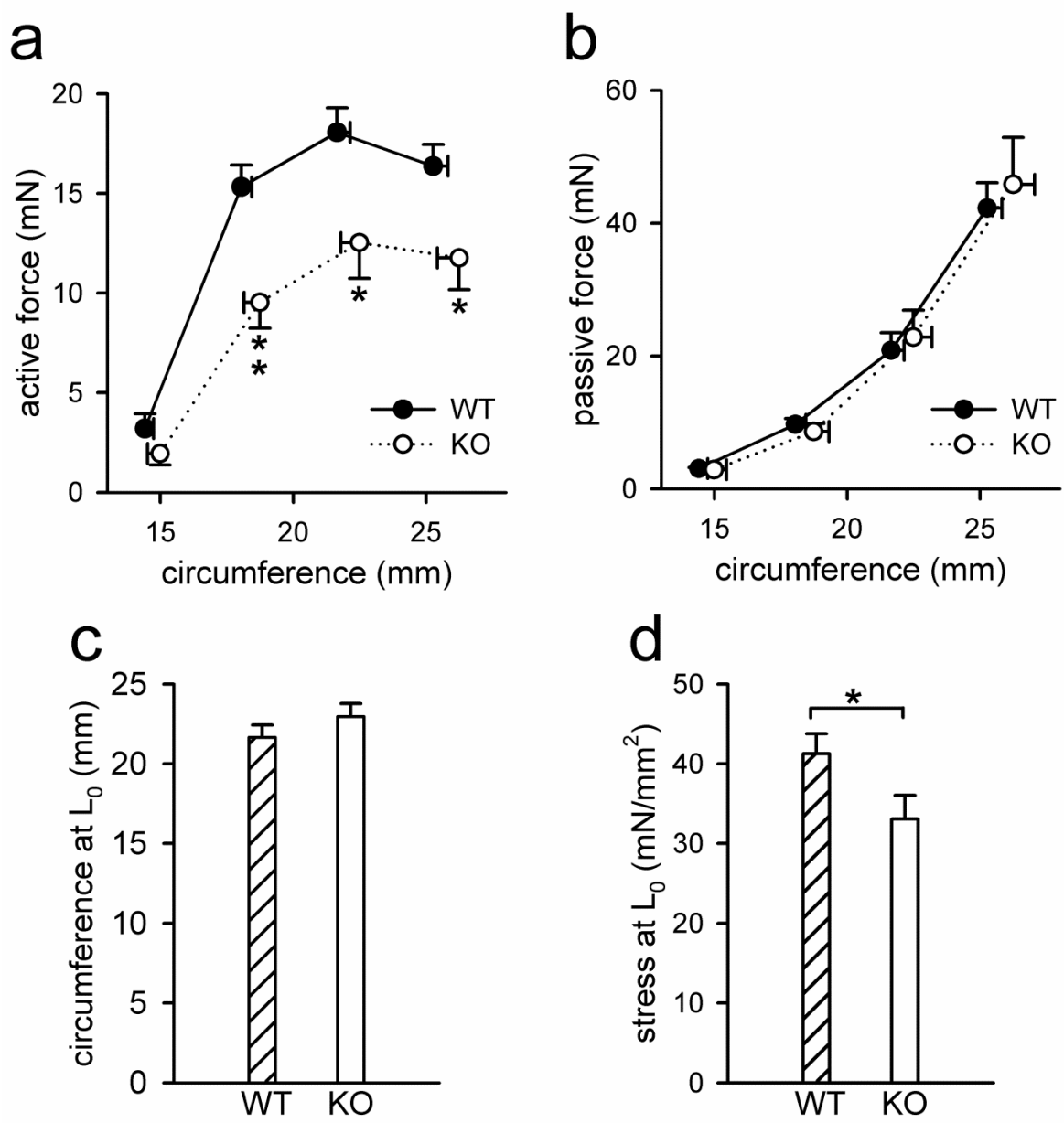
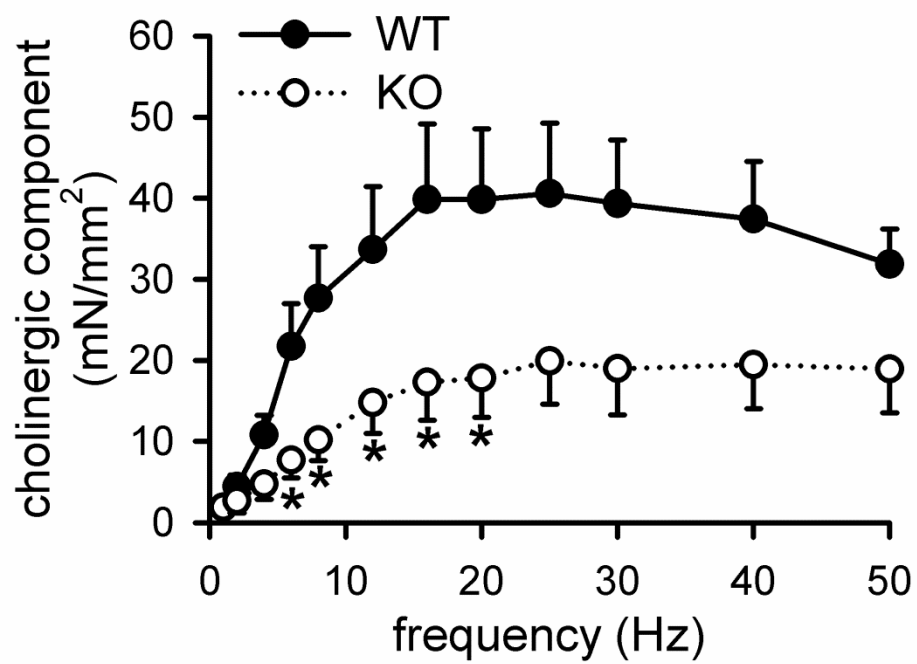


Figure 4

a



b

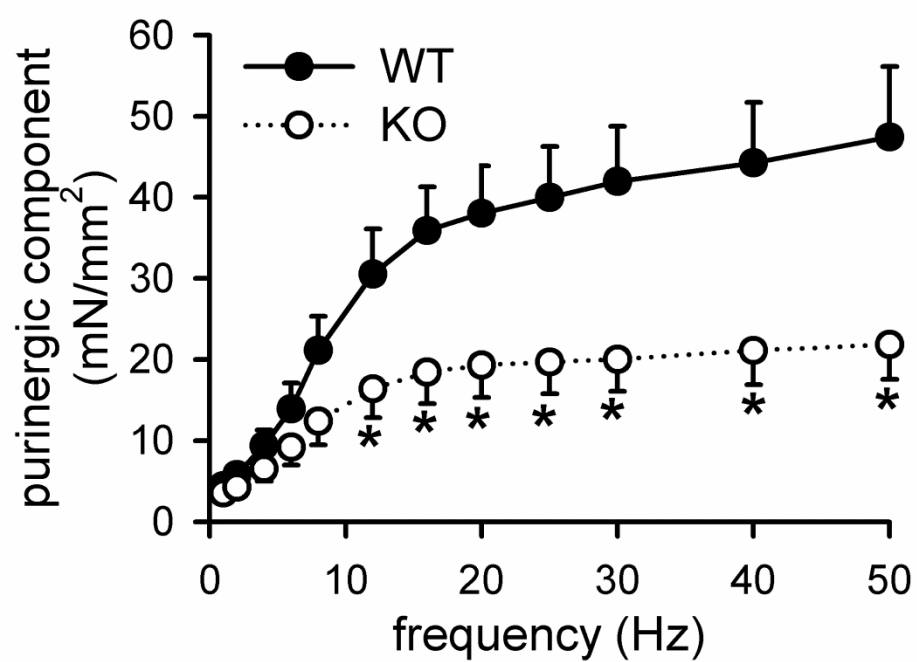


Figure 5

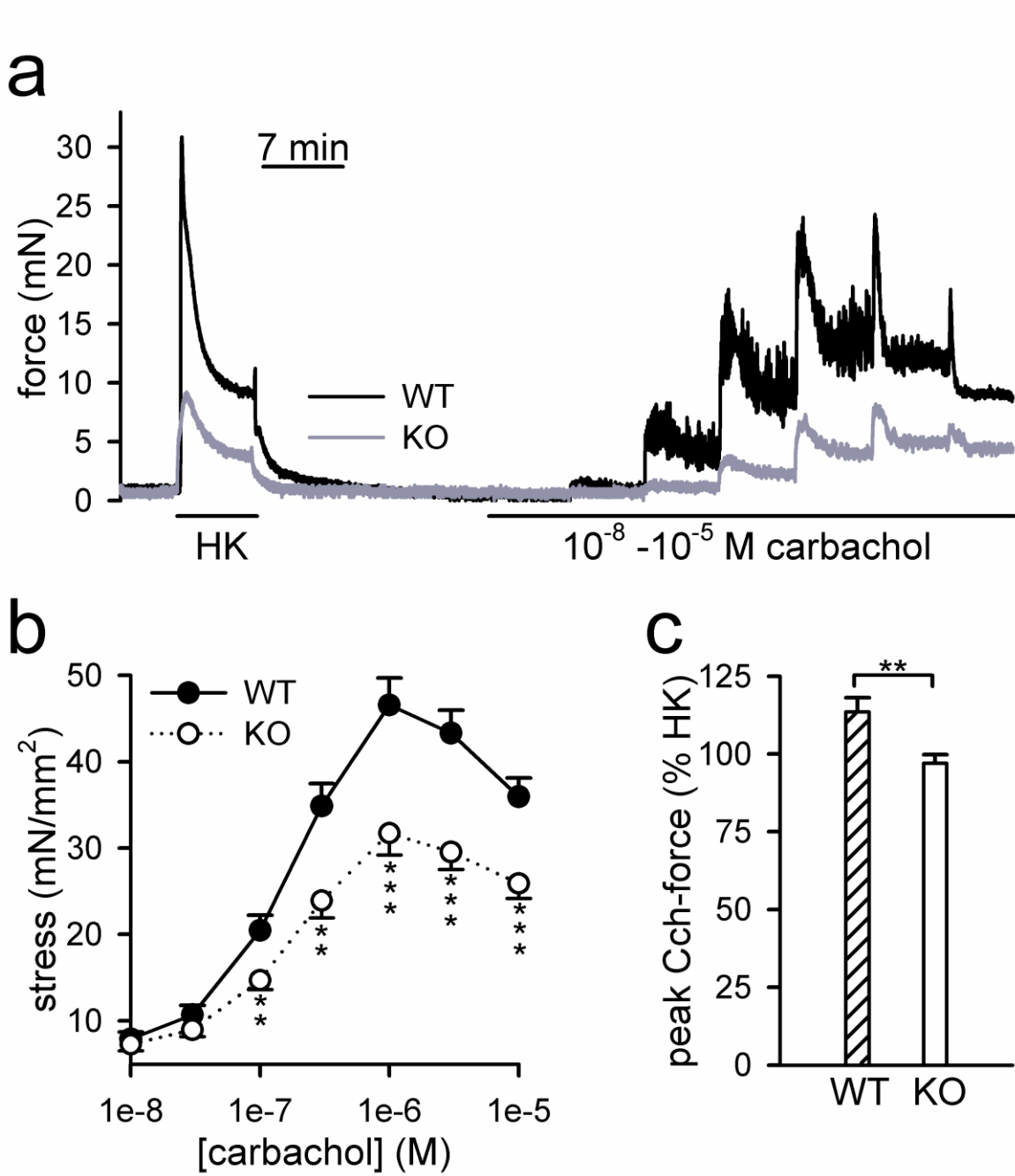


Figure 6

