Myocardin (MYOCD) is the founding member of a class of transcriptional co-activators that bind serum response factor to activate gene expression programs critical in smooth muscle (SM) and cardiac muscle development. Insights into the molecular functions of MYOCD have been obtained from cell culture studies and, to date, knowledge about \textit{in vivo} roles of MYOCD comes exclusively from experimental animals. Here, we defined an often lethal congenital human disease associated with inheritance of pathogenic \textit{MYOCD} variants. This disease manifested as a massively dilated urinary bladder, or megabladder, with disrupted SM in its wall. We provided evidence that monoallelic loss-of-function variants in \textit{MYOCD} caused congenital megabladder in males only, whereas biallelic variants were associated with disease in both sexes, with a phenotype additionally involving the cardiovascular system. These results were supported by co-segregation of \textit{MYOCD} variants with the phenotype in four unrelated families, by \textit{in vitro} transactivation studies where pathogenic variants resulted in abrogated SM gene expression, and finding megabladder in two distinct mouse models with reduced \textit{Myocd} activity. In conclusion, we have demonstrated that variants in \textit{MYOCD} result in human disease, and the collective findings highlight a vital role for MYOCD in mammalian organogenesis.
Loss-of-function variants in myocardin cause congenital megabladder in humans and mice

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

Myocardin (MYOCD) is the founding member of a class of transcriptional co-activators that bind serum response factor to activate gene expression programs critical in smooth muscle (SM) and cardiac muscle development. Insights into the molecular functions of MYOCD have been obtained from cell culture studies and, to date, knowledge about in vivo roles of MYOCD comes exclusively from experimental animals. Here, we defined an often lethal congenital human disease associated with inheritance of pathogenic MYOCD variants. This disease manifested as a massively dilated urinary bladder, or megabladder, with disrupted SM in its wall. We provided evidence that monoallelic loss-of-function variants in MYOCD caused congenital megabladder in males only, whereas biallelic variants were associated with disease in both sexes, with a phenotype additionally involving the cardiovascular system. These results were supported by co-segregation of MYOCD variants with the phenotype in four unrelated families, by in vitro transactivation studies where pathogenic variants resulted in abrogated SM gene expression, and finding megabladder in two distinct mouse models with reduced Myocd activity. In conclusion, we have demonstrated that variants in MYOCD result in human disease, and the collective findings highlight a vital role for MYOCD in mammalian organogenesis.
Introduction

Urinary tract and kidney malformations often result in termination of pregnancy after being detected on ultrasound screening, and these anomalies are also a major cause of renal failure in surviving children (1). While the genetic bases of kidney malformations are well-recognised (2), with pathogenic variants reported in transcription and growth factors that drive metanephric differentiation, the possible genetic causes of congenital ureter and bladder anomalies are much less clear (3). A striking urinary tract phenotype is megabladder, with a first trimester prevalence of 1:330-1670 (4). Megabladder accompanied by a thinned and wrinkled abdominal wall overlying the bladder is called prune belly syndrome (PBS). Megabladder and PBS are accompanied by kidney glomerular cysts considered to be secondary to fetal urinary flow obstruction (5). Some megabladders are associated with anatomically obstructed bladder outflow tracts and these bladders have increased smooth muscle (SM) in their walls. Other megabladders are examples of functional outflow obstruction, and have patent urethras and thin bladder walls (6). We hypothesised that mutations in genes affecting SM differentiation can cause megabladder and PBS.

Myocardin (MYOCD) is the founding member of a class of powerful transcriptional co-activators that bind to serum response factor (SRF) to activate cardiac and SM specific gene expression programs (7, 8). Complete loss of Myocd in mice causes embryonic lethality at mid-gestation due to failure of SM cell differentiation, whereas heterozygous knockout mice appear normal (9). Conditional mouse models subsequently defined the function of Myocd in postnatal development. Specifically, genetic ablation of Myocd in adult hearts causes heart failure due to loss of sarcomere structure and increased cardiomyocyte apoptosis (10). Genetic deletion of Myocd specifically in SM revealed that Myocd maintains vascular and visceral SM homeostasis postnatally (11). Despite these unique and important functions of Myocd in mice, no human genetic disorder associated with MYOCD variants has yet been defined. Here, we describe MYOCD loss-of-function variants in nine individuals from four
families with the megabladder/PBS spectrum, and we support these observations on urinary tract maldevelopment with Myocd mutant mouse models.

Results and Discussion
The index patient of Family A (II-2) (Fig. 1A) had a history of antenatal megabladder, and a bladder diverticulum was surgically resected in infancy. At 32 years of age, cardiac evaluation showed non-compaction cardiomyopathy and marked dilation (51 mm) of the aortic root. She also had an atrial septal defect, a ventricular septal defect (VSD), a patent ductus arteriosus and a bicuspid aortic valve. Her brother (II-1) had been diagnosed prenatally with megabladder and VSD, prompting clinical termination of pregnancy at midgestation. Autopsy revealed PBS, and histology showed disorganised SM bundles in the bladder and glomerular cysts in the kidneys (Fig. 2). The distal urethra was patent (Supplemental Fig.1) but the prostate and proximal urethra were not identified. A conclusion regarding anatomical obstruction was not possible because formal tests of urethral patency were not undertaken. The hindgut contained only a defined circular SM layer (Supplemental Fig. 2), and lacked the longitudinal layer that should be present at this gestational age. In contrast, two normal SM layers were present in the small intestine, and pulmonary artery SM appeared normal (Supplemental Fig. 2). Ultrasonography revealed no bladder or heart abnormalities in the parents. Whole-exome sequencing (WES) in index patient II-2 and Sanger sequencing in II-1 determined that both siblings carried compound heterozygous variants in MYOCD (p.[S229Qfs*17];[E530G], respectively called Family A mutation 1 and Family A mutation 2 in Fig. 1A). p.[S229Qfs*17] is predicted to create a premature stop codon and was paternally inherited, while the missense variant p.[E530G] is located in the functional leucine zipper (LZ) of the encoded protein (12, 13) and was maternally inherited (Fig. 1A and B). Both variants were absent from over 120,000 control exomes (14). WES in the index case failed to reveal pathogenic variants in genes known to cause megabladder, including ACTG2, CHRM3, HPSE2, LRIG2, MYH11 and MYLK (3, 15–17).
Next, we ascertained 22 additional families with megabladder or PBS of unknown etiology, identifying seven affected individuals from three unrelated families, all with heterozygous predicted loss-of-function MYOCD variants (Fig. 1A, Supplemental Table 1). In Family B, there were three male fetal deaths, all with PBS (Fig. 1A, C). WES revealed a heterozygous variant, c.343C>T in these three brothers, predicted to result in a premature stop p.[R115*]. This variant was also present in the unaffected mother, unaffected maternal grandmother and a healthy female sibling, each with normal bladder and heart imaging. The grandmother (II-3) reported a male stillbirth (III-4) of unknown cause in the third trimester. She had five siblings comprising four brothers who died antenatally (II-5, II6, II7 and II-8), each with a megabladder (further details unavailable), and one healthy sister (Fig. 1A). Family C’s first pregnancy was terminated after diagnosing PBS in the male fetus (Fig. 1A, C). Chromosomal microarray analysis revealed a heterozygous de novo 420kb deletion (chr17p12:hg19:12,172,568-12,609,597) encompassing the first two exons of MYOCD, including the start codon. In family D, three males had PBS: two died prenatally (III-1 and IV-1) and the other (III-4) was born and underwent kidney transplantation for end-stage renal failure (Fig. 1A). Sanger sequencing of MYOCD identified a heterozygous deletion of a single base c.1053-1054del resulting in a predicted frameshift p.[N351Kfs*19].

We tested the abilities of proteins encoded by MYOCD variants from Families A and B to activate the promoter of transgelin (Sm22 or Tagln), encoding a SM contractile protein. Western blotting revealed that the two predicted nonsense variants (p.[S229Qfs*17] Mutation A1 from Family A, and p.[R115*] Mutation B from Family B) produced a truncated protein, whereas the missense variant (E530G, Mutation A2 from Family A) produced full length protein (Supplemental Fig. 3). Neither nonsense variant resulted in activation of the Tagln-luciferase reporter, whereas the missense variant resulted in diminished activity versus wild-type MYOCD (Fig. 3A). The above results regarding the missense variant are consistent with previous reports that MYOCD homodimerizes through the LZ domain and that homodimerization facilitates stronger activation of SRF-dependent reporter genes (8, 12).
Myocd is normally not expressed in 10T1/2 fibroblasts but experimental overexpression in these cells activates the SM differentiation programme (8). We transfected 10T1/2 cells with either wild-type MYOCD or each of the three variants from Families A and B. As assessed by qPCR, wild-type MYOCD strongly induced endogenous expression of the SM transcripts Tagln, Myh11, Cnn1, Mylk and Acta2. Conversely, each of the three MYOCD variants resulted in either a lack of increased expression, or statistically significantly blunted responses (Fig. 3B).

To assess whether reduced MYOCD activity causes megabladder, we took advantage of a newly generated mutant mouse line carrying an allele, MyocdΔLZ, in which critical residues within the LZ of Myocd were specifically deleted (Fig. 4A and Supplemental Fig. 4A). Using primers that distinguish the wild-type from the LZ mutated transcript, we showed that both were detected in neonatal bladders of MyocdΔLZ/+ mice (Supplemental Fig. 4B). We crossed MyocdΔLZ/+ mice with those carrying a null allele of Myocd (Myocd−/−) (18). The alleles in the compound mutant offspring (MyocdΔLZ−/) therefore mimic the LZ and nonsense mutant alleles in family A. In contrast to homozygous null Myocd mutants (Myocd−/−)(9), compound heterozygous mutant offspring (MyocdΔLZ−/) survived to birth. In line with the human urinary tract malformations, MyocdΔLZ−/− mice developed grossly dilated bladders with little or no SM in their walls (Fig. 4A-E, Supplemental Fig. 4C and D). In MyocdΔLZ−/− mouse bladders, transcript levels of several Myocd target genes (i.e. Acta2, Myh11, Mylk, Tagln and Cnn1) were blunted versus wild-types (Supplemental Fig. 4E).

To gain further insight into the potential role of Myocd gene dosage in bladder malformations, we examined the megabladder mouse (mgb) generated by random insertion and translocation of a transgene into chromosome 11 (19). Transcriptional profiling in the bladders of these mice had already revealed that levels of Myocd transcripts were significantly reduced (20). Here, we identified the translocation breakpoint (together with four copies of translocated chr. 16 region) approximately 500kb upstream of the Myocd gene (Fig. 4F and Supplemental Fig. 5)
suggesting the presence of a regulatory Myocd enhancer. Next, we crossed Myocd<sup>mgb</sup>/ with Myocdm<sup>+/-</sup> mice (9) and demonstrated that compound mutant offspring (Myocd<sup>mgb</sup>/) had megablasts, providing further evidence that marked reduction of Myocd causes this phenotype (Fig. 4 G-J and Supplemental Fig 6-7). In addition, we observed patent ductus arteriosus in newborn Myocd<sup>mgb</sup>/ mice, but not in Myocdm<sup>+</sup> mice with just one allele mutated (Supplemental Fig. 8). By studying various Myocd mutants (Fig. 4K), we showed that a 70-80% reduction in Myocd mRNA in the bladder is sufficient to produce megabladder in mice. Interestingly, neonatal Myocd<sup>mgb</sup>/ mouse blasters have severely reduced transcripts levels of Myocd target genes, yet in aortas and hearts of these same mice the levels of Myocd target transcripts (apart from Myh11 in the aorta) is similar to heterozyous (Myocd<sup>mgb</sup>/) controls (Supplemental Fig. 9).

The bladder phenotypes in families carrying MYOCD variants are thus mimicked by two distinct mouse models with reduced Myocd activity. Supplemental Table 2 gives an overview of the phenotypes observed in the mouse lines. The collective results demonstrate that MYOCD plays a unique role in proper development of the bladder wall. Reduced Myocd activity resulted in little or no SM differentiation in the mouse blasters, and in disorganised SM bundles in the human fetal bladder. These SM defects would diminish the muscular force required to void urine from the bladder, resulting in the functional equivalent of lower urinary tract obstruction with severe bladder distension, ultimately culminating in kidney failure and death (19, 21).

Megabladder/PBS is a sex-limited trait with 95% male predominance, likely the result of differences in urethra and bladder development and length differences in urethra between males and females (4, 6). Additionally, sex hormones may play a role in defining the severity and progression of the disease, as clinical evidence demonstrates increased male susceptibility to acute and chronic kidney injury (22, 23). Indeed, in our study, seven of eight males proven to carry heterozygous loss-of-function MYOCD variants died before birth,
whereas all six female carriers with heterozygous loss-of-function variants appeared healthy. Consistent with this observation, male Myocd<sup>mgb/mgb</sup> mice are also more severely affected than females (Supplemental Table 2) (19). Notably, the only female with bladder disease in our study carried biallelic variants in MYOCD, suggesting that further reduction in MYOCD levels are needed to cause bladder phenotypes in females. This is supported by both Myocd mouse models where compound heterozygosity (i.e. Myocd<sup>mgb/-</sup> or Myocd<sup>Z/-</sup>) caused megabladder in either sex (Supplemental Table 2). An intriguing feature is the incomplete penetrance of bladder disease in a healthy male MYOCD mutation carrier in Family D. This may be caused by allelic imbalance, where the penetrance of a dominant loss-of-function mutation is determined by the expression level of the second allele, for instance due to variants in promoter or enhancer regions of MYOCD. Alternatively, redundancy with other MYOCD family members, such as MRTF-A that is also expressed in developing bladders (24), may affect penetrance.

Overall, we propose that we have identified a semi-dominant disorder (25), where heterozygous loss-of-function variants in MYOCD cause congenital megabladder, while biallelic loss-of-function MYOCD variants also cause a cardiovascular phenotype. Both biallelic carriers developed congenital heart defects, while the affected female was found, when investigated as an adult, to have severe dilation of the aortic root. A similar association of cardiac defects was observed in the Myocd<sup>mgb-/-</sup> mice ((26) and supplemental Fig. 8). Notably, a previous study already hinted at the possible involvement of MYOCD in megabladder, as it described one sporadic case with PBS and a 1.3Mb deletion of multiple genes, including MYOCD (27). Other SM-related genes have been implicated in PBS. These include variants in ACTA2, a MYOCD target gene, as well as MYH11 and MYLK, in which variants can cause visceral myopathy, a phenotype encompassing megabladder (3, 16). Moreover, each of these three genes has been associated with inherited thoracic aortic aneurysm and dissection (28). Notably, a SM-restricted deletion of Myocd in mice causes dilation of several visceral organs including the bladder, as well as dilation of the aorta (11).
Hence, there is compelling evidence that reduced MYOCD levels can result in urological and cardiovascular disease.

In conclusion, we demonstrate for the first time that variants in \textit{MYOCD} result in human disease. We propose that monoallelic loss-of-function variants in \textit{MYOCD} cause congenital megabladder in males, and that biallelic variants are associated with disease manifest in females which also involves the cardiovascular system. These findings not only have important implications for genetic counseling of families with megabladder, but also shed new light on bladder development and expand the pathophysiological spectrum of inherited SM disorders.

\textbf{Methods}

Experimental procedures are provided in Supplemental Methods.

\textbf{Study approval}

Blood samples for genetic testing were obtained upon written consent. Informed consent for DNA studies, clinical records, and use of ultrasound pictures and histological analysis of the terminated fetus of family A was obtained. Control human embryonic material, collected with maternal consent and ethical approval (REC 08/H0906/21+5), was sourced from the MRC and Wellcome Trust Human Developmental Biology Resource.

Mice were maintained according to NIH Guide for the Care and Use of Laboratory Animals.

\textbf{Author contributions}

ACH, GMB and AVP share first authorship and the order in which they are listed has been determined by workload. ACH, GMB, AVP, WGN, ASW and EEC designed the study. TBG, FML, IM, AMP, MLR, KDW, RG, LC and ED performed experiments. ACH, EO, SR, KDL, FB contributed clinical samples and clinical data. ACH, JME, GMB, AVP, WGN performed genetic
analysis. ACH, GMB, AVP, KMM, ARJ, BLB, WGN, ASW and EEC analysed experimental data. BLB, KMM and MLR contributed mouse lines. EJM and VMC were instrumental in interpretation of the data. ACH, AVP, ASW and EEC wrote the manuscript.

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References


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**Family A**
- Human: QGLGPP S TPIAVH
- Chimpanzee: QGLGPP S TPIAVH
- Rhesus: QGLGPP S TPIAVH
- Wolf: QGLGPL G NPIAVH
- Cow: QGLGSL G TPITGH
- Mouse: QVLGPL S TPIPVH
- Rat: QVLGPL S TPIPVH
- Chicken: QAVGQD S PPLPVP
- Zebrafish: MTKSQE N PPVPVP

**Family A mutation 1**
- p.S229Qfs*17
c.684dupC
- KQKVIN E LTWKLQ

**Family A mutation 2**
- p.E530G
c.1589A>G
- NEKIAL R PGPLEL

**Family B**
- p.R115*
c.343C>T
- PLSPVK N SFSGQT

**Family C**
- p.N351Kfs*19
c.1053-1054del TAD
- Leucine zipper
- SAP
- RPELs

**Family D**
- p.N351Kfs*19
c.1053-1054del

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**Figure 1**
- Human figures showing genetic markers and family trees for each family.
- Family A and B showing mutations with corresponding amino acid changes.
- Family C and D showing deletions and genetic markers.
- Images of embryos showing spinal and rib anomalies.

**Legend**
- MID: RPELs ++ α Q SAP Leucine zipper TAD
- p.R115*
p.p.S229Qfs*17
- p.E530G
- p.N351Kfs*19

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Figure 1. Identification of MYOCD variants in four families with congenital megabladder. (A) Pedigrees of four families presenting with congenital megabladder. Affected individuals are marked with black filled symbols. Available genotypes are shown beneath symbols. Slashed symbols denote deceased individuals. “p” with arrow denotes proband of the family. Gestational age is indicated above the symbol. N indicates normal bladder ultrasound. Grey filled symbols denote stillbirths with external features consistent with PBS. npe, normal prenatal echo. (B) Schematic diagram showing functional domains within MYOCD and the location of the identified mutations (7). Conservation of respective amino acid positions with the mutated residues highlighted. (C) Ultrasound images showing enlarged bladder of indicated fetuses of family B and C; asterisk denotes bladder.
Figure 2. Bladder and kidney abnormalities in Family A. (A) and (C) are taken from healthy midgestation fetuses. (B) and (D) are from the affected fetus from Family A. H&E staining from urinary bladders show transverse sections of muscle bundles (TSM) and longitudinal sections of muscle bundles (LSM) in the healthy and affected fetus. Note, however, that the bundles in the affected fetus appear disorganised and less compact compared to the well-defined muscle fibers in the control. (C) In a control fetal kidney, glomeruli (G) and tubules (T) are evident. (D) In the kidney from the affected fetus, glomeruli are cystic, with dilated Bowmans spaces (asterisks), a characteristic of fetal urinary flow obstruction. Bars are 20 µm.
Figure 3. **MYOCD** mutations abrogate activation of SM cell gene expression *in vitro*. (A) Mouse fibroblasts were transiently transfected for 48h with expression vectors for **MYOCD** or the indicated **MYOCD** mutants (Mutation A1: p.S229Qfs*17; Mutation A2: p.E530G; Mutation B: p.R115*) and a luciferase reporter linked to the *Transgelin* (Sm22) promoter (n=3/group). (B) Mouse fibroblasts were transfected with expression plasmids encoding **MYOCD** or the indicated mutants (n=4/group). An empty expression plasmid served as a control. RNA was isolated and SM gene expression (*Tagln*: transgelin; *Myh11*: Smooth muscle Myosin Heavy Chain 11; *Cnn1*: Calponin 1; *Mylk*: Myosin light chain kinase; *Acta2*: SM actin alpha 2) was measured by qPCR. GAPDH was used to normalize expression. Overexpression levels of **MYOCD** were comparable between conditions (Supplemental Fig. 3). A and B: *denotes p-value <0.01 compared to WT **MYOCD** according to One Way ANOVA with Dunnett’s multiple comparison test. Shown are representative experiments of 2 independent repeats.
Figure 4

Myocd\textsuperscript{ΔLZ} mouse model

- Δ 24 nt in LZ domain
- Ex10

Myocd\textsuperscript{Mgb} mouse model

- Translocation chr16 + transgene
- ~1.0 Mb
- Gm12295
- Myocd

Legend:

- In
- Bl
- Re
- E
- H
- J
- U
- D
- S
- DM
- P1 WT
- P1 Myocd\textsuperscript{ΔLZ}
- P1 Myocd\textsuperscript{Mgb}

Graph:

- Chart showing percentage of WT Myocd mRNA
- 0/6, 0/7, 0/4
- Megabladder phenotype:
Figure 4. Myocd loss-of-function in mice causes the megabladder phenotype. (A) Schematic representation of the MyocdΔLZ allele, in which 24 nucleotides are deleted in the LZ domain (p.I531_R539delinsM in NP_666498.2) (B-E), αSMA immunohistochemistry in one day old neonates from Myocd+/− and MyocdΔLZ/+ crosses. (C) Compound heterozygosity (MyocdΔLZ/−; reminiscent of the alleles present in the affected individuals in family A) results in wall thinning of the bladder and lack of SM cells compared to the WT bladder wall (B). (D,E) Higher magnifications of WT and MyocdΔLZ/− bladder walls showing lack of αSMA expressing muscle bundles in the putative detrusor layer, although expression appeared retained in myofibroblast-like cells in the lamina propria directly below the urothelium (U) and in the rectum (Re). (F) Schematic representation of the MyocdMgb allele. (G-J) Representative αSMA immunohistochemistry in postnatal day (P)1 bladder of wildtype (G,H) and MyocdMgb/− compound heterozygote (I,J) (from a cross of MyocdMgb/+ and Myocd+/− mice). Note the severe bladder distention and absent detrusor muscle in the MyocdMgb/− bladder. Bl: bladder, In: intestine, Re: rectum, U: urethelium; S: submucosa; DM: detrusor muscle. Scale bars are 500 µm in B, C, G and 100 µm in I, in D, E, H and J. (K) Myocd mRNA levels were quantified by qPCR using embryonic day (E)15 bladders of WT, Myocd+/−, MyocdMgb/+, MyocdMgb/mgb and MyocdMgb/− mice. The absolute numbers of embryos developing megabladder as a fraction of the total number of embryos analysed, is indicated above the graph and reveals a highly penetrant phenotype in the MyocdMgb/mgb and MyocdMgb/− mice.