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Supplementary Materials for

Deregulated Ca^{2+} cycling underlies the development of arrhythmia and heart disease due to mutant obscurin

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Supplementary Materials

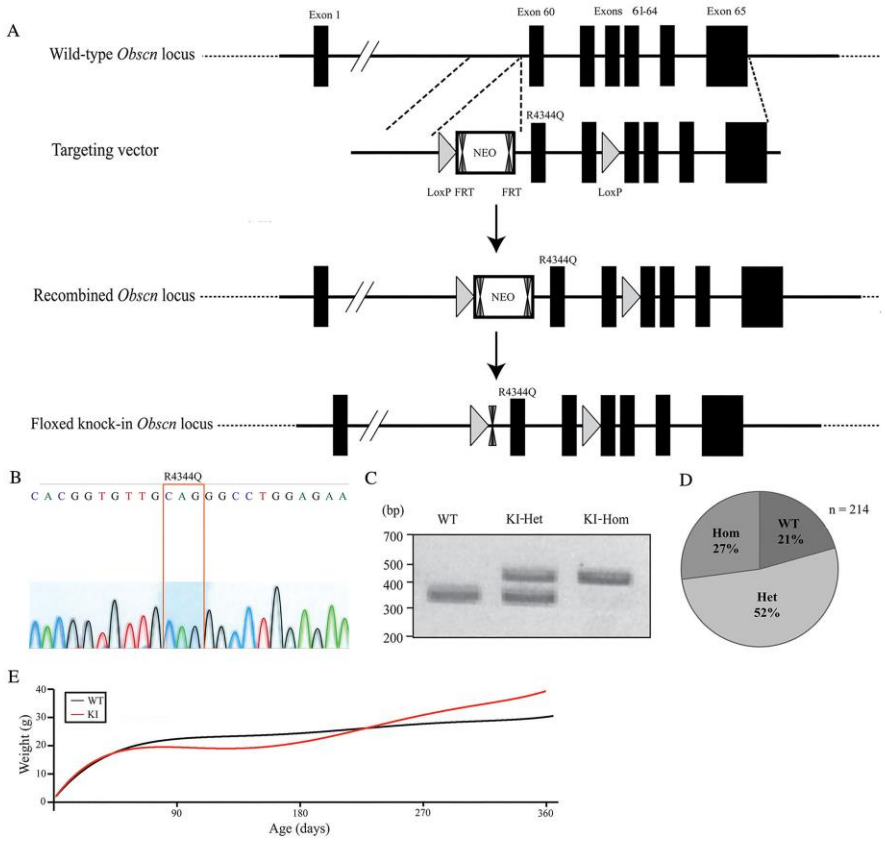

Figure S1

fig. S1. Generation of obscurin knock-in mice carrying the R4344Q mutation in Ig58. (**A**) Schematic diagram illustrating the targeting vector and the step-wise generation of obscurin knock-in mice. (**B**) DNA sequencing confirmed the successful incorporation of the R4344Q mutation in the genome of embryonic stem (ES) cells. (**C**) PCR analysis using genomic DNA extracted from the animal tails, and a primer set annealing to sequences in intron 59 located 5′ and 3′ to the proximal loxP and FRT sites confirmed deletion of the neomycin cassette and the three different possible genotypes. (**D**) Wild-type and knock-in mice were born according to Mendelian ratios. (**E**) Growth charts of wild-type and knock-in mice over 1-year. The average growth of homozygous female knock-in mice (n=5) tracked over the first year of their life reveals similar growth up to 3-months of age compared to age- and sex- matched wild-type (n=5)

littermates. Interestingly, between 3 and 6 months of age the knock-in mice exhibit a lag in their growth compared to wild-type littermates. This is not the case, however, at later ages (9-12 months) when the knock-in mice exhibit a greater growth rate compared to wild-type littermates.

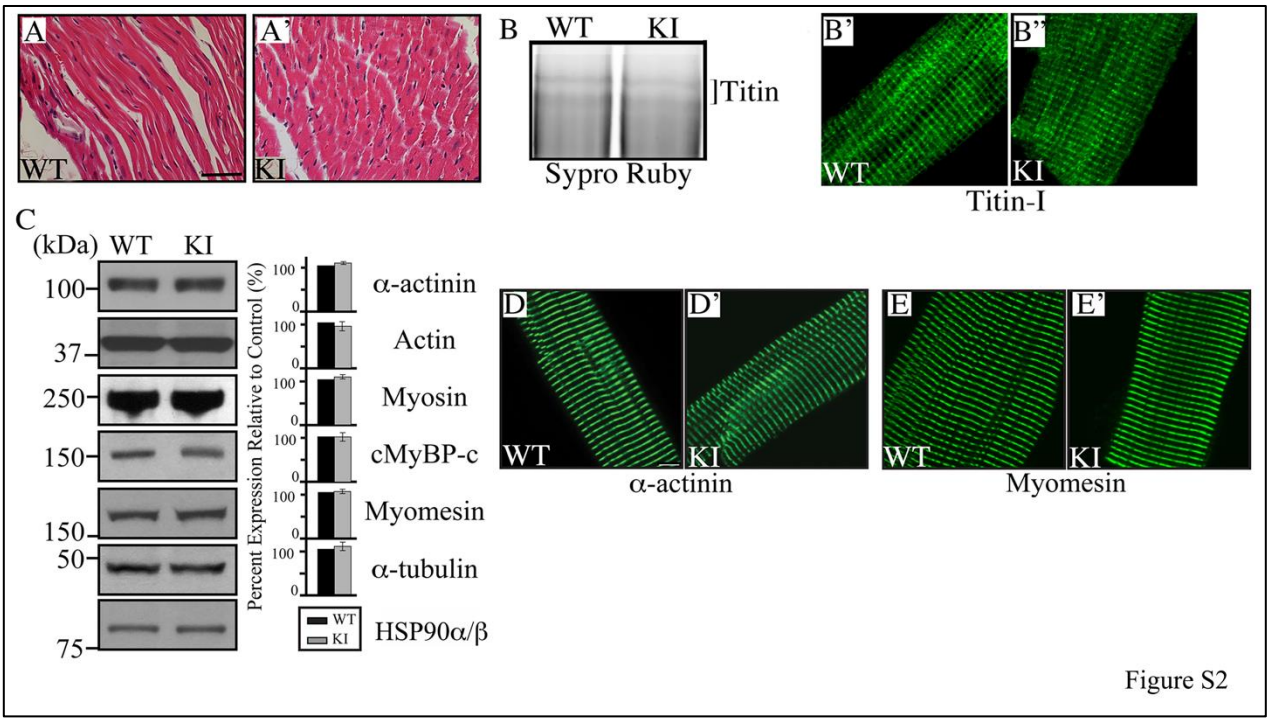

fig. S2. Sarcomeric organization is unaltered in 1-year-old homozygous knock-in female mice. (**A** to **A′**) Hematoxylin and eosin (H&E) staining of cardiac sections reveal no apparent structural alterations. (**B**) SYPRO® Ruby staining shows no altered expression for titin in knock-in lysates compared to control. (**B′** to **B″**) Similarly, titin localization is also unaltered in knock-in (KI) myocardia. (**C**) Representative immunoblots and relative quantification of the expression levels of sarcomeric proteins using densitometry showed no difference between wild-type and knock-in lysates prepared from left ventricles. HSP90α/β served as loading and normalization control and error bars represent SEM. (**D** to **E′**) Immunolabeling of sarcomeric proteins in wild-type (WT; D and E) and knock-in (KI; D′ and E′) cardiac sections revealed indistinguishable subcellular localizations.

table S1. Morphometric and echocardiographic analyses of sedentary wild-type and homozygous knock-in hearts.

Parameter	3-months		1-year	
	Wild-type	Knock-in	Wild-type	Knock-in
Female animals				
Whole Heart Mass /TL (mg/mm)†	*7.31±0.45*	*7.52±0.27*	*7.90±0.37*	*8.12±0.36*
LV Mass/TL (mg/mm)†	*5.03±0.28*	*5.22±0.23*	*5.39±0.38*	*5.56±0.23*
LV Anterior Wall Thickness; d (mm)	*0.89±0.05*	*0.83±0.08*	*0.90±0.07*	*0.90±0.08*
LV Anterior Wall Thickness; s (mm)	*1.26±0.07*	*1.17±0.08*	*1.32±0.12*	*1.23±0.19*
LV Internal Diameter; d (mm)	*3.46±0.12*	*3.72±0.06*	*3.42±0.12*	*3.28±0.08*
LV Internal Diameter; s (mm)	*2.44±0.17*	*2.73±0.14*	*2.12±0.15*	*1.94±0.21*
LV Posterior Wall Thickness; d (mm)	*0.67±0.03*	*0.67±0.05*	*0.80±0.05*	*0.85±0.14*
LV Posterior Wall Thickness; s (mm)	*0.99±0.07*	*0.93±0.07*	*1.16±0.04*	*1.16±0.15*
LV Absolute Wall Thickness (mm)	*1.56±0.07*	*1.50±0.11*	*1.71±0.11*	*1.30±0.26*
LV Relative Wall Thickness	*0.46±0.03*	*0.40±0.03*	*0.51±0.05*	*0.58±0.09*
LV End Diastolic Volume (μL)	*49.9±4.2*	*58.9±2.4*	*49.0±4.1*	*43.6±2.6*
LV End Systolic Volume (μL)	*22.2±3.6*	*28.1±3.5*	*16.0±2.8*	*12.9±3.5*
LV Ejection Fraction %	*57.2±4.3*	*52.8±4.1*	*69.2±3.2*	*70.6±8.0*
LV Fractional Shortening %	*29.8±2.9*	*26.8±2.6*	*38.5±2.4*	*40.9±6.2*
LV Stroke Volume (μL)	*27.8±1.8*	*30.8±1.2*	*33.0±1.8*	*30.7±3.7*
cLV Mass (mg)	*71.8±3.8*	*76.1±6.6*	*80.4±5.5*	*78.5±10.2*
cLV Mass/BW (mg/g)	*3.73±0.21*	*3.55±0.27*	*2.80±0.15*	*2.65±0.27*
Mitral Valve E Wave (mm/s)	*618±36*	*530±98*	*483±55*	*438±77*
Mitral Valve A Wave (mm/s)	*460±65*	*326±46*	*391±64*	*291±51*
Mitral Valve E/A	*1.57±0.31*	*1.60±0.15*	*1.37±0.13*	*1.53±0.19*
IVCT (ms)	*18.0±3.2*	*24.9±4.0*	*19.4±2.5*	*19.0±3.4*
IVRT (ms)	*23.9±3.3*	*33.5±8.2*	*18.4±1.4*	*20.4±2.1*
Mitral Valve Ejection Time (ms)	*43.9±2.5*	*46.2±5.8*	*38.8±2.5*	*43.0±5.8*
Myocardial Performance Index	*0.97±0.14*	*1.37±0.35*	*1.02±0.10*	*0.93±0.09*
Male animals				
Whole Heart Mass /TL (mg/mm)†	*10.31±0.64*	*8.58±0.36**	*10.61±0.36*	*11.53±0.73*
LV Mass/TL (mg/mm)†	*6.61±0.53*	*5.77±0.18*	*7.22±0.24*	*7.82±0.48*
LV Anterior Wall Thickness; d (mm)	*0.91±0.03*	*0.96±0.03*	*0.91±0.08*	*0.98±0.07*
LV Anterior Wall Thickness; s (mm)	*1.37±0.04*	*1.44±0.05*	*1.30±0.11*	*1.34±0.10*
LV Internal Diameter; d (mm)	*3.82±0.08*	*3.88±0.08*	*3.56±0.10*	*3.86±0.15*
LV Internal Diameter; s (mm)	*2.59±0.11*	*2.62±0.11*	*2.34±0.11*	*2.73±0.14**
LV Posterior Wall Thickness; d (mm)	*0.78±0.04*	*0.78±0.02*	*0.84±0.08*	*0.86±0.09*
LV Posterior Wall Thickness; s (mm)	*1.09±0.05*	*1.12±0.04*	*1.11±0.11*	*1.10±0.11*
LV Absolute Wall Thickness (mm)	*1.68±0.05*	*1.74±0.05*	*1.74±0.15*	*1.84±0.15*
LV Relative Wall Thickness	*0.44±0.02*	*0.45±0.02*	*0.49±0.04*	*0.48±0.04*
LV End Diastolic Volume (μL)	*63.3±3.2*	*65.9±3.4*	*53.6±3.8*	*66.0±6.0*
LV End Systolic Volume (μL)	*25.4±2.7*	*25.9±2.5*	*19.4±2.2*	*29.0±3.5**

LV Ejection Fraction %	*60.8±2.7*	*61.5±2.5*	*63.8±3.4*	*57.0±2.2*
LV Fractional Shortening %	*32.4±1.9*	*32.9±1.8*	*34.4±2.6*	*29.5±1.6*
LV Stroke Volume (μL)	*38.0±1.8*	*40.0±1.9*	*34.2±2.9*	*37.0±2.9*
cLV Mass (mg)	*94.0±4.4*	*101.2±4.6*	*91.3±12.6*	*111.6±14.0*
cLV Mass/BW (mg/g)	*3.25±0.16*	*3.35±0.14*	*2.29±0.24*	*2.67±0.26*
Mitral Valve E Wave (mm/s)	*680±42*	*756±50*	*560±44*	*494±55*
Mitral Valve A Wave (mm/s)	*473±39*	*511±34*	*409±41*	*342±47*
Mitral Valve E/A	*1.48±0.08*	*1.50±0.07*	*1.43±0.13*	*1.56±0.16*
IVCT (ms)	*22.0±2.6*	*20.8±3.2*	*15.2±2.4*	*14.4±1.7*
IVRT (ms)	*22.0±1.4*	*23.4±2.1*	*19.2±2.0*	*19.8±1.3*
Mitral Valve Ejection Time (ms)	*37.7±2.1*	*33.2±2.2*	*39.7±5.4*	*44.4±3.9*
Myocardial Performance Index	*1.24±0.16*	*1.51±0.26*	*1.05±0.30*	*0.81±0.07*

[†]These parameters were measured from specimen weighing during dissection; the remaining parameters were obtained via transthoracic echocardiography. Number of animals used for morphometric analysis during dissection: 3-months old wild-type females: n=9; 3-months old knock-in females: n=8; 1-year old wild-type females: n=8; 1-year old knock-in females: n=9; 3-months old wild-type males: n=4; 3-months old knock-in males: n=11; 1-year old wild-type males: n=9; 1-year old knock-in males: n=9; Number of animals used for transthoracic echocardiography: 3-months old wild-type females, n=8; 3-months old knock-in females, n=5; 1-year old wild-type females, n=11; 1-year old knock-in females, n=6; 3-months old wild-type males, n=13; 3-months old knock-in males, n=15; 1-year old wild-type males, n=9; and 1-year old knock-in males, n=12; ± Standard Error of the Mean (SEM); TL, Tibial Length; LV, Left Ventricle; d, diastole; s, systole; cLV mass: corrected Left Ventricle mass; BW, body weight; IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time. t-test, *p*-value set at $p<0.05$. Please note that in the 1-year old male population, LV internal diameter at systole and LV end systolic volume are significantly increased compared to wild-type controls; however, LV internal diameter at diastole and LV end diastolic volume are not significantly different. More importantly, additional parameters that often serve as markers for DCM, such as LV absolute and relative wall thickness, anterior and posterior wall thickness, ejection fraction, and fractional shortening are not altered either. Thus, we do not consider the increased LV internal diameter at systole and the increased end systolic volume substantial enough to indicate a DCM phenotype.

table S2. Morphometric and echocardiographic analyses of sham- and TAC-subjected wild-type and knock-in hearts.

	Wild-type		Obscurin Knock-in	
	Sham	**TAC**	**Sham**	**TAC**
Whole Heart Mass/TL (mg/mm)†	*6.62±0.32*	*10.11±0.28**	*6.99±0.29*	*11.46±0.62*^#*
LV Mass/TL (mg/mm)†	*4.73±0.43*	*8.03±0.24**	*5.24±0.30*	*9.53±0.66*^#*
LV Anterior Wall Thickness; d (mm)	*0.91±0.04*	*1.17±0.04**	*1.02±0.03**	*1.20±0.03*^*
LV Anterior Wall Thickness; s (mm)	*1.38±0.04*	*1.66±0.07**	*1.52±0.03**	*1.67±0.05*^*
LV Internal Diameter; d (mm)	*3.32±0.06*	*3.54±0.18*	*3.06±0.10**	*4.19±0.12*^#*
LV Internal Diameter; s (mm)	*1.94±0.07*	*2.55±0.25**	*1.63±0.09**	*2.74±0.21*^*
LV Posterior Wall Thickness; d (mm)	*0.81±0.04*	*1.23±0.04**	*0.92±0.03**	*1.16±0.04*^*
LV Posterior Wall Thickness; s (mm)	*1.16±0.02*	*1.55±0.05**	*1.34±0.07**	*1.40±0.03*#*
LV Absolute Wall Thickness (mm)	*1.69±0.04*	*2.43±0.08**	*1.95±0.03**	*2.46±0.04*^*
LV Relative Wall Thickness	*0.51±0.02*	*0.69±0.03**	*0.65±0.03**	*0.60±0.03*#*
LV End Diastolic Volume (µL)	*44.8±2.1*	*57.1±7.0*	*35.7±2.9**	*76.0±3.6*^#*
LV End Systolic Volume (µL)	*11.5±1.1*	*24.1±6.0*	*8.3±1.6*	*36.4±6.1*^*
LV Ejection Fraction %	*69.9±1.5*	*58.6±4.6**	*77.7±1.8**	*52.3±5.1*^*
LV Fractional Shortening %	*38.4±1.2*	*28.4±2.3**	*45.3±1.7**	*26.9±3.1*^*
LV Stroke Volume (µL)	*30.3±1.1*	*33.5±2.9*	*26.2±1.6*	*30.6±2.0^*
cLV Mass (mg)	*77.1±3.0*	*152.7±13.7**	*95.0±5.0**	*160.8±8.2*^*
cLV Mass/BW (mg/g)	*3.93±0.13*	*7.51±0.77**	*4.31±0.22*	*7.91±0.57*^*
Mitral Valve E Wave (mm/s)	*534±38*	*565±25*	*480±55*	*446±32#*
Mitral Valve A Wave (mm/s)	*413±19*	*348±40*	*385±56*	*368±21*
Mitral Valve E/A	*1.28±0.04*	*1.46±0.15*	*1.19±0.09*	*1.18±0.05*
IVCT (ms)	*17.9±1.8*	*17.9±2.6*	*14.9±2.4*	*16.8±2.7*
IVRT (ms)	*17.8±2.0*	*15.4±0.7*	*17.7±2.4*	*23.1±2.7#*
Mitral Valve Ejection Time (ms)	*49.3±3.9*	*45.3±3.6*	*42.4±4.5*	*40.6±5.1*
Myocardial Performance Index	*0.81±0.07*	*0.64±0.05*	*0.77±0.03*	*0.90±0.06#*

†These parameters were measured from specimen weighing during dissection; the remaining parameters were obtained via transthoracic echocardiography. Number of animals used for morphometric analysis during dissection: wild-type sham: n=5; wild-type TAC: n=7; knock-in sham: n=5; wild-type TAC: n=7; Data were obtained 8-weeks post-sham or post-TAC surgery; t-test: **p* <0.05, for the annotated group(s) compared with wild-type sham; t-test: ^*p* <0.05, for knock-in-TAC animals compared with knock-in-sham animals; t-test: #*p* <0.05, for knock-in-TAC animals compared with wild-type-TAC animals; Number of animals used for transthoracic echocardiography: wild-type-sham, n=11; knock-in-sham, n=10; wild-type-TAC, n=11; knock-in-TAC, n=12; TL, Tibial Length; LV, Left Ventricle; d, diastole; s, systole; cLV mass: corrected Left Ventricle mass; BW, body weight; IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; d, diastole; s, systole.

table S3. NMR-derived restraints and statistics of 20 NMR structures of wild-type Ig58.

	<20>	Best Structure
rmsd from distance constraints (Å)[2]		
total (1270)	0.027 ± 0.002	0.026
intraresidue (276)	0.005 ± 0.004	0.005
sequential ($\|i-j\|=1$) (345)	0.015 ± 0.004	0.012
medium range ($1<\|i-j\|\leq 1$) (109)	0.048 ± 0.007	0.045
long range ($\|i-j\|=1$) (472)	0.029 ± 0.003	0.029
hydrogen bonds (68)	0.049 ± 0.008	0.053
rmsd from exptl dihedral constraints (°)		
Φ, Ψ (169)	0.463 ± 0.142	0.690
rmsd from dipolar coupling restraints (Hz)		
D_{NH} (82)	0.74 ± 0.03	0.74
rmsd from exptl 13C chemical shifts		
13Ca (ppm)	1.34 ± 0.23	1.53
13Cb (ppm)	1.78 ± 0.19	1.73
rmsd from idealized geometry		
bonds (Å)	0.004 ± 0.001	0.004
angles (°)	0.691 ± 0.020	0.668
impropers (°)	0.618 ± 0.026	0.627
Lennard-Jones potential energy (kcal/mol)[3]	-350 ± 12	-340
Q-value[4]	0.27 ± 0.04	0.24
% most favorable region in the Ramachandran plot[5]	70.6 ± 2.9	72.2
rmsd of the mean structure (Å) [6]		
all backbone atoms (3-92)	0.471 ± 0.075	0.340
all heavy atoms (3-92)	1.088 ± 0.081	1.070

[1]The 20 ensemble structures, <20>, are the results of simulated annealing calculations. The best structure is the closest to the average structure. The values shown for the <20> are the mean ± standard deviation.

[2]None of the 20 structures has a distance violation > 0.4 Å or a dihedral angle violation of > 5°. The force constants used in the SA calculations are as follows: 1000 kcal mol $^{-1}$ Å^2 for bond length, 500 kcal mol^{-1} rad^{-2} for angles and improper torsions, 4 kcal mol^{-1} Å^{-4} for the quartic van der Waals (vdw) repulsion term (hard-sphere effective vdw set to 0.8 times their values in CHARMm parameters), 50 kcal mole^{-1} Å^{-2} for experimental distance constraints, 1 kcal mol^{-1} Å^{-2} for distance symmetry constraints, 0.5 kcal mol^{-1} ppm^{-2} for the ^{13}C chemical shift constraints, and 1.0 for the conformational database potential. The force constants (in kcal Hz^{-2}) used for dipolar coupling restraints is 0.60.

[3]Lennard-Jones van der Waals energies were calculated using CHARMm parameters and were not used in any stage of the structure determination.

[4]Q-values were determined by randomly removing 10% of all RDC values. To ensure accuracy, an ensemble of structures with a second randomly removed subset of RDCs was also run. The Q-value of this second set was similar to the first.

[5]PROCHECK was utilized to generate the Ramachandran plot.

[6]Backbone calculations include C$^{\alpha}$, N, and C′ atoms. Only residues 4339–4427 are included since no long-range NOE correlations were observed for residues 4337–4338 and 4428–4437.

table S4. Statistics of wild-type Ig58 crystal diffraction.

Parameters	Values
Wavelength (Å)	0.97918
Resolution range (Å)	37.64 - 2.411 (2.496 - 2.411)
Space group	P 61 2 2
Unit cell	44.482 44.482 177 90 90 120
Total reflections	11201 (1170)
Unique reflections	4329 (347)
Multiplicity	3.2 (3.4)
Completeness (%)	95.84 (80.14)
Mean I/sigma(I)	29.47 (3.11)
Wilson B-factor	66.54
R-merge	0.03344 (0.3173)
R-meas	0.04104
CC1/2	0.995 (0.931)
CC*	0.999 (0.982)
R-work	0.2463 (0.3529)
R-free	0.2649 (0.3225)
Number of non-hydrogen atoms	707
Macromolecules	706
Water	1
Protein residues	90
RMS(bonds)	0.020
RMS(angles)	2.08
Ramachandran favored (%)	84.6
Ramachandran allowed (%)	13.4
Ramachandran outliers (%)	0.0
Clash score	37.75
Average B-factor	81.40
Macromolecules	81.40
Solvent	77.70

The values in parentheses indicate numbers of restraints.

table S5. List of primers used for confirmation of gene targeting, animal genotyping, and site-directed mutagenesis.

Primer Name	Sequence	Expected Size of PCR product (bp)	Purpose
5'HR-Sense	5'CCATCCATTGTCCACTGATTATGCTCC3'	2068	To verify HR at the 5' end
5'HR-Anti-Sense	5'CCTGCTCTTTACTGAAGGCTCTTTACTATTGC3'		
3'HR-Sense	5'ATGCTCCAGACTGCCTTGGGAAAAG3'	6015	To verify HR at the 3' end and R4344Q mutation
3'HR-Anti-Sense	5'GTTTCATCTCCTTCCGCACTTTGTAACC3'		
Flp-Sense	5'ATCATAGAGCAGGACTGGACCGTAGCC3'	WT: 351 KI: 468	To verify NEO deletion and to genotype the animals
Flp-Anti-Sense	5'GTTTCATCTCCTTCCGCACTTTGTAACC3'		
R4344Q-Sense	5'AACACCGTTGTTCAGGGTCTGGAAAACGTTGAAGCG3'	NA	To introduce R4344Q into obscurin Ig58
R4344Q-Anti-sense	5'CGCTTCAACGTTTTCCAGACCCTGAACAACGGTGTT3'		

PCR: polymerase chain reaction; HR: homologous recombination; NEO: neomycin