Rothmund-Thomson Syndrome-like RECQL4 truncating mutations cause a haploinsufficient low bone mass phenotype in mice

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Abstract: 200
Number of Figures: 5

Conflict of Interest Statement: All authors declare no conflicts of interest.

Supporting Information

S1 Fig. Genotyping of point mutant alleles (A) Genomic DNA PCR plus MsI1 digest of *Recql4*$^{+/+}$ and *Recql4*$^{G522Efs/+}$. Two independent mice per genotype. (B) KASP genotyping of *Recql4*$^{+/+}$ and *Recql4*$^{R347X/+}$. HEX positive represents the R347X allele; FAM positive represents wild type allele. Two independent mice per genotype.

S2 Fig. RECQL4 does not localize with mitochondria and truncating RECQL4 mutations do not affect mitochondrial respiration. (A) Localization of mCherry (red) fused mouse RECQL4 and EGFP (green) fused human RECQL4 in Kusa4b10 cells. In the same cell lines, mitochondrial localization was determined by staining with Mitochondrial Staining Solution (MitoBlue, 1:500 diluted in HHBS). Images were colorized and merged. (B-E) Seahorse XF24-3 instrument analysis of oxygen consumption rate (OCR) as a reflection of mitochondrial respiration in HoxB8 immortalized *R26*-CreER^{T2} myeloid cells after tamoxifen-mediated *Recql4* deletion (day 4 after tamoxifen addition) in the following cell lines: (B) Δ/+; (C) Δ/K525A; (D) Δ/G522Efs; (E) Δ/R347X. Dotted lines represent isogenic controls, not treated with tamoxifen. Data expressed as mean ± SEM. No statistical significance achieved between the tamoxifen-treated and non-treated groups. The experiment was performed using triplicate wells and was independently executed two times using the same cell lines.

S3 Fig. The reduced size of the heterozygous *Recql4* mutants is not a result of a change in fat to lean mass ratio. Echo-MRI analysis of fat and lean percentage at ten weeks of age from males *Recql4*$^{+/+}$, *Recql4*$^{G522Efs/+}$, and *Recql4*$^{R347X/+}$ mice.

S4 Fig. Micro-CT analysis. Micro-CT measurements of germline 10-week old males *Recql4*$^{+/+}$, *Recql4*$^{K525A/K525A}$, *Recql4*$^{G522Efs/+}$, and *Recql4*$^{R347X/+}$ mice: (A) Bone volume.

(B) Cortical area. (C) Marrow area. Micro-CT measurements of 10-week old males *Osx*-Cre *Recql4*$^{fl/+}$, *Osx*-Cre *Recql4*$^{fl/K525A}$, *Osx*-Cre *Recql4*$^{fl/G522Efs}$, and *Osx*-Cre *Recql4*$^{fl/R347X}$ mice: (D) Bone volume. (E) Cortical area. (F) Marrow Area. Data expressed as mean ± SEM, Ordinary one-way ANOVA. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; n≥6 per genotype. Experiments were independently executed on separate cohorts, with results pooled for presentation. K=K525A; G=G522Efs; R=R347X.

S5 Fig. Heterozygous *Recql4* truncating mutations mildly affect myeloid progenitor populations. (A) Total leukocyte counts. (B) Hematopoietic stem cells (HSC) and primitive progenitors based on Lin-c-kit+Sca-1+CD105/CD150 staining per femur. (C) Pre-GM and GMP populations per femur. (D) HSC and primitive progenitors based on Lin-c-kit+Sca-1+CD34/Flt3 staining per femur. (E) Numbers of myelo-erythroid progenitors (MEP) per femur. (F) Erythroid (Pre-CFU-E, CFU-E) and megakaryocyte progenitor (MkP) frequency in the bone marrow and representative FACS plot. (G) Numbers of granulocytes and macrophages per femur. Data expressed as mean ± SEM, ordinary one-way ANOVA. *P<0.05; **P<0.01; ***P<0.001. Experiments were independently executed on separate cohorts, with results pooled for presentation. +/+=wild type, n=5; G=G522Efs, n=10; R=R347X, n=8; G/K=G522Efs/K525A, n=9; R/K=R347X/K525A, n=9.

S6 Fig. Effective deletion of *Recql4* floxed allele in murine mutants. (A) Genomic PCR from Hoxb8 immortalized *R26*-CreER^{T2} *Recql4*$^{fl/+}$, *R26*-CreER^{T2} *Recql4*$^{fl/K525A}$, *R26*-CreER^{T2} *Recql4*$^{fl/G522Efs}$, and *R26*-CreER^{T2} *Recql4*$^{fl/R347X}$ after addition of tamoxifen (400nM/mL) for 14 days demonstrating efficient gene deletion. True wild type (+/+) was used as control. (B-E) Independent replicate of proliferation curves of HoxB8 immortalized *R26*-CreER^{T2} myeloid cells without (fl) and with (Δ) tamoxifen-

mediated *Recql4* deletion in the following cell lines: (B) fl/+; (C) fl/K525A; (D) fl/G522Efs; (E) fl/R347X. Related to data in Figure 5A-5D.

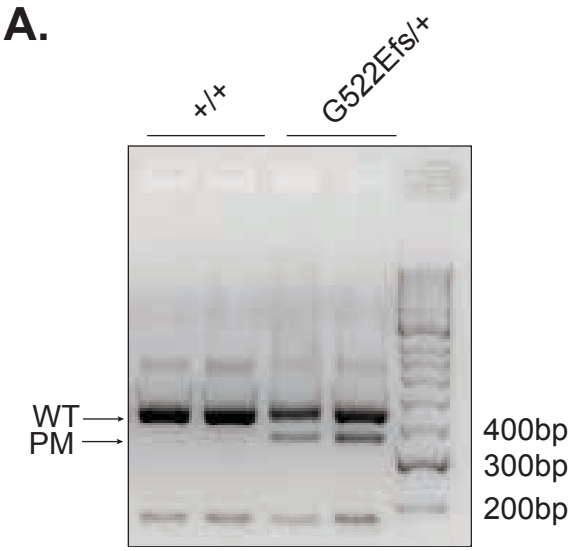
S7 Fig. Effective deletion of *Recql4* floxed alleles in cells expressing human constructs. Genomic PCR from Hoxb8 immortalized *R26*-CreER^{T2} *Recql4*$^{fl/f}$ cells expressing: (A) empty vector (control); or EGFP fused (B) wild type RECQL4; (C) R807Pfs; (D) Q757X; (E) L638P; (F) K508A; (G) C525Afs; (H) R350Gfs. Efficient gene deletion was achieved after the addition of tamoxifen (400nM/mL) for the indicated number of days.

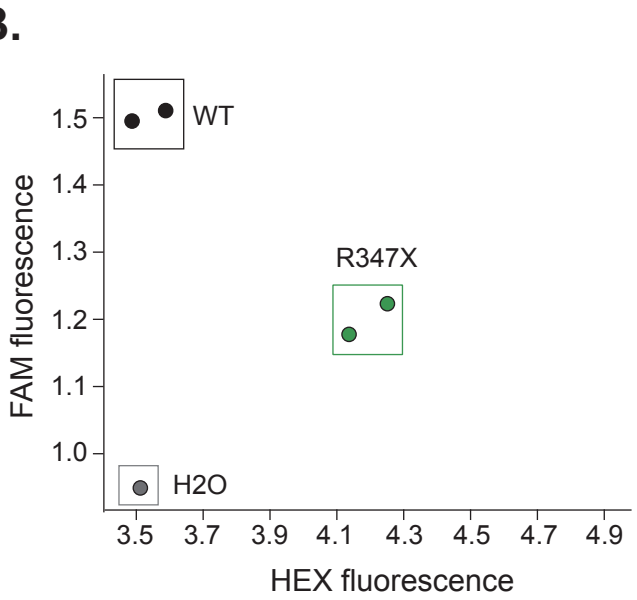
S8 Fig. Protein expression of human constructs. (A) Western blot from Kusa4b10 cells expressing EGFP fused RECQL4 wild type, K508A, R807Pfs, Q757X, L638P, C525Afs, and R350Gfs. Cells probed with anti-human/mouse RECQL4 (clone 3B1; top). The same blot re-probed with anti-Actin (bottom). (B) Expected protein product sizes.

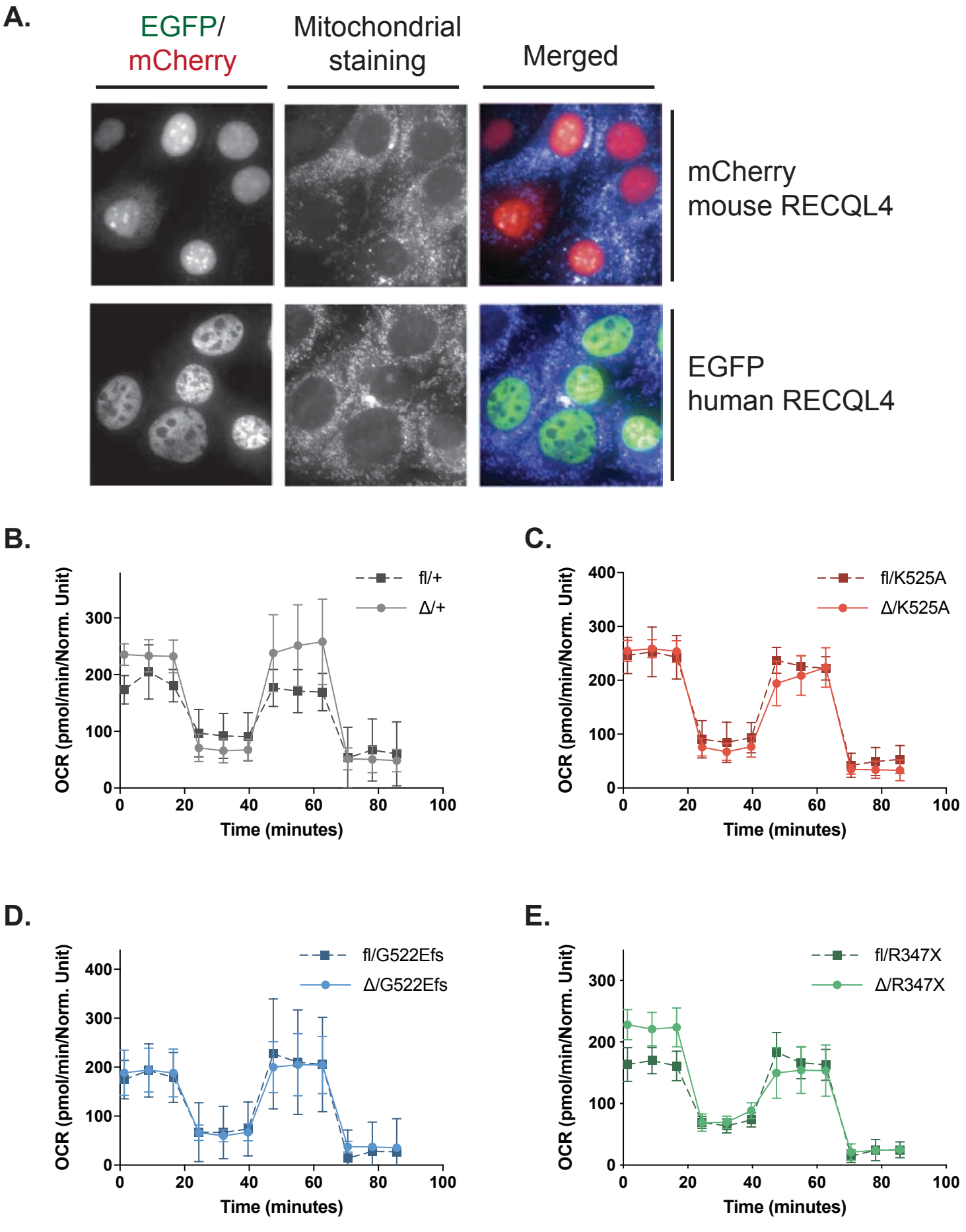
S9 Fig. Uncropped westerns for Figure 1D and Figure 1E.

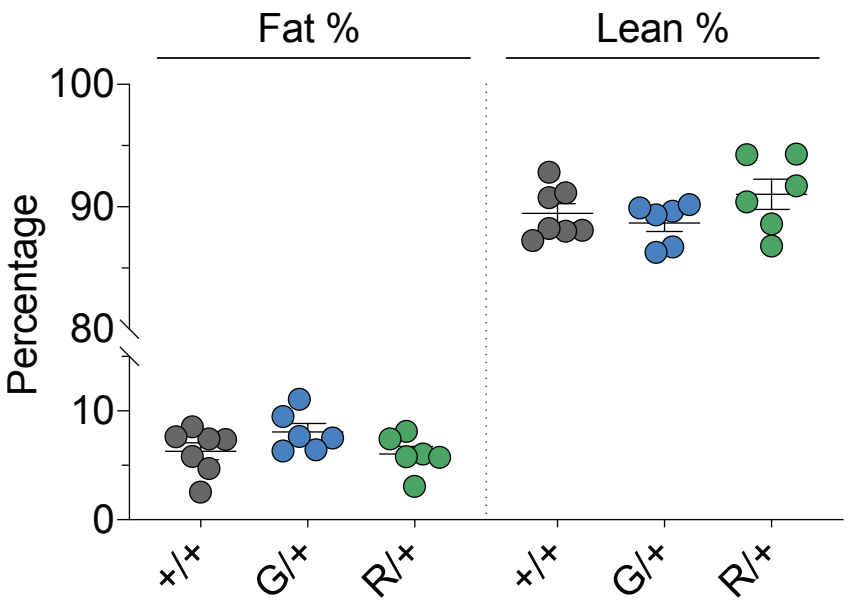
S1 Table. Tumors observed in mutant mice. List of tumors found during autopsy.

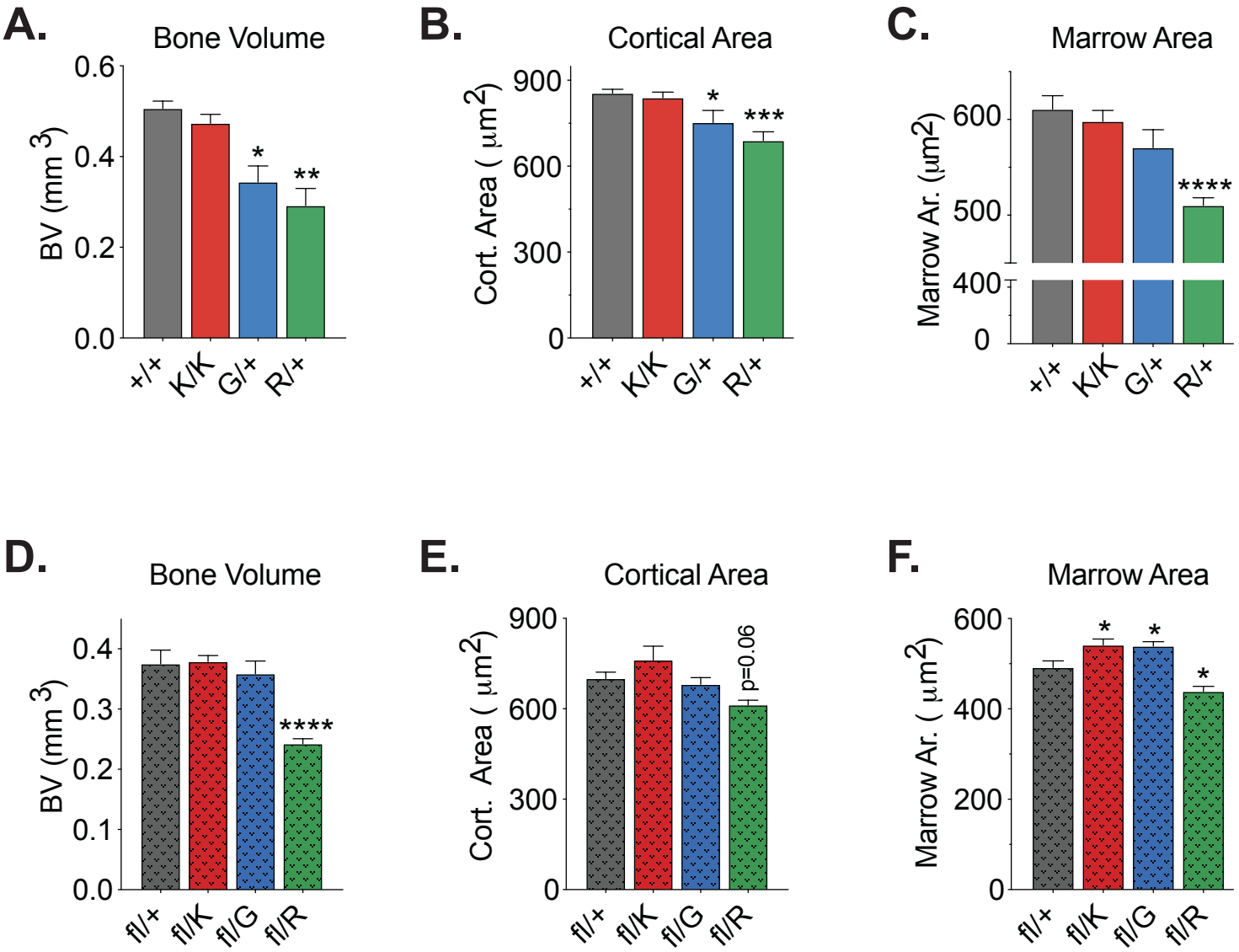
S2 Table. FACS Antibodies. List of antibodies used for flow cytometry in this study.

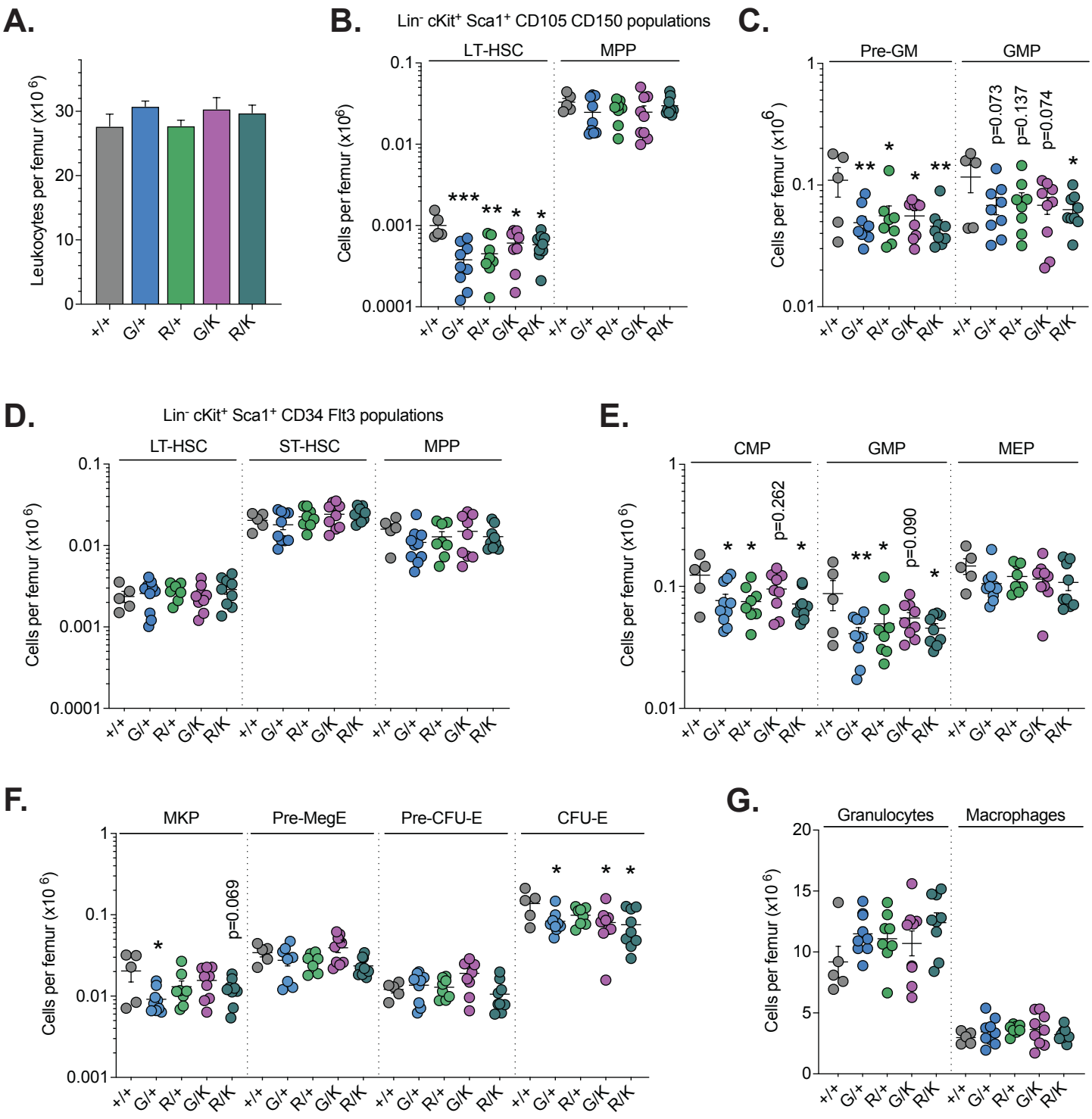

A.
+/+
G522Efs/+
WT
PM
400bp
300bp
200bp

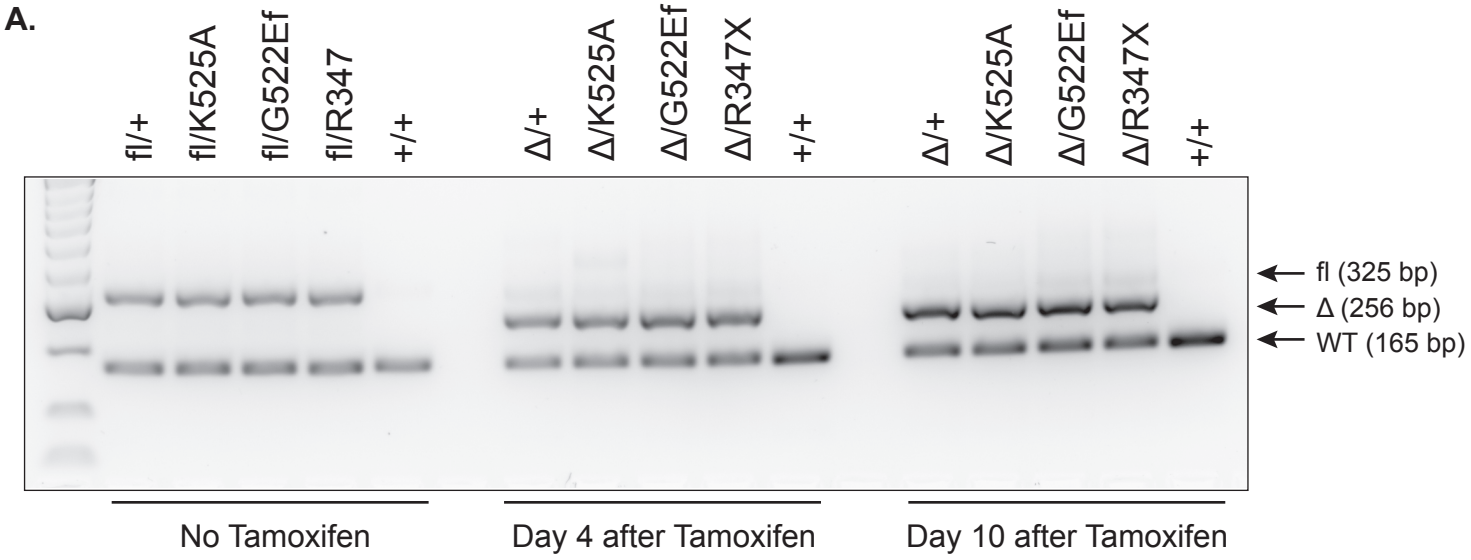

B.
WT
R347X
H2O
FAM fluorescence
HEX fluorescence
1.5
1.4
1.3
1.2
1.1
1.0
3.5
3.7
3.9
4.1
4.3
4.5
4.7
4.9

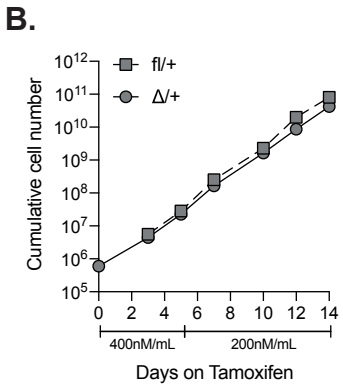

A.
EGFP/
mCherry
Mitochondrial
staining
Merged
mCherry
mouse RECQL4
EGFP
human RECQL4
B.
fl/+
Δ/+
OCR (pmol/min/Norm. Unit)
Time (minutes)
C.
fl/K525A
Δ/K525A
OCR (pmol/min/Norm. Unit)
Time (minutes)
D.
fl/G522Efs
Δ/G522Efs
OCR (pmol/min/Norm. Unit)
Time (minutes)
E.
fl/R347X
Δ/R347X
OCR (pmol/min/Norm. Unit)
Time (minutes)

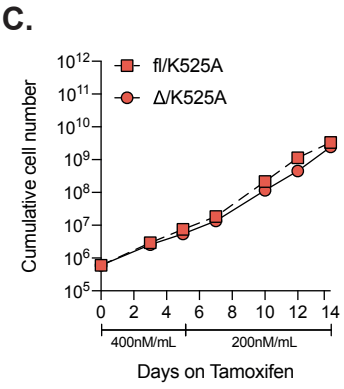

Fat %
Lean %
Percentage
100
90
80
10
0
+/+
G/+
R/+
+/+
G/+
R/+

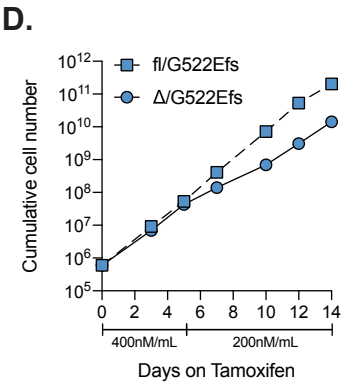

A.
Bone Volume
BV (mm3)
0.6
0.4
0.2
0.0
*
**
+/+
K/K
G/+
R/+
B.
Cortical Area
Cort. Area (μm2)
900
600
300
0
*
***
+/+
K/K
G/+
R/+
C.
Marrow Area
Marrow Ar. (μm2)
600
500
400
0
****
+/+
K/K
G/+
R/+
D.
Bone Volume
BV (mm3)
0.4
0.3
0.2
0.1
0.0
****
fl/+
fl/K
fl/G
fl/R
E.
Cortical Area
Cort. Area (μm2)
900
600
300
0
p=0.06
fl/+
fl/K
fl/G
fl/R
F.
Marrow Area
Marrow Ar. (μm2)
600
400
200
0
*
*
*
fl/+
fl/K
fl/G
fl/R

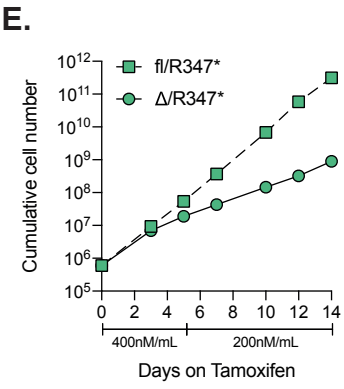

A.
Leukocytes per femur (x10 6)
0
10
20
30
+/+
G/+
R/+
G/K
R/K
B.
Lin- cKit+ Sca1+ CD105 CD150 populations
LT-HSC
MPP
Cells per femur (x10^6)
0.1
0.01
0.001
0.0001
***
**
*
*
C.
Pre-GM
GMP
Cells per femur (x10^6)
1
0.1
0.01
*
**
*
**
p=0.073
p=0.137
p=0.074
*
D.
Lin- cKit+ Sca1+ CD34 Flt3 populations
LT-HSC
ST-HSC
MPP
Cells per femur (x10 6)
0.1
0.01
0.001
0.0001
E.
CMP
GMP
MEP
Cells per femur (x10 6)
1
0.1
0.01
*
*
p=0.262
*
**
*
p=0.090
*
F.
MKP
Pre-MegE
Pre-CFU-E
CFU-E
Cells per femur (x10 6)
1
0.1
0.01
0.001
*
p=0.069
*
*
*
G.
Granulocytes
Macrophages
Cells per femur (x10 6)
20
15
10
5
0


A.
fl/+
fl/K525A
fl/G522Ef
fl/R347
+/+
Δ/+
Δ/K525A
Δ/G522Ef
Δ/R347X
+/+
Δ/+
Δ/K525A
Δ/G522Ef
Δ/R347X
+/+
fl (325 bp)
Δ (256 bp)
WT (165 bp)
No Tamoxifen
Day 4 after Tamoxifen
Day 10 after Tamoxifen

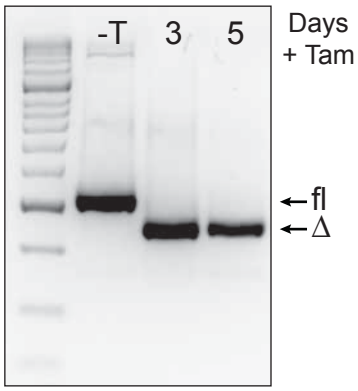

B.
fl/+
Δ/+
Cumulative cell number
Days on Tamoxifen
400nM/mL
200nM/mL


C.
fl/K525A
Δ/K525A
Cumulative cell number
Days on Tamoxifen
400nM/mL
200nM/mL

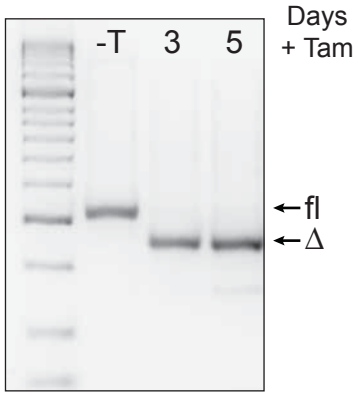

D.
fl/G522Efs
Δ/G522Efs
Cumulative cell number
Days on Tamoxifen
400nM/mL
200nM/mL


E.
fl/R347*
Δ/R347*
Cumulative cell number
Days on Tamoxifen
400nM/mL
200nM/mL

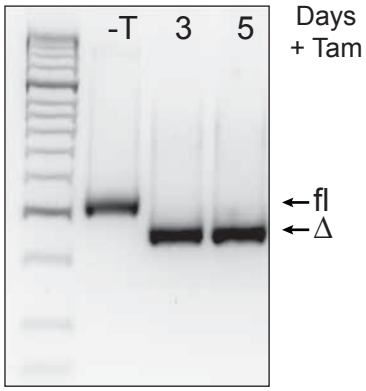
A. Empty vector (control)



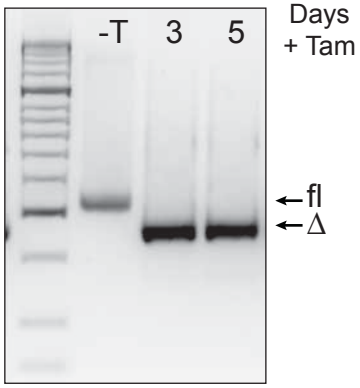
B. Wild type



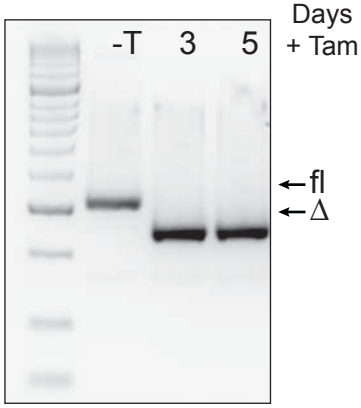
C. R807Pfs



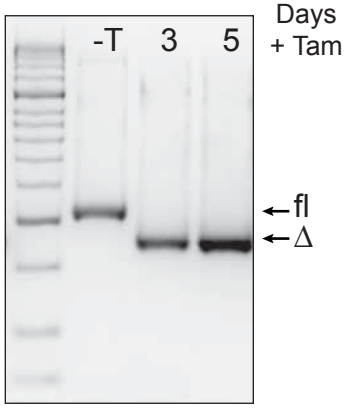
D. Q757X



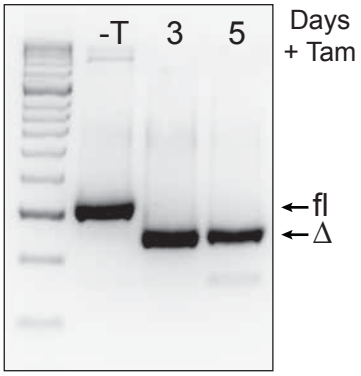
E. L638P



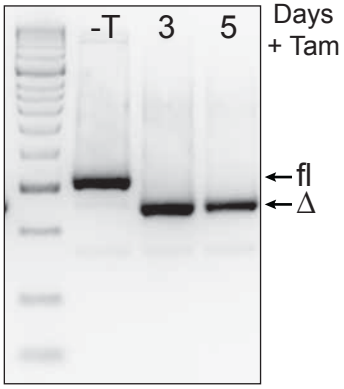
F. K508A



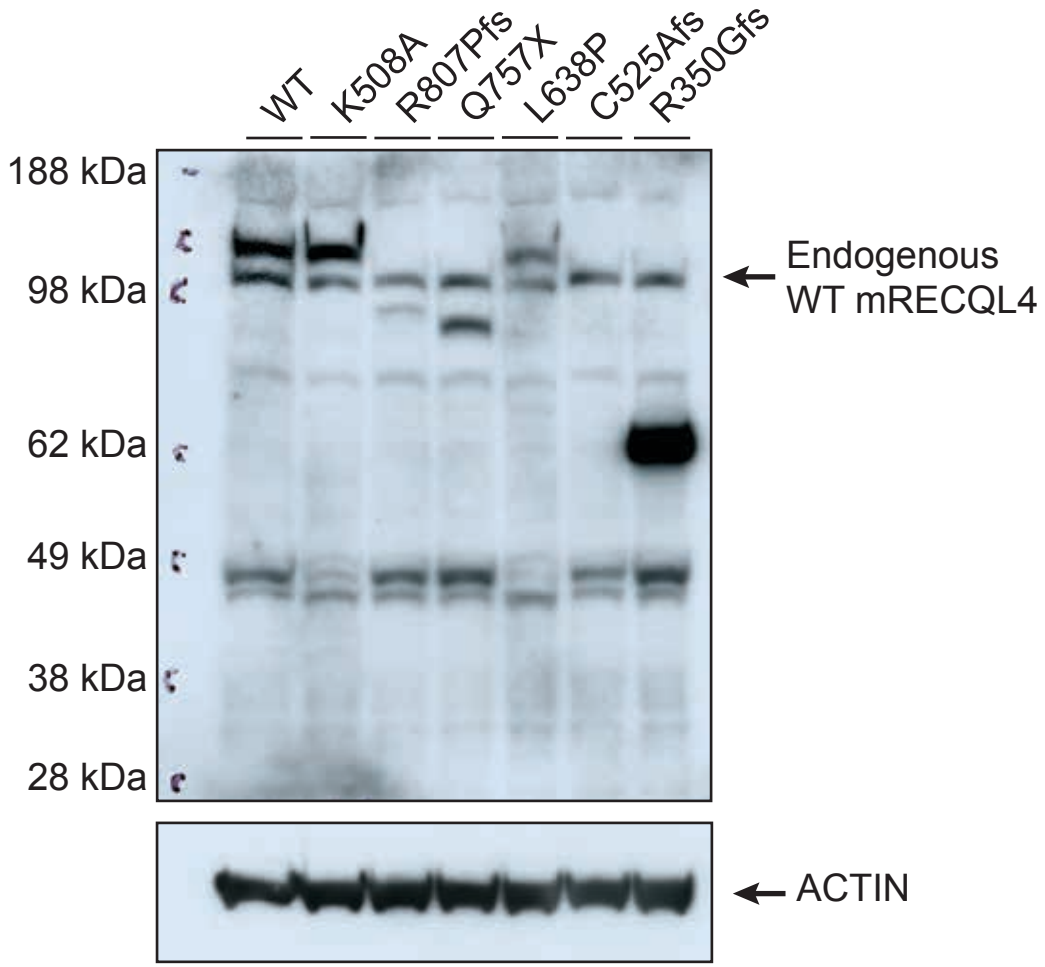
G. C525Afs



H. R350Gfs



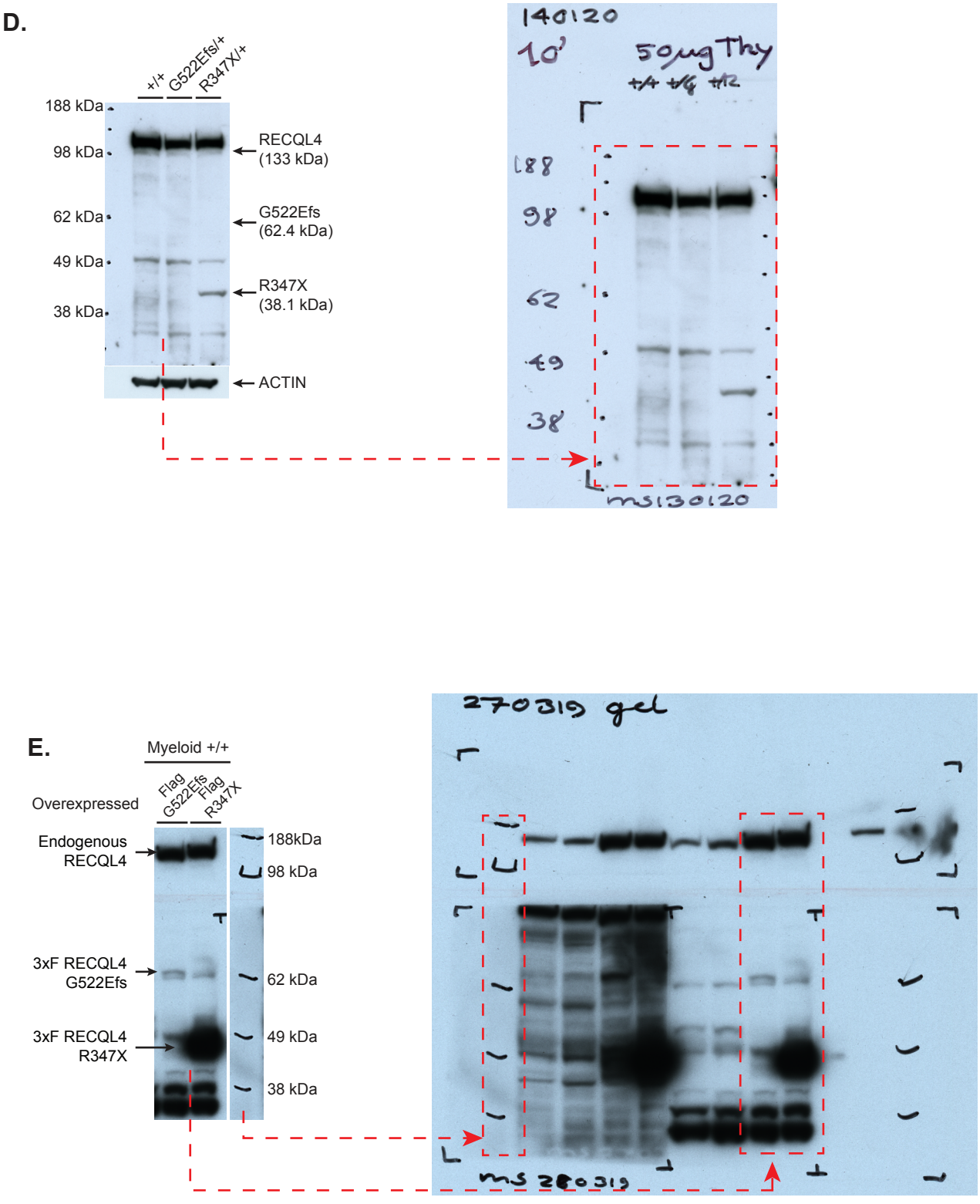
A.



B.

Human constructs	Expected protein size (kDa)
Endogenous mRECQL4 WT	135
EGFP hRECQL4 WT	161.8
EGFP hRECQL4 K508A	161.8
EGFP hRECQL4 R807Pfs	120.1
EGFP hRECQL4 Q757X	110.9
EGFP hRECQL4 L638P	161.8
EGFP hRECQL4 C525Afs	89.3
EGFP hRECQL4 R350Gfs	68.3

Uncropped westerns for Figure 1.



S1 Table. Tumors observed in mutant mice

Tumor type	Recql4$^{+/+}$	Recql4$^{K525A/+}$	Recql4$^{K525A/K525A}$	Recql4$^{G522/K525A}$	Recql4$^{R347X/K525A}$
Intestinal tumor	1	3	1	2	
Thymus tumor	2		2		1
Hepatic tumor	2	3	2	2	2
Lung tumor	1			1	
Spleen tumor				1	1
Mammary gland tumour			1		

S2 Table. FACS Antibodies (anti-mouse)

Antibody (clone)	Conjugate	Catalogue #	Company
Ter119 (TER-119)	PE	12-5921-83	Life Technologies Australia Pty Ltd/Thermo Fisher
CD71 (RI7 217.1.4)	APC	17-0711-82	Life Technologies Australia Pty Ltd/Thermo Fisher
B220/CD45R (RA3-6B2)	APC	17-0452-83	Life Technologies Australia Pty Ltd/Thermo Fisher
IgM (II/41)	Biotin	13-5790-82	Life Technologies Australia Pty Ltd/Thermo Fisher
CD43 (S7)	PE	553271	BD Pharmingen
CD19 (1D3)	PerCP-Cy5.5	45-0193-82	Life Technologies Australia Pty Ltd/Thermo Fisher
Mac1/CD11b (M1/70)	PE	12-0112-83	Life Technologies Australia Pty Ltd/Thermo Fisher
Gr-1/Ly6G (RB6-8C5)	PE-Cy7	25-5931-82	Life Technologies Australia Pty Ltd/Thermo Fisher
F4/80 (BM8.1)	APC	20-4801-U100	Tonbo Biosciences
CD4 (RM4-5)	eFluor-450	48-0042-82	Life Technologies Australia Pty Ltd/Thermo Fisher
CD8a/Ly-2 (53-6.7)	APC-eFluor 780	47-0081-82	Life Technologies Australia Pty Ltd/Thermo Fisher
TCRb (H57-597)	PE	12-5961-83	Life Technologies Australia Pty Ltd/Thermo Fisher
CD25 (PC61.5)	PE	12-0251-83	Life Technologies Australia Pty Ltd/Thermo Fisher
CD44 (IM7)	APC	17-0441-83	Life Technologies Australia Pty Ltd/Thermo Fisher
Sca-1 (D7)	APC	17-5981-82	Life Technologies Australia Pty Ltd/Thermo Fisher
c-Kit/CD117 (2B8)	APC-eFluor-780	47-1171-82	Life Technologies Australia Pty Ltd/Thermo Fisher
CD34 (RAM34)	eFluor-660	50-0341-82	Life Technologies Australia Pty Ltd/Thermo Fisher
CD135/Flk2/Flt3 (A2F10)	PE	12-1351-82	Life Technologies Australia Pty Ltd/Thermo Fisher
FcgR/CD16/32 (93)	PerCP-Cy5.5	45-0161-82	Life Technologies Australia Pty Ltd/Thermo Fisher
CD41 (MWReg30)	eFluor-450	48-0411-82	Life Technologies Australia Pty Ltd/Thermo Fisher
CD105 (MJ7/18)	PE-Cy7	120409	Biolegend
CD150 (TC15-12F12.2)	PE	115904	Biolegend
Streptavidin	BV 605	563260	BD Pharmingen