Bettini *et al.*, Figure S1

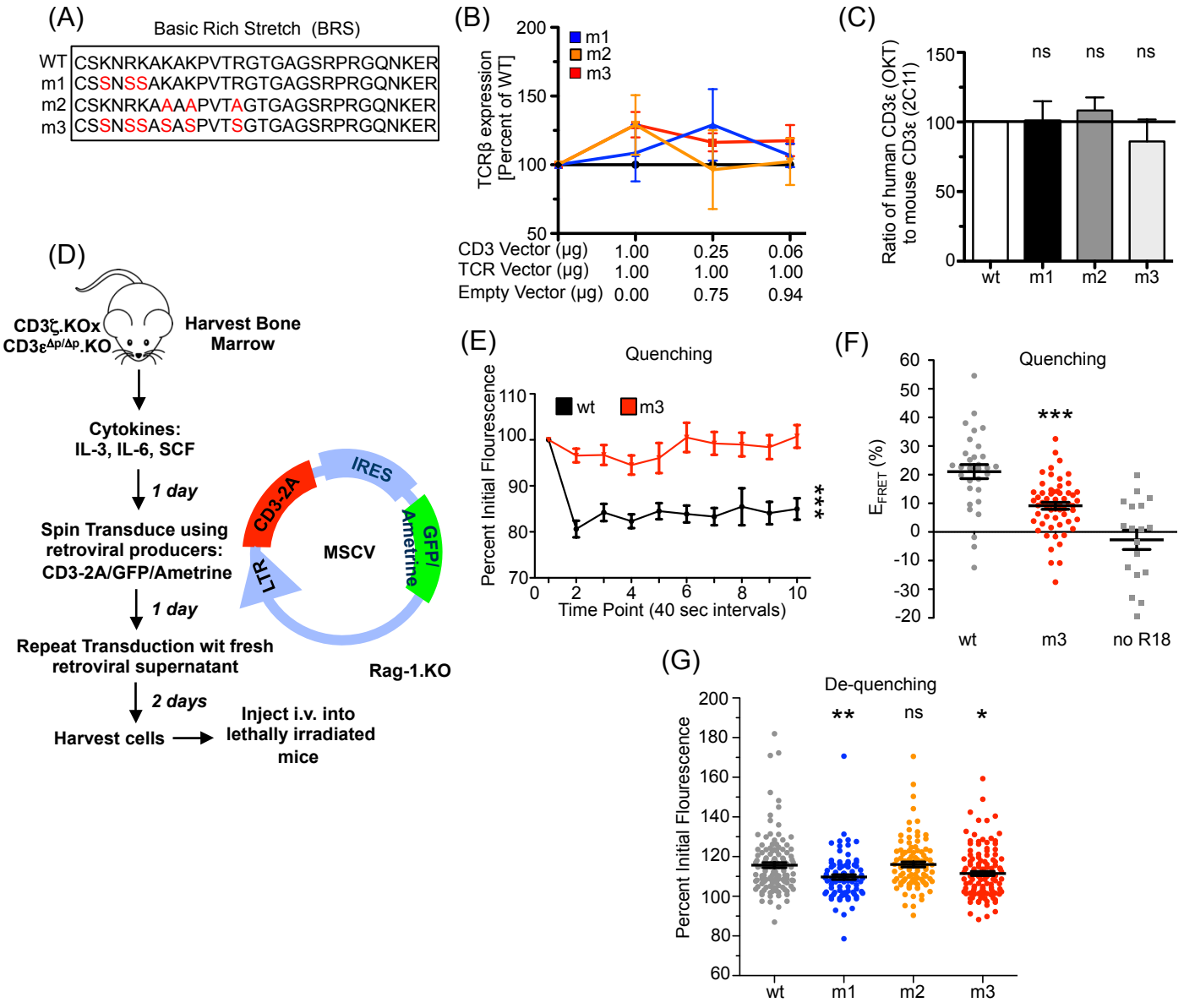


Figure S1: Mutations in the CD3ε basic rich stretch impair lipid binding *in vitro*

(A) Basic amino acids mutated to neutral charge are highlighted in red.

(B) 293T cells were transfected with titrating amounts of CD3ε chain plamsid DNA along with constant amount of AND TCR. TCR expression was normalized to CD3ε^{wt} chain. n=3 independent experiments. *p<0.05 for CD3ε^{m1} at 1µg CD3 vector.

(C)Phogrin-4 cells were transduced with chimeric CD3ε chain encoding the OKT human CD3ε ecto domain and mouse CD3ε cytosolic domain that encodes either m1, m2, m3 or wild-type basic anchor residues and empty vector. Efficiency of TCRβ/human CD3ε chain pairing or TCRβ/ mouse CD3ε chain pairing and expression was measured by OKT and 2C11 staining. The ratio of expression was determined for each experiment and compared to wild type. Shown is the mean of 4 independent experiments.

(D) Schematic representation of the CD3-2A constructs in the MSCV-IRES-GFP (pMIG) or the MSCV-IRES-Ametrine (pMIA) vector and a diagram depicting the retroviral stem cell gene transfer technique.

(E) Rag$^{-/-}$ mice were reconstituted with CD3εζ KO bone marrow transduced with either CD3ε^{wt}-TFP or CD3ε^{m3}-TFP retrovirus. After 5 weeks peripheral CD4^{+}CD25^{-} T cells were isolated by cell sorting. Lipid membrane dye (DIL) was added (quenching CD3ε-TFP in membrane) then at designated time, laser bleach the dye and calculate percent recovery of TFP in membrane (dequenching) (** p<0.001, *p<0.01 by one-way ANOVA, Dunn's Multiple Comparison Test).

(F)T cells expressing either AND TCR CD3ε^{wt}-TFP or CD3ε^{m3}-TFP were isolated as described in E. Quenching E_{FRET} (%) was calculated as the change in TFP fluorescence at the plasma membrane between pre- and post-R18 labeling scans. CD3ε^{m3}-TFP exhibits less quenching than CD3ε^{wt}-TFP (21% and 9.1%, respectively), while no TFP quenching is observed in the absence of R18 acceptor (no R18) (n=31 cells for CD3ε^{wt}-TFP; n=59 for CD3ε^{m3}-TFP; n=18 for no R18; *** p<0.0001 by unpaired two tailed t test).

(G) CD3ε-TFP fluorescence was determined and then a lipid membrane dye was added (time 0) to determine percent loss of TFP (Quenching). If CD3ε-TFP is in close proximity of the lipid dye, quenching will occur or in the case of laser bleaching, fluorescent de-quenching will occur. *** p<0.0001 by one-way analysis of variance (n=80-100 cells per group).

Bettini *et al.*, Figure S2

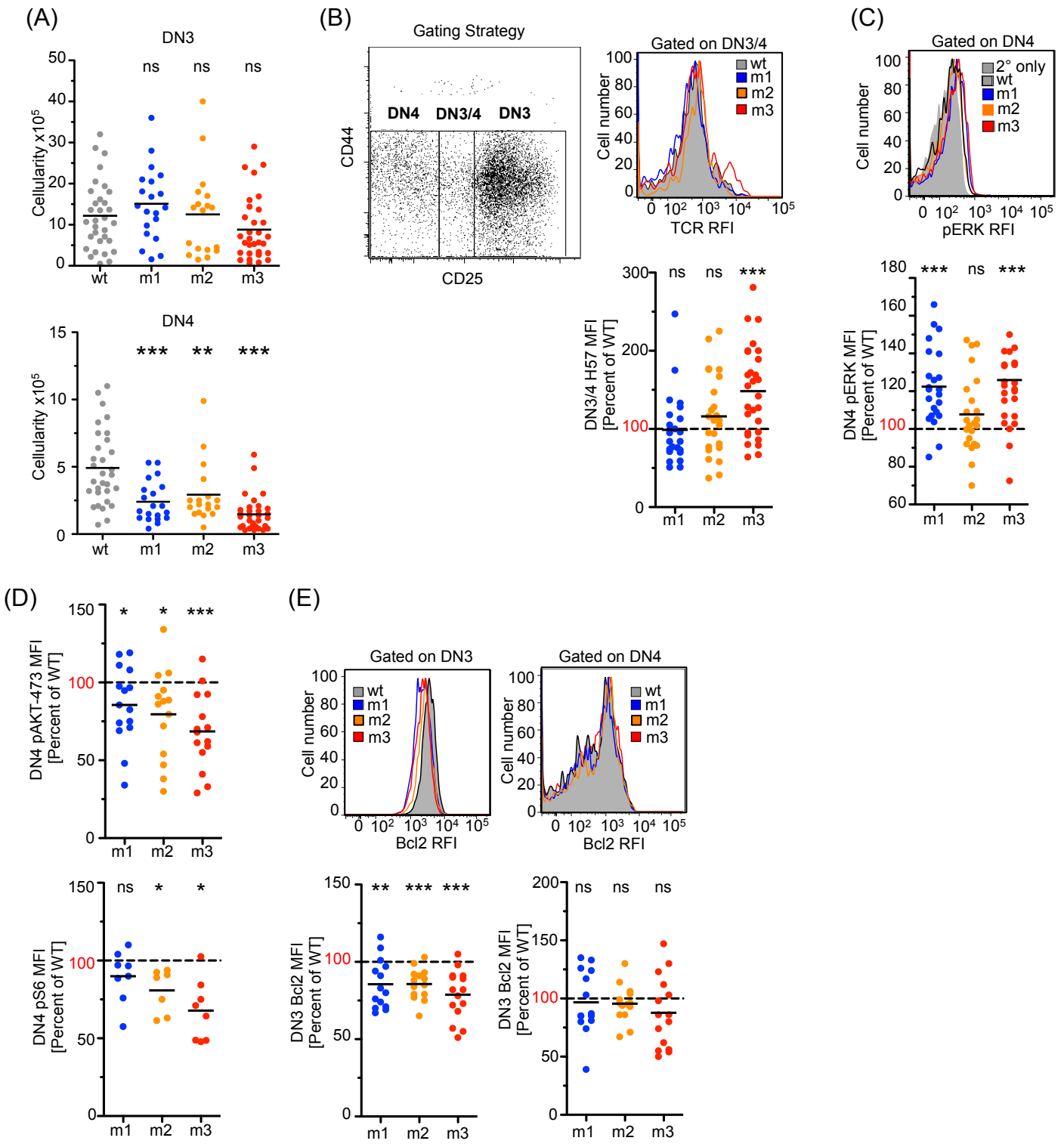


Figure S2: Mutations in the CD3ε basic rich stretch impact TCR signaling and survival

(A) Total cellularity was calculated for the DN3 and DN4 thymocyte population.
(B) Gating strategy for DN3/4 thymocytes. DN3/4 and DN4 thymocytes were analyzed for TCRβ expression (n=18-20 mice per group). RFI-Relative Fluorescent Intensity.
(C) DN4 thymocytes were analyzed for basal pERK and (n=18-20 per group). RFI-Relative Fluorescent Intensity.
(D) DN4 thymocytes were analyzed for basal pAKT-473 and pS6 (n=9-14 per group).
(E) DN3 and DN4 thymocytes were analyzed for Bcl-2 expression (n=14). RFI-Relative Fluorescent Intensity.

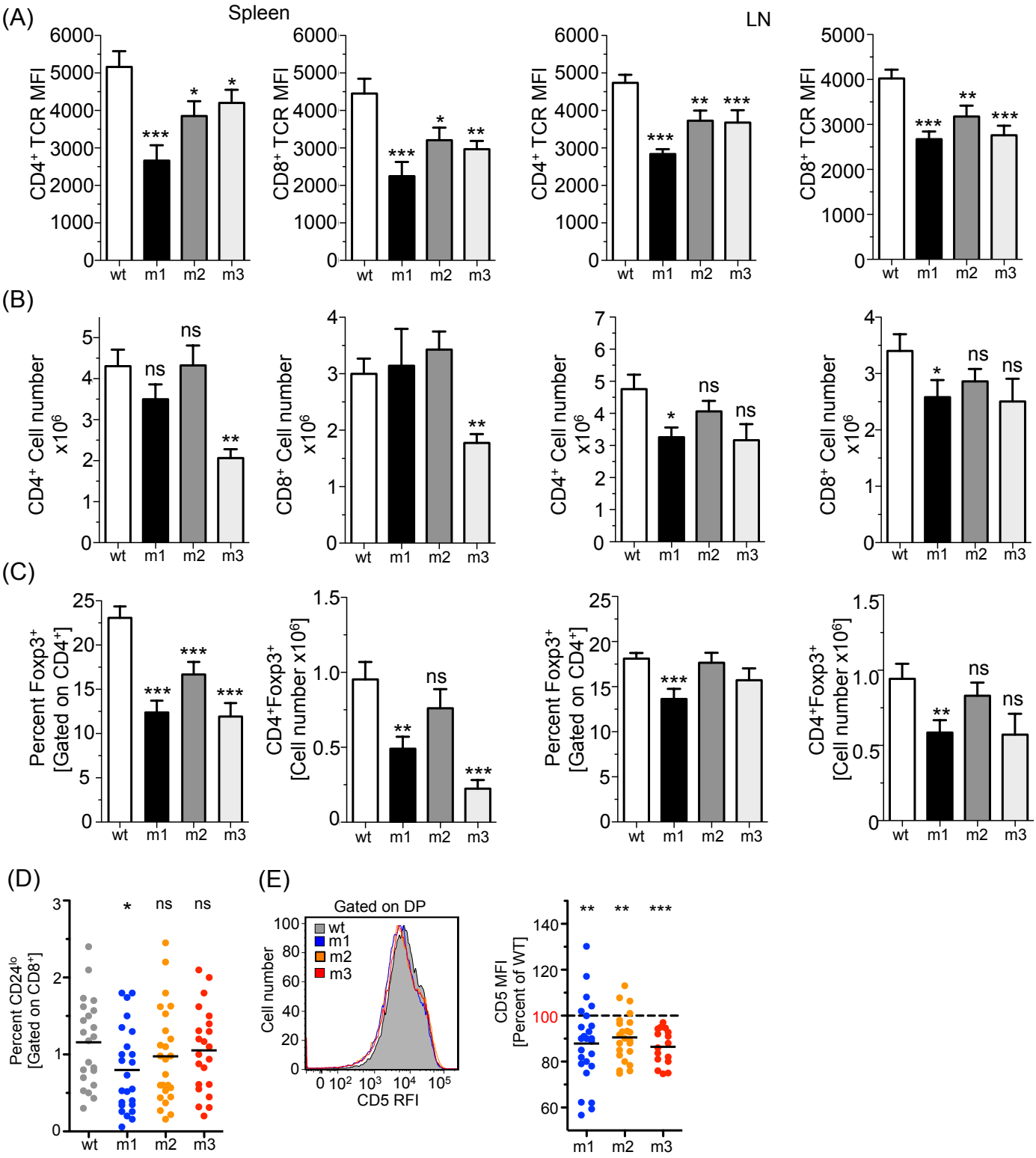


Figure S3: Mutations in the CD3ε basic rich stretch impact thymocyte maturation

(A) Peripheral CD4⁺ and CD8⁺ ratios and cell numbers were calculated for each *Rag*$^{-/-}$ mouse reconstituted with CD3ε^{wt} and CD3ε mutant transduced bone marrow

(B) The ratio and cell number of Foxp3⁺CD4⁺ cells found in the spleen and peripheral lymph nodes

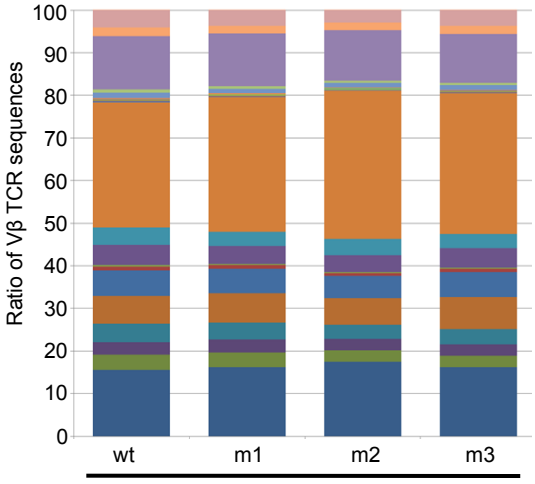
(C) TCR expression was measured for CD4⁺ and CD8⁺ T cells in the spleen and peripheral lymph nodes. Statistical Analysis was performed using Mann Whitney t-test.

(D) Total mature CD8 thymocyte ratios (CD24^{lo} CD8⁺) were calculate. Combined data are shown (n=24-31 mice per group) from at least 5 independent experiments

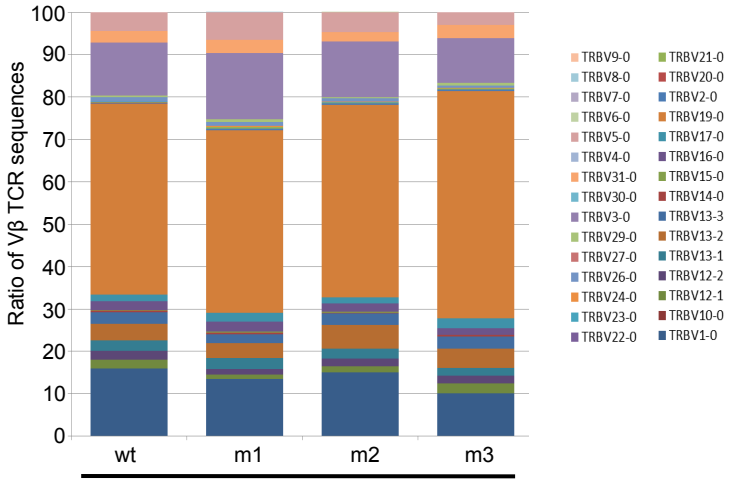
(E) Lower CD5 expression within the DP thymocytes further indicates lower basal TCR signaling in CD3ε mutant cells. CD4⁺CD8⁺ DP thymocytes were analyzed for CD5 expression. Representative and combined data shown (n=16-25 mice per group) from at least 5 independent experiments. RFI-Relative Fluorescent Intensity.

Bettini *et al.*, Figure S4

(A)



(B)



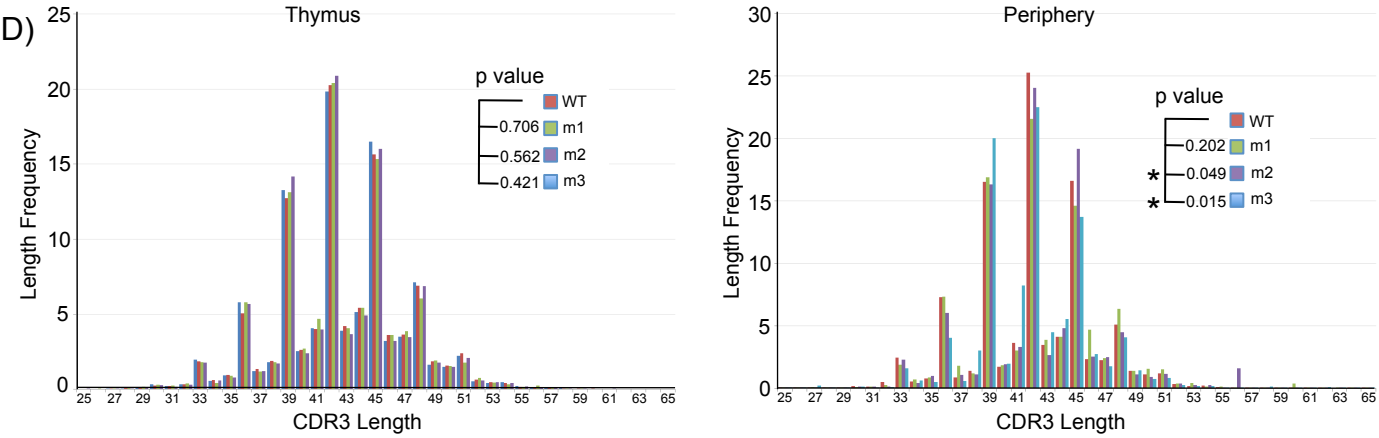
(C)

Number of productive reads

Mouse	wt	m1	m2	m3
1.	3,946,799	2,343,623	664,005	1,890,568
2.	799,416	1,449,476	1,382,081	2,382,024
3.	856,125	1,120,593	594,757	3,518,072

wt	m1	m2	m3
1,332,205	115,253	1,690,854	787,318
390,286	737,590	489,541	373,256
140,865	1,025,343	432,179	1,327,626

(D)



(E)

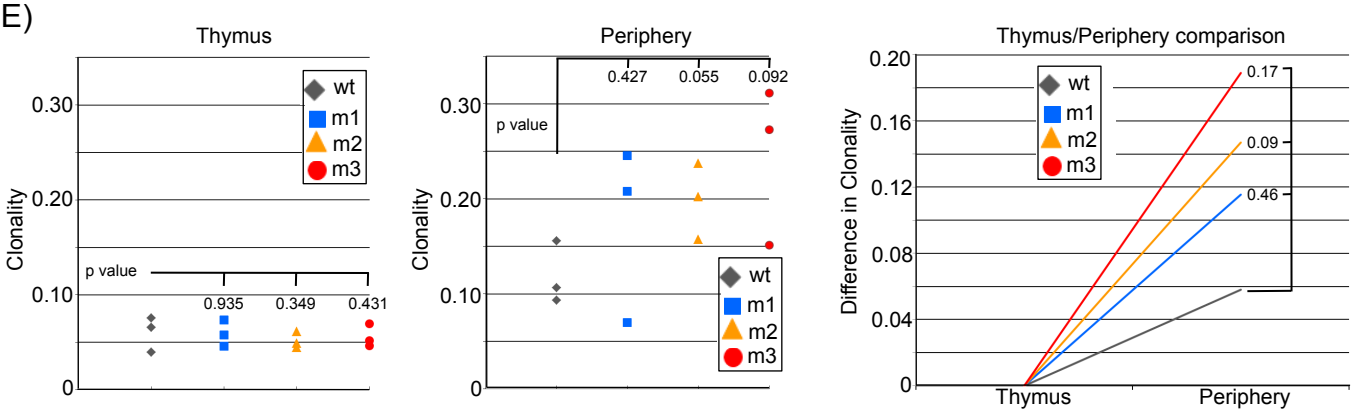


Figure S4: Mutations in the CD3ε basic rich stretch does not impact thymocyte diversity but impacts clonality

(A) CD4$^+$CD24^{lo}TCR$^+$ thymocytes and CD4$^+$CD25$^-$ TCR$^+$ spleen cells. (B) were sorted, pelleted and snap frozen then sent to Adaptive biotechnology for Vβ TCR sequencing. Ratios of each Vβ are indicated for individual mice in stacked bar graph (n=3 mice per group). (C) Total number of productive reads for each mouse in the thymus and periphery. (D) The average CDR3 length was determined for each group in the thymus and periphery. The average CDR3 amino acid length of the TCRs is significantly shorter (p<0.001) in peripheral CD4$^+$ CD3 T cells (p<0.001). (E) Clonality differences were determined between each group for each organ and by comparing matched thymus and peripheral samples (n=3 mice per group).