Statistics and Reproducibility

Figure 1
    b) n=4 biologically independent samples from guide redundancy. Statistics were derived using MAGeCK software[7] which utilized robust rank aggregation for gene level analyses. Data for this analysis can be found in Supplementary Table 1. This experiment was performed once.
    c) This experiment was performed once.
    d) n=3; except CD4 n=1 biologically independent samples. This experiment was performed four times with similar results. Usp22 ***P=0.0001, Atxn7l3 ***P=0.0003, Runx1 **P=0.0043, Cbfb ****P<0.0001 by one-way ANOVA with Dunnett's multiple comparison's test. This experiment was performed 5 times with similar results.
    e) This experiment was performed 5 times with similar results, with n=2 biologically independent samples each time.
    f) n=10 biologically independent samples. ****P<0.0001 by two-tailed paired t-test. This experiment was performed 5 times with similar results.

Figure 2
    a) This experiment was performed 3 times with similar results.
    b) This experiment was performed 3 times with similar results.
    c) n=4 biologically independent animals. Thy ***P=0.000570, pLN **P=0.003046, Spl ***P=0.000105 by Holm-Sidak's multiple comparisons test. This experiment was performed 3 times with similar results.
    d) n=5 biologically independent animals. ns indicates no significant difference, 1:0 P>0.9999, 1:16 P=0.1342, 1:8 *P=0.0161, 1:4 **P=0.0085 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 5 times with similar results.
    e) This experiment was performed 3 times with similar results.
    f) n=3 biologically independent animals. **P=0.0013 by two-tailed paired t-test. This experiment was performed 2 times with similar results.
    g) n=5 biologically independent animals. Statistics were derived using DESeq2 software which utilized negative binomial generalized linear models to test for differential expression. Source data for this analysis can be found in Supplementary Table 5. This experiment was performed once.
    h) H2BK120Ub ChIP: Usp22 WT n=2, Usp22 KO n=3 biologically independent animals, NTC RNP n=3, Usp22 RNP n=2, Rnf20 RNP n=2 biologically independent samples. This experiment was performed 2 times with similar results.
    i) n=3 biologically independent samples. ns indicates no significant difference. WT/NTC vs. WT/Rnf20 ns t=0.1943, WT/NTC vs. KO/NTC **t=4.997, WT/NTC vs. KO/Rnf20 ns t=0.7301, WT/Rnf20 vs. KO/NTC **t=5.191, WT/Rnf20 vs. KO/Rnf20 ns t=0.9245, KO/NTC vs. KO/Rnf20 *t=4.267 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 2 times with similar results.
    j) This experiment was performed 2 times with similar results.

Figure 3
a) Weeks 4, 5: n=3; Weeks 6, 7: WT n=7, KO n=9; Weeks 8, 9, 10: WT n=6, KO n=8; Weeks 11-19: WT n=3, KO n=2; Weeks 20-23: n=2 biologically independent animals. Statistics not shown due to low sample size after 10 weeks. Week 4 P=0.9828, Week 5 P=0.0029, Week 6 P=0.0008, Week 7 P=0.0003, Week 8 P=0.0004, Week 9 P=0.0089, Week 10 P=0.0112, Week 11 P=0.0022, Week 12 P=0.0002, Week 13 P=0.0002, Week 14 P=0.0061, Week 15 P=0.0037, Week 16 P=<0.0001, Week 17 P=0.0006, Week 18 P=0.0061, Week 19 P=0.0100, Week 20 P=0.0057, Week 21 P=0.0089, Week 22 P=0.0042, Week 23 P=0.0022. This experiment was performed 2 times with similar results.
b) This experiment was performed 2 times with similar results.
c) T naïve, WT Treg + T naïve: n=5; KO Treg + T naïve: n=4 biologically independent animals. Week 5, Week 7 *P=0.0159, Week 6 *P=0.0317 by two-tailed Mann Whitney test. This experiment was performed 2 times with similar results.
d) This experiment was performed 2 times with similar results.
e) Weeks 1-13: n=13; weeks 16-20: n=9 biologically independent animals. *P=0.0332, **P=0.0037 ***P=0.0007 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 2 times with similar results.
f) Days 3, 4, 19: WT n=5, KO n=4. Days 6-18: n=9 biologically independent animals. Day 16 **P=0.0013, Day 18 ***P=0.0002, Day 19 **P=0.0064 by two-way ANOVA with Sidak's multiple comparisons test.
g) WT: n=5, KO: n=4 biologically independent animals. This experiment was performed once.
h) WT: n=5, KO: n=4 biologically independent animals. LN CD4 P>0.9999, LN CD8 P=0.9998; Spl *P=0.0368, **P=0.0049 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed once.
i) WT: n=4, KO: n=3 biologically independent animals. ns indicates no significant difference. ns P=0.430, **P=0.0056 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed once.
j) WT: n=7, KO: n=5 biologically independent animals. *P=0.0406 by two-tailed paired t test. This experiment was performed once.
k) WT: n=7, KO: n=5 biologically independent animals. **P=0.0053 by two-tailed paired t test. This experiment was performed once.

Extended Data Figure 1
d-g) These experiments were performed once.

Extended Data Figure 2
b) Foxp3, NTC n=2; Usp22, Atxn7l3, Runx1, Cbfb n=3; CD4 n=1 biologically independent samples. This experiment was performed 5 times with similar results.
c) Foxp3, NTC n=2; Usp22, Atxn7l3, Runx1, Cbfb n=3; CD4 n=1 biologically independent samples. This experiment was performed 5 times with similar results.
d) NTC n=7; Usp22-3, Foxp3-2 n=3; Usp22-1/2, Atxn7l3-1, Cbfb-1/3, Runx1-3, Rnf20-1/2, Foxp3-1/3 n=2; Atxn7l3-2/3/4, Cbfb-2, Runx1-1/2/4, Rnf20-3 n=1 technically independent samples. Usp22-3 vs. NTC P=0.0021; Foxp3-2 vs. NTC

P=0.0074 by two-tailed unpaired t-test. This experiment was performed 2 times with similar results.

e) This experiment was performed 5 times with similar results.

f) n=10 biologically independent samples. **P=0.0029 by two-tailed paired t-test. This experiment was performed 5 times with similar results, n=2 each time.

g) n=10 biologically independent samples. ****P<0.0001 by two-tailed paired t-test. This experiment was performed 5 times with similar results, n=2 each time.

h) NTC n=7; USP22-2 n=10; USP22-1 n=8; USP22-3/4 n=6; RNF20-1, FOXP3-1, FOXP3-3 n=4; ATXN7L3-1/2/3, RNF20-2, FOXP3-2, USP22-1+RNF20-1, USP22-2+RNF20-3 n=2 biologically independent samples. USP22-1 ***P=0.0002; USP22-2 ****P<0.0001; USP22-3 *P=0.0365; USP22-4 P>0.9999; RNF20-1/3 P>0.9999; FOXP3-1 ****P<0.0001; FOXP3-3 P=0.0004 by one-way ANOVA with Sidak's multiple comparison's test. Statistics are based on comparison to non-targeting control (NTC). This experiment was performed 2 times with similar results.

i) USP22-1/2 n=4; USP22-3/4 n=2 biologically independent samples. P<0.0001 by simple linear regression. This experiment was performed 2 times with similar results.

j) FOXP3, USP22 n=8; ATXN7L3 n=2; NTC n=10; RNF20 n=3; USP22+RN20 n=3; CD4 n=4 biologically independent samples. FOXP3 **P=0.0041; USP22 **P=0.0022; ATXN7L3 P=0.6294; RNF20 P=0.7985; USP22+RNF20 P=9866; CD4 P=0.9943; USP22 vs. USP22+RNF20 **P=0.0094 by one-way ANOVA with Sidak's multiple comparison's test. Statistics are based on comparison to non-targeting control (NTC) except USP22 vs. USP22+RNF20. This experiment was performed 2 times with similar results.

Extended Data Figure 3

b) Source data can be found in **Supplementary Figure 1**. This experiment was performed for genotyping of $Usp22^{fl/fl}$ mice, 20+ times.

c) Source data can be found in **Supplementary Figure 1**. This experiment was performed 3 times with similar results.

d) n=4 biologically independent animals. Thy, Spl *P=0.026, pLN **P=0.005 by Holm-Sidak's multiple comparisons test. This experiment was performed 3 times with similar results.

e) Source data can be found in **Supplementary Figure 1**. This experiment was performed 2 times with similar results.

f) This experiment was performed 2 times with similar results.

g) n=5 biologically independent animals. 10ng/ul P=0.4165, 5ng/ul P=0.1078, 1ng/ul *P=0.0350 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 2 times with similar results.

h) This experiment was performed 5 times with similar results.

i) This experiment was performed 3 times with similar results.

j) n=6 biologically independent animals. WT-Control vs. KO-Control *P=0.0189, KO-Control vs. KO-Foxp3 **P=0.0024 by two-tailed paired t-test. This experiment was performed 3 times with similar results.

Extended Data Figure 4
   a) Source data can be found in **Supplementary Figure 1**. This experiment was performed 2 times with similar results.
   b) Source data can be found in **Supplementary Figure 1**. This experiment was performed 3 times with similar results.
   c) This experiment was performed 2 times with similar results.
      n=3 biologically independent animals. 15min P=0.3818, 30min P=0.1074, 45min *P=0.0114 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 2 times with similar results.

Extended Data Figure 5
   a) This experiment was performed 3 times with similar results.
   b) Thy n=3; pLN, Spl n=4 biologically independent animals. Thy *P=0.0348, Spl **P < 0.0078, pLN ****P < 0.0001 by Holm-Sidak's multiple comparisons test. This experiment was performed 3 times with similar results.
   c) Thy n=3; pLN, Spl n=4 biologically independent animals. Thy P=0.070, pLN P=0.729, Spl P=0.729 by Holm-Sidak's multiple comparisons test. This experiment was performed 3 times with similar results.
   d) n=2 biologically independent animals. Statistics were derived using DESeq2 software which utilized negative binomial generalized linear models to test for differential expression. Source data for this analysis can be found in **Supplementary Table 5**. This experiment was performed once.
   e) NTC n=2 biologically independent samples with 3 technically independent replicates for each; FOXP3 n=4 biologically independent samples (2 independent donors with 2 unique gRNAs) with 3 technically independent replicates for each; RNF20 n=2 biologically independent samples with 3 technically independent replicates for each; USP22+RNF20 n=3 biologically independent samples (2 independent donors with 2 unique gRNAs for 1 donor) with 3 technically independent replicates for each. FOXP3 ***P=0.0001, USP22 *P=0.0217, RNF20 P=0.9771, USP22+RNF20 *P=0.0355 by one-way ANOVA with Dunnett's multiple comparison's test. This experiment was performed once.
   f) n=2 biologically independent samples with 3 technically independent replicates for each, except NTC n=3 technically independent replicates. Day4-Foxp3 ****P<0.0001, Day4-Usp22 *P < 0.0136, Day4-Rnf20 P=0.9202, Day4-Usp22+Rnf20 P=0.2281, Day8-Foxp3 ****P<0.0001, Day8-Usp22 ****P<0.0001, Day8-Rnf20 **P=0.0037, Day8-Usp22+Rnf20 ***P=0.0004 by one-way ANOVA with Dunnett's multiple comparison's test. This experiment was performed once.
   g) Source data can be found in **Supplementary Figure 1**. This experiment was performed 3 times with similar results.
   i) n=3 biologically independent animals. TSS P>0.9999, CNS1 P=0.40 by two-tailed Mann-Whitney test. This experiment was performed 3 times with similar results.
   j) n=5 biologically independent animals. TSS **P=0.0079, CNS1 **P=0.0079 by two-tailed Mann-Whitney test. This experiment was performed 2 times with similar results.
   k) Usp22 ChIP-seq n=1 biologically independent animals, n=2 technically independent samples (see Methods). Rnf20 ChIP-seq n=2 biologically

independent samples. H2BK120Ub ChIP: Usp22 WT n=2, Usp22 KO n=3 biologically independent animals, NTC RNP n=3, Usp22 RNP n=2, Rnf20 RNP n=2 biologically independent samples. H2AK119Ub ChIP: NTC RNP n=2, Usp22 RNP n=2, Rnf20 RNP n=1 biologically independent samples. This experiment was performed once.

l) H2BK120Ub ChIP: Usp22 WT n=2, Usp22 KO n=3, NTC RNP n=3, Usp22 RNP n=2, Rnf20 RNP n=2 biologically independent samples. This experiment was performed once.

m) Usp22 ChIP-seq n=1 biologically independent samples, n=2 technically independent samples (see Methods). Rnf20 ChIP-seq n=2 biologically independent samples. H2BK120Ub ChIP: Usp22 WT n=2, Usp22 KO n=3 biologically independent animals, NTC RNP n=3, Usp22 RNP n=2, Rnf20 RNP n=2 biologically independent samples. H2AK119Ub ChIP: NTC RNP n=2, Usp22 RNP n=2, Rnf20 RNP n=1 biologically independent samples. Enhancer classifications described further in Methods. This experiment was performed once.

n) n=2 biologically independent samples. This experiment was performed 2 times with similar results.

Extended Data Figure 6

a) BL6 n=5, $Foxp3^{YFP-Cre}$ n=4, $Usp22^{fl/fl}Foxp3^{YFP-Cre}$ n=10 biologically independent animals. BL6 vs. $Foxp3^{YFP-Cre}$ P=0.3100, BL6 vs. $Usp22^{fl/fl}Foxp3^{YFP-Cre}$ *P=0.0347, $Foxp3^{YFP-Cre}$ vs. $Usp22^{fl/fl}Foxp3^{YFP-Cre}$ **P=0.0016 by one-way ANOVA with Tukey's multiple comparisons test. This experiment was performed 2 times with similar results.

b) This experiment was performed 2 times with similar results.

c) n=3 biologically independent samples. CD4 Eff **P=0.0028, CD4 Naive *P=0.0105, CD8 Eff **P=0.0011, CD8 Naïve ****P<0.0001 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 2 times with similar results.

Extended Data Figure 7

a) Source data can be found in **Supplementary Figure 1**. This experiment was performed 2 times with similar results.

b) This experiment was performed 2 times with similar results.

c) This experiment was performed 3 times with similar results.

d) WT n=3, KO n=4 biologically independent animals. LN CD4 **P=0.0016, LN CD8 ***P=0.0004, Spl CD4 *P=0.0213, Spl CD8 **P=0.0027 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 3 times with similar results.

e) This experiment was performed 3 times with similar results.

f) n=3 biologically independent animals. pLN and Spl ****P<0.0001 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 3 times with similar results.

g) This experiment was performed 2 times with similar results.

h) This experiment was performed 3 times with similar results.

i) Th1 WT n=5, Th1 KO n=4, Th2 n=4, Th17 n=3, iTreg n=4 biologically independent animals. Th1 P=0.949463. Th2 P=0.949463, Th17 P=0.099185, iTreg **P= 0.008 by two-way ANOVA with Holm-Sidak's multiple comparisons test. This experiment was performed 3 times with similar results.

Extended Data Figure 8
a) n=4 biologically independent animals. ***P=0.0006 by two-tailed unpaired t-test. This experiment was performed once.
b) n=4 biologically independent animals. *P=0.0124 by two-tailed unpaired t-test. This experiment was performed once.
c) WT n=5, KO n=4 except PD-1, PDL-1 n=3 biologically independent animals. Foxp3 P=0.06083, CD25 **P=0.0043, GITR ***P=0.0003, Helios ****P<0.0001, ICOS P=0.999, Nrp1 P=0.9998, CTLA-4 P=0.1430, PD-1/PD-L1 P>0.9999 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 2 times with similar results.
d) WT n=8, KO n=6 biologically independent animals. Ifng ***P < 0.0002, Grzb ***P < 0.0004, Cd8a ****P < 0.0001 by two-tailed unpaired t-test. This experiment was performed 2 times with similar results.
e) WT n=10, KO n=9 biologically independent animals. Days 0, 5, 9 P>0.9999, Day 15 P=0.9694, Day 21 P=0.8345, Day 24 *P=0.309, Day 26 ****P < 0.0001 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 2 times with similar results.
f) WT n=9, KO n=7, except CTLA-4 n=3 biologically independent animals. Foxp3 P=0.5057, CD25, GITR ****P < 0.0001, Helios P=0.9945, ICOS P>0.9999, Nrp1 P=0.9967, CTLA-4 P=0.9983 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed 2 times with similar results.
g) WT n=4, KO n=3 biologically independent animals. P=0.6465 by two-tailed unpaired t-test. This experiment was performed 3 times with similar results.
h) n=4 biologically independent animals. Day 5 P>0.9999, Day 8 P=0.9998, Day 11 P=0.6030, Day 14 P=0.2120, Day 16 ****P < 0.0001, Day 17 ***P=0.0004 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed once.
i) n=4 biologically independent animals. Foxp3, GITR, Helios, ICOS, Nrp1, CTLA-4, PD-L1 P>0.9999, CD25 *P=0.471, PD-1 P=0.1747 by two-way ANOVA with Sidak's multiple comparisons test. This experiment was performed once.
j) n=4 biologically independent animals. P=0.5492 by two-tailed unpaired t-test. This experiment was performed once.
k) WT n=6, KO n=2 biologically independent animals. This experiment was performed once.
l) WT n=6 except Foxp3, CD25, Nrp1 n=5, KO n=2 biologically independent animals. This experiment was performed once.
m) WT n=6, KO n=2 biologically independent animals. This experiment was performed once.

"Biologically independent samples" is defined as samples that are either from unique biological human donors and/or edited using a unique gRNA sequence.
"Biologically independent animals" is defined as individual mice.
"Technically independent samples" is defined as samples that are either from the same biological human donors and/or mouse and edited using the same gRNA sequence but the experiment was performed as an independent replicate.

Data exclusion
On principle, data were only excluded for failed experiments, reasons for which included suboptimal editing efficiency or technical challenges processing and isolating cells from tumors. In cases where experiments were performed multiple times, data was used from the experiments that were most accurately performed, analyzed and where data was reliably stored and retrieved with confidence.