

1 **Phospho-tuning immunity through Denisovan, modern human and mouse**
2 **TNFAIP3 gene variants**

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54 **Abstract:**

55 Resisting or tolerating microbes are alternative strategies to survive infection, but little is
56 known about the evolutionary mechanisms controlling this balance. Here, genomic analyses
57 of anatomically modern humans, extinct Denisovan hominins, and mice revealed a series of
58 missense variants in the immune response inhibitor A20 (encoded by *TNFAIP3*), substituting
59 non-catalytic residues of the ubiquitin protease domain to diminish I κ B-dependent
60 phosphorylation and activation of A20. Two A20 variants with partial phosphorylation
61 deficits appeared beneficial: one originating in Denisovans and introgressed in modern
62 humans throughout Oceania, and another in a mouse strain resistant to Coxsackievirus. By
63 contrast, a variant with 95% loss of phosphorylation caused spontaneous inflammatory
64 disease in humans and mice. Analysis of the partial phosphorylation variant in mice revealed
65 diminished tolerance of bacterial lipopolysaccharide or to poxvirus inoculation as trade-offs
66 for enhanced immunity.

67

68 **One Sentence Summary:**

69 Modern and ancient variants reveal a genetically tunable element for balancing immunity and
70 microbial tolerance.

71 **Main Text:**

72 Microbial resistance involves innate and adaptive immune responses that prevent, diminish,
73 or clear infection, often causing collateral damage to host tissues and increased energy
74 demands. Studies in plants¹, and more recently in animals^{2,3}, have shown that in some
75 circumstances it can be more efficient for a host to tolerate microbes rather than resist them.
76 Microbial tolerance involves homeostatic mechanisms to raise thresholds for initiating
77 immune responses, to physically separate microbes from host immune receptors, and to repair
78 damage caused directly by microbes or by collateral inflammation³⁻⁶. The genetic means by
79 which microbial resistance and tolerance are balanced remains incompletely understood.
80 Population genetic modelling predicts that resistance traits favor host polymorphism and
81 microbial evasion, whereas microbial tolerance traits tend towards fixation in hosts and
82 microbial mutualism⁷. High mortality in indigenous human populations of Oceania and the
83 Americas exposed to smallpox demonstrate how a tolerated pathogen in its adapted host can
84 cause devastating disease when introduced into non-adapted populations^{8,9}. An example in
85 animals can be seen for European rabbits exposed to myxoma poxvirus originating from
86 South American rabbits⁸. These cases illustrate the importance of fine-tuning microbial
87 immunity and tolerance during co-evolution of host and microbe, although little is known
88 about the molecular pathways which underpin it.

89 A binary perspective of the importance of balancing microbial immunity and tolerance comes
90 from Mendelian gene variants in mice and humans that completely inactivate the function of
91 one or both alleles of genes such as *CTLA4*, *IL10*, *FOXP3*, or *TNFAIP3*¹⁰⁻¹². These cause
92 severe pediatric autoimmune or inflammatory disease, particularly at mucosal barriers where
93 large microbial populations are normally tolerated, such as the microbial burden that drives
94 inflammatory pathology in mouse *Tnfaip3* deficiency⁶. Genome-wide and candidate
95 association studies in humans implicate variants at or near the *TNFAIP3* locus with
96 susceptibility to autoimmune disease¹³. In contrast to these disease-associated traits, few
97 examples of beneficial genetic adjustments that decrease microbial tolerance in favor for
98 heightened immunity exist.

99 A20, encoded by the *TNFAIP3* gene, promotes microbial tolerance as a negative regulator of
100 NF-κB signaling: an evolutionarily ancient and central pathway for activating innate and
101 adaptive immune responses¹³. A20 has multiple domains with inhibitory activity against NF-
102 κB, primarily preventing activation of the central IκB kinase (IKK) by upstream proteins
103 RIPK1, TRAF6 and NEMO. The A20 ovarian tumor (OTU) domain is a deubiquitin (DUB)
104 protease that cleaves activating K63-linked ubiquitin chains from RIPK1, TRAF6 or
105 NEMO¹⁴⁻¹⁶. The A20 zinc finger 7 domain (ZnF7) binds linear polyubiquitin to suppress IKK
106 activation, whereas ZnF4 promotes ligation of K48-linked ubiquitin chains to RIPK1,
107 triggering RIPK1 proteolysis^{14,17}. A20 feedback inhibition is induced at two levels: NF-κB
108 proteins directly induce *TNFAIP3* mRNA, and the inhibitory activities of A20 protein are
109 enhanced by IκKβ-induced serine phosphorylation near the ZnF domains, notably S381^{18,19}.

110 The role of the A20 OTU domain nevertheless remains enigmatic. The ZnF domains alone
111 are sufficient for NF-κB inhibitory function in cell-based studies^{20,21}, and mice homozygous
112 for *TNFAIP3* missense variants creating a catalytically inactive OTU domain have little^{15,18}
113 or no evidence²⁰ of excessive NF-κB signaling. Here we demonstrate that anatomically
114 modern human, archaic Denisovan, and mouse missense variants in the OTU domain
115 modulate A20 phosphorylation by IKK, serving as a genetically tunable element with
116 profound effects on the balance between microbial tolerance and immunity, and evidence of
117 introgression to high frequencies during human history.

118

119 **Modern human A20 OTU variants acquired from Denisovans**

120 Three convergent sequencing studies led us to a unique functional class of A20 missense
121 variants affecting the OTU domain, separate from the ubiquitin protease catalytic site (Fig.
122 1A, B; Extended Data Fig. 1). The most subtle allele, comprising T108A (rs376205580
123 g.138196008A>G) and I207L (rs141807543 g.138196957A>C) missense variants in *cis*, was
124 identified by whole genome sequencing in four of 85 families in Sydney. The majority of
125 individuals in our cohort carrying the T108A;I207L allele were healthy family members of
126 Māori or Pacific Islander ancestry. These variants were rare in a public variant collection
127 (gnomAD r2.0.2) but most frequent among individuals with an unassigned ancestry
128 (Extended Data Fig. 2A).

129 We traced the global distribution of the T108A;I207L allele in 279 individuals from the
130 Simons Genome Diversity Project dataset²², revealing high frequencies ranging from 25-75%
131 among people of Island Southeast Asia and Oceania, but an absence of the allele elsewhere in
132 the world (Extended Data Fig. 2B, Extended Data Table 1).

133 Unlike Africans or Eurasians, people in Island Southeast Asia and Oceania acquired up to 5%
134 of their genome from Denisovans: archaic hominins that interbred with modern humans
135 ~50,000 years ago migrating through Asia to settle the continent of Sahul (now Papua New
136 Guinea and Australia)²³⁻²⁶. Analysis of the high-coverage genome of a Denisovan finger
137 phalanx from a cave in the Altai Mountains of Siberia²⁷ revealed homozygous T108A;I207L
138 variants (Fig. 1C). Both variants were absent from the genome of a Neanderthal who had
139 inhabited the same cave²⁸ (Extended Data Table 2), suggesting that T108A and I207L arose
140 after the divergence of Denisovan and Neanderthal lineages 170,000-700,000 years ago²⁷.

141 Multiple Denisovan-derived genomic regions, including one encompassing *TNFAIP3*, bear
142 strong signatures of introgression in Papuans^{25,26,29}. By imputing haplotypes (Extended Data
143 Table 2) using genotype array data from 514 individuals from indigenous populations across
144 Island Southeast Asia and Oceania³⁰, we found evidence that the Denisovan *TNFAIP3*
145 haplotype has reached high frequencies in populations east of the Wallace Line, a 50 million
146 year-old faunal boundary separating organisms of Asiatic and Australian origin via deep
147 water channels between the two continental shelves (Fig. 1D, E and Extended Data Fig. 3)³¹.
148 Frequencies ranged from 0% in almost all populations west of the Wallace Line, but reached
149 100% in 8 Baining individuals from eastern Papua New Guinea. We observed the same
150 haplotype in 31/144 (22%) of exome-sequenced alleles in Martu Indigenous Australians from
151 the Pilbara region of Western Australia³², implying the haplotype enrichment occurred before
152 the isolation of Indigenous Australian and Papuan populations. The high frequency in
153 Polynesia indicates the Denisovan *TNFAIP3* haplotype was retained after admixture with
154 Austronesian farming populations expanding from mainland Asia starting ~4,000 years ago³³
155 in Eastern Indonesia and ~3,000 years ago in the Southwest Pacific³⁴.

156 Aside from the two missense variants in the introgressed Denisovan *TNFAIP3* haplotype,
157 many other non-coding variants could conceivably modulate transcription. Deep sequencing
158 of cDNA nevertheless revealed equal amounts of *TNFAIP3* mRNA from Denisovan and
159 modern human alleles in heterozygous leukocytes, with or without TNF α stimulation
160 (Extended Data Fig. 4). Of the two coding variants, T108A was not predicted
161 computationally to alter A20 function (Phred-scaled CADD score of 0.002) and occurs in
162 other vertebrate species (Extended Data Fig. 1). By contrast, I207 is invariant across most
163 jawed vertebrates, and I207L was predicted to be the most deleterious variant across the
164 Denisovan *TNFAIP3* haplotype (Phred-scaled CADD score: 23.2; Extended Data Fig. 3E).

166 **A mouse A20 OTU variant confers heightened resistance to Coxsackievirus**

167 The results above identify an ancient substitution in the A20 OTU domain as apparently
168 beneficial in human history. Further evidence that A20 OTU domain variants could be
169 beneficial came from another OTU substitution, I325N, identified in a genome-wide mouse
170 mutagenesis screen segregating with increased frequencies of circulating CD44^{hi}
171 activated/memory T cells and regulatory T cells (Fig. 2A) in otherwise healthy adult mice.
172 Detailed analysis revealed the I325N mutation also increased numbers of B cells in the spleen
173 and peritoneal cavity, and diminished I κ B α within B cells, CD8 T cells, NK cells and
174 dendritic cells (Fig. 2B and Extended Data Fig. 5). Macrophages immortalized from bone
175 marrow of I325N mutant mice produced more inflammatory cytokines in response to
176 lipopolysaccharide (LPS) than controls from wild-type mice (Extended Data Fig. 6). In
177 thymocytes, I325N increased TNF α -induced NF- κ B signaling in ways consistent with
178 diminished A20-mediated inhibition (Extended Data Fig. 7)¹⁸.

179 In wild-type mice transplanted with mixtures of mutant and wild-type A20 bone marrow, the
180 I325N mutation acted cell autonomously to increase B cell activation and proliferation by
181 LPS or antigen receptors, and to increase TCR and CD28-dependent formation of FOXP3 $^{+}$
182 CD4 $^{+}$ regulatory T cells and their Helios $^{+}$ FOXP3 $^{-}$ precursors within the thymus (Fig. 2C, D,
183 Extended Data Fig. 8). Surprisingly, I325N had a greater effect than the C103A OTU domain
184 mutation analyzed in parallel bone marrow transplant recipients, despite C103A completely
185 inactivating the polyubiquitin protease activity of A20^{15,20}, indicating I325N must diminish
186 additional inhibitory mechanisms.

187 Consistent with heightened cellular markers of immunity, I325N mutant mice had greater
188 resistance to Coxsackievirus B4 strain E2, a virus isolated from a human neonate with a
189 disseminated fatal infection causing extensive pancreatic necrosis^{35,36}. A virus dose that was
190 lethal for 90% of wild-type C57BL/6 mice was not lethal for *Tnfaip3*^{I325N/I325N} littermates, and
191 caused less mortality in *Tnfaip3*^{I325N/+} mice (Fig. 2E; Extended Data Fig. 9A). Mutant mice
192 had less infectious virus and viral RNA in the pancreas, less mRNA encoding immune
193 response cytokines IL-1 β and IFN β , less pancreatic necrosis, higher serum IL-6, and
194 preserved body-weight and euglycemia (Fig. 2F, G; Extended Data Fig. 9B-G).

195 The homogeneous genetic background of I325N mutant mice allowed testing if heightened
196 immunity imposed a subclinical cost or altered the insulin anabolic axis³⁷. *Tnfaip3*^{I325N/+} mice
197 were healthy, of normal weight, and fertile, producing homozygous mutant offspring at the
198 expected ratio. Homozygotes also appeared healthy, although their body weight was 5-20%
199 less than heterozygous or wild-type littermates (Fig. 3A), with histological analysis revealing
200 low-grade inflammation of the pancreatic islets, colon, kidney, and liver (Fig. 3B-C,
201 Extended Data Fig. 10). Pancreatic insulitis in I325N homozygotes was associated with a
202 50% reduction in beta cell mass (Fig. 3D), although random blood glucose levels and glucose
203 tolerance tests were normal (Extended Data Fig. 11A-E). Isolated *Tnfaip3*^{I325N/I325N} islets
204 exhibited normal basal insulin output but reduced insulin secretion when stimulated *in vitro*
205 (Extended Data Fig. 11F). Islet transplant and culture experiments showed the A20 mutation
206 acted within islet cells, exaggerating canonical and non-canonical NF- κ B signaling, lowering
207 insulin secretion, and increasing inflammatory cytokine production (Fig. 3E-H and Extended
208 Data Fig. 11G-I & 12)³⁸.

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212 **An allelic series of OTU domain variants with graded reductions of A20
213 phosphorylation**

214 The apparent beneficial effects of the T108A;I207L and I325N OTU domain substitutions
215 above contrasted with a third OTU domain substitution, C243Y, found as a family-specific
216 variant causing dominant Mendelian inflammation resembling Behçet's disease, with
217 childhood-onset oral and genital ulceration and skin inflammation³⁹. The biochemical basis
218 for the clinically penetrant effects of C243Y was obscure, because other similarly affected
219 cases of haploinsufficiency of A20 (HA20) result from nonsense or frameshift variants that
220 truncate or eliminate A20 protein¹².

221 Despite different clinical consequences, the three OTU domain variants formed a graded
222 biochemical allelic series when full-length A20 and IKK β were co-expressed in mouse
223 insulinoma cells and S381-phosphorylated A20 was compared with total A20 protein by
224 Western blotting. None of the variants affected A20 protein accumulation but each affected
225 IKK β -mediated S381 phosphorylation (Fig. 4A-C; Extended Data Fig. 13A). The pathogenic
226 C243Y variant caused the most severe loss of function, diminishing IKK β -mediated S381
227 phosphorylation to 5% of wild-type, whereas the Denisovan T108A;I207L variant diminished
228 phosphorylation the least (80% of wild-type) and the non-pathogenic I325N variant had an
229 intermediate effect (50% of wild-type). By contrast, the C103A catalytic site substitution did
230 not cause a significant decrease in A20 phosphorylation. Loss of S381 phosphorylation was
231 also observed by mass spectrometry of A20 purified from unstimulated human cells
232 transfected with A20^{I325N} compared to A20^{WT}, whereas A20^{C103A} exhibited normal
233 phosphorylation (Extended Data Fig. 13B).

234 S381-phosphorylated A20 predominantly migrated more slowly in SDS-PAGE than
235 unphosphorylated A20, either when tested by co-transfection with IKK β (Fig. 4A, B) or in
236 TNF α -stimulated peripheral blood leukocytes with endogenous A20 and IKK β (Fig. 4D,
237 S14). The ratio of this slowly migrating A20 species to the more rapidly migrating form was
238 markedly decreased in blood leukocytes from healthy donors heterozygous for the Denisovan
239 T108A;I207L variants, and further decreased in a healthy T108A;I207L homozygous donor,
240 compared to healthy controls without these variants (Fig. 4E). The ratio of phosphorylated-
241 S381 to fast-migrating A20 was also decreased (Extended Data Fig. 14F). TNF- α stimulated
242 leukocytes from variant carriers also had heightened expression of NF- κ B-induced transcripts
243 of *ICAM1*, *CXCL2*, *TNF*, and *TNFAIP3* itself (Extended Data Fig. 15), and a trend towards
244 reduced I κ B α protein levels (Extended Data Fig. 14H).

245 When A20 was measured for inhibition of an NF- κ B luciferase reporter in unstimulated or
246 TNF α -stimulated mouse insulinoma cells, inhibition was diminished in a graded fashion by
247 each OTU domain variant in the same order that these diminished S381 phosphorylation (Fig.
248 4F; Extended Data Fig. 13C). The deleterious C243Y variant was the most compromised
249 inhibitor of the series, almost as compromised as A20 with S381 substituted to non-
250 phosphorylatable alanine (S381A). Combining the intermediate I325N mutation with a
251 S381A mutation *in cis* did not cause a further decrease in A20 inhibition, consistent with
252 I325N having its effect upon phosphorylation. That conclusion was reinforced by combining
253 I325N *in cis* with a substitution of S381 to the phosphoserine mimetic glutamate (S381E),
254 which rescued the lost activity caused by I325N (Extended Data Fig. 13D, E). Testing the
255 two Denisovan variants individually, T108A had no measurable effect whereas I207L
256 diminished A20 inhibitory activity comparably to the two variants combined (Extended Data
257 Fig. 13F), supporting I207L as the functional variant within the introgressed Denisovan
258 haplotype.

259 To explore the structural and biochemical consequences of variants on the posterior surface
260 of the OTU domain, we focused on the intermediate I325N allele. Crystallographic structures
261 of A20 OTU domains with wild-type I325 or mutant N325 revealed no differences in features
262 with known functions, including the catalytic triad and ubiquitin-binding surface (Fig. 1B;
263 Extended Data Fig. 16A-C). I325N also did not alter the conserved posterior surface of the
264 OTU domain⁴⁰, including the β 3- β 4 loop containing C243 (Extended Data Fig. 16G),
265 although there were subtle shifts in the stem of the β 7- β 8 loop, which contains conserved
266 surface residues T321, T322 and L324 (Extended Data Fig. 16D-G). The β 7- β 8 loop itself is
267 disordered in all available OTU structures but, like the disordered loops in the unliganded S1
268 ubiquitin-binding site, it may undergo conformational changes upon binding a cognate
269 partner that are hindered by the I325N variant. Wild-type and variant OTU domains exhibited
270 similar thermal denaturation profiles (Extended Data Fig. 17A-C), and the corresponding
271 full-length proteins were comparably stable in cycloheximide-treated mammalian cells
272 (Extended Data Fig. 17D). I325N did not decrease DUB activity of bacterially expressed
273 OTU domain against K48-polyubiquitin *in vitro* (Extended Data Fig. 17E-G), but I325N
274 diminished K63-polyubiquitin DUB activity and K48-ubiquitin ligase activity when full
275 length A20 was expressed in human cells (Extended Data Fig. 17H, I) consistent with these
276 activities requiring S381 phosphorylation^{18,19}.

277

278 Shift from beneficial to detrimental loss of microbial tolerance

279 We next explored the possibility that partial loss of A20 phosphorylation and subclinical loss
280 of microbial tolerance might become detrimental in particular environmental or genetic
281 settings. In an experimental model for septic shock, I325N homozygous mice had increased
282 IL-6 production and high mortality following injection of a dose of bacterial LPS that was not
283 lethal in wild-type controls (Fig. 5A, B). In the rodent counterpart of smallpox, infection with
284 the orthopoxvirus ectromelia virus was tolerated and controlled by wild-type mice, yet
285 resulted in high mortality and higher viral titres in I325N homozygotes (Fig. 5C, D and
286 Extended Data Fig. 18). In bone marrow chimeric mice with a higher frequency of pancreatic
287 islet-reactive CD4⁺ T cells, autoreactive T cells escaped deletion and precipitated diabetes
288 when half the hematopoietic cells were homozygous for I325N (Extended Data Fig. 19A-C).
289 The rogue islet-reactive T cells were nevertheless derived equally from A20 wild-type or
290 I325N precursors, whereas I325N acted cell autonomously to increase MHC II and the T cell
291 costimulator CD86 on B cells and dendritic cells and to increase frequencies of germinal
292 centre B cells (Extended Data Fig. 19D-F).

293 While specific stressors were required to reveal detrimental effects of the intermediate I325N
294 variant, wasting disease occurred spontaneously in young C57BL/6 mice homozygous for the
295 severe C243Y variant. C243Y homozygotes were born at the expected Mendelian ratio but
296 compared to wild-type littermates failed to thrive, exhibiting a large reduction in body weight
297 and early lethality (Fig. 5E, F; Extended Data Fig. 20). Surviving C243Y homozygotes
298 showed intestinal pathology including loss of goblet cells responsible for sustaining the
299 mucin barrier between epithelium and microbes of the intestinal lumen, along with an
300 increased frequency of circulating CD44^{hi} activated/memory T cells (Extended Data Fig. 20).
301 C243Y homozygous mice thus resemble A20-null mice^{5,11}, paralleling the clinical similarity
302 between C243Y missense and A20 loss-of-function variants in heterozygous humans¹².

303 **Discussion**

304 Our findings reveal genetic and biochemical mechanisms for adaptively increasing immunity,
305 with trade-offs against microbial tolerance and anabolic metabolism that either remain
306 clinically silent or become detrimental in specific contexts. Three different missense alleles in
307 the N-terminal A20 OTU domain act, to different degrees, by diminishing A20
308 phosphorylation and tuning down A20's immune inhibitory activity. A rare human variant,
309 C243Y, diminishes phosphorylation almost entirely and shifts the balance away from
310 microbial tolerance to the extremes of immunity, resulting in severe inflammatory disease in
311 both mice and humans. This outcome is comparable with other human variants that eliminate
312 or truncate A20 protein from one *TNFAIP3* allele or engineered mouse variants that eliminate
313 A20 from both alleles^{11,12}. By contrast, I325N and I207L variants decrease A20
314 phosphorylation more modestly, without precipitating spontaneous inflammatory disease in
315 the mice or humans who harbor them. I325N in the mouse confers dramatically increased
316 resistance to an otherwise lethal dose of a Coxsackievirus. A similar resistance to microbial
317 pathogens may explain the beneficial effect of Denisovan I207L, as evidenced by its high
318 frequency in modern human populations east of the Wallace Line, who likely encountered
319 new infectious agents as they moved into environments with unique fauna and flora.

320 Previous studies in mice have conditionally deleted A20 in specific tissues, causing severe
321 inflammatory disease, or directly disabled individual enzymatic functions of A20 in all
322 tissues resulting in surprisingly little inflammation^{5,6,11,15,18,20}. By contrast, OTU domain
323 variants tuning phosphorylation regulate multiple ubiquitin-editing functions of A20^{18,19}, and
324 as shown here have greater effects on microbial tolerance and resistance *in vivo* than variants
325 affecting individual ubiquitin-editing activities. The C243Y and I325N variants involve
326 buried residues in two separate surface loops, β 3- β 4 and β 7- β 8 respectively (Extended Data
327 Fig. 16), on the highly conserved posterior surface of the OTU domain⁴⁰. Structural studies of
328 full-length A20 protein may illuminate how this surface relates to the C-terminal domain
329 carrying S381. Because the conserved surface bounded by C243Y and I325N is large, it
330 potentially offers many opportunities for substitution of buried or surface residues to tune
331 immunity and tolerance.

332 Our study of the intermediate loss-of-function A20 variant, I325N, provides two examples of
333 genetic trade-offs. The first was with glucose metabolism, where *Tnfaip3*^{I325N} had the
334 surprising effect within pancreatic islet beta cells of decreasing insulin secretion while
335 increasing inflammation. This is reminiscent of findings in *Drosophila* that insulin production
336 or action decreases during infection, diminishing body glycogen supplies through increased
337 FOXO activity, a transcriptional inducer of starvation responses³⁷. Resting insulin levels are a
338 good prognostic marker in human sepsis, and exogenous insulin treatment improves
339 outcome⁴¹. Subtly decreased A20 activity may enhance immune demands for energy and,
340 separately, help meet these energy demands by lowering insulin-induced anabolic growth,
341 both contributing to cachexia associated with chronic infection and inflammation.

342 The second example of a trade-off is the experimental demonstration that a beneficial trait in
343 one context can be deleterious in another. *Tnfaip3*^{I325N} conferred increased resistance to
344 Coxsackievirus, an important human enterovirus, but increased mortality to ectromelia virus,
345 a relative of variola and myxoma viruses. Higher ectromelia virus mortality in *Tnfaip3*^{I325N}
346 mice is reminiscent of the high mortality in indigenous populations of the Americas and
347 Oceania exposed to variola virus relative to Europeans⁸. The findings here suggest that the
348 devastation wrought by these and other microbes reflects selection during earlier
349 environmental conditions for lower microbial tolerance and a stronger immune response, with
350 A20 representing one critical determinant of this outcome.

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539

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548

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550 MEC, PB-A, JT, MW, OMS, AR, MJC, VG, MED, CCG. Tracing Denisovan haplotype in
551 human populations by OMS, PG, AR, MPC, CCG; peripheral blood analyses by NWZ, AR,
552 CCG, STG. I325N mutation: identification by SRW, CNJ, LLL, TW, CR, BW, CCG;
553 immune cell analysis by KH, SRD, YJ, NWZ, MY, EB, AE, MHS, GPN, FS, DEZ, AA,
554 IEW, STG, CCG; Coxsackievirus studies by NWZ, CL, JW, CK, STG; Ectromelia studies by
555 GC, GK; body weight, islet, LPS and inflammation studies by NWZ, SNW, EKM, JEV,
556 STG; protease activity and structural studies by KH, NWZ, DBL, DC, WP, IEW, BEC, PDM,
557 CJJ, BTP, AMB, STG, CCG. C243Y mouse strain production and analysis by NWZ, RB,
558 DZ, SNW, DC, JW, STG. A20 phosphorylation and NF- κ B luciferase analysis by NWZ, DC,
559 DWA, WP, IEW, CCG, STG.

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561 **Competing interests:** Authors declare no competing interests.

562

563 **Data and materials availability:** All data is available in the main text or the supplementary
564 materials.

565

566 **Extended Data:**

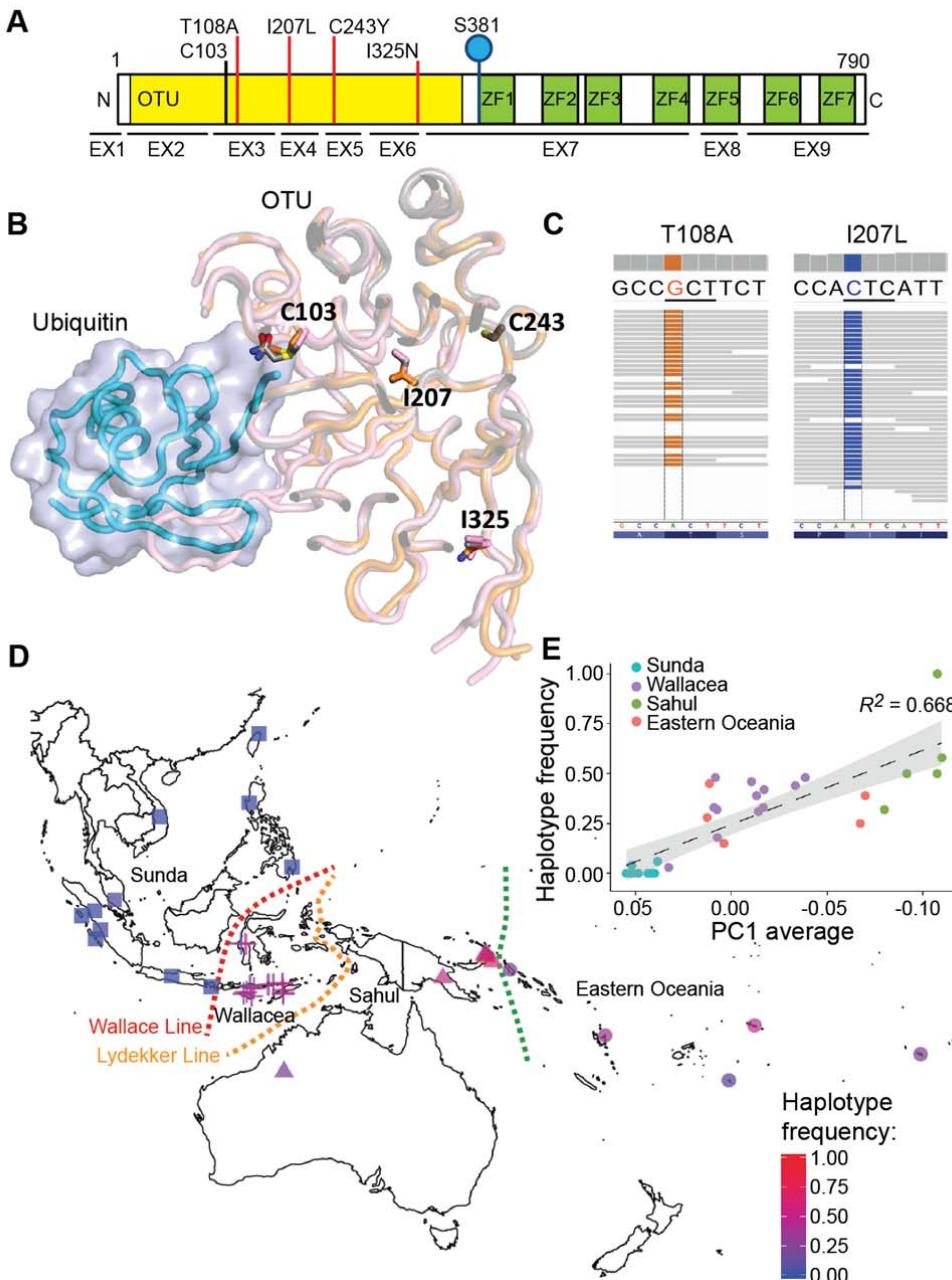
567 Materials and Methods

568 Extended Data Figures 1-20

569 Extended Data Tables 1-5

570 References (43-78)

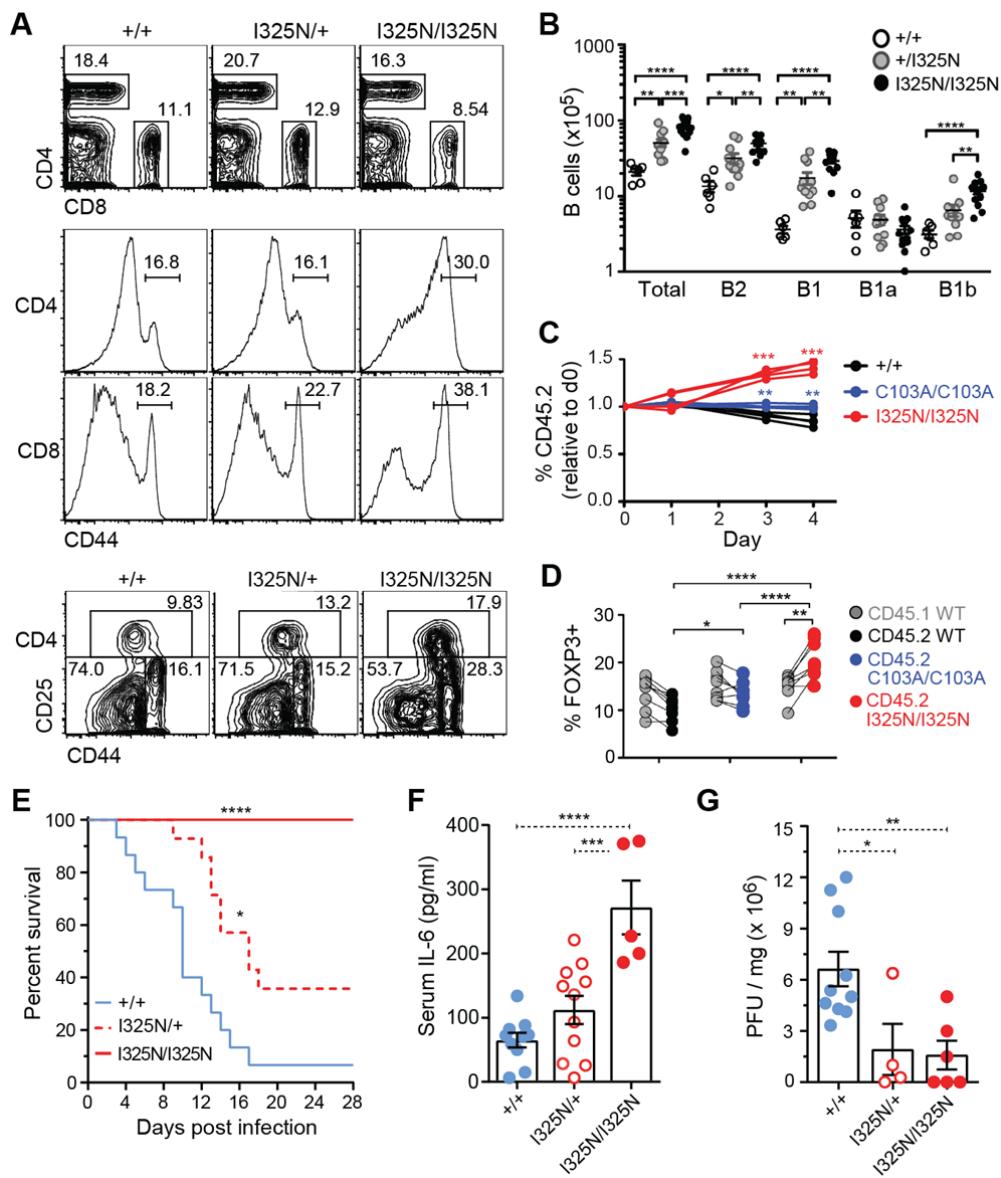
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573 **Fig. 1. Denisovan, modern human, and mouse variants substituting noncatalytic**
 574 **residues of the A20 OTU domain.** (A) Schematic of A20 protein encoded by the *TNFAIP3*
 575 *gene* (CCDS5187), showing exons (EX1-9), ovarian tumor domain (OTU), zinc fingers (ZF1-
 576 7), OTU catalytic residue (C103), S381 phosphorylation site, and missense variants studied
 577 here (red lines). (B) Location of variant residues in sliced structure 5LRX⁴² of A20 OTU
 578 domain (pink) complexed with ubiquitin (blue). Structures for WT and I325N OTU domains
 579 are superposed in grey and orange, respectively. (C) Read data of a high-coverage Denisovan
 580 genome²⁷ across *TNFAIP3* codons 108 and 207. (D) Imputed frequency of the Denisovan
 581 *TNFAIP3* haplotype in genotype array data from 514 individuals from indigenous
 582 populations across Island Southeast Asia and Oceania³⁰, and directly observed in exome data
 583 from 72 Martu indigenous Australians³². Different shape symbols group populations from

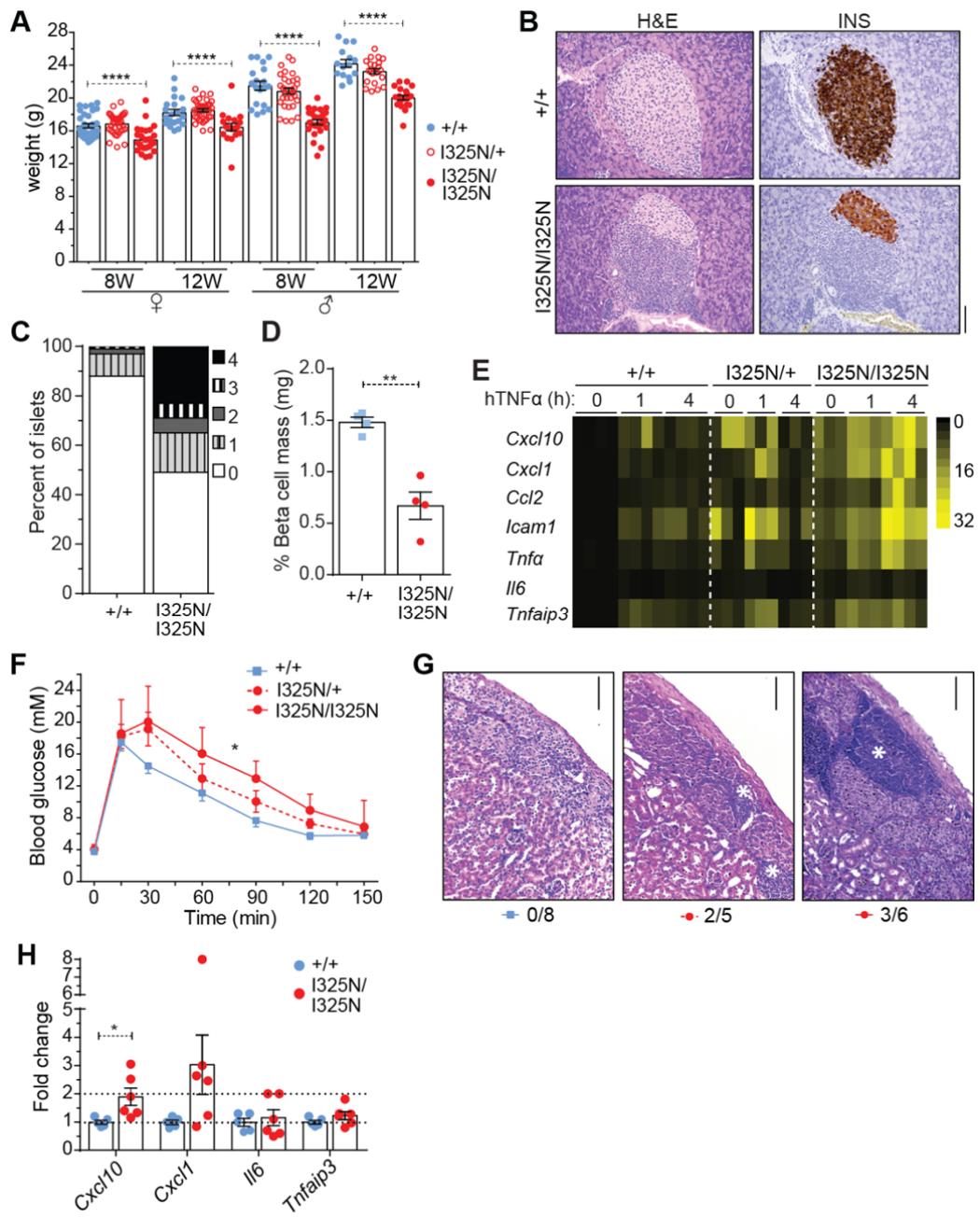
584 Sunda, Wallacea, Sahul and Eastern Oceania. **(E)** Imputed Denisovan *TNFAIP3* haplotype
585 frequency from populations described in (D) (filled circles) versus a surrogate estimate of
586 Papuan ancestry (principal component 1, Extended Data Fig. 3). Gray shading indicates 95%
587 confidence interval of linear regression line.
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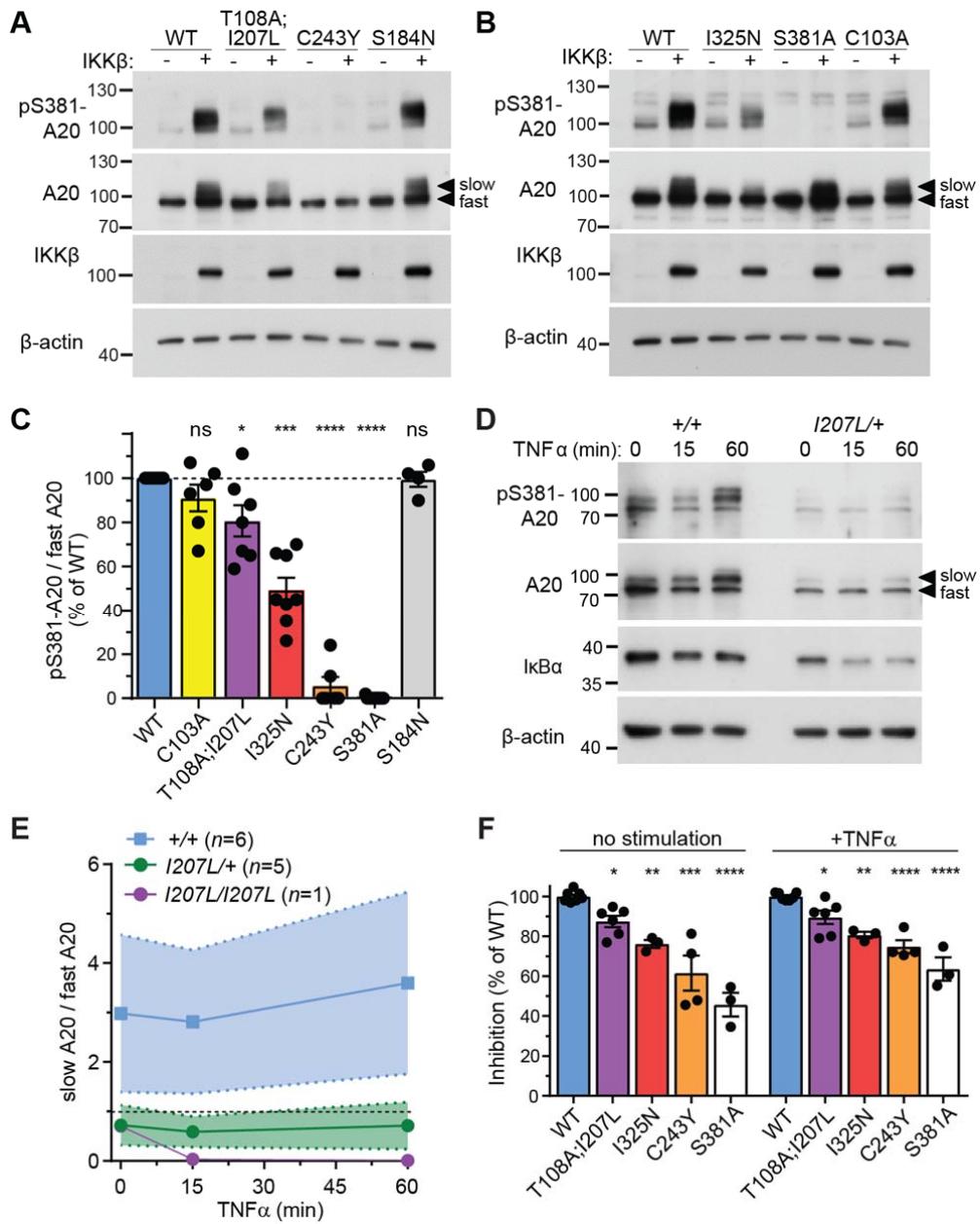
590 **Fig. 2. I325N OTU variant confers heightened immunity in mice.** (A) Representative flow
591 cytometric analysis of splenocytes, showing percentage of $CD4^+$ or $CD8^+$ T cells, and
592 percentage $CD44^{hi}$ activated/memory cells within these subsets or percentage $CD25^+$ $CD44^{int}$
593 regulatory T- cells or $CD25^-$ $CD44^{high}$ effector/memory T cells among $CD4^+$ cells. (B)
594 Numbers of indicated B cells in peritoneum of individual *Tnfaip3* mice (circles). (C, D)
595 Wild-type $CD45.1^+$ mice transplanted with equal mixtures of congenic bone marrow from
596 *Tnfaip3*^{+/+} $CD45.1^+$ donors and $CD45.2^+$ donors of *Tnfaip3*^{I325N/I325N}, *Tnfaip3*^{C103A/C103A} or
597 *Tnfaip3*^{+/+} genotypes. (E) Splenocytes from chimeras cultured with 0.1 μ g/ml LPS for 0-4
598 days, and the frequency of $CD45.2^+$ cells of the indicated *Tnfaip3* genotypes was measured
599 among B cells from individual chimeras (lines), expressed relative to their frequency on day
600 0. (D) Pairwise comparison of frequency of FOXP3⁺ cells amongst $CD45.2^+$ and $CD45.1^+$
601 $CD4^+$ splenocytes from the same chimera. (E-G) Female *Tnfaip3*^{+/+} ($n=15$), *Tnfaip3*^{I325N/+}
602 ($n=14$) or *Tnfaip3*^{I325N/I325N} ($n=10$) C57BL/6 mice were injected with Coxsackie B4 virus
603 strain E2 on day 0. (E) Kaplan-Meier survival data (Log-rank test). (F) Plasma IL-6 and (G)
604 virus titers (plaque forming units, PFU) per mg of pancreas on day 3. Error bars represent

605 SEM and one-way ANOVA used for significance analysis unless otherwise stated, $*P < 0.05$;
606 $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.



607
608 **Fig. 3. Subclinical inflammatory and metabolic consequences of the I325N variant. (A)**
609 Body weights of *Tnfaip3*^{+/+}, *Tnfaip3*^{I325N/+}, *Tnfaip3*^{I325N/I325N} mice of the indicated age and
610 sex. Statistical analysis by unpaired one-way ANOVA: ****P < 0.0001. **(B)** Hematoxylin
611 and eosin (H&E) and insulin (INS) stained pancreas sections (scale = 100 μ m) with **(C)**
612 cumulative insulitis scores (4 represents >75% islet mononuclear cell infiltration, and 0 an
613 absence of infiltrating cells) and **(D)** calculated beta cell mass for *Tnfaip3*^{+/+} and
614 *Tnfaip3*^{I325N/I325N} mice. **(E)** Expression of TNF α -induced genes in islets from *Tnfaip3*^{+/+},
615 *Tnfaip3*^{I325N/+}, *Tnfaip3*^{I325N/I325N} mice. Data represents 3 independent islet preparations with 3-
616 4 biological replicates. **(F)** Glucose tolerance test in diabetic wild-type mice transplanted with
617 *Tnfaip3*^{+/+}, *Tnfaip3*^{I325N/+} or *Tnfaip3*^{I325N/I325N} islets, N>8 mice per group. **(G)** H&E sections of
618 islet grafts at post-operative day 30 (scale = 50 μ m) with the fraction of grafts exhibiting
619 immune infiltrate shown below. **(H)** Islet grafts isolated on post-operative day 10 were

620 analysed for indicated mRNAs by RT-qPCR. *P* values represent Student's *t*-test unless
621 otherwise stated, **P* < 0.05; ***P* < 0.01; ****P* < 0.0001.

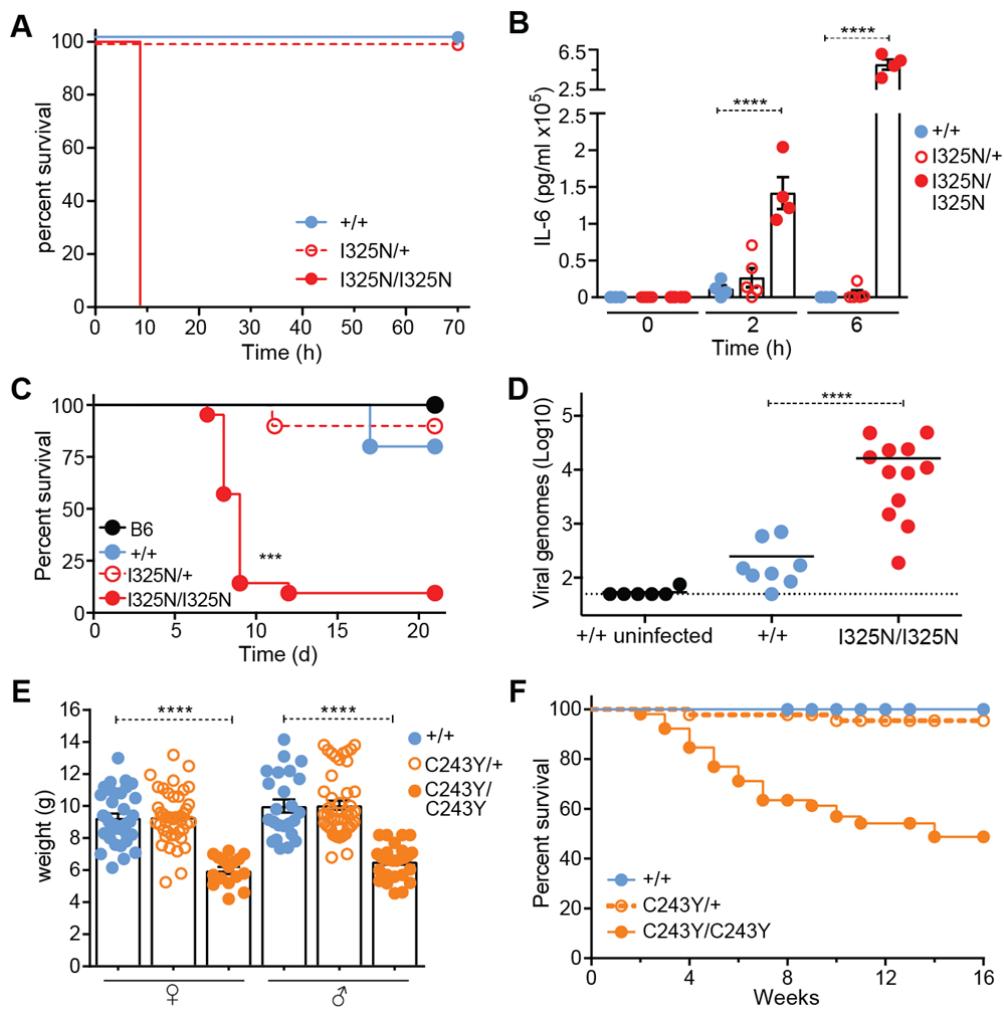


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Fig. 4. OTU domain variants decrease A20 phosphorylation and NF-κB inhibition. (A-C) Representative western blot of lysates from βTC3 mouse insulinoma cells transfected with vectors encoding wild-type (WT) A20 or the indicated A20 mutants, with or without co-transfected IKKβ, and probed with antibodies to phospho-Ser381 A20, total A20, IKKβ and β-actin. Molecular weight markers (kD) and slow- or fast-migrating A20 species are indicated. **(C)** Densitometric analysis from multiple independent experiments (dots) showing the ratio of pSer381-A20 to total fast migrating A20 in IKKβ-cotransfected cells, expressed relative to the ratio in cells with WT A20. **(D)** Representative western blot, probed with antibodies to the indicated proteins, of lysates from peripheral blood mononuclear cells from healthy donors with wild-type A20 or heterozygous for the Denisovan T108A;I207L haplotype, stimulated with TNFα for the indicated times. **(E)** Densitometric ratio of slow- to fast-migrating A20 species, showing mean and standard deviation from six normal donors, five T108A;I207L heterozygotes, and one T108A;I207L homozygote (Extended Data Fig.

636 14). **(F)** Inhibition of an NF- κ B luciferase reporter in β TC3 cells by co-transfection of WT
637 A20 or the indicated A20 mutants, expressed relative to wild-type A20 (Extended Data Fig.
638 13). Statistical analysis in C and F by one-way ANOVA: ns, $P > 0.05$; $*P < 0.05$; $**P < 0.01$;
639 $***P < 0.001$; $****P < 0.0001$.

640



641

Fig. 5. Shift from beneficial to detrimental effects in response to environmental or genetic stressors. Kaplan-Meier survival curves (A) and serum IL-6 concentration (B) in mice of the indicated genotypes given 50 μ g LPS by intraperitoneal injection at time=0. $N=16-18$ for each curve in (A). (C) Kaplan-Meier survival curves for sex-matched littermates of the indicated *Tnfaip3* genotypes and wild-type controls (B6) infected with Ectromelia virus. (D) Day 8 serum viral load assessed from mice in (C). (E) Weight of 3-week-old *Tnfaip3* $C243Y/C243Y$ mice and heterozygous or wild-type littermates. (F) Survival of *C243Y* homozygous mice ($n=52$) compared to heterozygous ($n=43$) or wild-type littermates ($n=41$). Statistical analysis by Students *t*-test and one-way ANOVA: *** $P < 0.001$, **** $P < 0.0001$.

651 **Materials and Methods**

652

653 **Human subjects**

654 Patients and healthy family members were recruited via the Clinical Immunogenomics
655 Research Consortium Australia and The Children's Hospital at Westmead, providing written
656 informed consent under protocols approved by relevant human research ethics boards.

657

658 **Genome sequencing**

659 Parent-proband trio genomes were sequenced on the Illumina HiSeq X platform using
660 DNA isolated from whole blood. Libraries were generated using either Illumina TruSeq
661 PCR-free or TrueSeq Nano.

662 Raw reads were aligned to the hs37d5 reference using BWA-MEM v0.7.10-r789
663 (arXiv:1303.3997 [q-bio.GN]), and sorted and duplicate-marked with Novosort v1.03.01
664 (Novocraft Technologies). The GATK suite v3.3-0-g37228af⁴³ was used for local indel
665 realignment and base quality score recalibration. gVCFs were generated with GATK
666 HaplotypeCaller, joint-called as trios using GATK GenotypeGVCFs, and variants
667 recalibrated using GATK Variant Quality Score Recalibrator (VQSR). Finally, VCF files
668 were annotated with Variant Effect Predictor (VEP) v76⁴⁴ using the LoFTEE⁴⁵ and dbNSFP
669 plugins.

670 Variants were packaged into databases using GEMINI (v0.18.3)⁴⁶ and imported into
671 Seave⁴⁷. All reported variants were manually inspected using IGV⁴⁸ to verify their
672 authenticity and validated by Sanger Sequencing.

673

674 **Population genetic analysis**

675 *TNFAIP3* genotypes were extracted from 279 publicly-available genomes from the
676 Simons Genome Diversity Project samples²², or exome data from 72 Martu indigenous
677 Australians³². In addition, a collection of 514 individuals from across mainland and Island
678 Southeast Asia, Papua New Guinea and Oceania was genotyped at 567,096 variants using the
679 Affymetrix Axiom Genome-Wide Human Array, with 538,139 autosomal variants with <5%
680 missing data kept for further analyses³⁰. Weir and Cockerham's estimated F_{ST} values were
681 calculated using VCFtools v0.1.14, and principal component analysis was performed using
682 PLINK v1.9. Genotypes were phased and imputed using the Michigan Imputation Server
683 v1.0.3 (<http://imputationserver.sph.umich.edu>), and compared to high-coverage Denisovan²⁷
684 and Altai Neanderthal²⁸ *TNFAIP3* haplotypes. Phred-scaled CADD scores⁴⁹ were calculated
685 for all PASS variants across the extended Denisovan haplotype with gnomAD allele
686 frequency <0.01.

687

688 **PBMC isolation and manipulation**

689 Human PBMCs were prepared using a Ficoll- Hypaque gradient and live count taken
690 using 0.1 % trypan blue. Cells were resuspended at 10^6 cells/ml in sterile freezing medium
691 (RPMI-1640, 10% DMSO, 50% FCS) in 1.5 ml Sarstedt cyrovials and stored in liquid
692 nitrogen. Human PBMCs were thawed in a 37°C water bath and added drop wise to pre-
693 warmed medium (RPMI-1640, 10% FCS, 100 U/ml P/S, 1% HEPES, 2 mM L-Glutamine,
694 2mM EDTA) and resuspended for culture at 37°C + 5% CO₂ for 1 h prior to stimulation with
695 recombinant human TNF α (R&D Systems). Following stimulation, cells were pelleted and
696 processed for immunoblotting or RT-qPCR, as described below.

697

698 **Mice**

699 The *Tnfaip3* *Lasvegas* strain (*Tnfaip3*^{I325N}) were generated by *N*-ethyl-*N*-nitrosourea
700 (ENU) mutagenesis of C57BL/6 mice, and propagated by backcrossing to C57BL/6. The

701 strain was maintained as heterozygous breeding pairs so that WT littermates could be used
702 for controls. To generate *Tnfaip3*^{C243Y} mice, a guide RNA with the sequence 5'-
703 GGGATATCTGTAACACTCC-3' was microinjected into C57BL/6 zygotes in combination
704 with Cas9 mRNA. Founder mice carrying the C243Y substitution were then crossed to
705 C57BL/6, and heterozygous offspring intercrossed to generate homozygous, heterozygous
706 and wild-type littermates for experimentation. Mouse lines were housed at the Australian
707 Phenomics Facility (Australian National University, Canberra, Australia), or at the Australian
708 BioResources Centre (ABR) (Moss Vale, NSW, Australia). *Tnfaip3*^{C103A¹⁸}, 3A9 TCR
709 transgenic⁵⁰ and insHEL transgenic⁵¹ mice have been described. Mice were genotyped for
710 transgenes and mutations by PCR and used 7–28 (typically 10-16) weeks after birth. For
711 TCR^{3A9} transgenic mouse experiments, mice were hemizygous for the 3A9 or insHEL
712 transgenes on the B10.BR and the B10.BR.SJL-*Ptprc*^a (CD45.1) backgrounds, and the
713 *Tnfaip3*^{I325N} mutation was backcrossed to B10.BR. To make chimeras, congenic B6.SJL-
714 *Ptprc*^a or B10.BR.SJL-*Ptprc*^a (CD45.1) mice were irradiated with two doses of 4.5 Gy 4 hrs
715 apart, and injected IV with mixtures of 1.8x10⁶ bone marrow cells from B6 or B10.BR SJL-
716 *Ptprc*^a mice and 1.8x10⁶ bone marrow cells from B6 (CD45.2) mutant or control mice, and
717 analyzed 8-14 weeks later. Animal studies were approved by the Garvan/St Vincent's or the
718 Australian National University Animal Ethics Committees. All procedures performed
719 complied with the Australian code of Practice for Care and Use of Animals for Scientific
720 Purposes.

721

722 Coxsackievirus infection model and virus quantification

723 Mice were intraperitoneally injected with normal saline (control) or 20 plaque forming
724 units (PFU) in 200 µl saline of the CVB4 E2 lab strain kindly provided by Prof. Malin
725 Flodström Tullberg (Karolinska Institutet, Solna, Sweden) grown in HeLa cells. Mice were
726 monitored daily and euthanized if they displayed gross signs of illness (e.g., ruffling,
727 hunching). The pancreas was harvested at indicated times for RT-qPCR and histopathology
728 analysis and viral titre determination. Serum was also collected by cardiac puncture for
729 measurement of IL-6 by ELISA (BD; OptEIATM Set Mouse IL-6), as per manufacturer's
730 instructions. Non-fasting blood glucose levels were measured at least twice-weekly using
731 Freestyle Lite Blood Glucose Test Strips (Abbott, Australia). Plaque assays were performed
732 to determine viral titres within the pancreas following infection. HeLa cells (0.6 x 10⁶/well)
733 were seeded in 2 ml complete medium (RPMI with 10% FCS, 2mM L-Glutamine, 100 U/ml
734 Penicillin, 100 µg/ml Streptomycin and 100 µg/ml Normocon) in 6 well plates and incubated
735 over night at 37°C. Infection samples were collected in RPMI, homogenized in a Dounce
736 tissue grinder and passed through a 22 µm filter, before preparing tenfold serial dilutions.
737 HeLa cells, at 90% confluence, were washed with 1X PBS and 400 µl of infectious
738 homogenate added to each well and incubated for 60 min at 37°C under gentle rocking.
739 Infectious media was removed and 3 ml of agar mix (2x 1.8% agar, 2x MEM containing 10%
740 FCS) was added to each well before incubating at 37°C for 3 days. Cells were then fixed with
741 Carnoy's reagent for 60 min and subsequently stained with 0.5% Crystal violet for 60 s. Wells
742 were extensively washed with H₂O and plaques counted.

743

744 Ectromelia virus model

745 Mice were inoculated subcutaneously with 10³ PFU Ectromelia virus (Moscow strain;
746 ATCC #VR-1374) in the flank of the left hind limb between the regio tarsi and regio pedis
747 under avertin anesthesia. Clinical scores were recorded daily, and animals weighed on days 0,
748 5 and every 2 days thereafter until day 21, and bled on days -1 and 8 to measure viral load.
749 Animals with a significant clinical score or 25% decrease of starting weight were euthanized
750 and considered dead the following day. Viral load was measured in blood by qPCR for

751 *ECTV-Mos-156* viral genomes and in organs by viral plaque assay as \log_{10} PFU/gram
752 tissue⁵².

753

754 Macrophage cultures

755 Bone marrow from femurs and tibiae was cultured 7 days in complete RPMI1640
756 medium with 10% fetal bovine serum, 100U/ml penicillin, 100 μ g/ml streptomycin, 2mM L-
757 glutamine (Life Technologies) and 50ng/ml recombinant human Macrophage colony-
758 stimulating factor (Peprotech). Cells were placed into 6-well plates for stimulation with
759 10ng/ml *Salmonella* Minnesota R595 (Re) ultra-pure LPS (List Biological) for different time-
760 points. RNA was extracted by TRIzol (Life Technologies), reverse transcribed to cDNA with
761 SuperScriptII (Life Technologies), *Cxcl1* and *Cxcl11* levels were measured using the Taqman
762 assay (Applied Biosystems). RNA levels relative to *Ef1a* were calculated with the delta Ct
763 values. Conditionally *Hoxb8*-immortalized bone-marrow progenitor cells were generated as
764 described⁵³.

765

766 LPS sepsis model

767 A low dose of 50 μ g LPS was administered by intraperitoneal injection. Mice were
768 monitored every 1 h for 10 h and sacrificed if ethical end-point reached. Monitoring was
769 conducted using body conditioning score and Grimace Scale (NC3Rs [National Centre for the
770 Replacement Refinement & Reduction of Animals in Research) approved by the Garvan/St
771 Vincent's Animal Ethics Committee. Monitoring continued twice daily for 7 days in
772 surviving mice, were weights and blood glucose levels were also measured. Serum was
773 collected by tail-tipping before LPS injection and 2 and 4 hours following injection for IL6
774 determination by ELISA (BD; OptEIATM Set Mouse IL-6), as per manufactures instructions.
775

776 Immunohistochemistry and beta cell area determination

777 Tissues were fixed in 10 % neutral buffered formalin (Sigma-Aldrich), paraffin
778 embedded and parallel sections (5 μ m) prepared. Sections were stained with hematoxylin and
779 eosin (H&E; Sigma-Aldrich) and for pancreatic tissue parallel sections stained for insulin
780 (purified rabbit anti-mouse insulin polyclonal antibody; 4590; Cell Signaling Technology).
781 Visualization of bound anti-insulin antibody was achieved using HRP-labelled polymer-
782 conjugated goat anti-rabbit IgG (Dako EnVision+ System), followed by counterstain with
783 hematoxylin. For pancreatic beta cell mass determination consecutive pancreatic serial
784 sections at 200 μ m intervals were stained for insulin and beta cell area quantified from total
785 area taken by insulin positive cells compared to non-positive tissue (ImageJ). Beta cell mass
786 (mg) was calculated by multiplying relative insulin-positive area by the mass of the isolated
787 pancreas before fixation. Images were captured using a Leica DM 4000 or Leica DM 6000
788 Power Mosaic microscope (Leica Microsystems).

789

790 Metabolic studies

791 Blood glucose levels (BGL) were determined using a FreeStyle Lite[®] glucometer and
792 blood glucose test strips (Abbott Diabetes Care) via tail tipping. Measurements were taken
793 from 8 and 12 week old male or female non-fasted mice. Intraperitoneal glucose tolerance
794 tests (IP-GTT) were conducted following an overnight fast (16 h) with access to water. The
795 following day mice were weighed and fasting blood glucose measurements taken.
796 Subsequently, mice were injected (IP) with 20% dextrose w/v (Sigma Aldrich) to a final
797 concentration of 2 g glucose per kg body weight (2g/kg). Blood glucose levels were
798 measured from the tail vein at 15, 30, 60 and 120 min post-glucose administration.
799 Intravenous (IV) GTT was conducted in a similar manner; however, glucose (1 g/kg) was
800 administered intravenously into the tail vein and blood glucose measurements taken at 0, 5,

801 10, 15, 20, 30, and 60 min post-injection. During the IV-GTT blood samples were also taken
802 for the determination of insulin content via ELISA, conducted as per manufactures
803 instructions (Cayman Chemical). Glucose-stimulated insulin secretion assay (GSIS) was
804 performed for islets *ex vivo* as described⁵⁴.
805

806 **Islet isolation, transplantation, and *in vitro* studies**

807 Islets were isolated as previously described⁵⁵, and counted for islet transplantation or *in*
808 *vitro* experiments using a Leica MZ9.5 stereomicroscope. Islets were transplanted under the
809 kidney capsule of diabetic B6 littermates as described⁵⁶. Diabetes was induced by IP
810 injection of 180 mg/kg streptozotocin (Sigma-Aldrich) dissolved in 0.1 M citrate buffer (pH
811 4.2) at a concentration of 20 mg/ml. Diabetes was determined as [blood glucose] \geq 16 mM on
812 two consecutive days measured by FreeStyle Lite® glucometer and Abbott Diabetes Care test
813 strips following tail tipping. Islet grafts were retrieved from recipients at indicated time points
814 post-transplantation for analysis of islet morphology, function, or degree of lymphocytic
815 infiltration by histology or gene expression by RT-qPCR. Gene expression in islet grafts was
816 calculated using the average WT Δ Ct value. Islets to be used for *in vitro* studies were cultured
817 overnight in islet overnight culture media (RPMI-1640, 20% FCS, 100 U/ml P/S, 2 mM L-
818 Glutamine) at 37°C + 5% CO₂.
819

820 **Flow cytometry**

821 Flow cytometric staining was performed as described in^{57,58}. Antibodies against the
822 following surface antigens were: CD4 (RM4-5), CD8 (53-6.7), CD21 (7G6), CD23 (B3B4),
823 CD25 (PC61.5), CD44 (IM7), CD69 (H1.2F3), CD93 (AA4.1), CD45.2 (104), B220 (RA3-
824 6B2), IgM (II/41), IgD (11-26). Splenocytes were labeled with CFSE (Invitrogen) and
825 cultured in RPMI1640 supplemented with 10% FCS, 2 mM L-glutamine, 55 μ M 2-
826 mercaptoethanol, and penicillin/streptomycin. B cells were stimulated with: F(ab')₂ goat anti-
827 mouse IgM (10 μ g/ml, Jackson Immunoresearch Laboratories), LPS from *E. coli* 055:B5
828 (10, 1, 0.1 μ g/ml, SIGMA), anti-CD40 (10 μ g/ml FGK4.5, BioXCell). When analysing 3A9
829 TCR transgenic cells single cell suspensions from thymus, spleen or pancreatic lymph node
830 were incubated for 30 min at 4°C in culture supernatant from the 1G12 hybridoma (specific
831 for TCR^{3A9}). Then, cells were pelleted by centrifugation and incubated for 30 min at 4°C in
832 FACS buffer containing assortments of fluorochrome- or biotin-conjugated monoclonal
833 antibodies against cell surface proteins. After washing in FACS buffer, cells were fixed and
834 permeabilised using the eBioscience Foxp3 staining buffers, then incubated with antibodies
835 specific for intracellular proteins and Brilliant Violet 605-streptavidin conjugate (BioLegend)
836 to detect biotin-conjugated antibodies. Antibodies were purchased from BD, eBioscience or
837 BioLegend. Data were acquired with LSR II flow cytometers (BD) and analyzed using
838 FlowJo software (Tree Star).
839

840 **CyTOF:**

841 Unstimulated spleen cells from four wild-type and four I325N-homozygous mutant mice
842 were individually labeled with mass-barcodes, mixed, permeabilized and stained with mass-
843 labeled antibodies to a panel of cell surface markers and intracellular proteins including I κ B α ,
844 and analyzed by CYTOF mass spectrometry^{59,60}. Spanning-tree Progression Analysis of
845 Density-normalized Events (SPADE)⁶¹ analysis was used to resolve leukocyte lineages and
846 subsets, and the relative intensity of I κ B α in each subset compared between mutant and wild-
847 type cell counterparts.
848
849
850

851 Real Time quantitative PCR (RT-qPCR)

852 Mouse islets were isolated and placed into 12-well non-tissue culture-treated plates (150-
853 200 islets/well; Fisher Scientific). Following an overnight culture islets were treated with
854 200 U/ml recombinant human (h) TNF α (R&D Systems) for 1, 4, or 24 h. Total RNA was
855 extracted using the RNeasy Plus Mini Kit (Qiagen) and reverse transcribed using Quantitect
856 Reverse Transcription Kit (Qiagen). Primers were designed using Primer3 software⁶² with
857 sequences obtained from GenBank and synthesized by Sigma Aldrich (Table 3, 4). PCR
858 reactions were performed on the LightCycler®480 Real Time PCR System (Roche) using the
859 FastStart SYBR Green Master Mix (Roche). Cyclophilin (CPH) was used as the
860 housekeeping gene and data analyzed using the $2^{\Delta\Delta CT}$ method. Initial denaturation was
861 performed at 95° C for 10 sec, followed by a three-step cycle consisting of 95° C for 15 sec
862 (4.8° C/s, denaturation), 63° C for 30 sec (2.5° C/sec, annealing), and 72° C for 30 sec (4.8°
863 C/s, elongation). A melt-curve was performed after finalization of 45 cycles at 95° C for 2
864 min, 40° C for 3 min and gradual increase to 95° C with 25 acquisitions/° C. Expression
865 differences were visualized using GraphPad or BAR Heatmapper plus tool.

866
867 Immunoblot analysis and immunoprecipitation

868 Primary islets were lysed in islet lysis buffer (50 mM Tris-HCl pH7.5, 1% Triton X,
869 0.27 M sucrose, 1 mM EDTA, 0.1 mM EGTA, 1 mM Na₃VO₄, 50 mM NaF, 5 mM
870 Na4P2O7, 0.1% β -mercaptoethanol; supplemented with EDTA-free protease inhibitor
871 [Roche]), β TC₃ cells were lysed with radioimmunoprecipitation (RIPA) buffer with SDS,
872 following relevant treatment with or without 200 U/ml of recombinant human (h) or mouse
873 (m) TNF α (R&D Systems). Protein concentration was measured using the Bradford assay
874 (Bio-Rad) and total protein (20-25 μ g) resolved on a 7 - 10% SDS PAGE gel and then
875 transferred to a nitrocellulose membrane, Immobilon-P® (Merck Millipore). Membranes
876 were incubated with anti-I κ B α (9242), anti-phospho-I κ B α (9256), anti-JNK (9252), anti-
877 phospho-JNK (9255), anti-I κ K α (2682), anti-phospho-I κ K α/β (16A6; 2697), anti-NIK
878 (4994), anti-NF- κ B2 p100/p52 (4882), anti-RelB (C1E4, 4922) (Cell Signaling Technology);
879 anti-beta-actin (AC15) (Sigma-Aldrich); or anti-S381-A20 (a kind gift by Professor Derek W.
880 Abbott¹⁹) followed by horseradish peroxidase (HRP)-labeled secondary antibody goat-anti-
881 mouse IgG Fc (Pierce Antibodies) or donkey-anti-rabbit IgG (GE Life Sciences). HRP
882 conjugates bound to antigen were detected and visualized by using an ECL detection kit (GE
883 Life Sciences).

884 Immunoprecipitation was conducted in thymocytes, which were lysed at 4° C in
885 immunoprecipitation buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 1%
886 Triton X 100, 5% glycerol) containing Complete® protease inhibitor and Phosphostop®
887 phosphatase inhibitors (Roche). Immunoprecipitation was then conducted by first preclearing
888 lysates with protein A/G-Sepharose (Thermo Fisher Scientific) for 1 h and then incubated with
889 anti-TNFR1 (ab7365) cross-linked beads or anti-A20 (59A426) antibody (Abcam) for 2 h at
890 4° C. Following incubation with only antibody 25 μ l of protein A/G beads were added and
891 incubated at 4° C on a roller overnight. Beads were washed 4 X with lysis buffer and then
892 eluted using low pH amide buffer for cross-linked beads or 30 μ l of Laemmli reducing gel-
893 loading sample buffer for non-cross linked beads. Samples were vortexed, heated to 100° C
894 for 5 min, cooled on ice for 10 min, and then loaded onto a 8 or 10% agarose gel for
895 immunoblotting for anti-A20 (56305/D13H3), anti-I κ B α (9242), anti-I κ K β (2684), anti-JNK
896 (9252), anti-TAK1 (52065), anti-phospho-I κ B α (9256), anti-phospho-JNK (9255), anti-
897 phospho-TAK1 (4536/90C7), anti-TNFR1 (3736C25C1) (Cell Signaling Technology), anti-
898 RIP1 (H-207) (Santa Cruz), anti-RIP1 (610458) (BD bioscience), anti-TAK1 (491840) (R&D
899 systems), or anti-beta-actin (AC15) (Sigma-Aldrich). For B cell stimulation, splenic B cells
900 purified by MACS with CD43 depletion were stimulated with anti-IgM F(ab')2 or LPS for

901 the indicated times. Cells were lysed with TNE buffer (1% Nonidet P-40, 20 mM Tris-HCl,
902 pH 8.0, 150 mM NaCl, 0.1 mM sodium orthovanadate, and complete protease inhibitor
903 [Roche]). The following antibodies were used: anti-I κ B α (9242), anti-TNFAIP3
904 (5630/D13H3) (Cell Signaling Technology), anti-A20 (A-12), anti-ubiquitin (P4D1) (Santa
905 Cruz) and anti-beta-actin (AC-15) (Sigma-Aldrich).

906

907 **In vitro reporter transfection studies**

908 Reporter assays were carried out as described previously^{63,64}. NF- κ B activity
909 experiments were conducted using β TC₃ cells transfected with 0.3 μ g of the NF- κ B.Luc
910 reporter (Promega) and 0.25 μ g CMV. β -galactosidase (a kind gift from Beth Israel Harvard
911 Medical School, Boston, MA). pcDNA vectors encoding human WT or variant A20
912 constructs or the empty pcDNA3.1 reporter were then added (0.3 μ g), and each well topped
913 with 0.15 pcDNA3.1 to make 1 μ g total DNA. Transfection was conducted using
914 Lipofectamine 2000 (Invitrogen). Following transfection cells were stimulated with 200 U/ml
915 of recombinant human (h) TNF α (R&D Systems). Luciferase activity was assayed in cell
916 lysates harvested 8 h post-stimulation, using a luciferase assay kit (Promega). Results were
917 normalized to β -galactosidase activity (Galactostar) to give relative luciferase activity.
918 Expression plasmids and reporters were obtained and maintained as described previously^{63,64}.
919

920 **Ubiquitination assays**

921 Wildtype A20, A20^{I325N} or A20^{C103A} OTU domain protein (1 μ g) purified from *E. coli*
922 was added to 2 μ g of K48-ubiquitin chains of mixed chain length (Ub₂-Ub₇) or to purified
923 tetra-ubiquitin (Ub₄) (#UC-230, UC-210, Boston Biochem). A20 OTU domains added to
924 mixed-length K48-ubiquitin chains were incubated in 100 μ l of deubiquitin buffer (50 mM
925 HEPES pH 8.0, 0.01% Brij-35, 3mM DTT) at 37° C with agitation at 400 rpm for 60 or 150
926 min in a benchtop incubator shaker. A20 OTU domains added to K48-tetra-ubiquitin chains
927 were incubated in 100 μ l of deubiquitin buffer (25 mM HEPES pH 8.0, 5 mM DTT, 5 mM
928 MgCl₂). At the indicated times, 20 μ l of reaction mixture was collected and the enzymatic
929 reaction stopped by addition of SDS sample buffer. Recombinant Flag-tagged full length
930 A20^{+/+} or A20^{I325N}, was expressed in HEK-293T cells and purified as described
931 previously^{14,17}. Full-length A20 deubiquitination reactions were performed using 100ng
932 recombinant A20, 500 ng of the indicated ubiquitin chain and DUB reaction buffer with or
933 without phosphatase inhibitor cocktail and were incubated for the indicated times at 37° C
934 with agitation at 1,000 rpm. Following incubation samples were placed on ice and 20 μ l
935 collected and added to SDS sample buffer to stop the reaction. Full-length ubiquitin ligase
936 assay was performed as previously described¹⁴. Samples were subjected to 1D SDS-PAGE
937 and immunoblotted for ubiquitin (clone P4D1; Santa Cruz or Cell Signaling Technology) or
938 A20 (clone 59A426; Abcam or A-12; Santa Cruz), as described above.
939

940 **OTU protein preparation and crystallisation**

941 The N-terminal OTU domains of human and mouse A20 (human WT and I325N,
942 residues 1-366; mouse WT and C103A, 1-360) were cloned into vector pGEX-6P-1 (GE
943 Healthcare), facilitating bacterial expression as a GST-fusion. Sequences were confirmed by
944 Sanger sequencing. Expression was performed in *E. coli* strain BL21 (DE3) Gold, where
945 cells were induced at an OD600 of 0.5 with 0.2 mM IPTG, followed by incubation at 20° C
946 overnight in LB medium. Cells were harvested by centrifugation, lysed by three cycles of
947 freeze-thaw and one cycle of pressure shock (human WT and I325N) or by sonication alone
948 (mouse WT and C103A). The GST-fusions was captured from the cleared lysate by passage
949 over GSH-Sepharose CL4B resin (GE). For purification of human WT and I325N, the resin
950 was washed (50 mM Tris (pH 8.8) 200 mM NaCl, 5 mM DTT, 1 mM EDTA), then the A20

951 component released by overnight incubation (4° C) with PreScission protease. Eluted A20
952 was stabilized by incubation with iodoacetamide (Sigma; 30 mM, 30 min at RT). The
953 reaction was terminated by addition of an equivalent amount of β -mercaptoethanol. For
954 purification of mouse WT and C103A protein, purified GST-OTU fusion protein was eluted
955 (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, 5 mM DTT, 10 mM glutathione) from the column
956 and the A20 OTU component was released by overnight incubation (4° C) with PreScission
957 protease. The cleavage products were subject to anion-exchange chromatography (HiTrap Q
958 FF; GE) where the OTU domain eluted as a single peak between 5 and 500 mM NaCl, 25
959 mM Tris-HCl pH 7.5. All proteins were then concentrated and subject to gel filtration
960 chromatography (ÄKTA S200 26/60, buffers as described above).

961 Crystals of both the A20^{I325N} variant (long triangular rods) and wild-type A20 (long rods)
962 OTU domains, grown under the same conditions; equal volumes of protein (2.7 mg/mL) and
963 well solution (50 mM CaCl₂, 100 mM MES (pH 6.0), 5% PEG1500) were combined in a
964 hanging drop setup. Crystals grew over several weeks at RT. Partial cryoprotection was
965 achieved by briefly (1-5 seconds) swimming crystals in a solution comprising equal volumes
966 of mother liquor and well solution doped with glycerol (25% v/v final) prior to being plunge
967 vitrified in liquid N₂. For the mouse wild-type A20, crystallization was achieved by vapor-
968 diffusion in hanging-drops at a protein concentration of 8 mg/mL in 1.8 - 2.4 M NaCl, 0.1 M
969 MES [pH 6-6.7]. Crystals were soaked in mother liquor containing 30% ethylene glycol for
970 one minute and immediately vitrified in a nitrogen cryo-stream.

971

972 Crystallographic data reduction and model refinement

973 Diffraction data using light of wavelength 0.9537 Å was collected at 100 K at beamline
974 MX2 at the Australian Synchrotron. Data were indexed and integrated with MOSFLM⁶⁵. The
975 spacegroups were scrutinized with POINTLESS, and the data scaled with AIMLESS^{66,67}, or
976 SCALA⁶⁸ accessed via the CCP4i software interface⁶⁹. Although grown under essentially the
977 same conditions, the human WT and I325N mutant A20 proteins crystallized in different
978 space groups. These data were highly anisotropic, resulting in poor completeness, low
979 multiplicity, and noisy electron density maps. In the case of the mouse OTU crystal, there
980 was significant thermal diffuse scattering. See Table 5 for data reduction and refinement
981 statistics.

982 Structures were solved by molecular replacement using PHASER⁷⁰. The search model
983 was the A-chain of PDB entry 3DKB, but stripped of surface loops that displayed
984 conformational variability in other PDB entries (2VFJ and 3ZJD). In the case of the human
985 I325N data the structure was originally solved in the space-group P3₁2 with 4 molecules in
986 the asymmetric unit. However, crystal packing and residual unaccounted-for electron density
987 suggested more molecules might be present. The structure was subsequently solved in the
988 lower symmetry P3₁ space group, with 6 molecules in the asymmetric unit, sensible packing
989 and no unaccounted-for density. The human wild-type A20 structure, solved as a control for
990 the iodoacetamide alkylation, also has 6 molecules (three dimers) in a different asymmetric
991 unit and space group. The mouse OTU structure was indexed and refined in the P3₂ space
992 group, with a dimer in the asymmetric unit. Restrained B-factor refinement, using local non-
993 crystallographic symmetry (NCS) restraints, was performed with REFMAC5⁷¹. For the
994 mouse A20 OTU structure TLS and restrained refinement was carried out using
995 phenix.refine⁷². Between rounds of refinement, electron density maps and composite OMIT
996 maps⁷³ and their fit to the model were examined using COOT⁷⁴. Amino acid side chains were
997 added/subtracted if suggested by difference map electron density. The active-site cysteine
998 (C103) was clearly identified through inspection of mFo-Dfc difference maps as the only
999 cysteine residue alkylated by the iodoacetamide treatment in both human structures (PDB
1000 residue descriptor YCM). All 6 molecules refined in each structure are highly similar in fold,

1001 both to themselves, and compared with each other. All molecules form dimers with
1002 neighboring molecules, as observed in other crystal structures. Structure validation was
1003 performed using the MOLPROBITY web server⁷⁵. The final human I325N, human WT, and
1004 mouse WT structures contain Ramachandran favored/outlier components of 88.64/0.00%,
1005 88.13/2.87 %, and 82.13/7.87 %, respectively.

1006

1007 Measurement of OTU domain thermal stability

1008 Purified A20 OTU domains (0.4 mg.mL⁻¹) were exchanged into 100 mM NaCl, 5 mM
1009 dl-dithiothreitol, 20 mM Na₂HPO₄ (pH 7.5). Circular dichroism data were collected with a
1010 Chirascan circular dichroism spectrometer (Applied Photophysics, UK) in a 1 mm cuvette.
1011 The protein was heated from 20° C to 90° C at a rate of 1° C min⁻¹ while ellipticity was
1012 monitored at 220 nm. The thermally induced unfolding of A20 OTU domains was not
1013 reversible. The unfolding of the A20 OTU domains was described by a two-state model⁷⁶
1014 (**Equation 1**).

1015

1016 **Equation 1**

$$y_{obs} = \frac{y_n + m_n T + (y_u + m_u T) \exp\left(\frac{\Delta H_{vH}}{R} \left(\frac{1}{T} - \frac{1}{T_{trs}}\right)\right)}{1 + \exp\left(\frac{\Delta H_{vH}}{R} \left(\frac{1}{T} - \frac{1}{T_{trs}}\right)\right)}$$

1017

1018 Where y_{obs} is the observed ellipticity, y_n and y_u are the ellipticity values observed for the
1019 native and unfolded states, respectively. The m_n and m_u values are the linear temperature
1020 dependencies of y_n and y_u . ΔH_{vH} is the apparent van't Hoff enthalpy, R is the universal gas
1021 constant, and T_{trs} is the temperature at which the population of unfolded protein is 50%.
1022 Curves were fit by non-linear regression using GraphPad Prism v6 (GraphPad Software).

1023

1024 Statistical methods

1025 Results are expressed as mean +/- standard error mean (SEM). Statistical analysis was
1026 performed using the Student's *t*-test or ANOVA were indicated.

1027 **Supplementary Text**

1028

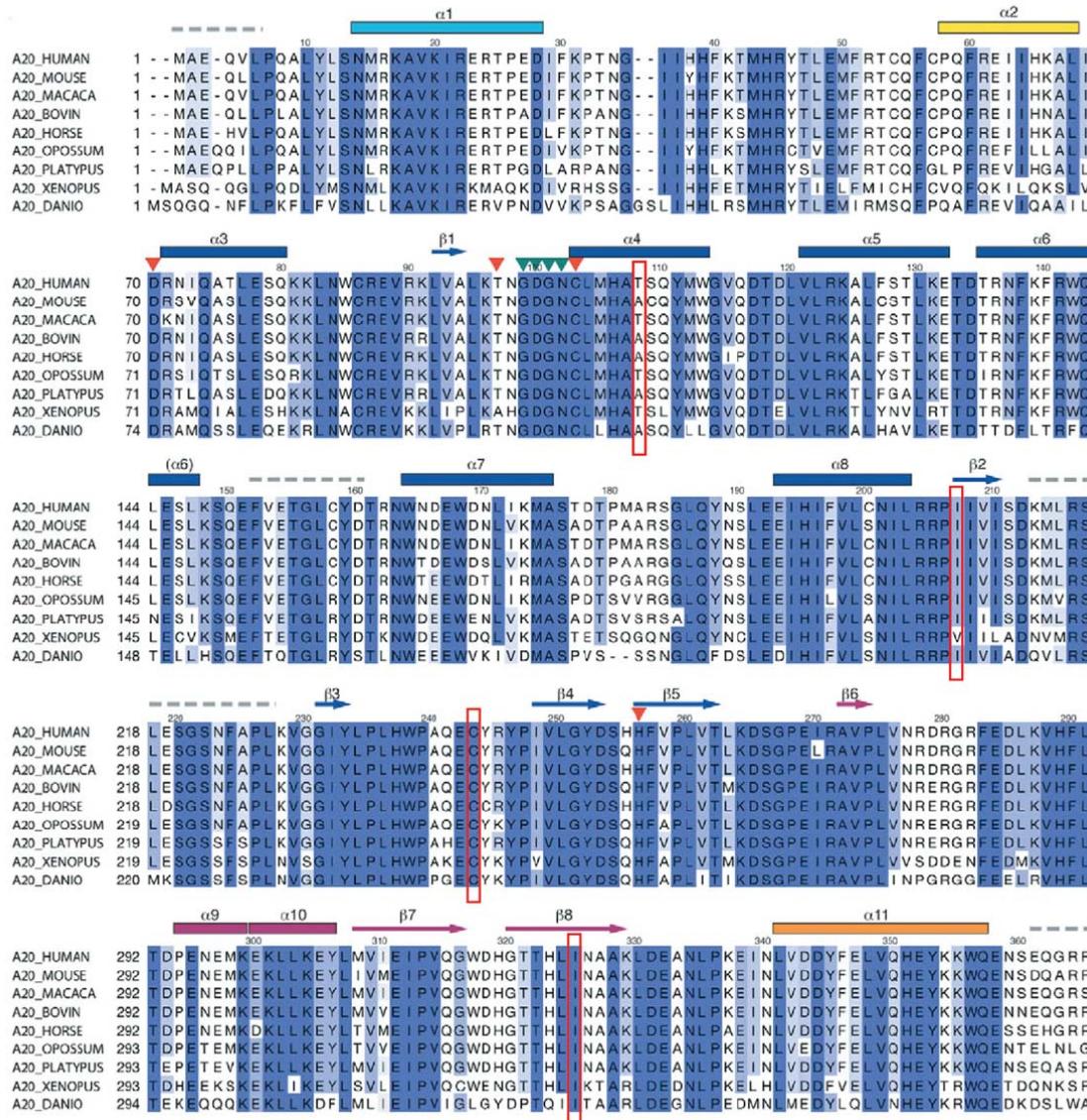
1029 Extended Figure 3 legend:

1030 **Fig.3. Subclinical inflammatory and metabolic consequences of the I325N variant. (A)**

1031 Body weights of 8 and 12 week old *Tnfaip3*^{+/+}, *Tnfaip3*^{I325N/+}, *Tnfaip3*^{I325N/I325N} male or
1032 female mice. **(B)** Representative H&E and insulin (INS) stained pancreas sections (note
1033 pancreatitis; scale = 100 μ m) with **(C)** cumulative insulitis scores (0 = no evidence of
1034 inflammation; 1 = mononuclear cells present, but not infiltrating islet; 2 = 0-25%
1035 mononuclear cell infiltration of islet [peri-insulitis]; 3 = >25% insulitis; 4 = >75% insulitis)
1036 and, **(D)** calculated beta cell mass for *Tnfaip3*^{+/+} and *Tnfaip3*^{I325N/I325N} mice. **(E)** Heat map
1037 depicting mRNA levels for islet early-immediate pro-inflammatory factors⁷⁷ measured by
1038 RT-qPCR from islets isolated from individual mice and treated with TNF α for the indicated
1039 times. Values normalised against 0 h *Tnfaip3*^{+/+} islets. Data represents 3 independent islet
1040 preparations with 3-4 biological replicates. **(F)** Islets isolated of the indicated genotypes were
1041 transplanted under the kidney capsule of syngeneic diabetic *Tnfaip3*^{+/+} recipients. When
1042 euglycemia was established comparably by mutant or wild-type islets (see Extended Data
1043 Fig. 12A), a glucose tolerance test was conducted. Following an overnight fast, recipients
1044 received 2 g/kg glucose (i.p.) and blood glucose monitored over time (min). Mean area under
1045 the curve was used for statistical comparison to wild-type. **(G)** At post-operative day 30, islet
1046 grafts were removed and analysed by H&E staining (scale bar = 50 μ m) with the fraction of
1047 islet grafts of each genotype exhibiting immune infiltrate shown below. **(H)** Islet grafts
1048 isolated on post-operative day 10 were analysed for indicated mRNAs by RT-qPCR. Gene
1049 expression was calculated using the average wild-type ΔCt value with each data point
1050 representing an individual graft. P values represent Student's *t*-test unless otherwise stated **P*
1051 < 0.05; ***P* < 0.01; ****P* < 0.0001.

1052 **Extended Data Figures and Tables**

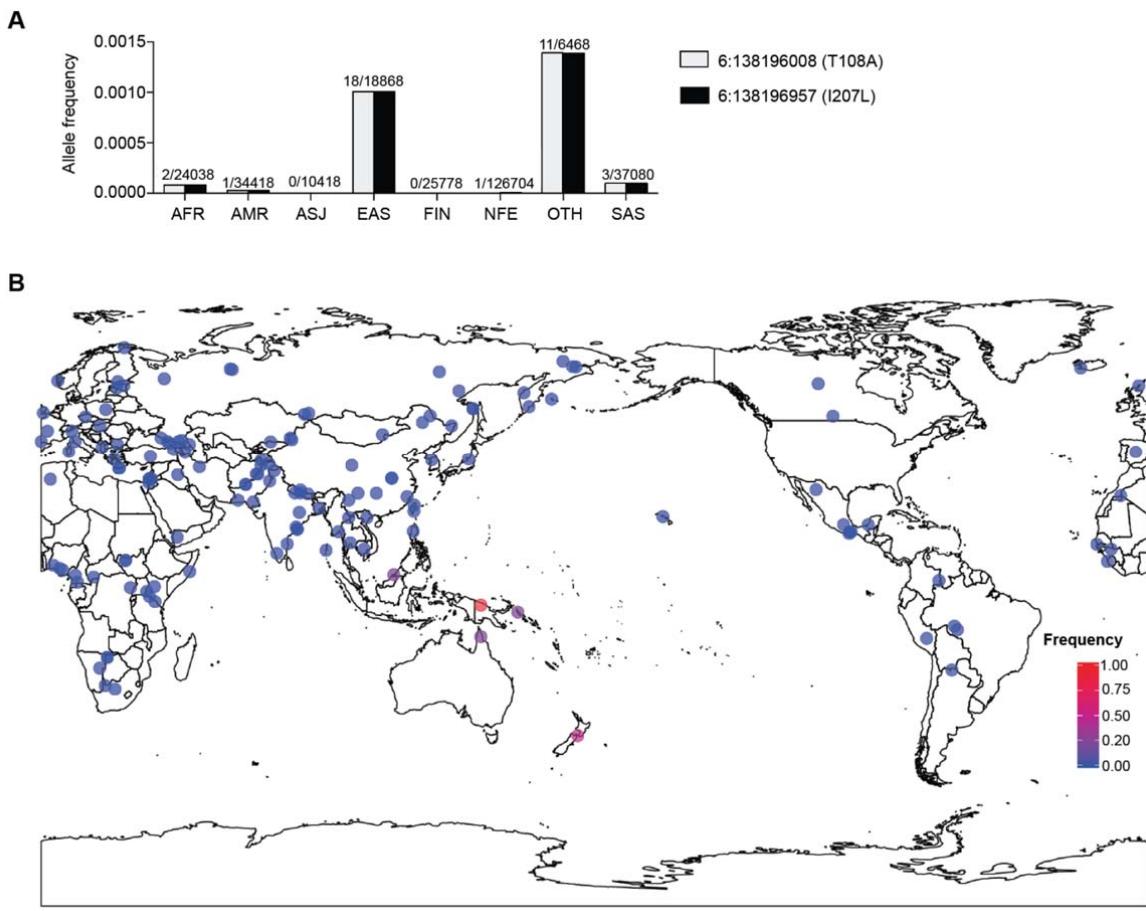
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1054

1055 **Extended Data Fig. 1.**

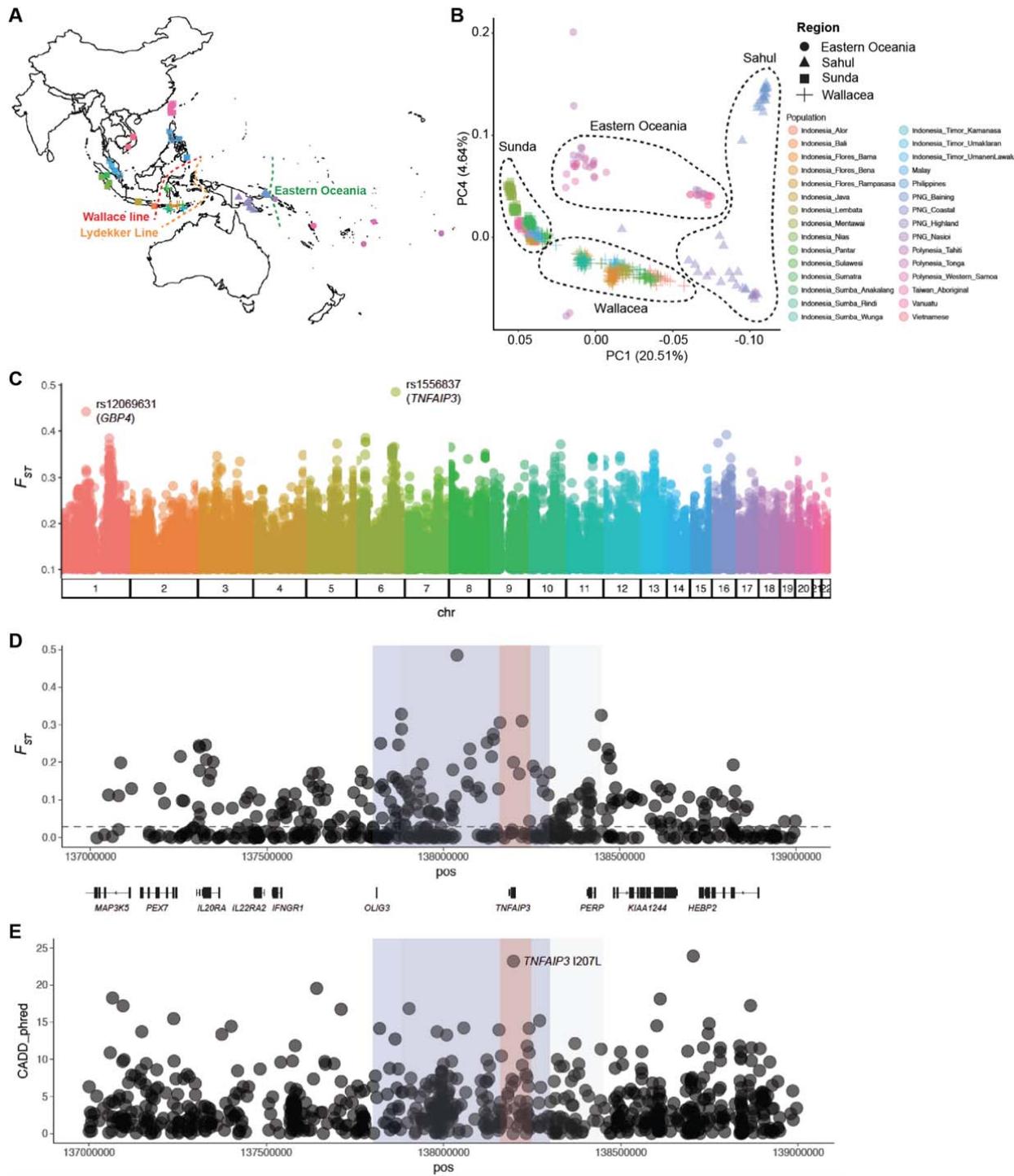
1056 **Evolutionary conservation of I207, C243 and I325 positions in A20 protein amongst**
 1057 **jawed vertebrates.** Aligned amino acid sequence and secondary structure elements of the
 1058 A20 OTU domain, from ⁴⁰. T108, I207, C243 and I325 residues are boxed in red.



1059

1060 **Extended Data Fig. 2.**

1061 **Global distribution of the T108A;I207L *TNFAIP3* haplotype.** (A) gnomAD r2.0.2
1062 population allele frequencies for the T108A and I207L missense variants in *TNFAIP3*, with
1063 allele counts above each bar (total as denominator). Populations are defined by principal
1064 component clustering of samples with individuals of known ancestry: those which do not
1065 cluster fall into the OTH group. AFR, African; AMR, Latino; ASJ, Ashkenazi Jew; EAS,
1066 East Asian; FIN, Finnish European; NFE, non-Finnish European; OTH, other; SAS, South
1067 Asian. The OTH population includes one homozygote, while all other alleles are
1068 heterozygous. (B) Frequency of the T108A;I207L *TNFAIP3* haplotype within 279 individual
1069 genomes from the Simons Genome Diversity Project²².



1071 **Extended Data Fig. 3.**

1072 **Population genetics of the Denisovan *TNFAIP3* haplotype.** (A) Geographic distribution of
 1073 samples used for population genetic analyses. Wallace Line (red)³¹, Lydekker Line (yellow).
 1074 Eastern Oceania is defined here as islands east of the Bismarck Archipelago (green line). (B)
 1075 Principal component analysis (PCA), with the proportion of variance explained by each PC
 1076 indicated on each axis (C) Genome-wide F_{ST} values between indigenous populations east and
 1077 west of the Wallace Line. (D) Maximum F_{ST} values on chromosome 6 scores span the

1078 *TNFAIP3* locus. Shaded regions correspond to peak F_{ST} values between populations east and
1079 west of the Wallace Line (grey, 6:137881500-138448062), a previously described haplotype
1080 of elevated Denisovan ancestry in Oceanians (blue, 6:137800000-138300000, ²⁵), and a
1081 putatively adaptively introgressed haplotype in Papuans (red, 6:138160925-138246514, ²⁹).
1082 (**E**) Phred-scaled CADD scores of all PASS variants with gnomAD allele frequency <0.01
1083 across the extended Denisovan haplotype ²⁷. Shading as per panel D.

1084

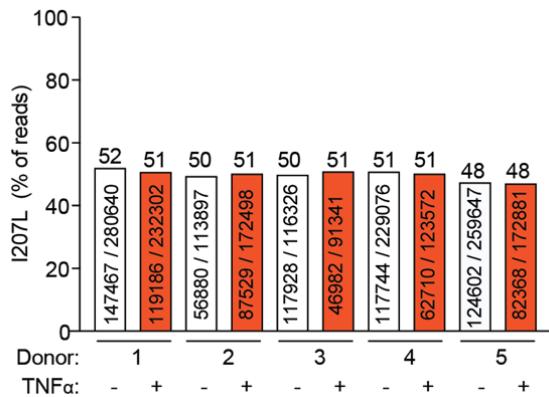
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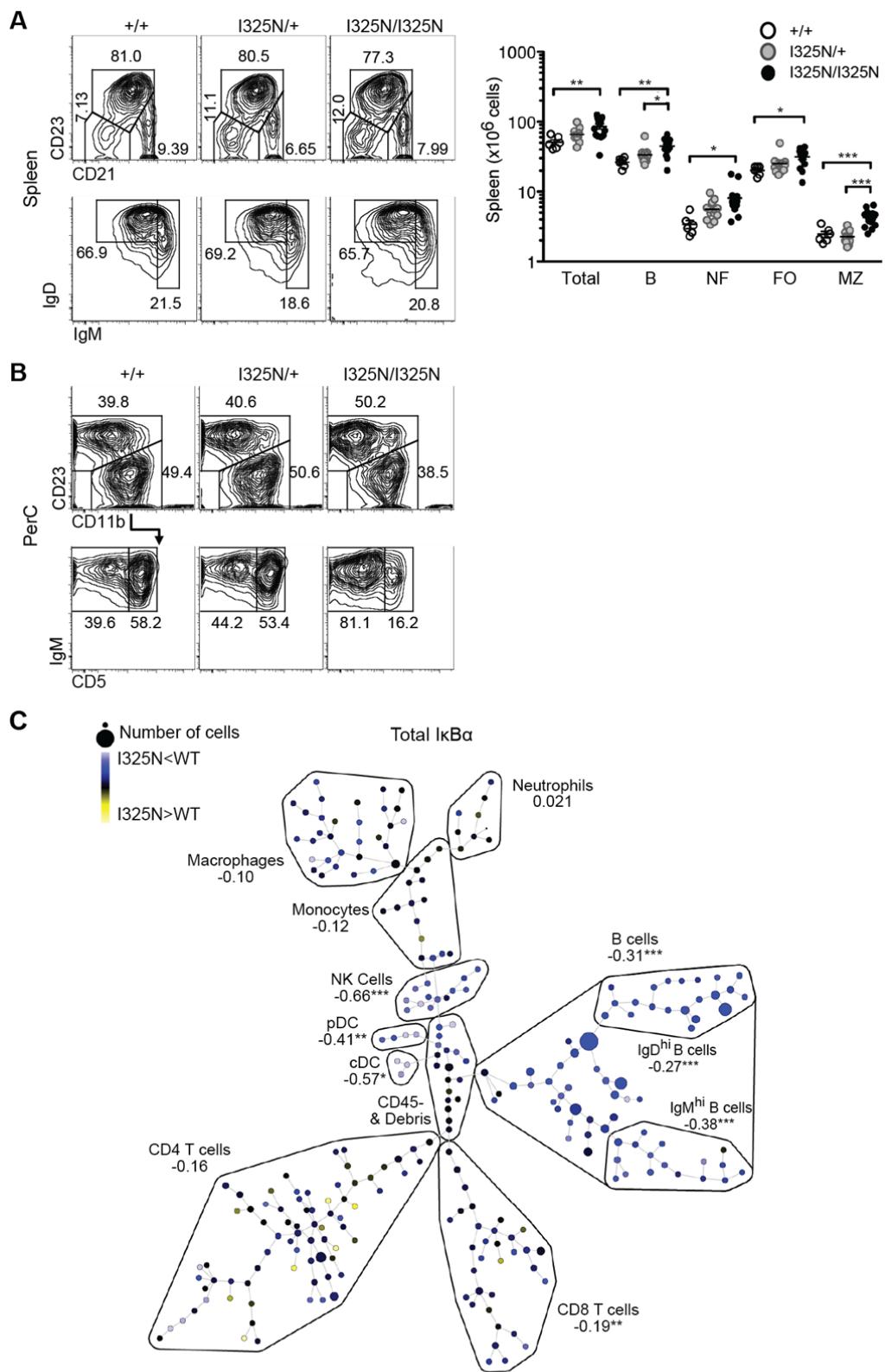
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1091 **Extended Data Fig. 4.**

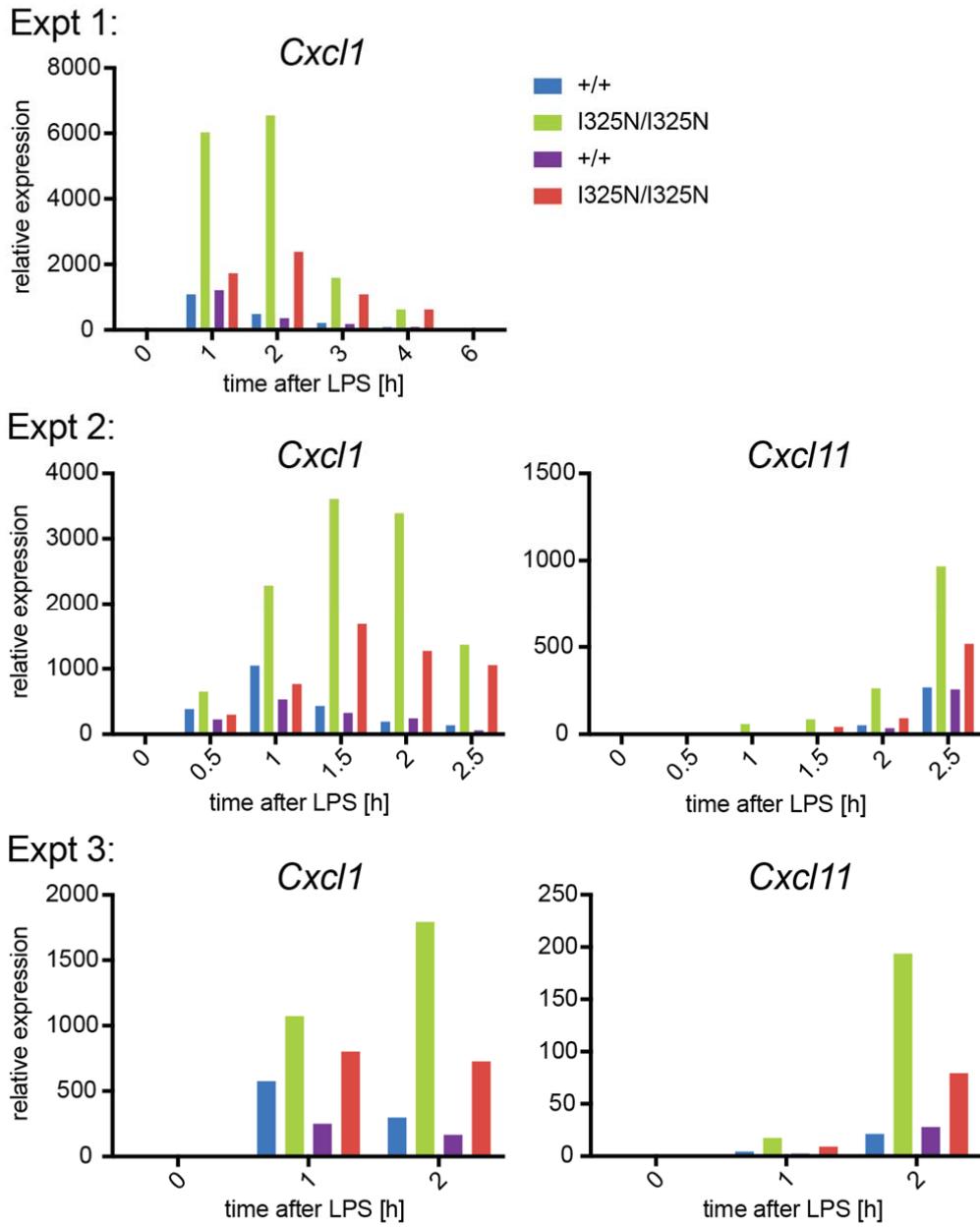
1092 **Relative abundance of Denisovan and modern human *TNFAIP3* mRNA from**
1093 **heterozygous leukocytes.** Peripheral blood mononuclear cells from five healthy
1094 T108A;I207L heterozygous donors were cultured with (+) or without (-) TNF α for 2 hours,
1095 mRNA isolated and converted to cDNA and amplified with primers in exon 3 and 4 on either
1096 side of the I207 codon. The products were deep sequenced on an Illumina MiSeq. Shown are
1097 the fraction and percentage of reads derived from the T108A;I207L Denisovan allele.



1098

1099 **Extended Data Fig. 5.**

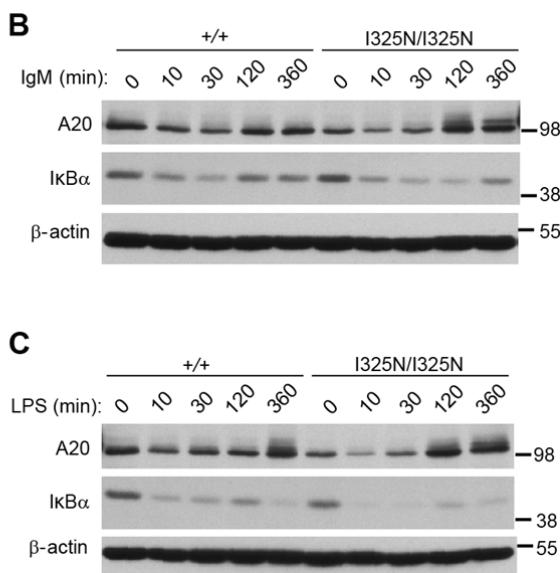
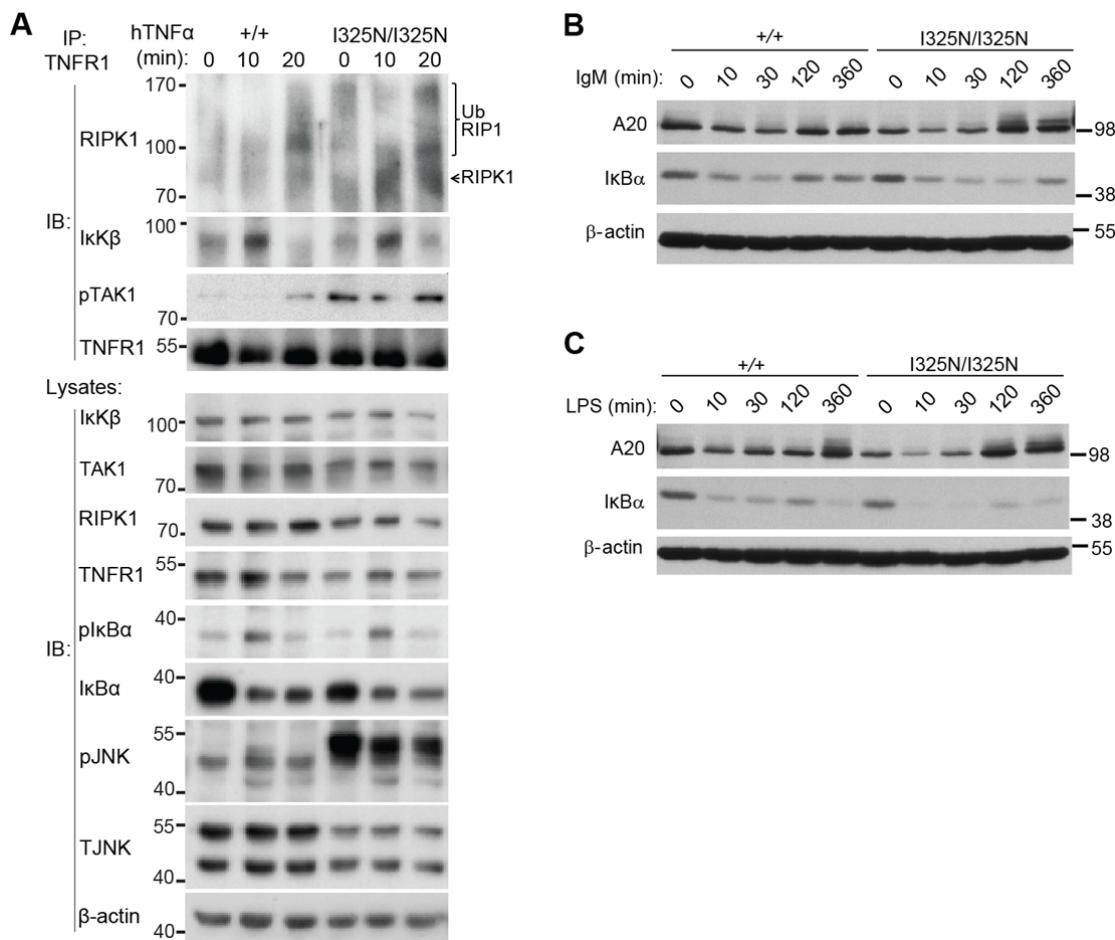
1100 **B cell lymphocytosis induced by the *Tnfaip3*^{I325N} mutation. (A)** Representative flow
1101 cytometry plots from mice of the indicated genotypes showing total frequency of splenic
1102 CD23⁺ and CD21⁺ positive cells (top panel). The bottom panel shows cells subsetted into
1103 IgD⁺ and IgM⁺ B cells. Note that the number of splenocyte B cell subsets is increased in
1104 *Tnfaip3*^{I325N/I325N} mice, shown to the right. **(B)** Representative flow cytometry plots from mice
1105 of the indicated genotypes showing the frequency of CD23⁺ and CD21⁺ positive cells in the
1106 peritoneal cavity (top panel). The bottom panel shows frequency of IgM⁺ and CD45⁺ B cells.
1107 Number of peritoneal cavity B cell subsets in mice of indicated genotypes is shown in Fig.
1108 2B. **(C)** CYTOF analysis of intracellular I κ B α in immune cells. Unstimulated spleen cells
1109 from *Tnfaip3*^{+/+} or *Tnfaip3*^{I325N/I325N} mice ($n=4$ per genotype) were individually labeled with
1110 mass-barcodes, mixed, permeabilized and stained with mass-labeled antibodies to a panel of
1111 cell surface markers and intracellular proteins including I κ B α , and analyzed by CYTOF mass
1112 spectrometry^{59,60}. Spanning-tree Progression Analysis of Density-normalized Events
1113 (SPADE; ⁶¹) analysis was used to resolve leukocyte lineages and subsets, and the relative
1114 intensity of I κ B α in each subset was compared between *Tnfaip3*^{I325N/I325N} and wild-type cell
1115 counterparts. Shown by color and numbers is the mean hyperbolic arcsine (arcsinh) ratio in
1116 minor and major leukocyte subsets. Note that I κ B α levels are reduced in B cell subsets
1117 indicating increased NF- κ B activation. Significant differences indicated by Student's *t*-test
1118 comparison of major subsets are marked: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



1119

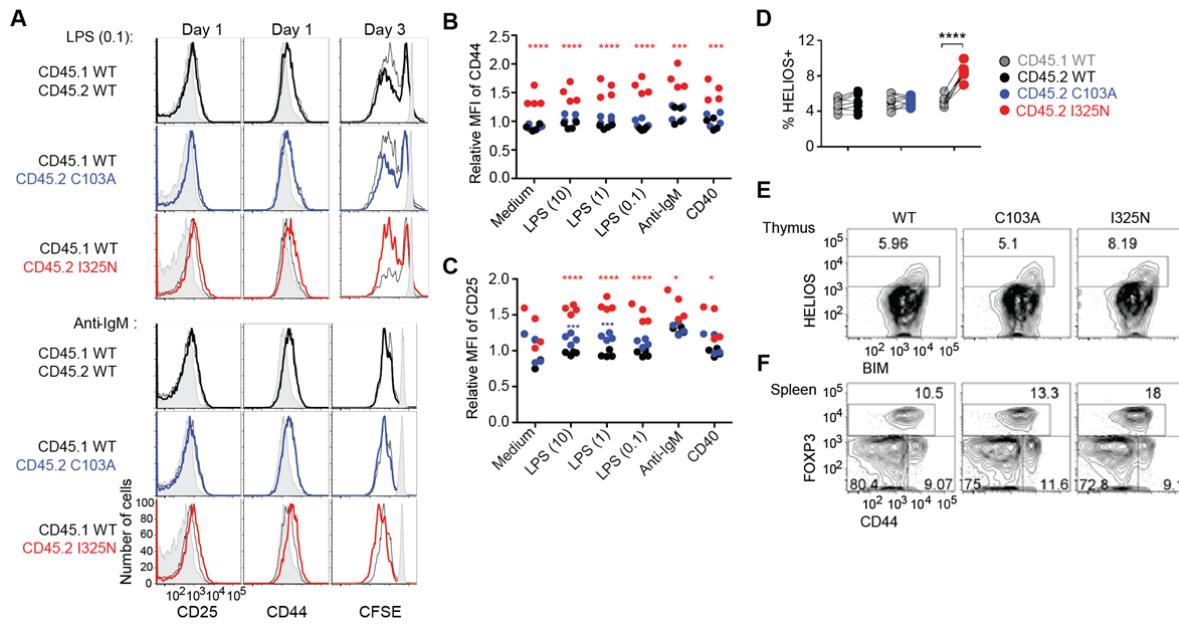
1120 **Extended Data Fig. 6.**

1121 **Exaggerated cytokine production by bone marrow-derived macrophages with the**
1122 ***Tnfaip3*^{I325N} mutation.** Bone marrow macrophages derived from conditionally *Hoxb8*-
1123 immortalized bone marrow progenitor cells were derived from two separate pairs of wild-
1124 type and I325N homozygous mice, stimulated with LPS (10ng/ml) for the indicated times,
1125 and *Cxcl1* or *Cxcl11* mRNA determined by qPCR relative to *Eif1a* expression. Data are
1126 shown from three independent experiments, each with two different mouse donors of each
1127 genotype.



1129 **Extended Data Fig. 7.**

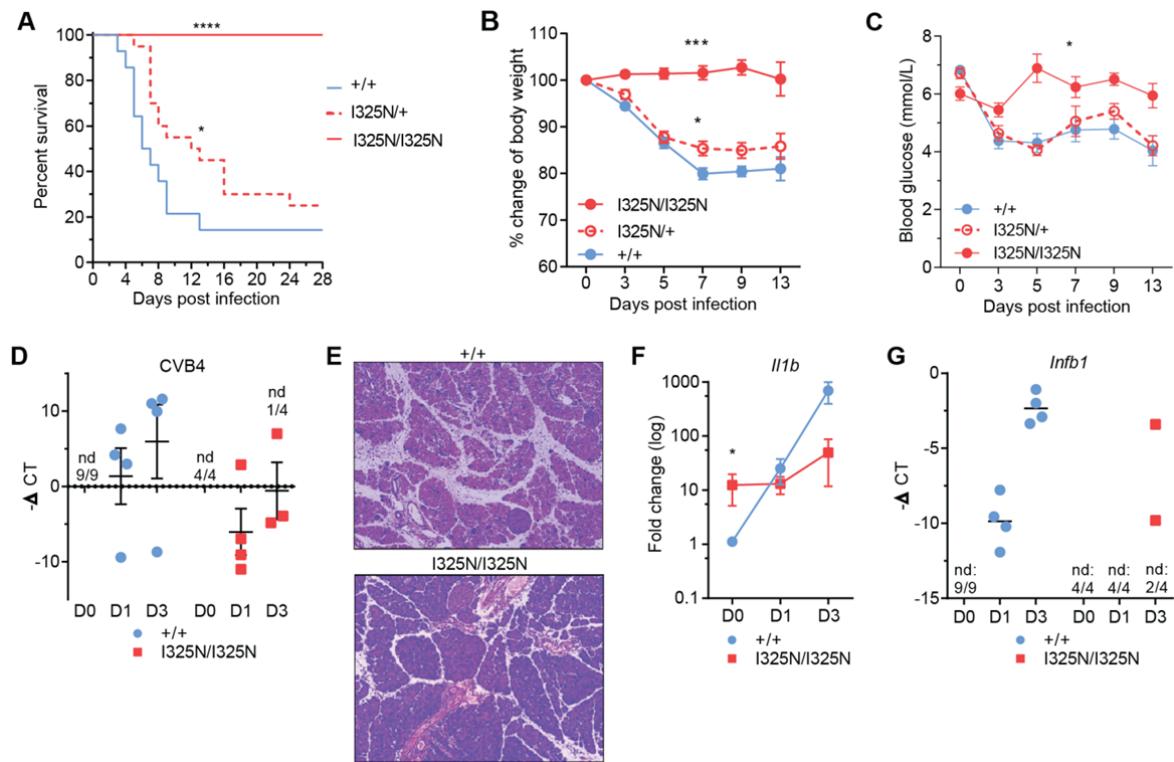
1130 **Prolonged NF- κ B signaling in thymocytes and B cells extracted from I325N variant**
 1131 **C57BL/6 mice. (A)** Representative immunoblot analysis (IB; $n=3$ independent experiments)
 1132 of canonical NF- κ B components following immunoprecipitation (IP) of TNFR1 from lysates
 1133 of *Tnfaip3*^{+/+} or *Tnfaip3*^{I325N/I325N} thymocytes treated with 200 U/ml hTNF α for the indicated
 1134 times. Following TNF α stimulation RIPK1 was recruited to the TNFR1 complex, with
 1135 increased high molecular weight polyubiquitinated forms of RIPK1 (UbRIP1) and auto-
 1136 phosphorylated transforming growth factor β activated kinase-1 [TAK1⁷⁸] in A20^{I325N/I325N}
 1137 thymocytes (A). Mutant thymocytes exhibited a modest increase in I κ B α degradation relative
 1138 to wild-type, and increased JNK phosphorylation (Lysate blots). A similar pattern of
 1139 enhanced TAK1 activation and JNK phosphorylation was seen in murine embryonic
 1140 fibroblasts and bone marrow derived macrophages expressing catalytically dead A20 OTU or
 1141 ZnF4 mutants¹⁸. **(B, C)** Wild-type (+/+) and I325N homozygous (I325N/I325N) *Tnfaip3*
 1142 splenic B lymphocytes were stimulated for the indicated times with anti-IgM (B), or
 1143 lipopolysaccharide (LPS) (C) and lysates analyzed by immunoblotting for A20, I κ B α and β -
 1144 actin (loading control). As observed for mutant thymocytes, B cells exhibited a modest
 1145 increase in I κ B α degradation relative to wild-type.



Extended Data Fig. 8.

Cell autonomous exaggeration of B cell activation and Treg formation by Tnfaip3 I325N more than C103A mutations.

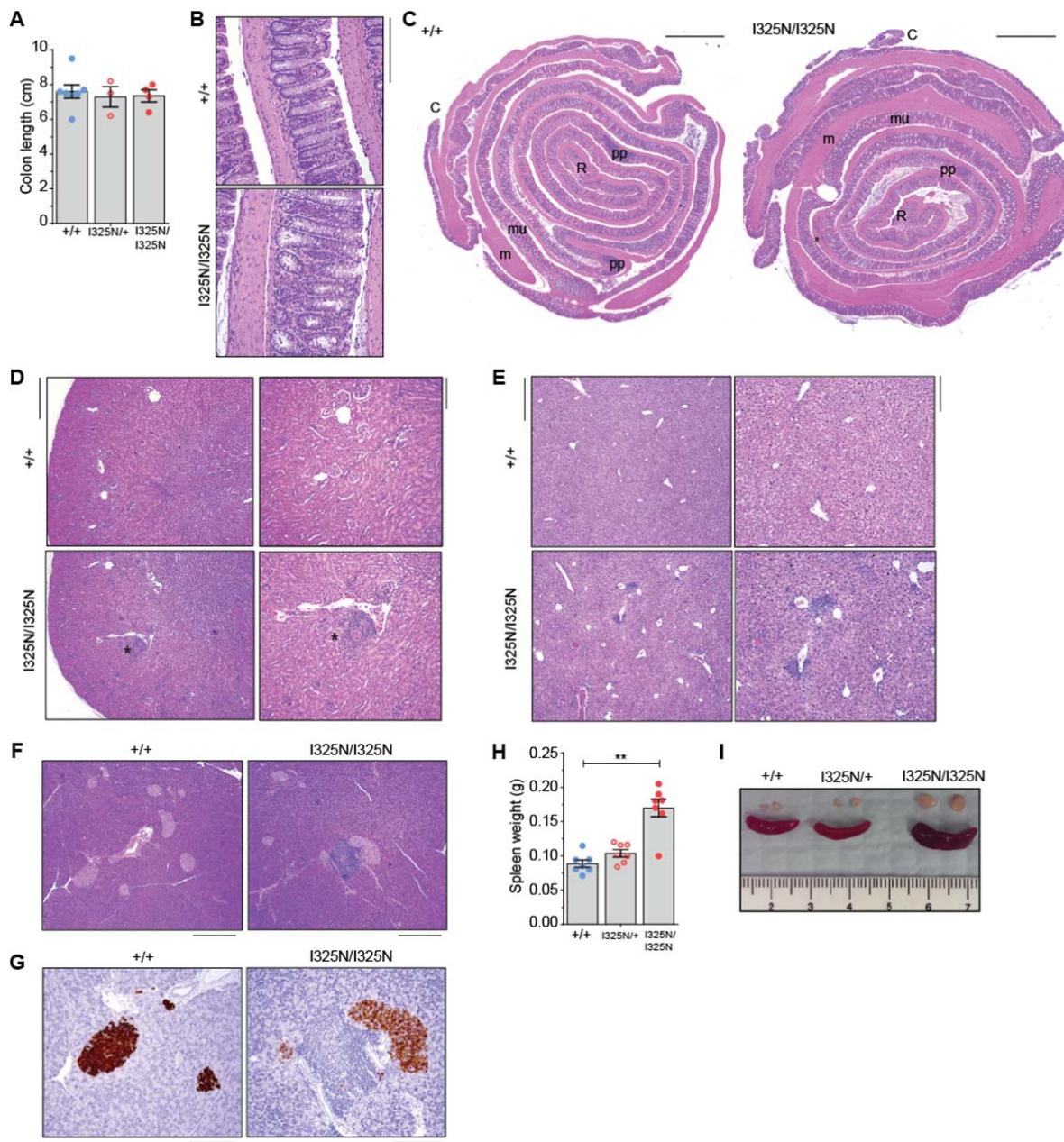
B6.CD45.1⁺ wild-type mice were transplanted with a congenic bone marrow mixture from *Tnfaip3*^{+/+} CD45.1⁺ donors, to provide wild-type lymphocytes as an internal control, and CD45.2⁺ donors of *Tnfaip3*^{I325N/I325N}, *Tnfaip3*^{C103A/C103A} or *Tnfaip3*^{+/+} genotypes. **(A)** Representative flow cytometric histograms of CD25 and CD44 expression on B cells from chimeric mice cultured for 1 day and CFSE dilution after 3 days with 0.1 ug/ml LPS or anti-IgM. Black or colored histograms, CD45.2⁺ B cells of the indicated *Tnfaip3* genotype; grey line unfilled histograms, CD45.1⁺ control B cells in the same stimulated culture; grey filled histograms, B cells in a parallel unstimulated culture. **(B, C)** Data from independent mixed chimeric animals showing relative mean fluorescence intensity (MFI) of CD25 or CD44 on CD45.2⁺ B cells of the indicated genotypes (red, I325N; blue, C103A; black, WT) compared to the co-cultured CD45.1⁺ wild-type B cells. Statistical analysis by ANOVA: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Figure 2C shows the percentage of CD45.2⁺ cells of the indicated genotypes among viable B cells, relative to starting percentage, in cultures from individual mixed chimera donors stimulated with 0.1 ug/ml LPS. **(D)** Pairwise comparison of percent HELIOS⁺ cells among the indicated CD45.2⁺ and CD45.1⁺ subsets of CD4⁺ CD8⁻ CCR7⁺ CD24⁺ FOXP3⁻ thymocytes from the same chimera. Analysis by paired Student's *t*-test; ****P < 0.0001. Compared to the C103A mutation, the I325N mutation exaggerated thymic formation of FOXP3⁺ CD4⁺ cells and their Helios⁺ FOXP3⁻ precursors, which depend on TCR signaling through CARD11 to NF- κ B. **(E, F)** Representative profiles gated on CD45.2⁺ cells of the indicated *Tnfaip3* genotypes. **(E)** Analysis of CD4⁺ CD8⁻ CCR7⁺ CD24⁺ FOXP3⁻ thymocytes, showing the percentage of immature medullary CD4 T cells induced by strong self-reactivity to express high levels of HELIOS and BIM. **(F)** Percentage of FOXP3⁺ CD44⁺ cells among CD4⁺ splenic T cells.



1173

1174 **Extended Data Fig. 9.**

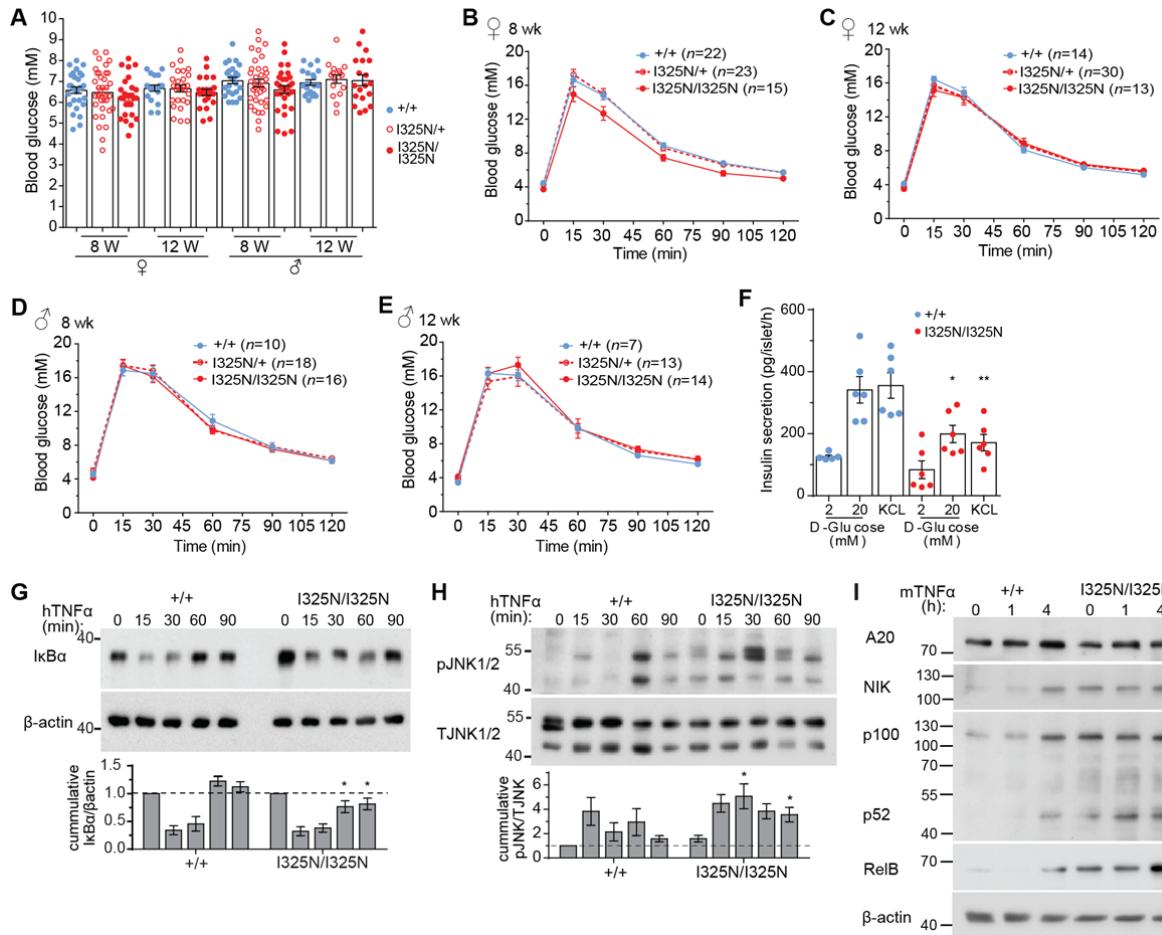
1175 **Increased resistance to Coxsackievirus B4 in A20 I325N mice.** Infection of C57BL/6 male
 1176 mice of the indicated *Tnfaip3* genotypes with 20 plaque forming units (PFU) of
 1177 Coxsackievirus B4 (CVB4) E2 strain by intraperitoneal injection. **(A)** Kaplan-Meier survival
 1178 curve showing a mean survival time of 6.5 and 12.5 days for *Tnfaip3*^{+/+} and *Tnfaip3*^{+/I325N}
 1179 mice, respectively. No *Tnfaip3*^{I325N/I325N} mice succumbed to infection. Significance
 1180 determined by Log-rank test. **(B)** Percent change in body weight after CVB4 infection and
 1181 **(C)** Blood glucose levels of mice with indicated *Tnfaip3* genotype, compared to wild-type by
 1182 area under the curve analysis. **(D)** CVB4 mRNA abundance on indicated days (D0-3) post
 1183 infection. **(E)** Hematoxylin & eosin stained sections of pancreas from *Tnfaip3*^{+/+} and
 1184 *Tnfaip3*^{I325N/I325N} mice at post-infection day 9. Note better preserved pancreatic architecture in
 1185 *Tnfaip3*^{I325N/I325N} mice. Scale bar = 200 μ m. **(F, G)** RTPCR analysis for inflammatory genes
 1186 *Il1b* (F) or *Infb1* (G) at post-infection day 0, 1 and 3. Error bars represent SEM and Student *t*-
 1187 test used for significance analysis unless otherwise stated, **P* < 0.05; ****P* < 0.001.



1188

1189 **Extended Data Fig. 10.**

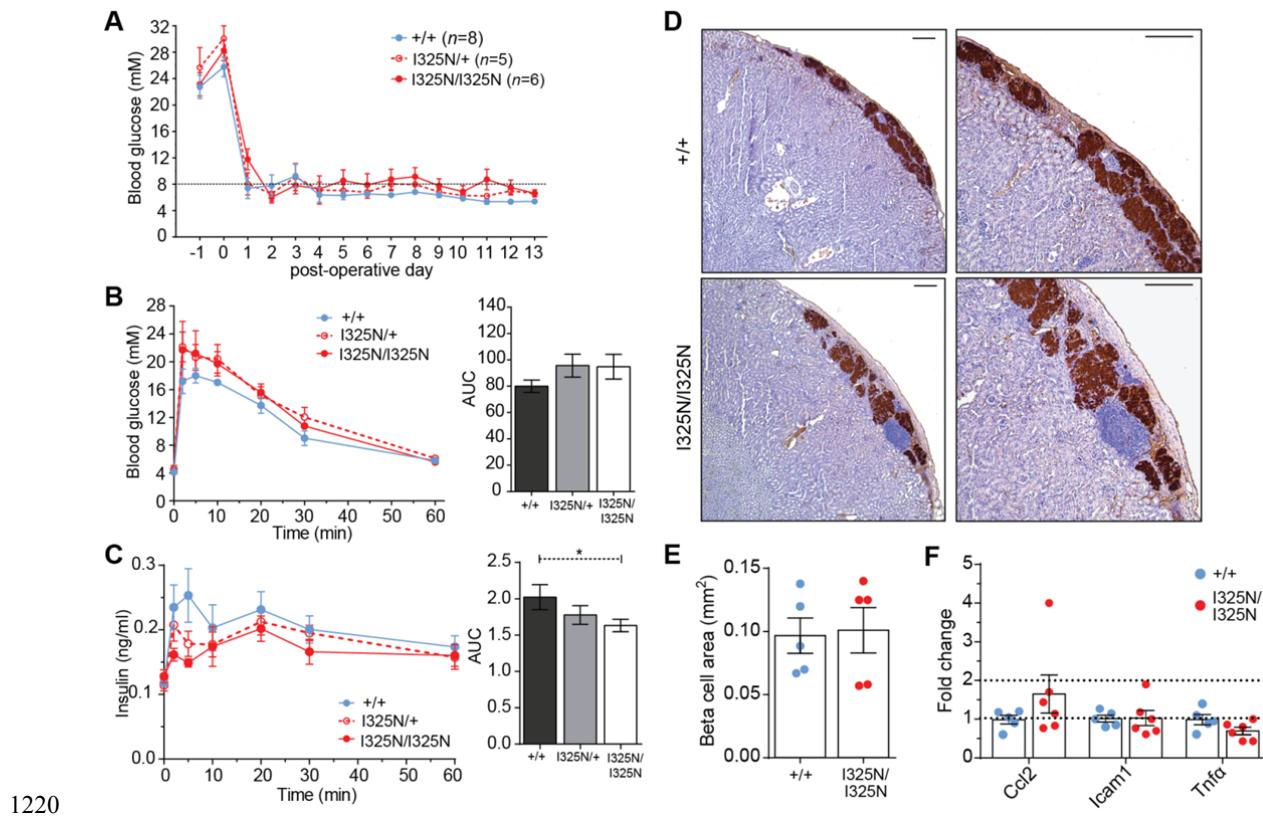
1190 **Histological analysis of tissues from A20^{I325N} mice.** (A) Colon length (cm) and (B)
1191 Hematoxylin and eosin (H&E) stained sections from *Tnfaip3*^{+/+} and *Tnfaip3*^{I325N/I325N} mice
1192 colon (20 X magnification; scale bar = 200 μ m) and (C) power-mosaic image of whole colon
1193 cross section (R=rectum; C=Caecum; m=muscularis; mu=mucosa; pp=Peyer's patches; scale
1194 = 1 mm). (D) H&E sections of kidney (4 X left images, scale bar = 500 μ m; 10 X right
1195 images, scale bar = 200 μ m) and (E) liver (4 X left images, scale bar = 500 μ m; 10 X right
1196 images, scale bar = 200 μ m). (F) H&E stained sections of pancreas (10 X images, scale bar =
1197 200 μ m) or, (G) insulin stained sections (20 X images, scale bar = 100 μ m). (H) Weight of
1198 spleens in grams (g). (I) Gross appearance of spleen and salivary glands from *Tnfaip3*^{+/+} and
1199 *Tnfaip3*^{I325N/I325N} mice. Images are representative from $n=4$ *Tnfaip3*^{+/+} and $n=4$
1200 *Tnfaip3*^{I325N/I325N} 16 week old mice. Error bars represent SEM, ** $P < 0.01$.

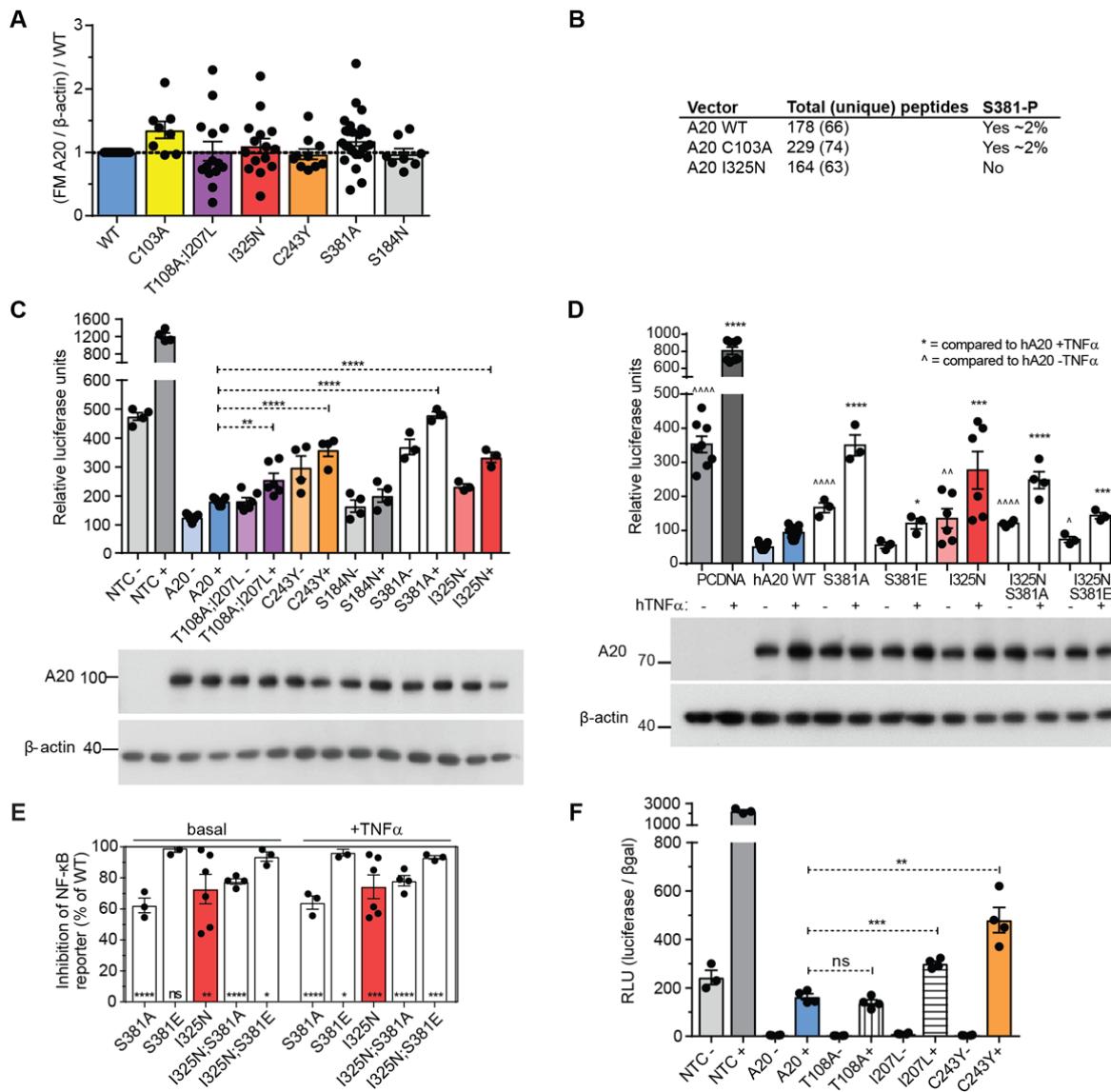


1201

1202 **Extended Data Fig. 11.**

1203 **Glucose homeostasis of A20^{I325N} mice and isolated islets.** (A) Random blood glucose levels
1204 of *Tnfaip3*^{+/+}, *Tnfaip3*^{I325N/+} or *Tnfaip3*^{I325N/I325N} female (♀) or male (♂) mice at 8 or 12 weeks
1205 of age. (B-E) Blood glucose levels (mM) were monitored following an intraperitoneal
1206 injection of glucose (2 g/kg) in 8 (B, D) or 12 week-old (C, E) mice. (F) Pancreatic islets
1207 were isolated from individual mice of the indicated A20 genotypes and incubated overnight.
1208 Following incubation an *in vitro* Glucose-Stimulated Insulin Secretion (GSIS) response in
1209 conditions of 2 mM, 20 mM D-glucose, or 25 mM KCl was conducted in separate groups of
1210 islets. Error bars represent SEM and Student's *t*-test used for significance between treatments,
1211 **P* < 0.05; ***P* < 0.01. (G-H) Pancreatic islets were isolated from individual mice of the
1212 indicated A20 genotypes, incubated overnight, and treated with 200 U/ml TNFα for the
1213 indicated times. (G) Representative immunoblot for IκBα and β-actin (loading control) or (H)
1214 phosphorylated JNK (pJNK) and total JNK (TJNK, loading control). Cumulative
1215 densitometry from 5 independent experiments is shown below (*n*=5 *Tnfaip3*^{+/+} and *n*=6
1216 *Tnfaip3*^{I325N/I325N} biological replicates). (I) Immunoblot for non-canonical NF-κB components
1217 NIK, p100/p52 and RelB; representative of 2 independent experiments. *Tnfaip3*^{I325N/I325N}
1218 islets exhibited increased activation of the non-canonical NF-κB pathway that can alter beta
1219 cell transcriptional programs to favor reduced insulin output³⁸.





1241

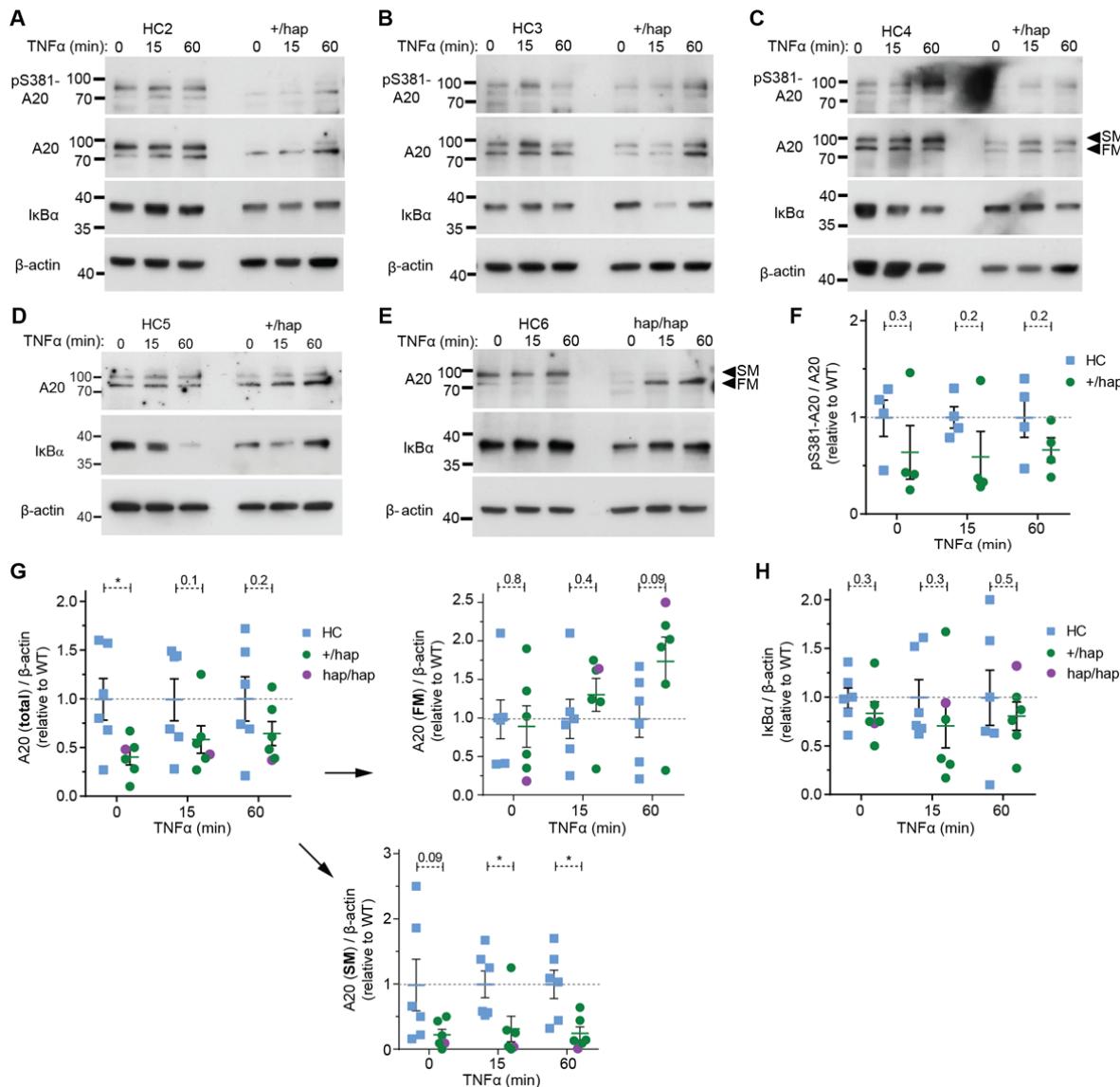
1242

Extended Data Fig. 13.

1243

In vitro analysis of missense variant effects on A20 control of NF-κB. (A) A20 accumulation in βTC3 mouse insulinoma cells transfected with wild-type (WT) A20, C103A A20, T108A;I207L A20, I325N A20, C243Y A20, S381A A20 or S184N A20 was determined by densitometry analysis of experimental immunoblots. Fast migratory (A20) 98 kDa A20 was corrected to loading control and compared to WT A20 of the same immunoblot [(FM A20/β-actin)/A20]. **(B)** Results of mass spectrometry analysis of full length WT A20, C103A mutant A20, or I325N mutant A20 expressed in and purified from HEK293 cells and analysed by mass spectrometry for phosphorylation of the indicated residues as per references ^{18,19}. **(C)** βTC3 cells co-transfected with an NF-κB.luciferase reporter and a CMV.βgal expression construct alone or with PCDNA3.1 encoding human wild-type A20 (hA20 WT; blue), A20 T108A/I207L (purple), A20 C243Y (orange), A20 S184N (grey), A20 I325N (red) or A20 S381A (white). Cells were stimulated with (+) 200 U/ml hTNFα for 8 h or left untreated (-). Data presented as relative luciferase units. Each column represents 3-5 independently transfected aliquots of cells within one experiment marked by circle symbols (representative of 3 independent experiments) and columns are arithmetic means. Statistical

1258 comparison by Student's *t*-test against WT hA20 (blue). **(D-F)** β TC₃ cells co-transfected with
1259 a NF- κ B.luciferase reporter and a CMV. β gal expression construct alone or with PCDNA3.1
1260 encoding human wild-type A20 (hA20 WT; blue), or (D) A20 I325N variant (I325N; red), or
1261 A20 with serine 381 substituted to non-phosphorylatable alanine (S381A or I325N S381A),
1262 or to phosphomimetic glutamate (S381E or I325N;S381E), (F) or A20 with the point variants
1263 T108A (vertical stripes), I207L (horizontal stripes) or C243Y (orange). Cells were stimulated
1264 with (+) 200 U/ml hTNF α for 8 h or left untreated (-). In (E) the two missense variants in the
1265 Denisovan haplotype, T108A and I207L, are tested individually (cross-hatched columns).
1266 Data presented as relative luciferase units. Each column represents mean of 3-6
1267 independently transfected aliquots of cells within one experiment marked by circle symbols
1268 (representative of 3 independent experiments) and columns are arithmetic means. Statistical
1269 comparison by Student's *t*-test against WT hA20 (blue) with TNF α (+) indicated by * or
1270 without (-) indicated by ^. In (F), data from (D) are presented as inhibition of NF- κ B reporter
1271 as a percent of WT A20. Error bars represent SEM, * P < 0.05; ** P < 0.01; *** P < 0.001;
1272 **** P < 0.0001.

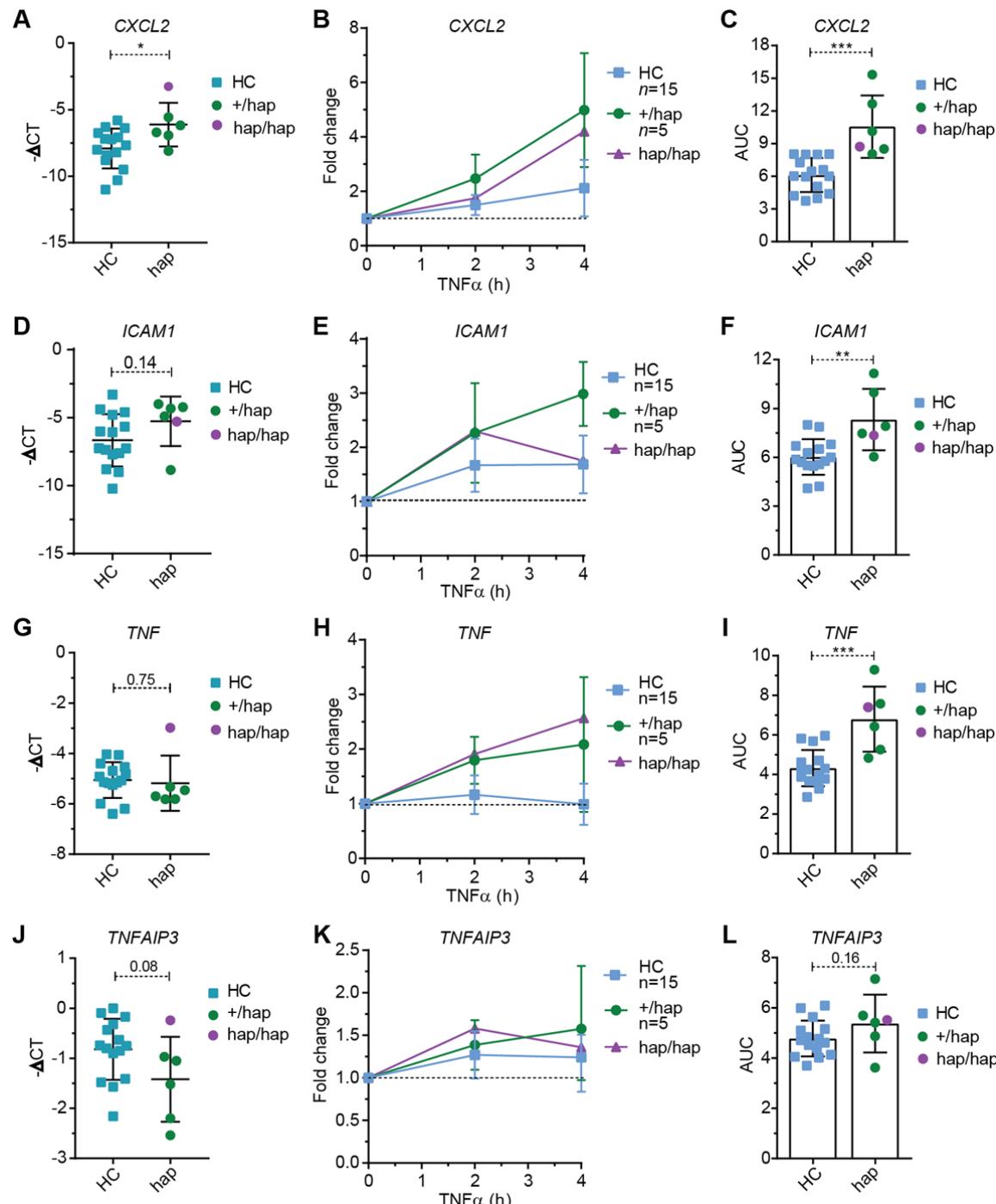


1273

1274 **Extended Data Fig. 14.**

1275 **Immunoblot analysis of peripheral blood mononuclear cells from healthy individuals**
 1276 **with the T108A I207L haplotype. (A-E)** Immunoblot analysis of peripheral blood
 1277 mononuclear cells (PBMC) from individuals with the Denisovan *TNFAIP3* haplotype (hap)
 1278 or without (healthy control donor; HC). PBMCs were left untreated or stimulated with
 1279 recombinant human TNF α (hTNF α) for 15 or 60 min. Proteins assessed include A20,
 1280 phosphoserine-A20 (pS381-A20), I κ B α and β -actin (loading control). **(F)** Densitometry
 1281 analysis of pS381-A20 levels in immunoblots A-C and Fig. 4D. Densitometry was calculated
 1282 by correcting to total A20 present and using the average value from samples without the
 1283 T108A,I207L haplotype (HC) to compare all samples. **(G)** Densitometry analysis of total
 1284 A20 levels in immunoblots A-C and Fig. 4D. Densitometry was calculated by correcting to β -
 1285 actin loading control and using the average WT value to compare all samples. **(G)** is further
 1286 divided into fast migrating (FM) or slow migrating (SM) A20. No significant difference is
 1287 observed for FM A20 between hap or HC individuals. In contrast, a significant difference is
 1288 observed between hap and HC individuals for SM A20, which correlates with phosphorylated
 1289 A20 in *in vitro* studies (Fig. 4D, E)^{18,19}; consistent with reduced phosphorylation for A20

1290 T108A;I2107L. **(H)** Densitometry analysis of I κ B α levels in immunoblots A-C and Fig. 4D.
1291 Densitometry was calculated by correcting to β -actin loading control and using the average
1292 value from samples without the T108A;I207L haplotype (HC) to compare all samples. Each
1293 symbol represents an individual lane in immunoblot. Error bars represent SEM and Student's
1294 *t*-test used for significance analysis unless otherwise stated, **P* < 0.05.

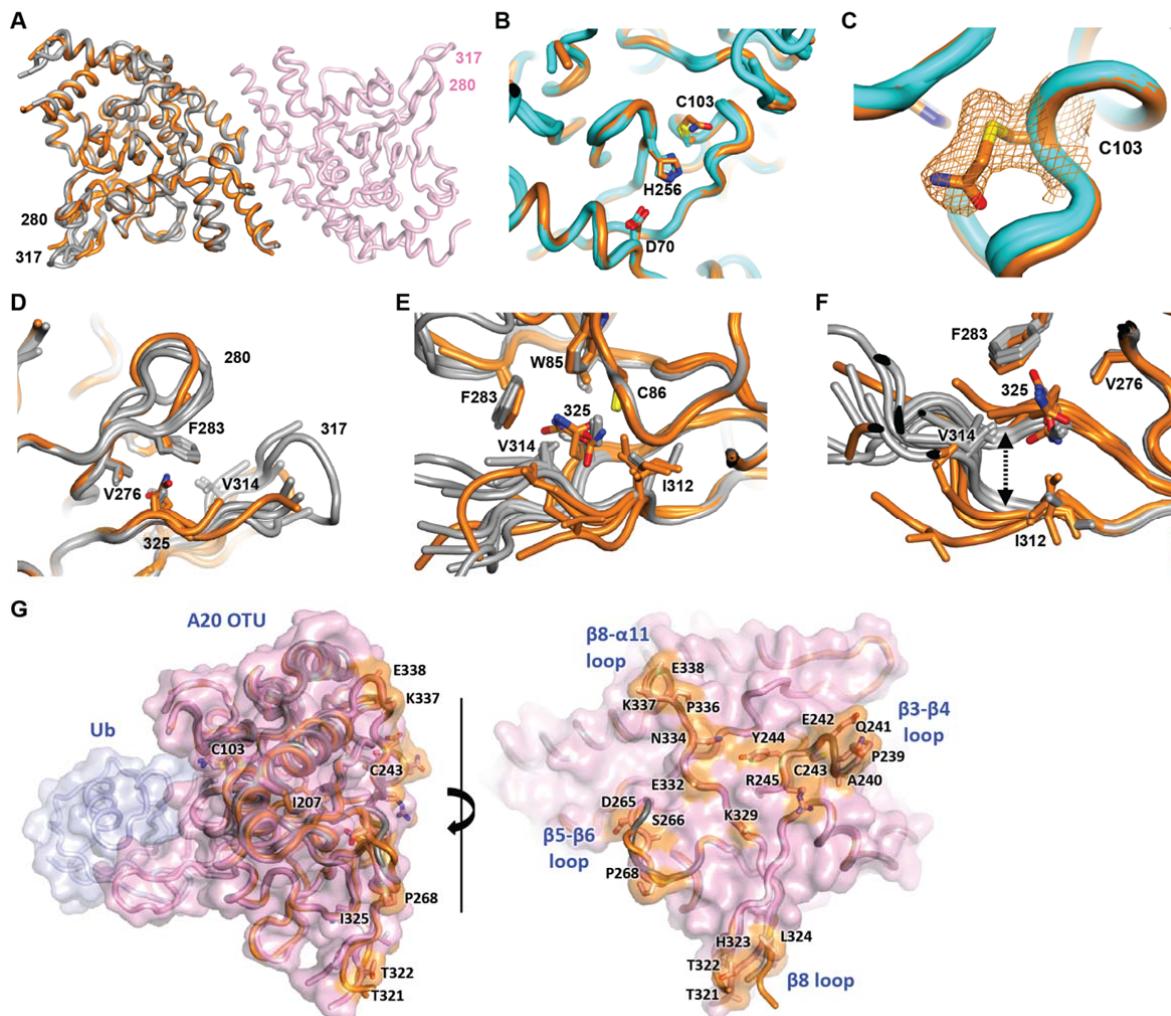


1295

1296 **Extended Data Fig. 15.**

1297 **Peripheral blood mononuclear cells from individuals with the Denisovan *TNFAIP3***
 1298 **T108A;I207L haplotype are hyper-inflammatory.** RT-PCR analysis of *CXCL2*, *ICAM1*,
 1299 *TNF*, and *TNFAIP3* genes expression from peripheral blood mononuclear cells (PBMCs)
 1300 from individuals heterozygous (green circles) or homozygous (purple circle) for the
 1301 Denisovan *TNFAIP3* haplotype (hap) and 15 healthy control donors (HCs; blue squares),
 1302 comparing negative ΔCT basal expression levels (A, D, G, J) or induction kinetics following
 1303 stimulation with recombinant human TNF α for 0, 2 or 4 h (B, E, H, K). Fold change was
 1304 determined by comparing stimulated values against non-stimulated PBMC preparations

1305 within the same group. Significance between groups was determined by area under the curve
1306 analysis (**C, F, I, L**). Error bars represent SD and Student's *t*-test used for significance
1307 analysis unless otherwise stated, * $P < 0.05$; *** $P < 0.001$.



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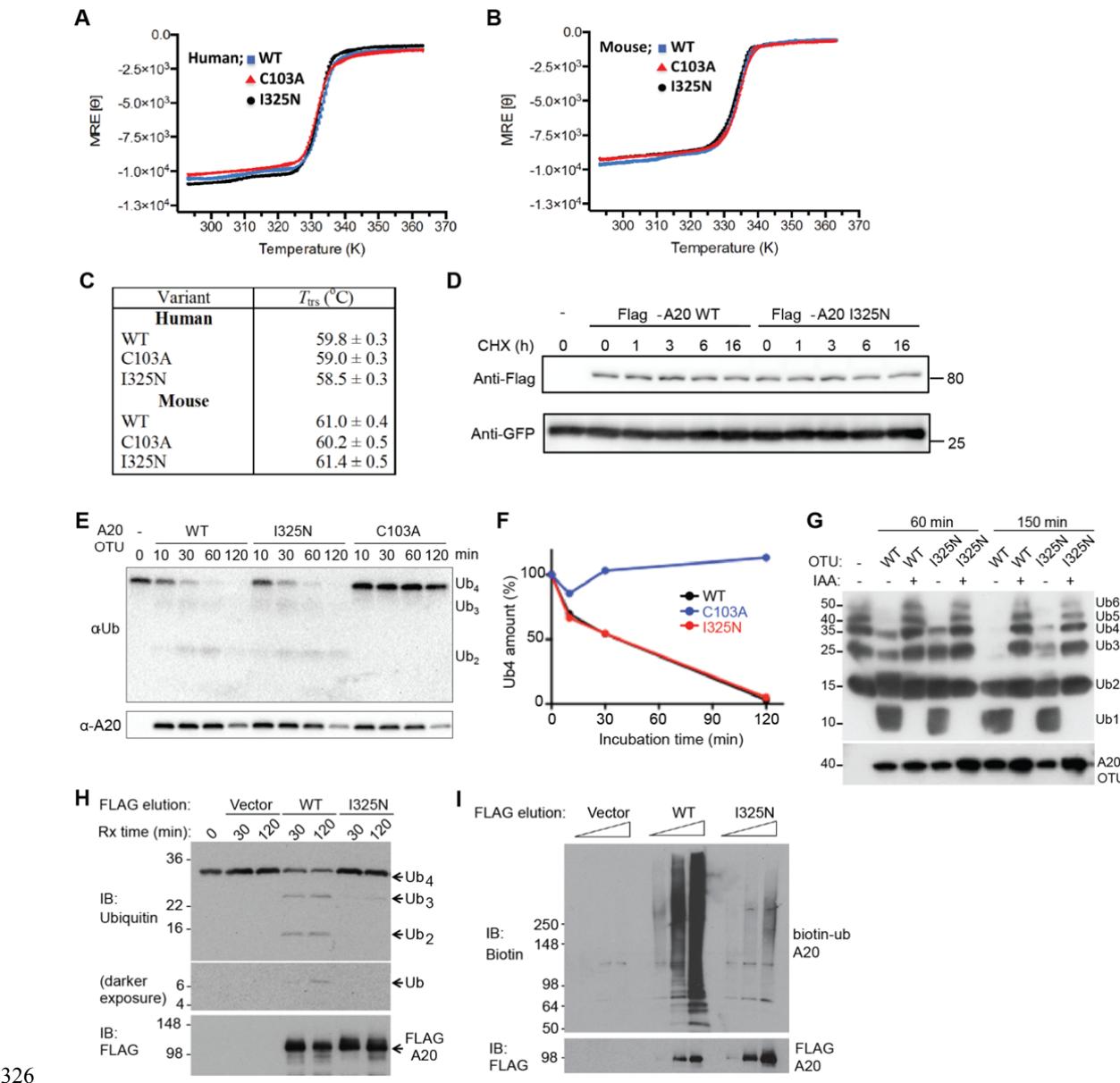
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Extended Data Fig. 16.

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1311

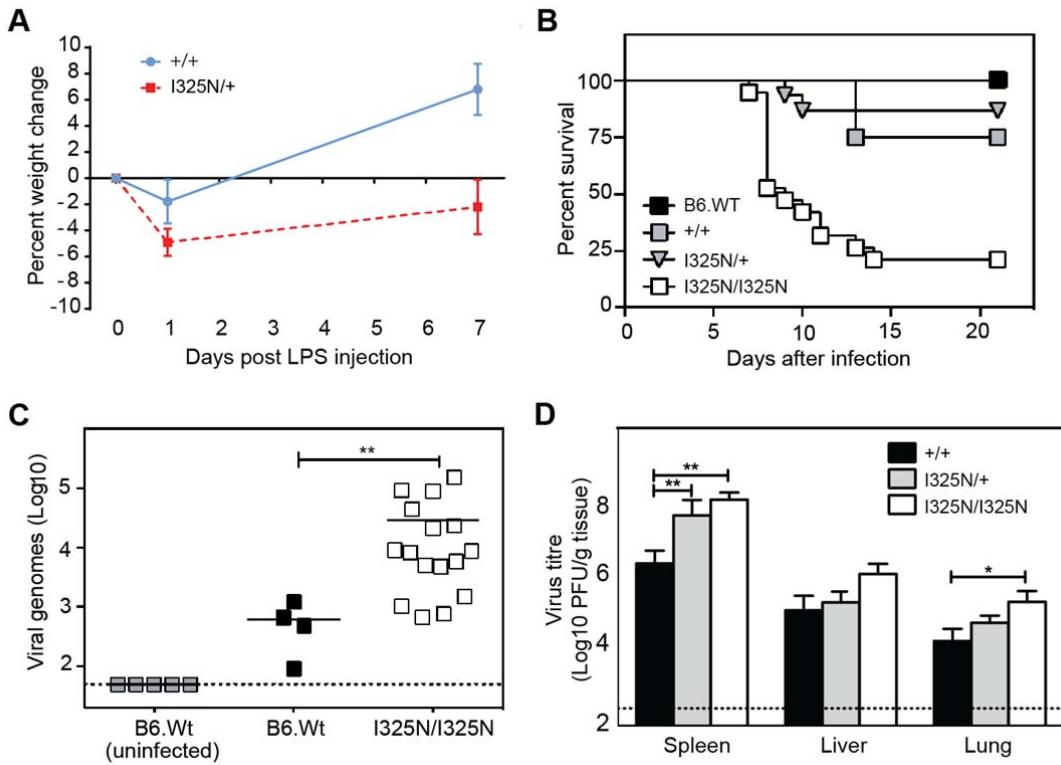
A20^{I325N} mutation subtly impacts the OTU structure. **(A)** Structures of human I325N variant (orange) and wild-type (WT; grey) A20 OTU domains superposed (each with 6 molecules per asymmetric unit). The dimer partner of one of these is shown in pink. Loop 317 and 280 are indicated on the periphery. **(B)** The I325N substitution does not alter the fold of the putative catalytic triad (orange cartoon and sticks) compared to previously published wild-type structures (cyan cartoon and sticks; PDB entries 3DKB, 2VFJ and 3ZJD). **(C)** The fold is additionally unperturbed by acetamidylation of C103 (orange mesh; 2mFo-DFc composite omit map contoured at 1.2 sigma). **(D-F)** The I325N substituted side-chain sits at the base of the $\beta_7-\beta_8$ loop containing residue 317, in a pocket also lined by the $\beta_6-\alpha_9$ loop containing residue 280 (D). Adjacent hydrophobic residues include W85, V276, F283, I312, V314 (E). The I325N substitution results in the base of the $\beta_7-\beta_8$ loop splaying apart (F, arrow), increasing $\beta_7-\beta_8$ loop disorder. **(G)** Structures of human I325N variant (orange) and wild-type (WT; grey) A20 OTU domains superposed on the wild-type OTU-ubiquitin structure 5LRX⁴². Conserved residues on the posterior surface are coloured orange. The $\beta_3-\beta_4$ loop containing the C243Y mutation and the structured base of the $\beta_7-\beta_8$ loop containing the I325N mutation are marked.



1327 **Extended Data Fig. 17.**

1328 **Molecular characterization of A20 I325N and A20 C103A.** (A-B) Thermal stability of
 1329 mouse and human WT, C103A, and I325N variants were examined using circular dichroism,
 1330 where mean residue ellipticity (MRE) was measured at 220 nm as a function of temperature
 1331 as solutions were heated at a rate of 1°C/min. Human (A) or mouse (B) A20 OTU residues 1-
 1332 370; WT (blue squares), C103A (red triangles), I325N (black circles). Curves were fit by
 1333 linear regression. (C) Unfolding transitions temperature (T_{trs}) of human A20 OTU variants
 1334 (residues 1-370) and mouse variants (residues 1-360), derived from the fit of the curves in
 1335 (A) and (B). (D) HEK293T cells were transfected with bicistronic retrovirus vector pMXs-
 1336 IRES-GFP, expressing FLAG-tagged mouse full-length TNFAIP3 wild-type or I325N. Each
 1337 group of transfected cells was replated 24 h after transfection and incubated with 10 μ M of
 1338 cycloheximide (CHX) for the indicated times before harvesting. Lysates were probed with
 1339 anti-FLAG and anti-GFP antibodies. (E, F) Reactions containing K48-linked tetra-ubiquitin
 1340 (Ub4) and *E. coli*-expressed and purified WT or I325N A20 OTU domain, or the catalytically

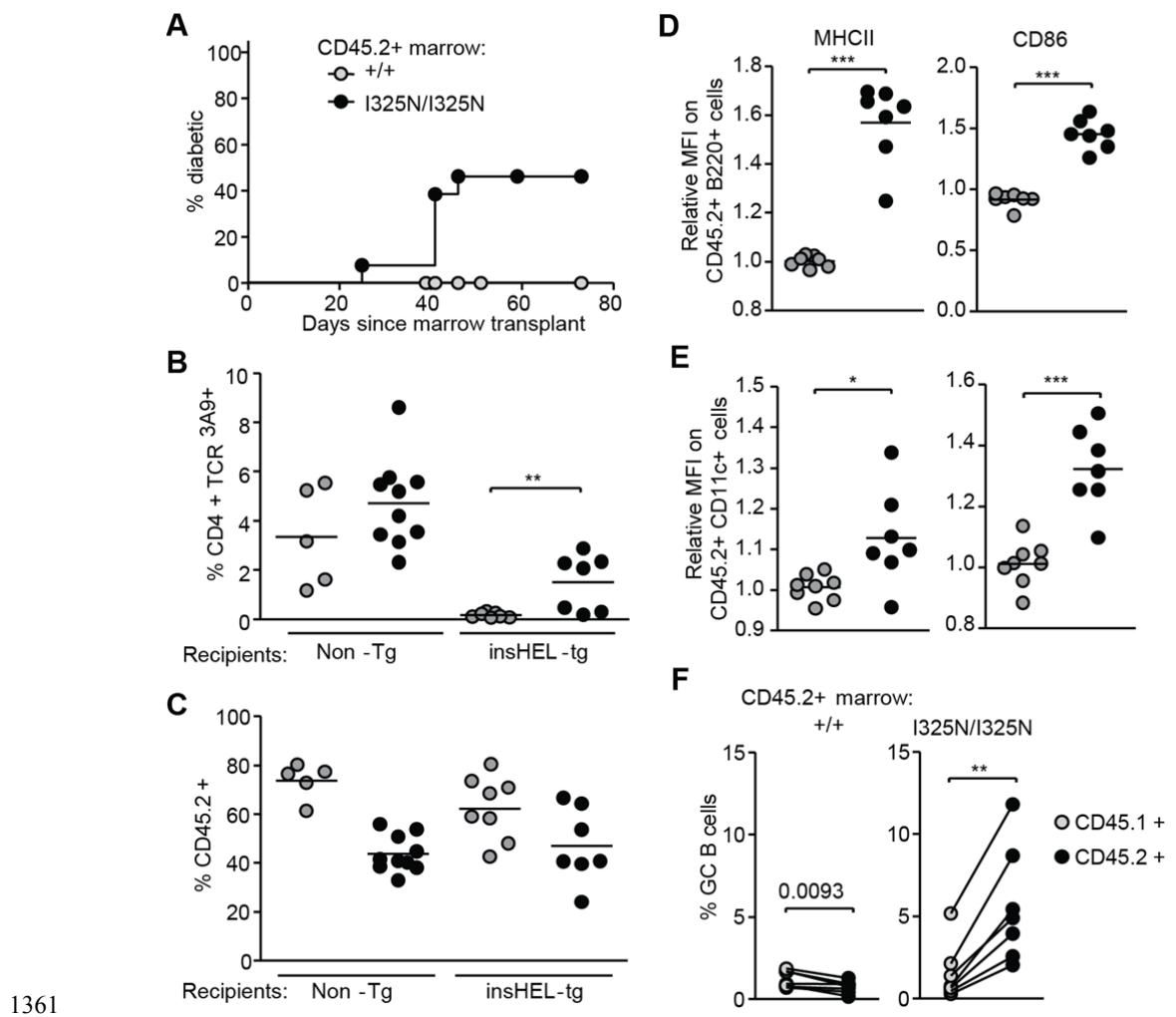
1341 deactivated A20 OTU domain, C103A, were incubated for the indicated times.
1342 Depolymerization of polyubiquitin was detected by immunoblotting for ubiquitin and A20;
1343 representative of two independent experiments. **(G)** Reactions containing mixed Ub₂-Ub₆
1344 K48-linked polyubiquitin chains and *E. coli*-expressed and purified WT or I325N A20 OTU
1345 domain ± iodoacetamide (IA) treatment were incubated at 37°C for 60 or 150 minutes.
1346 Depolymerization of polyubiquitin was detected by immunoblotting for ubiquitin and the N-
1347 terminus of A20; representative of 4 independent experiments. **(H, I)** Flag-tagged full length
1348 human A20 proteins (WT or I325N mutant) were expressed in HEK-293T cells and purified
1349 as previously described¹⁴. Purified A20 proteins were tested for K63-linked tetra-ubiquitin
1350 depolymerization (H) and *in vitro* ubiquitination assay (I).



1351

1352 **Extended Data Fig. 18.**

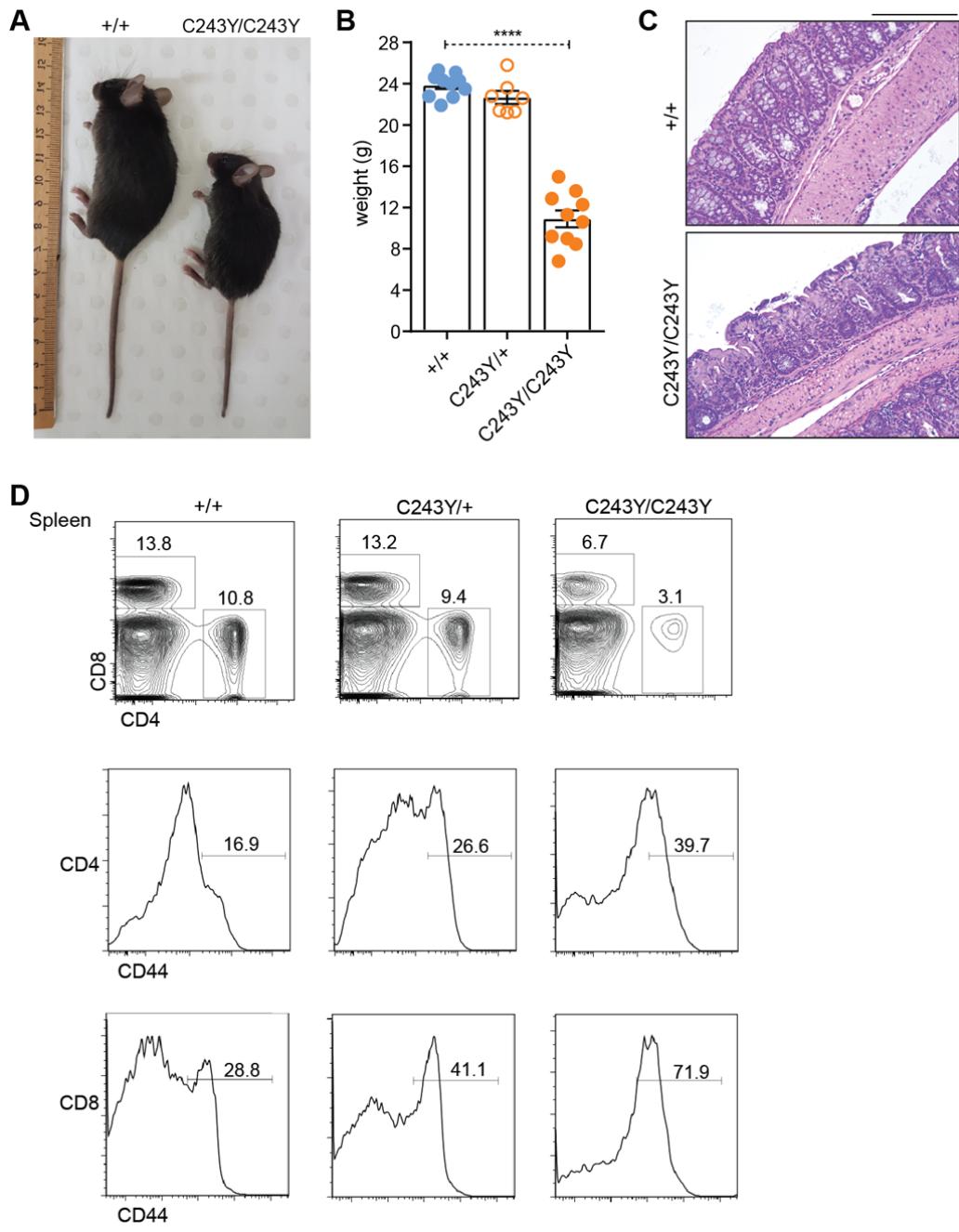
1353 **A20 I325N are susceptible to LPS injection and poxvirus infection.** (A) Daily body
 1354 weights for *Tnfaip3*^{+/+} or *Tnfaip3*^{I325N/+} mice administered 50 µg bacterial LPS by
 1355 intraperitoneal injection. (B-D) Ectromelia virus infection of B6 male siblings of the
 1356 indicated *Tnfaip3* genotypes and wild-type B6 control mice, showing (B) Kaplan-Meyer
 1357 survival plots and (C) viral load in blood on day 8; ***P* < 0.005 by unpaired *t* test. (D) Virus
 1358 load in spleen, liver and lung, respectively. Data expressed as mean ± SEM; data was log-
 1359 transformed and statistical significance determined by 2-way ANOVA; **P* < 0.05, ***P* <
 1360 0.01. Dotted lines in (C, D) denote limit of detection.



1361 **Extended Data Fig. 19.**

1362 **Diminished tolerance to self-antigen caused by mutant A20.** InsHEL-transgenic mice or
 1363 non-transgenic controls, both with wild-type A20 in pancreatic islets, were transplanted with
 1364 a congenic bone marrow mixture from *Tnfaip3*^{+/+} CD45.1⁺ TCR^{3A9}-transgenic donors, to
 1365 serve as an internal control, and CD45.2 TCR^{3A9}-transgenic donors with either
 1366 *Tnfaip3*^{I325N/I325N} (black circles) or *Tnfaip3*^{+/+} (grey circles) genotype. (A) Incidence of
 1367 diabetes in insHEL-transgenic recipients as a function of time after transplantation ($n=12$
 1368 chimeras for +/+ and 14 chimeras for I325N/I325N pooled from 2 separate experiments). (B)
 1369 Frequency of TCR^{3A9} positive CD4⁺ T cells among CD45.2⁺ *Tnfaip3*^{+/+} (grey circles) or
 1370 *Tnfaip3*^{I325N/I325N} (black circles) lymphocytes in pancreatic lymph nodes. Test recipients
 1371 expressed the insHEL antigen in pancreatic islets (insHEL-transgenic), while parallel non-
 1372 transgenic recipients lacked the autoantigen. (C) Percentage of CD45.2⁺ *Tnfaip3*^{+/+} (grey
 1373 circles) or *Tnfaip3*^{I325N/I325N} (black circles) cells among TCR^{3A9}⁺ CD4⁺ T cells in spleen. (D-
 1374 F) Analysis of insHEL transgenic recipients. Relative mean fluorescence intensity (MFI) of
 1375 staining for cell surface MHC class II or CD86 on CD45.2⁺ *Tnfaip3*^{+/+} (grey circles) or
 1376 *Tnfaip3*^{I325N/I325N} (black circles) B cells (D) or CD11c⁺ dendritic cells (E), compared to
 1377 CD45.1⁺ *Tnfaip3*^{+/+} B cells or dendritic cells in the same sample. (F) Frequency of germinal
 1378 center (GC) B cells among CD45.2⁺ B cells (black circles) compared to the frequency among
 1379 CD45.1⁺ *Tnfaip3*^{+/+} B cells in the same animal (linked by lines). Left panel, CD45.2
 1380 *Tnfaip3*^{+/+} marrow donor; right panel, CD45.2 *Tnfaip3*^{I325N/I325N} marrow donor. In (B-F),

1382 each symbol, or pair of symbols joined by a line, represents one chimeric mouse, all analyzed
1383 in a single experiment. Statistical analysis by 2-tailed Student's *t*-tests: **P* < 0.05; ***P* <
1384 0.01; ****P* < 0.001.
1385



1396 **Extended Data Table 1.**

1397 Allele frequencies of the Denisovan T108A;I207L haplotype in the Simons Genome
1398 Diversity Project²², as presented in (Extended Data Fig. 2B).

| Region | Denisovan <i>TNFAIP3</i> alleles | Total <i>TNFAIP3</i> alleles | Denisovan allele frequency |
|---------------------------|--|------------------------------------|----------------------------------|
| Africa | 0 | 88 | 0.00 |
| West Eurasia | 0 | 150 | 0.00 |
| Central Asia | 0 | 54 | 0.00 |
| South Asia | 0 | 78 | 0.00 |
| East Asia | 0 | 94 | 0.00 |
| Americas | 0 | 44 | 0.00 |
| Oceania & Island SE Asia: | 23 | 50 | 0.46 |
| (Brunei) | 1 | 4 | 0.25 |
| (Australia) | 1 | 4 | 0.25 |
| (Papua New Guinea) | 19 | 30 | 0.63 |
| (Bougainville) | 1 | 4 | 0.25 |
| (New Zealand) | 1 | 2 | 0.50 |

1399

1400 **Extended Data Table 2.**

1401 Polymorphic observed (black) and imputed (red) variants within a putatively adaptively
1402 introgressed haplotype in Melanesians (6:138160925-138246514, ²⁹). Ancestral, modern
1403 human (b37), Denisovan, and Altai Neanderthal genotypes are shown at each position for
1404 comparison. Genomic coordinates are based upon the hg19 reference.

| Chr | Position | rsID | Missense | Ancestral | b37 | Denisovan | Altai Neanderthal |
|-----|-----------|-------------|----------|-----------|-----|-----------|-------------------|
| 6 | 138161013 | rs6918329 | | G | A | G | G |
| 6 | 138174328 | rs2027276 | | C | A | A | C |
| 6 | 138190154 | rs3757173 | | G | A | A | G |
| 6 | 138195402 | rs596493 | | C | T | C | C |
| 6 | 138196008 | rs376205580 | T108A | A | A | G | A |
| 6 | 138196957 | rs141807543 | I207L | A | A | C | A |
| 6 | 138223082 | rs17066926 | | C | T | C | C |
| 6 | 138241185 | rs4895499 | | G | A | G | G |

1405

1406 **Extended Data Table 3.**

1407 Mouse primers used for qRT-PCR analysis of genes. Designed using Primer3 with sequences
1408 obtained from GenBank and synthesised by Sigma Aldrich (Australia). Primers from Roche
1409 Universal ProbeLibrary (UPL) System Assay are indicated.

| Gene | Primer sequence |
|-------------------------------|--|
| <i>Tnfaip3</i> | F-5'-CCTGTCACCAACGCTCCAAG-3' R-5'-ATTCCAGTCCGGTGGCAAG-3' |
| <i>Ccl2</i> | F- 5'-GGTCCCTGTATGCTTCTGG-3' R- 5'-CCTGCTGCTGGTATCCTCT-3' |
| <i>Cph2</i> | F- 5'-TGGACCAAACACAAACGGTTCC-3' R- 5'-ACATTGCGAGCAGATGGGGT-3' |
| <i>Cxcl1</i> | F- 5'-TGGCTGGGATTCACCTCAAG-3' R- 5'-TATGACTTCGGTTGGGTGCAG-3' |
| <i>Cxcl10</i> | F- 5'-GACGGGCCAGTGAGAATGAG-3' R- 5'-GTGTGTGCGTGGCTTCACTC-3' |
| <i>Icam1</i> | F- 5'-CCATGGGAATGTCACCAAGGA-3' R- 5'-ATCACGAGGCCACAATGAC-3' |
| <i>Il6</i> | F- 5'-TCCTTCCTACCCCAATTCCAA-3' R- 5'-TGGATGGTCTTGGCCTTAGCC-3' |
| <i>Il1b</i> UPL probe #38 | F- 5'-AGTTGACGGACCCAAAAG-3' R- 5'-AGCTGGATGCTCTCATCAGG-3' |
| <i>Rpl19</i> UPL probe #46 | F- 5'-CCACAAGCTTTCCCTTCG-3' R- 5'-GGATCCAACCAGACCTCTTT-3' |
| <i>Il6</i> UPL probe #28 | F- 5'-GCTACCAAATGGATATAATCAGGA-3' R- 5'-CCAGGTAGCTATGGTACTCCAGAA-3' |
| <i>CVB4</i> UPL probe #41 | F- 5'-CCCGGACCGAGTATCAATAA-3' R- 5'-CCGGGTAACGAACGGTT-3' |
| <i>Ifnb</i> UPL probe #78 | F- 5'-CACAGCCCTCTCCATCAACTA-3' R- 5'-CATTCCGAATGTTCGCCT-3' |

1410

1411 **Extended Data Table 4.**

1412 Human primers used for qRT-PCR analysis of genes. Designed using Primer3 with sequences
1413 obtained from GenBank and synthesised by Sigma Aldrich (Australia).

| | |
|----------------|---|
| <i>TNFAIP3</i> | F- 5'-AACGAACGGTGACGGCAAT-3' R- 5'-GAAGTCCACTCGGGCCAT-3' |
| <i>TNF</i> | F- 5'-TCCCCTGGAAAGGACACCAT-3' R- 5'GGGTTTGCTACAACATGGGCT-3' |
| <i>CXCL2</i> | F- 5'-AGGGAATTCACCTCAAGAACATCC-3' R- 5'-TGTGGCTATGACTTCGGTTGG-3' |
| <i>RPL13A</i> | F- 5'-CAAGCGGATGAACACCAAC-3' R- 5'-TGTGGGGCAGCATAACCTC-3' |
| <i>ACTB</i> | F- 5'-CATCGAGCACGGCATCGTCA-3' R- 5'-TAGCACAGCCTGGATAGCAAC-3'. |

1414

1415 **Extended Data Table 5.**

1416 Data collection and refinement statistics (molecular replacement).

| | I325N human (PDB code 5V3P) | WT human (PDB code 5V3B) | WT mouse (PDB code 5DQ6) |
|---|---------------------------------------|---------------------------------------|---|
| Data collection | | | |
| Space group | P3 ₁ | P2 ₁ | P3 ₂ |
| Cell dimensions | | | |
| <i>a, b, c</i> (Å) | 81.9, 81.9, 297.3 | 98.7, 80.9, 153.4 | 71.3, 71.3, 143.2 |
| <i>a, b, g</i> (°) | 90.0, 90.0, 120.0 | 90.0, 102.5, 90.0 | 90.0, 90.0, 120.0 |
| Resolution (Å) | 2.5-40.6 (2.50- 2.55) ^a | 3.0-34.56 (3.0- 3.10) ^a | 2.80-37.77 (2.80- 2.93) ^a |
| R_{pim} (all I+ & I-) ^b | 0.076 (0.471) | 0.056 (0.687) | 0.033 (0.398) |
| $I/s(I)$ | 7.8 (2.2) | 9.9 (1.5) | 12.0 (1.42) |
| $CC_{1/2}$ | 0.929 (0.713) | 0.994 (0.703) | 0.996 (0.782) |
| Completeness (%) | 99.7 (100.0) | 97.7 (99.1) | 96.07 (98.30) |
| Redundancy | 4.5 (4.6) | 5.3 (5.6) | 4.3 (4.3) |
| Wilson B (Å ²) | 47.7 | 79.0 | 90.8 |
| Refinement | | | |
| Resolution (Å) | 2.50-39.47 | 3.0-34.56 | 2.8-37.77 |
| No. reflections | 73040 | 44077 | 19279 |
| $R_{\text{work}} / R_{\text{free}}$ | 0.161/0.200 | 0.238/0.282 | 0.253/0.291 |
| No. atoms | | | |
| Protein | 17465 | 14207 | 5511 |
| Water | 21 | - | 26 |
| <i>B</i> factors average | | | |
| Protein | 55.4 | 77.3 | 122 |
| Water | 23.4 | - | 109 |
| R.m.s. deviations | | | |
| Bond lengths (Å) | 0.0115 | 0.0117 | 0.011 |
| Bond angles (°) | 1.54 | 1.53 | 1.43 |

1417