

1 **Missense mutations in the MLKL ‘brace’ region lead to lethal neonatal inflammation in mice**  
2 **and are present in high frequency in humans.**

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128 **Declaration of interests** – JMH, PAC, JMM and JS contribute to a project developing necroptosis  
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146 **SUMMARY**

147 We have isolated a mouse strain with a single missense mutation in the gene encoding MLKL, the  
148 essential effector of necroptotic cell death. The resulting substitution lies within the two-helix  
149 ‘brace’ and confers constitutive, RIPK3 independent, killing activity to MLKL. Mice homozygous  
150 for *Mlkl*<sup>D139V</sup> develop lethal inflammation within days of birth, implicating the salivary glands and  
151 pericardium as hotspots for necroptosis and inflammatory infiltration. The normal development of  
152 *Mlkl*<sup>D139V</sup> homozygotes until birth, and the absence of any overt phenotype in heterozygotes  
153 provides important *in vivo* precedent for the capacity of cells to clear activated MLKL. These  
154 observations offer an important insight into the potential disease-modulating roles of three  
155 common human *MLKL* polymorphisms that encode amino acid substitutions within or adjacent to  
156 the brace region. Compound heterozygosity of these variants is found at up to 12-fold the expected  
157 frequency in patients that suffer from a pediatric autoinflammatory disease, CRMO.

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161 **KEYWORDS**

162 Necroptosis, MLKL, programmed cell death, inflammation, Human missense genetic variation

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169 **INTRODUCTION**

170 Necroptosis is a form of programmed cell death associated with the production of pro-  
171 inflammatory cytokines, destruction of biological membranes and the release of intracellular  
172 Damage Associated Molecular Patterns (DAMPs) (Newton and Manning, 2016). Necroptosis  
173 depends on the activation of pseudokinase Mixed Lineage Kinase domain-Like (MLKL) by  
174 Receptor Interacting Protein Kinase 3 (RIPK3) (Murphy et al., 2013; Sun et al., 2012; Zhao et al.,  
175 2012). RIPK3-mediated phosphorylation of MLKL triggers a conformational change that  
176 facilitates the translocation to, and eventual irreversible disruption of, cellular membranes. While  
177 the precise biophysical mechanism of membrane disruption is still a matter of debate, it is  
178 consistently associated with the formation of an MLKL oligomer and the direct association of the  
179 four-helix bundle domain (4HB) of MLKL with membranes (Cai et al., 2014; Chen et al., 2014;  
180 Dondelinger et al., 2014; Hildebrand et al., 2014). In mouse cells, the expression of the murine  
181 MLKL 4HB domain alone (residues 1-125), 4HB plus brace helix (1-180), or the expression of  
182 phosphomimetic or other single site pseudokinase domain (PsKD) mutants is sufficient to induce  
183 membrane translocation, oligomerization and membrane destruction (Hildebrand et al., 2014;  
184 Murphy et al., 2013). While capable of disrupting synthetic liposomes when produced  
185 recombinantly, similarly truncated and equivalent single site (PsKD) mutant forms of human  
186 MLKL do not robustly induce membrane associated oligomerization and cell death without forced  
187 dimerization (Petrie et al., 2018; Quarato et al., 2016; Tanzer et al., 2016). Furthermore, both  
188 mouse and human MLKL mutants have been reported that have the capacity to form membrane  
189 associated oligomers, but fail to cause irreversible membrane disruption and cell death (Hildebrand  
190 et al., 2014; Petrie et al., 2018). Recent studies have revealed that necroptosis downstream of  
191 MLKL phosphorylation and membrane association can be modulated by processes that utilize the

192 Endosomal Sorting Complex Required for Transport (ESCRT) family of proteins. One model  
193 proposes a role for ESCRT in limiting necroptosis via plasma membrane excision and repair (Gong  
194 et al., 2017) while other models limit plasma membrane disruption by ESCRT-mediated  
195 endosomal trafficking and the release of MLKL in endosomes (Yoon et al., 2017) or the shedding  
196 of phosphorylated MLKL in extracellular vesicles (Zargarian et al., 2017).

197

198 In mice, the absence of MLKL does not appear to have obvious deleterious developmental or  
199 homeostatic effects (Murphy et al., 2013; Wu et al., 2013). However, genetic deletion of *Fadd*,  
200 *Casp8* or *Ripk1*, leads to inappropriate activation of MLKL and ensuing necroptosis during  
201 embryogenesis and is incompatible with life beyond embryonic day (E)10.5, E10.5 and 1-3 days  
202 post-natally, respectively (Dillon et al., 2014; Kaiser et al., 2014; Kelliher et al., 1998; Rickard et  
203 al., 2014b; Varfolomeev et al., 1998; Yeh et al., 1998; Zhang et al., 2011). Exploring the precise  
204 physiological consequences of inappropriate MLKL activation in these scenarios is complicated  
205 by the fact that FADD, Caspase-8 and RIPK1 also play important roles in cellular processes other  
206 than modulation of MLKL-induced necroptotic cell death (Alvarez-Diaz et al., 2016; Kaiser et al.,  
207 2011; Kang et al., 2004; Newton et al., 1998; Oberst et al., 2011; Rickard et al., 2014b).

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209 Aberrant levels of MLKL-dependent cell death contribute to disease in several genetic and  
210 experimental mouse models (Anderton et al., 2017; Dannappel et al., 2014; Hockendorf et al.,  
211 2016; Newton et al., 2016; Rickard et al., 2014a; Rickard et al., 2014b). In humans, *MLKL* mRNA  
212 and protein levels are positively correlated with survival of patients with pancreatic  
213 adenocarcinoma, cervical-, gastric-, ovarian- and colon- cancers (reviewed by (Lalaoui and  
214 Brumatti, 2017)). Interestingly, high levels of phosphorylated MLKL are associated with reduced

215 survival in esophageal and colon cancer patients (Liu et al., 2016b). Two missense *MLKL* somatic  
216 mutations identified in human cancer tissue have been found to confer a reduction in necroptotic  
217 function in cell-based assays (Murphy et al., 2013; Petrie et al., 2018). One recent study reported  
218 a significant enrichment of an ultra rare *MLKL* stop-gain gene variant p.Q48X in Hong Kong  
219 Chinese patients suffering from a form of Alzheimer's disease (Wang et al., 2018) however more  
220 common germline *MLKL* gene variants are only weakly associated with human disease in GWAS  
221 databases. In two recent studies, lethal immunodeficiency, arthritis and intestinal inflammation  
222 was reported in patients homozygous for ultra rare-loss of function RIPK1 mutations (Cuchet-  
223 Lourenco et al., 2018; Li et al., 2019), however to date, *MLKL* gene variants have not been directly  
224 implicated in any severe Mendelian forms of human disease.

225

226 We have identified a single base pair germline mutation of mouse *Mikl* that encodes a missense  
227 alteration to the *MLKL* mouse brace region and confers constitutive activation independent of  
228 upstream necroptotic stimuli. Given this mutant *Mikl* allele is subject to the same developmental  
229 and environmental controls on gene expression as wildtype *Mikl*, the postnatal lethality in these  
230 mice provides novel insight into the physiological and pathological consequences of dysregulated  
231 necroptosis. In parallel these findings inform the potential functional significance of three common  
232 human *MLKL* polymorphisms that encode non-conservative amino acid substitutions within, or in  
233 close proximity to, the brace helix that is mutated in the *Mikl*<sup>D139V</sup> mouse.

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## 238 RESULTS

### 239 Generation of a constitutively active form of MLKL.

240 An ENU mutagenesis screen was performed to identify mutations that ameliorate  
241 thrombocytopenia in *Mpl*<sup>-/-</sup> mice (Kauppi et al., 2008). A G<sub>1</sub> founder, designated *Plt15*, had a  
242 modestly elevated platelet count of 189x10<sup>6</sup>/mL compared to the mean for *Mpl*<sup>-/-</sup> animals  
243 (113±57x10<sup>6</sup>/mL) and yielded 19 *Mpl*<sup>-/-</sup> progeny. Ten of these mice had platelet counts over  
244 200x10<sup>6</sup>/mL, consistent with segregation of a dominantly acting mutation (**Fig. 1A**). Linkage  
245 analysis and sequencing (see Experimental Procedures) identified an A to T transversion in *Mkl1*  
246 that was heterozygous in all mice with an elevated platelet count (**Fig. 1B**). The *Mkl1*<sup>*Plt15*</sup> mutation  
247 results in a non-conservative aspartic acid-to-valine substitution at position 139. In the full length  
248 mMLKL structure D139 forms a salt bridge with an arginine residue at position 30 ( $\alpha$ 2 helix) of  
249 the MLKL four-helix bundle (4HB) domain (Murphy et al, 2013) (**Fig. 1C**). This salt bridge  
250 represents one of a series of electrostatic interactions between residues in helix  $\alpha$ 2 of the MLKL  
251 4HB domain and the two-helix ‘brace’ region. D139 of mouse MLKL is conserved in all MLKL  
252 orthologues in vertebrata reported to date (**Fig. 1D**). We have shown that the exogenous expression  
253 of the 4HB domain of murine MLKL alone is sufficient to kill mouse fibroblasts whereas  
254 exogenous expression of full length MLKL does not, indicating that this ‘electrostatic zipper’ may  
255 play an important role in suppressing the killing activity of the MLKL 4HB (Hildebrand et al.,  
256 2014). To determine if MLKL<sup>D139V</sup> exhibited altered ability to induce necroptotic cell death  
257 relative to MLKL<sup>Wt</sup>, we stably expressed these full length proteins under the control of a  
258 doxycycline-inducible promoter in immortalized mouse dermal fibroblasts (MDF) isolated from  
259 *Wt*, *Mkl1*<sup>-/-</sup>, *Ripk3*<sup>-/-</sup> or *Ripk3*<sup>-/-</sup>;*Casp8*<sup>-/-</sup> mice. While expressed at comparable levels, MLKL<sup>D139V</sup>  
260 induced markedly more death than MLKL<sup>Wt</sup>, on each of the genetic backgrounds tested (**Fig. 1E-**

261 **F, Supp. Fig. 1A).** This indicates that MLKL<sup>D139V</sup> is a constitutively active form of MLKL,  
262 capable of inducing necroptotic cell death independent of upstream signaling and phosphorylation  
263 by its activator RIPK3. Consistent with this interpretation, exogenous expression of MLKL<sup>D139V</sup>  
264 in *Ripk3*<sup>-/-</sup>; *Casp8*<sup>-/-</sup> MDFs was sufficient to induce the organelle swelling and plasma membrane  
265 rupture characteristic of TNF induced necroptosis when examined by Transmission Electron  
266 Microscopy (**Fig. 1G**).

267

### 268 **Constitutively active mouse MLKL causes a lethal perinatal inflammatory syndrome.**

269 To define the phenotypic consequences of constitutively active MLKL in the absence of any  
270 confounding effects resulting from *Mpl*-deficiency, all subsequent studies were performed on a  
271 *Mpl*<sup>+/+</sup> background. Homozygous *Mkl*<sup>D139V/D139V</sup> pups were born at expected Mendelian  
272 frequencies (**Supp. Table I**) and were ostensibly normal macroscopically and histologically at  
273 E19.5 (**Supp. Fig. 2A-D**). However, by 3 days of age, although outwardly indistinguishable from  
274 littermates (**Fig. 2A**), they exhibited reduced body weight (**Supp. Fig. 2B**) and failed to thrive,  
275 with a maximum observed lifespan of 6 days under conventional clean housing conditions. Like  
276 *Mkl*<sup>Wt/D139V</sup> mice, *Mkl*<sup>null/D139V</sup> compound heterozygotes were present at the expected frequency  
277 at P21 and developed normally to adulthood (**Supp. Table II**). Thus, the constitutive activity of  
278 MLKL<sup>D139V</sup> was not affected by the presence of normal MLKL protein suggesting it is the absolute  
279 allelic dose of *Mkl*<sup>D139V</sup> that determines perinatal lethality. To confirm that the phenotype of the  
280 ENU derived *Mkl*<sup>D139V</sup> mice was due to the *Mkl*<sup>D139V</sup> missense mutation, we independently  
281 generated *Mkl*<sup>D139V</sup> mice using CRISPR-Cas9 genomic editing. Homozygote CRISPR-  
282 *Mkl*<sup>D139V/D139V</sup> mice also died soon after birth (**Supp. Table III**).

283



284 Hematoxylin-Eosin stained-sections from both P2 and P3 *Mikl*<sup>D139V/D139V</sup> pups revealed multifocal  
285 acute inflammation characterized by neutrophilic infiltration, dilated blood vessels and edema  
286 (**Fig. 2B**) in the dermis and subcutis of the head and neck. These inflammatory features were not  
287 observed in *Mikl*<sup>Wt/Wt</sup> or *Mikl*<sup>Wt/D139V</sup> littermates, nor in *Mikl*<sup>-/-</sup> mice of the same age (**Supp. Fig.**  
288 **2I**). Cells of hematopoietic origin, revealed by immunohistochemical staining for CD45, were  
289 sparsely distributed throughout the lower head and neck and confined predominantly to a clearly  
290 delineated developing lymph node in *Mikl*<sup>Wt/Wt</sup> and *Mikl*<sup>Wt/D139V</sup> littermates (**Fig. 2C**). In contrast,  
291 CD45<sup>+</sup> cells were more numerous and distributed throughout the cutis, subcutis and salivary glands  
292 of *Mikl*<sup>D139V/D139V</sup> pups (**Fig. 2C**). A mixture of diffuse and focal inflammatory infiltration was also  
293 observed within the mediastinum and pericardial space of all P2/P3 *Mikl*<sup>D139V/D139V</sup> pups examined,  
294 as was a marked paucity of thymic cortical lymphocytes (**Fig. 2D, Supp. Fig 2E**), phenotypes not  
295 evident in E19.5 embryos (**Supp. Fig. 2D**). Apart from small foci of hepatocyte and enterocyte  
296 loss/necrosis evident in the livers and small intestines of some *Mikl*<sup>D139V/D139V</sup> pups examined (data  
297 not shown), no other lesions were observed by histopathology. Consistent with this inflammatory  
298 phenotype significantly elevated levels of several pro-inflammatory cytokines and chemokines  
299 were evident in the plasma of both E19.5 and P3 *Mikl*<sup>D139V/D139V</sup> pups (**Fig. 2E, F**). Blood glucose  
300 levels were normal (**Supp. Fig. 2 F, G**).

301

### 302 **Hematopoietic defects in *Mikl*<sup>D139V</sup> mice.**

303 Although blood cell numbers were unchanged in *Mikl*<sup>D139V/D139V</sup> pups at E19.5 relative to *Mikl*<sup>Wt/Wt</sup>  
304 and *Mikl*<sup>Wt/D139V</sup> littermates, by P3 significant deficits were evident in total white blood cell count,  
305 lymphocyte and platelet numbers (**Fig. 3A-C, Supp. Fig. 3A**). Similarly, the numbers of  
306 hematopoietic stem and progenitor cells were present at normal proportions in fetal livers of E18.5

307 *Mikl*<sup>D139V/D139V</sup> pups, although increased levels of intracellular ROS were uniformly evident (**Fig.**  
308 **3D-E, Supp. Fig. 3B**). By P2, deficits in CD150<sup>+</sup>CD48<sup>+</sup> and CD150<sup>+</sup>CD48<sup>-</sup> populations were  
309 present (**Fig. 3F**), accompanied by increased AnnexinV binding (which indicates either  
310 phosphatidyl serine exposure or plasma membrane rupture) in all lineages (**Fig. 3G**). In adult  
311 *Mikl*<sup>Wt/D139V</sup> mice, numbers of hematopoietic stem and progenitor cells were unaffected (**Fig. 3H**);  
312 however, upon myelosuppressive irradiation, recovery of hematopoietic cell numbers was delayed  
313 and characterized by increased expression of ROS and Annexin V (**Supp. Fig. 3C, D**). When  
314 challenged with the cytotoxic drug 5-fluorouracil (5-FU), blood cell recovery in *Mikl*<sup>Wt/D139V</sup> mice  
315 was similarly delayed (**Fig. 3I**). In competitive transplants in which test *Mikl*<sup>Wt/D139V</sup> or *Mikl*<sup>Wt/Wt</sup>  
316 marrow was co-injected with wild type competitor marrow in 10:1 excess, as expected, *Mikl*<sup>Wt/Wt</sup>  
317 marrow contributed to 90% of recipient blood cells 8 weeks after transplantation and maintained  
318 that level of contribution for 6 months (**Fig. 3J**). In contrast, *Mikl*<sup>Wt/D139V</sup> marrow performed poorly,  
319 contributing to 25% and 51% of recipient blood cells at these times (**Fig. 3J**). Similarly, while wild  
320 type fetal liver cells contributed to the vast majority of blood cells in irradiated recipients up to 6  
321 months after transplantation, cells from *Mikl*<sup>D139V/D139V</sup> embryos failed to compete effectively  
322 during this period (**Fig. 3K**). Heterozygote *Mikl*<sup>Wt/D139V</sup> fetal liver cells contributed poorly in the  
323 first month following the graft but recovered to contribute more after six months (**Fig. 3K**). Thus,  
324 while tolerated under steady-state conditions, heterozygosity of *Mikl*<sup>D139V</sup> is deleterious under  
325 conditions of hematopoietic stress. Bone marrow- derived HSCs from *Mikl*<sup>Wt/D139V</sup> adults and fetal  
326 liver- derived HSCs from *Mikl*<sup>Wt/D139V</sup> and *Mikl*<sup>D139V/D139V</sup> pups also formed fewer and smaller  
327 colonies in the spleens of lethally irradiated recipient mice after 8 days (**Supp. Fig. 3E**).

328

329 **Homozygous *Mkl*<sup>D139V</sup> fibroblasts are less sensitive to necroptotic stimuli and have low levels**  
330 **of MLKL protein.**

331 To examine if the constitutive activity of exogenously expressed MLKL<sup>D139V</sup> results in an  
332 enhanced propensity for necroptosis in cells that express MLKL<sup>D139V</sup> under the control of its  
333 endogenous promoter, we immortalized MDFs from *Mkl*<sup>Wt/Wt</sup>, *Mkl*<sup>Wt/D139V</sup> and *Mkl*<sup>D139V/D139V</sup>  
334 littermates and from *Mkl*<sup>-/-</sup> E19.5 pups. As expected, we observed no significant difference in the  
335 sensitivity of these cells to an apoptotic stimulus such as TNF plus Smac mimetic (**Fig. 4A**).  
336 However we observed a significant and consistent decrease in sensitivity to TNF induced  
337 necroptosis using three different pan-caspase inhibitors Q-VD-OPh, Z-VAD-fmk and IDUN-6556  
338 in a *Mkl*<sup>D139V</sup> dose dependent manner (**Fig. 4A**). While MDFs isolated from *Mkl*<sup>D139V/D139V</sup>  
339 homozygotes were up to 60% less sensitive to TNF-induced necroptosis compared to *Mkl*<sup>Wt/Wt</sup>  
340 MDFs, they were not completely resistant like *Mkl*<sup>-/-</sup> MDFs (**Fig. 4A**).

341  
342 Surprisingly, while there were no obvious differences in the levels of MLKL<sup>WT</sup> and MLKL<sup>D139V</sup>  
343 protein following inducible exogenous expression (**Fig. 1F**), MLKL was virtually undetectable by  
344 Western blot in *Mkl*<sup>D139V/D139V</sup> cells (**Fig. 4B**). There was, however, no significant reduction in  
345 *Mkl* transcript levels in these cells suggesting that this reduction was post-transcriptionally  
346 regulated (**Supp. Fig. 4A**). The reduction in MLKL<sup>D139V</sup> protein levels was also evident in whole  
347 body protein lysates prepared from E14 embryos (**Supp. Fig. 4B**). Lysates from E14 embryos also  
348 clearly show that *Mkl*<sup>Wt/D139V</sup> heterozygotes have intermediate levels of MLKL, reflecting the  
349 intermediate sensitivity of *Mkl*<sup>Wt/D139V</sup> MDFs to necroptotic stimuli (**Fig. 4A**).

350

351 **MLKL<sup>D139V</sup> and RIPK3-phosphorylated wildtype MLKL is turned over in a proteasome and**  
352 **lysosome dependent manner.**

353 Measuring the half-life of endogenously expressed MLKL<sup>D139V</sup> is not possible using conventional  
354 ‘pulse chase’ methods because this mutant protein induces necroptotic cell death, so we capitalized  
355 on our previous observation that an N-terminally FLAG-tagged MLKL 4HB forms a high  
356 molecular weight membrane-associated complex just like the untagged form, but, unlike the  
357 untagged version, does not kill cells (Hildebrand et al., 2014). Consistent with this observation, N-  
358 FLAG full-length mouse MLKL was phosphorylated by RIPK3 following stimulation with TSI,  
359 and formed a high molecular weight membrane associated complex, but did not induce cell death  
360 when inducibly expressed in *Mlkl*<sup>-/-</sup> MDFs (data not shown).

361  
362 Using this system we were able to measure the half-life of MLKL by inducing N-FLAG-MLKL<sup>WT</sup>  
363 or N-FLAG-MLKL<sup>D139V</sup> expression in *Mlkl*<sup>-/-</sup> MDFs for 15 hours in doxycycline then washing and  
364 culturing them in the absence of doxycycline for a further 2-24 hours. In the absence of a stimulus  
365 (UT), the levels of N-FLAG-MLKL<sup>WT</sup> remained consistent over the 24-hour period (**Fig. 4C**),  
366 indicating that wild type MLKL is a stable protein in MDFs. However, when these cells were  
367 treated with a necroptotic stimulus (TSI) the levels of wild type MLKL rapidly declined even  
368 though these cells were unable to undergo a necroptotic cell death. This indicates that RIPK3  
369 induced phosphorylation, oligomerization or translocation to the membrane induces turnover of  
370 MLKL in a cell death independent manner. Consistent with the fact that untagged MLKL<sup>D139V</sup>  
371 behaves as an auto-activated form of MLKL (**Fig. 1E**), the half-life of N-FLAG-MLKL<sup>D139V</sup> (4-6  
372 hours) was similar to the WT version stimulated with TSI (**Fig. 4C**). Thus, the absence of  
373 endogenously expressed MLKL<sup>D139V</sup> in E14 embryo lysates and cultured fibroblasts can be

374 attributed to the reduced post-translational stability of this mutant auto-activated form of the  
375 protein.

376

377 To determine which cellular mechanism(s) are required for the clearance of activated MLKL, we  
378 included a series of proteasome, lysosome and specific protease inhibitors during the ‘chase’  
379 period after doxycycline was withdrawn (schematic in **Fig. 4D**). The doses of these inhibitors were  
380 carefully titrated to minimize apoptotic cell death during the assay (**Supp. Fig. 4C**). Nevertheless,  
381 even at the very low doses used, the proteasome inhibitor PS341 reduced the clearance of TSI  
382 stimulated N-FLAG-MLKL<sup>WT</sup> (**Fig. 4D**). This protection was particularly evident when  
383 specifically probing for phospho(p)-MLKL. Chloroquine, Bafilomycin and NH<sub>4</sub>Cl also partially  
384 protected against p-MLKL clearance (**Fig. 4D**). These agents have multifaceted actions, but  
385 interfere with the processes of lysosomal acidification and/or the fusion of  
386 autophagosomes/endosomes with lysosomes and thus prevent protein degradation by lysosomal  
387 proteases. Loss of total N-FLAG-MLKL<sup>D139V</sup> was also prevented by PS341, however it was not  
388 possible to probe for p-MLKL as this activated form of MLKL is not phosphorylated in this assay  
389 due to the absence of TSI stimulation (**Fig. 4E**).

390

391 The reduced half-life of activated MLKL supports recent findings by others that mechanisms exist  
392 for the clearance of activated forms of MLKL (Gong et al., 2017; Yoon et al., 2017; Zargarian et  
393 al., 2017). Based on these findings we hypothesized that this MLKL-clearance mechanism limits  
394 the capacity of MLKL<sup>D139V</sup> to kill *Mkl1*<sup>D139V</sup> hetero and homozygote cells in culture and *in vivo* by  
395 maintaining protein levels below a critical threshold. To test whether this protective mechanism  
396 could be overwhelmed, we incubated MDFs with agents that have been shown to induce *Mkl1*

397 expression (TNF, interferons (IFN)  $\beta$  and  $\gamma$ ) (Rodriguez et al., 2016; Rusinova et al., 2013; Tanzer  
398 et al., 2017; Thapa et al., 2013), or inhibit its turnover (proteasome and lysosome inhibitors).  
399 MLKL<sup>D139V</sup> protein in untreated *Mkl1*<sup>D139V/D139V</sup> MDFs was undetectable by Western blot but  
400 became faintly detectable following stimulation with such stimuli (**Fig. 4B & Supp. Fig. 4D**). This  
401 correlates with moderate but statistically significant increases in cell death (particularly when  
402 compared with the lack of sensitivity to conventional necroptotic stimuli (**Fig. 4A**)), when exposed  
403 to IFN $\beta$  alone and in combination with proteasome or lysosome inhibitors (**Fig. 4F**). An allele-  
404 dose dependent sensitivity is also evident in primary MDFs (**Supp. Fig. 4E**). Together, these  
405 experiments provide evidence for the existence of steady-state MLKL surveillance and turn-over  
406 mechanisms that suppress cell death by lowering the abundance of activated MLKL below a killer  
407 threshold – both at the cellular *and* whole animal level.

408  
409 Interestingly, genetic deletion of *Tnfr1*, *Myd88* and *Ifnar* did not provide any extension to the  
410 lifespan of *Mkl1*<sup>D139V</sup> homozygote pups (**Table I**), indicating that the removal of any one of these  
411 routes to NF- $\kappa$ B- and interferon- mediated gene upregulation is not sufficient to protect against a  
412 double allelic dose of *Mkl1*<sup>D139V</sup>. Similarly, combined genetic deletion of *Casp8* and *Ripk3* did not  
413 rescue or extend the life of *Mkl1*<sup>D139V/D139V</sup> mice, indicating that post-natal death is not mediated by  
414 bystander extrinsic apoptotic cell death that may occur secondary to initial waves of MLKL<sup>D139V</sup>-  
415 mediated necroptosis and associated inflammatory cytokine release (**Table I**). To test whether the  
416 death of *Mkl1*<sup>D139V/D139V</sup> neonates was mediated by activation of the inflammasome we also crossed  
417 this line with the *Caspase 1/11* null mouse strain (Kuida et al., 1995; Li et al., 1995). This did not  
418 enhance the lifespan of *Mkl1*<sup>D139V/D139V</sup> pups (**Table I**).

419

420 **Three of the four most frequent missense gene variants in human *MLKL* encode amino acid**  
421 **substitutions within or immediately adjacent to the brace region.**

422 Given the severe inflammatory phenotype of murine *Mkl<sup>D139V/D139V</sup>* neonates and the significant  
423 defects in stress hematopoiesis observed in murine *Mkl<sup>Wt/D139V</sup>* adults, we explored the prevalence  
424 of brace region variation in human *MLKL*. Examination of the gnomAD database (Lek et al.,  
425 2016), which contains human *MLKL* exome or genome sequence data from a total of over 141,456  
426 individuals revealed that the second and third highest frequency human *MLKL* missense coding  
427 variants; rs34515646 (R146Q) and rs35589326 (S132P), alter the same brace helix (**Table II, Fig.**  
428 **5A**). The 4<sup>th</sup> most common human *MLKL* polymorphism, rs144526386 (G202\*V) is a missense  
429 polymorphism identified exclusively in the context of a shorter splice isoform of *MLKL* (\*) named  
430 ‘*MLKL2*’ (Arnez et al., 2015) (**Table II, Fig. 5B**). The full length canonical transcript of *MLKL*  
431 encodes a 471 amino acid protein, while alternatively spliced *MLKL2* encodes an isoform of  
432 *MLKL* that is 263 amino acids long and is missing a large portion of the pseudokinase domain  
433 which functions to repress the killing potential of the 4HB domain (Cai et al., 2014; Chen et al.,  
434 2014; Dondelinger et al., 2014; Hildebrand et al., 2014) and recruit co-effectors like RIPK3 and  
435 HSP90 ((Jacobsen et al., 2016; Petrie et al., 2018). Glycine202\* is encoded by an extension to  
436 exon 9 that is unique to the *MLKL2* splice isoform (**Fig. 5A, B**).

437  
438 While the amino acid substitution *MLKL<sup>R146Q</sup>* is classified as ‘tolerated’ and ‘benign’ by  
439 SIFT/POLYPHEN 2 algorithms (Adzhubei et al., 2013; Sim et al., 2012) (**Supp. Table IV**), R146  
440 of human *MLKL* shows NMR chemical shift perturbations in the presence of the negatively  
441 charged phospholipids IP3 and IP6, indicating a possible role in membrane association and  
442 disruption (Dovey et al., 2018; Quarato et al., 2016). Ser-132 lies at the intersection of a dynamic

443 disordered loop and the first structured residue of the conserved brace helix 1 (**Fig. 5A**) (Murphy  
444 et al., 2013; Petrie et al., 2018; Su et al., 2014). A Serine-to-Proline substitution at this position is  
445 predicted to significantly impact the conformation of the immediately adjacent W133 (brace helix)  
446 and in turn, the closely situated W109 (4 helix bundle) (**Supp. Fig. 5A**). When mapped to a model  
447 of MLKL splice-isoform 2 (Arnez et al., 2015) Glycine 202\* is predicted to be on an isoform 2-  
448 specific helix and to form an interface along with S132 and R146 of brace helix 1. While the  
449 precise structural consequence of these three brace polymorphisms is unknown, modelling of  
450 human MLKL predicts that disruption in the brace region favours adoption of an activated  
451 conformation (Petrie et al, 2018). Consistent with this prediction, the murine equivalent of the  
452 human S132P variant, mMLKL<sup>S131P</sup>, formed high molecular weight membrane-associated  
453 complexes and killed MDFs in the absence of a necroptotic stimulus (**Fig. 5 C, D**) when expressed  
454 at close to endogenous levels (**Supp. Fig. 5B**).

455

456 **MLKL brace helix variants appear *in trans* at a higher frequency in a cohort of CRMO**  
457 **patients than in healthy controls.**

458 To investigate if human MLKL brace region polymorphisms play a role in human  
459 autoinflammatory disease we examined their frequency in cohorts suffering from Ankylosing  
460 Spondylitis (AS), chronic recurrent multifocal osteomyelitis (CRMO), Guillain Barre Syndrome  
461 (GBS) and Synovitis, Acne, Pustulosis, Hyperostosis and Osteitis (SAPHO) Syndrome. The  
462 individual minor allele frequencies of R146Q, S132P and G\*202V are not enriched in these disease  
463 cohorts relative to healthy controls when population distribution is accounted for (**Supp. Tables**  
464 **IV and V**). However these alleles occur *in trans* (making ‘compound heterozygotes’ – schematic  
465 in **Fig. 5E**) in 3 out of 128 CRMO patients. This is 29 times the frequency that these combinations



466 are observed in healthy NIH 1000 genomes samples (where there are only 2 compound  
467 heterozygotes for these polymorphisms out of 2504 healthy individuals sequenced), or at 10-12  
468 times the frequency when only European CRMO patients and two separate healthy European  
469 control populations were compared (**Table III**).

470

## 471 **DISCUSSION**

472 In contrast to apoptosis, necroptosis is widely held to be an inflammatory form of cell death.  
473 However, definitive evidence for this proposition has yet to emerge. Because MLKL is activated  
474 by inflammatory stimuli such as TNF it is very difficult to separate cause from effect. The  
475 identification of an auto-activating mutant of MLKL (*Mkl<sup>D139V</sup>*) in mice has allowed us to explore  
476 the consequences of inappropriate necroptosis in the absence of such confounding factors.  
477 Furthermore it has led to significant insights into developmental processes sensitive to MLKL  
478 activation and into physiological mechanisms that exist to neutralize activated MLKL. These  
479 turnover mechanisms critically control cell fate, determining whether auto-active MLKL<sup>D139V</sup> is  
480 present at a sufficient level to promote cell death.

481 While MLKL phosphorylation might serve as an immuno-histochemical marker for necroptosis  
482 ordinarily, in the *Mkl<sup>D139V</sup>* mice it is not possible to pinpoint exactly which cell type/s undergo  
483 necroptosis. Nevertheless, the presence of high levels of circulating pro-inflammatory cytokines  
484 in *Mkl<sup>D139V/D139V</sup>* pups at E19.5 relative to *Mkl<sup>Wt/Wt</sup>* and *Mkl<sup>Wt/D139V</sup>* littermates suggests that  
485 necroptosis and ensuing inflammation occurs in the sterile *in utero* environment. This is not  
486 enough to overtly retard prenatal development or affect hematopoietic cell populations other than  
487 by moderately reducing circulating platelet levels. However, upon birth and/or exposure to the  
488 outside environment the capacity of homozygous *Mkl<sup>D139V/D139V</sup>* pups to suppress *MLKL<sup>D139V</sup>*

489 activity appears overwhelmed and they die within days of birth. This is clearly a dose-dependent  
490 effect because both *Mlkl*<sup>D139V/Wt</sup> and *Mlkl*<sup>D139V/null</sup> heterozygous mice are viable. We therefore  
491 speculate that transcriptional upregulation of *Mlkl*<sup>D139V</sup> overwhelms the turnover and/or membrane  
492 repair mechanisms that counteract MLKL activation (Gong et al., 2017; Yoon et al., 2017). Post-  
493 natal death cannot be prevented by combined deficiencies in *Ripk3* and *Casp8* or indeed deficiency  
494 of any other inflammatory gene that we tested, including *Tnfr1*, *Myd88* or *Ifnar*. This further  
495 supports the idea that excessive MLKL-induced necroptosis can generate an inflammatory  
496 response in the absence of other inflammatory mediators. Difficulty with suckling due to  
497 inflammatory infiltration of the head and neck and resulting failure to thrive is one possible  
498 explanation for the lethality. However, the narrow window of mortality for *Mlkl*<sup>D139V/D139V</sup> pups  
499 and marked pericardial immune infiltration make heart failure another potential cause of sudden  
500 neonatal death.

501  
502 One of the most unexpected findings from our study is the physiological importance of endogenous  
503 mechanisms that limit the ability of activated MLKL to kill cells. While others have recently shown  
504 that an ESCRT dependent repair mechanism can help protect membranes from limited MLKL  
505 damage it was not feasible to demonstrate the physiological relevance of this finding (Gong et al.,  
506 2017; Yoon et al., 2017). Our data suggest both proteasomal and lysosomal mechanisms also exist  
507 to dispose of activated MLKL. While proteasomal degradation is usually considered to be  
508 cytoplasmic and completely separate from lysosomal degradation, it was notable that low doses of  
509 either the proteasome inhibitor, PS341, or chloroquine (that inhibits lysosome acidification)  
510 limited p-MLKL degradation to very similar extents. This creates the possibility that these  
511 mechanisms or the previously described ESCRT mechanism intersect. Finally, the ability of these

512 mechanisms to hold heterozygous levels of active MLKL in check without deleterious  
513 consequences *in vivo* supports the idea that direct inhibition of activated MLKL may be an  
514 effective means to therapeutically prevent unwanted necroptotic cell death.

515

516 The *Mlkl*<sup>D139V</sup> brace mutant mouse strain may be a useful model to study the role of necroptosis in  
517 human health and disease. According to current allele frequencies in gnomAD, up to 8% of  
518 individuals globally are heterozygous for missense *MLKL* gene variants within the brace-coding  
519 region. This percentage of people with brace variants indicates that this region is highly tolerant  
520 to missense mutation (**Fig. 5F, Supp. Fig. 5C**). High tolerance to missense variation in a coding  
521 sequence is often used to filter out potential pathogenic variants in human genetic studies because  
522 it indicates that such variations are likely to be functionally neutral (Traynelis et al., 2017).  
523 However, the first brace helix is both highly evolutionarily conserved yet also tolerant of missense  
524 mutations in the human population (**Fig. 1D, Fig. 5F,G**). Furthermore *in vivo* and *in vitro* data  
525 show that amino acid substitutions in the brace region have profound effects on MLKL function  
526 (Davies et al., 2018; Quarato et al., 2016). Therefore, overlaid with structural, biochemical, cell  
527 and animal-based evidence of function, it is tempting to speculate that these human MLKL brace  
528 region variants have accumulated not simply by chance, but through positive evolutionary  
529 selection. While defective emergency hematopoiesis is likely to be subject to *negative* evolutionary  
530 selection, *Mlkl*<sup>D139V</sup> mouse-derived HSCs are only defective following chemo- or radio-ablation.  
531 Given that these forms of HSC depletion are unlikely to have been a significant selective force  
532 during human evolution, we speculate that these human brace polymorphisms have achieved high  
533 frequencies in the human population because they have conferred a selective advantage to  
534 infectious disease. Evidence for positive selection has been found for over 300 immune-related

535 gene loci and many of these have been found to be associated with the incidence of autoimmune  
536 and autoinflammatory disease in modern humans (Gutierrez-Arcelus et al., 2016; Ramos et al.,  
537 2015). Many of these variants have also been mechanistically linked to defense against a particular  
538 pathogen (Karlsson et al., 2014; Ramos et al., 2015). While increased numbers and examination  
539 of independent cohorts will be required to confirm the statistical enrichment of human MLKL  
540 brace variants occurring in *trans* in CRMO, this patient cohort offers a tantalizing first clue into  
541 their potential as modifiers of complex/polygenic inflammatory disease.

542

## 543 **EXPERIMENTAL PROCEDURES**

### 544 **Mice**

545 All mice were backcrossed to C57BL/6 mice for >10 generations or generated on a C57BL/6J  
546 background. *Mlkl*<sup>-/-</sup>, *Tnfr1*<sup>-/-</sup>, *Myd88*<sup>-/-</sup>, *IFNAR1*<sup>-/-</sup>, *Ripk3*<sup>-/-</sup>, *Casp8*<sup>-/-</sup> and *Casp1/Casp11*<sup>-/-</sup> mice were  
547 generated as described (Adachi et al., 1998; Beisner et al., 2005; Hwang et al., 1995; Kuida et al.,  
548 1995; Li et al., 1995; Murphy et al., 2013; Newton et al., 2004; Peschon et al., 1998). Mice  
549 designated as E19.5 were obtained by Caesarean section from mothers that received progesterone  
550 injections at E17.5 and E18.5. An independent mouse strain that carried the D139V mutation in  
551 the *Mlkl* gene (MLKL<sup>D139V</sup> CRISPR) was generated using CRISPR/Cas9 as previously described  
552 (Wang et al., 2013). Briefly, one sgRNA of the sequence GGAAGATCGACAGGATGCAG  
553 (10ng/μl), an oligo donor of the sequence  
554 ATTGGAATACCGTTTCAGATGTCAGCCAGCCAGCATCCTGGCAGCAGGAAGATCGA  
555 CAGGTTGCAGAAGAAGACGGgtgagtctcccaaagactgggaaagagtaggccagggttggggtagggtagg  
556 (10ng/uL) and Cas9 mRNA (5ng/μL) were injected into the cytosol of C57BL/6J zygotes. Mice  
557 were sequenced across the mutated region to confirm incorporation of the altered codon and

558 analysis was performed after at least 2 back-crosses to C57BL/6. The relevant Animal Ethics  
559 Committee approved all experiments.

560

## 561 **Linkage analysis**

562 We mapped the chromosomal location of the *Plt15* mutation by mating affected mice to 129/Sv  
563 *Mpl*<sup>-/-</sup> mice to produce N<sub>2</sub> (backcross) and F<sub>2</sub> (intercross) generations. A genome wide scan using  
564 20 N<sub>2</sub> mice with the highest platelet counts ( $287 \pm 74 \times 10^6/\text{ml}$ , compared with  $133 \pm 75 \times 10^6/\text{ml}$  for  
565 the overall population, **Fig. 1A**) localized the mutation to a region of chromosome 8 between  
566 *D8Mit242* and *D8Mit139* and linkage to this region was then refined. Analysis of the F<sub>2</sub> population  
567 revealed a significant reduction in the frequency of mice homozygous for *C57BL/6* alleles in this  
568 interval (e.g. *D8Mit200* 3/81 F<sub>2</sub> mice homozygous *C57BL/6*,  $p=2.2 \times 10^{-5}$   $\chi^2$ -test), suggesting the  
569 *Plt15* mutation results in recessive lethality. The refined 2.01 Mb interval contained 31 annotated  
570 genes, only five of which appeared to be expressed both in the hematopoietic system and during  
571 embryogenesis (<http://biogps.gnf.org/>): *Dead box proteins 19a* and *19b* (*Ddx19a* and *Ddx19b*),  
572 *Ring finger and WD repeat domain 3* (*Rfwd3*), *Mixed lineage kinase domain like* (*Mkl1*), and *WD40*  
573 *repeat domain 59* (*Wdr59*). Sequencing identified a single mutation, an A to T transversion in *Mkl1*  
574 that was heterozygous in all mice with an elevated platelet count.

575

## 576 **Reagents**

577 *Antibodies*; Rat-anti mRIPK3 and rat anti-mMLKL 8F6 (selected for affinity to residues 1-30 of  
578 mouse MLKL) and rat anti-MLKL 3H1 (MLKL brace region) were produced in-house. Anti-Pro  
579 Caspase 8 (#4927) and GAPDH (#2113) were purchased from Cell Signaling Technology. Anti-  
580 mouse P-MLKL (ab196436) and anti-Actin (ab5694) were purchased from Abcam. Anti-VDAC

581 (AB10527) was purchased from Millipore. FC-hTNF was produced in house and used at a final  
582 concentration of 100ng/mL. Recombinant mouse IFN $\gamma$  and  $\beta$  were purchased from R&D Systems  
583 (Minneapolis, MN, USA) Q-VD-OPh and ZVAD were purchased from MP Biomedicals (Seven  
584 Hills, NSW, Australia). Smac mimetic also known as Compound A, and the caspase inhibitor IDN-  
585 6556 were a gift from TetraLogic (Malvern, PA, USA). Propidium iodide, doxycycline, and  
586 bafilomycin were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

587

### 588 **Cell line generation and culture.**

589 Primary mouse dermal fibroblasts were prepared from skin taken from the head and body of E19.5  
590 pups delivered by C-section or from the tails of adult mice as described (Etemadi et al., 2013).  
591 Primary MDFs were immortalized by stable lentiviral transduction with SV40 large T antigen.  
592 Immortalized MDFs were stably transduced with exogenous mouse and human MLKL cloned into  
593 the pFTRE 3G vector, which was generated by Toru Okamoto, and allows doxycycline- inducible  
594 expression as described (Murphy et al., 2013). Cells were maintained in culture as previously  
595 described (Tanzer et al., 2017).

596

### 597 **Cell death assays**

598 Cell death assays were performed as described previously using  $5 \times 10^4$  MDFs per well in 24 well  
599 tissue culture plates (Murphy et al., 2013). Doxycycline (20 ng/mL) was added together with death  
600 stimuli. Fc-hTNF was produced in house and used at 100ng/mL, Compound A Smac mimetic and  
601 IDN6556 were used at 500 nM and 5  $\mu$ M respectively. ZVAD and QVD-OpH were used at 25  $\mu$ M  
602 and 10  $\mu$ M respectively. Mouse and human interferons gamma and beta were used at 30 ng/mL,  
603 PS341 and MG132 at 2 nM and 200 nM respectively and Bafilomycin at 300 nM.

604

### 605 **MLKL turn-over assays**

606  $5 \times 10^4$  MDFs per well were plated in 24 well tissue culture plates and allowed to settle.  
607 Doxycycline (20 ng/mL) +/- TNF, Smac Mimetic and IDN6556 was added. After 15 hr, 'no dox'  
608 and '0' wells were harvested. Media was removed from remaining wells and cells were washed  
609 with PBS and fresh media containing IDN6556 was re-added. Wells were then harvested 2, 4, 6,  
610 8 and 24 hours from this point. Cells were harvested by direct lysis in reducing SDS-PAGE lysis  
611 buffer.

612

### 613 **MLKL protection assays**

614  $5 \times 10^4$  MDFs per well were plated in 24 well tissue culture plates and allowed to settle.  
615 Doxycycline (20 ng/mL) was added. After 18 hrs, 'no dox' and 'T<sub>0</sub>' samples were harvested.  
616 Media was removed and cells washed before addition of fresh media containing TSI or IDN alone  
617 for 3 hrs. Cells were washed again and media restored with IDN6556 alone (UT), or IDN6556 +  
618 inhibitor (MG132 (200 nM), PS341 (10-40 nM), Chloroquin (50  $\mu$ M), Bafilomycin (300 nM),  
619 Ca-074 Me (20  $\mu$ M), TLCK (100  $\mu$ M) and AEBSF (100  $\mu$ M)) for a further 21 hours. Cells were  
620 harvested by direct lysis in reducing SDS-PAGE lysis buffer.

621

### 622 **Transmission Electron Microscopy**

623 Murine dermal fibroblasts prepared from mice of the indicated genotypes were untreated or  
624 stimulated with the indicated agents for the indicated hours. Then, cells were fixed with 2%  
625 glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, postfixated with 2% OsO<sub>4</sub>, dehydrated in  
626 ethanol, and embedded in Epok 812 (Okenshoji Co.). Ultrathin sections were cut with an

627 ultramicrotome (ultracut N or UC6: Leica), stained with uranyl acetate and lead citrate, and  
628 examined with a JEOL JEM-1400 electron microscope. The viability of a portion of these cells  
629 was determined by measuring LDH release as described previously (Murai et al., 2018).

630

### 631 **Mouse histopathology**

632 Caesarian-sectioned E19.5 and Day P2/3 pups were euthanized by decapitation and fixed in 10%  
633 buffered formalin. 5 µm coronal sections were taken at 200 µm intervals for the full thickness of  
634 the head, 5 µm sagittal sections were taken at 300 µm intervals for the full thickness of the body.  
635 A thorough examination of these sections was performed by histopathologists Aira Nuguid and  
636 Tina Cardamome at the Australian Phenomics Network, Melbourne. Findings were confirmed by  
637 Veterinary Pathologist Prof. John W. Finney, SA Pathology, Adelaide and clinical Pathologist  
638 Prof. Catriona McLean, Alfred Hospital, Melbourne.

639

### 640 **Measurement of relative thymic cortical thickness**

641 Representative images of thymus sections were analysed to determine relative cortical thickness  
642 using ImageJ. Briefly, medullary areas were identified on the basis of H and E staining and  
643 removed from the larger thymus structure using the Image J Image Calculator function to isolate  
644 the cortical region. The thickness of the cortical region, defined by the radius of the largest disk  
645 that can fit at a pixel position, was determined using the Local Thickness plugin in ImageJ  
646 ([http://www.optinav.info/Local\\_Thickness.htm](http://www.optinav.info/Local_Thickness.htm)).

647

648

649



650 **Immunohistochemistry**

651 Following terminal blood collection, P0 and P3 pups were fixed for at least 24 hours in 10%  
652 buffered formalin and paraffin embedded before microtomy. Immunohistochemical detection of  
653 Cleaved caspase 3 (Cell Signaling Technology #9661) and CD45 (BD) was performed as described  
654 previously (Rickard et al., 2014b).

655

656 **Cytokine quantification**

657 All Plasma was stored at -80°C prior to cytokine analyses. Cytokines were measured by Bioplex  
658 Pro mouse cytokine 23-plex assay (Bio- Rad #M60009RDPD) according to manufacturer's  
659 instructions. When samples were designated '<OOR' (below reference range) for a particular  
660 cytokine, they were assigned the lowest value recorded for that cohort (as opposed to complete  
661 exclusion or inclusion as 'zero' which would artificially inflate or conflate group averages  
662 respectively). Values are plotted as fold change relative to the mean value for the *Wt/Wt* samples,  
663 and p values were calculated in Microsoft Excel using a 2 tailed TTEST, assuming unequal  
664 variance. Data is only shown for cytokines that displayed statistically significant differences  
665 between genotypes at either of or both day E19.5 and day P3.

666

667 **Hematological Analysis**

668 Blood was collected from P0 and P3 pups into EDTA coated tubes using heparinized glass  
669 capillary tubes from the neck cavity immediately after decapitation. After centrifugation at 500G  
670 for 5 min, 5-15  $\mu$ L of plasma was carefully removed and this volume was replaced with PBS.  
671 Blood cells were resuspended and diluted between 8-20 fold in DPBS for automated blood cell  
672 quantification using an ADVIA 2120 hematological analyzer within 6 hours of harvest. Blood was

673 collected from adult mice retro-orbitally into tubes containing EDTA and analyzed using an  
674 ADVIA120 automated hematological analyzer (Bayer).

675

## 676 **Transplantation Studies**

677 Donor bone marrow or fetal liver cells were injected intravenously into recipient *C57BL/6-*  
678 *CD45<sup>Ly5.1/Ly5.2</sup>* mice following 11Gy of gamma-irradiation split over two equal doses. Recipient  
679 mice received neomycin (2 mg/mL) in the drinking water for 4 weeks. Long term capacity of stem  
680 cells was assessed by flow cytometric analysis of donor contribution to recipient mouse peripheral  
681 blood and/or hematological organs up to 6 months following engraftment. Recovery from  
682 cytotoxic insult was assessed by automated peripheral blood analysis at regular times following  
683 treatment of mice with 150 mg/kg 5-fluorouracil (5-FU).

684

## 685 **Flow Cytometry**

686 To analyze the contribution of donor and competitor cells in transplanted recipients, blood cells  
687 were incubated with a combination of the following antibodies: Ly5.1-PE, Ly5.2-FITC, Ly5.2-  
688 biotin or Ly5.2 PerCPCy5.5 (antibodies from Becton Dickenson, Ca). If necessary, cells were  
689 incubated with a streptavidin PECy5.5 (BD), mixed with propidium iodide (Sigma) and analysed  
690 on a LSRI (BD Biosciences) flow cytometer. To analyse the stem- and progenitor cell  
691 compartment, bone marrow cells were incubated with biotinylated or Alexa700 conjugated  
692 antibodies against the lineage markers CD2, CD3, CD4, CD8, B220, CD19, Gr-1 and Ter-119.  
693 For stem and progenitor cell detection antibodies against cKit, Sca-1, CD48, AnnexinV, CD105,  
694 Fc  $\gamma$  RII/III or CD135 in different combinations (see antibody list for details). Finally

695 FluoroGold (AAT Bioquest Cat#17514) was added for dead cell detection. Cells were then  
696 analysed on LSRII or Fortessa1 (BD Biosciences) flow cytometers.

697

### 698 **Reactive Oxygen Species (ROS) detection**

699 ROS was detected by using Chloromethyl-H<sub>2</sub>DCFDA dye according to the manufacturer's  
700 instructions (Invitrogen Cat#C6827). In brief, bone marrow cells were loaded with 1 μM  
701 Chloromethyl-H<sub>2</sub>DCFDA for 30 minutes at 37°C. Loading buffer was then removed, and cells  
702 were placed into 37°C StemPro-34 serum free medium (ThermoFisher Cat#10639011) for a 15  
703 minute chase period. After incubation cells were placed on ice and stained with surface  
704 antibodies suitable for FACS analysis. Cells were analysed using a LSRII flow cytometer  
705 (Becton Dickinson).

706

### 707 **Quantitative PCR**

708 RNA was prepared using Trizol (Invitrogen) according to the manufacturer's instructions and  
709 10 μg was used for first strand cDNA synthesis using SuperScript II (Life Technologies). ~0.5 μg  
710 of cDNA was then used in a TaqMan PCR reaction with Universal PCR mastermix and murine  
711 Mlkl (Mm1244222\_n1) and GAPDH (Mm99999915\_m1) Taqman probes (ThermoFisher) on an  
712 ABI 7900 Fast Real-Time PCR instrument (Applied Biosystems). Mlkl expression relative to  
713 GAPDH control was determined using SDS version 2.3 program (Applied Biosystems) and  
714 expressed as  $\Delta$  CT values.

715

716

717

718 **Statistics (Mouse and cell-based assays)**

719 Please consult figure legends for description of error bars used. All P values were calculated in  
720 Microsoft Excel or Prism using an unpaired, two tailed t-test, assuming unequal variance.

721 \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\* $p \leq 0.005$

722

723 **Whole Exome Sequencing**

724 DNA from CRMO probands and their family members (when available) was purified from saliva  
725 or blood and prepared for whole exome sequencing (WES). The samples underwent WES at  
726 several different times, enriched using the Agilent SureSelect Human All Exon V4, V5 or  
727 V6+UTR (Agilent Technologies) before sequencing at either Otogenetics, Inc (Atlanta, GA),  
728 Beckman Coulter Genomics (Danvers, MA), or at the University of Iowa Genomics Core (Iowa  
729 City, IA). The fastq files were quality-checked and processed to vcf format as described previously  
730 (Cox et al., 2017). Variants for all samples were called together using GATK's Haplotype Caller  
731 (McKenna et al., 2010) and were recalibrated and hard-filtered in GATK as described previously  
732 (Cox et al., 2017). Variants were annotated with minor allele frequencies (MAFs) from 1000  
733 genomes (Genomes Project et al., 2015), ExAC and gnomAD (Lek et al., 2016) and with  
734 information regarding the effect of each variant using SNPSift/SNPEff (Cingolani et al., 2012).  
735 The databases used for annotation were dbNSFP2.9 (Liu et al., 2016a) (for MAFs) and GRCh37.75  
736 for protein effect prediction.

737

738 **Ancestry Determination**

739 Ancestry was determined for each CRMO proband using the LASER software package (Wang et  
740 al., 2014). A vcf file including ten probands at a time was uploaded to the LASER server and the

741 TRACE analysis was selected using the Worldwide panel. For probands with indeterminate  
742 ancestry using the Worldwide panel, the European and Asian panels were used. Principal  
743 component values for each proband were plotted using R Statistical Software and the code  
744 provided in the LASER package.

745

#### 746 **MLKL variant quantification**

747 *1000 Genomes*: Vcf files from 1000 genomes were annotated and filtered as described previously  
748 (Cox, 2018). Values for MLKL variants rs35589326 (S132P), rs34515646 (R146Q), and  
749 rs144526386 (G202V) as well as all MLKL coding variants were queried and tabulated for allele  
750 and genotype count for participants of all ancestry (n=2504), and for those of European ancestry  
751 (n=503). Compound heterozygous variants were evident due to the phasing of all variants in the  
752 1000 genomes dataset. *CRMO*: Allele and genotype counts for all MLKL coding variants were  
753 tabulated in probands of European ancestry (n=101) and for all probands (n=128). Compound  
754 heterozygous variants were identified using parental sequence data. *AS*: DNA from all subjects in  
755 AS cohort were genotyped using the Illumina CoreExome chip following standard protocols at the  
756 Australian Translational Genomics Centre, Princess Alexandra Hospital, Brisbane. Bead intensity  
757 data was processed and normalized for each sample and genotypes called using the Illumina  
758 Genome Studio software. All the samples listed in the table have been passed quality control  
759 process. *GB*: Genotyping was performed in an ISO15189-accredited clinical genomics facility,  
760 Australian Translational Genomics Centre (ATGC), Queensland University of Technology. All  
761 samples were genotyped by Illumina HumanOmniExpress (OmniExpress) BeadChip (Blum et al.,  
762 2018). *QUT controls*: A collection of healthy control data of verified European ancestry from  
763 various cohort studies, compiled by the Translational Genomics Group, QUT and typed on an

764 Illumina CoreExome microarray. Includes data from the The UK Household Longitudinal Study,  
765 led by the Institute for Social and Economic Research at the University of Essex and funded by  
766 the Economic and Social Research Council. The survey was conducted by NatCen and the  
767 genome-wide scan data were analysed and deposited by the Wellcome Trust Sanger Institute.  
768 Information on how to access the data can be found on the understanding Society website  
769 <https://www.understandingsociety.ac.uk/> .

770

### 771 **Statistical Analysis (Human data)**

772 Statistical comparisons were performed at the level of allele frequency or the level of compound  
773 heterozygote sample frequency using either a Fisher's exact test or a Chi-Squared test with Yates  
774 correction as specified under each table. Compound heterozygous variants were quantified and  
775 compared at the individual rather than the allelic level, where individuals with and without  
776 qualifying variants were compared at the allelic level.

777

### 778 **Web resources**

779 gnomAD – <https://gnomad.broadinstitute.org/>

780 <http://asia.ensembl.org>

781 OrthoDB - <https://www.orthodb.org>

782 CADD - <https://cadd.gs.washington.edu/>

783

784 Clustal Omega - <https://www.ebi.ac.uk/Tools/msa/clustalo/>

785 WEBLOGO - <https://weblogo.berkeley.edu/logo.cgi>

786 Missense Tolerance Ratio (MTR) Gene Viewer - <http://biosig.unimelb.edu.au/mtr-viewer>

787

788 UK biobank - <https://www.ukbiobank.ac.uk>

789

790 **FIGURE LEGENDS**

791 **Figure 1: Murine MLKL<sup>D139V</sup> is a constitutively active form of MLKL**

792 (A) Platelet counts from *Mpl*<sup>-/-</sup> mice and offspring from matings between *Plt15* mice and *Mpl*<sup>-/-</sup>  
793 mice on a C57BL/6 or mixed C57BL/6:129/Sv background used for linkage analysis (Mixed N<sub>2</sub>).

794 (B) A missense mutation (D139V) in the second exon of *Mlkl* was identified in DNA isolated from  
795 *Plt15* mutant mice. DNA sequence is shown for a wild type (top), a heterozygous mutant (middle),  
796 and a homozygous mutant (bottom). (C) Aspartate 139 contributes to an ‘electrostatic zipper’

797 joining brace helix 1 and the 4HB α2 helix of mouse MLKL (PDB code 4BTF) (Murphy et al.,  
798 2013). (D) Sequence logo of the MLKL brace domain generated from a multiple sequence

799 alignment of all Vertebrata MLKL sequences (257) available on OrthoDB. (E) Mouse dermal  
800 fibroblasts (MDFs) of indicated genotypes were stably transduced with *Mlkl*<sup>Wt</sup> and *Mlkl*<sup>D139V</sup>

801 lentiviral constructs and expression was induced with doxycycline (dox) for 21 hrs. PI-positive  
802 cells were quantified by flow cytometry. Means ± SEM are plotted for between 4-8 experiments

803 (a combination of biological repeats and independent experiments) for each genotype with the  
804 exception of *R3*<sup>-/-</sup>*C8*<sup>-/-</sup> + *Mlkl*<sup>Wt</sup> (n=2, ± SD). (F) Western blot analysis of whole cell lysates taken

805 6 hours post doxycycline induction for analysis of MLKL, RIPK3 and pro-caspase 8 expression.

806 (G) Transmission electron micrographs of MDFs stimulated as indicated. Images selected are  
807 representative of 2-3 independent analyses. TBZ; TNF + Birinapant + Z-VAD-FMK.

808

809 **Figure 2: Homozygous *Mlkl*<sup>D139V</sup> neonates exhibit dispersed inflammation and secondary**  
810 **lymphoid organ hypoplasia throughout the head, neck and mediastinum.** (A) Macroscopic

811 appearance of *Mlkl*<sup>Wt/Wt</sup>, *Mlkl*<sup>Wt/D139V</sup> and *Mlkl*<sup>D139V/D139V</sup> mice at postnatal day 3. (B) Coronal

812 section of mouth and neck region of postnatal day 2 litter mates stained with haematoxylin and

813 eosin (H&E). Dilated blood vessels and edema are indicated by arrows. (C) Serial mandible  
814 sections from postnatal day 3 litter mates stained with H&E and anti-CD45. Inset black boxes are  
815 magnified in right panel. SL, sublingual gland. SM, submandibular gland. Images representative  
816 of n=3-4 P3 pups per genotype. (D) H&E stained sections from mediastinum of postnatal day 2  
817 litter mates. Thymic cortical thinning and pericardial infiltration are indicated by arrows. For full  
818 anatomical annotations for B and D see **Supp. Fig. 2H**. (B) and (D) representative of n= 5-6 P2  
819 pups examined. Multiplex measurement of plasma cytokines levels at E19.5 (E) and postnatal day  
820 3 (F). Error bars represent mean  $\pm$  SD of indicated numbers of independent pups per genotype  
821 sampled.

822

823 **Figure 3: Alterations in hematopoietic cells and defective emergency hematopoiesis in**  
824 ***Mikl*<sup>D139V</sup> mice.** (A-C) Absolute white blood cell (WBCB) and lymphocyte numbers in the  
825 peripheral blood of E19.5 and P3 pups, n indicated. (D-G) Proportions of HSC (Lineage<sup>-</sup>Sca-1<sup>+</sup>c-  
826 kit<sup>+</sup> (LSK) CD150<sup>+</sup> CD48<sup>-</sup>), MPP (LSK CD150<sup>-</sup> CD48<sup>-</sup>), HPC-1 (LSK CD150<sup>-</sup> CD48<sup>+</sup>) and HPC-  
827 2 (LSK CD150<sup>+</sup> CD48<sup>+</sup>)(Oguro et al., 2013) in E18.5 fetal liver (D) and P2 bone marrow cells (F).  
828 Levels of ROS in E18.5 fetal liver cell populations (E) and AnnexinV expression in P2 bone  
829 marrow cell populations (G) are shown relative to *Mikl*<sup>Wt/Wt</sup>. *Mikl*<sup>Wt/Wt</sup> -blue bar, *Mikl*<sup>Wt/D139V</sup> -red  
830 bar, *Mikl*<sup>D139V/D139V</sup> -green bar. Values from all independent animals sampled are plotted (n=2-18).  
831 (H) Numbers of HSC (Lineage<sup>-</sup>Sca-1<sup>+</sup>c-kit<sup>+</sup> (LSK) CD150<sup>+</sup> CD48<sup>-</sup>), MPP (LSK CD150<sup>-</sup> CD48<sup>-</sup>),  
832 HPC-1 (LSK CD150<sup>-</sup> CD48<sup>+</sup>) and HPC-2 (LSK CD150<sup>+</sup> CD48<sup>+</sup>) in adult bone marrow from  
833 *Mikl*<sup>Wt/Wt</sup> and *Mikl*<sup>Wt/D139V</sup> mice, n indicated. Error bars in A-G represent mean  $\pm$  SD. (I) Numbers  
834 of red and white blood cells and platelets in *Mikl*<sup>Wt/Wt</sup> and *Mikl*<sup>Wt/D139V</sup> mice after treatment with  
835 150mg/kg 5FU or saline. Means  $\pm$  SEM from one experiment in which three mice were sampled



836 at each time point for each treatment group, similar results were obtained in an independent cohort.  
837 (J) Bone marrow cells ( $2 \times 10^6$ ) from *Mkl<sup>Wt/Wt</sup>* or *Mkl<sup>Wt/D139V</sup>* mice on a CD45<sup>Ly5.2</sup> background were  
838 mixed with  $2 \times 10^5$  wild type CD45<sup>Ly5.1</sup> competitor bone marrow cells and transplanted into  
839 irradiated CD45<sup>Ly5.1/Ly5.2</sup> recipients. Peripheral blood mononuclear cells from recipient mice were  
840 analysed after 56 days and then again at 180 days. Host contribution (CD45<sup>Ly5.1/Ly5.2</sup>) is depicted  
841 in gray, competitor (CD45<sup>Ly5.1</sup>) in white, and test (CD45<sup>Ly5.2</sup>) in black. The mean and standard  
842 error of the mean (SEM) are shown for 3 donors per genotype and 3-5 recipients per donor. (K)  
843  $2 \times 10^6$  fetal liver cells (CD45<sup>Ly5.2</sup>; *Mkl<sup>Wt/Wt</sup>*, *Mkl<sup>Wt/D139V</sup>* or *Mkl<sup>D139V/D139V</sup>*) were transplanted into  
844 lethally irradiated recipients (CD45<sup>Ly5.1/Ly5.2</sup>) together with  $2 \times 10^5$  competitor bone marrow cells  
845 (CD45<sup>Ly5.1</sup>). Contribution to peripheral blood mononuclear cells was assessed 28 days after  
846 transplantation, and again at 180 days. Host contribution (CD45<sup>Ly5.1/Ly5.2</sup>) is depicted in gray,  
847 competitor (CD45<sup>Ly5.1</sup>) in white, and test (CD45<sup>Ly5.2</sup>) in black. Mean  $\pm$  SEM are shown (2-10  
848 donors per genotype, 2-6 recipients per donor).

849

850 **Figure 4: MLKL<sup>D139V</sup> and activated MLKL<sup>WT</sup> are cleared from cells via a mechanism that**  
851 **requires proteasome function and lysosomal acidification.** MDFs were isolated from *Mkl<sup>Wt/Wt</sup>*,  
852 *Mkl<sup>Wt/D139V</sup>*, *Mkl<sup>D139V/D139V</sup>* or *Mkl<sup>-/-</sup>* pups, immortalized and stimulated as indicated for 21 hrs for  
853 quantification of PI-positive cells using flow cytometry (A), or for 4 hrs for western blot analysis  
854 (B). *Mkl<sup>-/-</sup>* MDFs were stably transduced with doxycycline-inducible FLAG-MLKL<sup>WT</sup> and  
855 FLAG-MLKL<sup>D139V</sup> constructs to examine MLKL protein stability after doxycycline withdrawal  
856 (C) and in the presence of indicated compounds (D) and (E). (F) Immortalized MDFs from (A)  
857 stimulated as indicated for 21 hrs for quantification of PI-positive cells using flow cytometry. (A)

858 and (F) represent mean  $\pm$  SEM of 2-6 independent experiments. B-E are representative images of  
859 at least 3 independent experiments.

860

861 **Figure 5. Three of the four highest frequency missense human MLKL SNPs encode non -**

862 **conservative amino acid substitutions within or adjacent to the brace helix region.** (A) S132

863 and R146 (magenta) are located on either side of D140 (yellow - equivalent to mouse D139) in the

864 first human MLKL brace helix. Alternate amino acids encoded by human polymorphisms

865 indicated in parentheses. (B) G202 is predicted to be on an  $\alpha$  helix unique to MLKL splice-isoform

866 2 and to form an interface along with S132 and R146. The mouse equivalent of human rs35589326

867 (hMLKL<sup>S132P</sup>), mMLKL<sup>S131P</sup>, spontaneously forms membrane-associated high molecular weight

868 complexes following Blue Native (BN) PAGE (C) and kills MDFs (D) in the absence of extrinsic

869 necrotic stimuli when expressed in mouse dermal fibroblasts for 6 (C) and 21 hrs respectively

870 (D). C; cytoplasmic fraction, M; crude membrane fraction, TSI; TNF, Smac-mimetic and

871 IDN6556. (E) Schematic showing brace helix variant combinations identified as alleles *in trans*

872 in 3 CRMO patients. (F) MTRs are mapped onto the structure of MLKL to show regions that have

873 low tolerance to missense variation in the human population (red) and regions that have increased

874 tolerance to missense variation (blue), normalized to the gene's MTR distribution. (G)

875 Evolutionary conservation Multiple sequence alignment (MSA) conservation scores are mapped

876 onto the structure of MLKL to show regions that are highly conserved through evolution (red) and

877 regions that are less conserved through evolution (blue). (C) is representative of 2 independent

878 experiments, (D) mean  $\pm$  SEM of 4-5 independent experiments.

879

880

881 **SUPPLEMENTARY FIGURE LEGENDS**

882 **Supp. Fig. 1**

883 (A) Viability of non-transduced *Wt* MDFs or *RIPK3<sup>-/-</sup>*, *Caspase8<sup>-/-</sup>* MDFs expressing dox-  
884 inducible *Mlkl<sup>Wt</sup>* or *Mlkl<sup>D139V</sup>* was monitored by measuring LDH release at the indicated time points  
885 post addition of TNF (T), Birin pant (B) and ZVAD-fmk (Z) or doxycycline. These conditions  
886 correspond to those used for TEM analyses (**Fig. 1G**).

887

888 **Supp. Fig. 2**

889 (A) Macroscopic appearance of E19.5 pups of indicated genotypes after Caesarean delivery. (B)  
890 Body weights of *Mlkl<sup>Wt/Wt</sup>*, *Mlkl<sup>Wt/D139V</sup>*, *Mlkl<sup>D139V/D139V</sup>* mice at E19.5 and postnatal Day 3. (C)  
891 Serial mandible sections from E19.5 pups stained with H&E and anti-CD45. (D) H&E and anti-  
892 CD45 or cleaved caspase-3 (CC3) stained section of E19.5 mediastinum. (E) Serial sections of  
893 thymi from postnatal day 3 pups stained with H&E and anti-CD45 and quantification of thymic  
894 cortical thickness. (F-G) Blood glucose measured at E19.5 and postnatal day 3 (non-fasting)  
895 plotted as mean  $\pm$  SEM for n=3-39 pups per genotype. (H) Anatomical annotation of head and  
896 mediastinum of postnatal day 2 *Mlkl<sup>Wt/Wt</sup>* pup. (I) Coronal section of postnatal day 2 pup  
897 mouth/neck region and mediastina stained with H&E.

898

899 **Supp. Fig. 3**

900 (A) Numbers of red blood cells (RBC), neutrophils and mean platelet volume in the peripheral  
901 blood of E19.5 and P3 pups. (B) Common myeloid progenitors (CMP, Lineage<sup>-</sup>IL7R $\alpha$ <sup>-</sup>Sca1<sup>-</sup>  
902 cKit<sup>+</sup>CD34<sup>+</sup>Fc $\gamma$ RII/III<sup>-</sup>), granulocyte-macrophage progenitors (GMP, Lineage<sup>-</sup>IL7R $\alpha$ <sup>-</sup>  
903 cKit<sup>+</sup>Sca1<sup>-</sup>CD150<sup>-</sup>Endoglin<sup>-</sup>Fc $\gamma$ RII/II<sup>+</sup>), Colony-forming units-erythroid (CFU-E, Lineage<sup>-</sup>

904 IL7R $\alpha$ <sup>-</sup>cKit<sup>+</sup>Sca1<sup>-</sup>CD150<sup>-</sup>Fc $\gamma$ RII/III<sup>-</sup>Endoglin<sup>hi</sup>), and megakaryocyte-erythroid progenitors  
905 (MegE, Lineage-IL7R $\alpha$ <sup>-</sup>Sca1<sup>-</sup>cKit<sup>+</sup>CD150<sup>+</sup>Endoglin<sup>low</sup>Fc $\gamma$ RII/III<sup>-</sup>) in E18.5 fetal livers, P2 bone  
906 marrow cells and adult bone marrow, presented as a percent from Lin-cKit<sup>+</sup>Sca1<sup>-</sup> cell fractions.  
907 Mean  $\pm$  SD, n=3-6 (E18.5), n=9-11 (P2 BM), n=9 (adult BM) per genotype. (C) Recovery of red  
908 blood cells, white blood cells, platelets and bone marrow progenitor cells (Lineage-Sca-Kit<sup>+</sup>) in  
909 *Mkl<sup>Wt/Wt</sup>* and *Mkl<sup>Wt/D139V</sup>* mice following 375 Rad whole body irradiation. (D) Relative amount  
910 of ROS and AnnexinV in LSK and progenitor cells was determined 7 days post irradiation. (E)  
911 Bone marrow (BM) or fetal liver (FL) cells ( $7.5 \times 10^4$  -  $3 \times 10^5$ ) from mice of the indicated  
912 genotypes (*Mkl<sup>Wt/Wt</sup>*, *Mkl<sup>Wt/D139V</sup>* or *Mkl<sup>D139V/D139V</sup>*) were transplanted into lethally irradiated  
913 recipients and spleens were removed for enumeration of CFU-S after 8 days. Mean  $\pm$  SEM from  
914 2-8 donors. Spleens taken from recipients of *Mkl<sup>Wt/Wt</sup>* or *Mkl<sup>Wt/D139V</sup>* bone marrow ( $7.5 \times 10^4$  or  
915  $3.0 \times 10^5$  cells transplanted respectively) were photographed to detail the size and number of  
916 colonies. *Mkl<sup>Plt15/+</sup>* cells generated very small colonies at low frequency (arrows).

917

#### 918 **Supp. Fig. 4**

919 (A) Mouse *Mkl* mRNA levels quantified using TaqMan probes. (B) E14.5 whole embryo lysates  
920 from 3 pups per genotype were probed by western blot for relative MLKL protein levels. (C)  
921 Viability of cells following 21 hr incubation with inhibitors used in **Fig 4D-E**. Representative of  
922 3 similar experiments. (D) MDFs were treated as indicated for 21 hours. Whole cell lysates were  
923 analysed by western blot for levels of MLKL. (E) Primary MDFs were isolated from *Mkl<sup>Wt/Wt</sup>*,  
924 *Mkl<sup>Wt/D139V</sup>* or *Mkl<sup>D139V/D139V</sup>* mice and stimulated as indicated for 21hrs for quantification of PI  
925 positive cells using flow cytometry.

926

927

928 **Supp. Fig. 5**

929 (A) A proline in position 132 of human MLKL is predicted to significantly impact the  
930 conformation of the immediately adjacent W133 (brace helix) and in turn, the closely situated  
931 W109 (4 helix bundle). (B) MDFs stably transduced with doxycycline inducible constructs  
932 expressing mouse MLKL<sup>S131P</sup> were analysed by western blot for MLKL levels after 4 hrs dox  
933 induction. (C) Missense Tolerance Ratio (MTR) distribution for human MLKL using gnomAD  
934 exome data.

935

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Figure 1. mMLKL<sup>D139V</sup> is a constitutively active form of murine MLKL.

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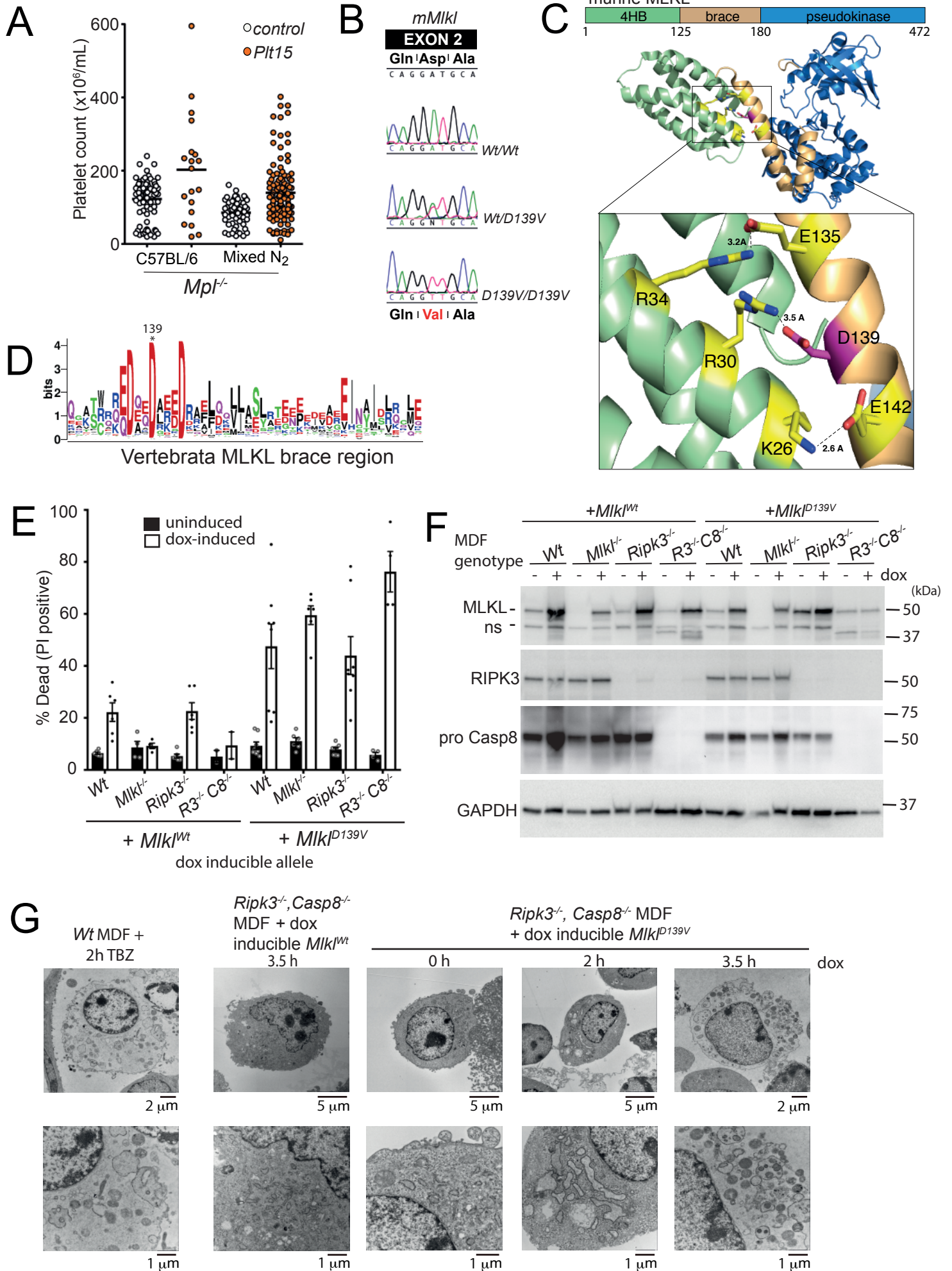




Figure 2. Homozygous *Mikl*<sup>D139V</sup> neonates exhibit dispersed inflammation and secondary lymphoid organ hypoplasia throughout the head, neck and mediastinum.

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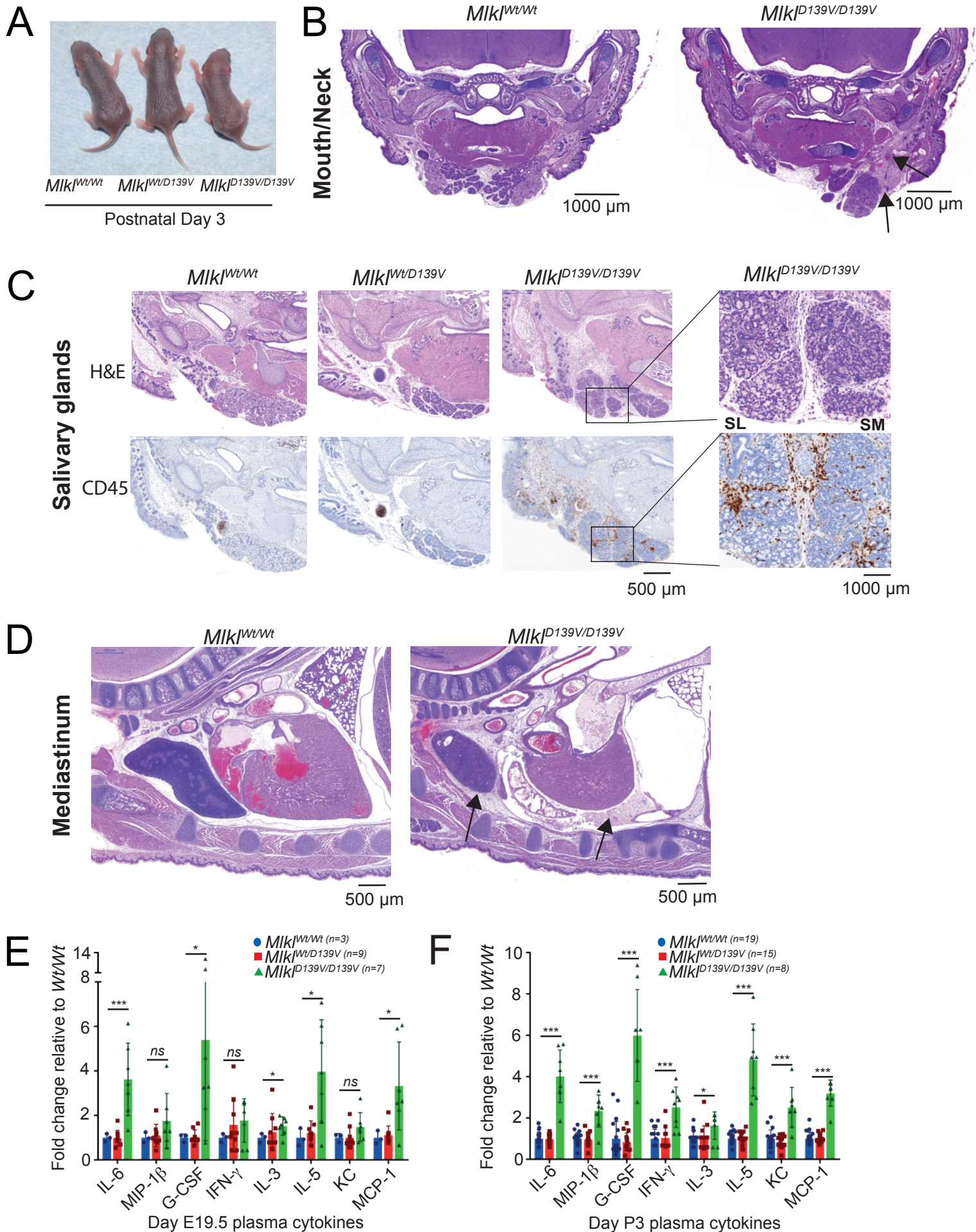


Figure 3. Alterations in hematopoietic cells and defective emergency hematopoiesis in *Mikl*<sup>D139V</sup> mice.

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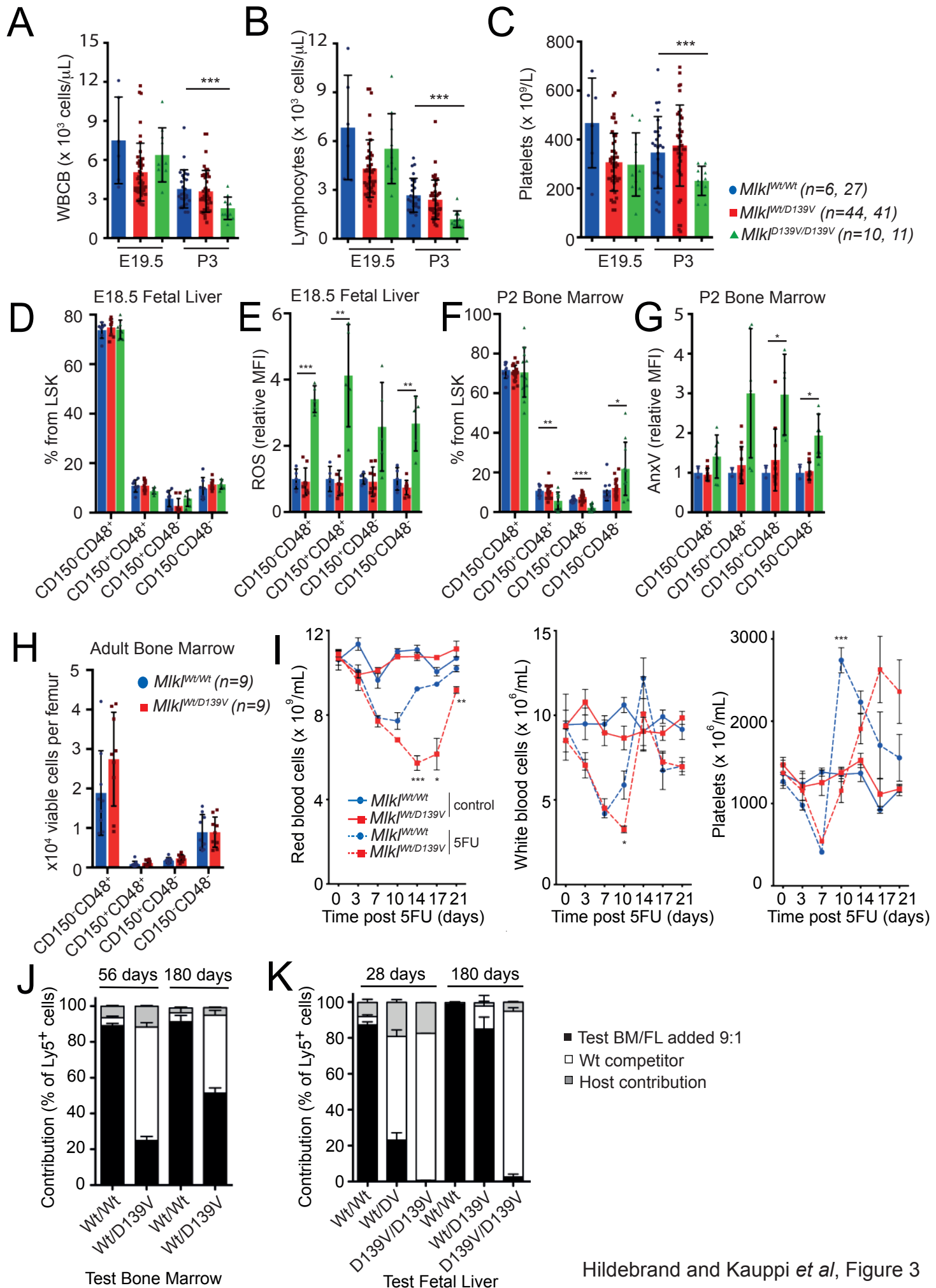


Figure 4. MLKL<sup>D139V</sup> and activated MLKL<sup>WT</sup> is cleared from cells via a mechanism that requires proteasome function and lysosomal acidification.

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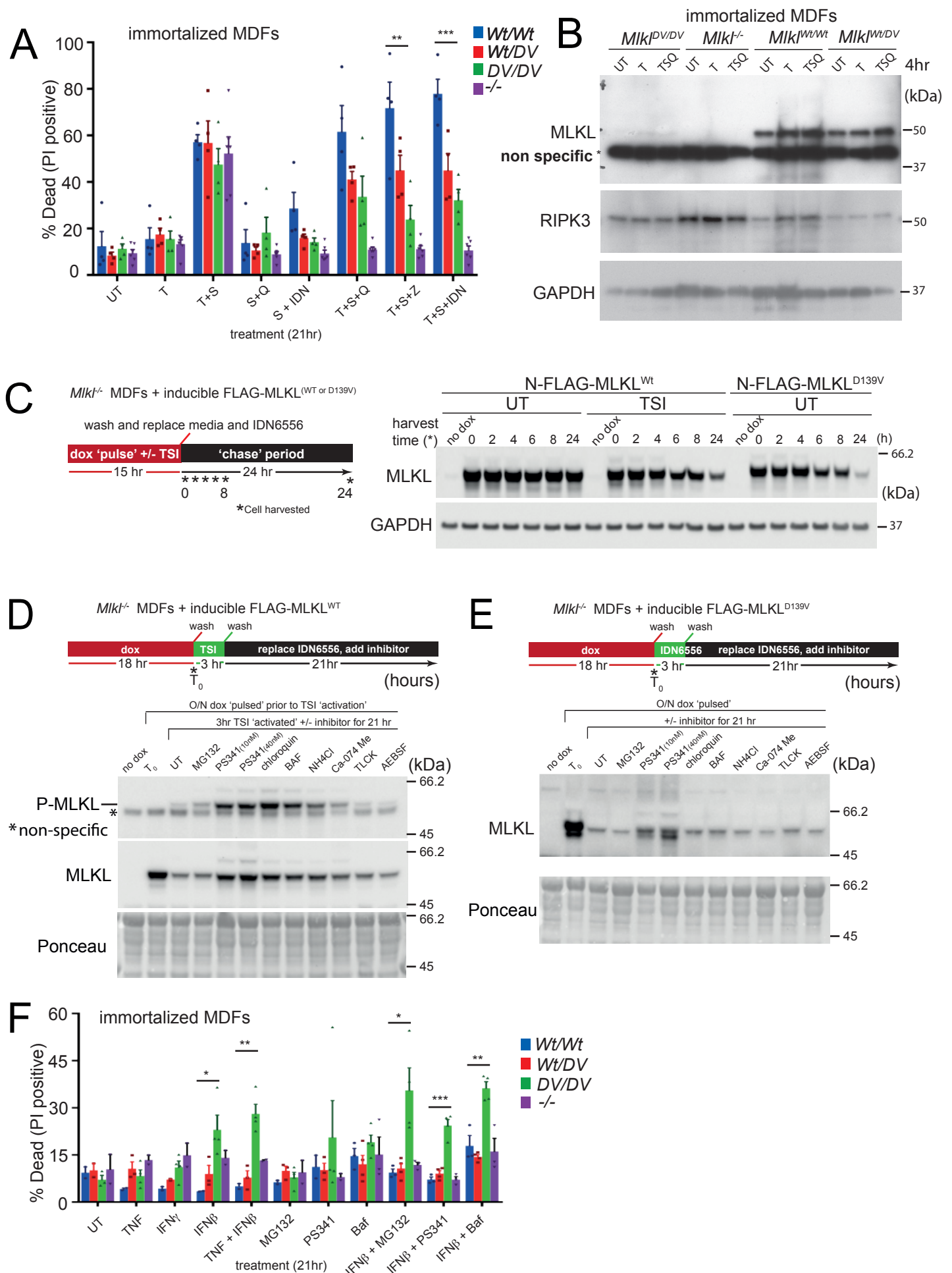




Table I. Postnatal lethality in *Mkl<sup>D139V</sup>* homozygotes is independent of *Tnfr1*, *Myd88*, *Ripk3*, *Casp8*, *Casp1* and *Casp11*.  
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*Mkl<sup>Wt/D139V</sup> x Mkl<sup>Wt/D139V</sup>*

stage genotyped	E14	E18	postnatal day 21						
	Wt	Wt	Wt	<i>Tnfr1</i> <sup>-/-</sup>	<i>Myd88</i> <sup>-/-</sup>	<i>Ripk3</i> <sup>-/-</sup> , <i>C8</i> <sup>+/-</sup>	<i>Ripk3</i> <sup>-/-</sup> , <i>C8</i> <sup>-/-</sup>	<i>Ifnar</i> <sup>-/-</sup>	<i>C1</i> <sup>-/-</sup> , <i>C11</i> <sup>-/-</sup>
C57BL/6 genetic background									
<i>Mkl<sup>Wt/Wt</sup></i>	58 (39)	7 (10)	15 (11)	19 (15)	3 (2)	10(6)	2(2)	15 (11)	1(1)
<i>Mkl<sup>Wt/D139V</sup></i>	70 (78)	17 (20)	30 (22)	41 (30)	6 (4)	14(12)	5(4)	30 (22)	2(2)
<i>Mkl<sup>D139V/D139V</sup></i>	28 (39)	13 (10)	0 (11)	0 (15)	0 (2)	0 (6)	0(2)	0 (11)	0(1)
total # genotyped	156	37	45	60	9	24	7	45	3

( ) number of pups expected from mendelian segregation, calculated from total number of pups that genotyped, rounded to nearest whole number.

Table II. Human *MLKL* brace helix polymorphism frequency

Feature	human MLKLSNP		
	<i>R146Q- rs34515646</i>	<i>S132P- rs35589326</i>	<i>G202*V- rs144526386</i>
CADD Score (phred-scaled)	0.407	6.381	3.825
UK Biobank- Total MAF (n)	0.0253 (487,658)	0.0161 (487,625)	0.0147 (487,488)
gnomAD- Total MAF (n)	0.0152 (141,339)	0.0138 (141,442)	0.01228 (141,400)
gnomAD- Highest MAF (n) population	0.0252 (64,541) European (Non-Finnish)	0.0311 (5,185) Ashkenazi Jewish	0.0245 (5,184) Ashkenazi Jewish
1000 genomes- Total MAF (n)	0.0052 (2,504)	0.0088 (2,504)	0.0102 (2,504)
1000 genomes- Highest MAF (n) population	0.018 (503) European	0.024 (489) South Asian	0.021(503) European

*n* - number of individuals sequenced  
 MAF - Minor Allele Frequency -count  
 \*alternate transcript

Table III. Human *MLKL* brace helix compound heterozygotes in CRMO vs Healthy Controls

Population	Frequency of relevant compound Hets			2 tailed p value	
	CRMO (n)	Healthy controls (n)	CRMO:Healthy <sup>a</sup>	Fisher's exact	χ square with Yates
Global	0.023 (3/128)	0.0008 (2/2504*) NIH 1KG	29:1	0.001	0.0001
European	0.02 (2/101)	0.002 (1/503*) NIH 1KG	10:1	0.074	0.1215
European	0.02 (2/101)	0.0017 (25/14,542*) QUT controls	12:1	n/a	0.0022

Frequency of CRMO patients that are compound heterozygotes (see Supp. Fig 5c for schematic)

*n* - number of individuals sequenced

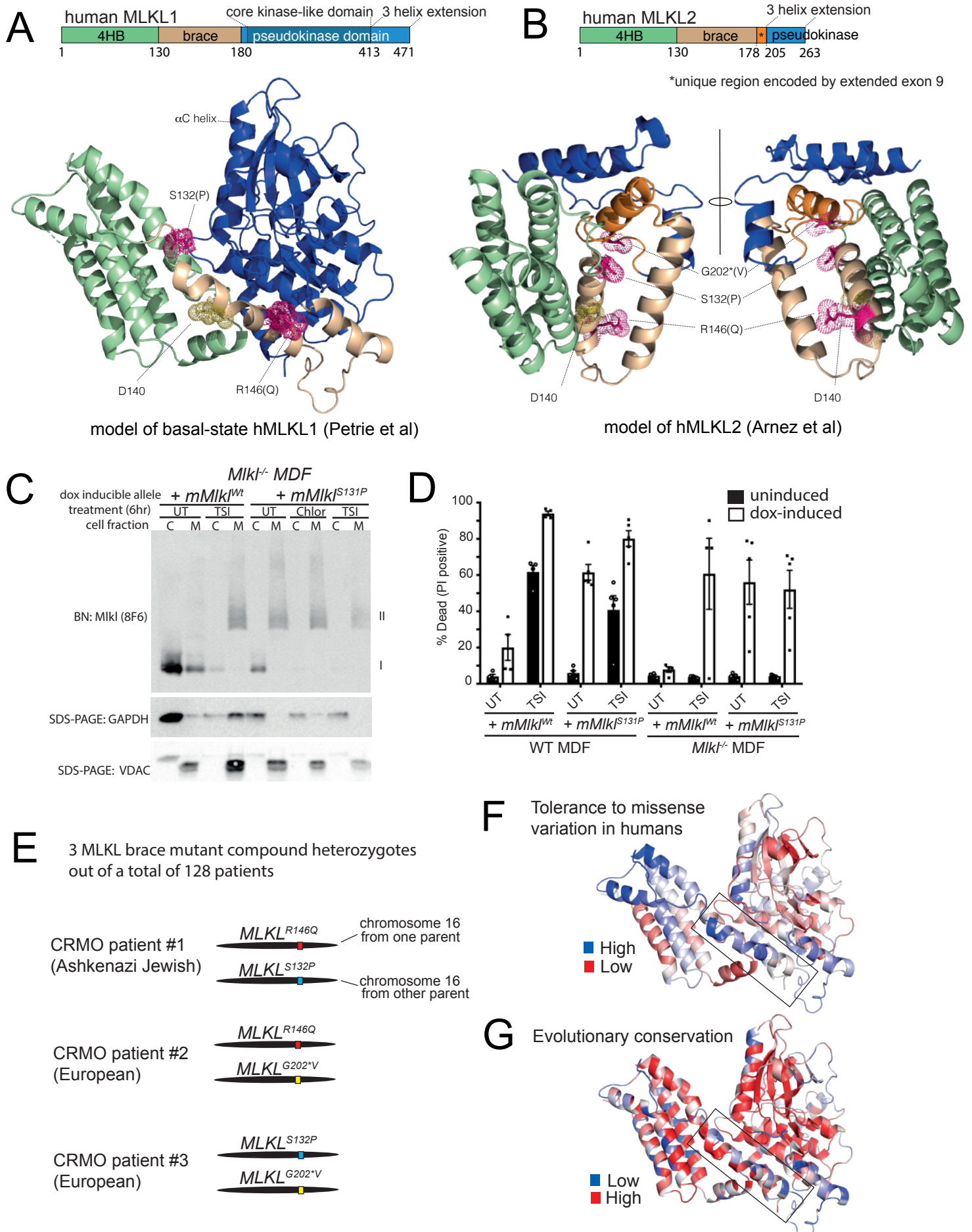
# frequency of brace variant combinations found in CRMO Patients 1 (Asian), 2 and 3 (European)

\*frequency of brace variant combinations found in European CRMO Patients (2 and 3)

<sup>a</sup>frequency ratio rounded to nearest whole number

Figure 5. Three out of the four highest frequency missense human MLKL SNPs encode non conservative amino acid changes within the brace helix region.

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Age	Number of litters	Number of mice	<i>Mikl<sup>Wt/Wt</sup></i>	<i>Mikl<sup>Wt/D139V</sup></i>	<i>Mikl<sup>D139V/D139V</sup></i>
E13.5	1	8	2(2)	3(4)	3(2)
E14.5	22	156	58(39)	70(78)	28(39)
E16.5	3	27	5(7)	19(14)	3(7)
E17.5	2	23	7(6)	9(12)	8(6)
E18.5	5	39	7(10)	17(20)	13(10)
P21	12	45	15(11)	30(23)	0(11)

Embryos from matings between *Mikl<sup>Wt/D139V</sup>* and *Mikl<sup>Wt/D139V</sup>* mice were genotyped at the gestational (E) or postnatal (P) age indicated (days). Observed numbers of *Mikl<sup>Wt/Wt</sup>*, *Mikl<sup>Wt/D139V</sup>* and *Mikl<sup>D139V/D139V</sup>* embryos tabulated with numbers expected from Mendelian inheritance (in the absence of lethality) indicated in parentheses.

Hildebrand and Kauppi *et al*, Supp. Table 1

Table SII. Outcome of *Mikl<sup>Wt/D139V</sup> × Mikl<sup>null/null</sup>* cross

Age	Number of litters	Number of mice	<i>Mikl<sup>Wt/null</sup></i>	<i>Mikl<sup>D139V/null</sup></i>
P21	8	40	19 (20)	21 (20)

Surviving progeny from matings between *Mikl<sup>null/null</sup>* and *Mikl<sup>Wt/D139V</sup>* mice were genotyped at postnatal day 21. Observed numbers are tabulated, with numbers expected from mendelian inheritance in the absence of lethality indicated in parentheses.

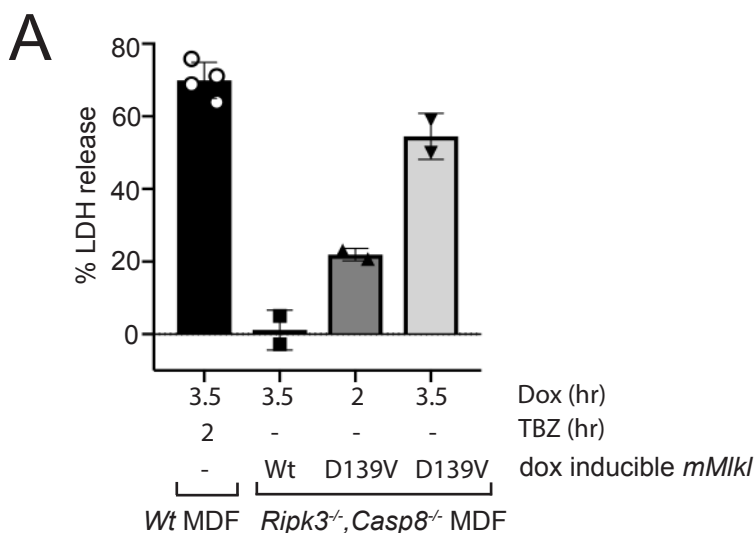
Hildebrand and Kauppi *et al*, Supp. Table II

Table SIII. Outcome of CRISPR-*Mikl<sup>Wt/D139V</sup> × CRISPR-*Mikl<sup>Wt/D139V</sup>** cross

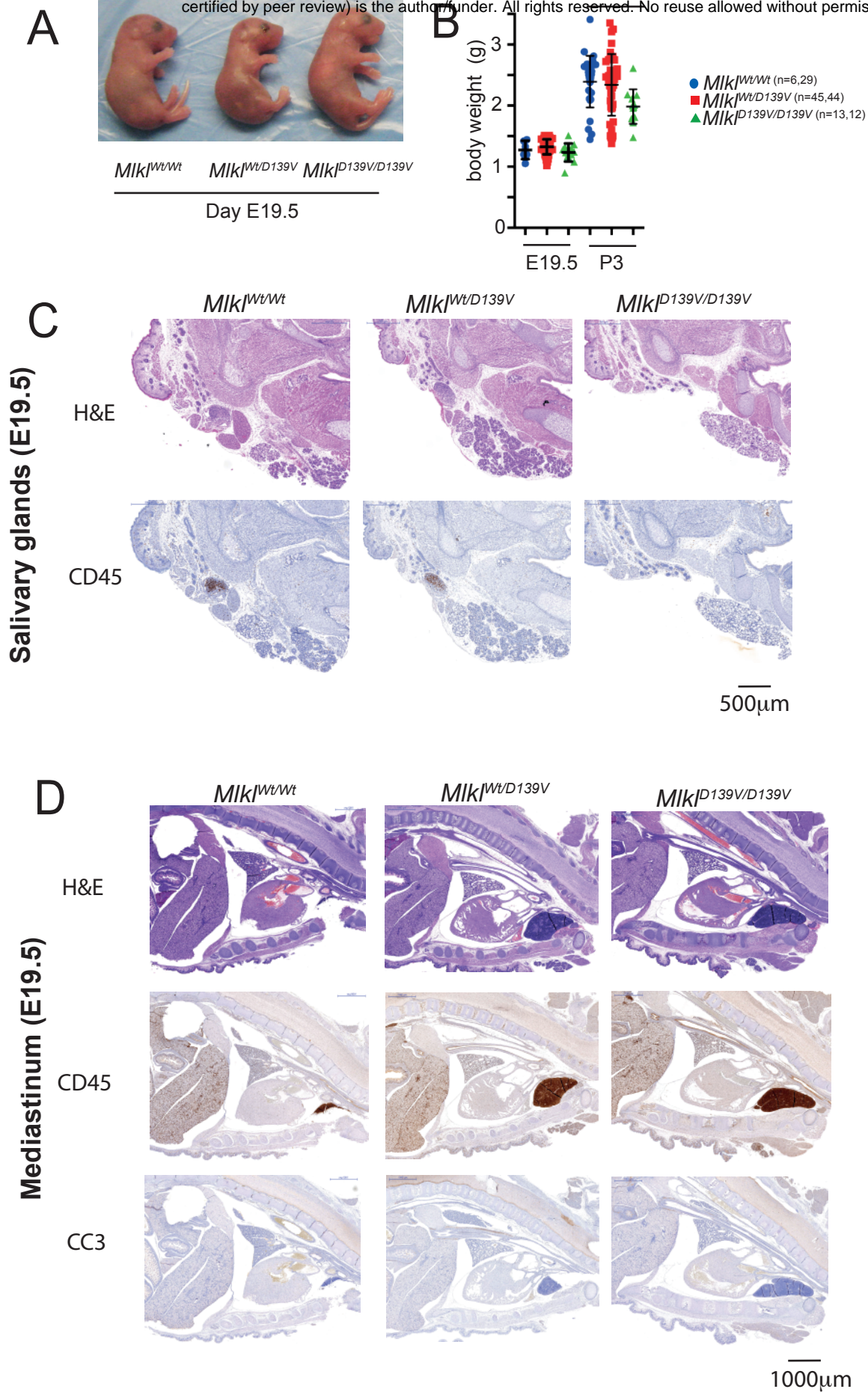
Age	Number of litters	Number of mice	<i>Mikl<sup>Wt/Wt</sup></i>	<i>Mikl<sup>Wt/D139V</sup></i>	<i>Mikl<sup>D139V/D139V</sup></i>
P21	7	36	12 (9)	24 (18)	0 (9)

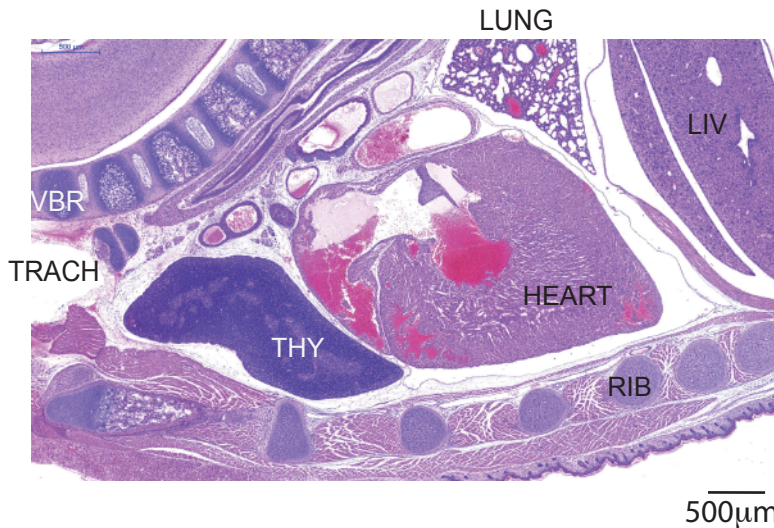
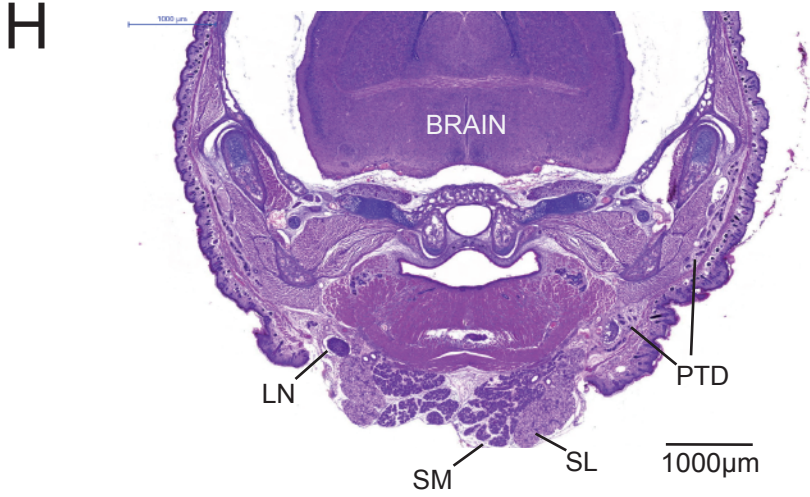
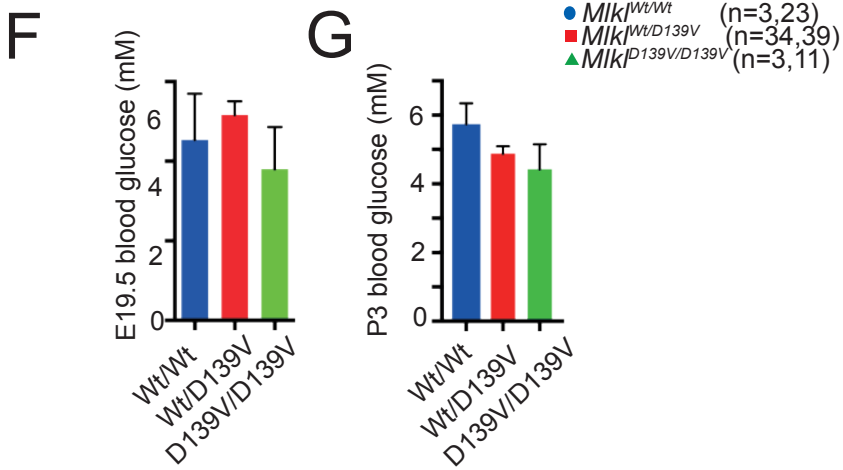
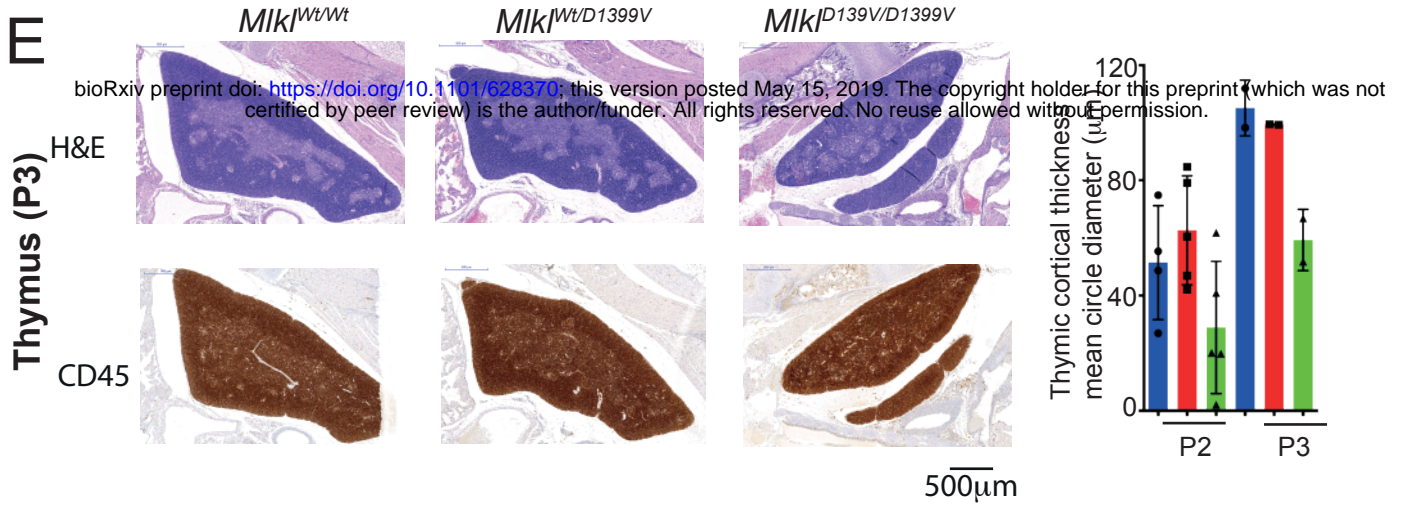
Surviving progeny from matings between CRISPR-induced *Mikl<sup>Wt/D139V</sup>* mice were genotyped at postnatal day 21. Observed numbers are tabulated, with numbers expected from mendelian inheritance in the absence of lethality indicated in parentheses.

Hildebrand and Kauppi *et al*, Supp. Table III



Hildebrand and Kauppi *et al*, Supp. Figure 1



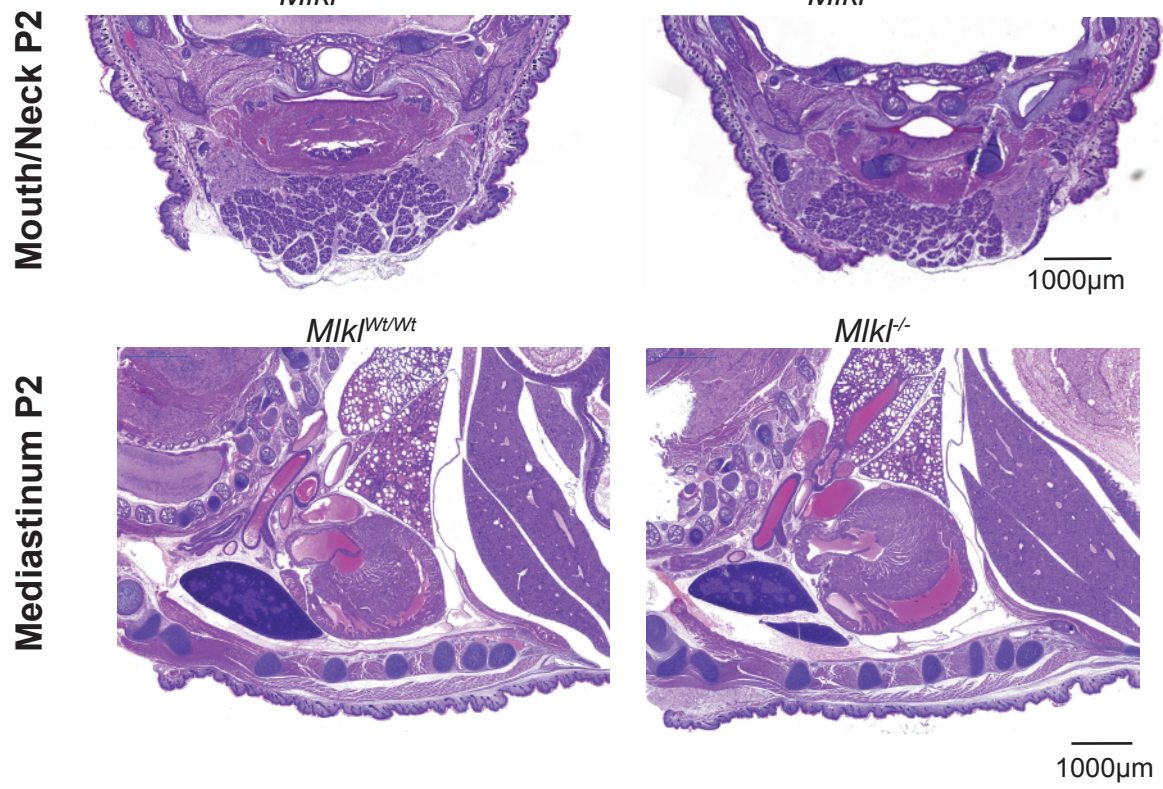


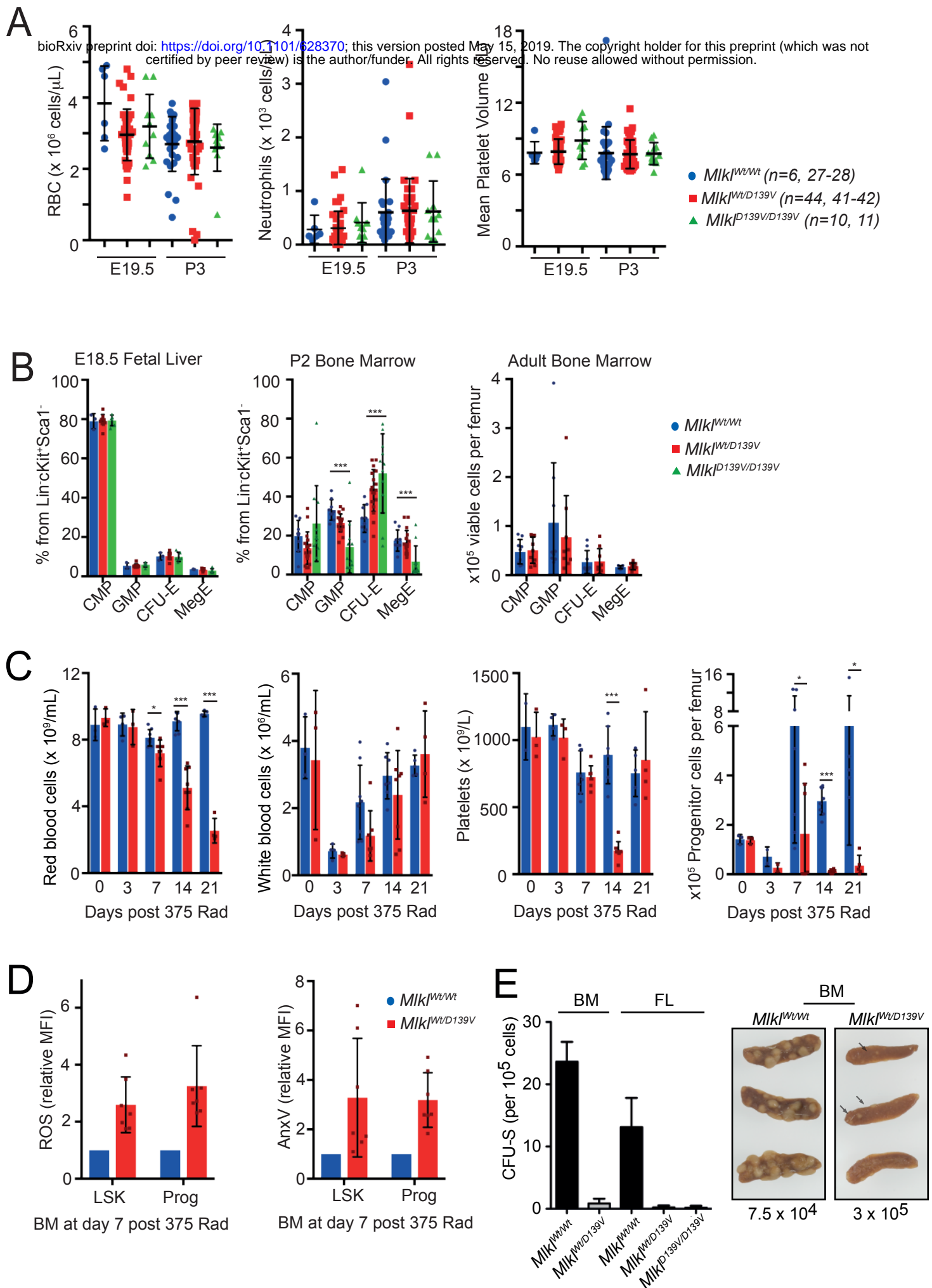
#### Annotation

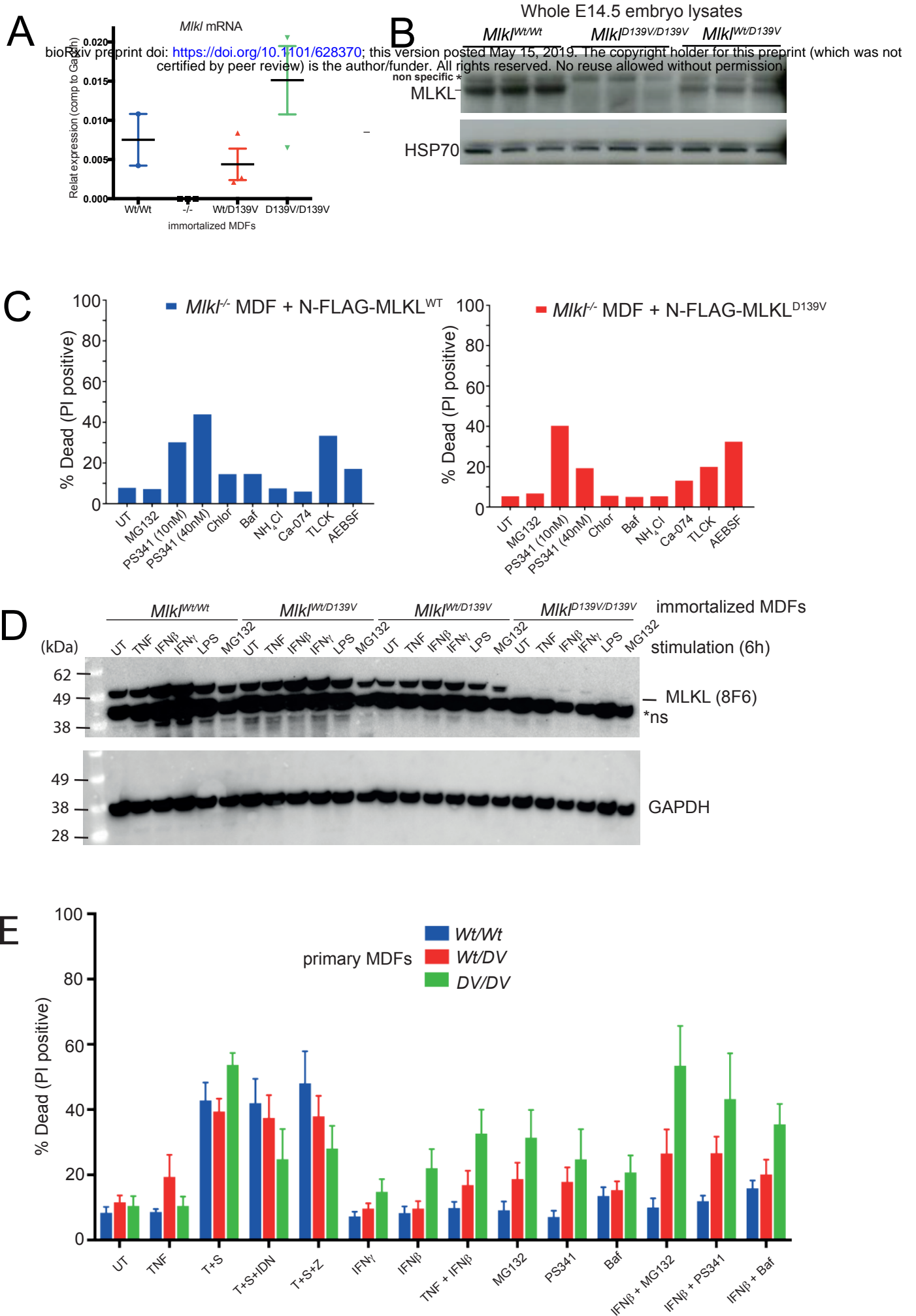
LN - Lymph node  
 SM - Submandibular gland  
 SL - Sublingual gland  
 PTD - Parotid gland  
 BRAIN - Brain

TRACH - Trachea  
 VBR - Vertebra  
 THY - Thymus  
 HEART - Heart  
 LIV - Liver  
 RIB - Rib  
 LUNG - Lung









Supp. Table IV. Human *MLKL* brace helix variants - individual MAFs in CRMO vs Healthy Con-

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Feature	<i>R146Q</i> - rs34515646	<i>S132P</i> - rs35589326	<i>G202*V</i> - rs144526386
1000 genomes- EU MAF (n)	0.0179 (503)	0.0149 (503)	0.0209 (503)
U.Iowa CRMO cohort Total MAF (n)	0.0273 (128)	0.0234 (128)	0.0234 (128)
U.Iowa CRMO cohort EU MAF (n)	0.0347 (101)	0.0198 (101)	0.0198 (101)
U.Iowa CRMO TOTAL vs 1000 genomes Total MAF	p= 0.0009	p= 0.0329	p= 0.057
U.Iowa CRMO EU vs 1000 genomes EU MAF	p= 0.1687	p= 0.5423	p= 0.99
SIFT Score (classification)	MLKL1- 0.569 (TOLERATED) MLKL2- 0.536 (TOLERATED)	MLKL1- 0.25 (TOLERATED) MLKL2- 0 (DELETERIOUS)	MLKL2- 0.069 (TOLERATED)
POLYPHEN-2 Score (classification)	MLKL1- 0.114 (BENIGN)	MLKL1- 0.996 (PROBABLY DAMAGING)	n/a

*n* - number of unrelated individuals sequenced

MAF - Minor Allele Frequency -count

\*alternate transcript

*p* - 2-tailed fisher's exact *p*-value by comparing the allele counts in cases and controls

Supp. Table V. Human *MLKL* brace helix individual MAFs in AS, GB and SAPHO vs Healthy Controls

Disease	MIKI SNP	Disease MAF (n)	matched healthy control MAF (n)	<i>p</i> value
Ankylosing Spondylitis	<i>R146Q</i>	0.0274 (8244) imputed	0.0255(14542)	0.227
	<i>S132P</i>	0.017 (8244) genotyped	0.0165 (14542)	0.699
	<i>G202*V</i>	0.0144 (8244) genotyped	0.0155 (14542)	0.385
Guillain-Barre syndrome	<i>R146Q</i>	0.0084 (178) imputed	0.0255(14542)	0.328
	<i>S132P</i>	N/A ( <i>INFO</i> score <0.6) imputed	0.0165 (14542)	N/A
	<i>G202*V</i>	0.0112 (178)	0.0155 (14542)	0.665
SAPHO	<i>R146Q</i>	0.0227 (22)	0.0052 (2,504)	0.960
	<i>S132P</i>	0.0227 (22)	0.0088 (2,504)	0.327
	<i>G202*V</i>	N/A	0.0102 (2,504)	N/A

*n* - number of unrelated individuals sequenced

MAF - Minor Allele Frequency-count

N/A - not available

*p* = chi-square test (with Yates' continuity correction) by comparing the allele counts in cases and controls

