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1 **Zeb1 represses TCR signaling, promotes proliferation in T cell progenitors and**
2 **is essential for NK1.1⁺ T cell development**

3

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33 **Abstract**

34 T cell development proceeds under the influence of a network of transcription factors
35 (TFs). The precise role of Zeb1, a member of this network remains unclear. Here, we
36 report that Zeb1 expression is induced early on during T cell development at the
37 Cd4-Cd8- double negative (DN) stage 2. Zeb1 expression further augments at the
38 Cd4+Cd8+ double positive (DP) stage before decreasing in more mature subsets.
39 We performed an exhaustive characterization of T cells in *Cellophane* mice that bear
40 *Zeb1* hypomorphic mutations. The *Zeb1* mutation, profoundly affected all thymic
41 subsets, specially DN2 and DP cells. Zeb1 promoted survival and proliferation in both
42 populations in a cell-intrinsic manner. In the periphery of *Cellophane* mice, the
43 number of conventional T cells was near normal, but invariant iNKT cells, NK1.1⁺ γδ
44 T cells and Ly49⁺ Cd8 T cells were virtually absent. This suggested that Zeb1
45 regulates the development of unconventional T cells from DP progenitors. A
46 transcriptomic analysis of WT and *Cellophane* DP revealed that Zeb1 regulated the
47 expression of multiple genes involved in cell cycle and TCR signaling, possibly in
48 cooperation with Tcf1 and Heb. Indeed, *Cellophane* DP displayed stronger signaling
49 than WT DP upon TCR engagement in terms of calcium response, phosphorylation
50 events and expression of early genes. Thus, Zeb1 is a key regulator of cell cycle and
51 TCR signaling during thymic T cell development. We propose that thymocyte
52 selection is perturbed in Zeb1-mutated mice, in a way that does not allow the survival
53 of unconventional T cell subsets.

54 **Introduction**

55 T cell development occurs in the thymus and starts from immature thymocytes that
56 are double negative (DN) for Cd4 and Cd8 expression. The DN population can be
57 subdivided into four subsets, DN1-DN4, depending on the expression of the cell
58 surface molecules Cd44 and Cd25 (for a review, see [1]). DN1 cells (Cd44⁺Cd25⁻)
59 are the most immature progenitors and retain the ability to differentiate into non-T cell
60 lineages. In DN2 cells (Cd44⁺Cd25⁺), the expression of RAG1/2 is induced, which
61 promotes the rearrangements of gene segments encoding for TCR- β , TCR- γ , and
62 TCR- δ subunits. In DN3 cells (Cd44⁻Cd25⁺) the TCR β -chain associates with the pre-
63 TCR α -chain and Cd3 subunits to form the pre-TCR complex; the pre-TCR allows β -
64 selection to occur. During β -selection, DN3 cells with productive TCR β
65 rearrangements receive survival and proliferative signals and mature into the DN4
66 (Cd44⁻Cd25⁻) stage. DN4 thymocytes then develop into Cd4⁺Cd8⁺ double-positive
67 (DP) stages [2].

68 At the DP stage, a succession of events takes place and determines the fate
69 of developing T cells; including rearrangement of the T cell antigen receptor (TCR)
70 alpha locus, association of the $\alpha\beta$ T cell receptor, and subsequent thymic selection.
71 In general, high affinity interactions between the $\alpha\beta$ TCR and self-peptide-MHC
72 complexes (pMHC) presented by different thymic cells lead to negative selection and
73 elimination of self-reactive thymocytes while low affinity interactions result in positive
74 selection and development of Cd4 or Cd8 single positive (SP) T cells [3–5]. In spite
75 of this general rule, regulatory T cells (Tregs) and invariant NKT cells (iNKT) receive
76 stronger TCR signals than conventional T cells during their development [6], as a
77 result of selection by agonist self-antigens. iNKT cells are a subset of innate like T
78 cells with a single invariant TCR α chain (V α 14-J α 18 in mice) and a limited repertoire
79 of TCR β chains (V β 8.2, V β 7, or V β 2) that recognize glycolipid antigens bound to
80 Cd1d, a non-polymorphic MHC molecule [7]. Their development includes discrete
81 stages (stages 0-3) that can be discriminated by Cd44 and NK1.1 expression [8].
82 Three functionally distinct iNKT cell subsets have also been identified; iNKT1 cells
83 express T-bet and mainly secrete IFN- γ ; iNKT2 cells express Gata3 and Plzf and
84 secrete IL-4 and IL-13; iNKT17 express Ror γ t and secrete IL-17. The TCR signal
85 strength during selection governs the development of iNKT cell subsets, with strong
86 signals promoting iNKT2 and iNKT17 development [9,10]. A large number of
87 molecules regulate the strength of the TCR-derived signaling cascade. TCR signal

88 strength can also be modulated at the transcriptional level by transcription factors
89 (TFs) such as Sox4 [11], or at the post-transcriptional level by miR-181[12,13]. The
90 loss of either blocks iNKT cell development. Mechanistically, miR-181a regulates the
91 expression of multiple phosphatases and other proteins to boost TCR signaling as
92 well as cell metabolism [12,13]. Interestingly, mice expressing a hypomorphic form of
93 Zap70, a major TCR-proximal kinase, also have impaired developmental maturation
94 of $\gamma\delta$ T cells, suggesting that innate-like T cell subsets are particularly dependent on
95 a tight regulation of TCR signal strength for their development [14].

96 A dense network of TFs have been shown to regulate T cell development [15].
97 Early commitment is dependent on Notch signals [16], which induce many TFs and
98 maintain their expression throughout T cell development. Among those factors, the E
99 protein family factors E2a, Tcf1 (encoded by *Tcf7*) and Heb (encoded by *Tcf12*) [17]
100 induce the expression of TCR components and balance the survival and proliferation
101 of thymocytes [18]. Many other TF such as Gata3, Myb, Runx1, and Bcl11b also
102 cooperate with E proteins at different developmental stages and further establish T
103 cell identity [15,18].

104 Zeb family of TFs consist of Zeb1 and Zeb2. They are best known for their role
105 in epithelial-to-mesenchymal transition (EMT). EMT programs operate at different
106 stages of embryonic development and are downstream of Wnt, TGF- β , Bmp, Notch,
107 and other signaling pathways [19]. *Zeb1*^{-/-} mice exhibit multiple developmental
108 defects and die at birth [20]. In pathological settings, activation of EMT programs
109 contributes to fibrosis and cancer metastases [21]. Zeb1 and Zeb2 are highly
110 homologous and are characterized by two clusters of zinc fingers expressed on the
111 protein extremities. They also contain a homeodomain, a Smad-binding domain and
112 can interact with many other TFs [22]. Zeb1 and Zeb2 are also expressed in a tightly
113 regulated manner in the immune system and regulate cell differentiation [23]. We and
114 others have previously shown that Zeb2 regulated terminal NK cell [24] and effector
115 Cd8 T cell differentiation [25,26]. Mutated mice expressing a truncated form of Zeb1,
116 that removes the C-terminal zinc finger clusters at C727, have a small and
117 hypocellular thymus caused by a reduction in early T-cell precursors [27]. In
118 *Cellophane* mutant mice, a T \rightarrow A mutation in the seventh exon of *Zeb1* replaces the
119 tyrosine at position 902 with a premature stop codon [28]. The resulting mRNA
120 encodes a truncated protein lacking the C-terminal zinc finger domain, which is

121 predicted to be hypomorphic. *Cellophane* homozygote mice have small hypocellular
122 thymi with fewer DP thymocytes. However, the mechanism of Zeb1 action during T
123 cell development, and its role in mature T cell subsets remain unclear. Here, we
124 show that *Cellophane* homozygous mice virtually lack several peripheral T cell
125 subsets including iNKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49 expressing Cd8 T cells. This
126 specific defect in innate-like T cells is caused by a cell-intrinsic role of Zeb1 in T cell
127 development. We show that Zeb1 expression is maximal at the DN2 and DP stages
128 of T cell development. Furthermore Zeb1 regulates the transition to the SP stage by
129 promoting cell proliferation, survival and repressing the expression of various
130 molecules involved in the strength of TCR signaling. Therefore, we propose that
131 Zeb1 is a key regulator of thymocyte selection, essential for the development and
132 survival of innate-like T cell subsets undergoing agonist-type selection.

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136

137 **Results**

138

139 **Zeb1 is highly expressed at the DN2 and DP stages of T cell development**

140 To study the role of Zeb1 in T cell development, we sorted thymocyte subsets and
141 measured Zeb1 transcript levels by semi-quantitative (Q) RT-PCR (reverse
142 transcription polymerase chain reaction). As shown in Figure 1A, Zeb1 transcript
143 levels were low in DN1, started to increase at the DN2 stage and were maximal in
144 DP thymocytes. This expression then decreased as T cells underwent selection and
145 matured into either conventional T cells, or iNKT cells. Interestingly, the expression of
146 Zeb2 was somewhat reciprocal to that of Zeb1, with high expression in early thymic
147 progenitors (DN1 to DN4) and lowest expression in DP (Figure 1B). This pattern of
148 expression was matched by data from the Immgen consortium [29] (Figure S1A).
149 Thus, as seen for memory T cells [30], Zeb1 and Zeb2 have reciprocal patterns of
150 expression in thymocytes. We used the Immgen web browser to search for co-
151 regulated genes across different immune subsets. The E-protein Heb (encoded by
152 *Tcf12*) was in the top 3 genes found to be co-regulated with Zeb1 (Figure S1B) [29].
153 Heb is well known for its important role throughout T cell development [31], and
154 especially at the DP stage[32], further pointing to Zeb1 as a potential regulator of this
155 developmental stage. We also analyzed the expression of Zeb1 protein in total
156 thymocytes (80% of which are composed of DP). Zeb1 was strongly expressed in
157 WT but not *Cellophane* thymocytes. Mutant mice only expressed reduced quantities
158 of a truncated form of Zeb1 (Figure 1C).

159

160 **Impaired development of both conventional and unconventional T cells in** 161 ***Cellophane* mice**

162 To define the impact of the *Cellophane* mutation on T cell development, we analyzed
163 the T cell composition in the thymus, spleen and lymph nodes of *Cellophane* mice.
164 The cell numbers in spleen, and liver were normal while the number of lymphocytes
165 was reduced in lymph nodes (LN) (Figure 2A). As shown by previous observations
166 [28] we also observed a strong decrease in cell numbers from thymus in *Zeb1*-
167 mutated mice (Figure 2A). This decreased number affected all subsets defined by
168 Cd4, Cd8, Cd44 and Cd25 expression (Figure 2B-C). The Cd4⁺Cd8⁺ DP thymocytes
169 and DN2 populations also decreased in frequency within *Cellophane* thymocytes

170 (Figure 2B-C). In the LN and spleen, the percentage of Cd4 T cells, and Cd8 T cells
171 were reduced (Figure 2D-E) and the proportion of memory-phenotype Cd44⁺ T cells
172 from Cd8 T cells was decreased by nearly 30% (Figure 2F).

173 We then investigated the development of unconventional T cell subsets. We
174 observed a drastic decrease in the frequency and in the number of iNKT cells as well
175 as NK1.1⁺ $\gamma\delta$ T cells in *Cellophane* mice compared to littermate controls (Figure 3A-
176 D). This decrease affected all organs from *Cellophane* mice (Figure 3A-D). iNKT cells
177 were mainly affected at stage 3 (Figure 3E). To complete our analysis, we also
178 studied memory-phenotype Ly49⁺ Cd8 T cells which are thought to arise “naturally” in
179 the thymus, without antigenic experience [33]. All Ly49⁺ Cd8 T cell populations were
180 also strongly decreased in LN and spleen from *Cellophane* mice, both in terms of
181 frequencies and numbers, and irrespective of the inhibitory Ly49 receptor analyzed
182 (Ly49A, Ly49F or Ly49G2) (Figure 3F-G).

183 Altogether, these data confirm the important role of Zeb1 in early T cell
184 development. We also demonstrate an essential and specific role of Zeb1 in the
185 development of peripheral T cell subsets expressing NK cell markers such as iNKT
186 cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49⁺ Cd8 T cells.

187

188 **Cell intrinsic role of Zeb1 in thymic progenitors and T cell development**

189 Zeb1 is also required for the development and expression of non-hematopoietic
190 tissues and cell types [20]. To test if Zeb1 had an intrinsic role in T cell development,
191 we generated chimeric mice by reconstituting sub-lethally irradiated Ly5a (Cd45.1)
192 mice with the BM from *Cellophane* (Cd45.2) or “WT” Ly5a x C57BL/6 (Cd45.1/2)
193 mice. In the thymus of chimeric mice, the frequency of DN2 and DP was strongly
194 decreased for *Cellophane* BM-reconstituted mice compared to WT BM-reconstituted
195 mice, while the proportion of other populations defined by Cd4 and Cd8 was
196 increased (Figure 4A). iNKT cells and Ly49⁺ T cell subsets were also strongly
197 reduced in the peripheral organs of *Cellophane*→Ly5a BM chimeric mice compared
198 to WT→Ly5a chimera indicating that *Zeb1* regulated T cell development intrinsically
199 (Figure 4B-C). Cell numbers for all thymic T cell subsets and for peripheral iNKT cells
200 and Ly49⁺ T cells were decreased in *Cellophane*→Ly5a BM chimeric mice compared
201 to WT→Ly5a chimera (Figure S2). NK1.1⁺ $\gamma\delta$ T cells were not analyzed because
202 many of them are derived from fetal precursors [34], and are not reconstituted in BM

203 chimera mice. To further test the role of Zeb1 in the environment of developing T
204 cells, we also made different BM chimeric mice using WT and *Cellophane* mice as
205 both recipients and BM donors (WT→WT, WT→*Cellophane*, *Cellophane*→WT and
206 *Cellophane*→*Cellophane*). As shown in Figure S3A-B, the frequencies and numbers
207 of DN and DP subsets were determined by the genotype of the BM donor and not by
208 that of the host genotype. Similar conclusions could be reached upon examination of
209 the frequency and number of NKT cells in the thymus and in the liver (Figure S3C).

210 We then generated mixed BM chimera by reconstituting lethally irradiated
211 Ly5a mice with a 1:1 mixture of BM from *Cellophane* and Ly5a x C57BL/6 (WT) mice.
212 *Cellophane* T cell progenitors had a poor competitive fitness in BM chimeric mice
213 (Figure 4D). Indeed, the percentage of cells originating from the *Cellophane* BM
214 progenitors was already low in DN and further decreased when transitioning between
215 DN and DP stages (Figure 4D). In the periphery of mixed BM chimeric mice, we
216 found that the frequency of iNKT and Ly49⁺ Cd8 T cells was strongly reduced
217 amongst *Cellophane* compared to WT lymphocytes (Figure 4E-F), thus revealing the
218 role of Zeb1 in T cell development is cell-intrinsic, and not due to a defective stromal
219 environment. Of note, we also analyzed the reconstitution of myeloid cells as control.
220 In the spleen, on average 20% of macrophages, 25% of dendritic cells and 28% of
221 neutrophils were of *Cellophane* origin (Figure S3D), suggesting that Zeb1 regulated
222 the development of all hematopoietic subsets, perhaps by regulating multipotent
223 progenitors. However, the most important effects were observed for thymocytes and
224 peripheral T cell subsets expressing NK cell markers.

225

226 **Reduced survival and proliferation of *Cellophane* DN2 and DP cells**

227 The decreased cellularity of the *Cellophane* thymi could be due to a reduced
228 proliferation or increased apoptosis of thymocytes. To address this point, we first
229 compared the survival of WT and *Cellophane* thymocytes upon *ex vivo* culture. We
230 found that *Cellophane* DN2, DN3 and DN4 had a reduced *ex vivo* viability compared
231 to their WT counterparts (Figure 5A). Moreover, after 24 or 48 h in culture,
232 *Cellophane* DN2, DN3, DN4 and DP were also less viable than controls (Figure 5A).

233 Next, we compared *in vivo* proliferation of WT and *Cellophane* thymocytes, as
234 measured by EdU incorporation. *Cellophane* DN2 and DP proliferated less than their
235 WT counterpart, while *Cellophane* proliferated more than WT DN3 (Figure 5B). Ki67

236 staining corroborated our data obtained with EdU incorporation (Figure 5C-D). As all
237 DP cells were Ki67 positive, we reported only changes in the MFI (Figure 5D).

238 Thus, the *Cellophane* mutation affects both the survival and proliferation of
239 developing DN2 and DP thymocytes, which could account for the decreased number
240 of cells in the *Cellophane* thymi.

241

242 **Zeb1 tunes TCR signal strength**

243 To gain insight in the mechanism of Zeb1 function, we focused on DP as they
244 expressed the highest level of Zeb1 among thymocytes (Figure 1A). We first
245 compared the expression of membrane proteins involved in thymocyte selection in
246 *Cellophane* vs WT DP cells by flow cytometry. *Cellophane* DP expressed higher
247 levels of Cd69, Cd25, and Cd5 compared to WT thymocytes (Figure 6A). Nur77 is
248 an early response gene expressed in T cells within hours after TCR stimulation. We
249 observed an increase in the intracellular expression for Nur77 which correlated to
250 TCR β levels in *Cellophane* compared to WT DP thymocytes (Figure 6A). Of note
251 similar levels of Cd4 and Cd8 were measured (Figure 6A).

252 Next, we focused our attention on thymic iNKT cell subsets. The mouse
253 thymus is known to contain at least three iNKT subsets, ie iNKT1, iNKT2 and iNKT17
254 that are thought to have distinct roles in the immune response [35]. NKT1 cells
255 correspond mainly to stage 3 NKT cells. TCR signal strength governs the
256 development of iNKT cell subsets in the thymus, in which high signal strength is
257 necessary for iNKT2 and iNKT17 development [9,10]. We examined iNKT cell
258 subsets by staining for Plzf and Ror γ t [36] in *Cellophane* mice vs WT mice. Results in
259 Figure 6B show a significant increase in the representation of iNKT2 and iNKT17 and
260 a decrease in that of iNKT1 in *Cellophane* compared to control mice. These data
261 evocated an increase in the TCR signal from DP progenitors of iNKT cells in
262 *Cellophane* mice. The change in iNKT subsets was associated with subtle changes
263 in the TCR repertoire as assessed by measuring the frequency of V β 8, V β 7 and V β 2
264 positive cells amongst iNKT cells of each genotype. We observed a 2-fold increase in
265 V β 7 usage in *Cellophane* mice (Figure 6C). This could reflect the increase in iNKT2
266 cells as a previous article showed that V β 7 was more often associated with iNKT2
267 cells [9]. Thymic *Cellophane* iNKT cells expressed normal levels of transcription
268 factors T-bet and Egr2 but strongly reduced levels of Cd4 (Figure 6D). Since Cd4 is

269 known to sustain TCR signal strength [5], the selection of Cd4^{low} iNKT cells in
270 *Cellophane* mice could reflect an adaptation to overt TCR signaling in *Cellophane*
271 DP .

272 We then analyzed specifically TCR signaling in developing thymocytes. We
273 started by measuring the phosphorylation (p) level of a series of signaling proteins
274 involved in TCR-mediated activation, either at steady-state in freshly isolated
275 thymocytes, or following TCR engagement by crosslinking anti-Cd3 antibodies. To
276 minimize the experimental variation, we used a barcoding strategy that allowed
277 stimulating and then staining WT and *Cellophane* thymocytes simultaneously (see
278 methods). Results in Figure 6E-F showed an increase in pAkt (Ser473), ribosomal
279 protein pS6, and to a lesser extent of pErk, either at steady state or following TCR
280 engagement in *Cellophane* DP compared to WT DP. Thus, the Mapk and PI3K/Akt
281 pathways are more active in thymocytes undergoing selection in *Cellophane*
282 compared to control mice. To complement this analysis, we also assessed the
283 calcium response of DP from both genotypes in response to TCR engagement. As
284 shown in Figure 6G-H, this response was stronger for *Cellophane* DP compared to
285 control DP both in terms of intensity (peak) and duration (area under the curve, AUC).
286 Thus, Zeb1 tunes signal strength downstream TCR engagement at the DP stage and
287 *Cellophane* DP have increased TCR signaling. The latter may increase negative
288 selection and therefore account for defective T cell development in *Cellophane* mice.
289 We also measured preTCR signaling in DN cells following stimulation with anti-CD3
290 antibodies. This analysis did not reveal any difference between WT and *Cellophane*
291 DN cells (data not shown).

292

293 **Zeb1 broadly shapes transcription at the transition DP→SP to promote** 294 **proliferation and repress TCR signaling**

295 To further uncover the mechanisms of Zeb1 function during T cell development, we
296 performed RNA-seq to compare WT and *Cellophane* DP transcriptomes. We found
297 538 differentially expressed genes (DEGs, p-value<0.05, Log2 Fold change>1). 204
298 genes were increased and 334 were down regulated in *Cellophane* DP compared to
299 WT DP. These data reveal that Zeb1 broadly shapes the genetic program of
300 developing thymocytes (Figure 7A and Table S1). For some of the DEGs identified,
301 antibodies were available, and we were thus able to confirm higher expression of
302 Foxo1, Ms4a4b, Itgb7, Ccr7 and Ccr4 in *Cellophane* compared to control DP cells

303 and reciprocally we observed lower Cd81 expression in *Cellophane* DP vs WT
304 (Figure 7B).

305 Next, we queried the Immgen database to retrieve the expression profile of the
306 Zeb1-regulated geneset (induced or repressed) across all thymocyte subsets.
307 Interestingly, genes down regulated in *Cellophane* DP (ie normally induced by Zeb1)
308 correspond to genes that are normally expressed at high levels in early T cell
309 progenitors and low levels in mature T cells (Figure 7C). Their expression level
310 normally drops at the DP to SP transition, at the time when Zeb1 is highly expressed.
311 Genes up regulated in *Cellophane* DP correspond to genes that follow the reciprocal
312 pattern of expression (Figure 7C). This pattern of expression also correlates with cell
313 proliferation and TCR responsiveness in thymocytes. Indeed, irrespective of mouse
314 genotype, SP T cells are much more responsive to TCR signaling mediated calcium
315 responses than DP, but reciprocally do not cycle as much as DP (data not shown).
316 This suggests that Zeb1 promotes cell proliferation and represses TCR signaling
317 specifically at the DP stage, presumably to ensure proper selection.

318 A functional annotation analysis of DEGs in our RNAseq analysis using
319 “Metascape” [37] highlighted the cell cycle as the most down regulated biological
320 process in *Cellophane* DP compared to control DP (Figure 7D, and Table S2),
321 confirming findings in Figure 3. Pathways linked to $\text{I-fn}\gamma$ (but also type I-Ifn, Table S2),
322 antigen presentation, leukocyte differentiation and apoptosis were significantly
323 associated with genes up regulated in *Cellophane* DP compared to WT (Figure 7D).
324 Of note, a modest but significant enrichment for genes involved in the calcium
325 response was also associated with these genes, corroborating our data in Figure 6.
326 To further annotate this dataset, we also performed individual Pubmed searches,
327 looking for connections between genes up regulated in *Cellophane* DP compared to
328 control DP and “T cell activation” or “TCR signaling” or “T cell development”.
329 Interestingly this analysis showed more than 25% of the genes in the list had a
330 known role in T cell activation or TCR signaling; and 10% had a role in T cell
331 development, as defined using loss-of-function mouse strains (Table S3). Moreover,
332 we used the STRING database of physical and functional protein interactions [38] to
333 further annotate genes up or down regulated in *Cellophane* DP compared to control
334 DP. In particular, we used the Pubmed module that searches for enrichment for gene
335 lists in articles in Pubmed. This unbiased analysis showed that genes up regulated in
336 *Cellophane* DP were significantly enriched for genes involved in negative selection

337 [39] or T cell maturation regulated by Bcl11b [40] (Table S4), which corroborated with
338 our manual PubMed searches.

339

340 **Chromatin regions remodeled at the DP stage contain Zeb1 binding motifs**

341 Next, we wanted to determine if Zeb1 could regulate chromatin remodeling at the DP
342 stage of T cell development. For this, we took advantage of a recently published
343 large-scale analysis of chromatin accessibility and gene expression across 86
344 immune subsets including T cell developmental stages in the thymus [41]. In this
345 study, *in silico* predictions pointed to Zeb1 as one of the few TF whose expression
346 correlated with modifications of chromatin accessibility during thymic T cell
347 development, and for which the corresponding chromatin regions contained sites
348 predicted to be bound by Zeb1. Other TFs in this category included Gata3, Tcf7, Lef1,
349 Tcf12 and Zfp740 (Figure S4A), and especially Tcf7 and Tcf12 whose role in T cell
350 development has been well established [32]. We retrieved open chromatin regions
351 (OCRs) for which Zeb1 motifs were discovered in this study and whose accessibility
352 changed during T cell development (see corresponding clusters in Figure S4B) and
353 compared the list of corresponding genes with DEGs identified in our own study
354 between *Cellophane* and WT DP. We found an important overlap between both lists
355 that included many of the genes previously highlighted in our analysis (Figure 7E, p-
356 value=1.839413e-46). Altogether, these data suggest that Zeb1 is a direct
357 transcriptional regulator of T cell development, especially operating at the DP to SP
358 transition to promote proliferation and ensure proper selection.

359

360 **Discussion**

361 Here, we demonstrated that Zeb1 is essential for the transition through the DN2 and
362 DP stages of T cell development as well as for the differentiation of iNKT cells,
363 NK1.1⁺ $\gamma\delta$ T cells and Ly49⁺ Cd8 T cells. Mechanistically, Zeb1 regulates the
364 expression of a number of genes that were notably involved in cell proliferation or in
365 TCR signaling at the DP stage. In *Cellophane* mice, these events may perturb thymic
366 development and selection in a way that does not allow the production of NK1.1⁺ and
367 Ly49⁺ T cell subsets.

368 Zeb1 expression was found to increase at the DN2 stage and to be maximal at
369 the DP stage of T cell development. Accordingly, we found a decrease in frequency
370 of DN2 and DP thymocytes in *Cellophane* mice. This could be accounted for by a
371 cell-intrinsic role for Zeb1 in DN2 and DP proliferation. A number of genes involved in
372 the cell cycle were differentially expressed between WT and *Cellophane* DP as
373 revealed by our RNAseq analysis. There is also a strong link in the literature between
374 Zeb1 and cell proliferation in cancer. In particular, Zeb1 interacts with many TFs
375 involved in the regulation of cell growth such as Smad TFs which is downstream of
376 several growth factor pathways [22]. Moreover, Zeb1 is known to repress cyclin-
377 dependent kinases during EMT [21]. However, Cdkn2c and Cdkn3 were decreased
378 by Zeb1 in DP thymocytes, suggesting differential roles of Zeb1 in epithelial versus
379 lymphoid cells. The decreased proliferation of DN2 and DP is expected to have
380 important consequences on the general thymic output. Indeed, we found decreased
381 numbers of peripheral T cells in *Cellophane* mice. However, this defect was much
382 more pronounced for iNKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49⁺ Cd8 T cells. In
383 particular, iNKT cells were virtually absent from the periphery. This altered
384 development was associated with an increased TCR signaling at the DP stage, which
385 was verified by increased basal levels of Cd5 and Nur77 but also increased mTOR
386 activity and calcium flux upon Cd3 engagement. iNKT cells are known to receive
387 stronger TCR signals than conventional T cells during their development [6]. Thus,
388 increased TCR signaling in *Cellophane* DP could trigger cell death i.e. via negative
389 selection of iNKT precursors. The increased negative selection of iNKT cells has
390 already been shown to occur in mice in which transgenic TCR- β chain conferred high
391 affinity for self-lipid/Cd1d complexes when randomly paired with V α 14-J α 18
392 rearrangements [42]. Thus, increased negative selection could impair the
393 development of iNKT cells in *Cellophane* mice, and perhaps that of other T cell

394 subsets expressing NK cell markers. Indeed, strong TCR-mediated signals are also
395 important for $\gamma\delta$ T cell development [43,44]. In particular, NK1.1⁺ $\gamma\delta$ T cells have an
396 oligoclonal TCR repertoire and accumulate in mouse models of decreased TCR
397 signaling [45], suggesting that this subset of $\gamma\delta$ cells can also be negatively selected.
398 The ontogeny of Ly49⁺ Cd8 T cells is not very well known, but our data suggest that
399 their development and selection could share common mechanisms with that of
400 NK1.1⁺ T cells. How does Zeb1 regulate TCR signal strength? *Cellophane* DP
401 thymocytes expressed higher levels of TCR β than control DP, and this could certainly
402 lead to higher TCR signaling. Moreover, the RNAseq analysis we performed
403 suggested multiple connections between Zeb1 and signal transduction through the
404 TCR. For example, multiple members of the GTPases of the IMMunityAssociated
405 Proteins (GIMAP) family (GIMAP5 and GIMAP8) were up regulated in *Cellophane*
406 DP. Interestingly, Gimap5 enhances calcium influx following TCR stimulation [46].
407 Several members of the family of four-pass membrane receptors Ms4a are also up
408 regulated in *Cellophane* DP and could reinforce TCR signaling. Indeed, transduction
409 of Ms4a4b into naive T cells can heighten their sensitivity to antigen through a
410 process that could involve association with costimulatory molecules [47]. Several
411 phosphatases and kinases are also deregulated in *Cellophane* DP and could
412 perhaps alter TCR signaling. In particular the expression of Pyk2 (encoded by *Ptk2b*)
413 or that of Rasgrp4, Rasl1 and Rasa3 could all contribute to higher TCR signaling via
414 the calcium flux or the Mapk pathway.

415 A series of TF were also deregulated in *Cellophane* DP, with a notable up
416 regulation of JunB, Jun, Atf6, Foxo1, Stat4 or Irf7/9. JunB and Jun are essential
417 components of AP-1 TFs and are typically induced downstream of TCR stimulation.
418 Similar to Nur77, they could represent surrogate markers of increased TCR signaling
419 in *Cellophane* DP. The derepression of Foxo1 could in part account for altered T cell
420 development as it regulates Ccr7, Cd62L and S1pr1 via Klf2 [48]. The lack of Klf2
421 control could perturb, perhaps accelerate, the normal migration of developing
422 thymocytes in the medullar region where negative selection occurs. Foxo1 deletion in
423 thymocytes was reported to decrease the number of DP thymocytes, and Foxo1
424 deficient peripheral T cells seem to be refractory to TCR stimulation through
425 unknown mechanisms [49]. Moreover, up regulation of Foxo1 in *Cellophane* DP
426 could in part explain the opposite changes in cell proliferation observed in these cells

427 compared to control DP, since Foxo TFs are known to promote stem cell quiescence
428 [50] and clearly contribute to the regulation of cell division, survival and metabolism in
429 T cells [51]. A recent study showed that the transcriptional repressor Gfi1 is important
430 to maintain Foxo1 expression at low levels in DP thymocytes [52]. In the absence of
431 Gfi1, there was premature expression of genes normally expressed in mature T cells
432 and accelerated maturation of DP to SP thymocytes, largely attributable to Foxo1 de-
433 repression. Zeb1 and Gfi1 could therefore cooperate to repress Foxo1.

434 There are many similarities in the phenotype of *Tcf12* deficient [53] and
435 *Cellophane* mutant mice, in particular susceptibility of DP to cell death and impaired
436 development of iNKT cells. Moreover, microarray data from the Immgen consortium
437 suggest that *Tcf12* and *Zeb1* are strongly co-regulated and ATAC-seq data predict
438 that they control chromatin accessibility during thymic T cell development together
439 with *Gata3*, *Tcf1*, *Lef1* and *Zfp740* (Figure S4 and [41]). Altogether these data
440 suggest a strong functional link between *Zeb1* and *Heb* (encoded by *Tcf12*), and
441 perhaps *Tcf1*, which acts in coordination with *Heb* [32]. The fact that *Zeb1* is known
442 to bind tandem E-box motifs, suggests a possible competition between *Zeb* members
443 and E-proteins for those genes regulated by tandem E-boxes. Such a competition
444 has been previously established at least in the context of the CD4 enhancer which is
445 repressed by *Zeb1*, through a competition with *Heb* for E-box binding [54]. Moreover,
446 *Zfh-1* and *Daughterless*, the drosophila homologs of *Zeb1* and *Tcf12*, are also known
447 to compete for the same genomic sites [55]. The deletion of *Tcf12* and *Tcf1* in
448 thymocytes has the opposite phenotype as *Cellophane Zeb1* mutation in terms of DP
449 proliferation [32]. This suggests that *Heb* and *Zeb1* could have partially antagonistic
450 activities in the regulation of genes bearing tandem E-box elements. A competition
451 between *Zeb1* and E-proteins has already been suggested in the control of *GATA3*
452 expression in human CD4 T cells [56]. *Cellophane* mice express a truncated form of
453 *Zeb1* that is also expressed at lower levels than WT *Zeb1*. As the phenotype of these
454 mice is milder than that of *Zeb1*^{-/-} mice, we assumed that the *Cellophane* mutation
455 was hypomorphic. However, we cannot exclude that *Cellophane Zeb1* can retain
456 some DNA binding and therefore act as a dominant negative molecule by preventing
457 binding of E-box proteins. Further work will be needed to precisely map the
458 interactions between *Zeb* TFs and E proteins. This regulatory network may also
459 include inhibitor of differentiation genes *Id2* and *Id3*, as these TFs bind and inactivate
460 E-proteins, thereby regulating their function. Moreover, the deficiency in *Id3* has the

461 same impact on NK1.1+ $\gamma\delta$ T cells as a deficiency in TCR signaling [45], which links
462 both events.

463 *Zeb1* genomic region is frequently deleted in cutaneous T cell lymphomas
464 (CTCL) [57]. These deletions are often associated with genetic mutations in
465 components of the TCR signaling machinery (recurrent alterations in *Card11*, *Plcg1*,
466 *Lat*, *Rac2*, *Prkcq*, *Cd28* and genes that encode calcium channel subunits). This
467 observation, together with our own data showing a role for *Zeb1* in repressing TCR
468 signaling, suggest that *Zeb1* deletion could promote lymphomagenesis by releasing
469 TCR signaling natural brakes. Of note, a previous study proposed an essential role of
470 IL-15 in CTCL development, and showed that IL-15 expression was depressed in
471 patients with CTCL due to promoter hypermethylation and in the failure of *Zeb1* to
472 have access to and repress the IL-15 regulatory region [58]. However, IL-15
473 expression was not detected in developing thymocytes in our RNAseq analysis,
474 excluding the possibility that the IL-15 pathway could play a role in the *Zeb1*
475 mechanism of action in T cell development. Yet, we found that the transcriptional
476 response to different cytokines such as interferons or IL-6 was increased in
477 *Cellophane* DP (table S2). *Zeb1* may therefore normally repress the response to
478 these cytokines, presumably to ensure proper selection. TGF- β is a known regulator
479 of iNKT cell development [59] and that it promotes early differentiation and prevents
480 apoptosis of developing iNKT cells. A recent study showed that *Zeb1* was induced by
481 TGF- β in conventional Cd8 T cells stimulated through the TCR and was essential for
482 memory T cell survival and function [30]. Although we failed to detect any effect of
483 recombinant TGF- β on *Zeb1* expression in thymocytes (data not shown), it would be
484 interesting to address this question *in vivo* using appropriate genetic models.

485 In summary, *Zeb1* is an essential member of the TF network that regulates T
486 cell development and selection at the DN2 and DP stages. Furthermore, we have
487 also shown that *Zeb1* allows the development of iNKT cells and other T cell subsets
488 expressing NK cell markers by regulation of cell cycle and TCR signaling in
489 developing thymocytes.

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497 **Material and Methods**

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499 **Mice**

500 Mice at 8 to 24 weeks were used. Wild-type C57BL/6 mice were purchased from
501 Charles River Laboratories (L'Arbresle). *Cellophane* mice were previously described
502 [28], littermate mice were used as controls. This study was carried out in accordance
503 with the French recommendations in the Guide for the ethical evaluation of
504 experiments using laboratory animals and the European guidelines 86/609/CEE. All
505 experimental studies were approved by the bio-ethic local committee CECCAPP.
506 Mice were bred in the Plateau de Biologie Expérimentale de la Souris (ENS, Lyon).

507

508 **Bone marrow chimeric mice**

509 8- to 10-week-old Ly5a mice were anesthetized with ketamine/xylazine before
510 irradiation at 9 Gray with a X-ray irradiator XRAD-320. After irradiation, they were
511 intravenously injected with $2-5 \times 10^6$ cells collected from either wild type or mutant
512 murine bone marrow, or a mix of both (as indicated in the figures). Immune cell
513 reconstitution was analyzed 8 weeks post BM injection.

514

515 **Flow cytometry**

516 Single-cell suspensions of the thymus, spleen and liver were used for flow cytometry.
517 Cell viability was measured using Annexin-V (BD Biosciences)/live-dead fixable
518 (eBiosciences) staining. Intracellular stainings for TFs were performed using Foxp3
519 kit (ebioscience). Lyse/Fix and PermIII buffers (BD Biosciences) were used for
520 intracellular staining of phosphorylated proteins. Flow cytometry was carried out on a
521 FACS Canto, a FACS LSR II, or a FACS Fortessa (Becton-Dickinson). Data were
522 analysed using FlowJo (Treestar). Antibodies were purchased from eBioscience, BD
523 biosciences, R&D Systems, Beckman-Coulter, Miltenyi or Biolegend. We used the
524 following antibodies: anti-mouse Cd3 (clone 145-2C11), anti-mouse Cd4 (clone
525 GK1.5), anti-mouse Cd8 (clone 53-6.7), anti-mouse TCR β (clone H57-597), anti-
526 mouse Cd69 (clone H1.2F3), anti-mouse TCR $\gamma\delta$ (clone GL3), anti-mouse NK1.1
527 (clone PK136), anti-mouse Cd24 (clone M1/69), anti-mouse Cd44 (clone IM7), anti-
528 mouse Cd27 (clone LG.7F9), anti-mouse TCRV β 2 (clone B20.6), anti-mouse
529 TCRV β 7 (clone TR310), anti-mouse TCRV β 8.1/8.2 (clone KJ16-133), anti-mouse

530 Ly49A (clone A1), anti-mouse Ly49E/F (clone REA218), anti-mouse Ly49G2 (clone
531 4D11), anti-mouse Cd45.1 (clone A20), anti-mouse Cd45.2 (clone 104), anti-mouse
532 Nur77 (clone 12.14), anti-mouse Ccr7 (clone 4B12), anti-mouse Cd5 (clone 53-7.3),
533 anti-mouse Cd81 (clone Eat-2), anti-mouse Cd53 (clone OX79), anti-mouse Lpam-1
534 (clone DATK-32), anti-mouse Foxo1 (clone C29H4), anti-mouse Ms4a4b (clone
535 444008), anti-mouse Cd74 (clone In1/Cd74), anti-mouse Tbet (clone 4B10), anti-
536 mouse Egr2 (clone erongr2), anti-mouse Plzf (clone Mags.21F7), anti-mouse Ror γ
537 (clone AFKJS-9), anti-mouse pErk (clone 20A), anti-mouse pAkt (Ser473) (clone
538 M89-61), anti-mouse pS6 (clone D57.2.2E). For staining of iNKT cells, phycoerythrin
539 (PE)-conjugated PBS-57 loaded on mouse Cd1d tetramers (mCd1d/PBS-57) were
540 obtained from Tetramer Core Facility of the National Institute of Health.

541

542 **Measurement of *in vivo* cell proliferation and *ex vivo* survival**

543 Mice were given one intraperitoneal injection of 0.2 mg EdU (BD Bioscience). 12
544 hours after EdU injection, mice were sacrificed and organs harvested. Cells derived
545 from the thymus were stained with antibodies specific for cell surface antigens as
546 described above. After fixation and permeabilization, cells stained with FITC anti-EdU
547 antibody and 7-AAD (BD Pharmingen), according to manufacturer instructions. EdU
548 incorporation for different cell populations was measured by flow cytometry.

549 For the measurement of viability, we stained thymocyte suspensions with Annexin-V
550 (BD Biosciences), 7-AAD and coupled with other surface markers Cd4, Cd8, Cd69,
551 TCR β , Cd25, Cd44, either *ex vivo* or 24, 48 or 72 h after *in vitro* culture in complete
552 medium.

553

554 **Cell sorting and RNA preparation**

555 Lymphocytes were obtained from the thymus. Immune cell population including DN1-
556 4, DP, single positive CD4⁺ and CD8⁺, iNKT cells were stained in combination with
557 other cell specific markers Cd4, Cd8, Cd69, TCR β , Cd25, Cd44 and mCd1d/PBS-57
558 and subsequently sorted into different subsets using a FACSAria Cell Sorter (Becton-
559 Dickinson, San Jose, USA). Purity of sorted cell populations was over 98% as
560 validated by flow cytometry. Sorted cells were lysed using Trizol reagent (Invitrogen)
561 or RLT buffer from the RNeasy Micro kit (Qiagen) and RNA was extracted according
562 to the manufacturer's instructions.

563

564 **Quantitative RT-PCR**

565 We used High capacity RNA-to-cDNA kit (applied biosystem, Carlsbad, USA) or
566 iScript cDNA synthesis kit (BioRad) to generate cDNA for RT-PCR. PCR was carried
567 out with a SybrGreen-based kit (FastStart Universal SYBR Green Master, Roche,
568 Basel, Switzerland) or SensiFast SYBR No-ROX kit (Bioline) on a StepOne plus
569 instrument (Applied biosystems, Carlsbad, USA) or a LightCycler 480 system
570 (Roche). Primers were designed using the Roche software. We used the following
571 primers for mouse QPCR: Zeb1 forward primer, 5'- GCCAGCAGTCATGATGAAAA-
572 3'; Zeb1 reverse primer, 5'- TATCACAATACGGGCAGGTG-3'; Zeb2 forward primer,
573 5'-CCAGAGGAAACAAGGATTTTCAG-3'; Zeb2 reverse primer, 5'-AGGCCTGACATG
574 TAGTCTTGTG-3'; Gapdh forward primer, 5'-GCATGGCCTTCCGTGTTTC-3'; Gapdh
575 reverse primer, 5'- TGTCATCATACTTGGCAGGTTTCT-3'. The relative expression
576 of Zeb1 and Zeb2 were normalized to Gapdh expression.

577

578 **Western blotting**

579 Cells were lysed in NP40 lysis buffer (20mM Tris, HCl pH7.4; 150mM NaCl; 2mM
580 EDTA; 1% NP40) containing protease inhibitors for 30min on ice. Supernatant was
581 collected following 10min centrifugation at 12 000g at 4°C and protein concentration
582 was quantified by µBCA quantification kit (Thermo Fisher scientific). Fifty µg of total
583 cellular protein from thymus were incubated during 5 minutes at 95°C. Protein
584 samples were separated by electrophoresis using Novex 4–12% Tris-Glycine gels
585 (Life Technologies) for 1 hour at 120V. Proteins then were transferred on a PVDF
586 membrane (Bio-rad). After blocking with PBS 0.1% tween and 5% milk for 1 hour,
587 membranes were probed with the following primary antibodies: anti-Gapdh (Cell
588 Signaling Technology, 2118), anti-Zeb1 (Cell Signaling Technology, 3396, **raised**
589 **against a peptide around Asp846, the Cellophane mutation truncating the protein**
590 **after Tyr902**) over night at 4°C. Membranes were washed three times with PBS 0.1%
591 tween, secondary antibodies were added for one hour at RT. Anti-Rabbit and anti-
592 mouse HRP conjugate secondary antibodies were provided by Jacson
593 Immunoresearch. Proteins were revealed with Chemiluminescence Western
594 Lightening Plus kit (Perkin-Elmer).

595

596 **RNAseq analysis**

597 Thymic suspensions were stained in combination with anti-Cd3, anti-Cd4, anti-Cd8,
598 anti-Cd69, anti-TCR β and subsequently sorted into different subsets using a
599 FACS Aria Cell Sorter (Becton-Dickinson, San Jose, USA). Purity of sorted cell
600 populations was over 98% as measured by flow cytometry. RNA libraries were
601 prepared as previously described [60]. Briefly, total RNA was purified from 5×10^4
602 sorted thymocytes using the Direct-Zol RNA microprep kit (Zymo Research)
603 according to manufacturer instructions and was quantified using QuantiFluor RNA
604 system (Promega). 1 μ l of 10 μ M Oligo-dT primer and 1 μ l of 10mM dNTPs mix were
605 added to 0.15ng of total RNA in a final volume of 2.3 μ l. Oligo-dT were hybridized
606 3min at 72°C and reverse transcription (11 cycles) was performed. PCR pre-
607 amplification was then conducted using 16 cycles. cDNA were purified on AmpureXP
608 beads (Beckman Coulter) and cDNA quality was checked on D5000 screen tape and
609 analysed on Tape station 4200 (Agilent). Three ng of cDNA were tagmented using
610 Nextera XT DNA sample preparation kit (Illumina). Tagged fragments were further
611 amplified and purified on AmpureXP beads (Beckman Coulter). Tagged library quality
612 was checked on D1000 screen tape and analyzed on Tape station 4200 (Agilent).
613 Sequencing was performed by the GenomEast platform, a member of the “France
614 Génomique” consortium (ANR-10-INBS- 0009), on an Illumina HiSeq 4000
615 sequencing machine (read length 1x50nt).

616

617 **Measurements of TCR signaling**

618 ***Calcium response***

619 Thymocytes were first barcoded with anti-Cd45 coupled with different fluorochromes
620 for WT and *Cellophane* respectively and then stained at RT with fluorescent anti-Cd4
621 and anti-Cd8, anti-Cd69, anti-TCR β , anti-Cd25 and anti-Cd44 antibodies, followed by
622 a staining with Indo-1 (1 μ M, Life Technologies) at 1×10^7 cells/ml for 30 min at 37°C
623 and two times' washing at 4°C. Cells were resuspended in the RPMI medium (0.2%
624 BSA and 25mM HEPES) and were placed at 37°C for 5-10 min prior to acquisition.
625 Samples were acquired on a LSR II (BD) as following steps: 15 s baseline acquisition,
626 addition of anti-Cd3 biotin (2C11, 10 μ g/ml), acquisition for 1 min 30 s, addition of
627 Streptavidin (Life Technologies, 10 μ g/ml) and, acquisition for another 3-5 min.

628

629 ***Phosphorylation events***

630 Different samples corresponding to the different mice were barcoded by labeling
631 them with a series of anti-Cd45 antibodies coupled with different fluorochromes. For
632 phospho-flow stainings, 3×10^6 mixed thymocytes were stained using biotinylated Cd3
633 (2C11, 5 μ g/ml) and other surface markers for 15 min, followed by streptavidin (Life
634 Technologies, 10 μ g/ml) stimulation and fixed by addition of 10 volumes of Lyse/Fix at
635 the indicated time point. The level of pErk, pS6 or pAkt were normalized by the mean
636 fluorescence intensity (MFI) that was detected in the non-stimulated condition
637 (regarded as 100%) for each mouse.

638

639 ***In silico* analyses**

640 Functional annotations of DEGs were performed using Metascape [37] or STRING
641 [38] using default parameters. Additionally, we used several functionalities of the
642 Immgen database browsers [29] to generate some of the figures in the
643 supplementary information.

644

645 **Statistical analysis**

646 Statistical analyses were performed using Prism 5 (Graph-Pad Software). Two tailed
647 unpaired t-test, paired t-test, and ANOVA tests with Bonferroni correction were used
648 as indicated. We used the hypergeometric test and Benjamini-Hochberg p-value
649 correction algorithm to calculate if the enrichment of the overlap between gene lists
650 were statistically significant.

651

652

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667

668

669 **Author contributions**

670 JZ, AB, MW, DL, DEC, ALM, AR and AM performed experiments. RS and QM
671 performed in silico analyses. JC, LG, YGH provided reagents, conceptual insight and
672 helped writing the paper. TW wrote the paper and supervised the work.

673

674

675 **Conflict of interest**

676 The authors declare no conflict of interest

677

678 **Supplementary information** accompanies the manuscript on *Cellular & Molecular*
679 *Immunology's* website (<http://www.nature.com/cmi/>)

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833 length RNA-seq from single cells using Smart-seq2. *Nat Protoc* 2014; **9**:171–181.
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837 **Figure legends**

838

839 **Figure 1: Zeb1 expression in WT and *Cellophane* thymocytes**

840 (A-B) RT-PCR analysis of RNA from sorted thymocyte subsets isolated from
841 C57BL/6 mice, as indicated. Results are presented relative to expression of the
842 control gene *Gapdh*. (C) WB analysis of Zeb1 expression in total thymocytes of WT
843 and *Cellophane* mice, as indicated. Data are representative of three independent
844 experiments with 3 to 6 mice (A-B) or three independent experiments with three mice
845 (C).

846

847 **Figure 2: Cellularity and proportion of T cell subsets in *Cellophane* mice.**

848 (A) Total cell number from the thymus, spleen, liver and LN. (B) Percentages and
849 absolute numbers of indicated T cell subsets (DN, DP, Cd4 SP and Cd8 SP) in the
850 thymus. (C) Percentages and absolute numbers of indicated DN subsets defined by
851 Cd44 and Cd25 expression in the thymus. (D-E) Percentages and absolute numbers
852 of (D) Cd4 T cells and (E) Cd8 T cells. (F) Percentages and absolute numbers of
853 CD8⁺ Cd44⁺ memory T cell in LN and spleen. Each dot represents an individual
854 mouse. Data are pooled from 7 to 8 mice in four experiments (A-E) or 3 to 6 mice in
855 two experiments (F). The statistical analysis was performed using unpaired Student *t*
856 test.

857

858 **Figure 3: *Cellophane* mice have decreased numbers of thymic DP and lack
859 iNKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49⁺ Cd8 cells.**

860 (A) Flow cytometry analysis of Cd1d-tet⁺ iNKT cells (black gate) from thymus, spleen,
861 and liver in wild-type (WT) and *Cellophane* (Cello) homozygous mice as indicated.
862 (B) Percentage and absolute numbers of iNKT cells (mCd1d/PBS-57⁺ TCR β ⁺) in
863 thymus, spleen, and liver. (C) Flow cytometry analysis of NK1.1⁻ and NK1.1⁺ $\gamma\delta$ T
864 cells (black square gates) from the spleen of WT and *Cellophane* mice. (D)
865 Percentage within total $\gamma\delta$ T cells and absolute numbers of NK1.1⁻ and NK1.1⁺ $\gamma\delta$ T
866 cells in spleen and LN. (E) Percentage within total thymic iNKT of stages 0-3, as
867 defined by Cd24, Cd44 and NK1.1 expression. (F) Flow cytometry analysis of Cd8⁺
868 Cd44⁺ Ly49A⁺ / Ly49F⁺ / Ly49G2⁺ T cells (black square gates) from LN and spleen in
869 WT and *Cellophane* mice. (G) Percentage within Cd8⁺Cd44⁺ T cells and absolute
870 numbers of Cd8⁺ Cd44⁺ Ly49⁺ T cells in spleen and LN. Each dot represents an

871 individual mouse. Data are representative of 2-4 independent experiments with 6
872 mice in total for each panel. The statistical analysis was performed using unpaired
873 Student *t* test.

874

875

876 **Figure 4: The role of *Zeb1* in T cell development is cell-intrinsic.**

877 (A) Flow cytometry analysis of the indicated thymocyte subsets from BM chimeras.
878 Frequencies are shown for each subset. Recipients were Ly5a mice (Cd45.1), and
879 donor BM was either from Ly5a x C57BL/6 (Cd45.1/2) or *Cellophane* (Cd45.2). (B)
880 iNKT cell and (C) Cd8⁺ Ly49⁺ T cell frequencies in the spleen, liver or LN from the
881 same BM chimera described in A. (D) DN1-4, DP, Cd4⁺ and Cd8⁺ cell reconstitution
882 in thymus of competitive BM chimeras. Recipients were Ly5a mice (Cd45.1) and
883 donor BM was a 1:1 mix of Ly5a x C57BL/6 (Cd45.1/2) and *Cellophane* (Cd45.2). 8
884 weeks after BM transplantation, cells were analyzed by flow cytometry. The number
885 above the red bar shows the percentage of indicated cells originating from the
886 *Cellophane* BM. (E-F) Frequency of iNKT cells (E) and Ly49⁺ cells within Cd8⁺ Cd44⁺
887 T cells in spleen, liver or LN from BM chimera described in D. Each dot represents an
888 individual mouse. Results show the pooled data from two to three independent
889 experiments for a total of 2 to 6 mice per group in each panel. Statistical analyses
890 were performed using unpaired Student *t* test.

891

892

893 **Figure 5: *Cellophane* DP cells have reduced survival and proliferation.**

894 (A) Percentages of Annexin V-negative cells (live cells) by wild-type and *Cellophane*
895 thymocytes cultured for 0-72h. Each graph shows a different subset, as indicated (B)
896 EdU incorporation of wild-type and *Cellophane* thymocytes after a 12-hour *in vivo*
897 pulse of 0.2 mg EdU. (C) Frequencies of Ki67⁺ cells in DN1-4 subsets from WT and
898 *Cellophane* mice. (D) Ki67 nuclear staining in DP thymocytes from WT and
899 *Cellophane* mice. Bar graphs (right panel) show mean \pm SD fluorescence intensity
900 (MFI). Each dot represents an individual mouse. Data are pooled from three
901 independent experiments with three mice in each group (A-D). Statistical analysis
902 was performed using unpaired Student *t* test.

903

904 **Figure 6: *Cellophane* DP thymocytes display increased TCR signaling.**

905 (A) Flow cytometry analysis of Cd69, Cd25, Cd5, Nur77, Cd4, Cd8 and Tcrβ
906 expression (mean fluorescence intensity) in DP thymocytes of wild-type and
907 *Cellophane* mice. (B) Left panels: Representative flow cytometry analysis of iNKT1
908 (Plzf lo, Roryt-, Tbet+), iNKT2 (Plzf hi, Roryt-, Tbet-), and iNKT17 (Plzf int, Roryt+,
909 Tbet-) subsets in wild-type and *Cellophane* mice (as defined by the gating strategy
910 shown in the upper-panel). Right panel: graphs of the percentage of iNKT subsets
911 within total iNKT. (C) The TCRVβ repertoire of thymic iNKT cells was analyzed by
912 flow cytometry. Bar graphs show mean±SD frequencies of individual Vβ chain
913 within iNKT cells. (D) Expression of Cd4, Tbet, Egr2 and Plzf in iNKT cells as
914 measured by flow cytometry (mean fluorescence intensity). (E) Phosphorylation
915 levels of Erk, S6 and Akt (S473) in resting DP thymocytes from WT and *Cellophane*
916 mice. (F) Phosphorylation levels of Erk, S6 and Akt (Ser473) in DP cells stimulated
917 with anti-Cd3 antibody for the indicated time. Results are normalized to the non-
918 stimulated condition (ie basal level, 100%) for each group. (G) Representative
919 histogram overlay showing the Ca²⁺ flux in thymic DP cells from wild-type or
920 *Cellophane* mice stimulated with anti-Cd3 antibodies, as measured by flow cytometry.
921 Thymocytes were activated following incubation with biotinylated anti-Cd3 (Arrow)
922 followed by cross-linking with streptavidin (Arrowhead). (H) Dot plots showing the
923 AUC (Area under curve) and Ca²⁺ peak. Statistical analysis was performed using
924 unpaired Student *t* test. Each dot represents an individual mouse. Data are pooled
925 from three experiments with a total of 8 mice in each group (A) or three experiments
926 with 6 mice (B), or three mice (C), or two experiments with 6 mice (D-F) or three
927 experiments with 3 mice (G-H).

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929

930 **Figure 7: RNAseq analysis of *Zeb1*-regulated genes.** (A) Volcano-plot representing
931 -log₁₀ (adjusted p-value) as a function of the estimated log₂ fold-change. Significant
932 genes were selected using the following thresholds: adjusted p-value lower than 0.05
933 and absolute value of log₂ Fold-Change greater than 1. For significant genes (plotted
934 in red), a selection of gene names is displayed. Results are representative of
935 biological replicates in each group. (B) Flow cytometry analysis of several genes
936 identified by RNA-seq either up-regulated (Foxo1, Ms4a4b, integrin α4:β7, Ccr7,
937 Ccr4, Cd74, Cd53,) or downregulated (Cd81) in *Cellophane*. Data are pooled from
938 two independent experiments with a total of 6 mice. (C) Expression level of two

939 selected gene list (200 genes for each list) - either upregulated (WT<Cello) or
940 downregulated (WT>Cello) in *Cellophane* with fold change>2 and p-value<0.05 - in
941 different $\alpha\beta$ T cell subsets, as indicated. Graphs are adapted from the Immgen
942 website. (D) Pathway analysis of DEGs in (A) using Metascape. Selected terms are
943 shown among the most significant ones. (E) Venn diagram showing the overlap
944 between DEGs in (A) and genes located in the 100kb around OCRs containing Zeb1
945 motifs, and for which chromatin accessibility changed during T cell development
946 (corresponding clusters can be visualized in Figure S4B). The genes upregulated in
947 WT are shown in red while the downregulated are shown in blue.

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952 **Supplementary figure legends**

953
954 **Table S1:** Genes differentially expressed between control and *Cellophane* DP.
955 Filters: Adjusted p-value<0.05, Fold change>2.

956

957 **Table S2:** Pathway analysis of genes up regulated or down regulated in *Cellophane*
958 DP compared to control DP, using “metascape”.

959

960 **Table S3:** Functional annotation of genes up regulated in *Cellophane* DP compared
961 to control DP. Individual Pubmed queries were performed to search for association
962 between corresponding genes and T cell activation or T cell development. A
963 summary of the association and the reference of the article are provided.

964

965 **Table S4:** Functional annotation of genes up regulated in *Cellophane* DP compared
966 to control DP using the Pubmed module search in “String”.

967

968 **Figure S1: Zeb1 is highly expressed in DP and co-regulated with Tcf12.**

969 (A) Expression profile of Zeb1 and Zeb2 mRNA level in thymic $\alpha\beta$ T cell subsets.
970 Image is adapted from Immgen (<http://www.immgen.org/>). The red color indicates
971 higher expression. (B) Genes for which the expression pattern in $\alpha\beta$ T cell subsets is
972 highly correlated with that of Zeb1. The figure is adapted from data on the Immgen
973 browser (<http://www.immgen.org/>).

974

975 **Figure S2:** Decreased numbers of thymocyte subsets and peripheral NKT cells in
976 *Cellophane*→Ly5a BM chimeric mice

977 Cell numbers calculated for the panels A-C in Figure 4, as indicated.

978

979 **Figure S3: The role of Zeb1 in T cell development is cell-intrinsic.** (A-C) Flow
980 cytometry analysis of indicated thymocyte subsets or thymus/liver NKT cells from BM
981 chimeras. Recipients were C57BL/6 (WT) mice or *Cellophane*, as indicated, and
982 donor BM was either Ly5a x C57BL/6 (WT) or *Cellophane*, as indicated. Graphs
983 show the mean±SD frequency or number of 3-4 mice in 2 experiments. (D) Mixed
984 BM chimeric mice described in Figure 4D were analyzed by flow cytometry for the

985 indicated subsets. Results show the BM donor origin in percentage for each gated
986 subset. Mean +/-SD of 6 mice.

987

988 **Figure S4: Zeb1 regulates epigenetic remodeling during T cell development.** (A)
989 Snapshot of Figure 5 in Yoshida et al [41], highlighting the cluster of transcription
990 factors predicted to regulate chromatin accessibility during T cell development. (B)
991 Boxplot graphs showing the mean accessibility of different open chromatin regions
992 (OCRs), as determined by ATACseq by Yoshida *et al*, in developing T cell subsets,
993 as indicated. All OCRs contain Zeb1 binding sites.

Figure 1

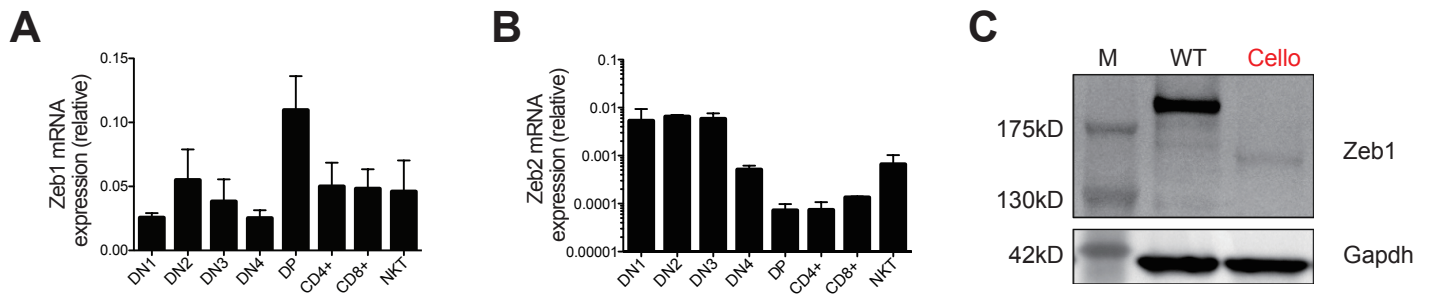


Figure 2

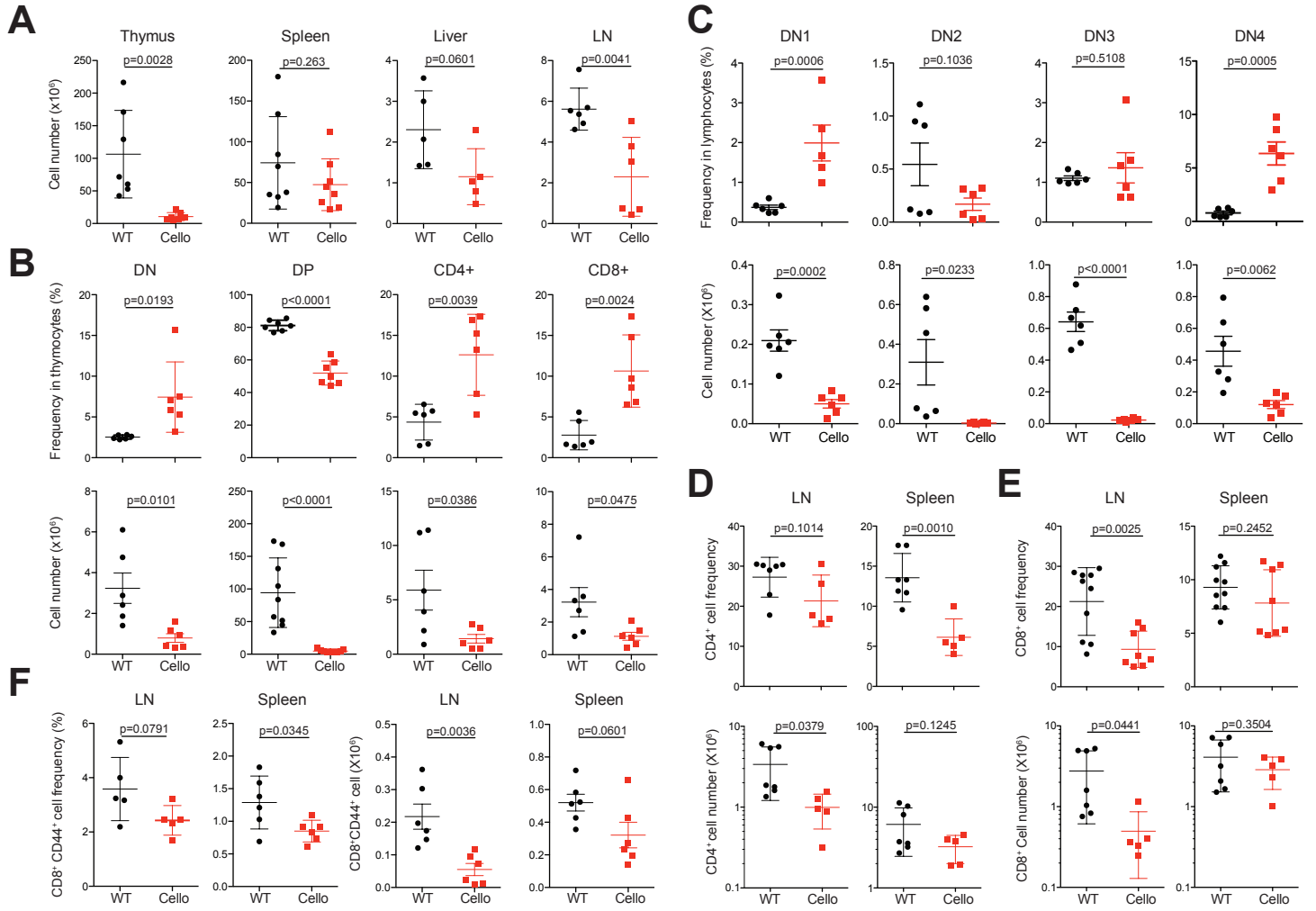


Figure 3

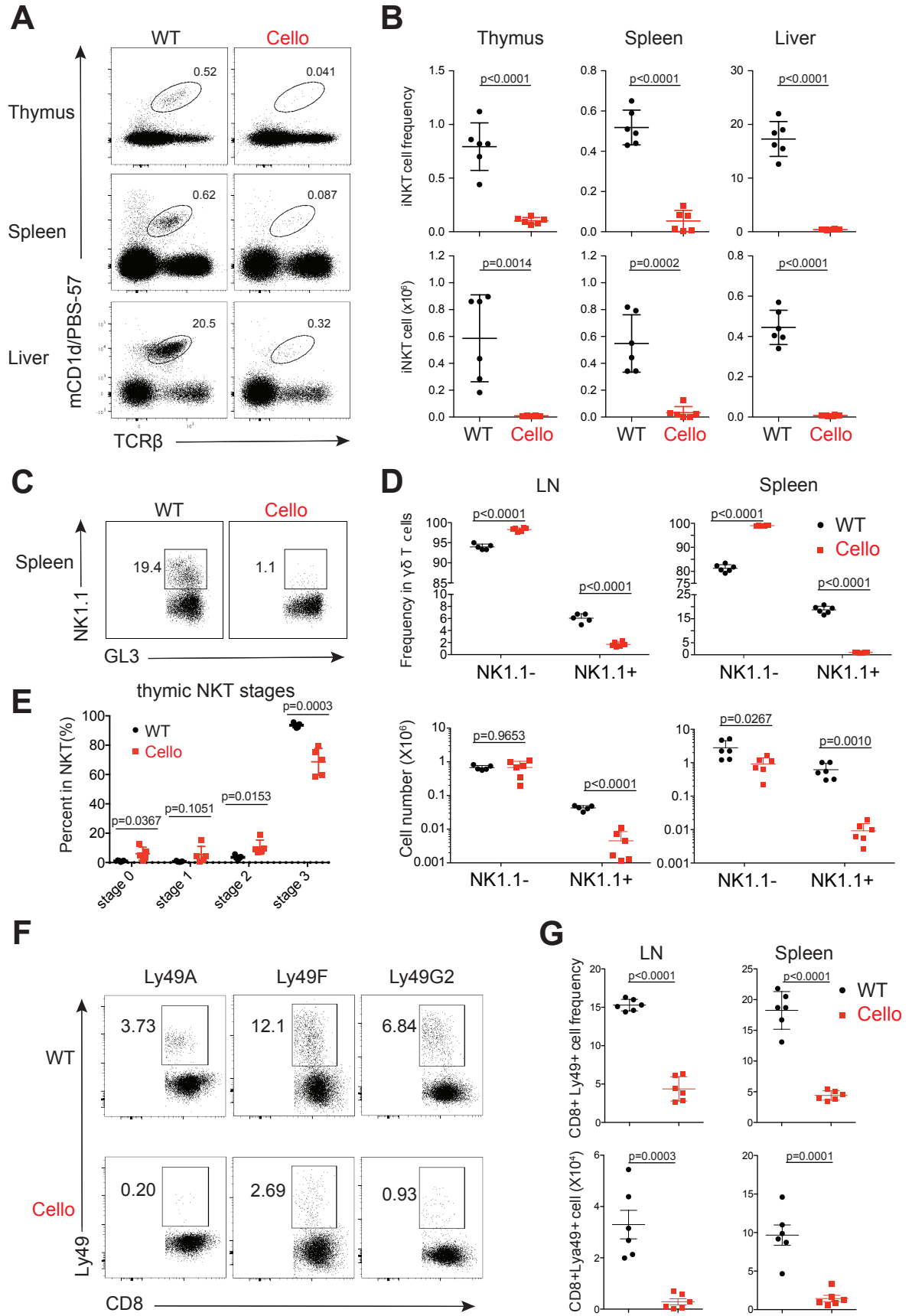


Figure 4

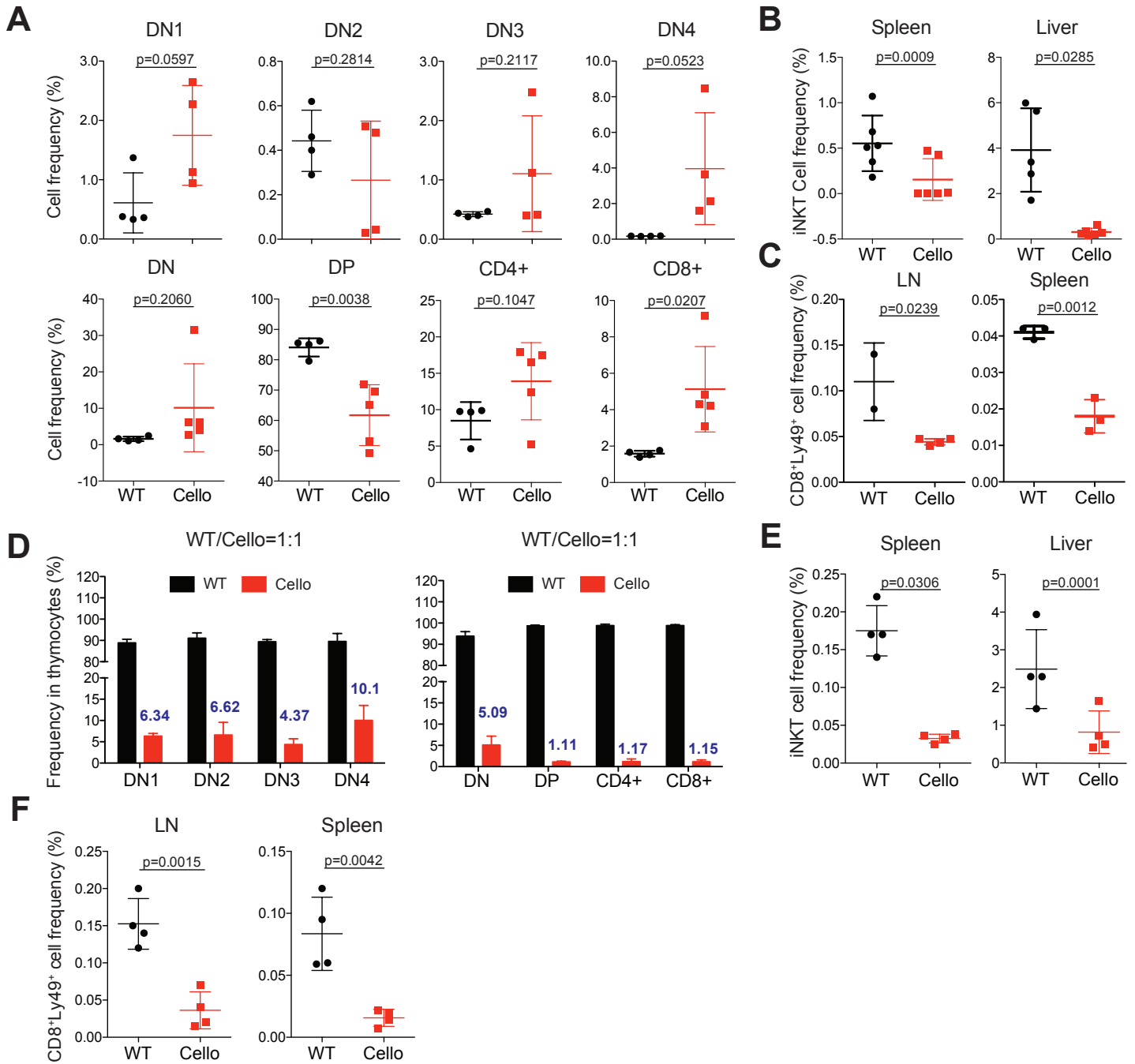


Figure 5

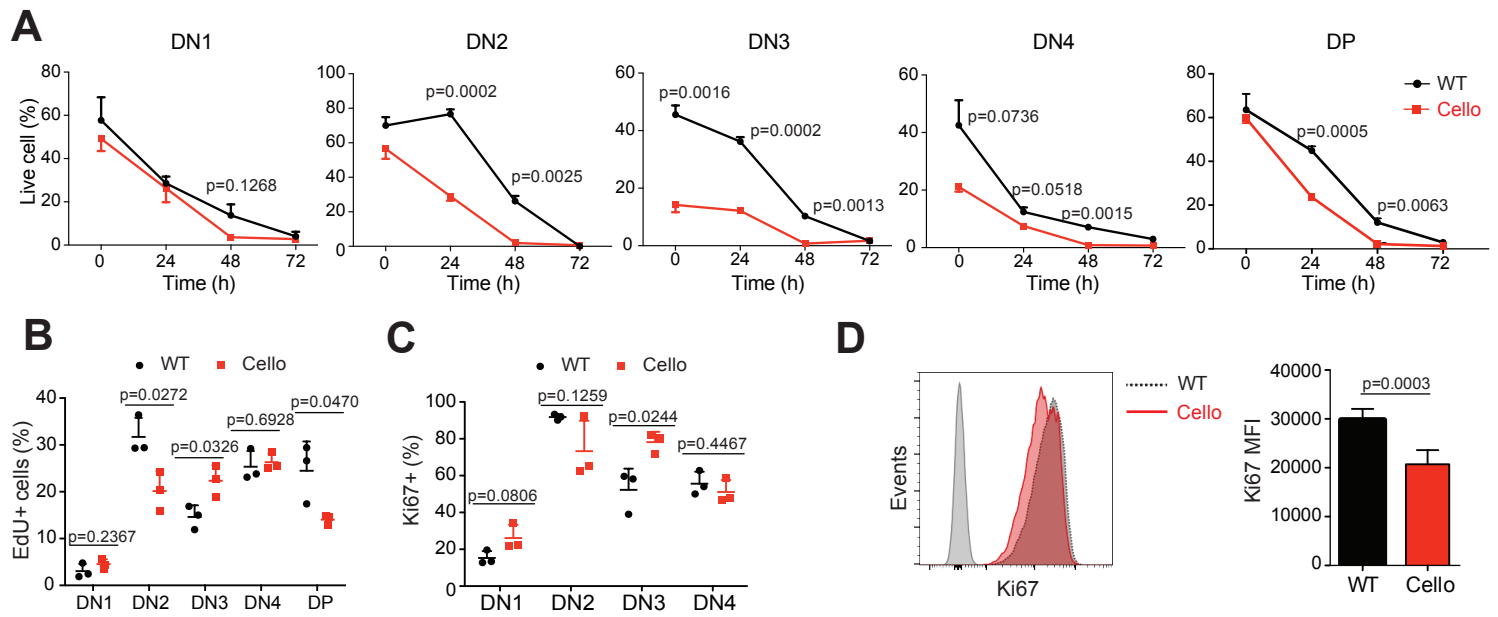


Figure 6

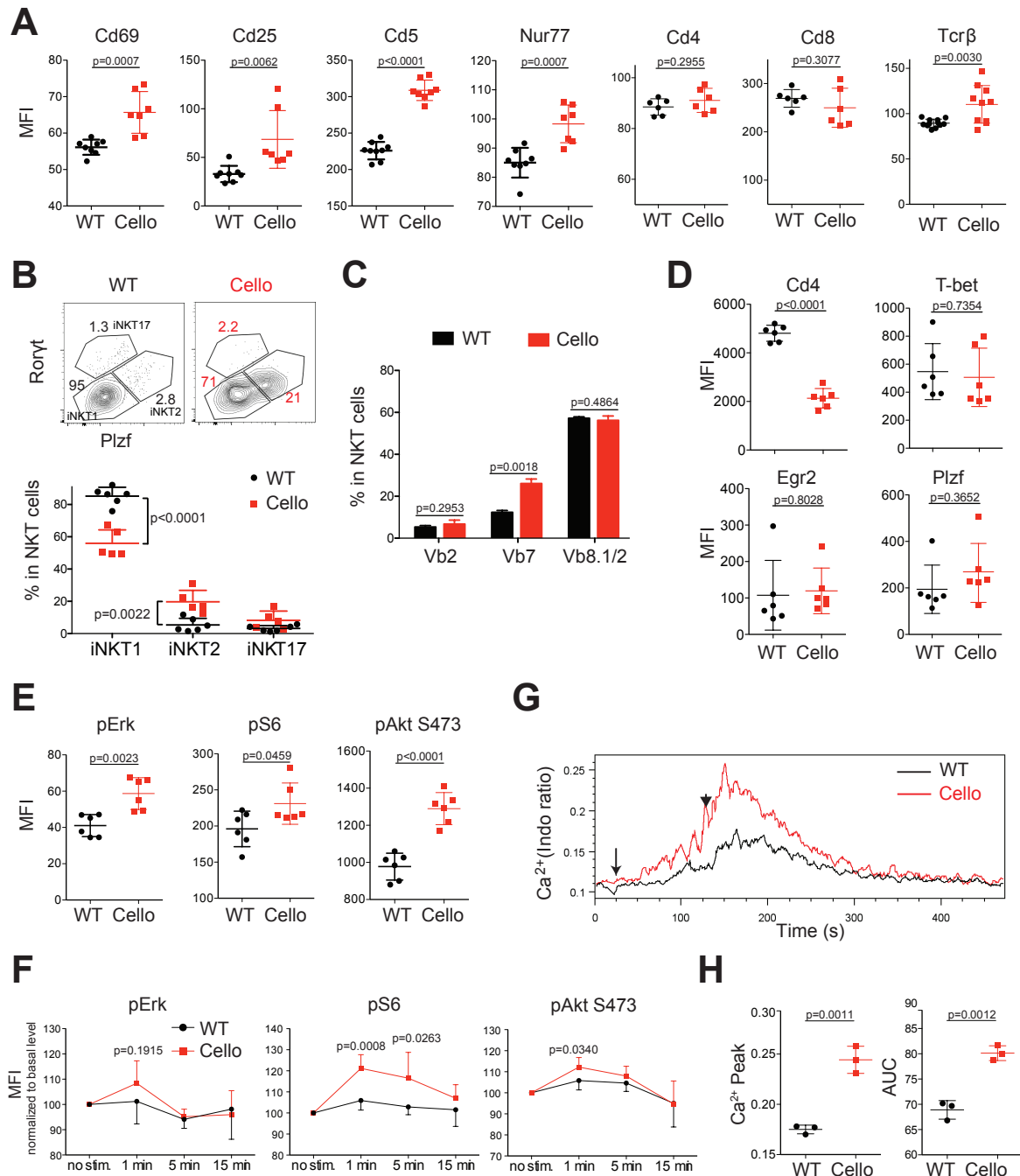
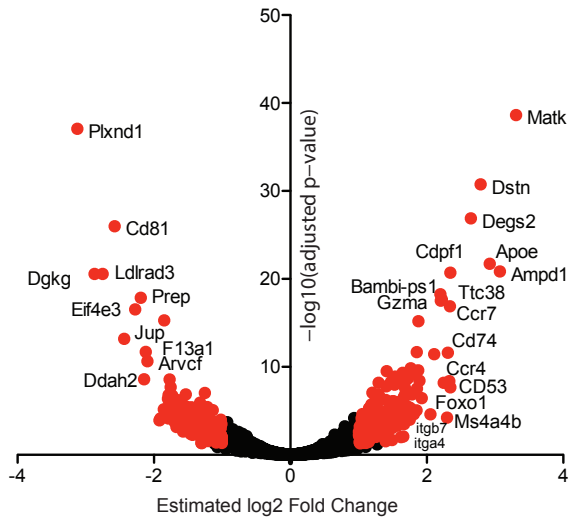
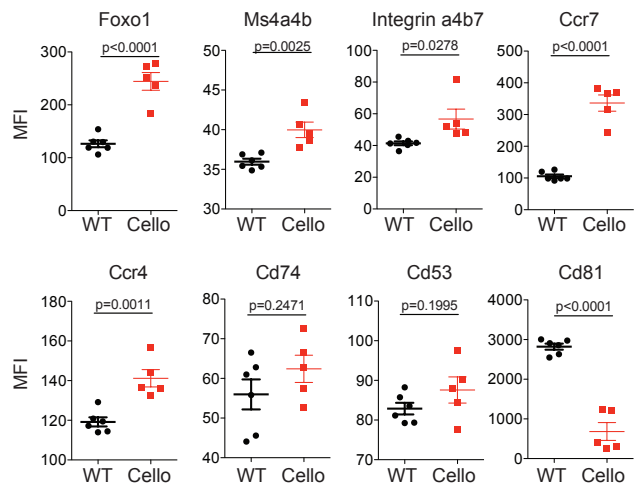


Figure 7

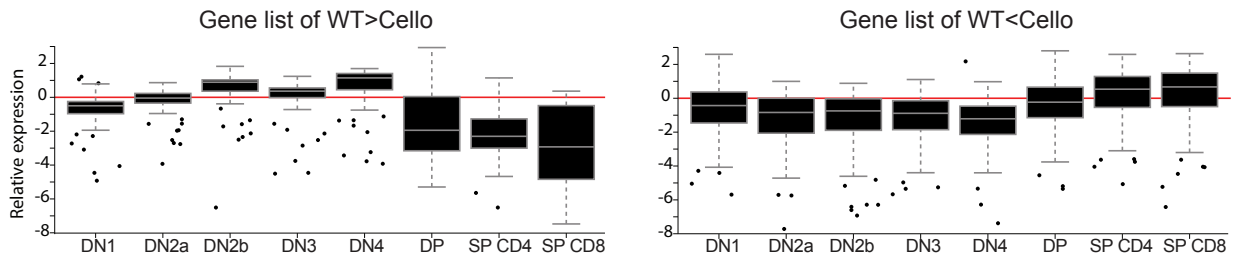
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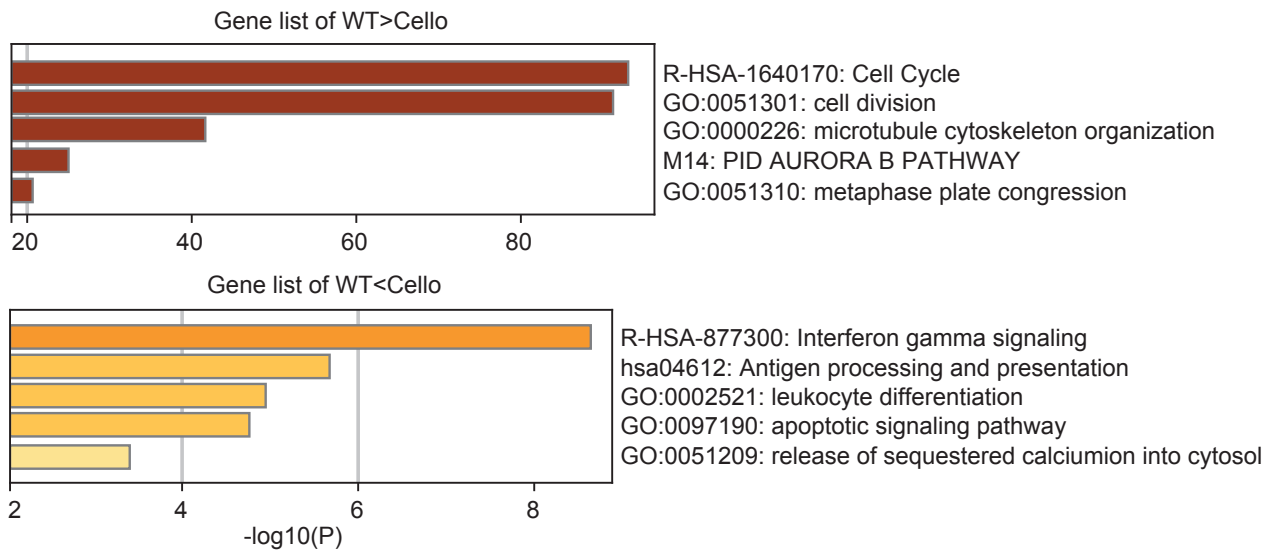
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