

Zeb1 represses TCR signaling, promotes the proliferation of T cell progenitors and is essential for NK1.1+ T cell development

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- 2 is essential for NK1.1⁺ T cell development
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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⁺ y δ 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. А 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-y; iNKT2 cells express Gata3 and Plzf and 84 secrete IL-4 and IL-13; iNKT17 express Roryt 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, and other signaling pathways [19]. Zeb1^{-/-} mice exhibit multiple developmental 107 defects and die at birth [20]. In pathological settings, activation of EMT programs 108 109 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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137 Results

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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

(Figure 2B-C). In the LN and spleen, the percentage of Cd4 T cells, and Cd8 T cells
were reduced (Figure 2D-E) and the proportion of memory-phenotype Cd44⁺ T cells
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-hematopoeitic 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 increased (Figure 4A). iNKT cells and Ly49⁺ T cell subsets were also strongly 196 197 reduced in the peripheral organs of Cellophane-Ly5a BM chimeric mice compared 198 to WT \rightarrow 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 to WT \rightarrow Ly5a chimera (Figure S2). NK1.1⁺ $\gamma\delta$ T cells were not analyzed because 201 202 many of them are derived from fetal precursors [34], and are not reconstituted in BM

chimera mice. To further test the role of Zeb1 in the environment of developing T cells, we also made different BM chimeric mice using WT and *Cellophane* mice as both recipients and BM donors (WT \rightarrow WT, WT \rightarrow *Cellophane*, *Cellophane* \rightarrow WT and *Cellophane* \rightarrow *Cellophane*). As shown in Figure S3A-B, the frequencies and numbers of DN and DP subsets were determined by the genotype of the BM donor and not by that of the host genotype. Similar conclusions could be reached upon examination of 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

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

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

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

Thus, the *Cellophane* mutation affects both the survival and proliferation of developing DN2 and DP thymocytes, which could account for the decreased number 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 TCRβ 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 Roryt [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

known to sustain TCR signal strength [5], the selection of Cd4 ^{low} iNKT cells in *Cellophane* mice could reflect an adaptation to overt TCR signaling in *Cellophane*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 minimize the experimental variation, we used a barcoding strategy that allowed 276 277 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

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

295 To further uncover the mechanisms of Zeb1 function during T cell development, we performed RNA-seq to compare WT and Cellophane DP transcriptomes. We found 296 297 538 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

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

305 Next, we gueried 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 genotype, SP T cells are much more responsive to TCR signaling mediated calcium 314 315 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 RNAseg 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 $Ifn\gamma$ (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 with338 our manual PubMed searches.

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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.

360 Discussion

Here, we demonstrated that Zeb1 is essential for the transition through the DN2 and DP stages of T cell development as well as for the differentiation of iNKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49⁺ Cd8 T cells. Mechanistically, Zeb1 regulates the expression of a number of genes that were notably involved in cell proliferation or in TCR signaling at the DP stage. In *Cellophane* mice, these events may perturb thymic development and selection in a way that does not allow the production of NK1.1⁺ and 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 particular, iNKT cells were virtually absent from the periphery. This altered 383 384 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 Va14-Ja18 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 Daugtherless, 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 mice is milder than that of Zeb1^{-/-} mice, we assumed that the Cellophane mutation 454 455 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 guestion *in vivo* using appropriate genetic models.

In summary, Zeb1 is an essential member of the TF network that regulates T cell development and selection at the DN2 and DP stages. Furthermore, we have also shown that Zeb1 allows the development of iNKT cells and other T cell subsets expressing NK cell markers by regulation of cell cycle and TCR signaling in 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 2-5x10^6 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 TCRβ (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 TCRVβ2 (clone B20.6), anti-mouse 529 TCRVβ7 (clone TR310), anti-mouse TCRVβ8.1/8.2 (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 Roryt 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.

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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.

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

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554 Cell sorting and RNA preparation

555 Lymphocytes were obtained from the thymus. Immune cell population including DN1-4, DP, single positive CD4⁺ and CD8⁺, iNKT cells were stained in combination with 556 557 other cell specific markers Cd4, Cd8, Cd69, TCRβ, 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.

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'-CCAGAGGAAACAAGGATTTCAG-3': Zeb2 reverse primer, 5'-AGGCCTGACATG 574 TAGTCTTGTG-3'; Gapdh forward primer, 5'-GCATGGCCTTCCGTGTTC-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). Fifity 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 membrane (Bio-rad). After blocking with PBS 0.1% tween and 5% milk for 1 hour. 586 587 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-TCRB and subsequently sorted into different subsets using a 599 FACSAria 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). 1µl of 10µM Oligo-dT primer and 1µl of 10mM dNTPs mix were 605 added to 0.15ng of total RNA in a final volume of 2.3µl. 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 guality 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 HiSeg 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-TCR8, anti-Cd25 and anti-Cd44 antibodies, followed by a staining with Indo-1 (1µM, Life Technologies) at 1x10⁷ cells/ml for 30 min at 37°C 622 623 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 LSRII (BD) as following steps: 15 s baseline acquisition, 626 addition of anti-Cd3 biotin (2C11, 10µg/ml), acquisition for 1 min 30 s, addition of 627 Streptavidin (Life Technologies, 10µg/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 phospho-flow stainings, 3x10⁶ mixed thymocytes were stained using biotinylated Cd3 632 633 (2C11, 5µg/ml) and other surface markers for 15 min, followed by streptavidin (Life 634 Technologies, 10µg/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

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

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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.

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668

669 Author contributions

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

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675 Conflict of interest

676 The authors declare no conflict of interest

678 **Supplementary information** accompanies the manuscript on *Cellular & Molecular*

679 *Immunology*'s website (http://www.nature.com/cmi/)

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- 837 Figure legends
- 838

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

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

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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 t856 test.

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Figure 3: *Cellophane* mice have decreased numbers of thymic DP and lack iNKT cells, NK1.1⁺ γδ T cells and Ly49⁺ Cd8 cells.

(A) Flow cytometry analysis of Cd1d-tet⁺ iNKT cells (black gate) from thymus, spleen, 860 861 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⁺ $v\delta$ 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⁺ Lv49⁺ T cells in spleen and LN. Each dot represents an

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

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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⁺ Lv49⁺ 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.

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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+/-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.

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

905 (A) Flow cytometry analysis of Cd69, Cd25, Cd5, Nur77, Cd4, Cd8 and Tcr β 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 TCRV β 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 histogram overlay showing the Ca²⁺ flux in thymic DP cells from wild-type or 919 920 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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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 α 4; β 7, 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 legends953

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

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

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

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Table S4: Functional annotation of genes up regulated in *Cellophane* DP comparedto 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 (<u>http://www.immgen.org/</u>). 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 (<u>http://www.immgen.org/</u>).

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

976 Cellophane-Lysa BM chimeric mice 977 Cell numbers calculated for the panels A-C in Figure 4, as indicated.

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Figure S3: **The role of Zeb1 in T cell development is cell-intrinsic**. (A-C) Flow cytometry analysis of indicated thymocyte subsets or thymus/liver NKT cells from BM ohimeras. Recipients were C57BL/6 (WT) mice or *Cellophane*, as indicated, and donor BM was either Ly5a x C57BL/6 (WT) or *Cellophane*, as indicated. Graphs show the mean+/-SD frequency or number of 3-4 mice in 2 experiments. (D) Mixed 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.

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





Figure 3









Figure 7

