



HAL
open science

De novo generation of the NPM-ALK fusion recapitulates the pleiotropic phenotypes of ALK+ ALCL pathogenesis and reveals the ROR2 receptor as target for tumor cells

Loélia Babin, Alice Darchen, Elie Robert, Zakia Aid, Rosalie Borry, Claire Soudais, Marion Piganeau, Anne de Cian, Carine Giovannangeli, Olivia Bawa, et al.

► To cite this version:

Loélia Babin, Alice Darchen, Elie Robert, Zakia Aid, Rosalie Borry, et al.. De novo generation of the NPM-ALK fusion recapitulates the pleiotropic phenotypes of ALK+ ALCL pathogenesis and reveals the ROR2 receptor as target for tumor cells. *Molecular Cancer*, 2022, 21 (1), pp.65. 10.1186/s12943-022-01520-0 . mnhn-03868576

HAL Id: mnhn-03868576

<https://mnhn.hal.science/mnhn-03868576>

Submitted on 25 Nov 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

[Click here to view linked References](#)

1 ***De novo* generation of the NPM-ALK fusion recapitulates the pleiotropic phenotypes of ALK+**
2 **ALCL pathogenesis and reveals the ROR2 receptor as target for tumor cells**

3
4 4 Loélia Babin^{#,1}, Alice Darchen^{#,1}, Elie Robert², Zakia Aid², Rosalie Borry¹, Claire Soudais³, Marion
5 5 Piganeau⁴, Anne De Cian⁴, Carine Giovannangeli⁴, Olivia Bawa⁵, Charlotte Rigaud⁶, Jean-Yves
6 6 Scoazec⁷, Lucile Couronné⁸, Layla Veleau⁹, Agata Cieslak⁹, Vahid Asnafi⁹, David Sibon⁹, Laurence
7 7 Lamant¹⁰, Fabienne Meggetto¹⁰, Thomas Mercher^{2†}, and Erika Brunet^{1†}

8
9
10 ¹ Institut Imagine, INSERM UMR 1163, Laboratory of the « Genome Dynamics in the Immune
11 System », Équipe Labellisée La Ligue Nationale Contre Le Cancer, Université de Paris, Université Paris
12 Saclay, Paris, France

13 ² INSERM Unité 1170 (U1170), Gustave Roussy Cancer Campus, Equipe labellisée Ligue Nationale
14 Contre le Cancer, Université Paris Saclay, Programme PEDIAC, OPALE Carnot Institute, Villejuif,
15 94805, France

16 ³ Institut Imagine, INSERM UMR1163, Laboratory of Lymphocyte Activation and Susceptibility to
17 EBV infection, Université de Paris, Paris, France

18 ⁴ Museum National d'Histoire Naturelle, INSERM U1154, CNRS UMR 7196, Sorbonne Universités,
19 43 rue Cuvier, Paris, F-75231, France

20 ⁵ PETRA platform, Gustave Roussy, AMMICa, CNRS-UMS 3655 Inserm US23, University Paris
21 Saclay, Villejuif, 94805, France

22 ⁶ Department of Pediatric and Adolescent Oncology, Gustave Roussy, Villejuif, 94805, France

23 ⁷ Department of Pathology, Gustave Roussy; AMMICa CNRS UMS3655 Inserm US23; Université Paris
24 Saclay, Villejuif, 94805, France

25 ⁸ Laboratory of Onco Hematology, Hôpital Necker- Enfants Malades, Assistance Publique
26 Hôpitaux de Paris (APHP); Laboratory of Cellular and Molecular Mechanisms of Hematological
27 Disorders and Therapeutical Implications, INSERM U1163, Imagine Institute; University of Paris,
28 Paris, France

29 ⁹ Université de Paris, Institut Necker-Enfants Malades (INEM), INSERM U1151, and Laboratory of
30 Onco-Hematology, AP-HP Hôpital Necker Enfants-Malades, Paris, France

31 ¹⁰ Inserm, UMR1037 CRCT, Université Toulouse III-Paul Sabatier, CNRS, ERL5294 CRCT,
32 Laboratoire d'Excellence Toulouse Cancer-TOUCAN, Équipe Labellisée La Ligue Nationale Contre Le
33 Cancer, F-31000 Toulouse, France

34
35 # These authors contributed equally to this study

36 †Co-corresponding authors

37
38 **Correspondance :**

39 erika.brunet@inserm.fr

40 Institut Imagine, INSERM UMR1163

41 24, Boulevard du Montparnasse

42 75015 Paris, France

43 Phone +33 1 42 75 44 31

44
45 thomas.mercher@inserm.fr

46 Institut Gustave Roussy, INSERM U1170

47 114, rue Édouard-Vaillant

48 94805 Villejuif, France

49 Phone + 33 1 42 11 44 83

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 **ABSTRACT**

2
3 **Background:** Anaplastic large cell lymphoma positive for ALK (ALK+ ALCL) is a rare type of non-
4 Hodgkin lymphoma. This lymphoma is caused by chromosomal translocations involving the anaplastic
5 lymphoma kinase gene (ALK). In this study, we aimed to identify mechanisms of transformation and
6 therapeutic targets by generating a model of ALK+ ALCL lymphomagenesis *ab initio* with the specific
7 NPM-ALK fusion.

8 **Methods:** We performed CRISPR/Cas9-mediated genome editing of the NPM-ALK chromosomal
9 translocation in primary human activated T lymphocytes.

10 **Results:** Both CD4+ and CD8+ NPM-ALK-edited T lymphocytes showed rapid and reproducible
11 competitive advantage in culture and led to *in vivo* disease development with nodal and extra-nodal
12 features. Murine tumors displayed the phenotypic diversity observed in ALK+ ALCL patients, including
13 CD4+ and CD8+ lymphomas. Assessment of transcriptome data from models and patients revealed
14 global activation of the WNT signaling pathway, including both canonical and non-canonical pathways,
15 during ALK+ ALCL lymphomagenesis. Specifically, we found that the WNT signaling cell surface
16 receptor ROR2 represented a robust and genuine marker of all ALK+ ALCL patient tumor samples.

17 **Conclusions:** In this study, *ab initio* modeling of the ALK+ ALCL chromosomal translocation in mature
18 T lymphocytes enabled the identification of new therapeutic targets. As ROR2 targeting approaches for
19 other cancers are under development (including lung and ovarian tumors), our findings suggest that
20 ALK+ ALCL cases with resistance to current therapies may also benefit from ROR2 targeting strategies.

21
22 **KEYWORDS**

23 ALK+ ALCL, lymphoma, NPM-ALK fusion, CRISPR/Cas9 models, WNT, ROR2, biomarker,
24 therapeutic targets

26 BACKGROUND

1 27 Anaplastic large cell lymphoma with anaplastic lymphoma kinase gene translocations (ALK+
2 ALCL) is a mature T-cell lymphoma that primarily affects lymph nodes, but also gives rise to tumors in
3 28 extranodal organs and occasional characteristic skin lesions. Although the majority of patients recover
4 29 with first line chemotherapy, 10% to 30% of patients relapse with a poor prognosis [1-4].
5 30

6 31 In terms of immune phenotypes, ALK+ ALCL tumor cells are primarily characterized by
7 32 constant CD30 antigen expression in addition to a variety of other immunologic phenotypes
8 33 (summarized in [5]). While the majority of ALK+ ALCL tumors are CD4+ (approx. 70%),
9 34 approximately 10% of patient samples exhibit a CD8+ phenotype. Null T-cell phenotypes and rarer
10 35 CD4+CD8+ phenotypes have also been described. Only 4% of ALK+ ALCL patient cells express T-
11 36 cell receptors (TCR) at the cell surface, whereas rearrangements at the genomic TCR locus have often
12 37 been observed (>75%) [6]. The majority of ALK+ ALCL tumor cells have been shown to express at
13 38 least one T-cell specific marker. This pleiotropic combination of markers observed in ALK+ ALCL has
14 39 rendered the identification of the cell of origin difficult, with reports proposing either a thymic or a
15 40 peripheral origin [6-8].
16 41

17 42 Between 60% and 80% of ALK+ ALCL cases harbor the NPM-ALK chromosomal
18 43 translocation, which leads to constitutive activation of the ALK protein. Uncontrolled activation of ALK
19 44 induces several cascades of signaling pathways, including phosphorylation of STAT3, which is
20 45 important for the maintenance of the malignant phenotype [9-11].
21 46

22 47 The oncogenic potential of the *NPM-ALK* fusion gene was first demonstrated *in vitro* using
23 48 murine cell lines and primary cells. Several *in vivo* approaches in mice have failed to phenocopy human
24 49 ALK+ ALCL and rather often yield B-cell lymphomas [12-16]. More recently, transduction of primary
25 50 human CD4+ T lymphocytes with the *NPM-ALK* transgene has led to *in vitro* transformation of the cells
26 51 [7, 8, 17] and *in vivo* tumor formation [7, 17]. However, lentiviral expression does not precisely
27 52 reproduce gene dosage nor the spatiotemporal variations in gene expression associated with the various
28 53 stages of differentiation. Moreover, these models lack other potentially important oncogenic features
29 54 resulting from chromosomal translocation formation, including the reciprocal fusion gene, potential
30 55 haplo-insufficiency of NPM1 or chromatin states resulting from the repositioning of translocated
31 56 chromosomes. As high expression levels of NPM-ALK is toxic [18] and a limited number of clones are
32 57 able to grow despite high transduction efficiency, it is likely that prolonged selection is required to
33 58 establish the appropriate conditions in these models. Interestingly, transplantation of CRISPR/Cas9-
34 59 edited cells from murine fetal liver hematopoietic stem cells into mice led to the development of CD30+
35 60 T-cell lymphomas with spleen and secondary organ involvement. However, no nodal tumors were
36 61 detected, and cells were primarily CD3+ and CD4+CD8+ (uncommon immunophenotypes found in
37 62 humans).
38 63
39 64
40 65

61 In the present study, we demonstrate high-efficacy transformation of primary human activated
62 T lymphocytes upon CRISPR/Cas9-mediated engineering of the NPM-ALK translocation. Translocated
63 human T lymphocytes increased survival for over three months and presented with various phenotypes.
64 Murine recipients transplanted with the human NPM-ALK lymphocytes developed systemic disease
65 within a median of three months, with nodal, extranodal and sporadic skin involvement, thus resembling
66 the disease observed in humans. Interestingly, we found that tumor formation recapitulated the immune
67 phenotypic diversity (including the less common CD8+ tumors), the histological pattern (large and small
68 tumor cells) and the transcriptomic signature of ALK+ ALCL patient cells. Transcriptomic analysis
69 using this oncogenic model revealed specific activation of the WNT signaling pathways in ALK+
70 ALCL, which was further confirmed in patient data. Furthermore, gene analysis of the progression from
71 wild type lymphocytes into tumors revealed that the ROR2 transmembrane receptor was upregulated
72 during oncogenesis. As the ROR2 transmembrane receptor is exclusively expressed in adult tissue, it
73 represents a promising surface target to treat ALK+ ALCL patients.
74 Overall, we have established a unique efficient genome-editing strategy to model gene fusions in
75 primary human lymphocytes and characterized the steps of normal T-cell progression into transformed
76 ALK+ lymphomas *in vivo*. The ROR2 cell surface receptor represents a diagnostic alternative and
77 potential therapeutic target for ALCL patients with resistance to chemotherapy.

78 **METHODS**

79 **Primary cells and cell lines**

80 PBMCs were isolated using SepMate™-50 (IVD) (StemCell Technologies #85450) following
81 manufacturer's instructions. PBMCs were activated 5 to 7 days on coated plates with anti-CD3-OKT3
82 (Biolegend #317325 RRID: AB_11147370) and 1 ng/uL anti-CD28 (eBioscience #16-0289-81 RRID:
83 AB_468926) in RPMI medium (Invitrogen) supplemented with 20% heat-inactivated Fetal Bovin Serum
84 (GIBCO). After activation cells were directly transfected with the RiboNucleoProtein RNP/Cas9
85 complex. The patient-derived xenograft (PDX) model was obtained from a tumor biopsy of a patient
86 with newly diagnosed ALK+ ALCL and cells were engraved subcutaneously in NSG mice by the team
87 of D. Sibon. ALK+ ALCL cell lines and patient-derived xenograft (PDX) were cultivated in RPMI
88 (Invitrogen) medium supplemented with 20% heat inactivated Fetal Bovine Serum (GIBCO).

89 **CRISPR/Cas9 transfection and translocation frequency**

90
91 T lymphocytes were transfected at 5 to 7 days post CD3/CD28 activation, with the RNP/Cas9 complex
92 using the 4D Nucleofector Amaxa technology (Lonza) (using the gRNA^{NPM} and gRNA^{ALK} and the Cas9
93 protein (quantity ratio 2:1) and as described in [19]). IL-2 (40U/mL) was added in the media once at the
94 time of transfection but never used afterwards. Transfected cells were long term maintained in 20%
95 heat-inactivated FBS complemented RPMI medium. gRNA sequences are listed in Supplementary
96 Materials.
97

98

1 99

2

3 100 **TCR analysis**

4
5 101 TCR γ analysis was performed as previously described [20].

6
7 102

8 103 **PCR-based translocation detection and frequency**

9
10 104 Serial dilutions of DNA from transfected cells enable the assessment of translocation frequency as
11
12 105 previously described in Supplementary Reference [21]. Primer sequences are listed in Supplementary
13
14 106 Materials.

15
16 107

17 108 **NGS breakpoint junction sequencing**

18
19 109 The first-round of PCR was performed using primers containing adapter sequences. The second round
20
21 110 of PCR was performed using primers containing barcode sequences. PCR products were sequenced
22
23 111 using 2x100 cycles (paired-end reads, 100 nucleotides) on the Illumina NovaSeq6000 instrument
24
25 112 (Illumina). Sequences were analyzed using previously described software to identify indels and
26
27 113 microhomology [22] and we analyzed junction sequences with at least 2 reads. Primer sequences are
28
29 114 listed in Supplementary Materials.

30 115

31 32 116 **RNA-seq and bioinformatics analysis**

33
34 117 Sequencing was carried out using 2x100 cycles (paired-end reads, 100 nucleotides) for all samples on
35
36 118 the Illumina NovaSeq6000 instrument. Reads were quantified with salmon v0.14.1 (genome GRCh38)
37
38 119 and differential analysis was performed using the R package DESeq2 (R version 4.0.3 and DESeq2
39
40 120 version 1.30.1). No statistical methods were used to predetermine sample size. RNAseq experiments
41
42 121 were performed in triplicates. All GSEA analyses (version 4.1.0) were performed using the pre-ranked
43
44 122 mode because of the weak number of samples for each condition in data coming from the model. In
45
46 123 order to identify genes harboring a strong progressive up-regulation in the model's dataset, genes that
47
48 124 harbored an overexpression associated to a LFC2 higher than 2 (and obligatorily associated with an
49
50 125 adjusted p-value lower than 5%) both between conditions WT (wild type) and NPM-ALK *in vitro* as
51
52 126 well as between conditions NPM-ALK *in vitro* and NPM-ALK *in vivo* were selected.

50 127

51 52 128 **Animal experiments**

53
54 129 NSG immunodeficient mice (NOD.Cg-Prkdc(scid) Il2rg(tm1Wjl)/SzJ (the Jackson Laboratory, Bar
55
56 130 Harbor, ME, USA) were maintained at the Gustave Roussy preclinical facility and NOD/SCID Gamma
57
58 131 (NSG NOD-prkdcscid) mice (Janvier Labs) for subcutaneous experiments were housed at the CRCT
59
60 132 facility. For xenograft tumor assay, a total of 3×10^6 ALKIma1 cells were injected subcutaneously into

60

61

62

63

64

65

133 both flanks of 5-week-old female NSG mice as described [7]. For intravenous injections, 8 to 12-weeks
1 134 old NSG mice were irradiated at 1.5 Gy, and 0.7 to 3 million human cells were injected intravenously
2
3 135 (i.v.). Disease progression was monitored by flow cytometry of mouse peripheral blood drawn
4
5 136 periodically by submandibular bleeds. Mice were sacrificed when engraftment reached at least 30% or
6
7 137 upon reaching a defined disease endpoint.

8 138

9
10 139 **Histological analysis**

11 140 Subcutaneous tumors or organs were excised and sections were fixed in 10% neutral buffered formalin
12
13 141 and embedded in paraffin for staining with H&E. For histological analyses, sample organs were stained
14
15 142 with hematoxylin and eosin. Briefly, the slides were heat-treated for antigen retrieval using CC1 buffer
16
17 143 (pH8) and incubated with pre-diluted primary antibodies to anti-ALK1 (clone ALK-01), anti-CD30
18 144 (clone Ber-H2), anti-CD4 (clone SP35) and anti-CD3 (clone 2GV6) (all from Ventana, Roche
19
20 145 Diagnostics) and anti-ROR2 (Abcam #ab218105). Epitopes were subsequently visualized using the
21
22 146 OptiView DAB detection method (Ventana, Roche Diagnostics) and nuclei were counterstained with
23 147 haematoxylin. For interpretation, the slides were evaluated by light microscopy.

24
25 148

26 149 Additional materials and methods are described in the supplemental Material.

27
28 150

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

61

62

63

64

65

151 RESULTS

152 Generation of t(2;5)(p23;q35) in human primary T lymphocytes induces efficient growth 153 and proliferation of cells through endogenous NPM-ALK activation.

154 To recapitulate the t(2;5)(p23;q35) translocation *in situ*, CD3 and CD28 activated T
155 lymphocytes isolated from peripheral blood mononuclear cells (PBMCs) from healthy donors were
156 transfected with the ribonucleic protein (RNP)/Cas9 complexes with gRNA^{NPM1} (targeting the *NPM1*
157 gene) and gRNA^{ALK} (targeting the *ALK* gene) (**Fig. 1A**). Transfected cells were maintained in IL-2-free
158 medium for long-term culture. A total of 15 independent donors were used in this study (**Supplementary**
159 **Table S1**). This strategy led to efficient generation of *NPM-ALK* genomic fusions (translocation
160 frequency > 1.7 % at day 5 post-transfection) (**Fig. 1B**). In control lymphocytes, we observed rapid cell
161 proliferation arrest in culture medium without addition of IL-2 as previously reported [23] (**Fig. 1C** and
162 **Supplementary Fig. S1A**). By contrast, CD4⁺ and CD8⁺ activated T lymphocytes treated with
163 gRNA^{NPM1} and gRNA^{ALK} started to proliferate 12 to 15 days after transfection (**Fig. 1C** and
164 **Supplementary Fig. S1A**). Accordingly, translocation frequency rapidly increased to 100%, thereby
165 demonstrating positive selection of cells carrying the t(2;5)(p23;q35) translocation (hereafter referred to
166 as “NA cells”) (**Fig. 1B**). We obtained a similar result using a different pair of gRNAs, thus indicating
167 that cell proliferation and positive selection were due to the translocation (**Supplementary Fig. S1B**).
168 We confirmed the formation of the t(2;5)(p23;q35) chromosomal translocation in every NA cell by
169 fluorescence *in situ* hybridization (FISH) analysis (**Fig. 1D**). NA cells expressed a functionally active
170 form of NPM-ALK (constitutive ALK and STAT3 phosphorylation) (**Fig. 1E**).

171 We further confirmed the efficacy of our approach by sequencing the breakpoint junctions from
172 the pool of NA cells. Strikingly, approximately 100 distinct fusion sequences were amplified at day 5,
173 which were still detected at day 15 post-transfection, thereby demonstrating the generation of highly
174 polyclonal cultures (**Fig. 1F** and **Supplementary Table S2**). The breakpoint junctions showed small
175 deletions/insertions (median size: -4 bp for Der2 and +4 bp for Der5) with few microhomologies
176 (median size of microhomology: < 2 bp, **Fig. 1G** and **Supplementary Table S2**), typical of the classical
177 non-homologous end joining repair pathway, the predominant translocation mechanism in human cells
178 after double-strand breaks [24] (**Fig. 1G, Supplementary Table S2**). TCR analysis of DNA from NA
179 cells at day 15 showed multiple TCR peaks and, together with the variety of breakpoint junctions
180 detected, ascertained the polyclonal nature of the edited NA lymphocytes (**Fig. 1F to I**). NA cells from
181 the four donors were maintained in culture medium without IL-2 for three months. At this time point, 1
182 to 2 different translocation breakpoint junctions were detected, indicating an expected selection of a few
183 clones during culture (**Supplementary Fig. S1C**). Furthermore, 13 experiments from independent
184 donors led to positive selection and expansion of NA cells for at least 1.5 months (summarized in
185 **Supplementary Table S1**), while viable control cells were not detected after 18 days. Overall, the

186 results demonstrate that this strategy leads to quick, reproducible and efficient CRISPR/Cas9-mediated
187 engineering of the t(2;5)(p23;q35) in pre-activated human primary T lymphocytes.

189 **A t(2;5)(p23;q35) engineered cell line (ALKIma1) represents a novel model of ALK+ ALCL**

190 We next transfected purified CD4+ lymphocytes, which represent the most typical tumor
191 phenotype in ALK+ ALCL, and were able to express active NPM-ALK protein as early as 6 days post-
192 transfection (**Fig. 2A**). We established a stable cell line from Donor #11 (hereafter referred to as
193 ALKIma1 cells, **Supplementary Table S1**). ALKIma1 cells grew for more than 100 days, presented
194 with increased phosphorylation of NPM-ALK (**Fig. 2A**), typical ALCL morphology with large cells and
195 a large nucleus (**Fig. 2B**) and showed robust telomerase activity, in agreement with the immortalized
196 phenotype (**Supplementary Fig. S1D**).

197 Cytogenetic analysis of ALKIma1 cells revealed a nearly diploid karyotype with the typical reciprocal
198 t(2;5)(p23;q35) translocation (**Fig. 2C**). After four months in culture, ALKIma1 cells, which are CD4+
199 and CD3-, displayed high levels of CD30 (**Fig. 2D**). These cells were clonal with a single sequence for
200 each translocation breakpoint and a clonal TCR (**Fig. 2E**). Importantly, ALKIma1 cells expressed
201 functional p53 with G1/S checkpoint activation (**Supplementary Fig. S1E, F**) as found in most ALK+
202 ALCL patient tumors (p53 is mutated in <10% of cases) [25]. We also showed functional activation of
203 the downstream STAT3 pathway (**Fig. 2A, F**) and inhibition of NPM-ALK phosphorylation leading to
204 cell death upon treatment with crizotinib, a specific ALK inhibitor used in the clinic (**Fig. 2F, G**) [26].

205 To evaluate whether the ALKIma1 cell line gives rise to tumors *in vivo*, we performed
206 subcutaneous xenotransplantation in immunodeficient NSG mice (**Fig. 2H**). Rapid tumor growth was
207 observed in recipient animals (6 tumors, 3 mice). All mice exhibited skin nodules without dermis and
208 subcutis hyperplasia (**Supplementary Fig. S1G**). Histological analysis of the subcutaneous tumors
209 showed ALK+ ALCL cell morphology (primarily common type cells with a group of small cells).
210 Immunohistochemistry (IHC) experiments confirmed robust nuclear and cytoplasmic expression of
211 ALK typical of the NPM-ALK translocation and CD30 expression (**Fig. 2I**). The cells were CD4+,
212 primarily CD3-, CD2+, CD5+, CD7+, CD20-, GranzymeB- and Perforin+, a representative phenotype
213 of ALK+ ALCL tumors (**Fig. 2I** and **Supplementary Fig. S1H**). These results show that the
214 CRISPR/Cas9-generated ALKIma1 cell line represents a novel and *bona fide* cellular model of ALK+
215 ALCL tumors.

217 **Induction of t(2;5)(p23;q35) recapitulates the phenotypic diversity observed in ALK+ ALCL** 218 **patients**

219 ALK+ ALCL patients are characterized by various immune phenotypes. Most tumors are CD4+,
220 although tumors can also exhibit a CD8+ phenotype (10%), a null T-cell or a CD4+CD8+ phenotype
221 (rare). During *in vitro* expansion of mixed CD4+ and CD8+ T lymphocyte populations, CD8+ cells were
222 selected overtime (**Fig. 3A** and **Supplementary Fig. S2A**). NA cells obtained after editing of unsorted

223 CD4+ and CD8+ activated lymphocytes showed a similar bias toward CD8+ NA cell selection (**Fig. 3A**,
224 **Supplementary Fig. S2B**). Interestingly, both CD4+ and CD8+ NA cells showed a similar increased
225 expression of the marker CD30 at 10 days post-transfection, while wild type lymphocytes displayed
226 decreased CD30 expression levels (**Fig. 3B**). We also observed a CD3- and CD30+ specific cell
227 population reminiscent of typical ALK+ ALCL cells (**Fig. 3C**). Furthermore, we detected a CD4-CD8-
228 cell population mostly comprised of CD30+ cells (**Fig. 3C**), which is a phenotype also found in primary
229 ALCL tumors [27]. According to our results, editing of primary human T lymphocytes led to a variety
230 of immune phenotypes, as found in ALK+ ALCL patients. After CD4+ cell sorting and transfection with
231 gRNA^{NPM1} and gRNA^{ALK}, we also obtained a collection of CD4+ NA cells from independent experiments
232 (e.g., NA1, NA2 and NA3 cells, **Fig. 3D, E** and **Supplementary Fig. S3A**). Overall, we were able to
233 recapitulate *in vitro* most of the different immune phenotypes found in ALK+ ALCL.

234 235 **Early polyclonal NPM-ALK-translocated T lymphocytes induce *in vivo* disease development with** 236 **diverse phenotypes, including CD8+ tumors**

237 To avoid possible clonal selection bias upon long-term *in vitro* culture, we injected NSG
238 recipient mice with NA cells early after transfection, when engineered NA cells were polyclonal with
239 heterogeneous expression of the various markers. We performed intravenous injections into mice to
240 assess the capacity of our model to yield disseminated disease *in vivo* (**Fig. 4A**). First, we injected the
241 NA1, NA2 and NA3 cells into mice after one month in culture (**Fig. 3D, E**). At the time of injection,
242 the NA1 and NA3 cells were CD4+ with a small population of CD30+ cells, while the NA2 cells were
243 CD30- with a small population of CD4+CD8+ cells (**Fig. 3E**). CD45+ human cells were detected in the
244 blood samples as soon as six weeks post-injection, with the exception of the NA3 cells (**Supplementary**
245 **Fig. S3B**). In terms of latency, mice injected with NA1 and NA2 cells (primary and secondary
246 recipients) developed tumors that reached ethical endpoint criteria within 86-88 days (**Fig. 4B**). The
247 NA3 cells did not cause disease in any recipient mouse (7/7) during the follow-up at 14 months
248 (**Supplementary Table S1**). Interestingly, the mice injected with NA1 and NA2 cells developed
249 disseminated disease in lymph nodes and extranodal organs (liver, spleen and lungs) (**Fig. 4C, E**,
250 **Supplementary Fig. S3C to E**). We also observed skin involvement as reported in ALK+ ALCL
251 patients (**Fig. 4E** and **Supplementary Fig. S4A**). Tumor cells isolated from the various organs were
252 primarily CD3- but expressed ALK (high expression levels; both nuclear and cytoplasmic), CD30 and
253 PDL1 (**Fig. 4C, 4E**, and **Supplementary Fig. S3D, E**). Interestingly, the NA2 cells [initially CD30-
254 (**Fig. 3E**)] also yielded tumors with high levels of CD30 expression (**Supplementary Fig. S3E**). Tumor
255 cells from NA1 cells that were reinjected into secondary recipients led to tumor development in
256 lymphoid organs (**Fig. 4A, 4B** and **Supplementary Fig. S3F**).

257 Morphologically, mice developed tumors and skin nodules with a dense infiltrate of both small
258 and large cells, which is referred to as mixed pattern in human pathology. Large cells had the attributes

259 of so-called “hallmark” ALCL cells with a kidney-shaped nucleus and abundant cytoplasm. Small cells
1 260 exhibited an irregular chromatic nucleus centrally located within a pale cytoplasm, referred to as “fried
2 261 egg” cells (example of H&E staining in **Fig. 4E** and **Supplementary Fig. S4A**). Histological analysis
3 262 confirmed high expression levels of the CD30 marker (**Fig. 4E, Supplementary Fig. S4A**). The NA1
4 263 and NA2 cells were oligoclonal at the time of injection, as shown by TCR analysis (**Supplementary**
5 264 **Fig. S4B**). Unexpectedly, the NA2 cells led to CD4+CD8+ tumor formation in one of the mice
6 265 (**Supplementary Fig. S4A**), which probably arose from the few double positive cells present at the time
7 266 of injection (**Fig. 3E**).

13 267 We then injected NA cells as early as 15 days after transfection, when 100 clones were detected
14 268 (**Fig. 1F**) (hereafter referred to as NA_E cells, “-E” for early, **Fig. 4A**). For this experiment, we injected
15 269 either a mixed population of CD4+ cells and CD8+ cells (NA4_E) or a pure population of CD8+ cells
16 270 (NA5_E^{CD8}) (**Supplementary Fig. S5A**). High translocation frequency was noted at the time of injection
17 271 (**Supplementary Fig. S5B**). We injected 0.7 million cells for each condition (Donors 14 and 15, n=3
18 272 and n=4 recipient mice, respectively, **Supplementary Table S1**). At 6-8 weeks post injection, human
19 273 CD45+ cells were detectable in the blood of 2/3 NA4_E and all four NA5_E^{CD8} recipient mice
20 274 (**Supplementary Fig. S5C**). All CD45+ mice developed disseminated disease; the mice reached the
21 275 ethical endpoint criteria within 118 days (**Fig 4B, F, G, Supplementary Fig. S5D**). One mouse injected
22 276 with NA4_E cells (#925, **Fig. 4F** and **Supplementary Fig. S6A**) developed exclusively a CD4+
23 277 phenotype, while the other mouse (#923, **Supplementary Fig. S5D** and **S6A**) exhibited a mixed
24 278 phenotype in the spleen (with CD8+CD4- and CD8-CD4+ cell infiltration) and pure CD8+CD4- cell
25 279 infiltration in the lymph nodes (**Supplementary Fig. S5D**). Notably, DNA extracted from the spleen
26 280 from this latter mouse revealed two different fusion points, thus indicating engraftment of independent
27 281 clones (**Supplementary Fig. S6B**). All four mice injected with NA5_E^{CD8} cells developed CD8+CD4-
28 282 tumors with either CD3- cells or CD3+ cells, as observed in CD8+ ALCL tumor cells [28] (**Fig. 4F, G,**
29 283 **Supplementary Fig. S5D**). This result indicates that NA cells can yield *de novo* CD8+ tumors as found
30 284 in 10% of patients, which constitutes a unique model of CD8+ ALK+ ALCL tumorigenesis.

285 286 **Expression profiles from NPM-ALK translocated models reveal activation of canonical and non- 287 canonical WNT pathways in ALK+ ALCL patient samples**

288 To gain insight into the molecular mechanism of NPM-ALK-mediated transformation, we
289 analyzed the transcriptomes at an early *in vitro* stage and in *in vivo* models. We performed RNAseq
290 analysis of three groups: 1-*in vitro* wild type CD4+ activated lymphocytes [n=3], 2-*in vitro* NPM-ALK-
291 engineered CD4+ lymphocytes [n=3] and 3-*in vivo*-derived CD4+ lymphoma cells purified by flow
292 cytometry to isolate CD3+ [n=3] and CD3- populations [n=3]. Principal component analysis clustering
293 showed that the three groups could be clearly segregated (**Fig. 5A**). Principal component analysis
294 focused on CD3+ and CD3- tumor cells could discriminate CD3+ cells from CD3- cells but mostly on
295 the second axis (**Supplementary Fig. S7A, Supplementary Tables S3 and S4**) and CD3- cells showed

296 enrichment primarily in cell cycle gene sets (**Supplementary Fig. S7B**). Based on these minor
297 differences between CD3⁻ and CD3⁺ cells, gene signatures from *in vivo* models were later assessed by
298 combining CD3⁻ and CD3⁺ samples. Using differential gene lists, we determined whether the molecular
299 basis of the models resembled that of ALK⁺ ALCL patients by performing reciprocal enrichment
300 analyses using transcriptome data from our models and published ALK⁺ ALCL patient data [7]. First,
301 we assessed upregulated or downregulated gene signatures obtained from our models (gene lists of the
302 200 most deregulated genes) via gene set enrichment analysis (GSEA) on transcriptomes from ALK⁺
303 ALCL patients compared with reactive lymph nodes (controls). Consistently, both *in vitro* and *in vivo*
304 upregulated gene signatures showed significant enrichment in the ALK⁺ ALCL patient samples, and
305 both downregulated gene signatures showed enrichment in the reactive lymph node samples (**Fig. 5B**).
306 Furthermore, the 200 most upregulated genes in the ALK⁺ ALCL patient samples (compared with
307 reactive lymph nodes) were also enriched *in vitro* and *in vivo* in NA cells (**Fig. 5C**).

308 To identify commonly deregulated pathways in *in vitro* cells, *in vivo* models and patient
309 samples, we intersected the significantly enriched Reactom and Hallmark gene lists for each of the three
310 groups compared with the respective controls (**Fig. 5D, Supplementary Tables S5 to S8**). The WNT
311 signaling pathway and WNT genes showed consistent enrichment in both NA models and patients (**Fig.**
312 **5D, E**). Among the "extracellular matrix" gene list (**Fig. 5D**), 29 genes primarily encoding collagens
313 and metalloproteinases were commonly enriched in the three groups (**Supplementary Fig. S7C** and
314 **Supplementary Table S9**). The ALK⁺ cells and a PDX model displayed β -CATENIN translocated
315 in the nucleus, indicating functional activation of the canonical WNT pathway (**Fig. 5F**). We found that
316 canonical and non-canonical WNT pathway gene sets were enriched in NA cells and patient cells
317 compared with controls (**Fig. 5G** and **Supplementary Fig. S7D**), including canonical (e.g. CTNNB1,
318 FDZs) and non-canonical (e.g. ROR2, RAC1) WNT pathway genes. Overall, transcriptomes from our
319 models ascertained that they comparably reproduced molecular characteristics of ALK⁺ ALCL patient
320 cells and displayed global upregulation of the WNT pathways.

321 **ROR2 is a robust marker of ALK⁺ tumor cells in ALK⁺ ALCL patients**

322 We hypothesized that genes significantly upregulated during the early steps of transformation
323 (*in vitro* NA models) and further upregulated in fully transformed cells (*in vivo* NA models) would
324 provide insight into key pathological pathways and potential therapeutic targets. To identify such
325 upregulated genes, we applied a twofold enrichment threshold to compare *in vitro* NA cells vs. activated
326 T lymphocytes and a subsequent twofold threshold to compare *in vivo* vs. *in vitro* NA cells. Accordingly,
327 we identified 24 genes that were consistently upregulated upon transformation (**Fig. 6A**). Importantly,
328 expression of these 24 genes efficiently distinguished ALK⁺ ALCL patients apart from controls (**Fig.**
329 **6B**), thus demonstrating that this molecular signature established from our models was relevant to
330 identify ALK⁺ ALCL samples. The transmembrane ROR2 surface receptor was identified as clearly
331

332 upregulated (**Fig. 6A, 6B** and **Supplementary Fig. S7E**). ROR2 protein is nearly absent from adult
1 333 tissues [29] and only weakly expressed in activated T lymphocytes (**Fig. 6A to 6D**). Using western blot
2 334 analysis, we confirmed ROR2 expression in ALK+ ALCL in the ALKIma1 cell line and various ALK+
3 335 ALCL cells, including a PDX model (**Fig. 6C**). Interestingly, NA cells expressed ROR2 at early stages
4 336 during transformation (at 16 days, **Fig. 6D**). Immunofluorescence experiments show that ROR2 is
5 337 markedly expressed at the membrane in the ALKIma1 cell line and PDX patient cells (**Fig. 6E**). As
6 338 shown from our RNAseq data, all patient samples (39/39) expressed significantly increased levels of
7 339 ROR2 (**Fig. 5H, 6B**). ROR2 is implicated in the WNT pathways and has been reported to drive both the
8 340 canonical and non-canonical WNT pathways in cancer cells [30]. Interestingly, the WNT5B, WNT7B
9 341 and WNT11 ligands were also upregulated in patients (**Fig. 5E**), which positively correlated with ROR2
10 342 expression levels (**Supplementary Fig. S7F**).

18 343 As ALCL diagnosis is largely based on histological analysis, we performed IHC assessment of
19 344 ROR2 expression in spleen and lymph node sections derived from our NA models. We observed robust
20 345 ROR2 expression with marked membrane expression (**Fig. 5F, Supplementary Fig. S7G**). We
21 346 performed ROR2 IHC on biopsies from two independent cohorts of ALCL patient samples (cohort #1:
22 347 P1 to P27 and cohort #2: P28 to P44) for which the ALK status was established using IHC and/or
23 348 RNAseq. The samples were derived from an unselected cohort of ALCL patients (P1-P27) or from a
24 349 cohort of patients with aggressive refractory disease (P28-P44). The analysis revealed positive
25 350 expression of ROR2 in 40/44 patient samples (**Table 1**). In agreement with our expression data, nearly
26 351 all ALK+ ALCL samples were ROR2 positive (37/38), with clearly discernible expression in the
27 352 membrane. A large proportion of tumor cells expressed ROR2 (often 80% to 100% for 30/38 samples)
28 353 and expression intensity was ranked from null (0) to high (3)(**Fig. 6G, Table 1 and Supplementary**
29 354 **Fig. S7H**). All chemotherapy-resistant patient samples displayed ROR2 expression. Furthermore, ROR2
30 355 expression was observed in four patient samples harboring alternative ALK fusions with other or
31 356 unknown ALK partners (**Fig. 6G and Table 1**), thus indicating that ROR2 is a genuine marker of ALK+
32 357 ALCL regardless of the fusion partner. Finally, three of the six tumor samples that did not express ALK
33 358 were positive for ROR2, including two of four ALK- ALCL samples, thus indicating that aberrant ROR2
34 359 expression represents a marker for other types of ALK- human lymphoma (**Fig. 6G and Table 1**).

46 360 According to the human expression data from the human cell atlas immune system
47 361 (www.Immgen.org), we confirmed that a variety of immune cells express low levels of ROR2
48 362 (**Supplementary Fig. S8A**). Reanalysis of published ROR2 expression datasets (GSE6338, GSE14879,
49 363 GSE19069 and GSE65823)) displayed higher ROR2 expression levels in ALK+ ALCL samples
50 364 compared with other peripheral T-cell lymphoma samples (**Supplementary Fig. S8B to D**). We
51 365 hypothesized that samples in the dataset GSE19069, which are characterized by reduced ROR2
52 366 expression levels in ALK+ ALCL, were comprised of tissues with fewer tumor cell infiltrates
53 367 (**Supplementary Fig. S8D**). Further supporting our ALK- ALCL histology data, increased ROR2
54 368 expression was recurrently found in other independent ALK- ALCL patient samples (**Supplementary**

369 **Fig. S8 D to F)** and in a few other T cell lymphomas (**Supplementary Fig. S8D**). In particular, an
1 370 independent dataset displayed >50% of ALK- ALCL with high ROR2 expression without concomitant
2
3 371 higher ALK expression levels (**Supplementary Fig. S8E**), further indicating that ROR2 is an
4
5 372 independent marker for human ALCL.

6 373 Overall, these results demonstrate that the molecular characterization of the evolution from
7
8 374 normal T lymphocytes to *in vivo* NA models enabled the identification of ROR2 as a relevant cell surface
9
10 375 marker of ALK+ ALCL tumor cells.

11 376

12

13 377 **DISCUSSION**

14 378 *De novo* generation of the pathognomonic t(2;5)(p23;q35) translocation presents significant
15 379 advantages over other overexpression model approaches, as fusion oncogene expression is controlled
16 380 by the human chromatin environment and subjected to endogenous regulatory elements upon
17
18 381 transformation. These NA models provide a reliable framework to analyze cells from the initial process
19
20 382 of oncogenic chromosomal rearrangement including the early steps of transformation, with pre-
21
22 383 transformed cells as early as 15 days (**Fig. 1B, C**).

23 384 Generation of the NPM-ALK translocation enabled reproduction of the heterogeneous ALK+
24
25 385 ALCL tumor phenotype. Interestingly, the panel of independent ALK+ ALCL tumors (early injections
26
27 386 in mice) did not always yield *in vitro* stable NA cell lines (**Supplementary Table S1**). While *in vitro*
28
29 387 NA cells were maintained independently with various CD30 levels, all tumors expressed high levels of
30
31 388 CD30. These data indicate a strong positive *in vivo* selection of CD30. Interestingly, expression data
32
33 389 from CD3+ and CD3- populations within the same tumor revealed that cell cycle genes were enriched
34
35 390 in CD3- cells, which is associated with increased proliferative activity rather than a differentiation state.
36
37 391 Furthermore, we observed CD8+ tumors that reflected the oncogenic potential of the NPM-ALK-
38 392 associated translocation in CD8+ T lymphocytes.

39
40 393 Additionally, these models displayed several other clinical features observed in ALK+ ALCL patients.
41
42 394 Indeed, patients most often develop tumors in lymph nodes, followed by extranodal sites (60%) and
43 395 typical skin lesions (up to 25% for pediatric ALK+ ALCL) [1, 2]. Accordingly, mice injected with NPM-
44
45 396 ALK translocated cells developed tumors in lymph nodes, splenomegaly, lung infiltrations and skin
46
47 397 nodules. Finally, tumor cells from NA models also recapitulated the various cellular morphologies
48
49 398 observed in patients including large ALCL common type cells, with a group of small cells. Of note,
50 399 consistent with the lack of recurrent additional genetic alteration reported in ALK+ ALCL patients,
51
52 400 analysis of variants using RNAseq data from our NA murine tumors did not identify obvious additional
53
54 401 driver events using the Cancer Genome Interpreter. Indeed, while some putative driver events were
55 402 found, it is worth noting that none of the alterations are recurrent and most of the variants correspond to
56
57 403 known SNP listed in dbSNP or other databases (see **Supplementary Table S10**). These results suggest
58 404 that recurrent additional mutation is not required for NPM-ALK tumorigenesis in these models.

405 Molecular analysis of these models revealed that the activation of both the canonical and non-
1 406 canonical WNT pathways is relevant to ALK+ ALCL patient tumors. The WNT pathway, which
2 407 coordinates multiple biological processes such as development, stemness, tissue regeneration and
3 408 homeostasis, has been associated with metastasis, cancer stem cells and immune control [31-34].
4 409 Simultaneous activation of the two WNT pathways has been reported in colon cancer cells [35], and the
5 410 WNT/ β -catenin pathway is upregulated in a highly tumorigenic subpopulation of ALK+ ALCL cell
6 411 lines [36]. Our data further indicate that an imbalance in WNT pathway activation occurs in ALK+
7 412 ALCL. Evidence has shown that the WNT pathways acts in oncogenic signaling to promote immune
8 413 evasion in tumors [37, 38]. Overactivation of the WNT pathway in ALK+ ALCL tumors could inhibit
9 414 the activity of therapeutics, such as anti-PD1 therapy (now in clinical trials) as suggested for melanoma
10 415 treatment [37]. Furthermore, cancer-specific WNT inhibitors are under development [39] and could
11 416 benefit ALK+ ALCL patients.

12 417 Analyzing our models, we identified a gene signature of 24 upregulated genes that efficiently
13 418 distinguished ALK+ ALCL patient samples from controls. The ROR2 membrane receptor was highly
14 419 upregulated in 100% of patient samples, which was validated by histological assessment. ROR2 has
15 420 been shown to act in both canonical and non-canonical pathways [30]. Notably, we found that several
16 421 WNT ligands were upregulated in ALK+ ALCL cells, which positively correlated to ROR2 expression
17 422 levels (**Fig. 5E, Supplementary Fig. S7F**). WNT5b and WNT11 have been shown to regulate the non-
18 423 canonical WNT pathway in non-hematological cancers (for review see [32]). WNT5B has been shown
19 424 to interact with ROR2 in osteosarcoma [40], while preliminary results indicate that WNT11 could be a
20 425 ligand of ROR2 in breast cancer [41]. These data suggest that alteration of both WNT ligands and their
21 426 respective receptors may provide an autocrine mechanism to maintain lymphoma cells. ROR2 has been
22 427 shown to control cell migration in mice and *C. Elegans* [42, 43]. Overexpression of ROR2 in
23 428 osteosarcoma [40], melanoma [44] and breast cancer [45] has been proposed to play a role in cell
24 429 migration, cell proliferation and spatiotemporal control of tumor cell heterogeneity [46]. Indeed,
25 430 deciphering the function of ROR2 and its relationship with WNT pathway activation in migration and
26 431 metastatic processes would provide further insight into ALK+ ALCL pathophysiology.

27 432 ROR2 is a membrane receptor with a very restricted pattern of expression, which is increased
28 433 during early embryogenesis and decreased during ontogeny to almost undetectable levels in most adult
29 434 organs [30]. Thus, the distinct ROR2 expression pattern in ALK+ ALCL patient samples indicates that
30 435 ROR2 and downstream oncogenic pathways may represent potential therapeutic targets. This study also
31 436 highlights the potential to use ROR2 as an immune marker to target ALCL cells. In this regard, the
32 437 current phase 1-2 trials of ADC antibody and CART targeting ROR2 for other tumor types (e.g., NSCLC
33 438 and ovarian tumors: ClinicalTrials.gov #NCT03504488, NCT03960060, NCT03393936, [30]) may also
34 439 benefit ALK+ ALCL patients.

40 441 CONCLUSION

442 In conclusion, this work demonstrates that *ab initio* modeling of NPM-ALK specific
1 443 chromosomal translocation in mature T lymphocytes provides unique models to identify novel
2
3 444 therapeutic targeting approaches. Our model enabled the identification of new candidate genes.
4
5 445 Remarkably, we found that ROR2 may represent a genuine biomarker of ALK+ ALCL that may be
6
7 446 targeted to treat patients.
8

9 10 448 **LIST OF ABBREVIATIONS**

11 449 **ALK:** Anaplastic lymphoma kinase

12
13 450 **ALK+ ALCL:** Anaplastic large cell lymphoma ALK positive

14
15 451 **NA cells:** Cells positive for the NPM-ALK-associated translocation

16 452 **PBMC:** Peripheral blood mononuclear cell

17
18 453 **CRISPR:** Clustered Regularly Interspaced Short Palindromic Repeats

19
20 454 **Cas9:** CRISPR-associated protein 9

21
22 455 **gRNA:** Guide RNA

23 456 24 25 457 **DECLARATIONS**

26 458 **Ethics approval and consent to participate**

27 459 Experimental procedures were specifically approved by the INSERM CEEI/IRB ethics committee (Avis
28
29 460 n°20-664). PBMCs were isolated from healthy donor blood (under agreement EFS C CPSL UNT N°
30
31 461 20/EFS/005). Animal care and use for this study were performed in accordance with the
32
33 462 recommendations of the European Community (2010/63/UE) for the care and use of laboratory animals.
34
35 463 All experiments were approved by the French National Animal Care and Use Committee (CEEA 26:
36
37 464 #2017122111548235_v2, the Gustave Roussy preclinical facility, Villejuif, France). All experiments
38
39 465 were approved by the French National Animal Care and Use Committee (APAFIS# 6725-
40 466 2016091415495765-v8, CRCT Toulouse France). The use of tissue samples has been approved by our
41
42 467 institutional ethics committee (patient collection samples: DC-2020-4074; AC-2020-4031 (CRB of
43 468 CHU Toulouse)).

44 469 45 470 46 47 471 **Consent for publication**

48
49 472 Not applicable

50 473 51 52 474 **Availability of data and materials**

53 475 RNA-seq data generated in this study were deposited in the ArrayExpress database (E-MTAB-10924).

54
55 476 RNA-seq data for ALCL patients were previously published [7].

56
57 477 Other raw data from this study are available on Mendeley reserved DOI: doi:10.17632/x5fyb55w9x.1
58
59
60

478

1 479 **Competing interests**

2
3 480 The following authors (EB, TM, LB, ER, AD, FG and LL) declare a European patent application for
4
5 481 “Methods For The Treatment Of Anaplastic Large Cell Lymphoma” (reference: BRUNET21072MC/
6
7 482 BTL, April 9, 2021, application number EP21305467, applicants INSERM, and IMAGINE Institute,
8 483 France).

9
10 484
11 485 **Funding**

12
13 486 This work was supported by INSERM, Institut National du Cancer and Cancéropôle Ile de France (grant
14
15 487 2016-1-PL BIO-07-1), Ligue Contre le Cancer [“Équipes Labellisées” to E. B. (PI: de Villartay) ,T.M.
16 488 and F.M.], Fondation ARC (M.P. and L.B.), Fondation pour la Recherche Médicale (Z.A., FRM-
17
18 489 ING20150532273). T.M. is supported by Sites de Recherche Intégrée sur le Cancer (SIRIC)-SOCRATE
19
20 490 (INCa-DGOS-INSERM 12551) and the PEDIAC consortium (INCA_15670).

21 491
22
23 492 **Authors' contributions**

24
25 493 L.B., A.D., Z.A., R.B., C.S., M.P. and A.C. carried out the experiments. E.R. performed the
26
27 494 bioinformatic analyses and processed the sequencing data. A.D.C., and C.G. produced and/or provided
28 495 key reagents. L.C. analyzed the karyotypes of the ALK1ma1 cells. L.L., O.B, C.R. and J.Y.S provided
29
30 496 and analyzed IHC patient samples. L.L and F.M. provided patient sequencing data. D.S, L.V. and V.A.
31
32 497 provided cells and valuable expertise. T.M. and E.B. originally conceived the project, designed the
33 498 experiments and supervised the work. L.B, A.D., T.M. and E.B. wrote the paper with the help of D.S.
34
35 499 and F.M., and all authors reviewed and agreed on the final manuscript.

36 500
37
38 501 **Acknowledgements**

39
40 502 We thank the Imagine sequencing (C. Bole), Imagine cytometry (O. Pellé), Imagine bioinformatic (C.
41
42 503 Masson) and Imagine microscopy (M. Garfa-Traore) platforms for helping with cell analysis and image
43 504 analysis. We thank Maxime Heintzé, Patrick Revy and Jean-Pierre de Villartay and each member of the
44
45 505 ‘Genome Dynamics in the Immune System’ lab for scientific discussions and technical support. We
46
47 506 thank Véronique Minard for support with clinical samples/data and Gustave Roussy flow cytometry
48 507 (PFIC) and preclinical (PFEP) platforms. The authors accessed data assembled by the ImmGen
49
50 508 consortium for this study.

51 509
52
53 510 **REFERENCES**

- 54
55 511 1. Mussolin L, Le Deley MC, Carraro E, Damm-Welk C, Attarbaschi A, Williams D, Burke A, Horibe
56 512 K, Nakazawa A, Wrobel G, et al: **Prognostic Factors in Childhood Anaplastic Large Cell**
57 513 **Lymphoma: Long Term Results of the International ALCL99 Trial.** *Cancers (Basel)* 2020, **12.**
58 514 2. Sibon D, Nguyen DP, Schmitz N, Suzuki R, Feldman AL, Gressin R, Lamant L, Weisenburger DD,
59 515 Rosenwald A, Nakamura S, et al: **ALK-positive anaplastic large-cell lymphoma in adults: an**
60 516 **individual patient data pooled analysis of 263 patients.** *Haematologica* 2019, **104**:e562-e565.

- 517 3. Morel A, Brière J, Lamant L, Loschi M, Haioun C, Delarue R, Tournilhac O, Bachy E, Sonet A,
1 518 Amorim S, et al: **Long-term outcomes of adults with first-relapsed/refractory systemic**
2 519 **anaplastic large-cell lymphoma in the pre-brentuximab vedotin era: A LYSA/SFGM-TC**
3 520 **study.** *Eur J Cancer* 2017, **83**:146-153.
- 4 521 4. Lowe EJ, Reilly AF, Lim MS, Gross TG, Saguilig L, Barkauskas DA, Wu R, Alexander S, Bollard
5 522 CM: **Brentuximab vedotin in combination with chemotherapy for pediatric patients with**
6 523 **ALK+ ALCL: results of COG trial ANHL12P1.** *Blood* 2021, **137**:3595-3603.
- 7 524 5. Swerdlow S: **WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.** 2017
8 525 6. Malcolm TI, Villarese P, Fairbairn CJ, Lamant L, Trinquand A, Hook CE, Burke GA, Brugieres L,
9 526 Hughes K, Payet D, et al: **Anaplastic large cell lymphoma arises in thymocytes and requires**
10 527 **transient TCR expression for thymic egress.** *Nat Commun* 2016, **7**:10087.
- 11 528 7. Congras A, Hoareau-Aveilla C, Caillet N, Tosolini M, Villarese P, Cieslak A, Rodriguez L, Asnafi
12 529 V, Macintyre E, Egger G, et al: **ALK-transformed mature T lymphocytes restore early thymus**
13 530 **progenitor features.** *J Clin Invest* 2020, **130**:6395-6408.
- 14 531 8. Pawlicki JM, Cookmeyer DL, Maseda D, Everett JK, Wei F, Kong H, Zhang Q, Wang HY, Tobias
15 532 JW, Walter DM, et al: **NPM-ALK-Induced Reprogramming of Mature TCR-Stimulated T Cells**
16 533 **Results in Dedifferentiation and Malignant Transformation.** *Cancer Res* 2021, **81**:3241-3254.
- 17 534 9. Amin HM, McDonnell TJ, Ma Y, Lin Q, Fujio Y, Kunisada K, Leventaki V, Das P, Rassidakis GZ,
18 535 Cutler C, et al: **Selective inhibition of STAT3 induces apoptosis and G(1) cell cycle arrest in**
19 536 **ALK-positive anaplastic large cell lymphoma.** *Oncogene* 2004, **23**:5426-5434.
- 20 537 10. Chiarle R, Simmons WJ, Cai H, Dhall G, Zamo A, Raz R, Karras JG, Levy DE, Inghirami G: **Stat3**
21 538 **is required for ALK-mediated lymphomagenesis and provides a possible therapeutic target.**
22 539 *Nat Med* 2005, **11**:623-629.
- 23 540 11. Zamo A, Chiarle R, Piva R, Howes J, Fan Y, Chilosi M, Levy DE, Inghirami G: **Anaplastic**
24 541 **lymphoma kinase (ALK) activates Stat3 and protects hematopoietic cells from cell death.**
25 542 *Oncogene* 2002, **21**:1038-1047.
- 26 543 12. Turner SD, Tooze R, Maclennan K, Alexander DR: **Vav-promoter regulated oncogenic fusion**
27 544 **protein NPM-ALK in transgenic mice causes B-cell lymphomas with hyperactive Jun kinase.**
28 545 *Oncogene* 2003, **22**:7750-7761.
- 29 546 13. Chiarle R, Gong JZ, Guasparri I, Pesci A, Cai J, Liu J, Simmons WJ, Dhall G, Howes J, Piva R,
30 547 Inghirami G: **NPM-ALK transgenic mice spontaneously develop T-cell lymphomas and plasma**
31 548 **cell tumors.** *Blood* 2003, **101**:1919-1927.
- 32 549 14. Jager R, Hahne J, Jacob A, Egert A, Schenkel J, Wernert N, Schorle H, Wellmann A: **Mice**
33 550 **transgenic for NPM-ALK develop non-Hodgkin lymphomas.** *Anticancer Res* 2005, **25**:3191-
34 551 3196.
- 35 552 15. Turner SD, Merz H, Yeung D, Alexander DR: **CD2 promoter regulated nucleophosmin-**
36 553 **anaplastic lymphoma kinase in transgenic mice causes B lymphoid malignancy.** *Anticancer Res*
37 554 2006, **26**:3275-3279.
- 38 555 16. Giuriato S, Foisseau M, Dejean E, Felsher DW, Al Saati T, Demur C, Ragab A, Kruczynski A, Schiff
39 556 C, Delsol G, Meggetto F: **Conditional TPM3-ALK and NPM-ALK transgenic mice develop**
40 557 **reversible ALK-positive early B-cell lymphoma/leukemia.** *Blood* 2010, **115**:4061-4070.
- 41 558 17. Zhang Q, Wei F, Wang HY, Liu X, Roy D, Xiong QB, Jiang S, Medvec A, Danet-Desnoyers G,
42 559 Watt C, et al: **The potent oncogene NPM-ALK mediates malignant transformation of normal**
43 560 **human CD4(+) T lymphocytes.** *Am J Pathol* 2013, **183**:1971-1980.
- 44 561 18. Cecccon M, Merlo MEB, Mologni L, Poggio T, Varesio LM, Menotti M, Bombelli S, Rigolio R,
45 562 Manazza AD, Di Giacomo F, et al: **Excess of NPM-ALK oncogenic signaling promotes cellular**
46 563 **apoptosis and drug dependency.** *Oncogene* 2016, **35**:3854-3865.
- 47 564 19. Sole A, Grossetête S, Heintzé M, Babin L, Zaïdi S, Revy P, Renouf B, De Cian A, Giovannangeli
48 565 C, Pierre-Eugène C, et al: **Unraveling Ewing Sarcoma Tumorigenesis Originating from Patient-**
49 566 **Derived Mesenchymal Stem Cells.** *Cancer Res* 2021, **81**:4994-5006.
- 50 567 20. Derriex C, Trinquand A, Bruneau J, Verkarre V, Lhermitte L, Alcantara M, Villarese P, Meresse
51 568 B, Sibon D, Hermine O, et al: **A Single-Tube, EuroClonality-Inspired, TRG Clonality Multiplex**
52 569 **PCR Aids Management of Patients with Enteropathic Diseases, including from Formaldehyde-**
53 570 **Fixed, Paraffin-Embedded Tissues.** *J Mol Diagn* 2019, **21**:111-122.
- 54 571 21. Renouf B, Piganeau M, Ghezraoui H, Jasin M, Brunet E: **Creating cancer translocations in human**
55 572 **cells using Cas9 DSBs and nCas9 paired nicks.** *Methods Enzymol* 2014, **546**:251-271.

- 573 22. Renaud JB, Boix C, Charpentier M, De Cian A, Cochennec J, Duvernois-Berthet E, Perrouault L,
1 574 Tesson L, Edouard J, Thinard R, et al: **Improved Genome Editing Efficiency and Flexibility Using**
2 575 **Modified Oligonucleotides with TALEN and CRISPR-Cas9 Nucleases.** *Cell Rep* 2016, **14**:2263-
3 576 2272.
- 4 577 23. Levine BL, Bernstein WB, Connors M, Craighead N, Lindsten T, Thompson CB, June CH: **Effects**
5 578 **of CD28 costimulation on long-term proliferation of CD4+ T cells in the absence of exogenous**
6 579 **feeder cells.** *J Immunol* 1997, **159**:5921-5930.
- 7 580 24. Ghezraoui H, Piganeau M, Renouf B, Renaud JB, Sallmyr A, Ruis B, Oh S, Tomkinson AE,
8 581 Hendrickson EA, Giovannangeli C, et al: **Chromosomal translocations in human cells are**
9 582 **generated by canonical nonhomologous end-joining.** *Mol Cell* 2014, **55**:829-842.
- 10 583 25. Rassidakis GZ, Thomaidis A, Wang S, Jiang Y, Fourtouna A, Lai R, Medeiros LJ: **p53 gene**
11 584 **mutations are uncommon but p53 is commonly expressed in anaplastic large-cell lymphoma.**
12 585 *Leukemia* 2005, **19**:1663-1669.
- 13 586 26. Mosse YP, Voss SD, Lim MS, Rolland D, Minard CG, Fox E, Adamson P, Wilner K, Blaney SM,
14 587 Weigel BJ: **Targeting ALK With Crizotinib in Pediatric Anaplastic Large Cell Lymphoma and**
15 588 **Inflammatory Myofibroblastic Tumor: A Children's Oncology Group Study.** *J Clin Oncol*
16 589 2017, **35**:3215-3221.
- 17 590 27. Juco J, Holden JT, Mann KP, Kelley LG, Li S: **Immunophenotypic analysis of anaplastic large**
18 591 **cell lymphoma by flow cytometry.** *Am J Clin Pathol* 2003, **119**:205-212.
- 19 592 28. Shen J, Medeiros LJ, Li S, Wang SA, Lin P, Khanlari M, Iyer SP, Yin CC, Tang G, Jorgensen JL,
20 593 et al: **CD8 expression in anaplastic large cell lymphoma correlates with noncommon**
21 594 **morphologic variants and T-cell antigen expression suggesting biological differences with**
22 595 **CD8-negative anaplastic large cell lymphoma.** *Hum Pathol* 2020, **98**:1-9.
- 23 596 29. Yoda A, Oishi I, Minami Y: **Expression and function of the Ror-family receptor tyrosine kinases**
24 597 **during development: lessons from genetic analyses of nematodes, mice, and humans.** *J Recept*
25 598 *Signal Transduct Res* 2003, **23**:1-15.
- 26 599 30. Menck K, Heinrichs S, Baden C, Bleckmann A: **The WNT/ROR Pathway in Cancer: From**
27 600 **Signaling to Therapeutic Intervention.** *Cells* 2021, **10**.
- 28 601 31. Azbazdar Y, Karabicici M, Erdal E, Ozhan G: **Regulation of Wnt Signaling Pathways at the**
29 602 **Plasma Membrane and Their Misregulation in Cancer.** *Front Cell Dev Biol* 2021, **9**:631623.
- 30 603 32. Chen Y, Chen Z, Tang Y, Xiao Q: **The involvement of noncanonical Wnt signaling in cancers.**
31 604 *Biomed Pharmacother* 2021, **133**:110946.
- 32 605 33. Gajos-Michniewicz A, Czyz M: **WNT Signaling in Melanoma.** *Int J Mol Sci* 2020, **21**.
- 33 606 34. Xu X, Zhang M, Xu F, Jiang S: **Wnt signaling in breast cancer: biological mechanisms,**
34 607 **challenges and opportunities.** *Mol Cancer* 2020, **19**:165.
- 35 608 35. Flores-Hernandez E, Velazquez DM, Castaneda-Patlan MC, Fuentes-Garcia G, Fonseca-Camarillo
36 609 G, Yamamoto-Furusho JK, Romero-Avila MT, Garcia-Sainz JA, Robles-Flores M: **Canonical and**
37 610 **non-canonical Wnt signaling are simultaneously activated by Wnts in colon cancer cells.** *Cell*
38 611 *Signal* 2020, **72**:109636.
- 39 612 36. Wu C, Zhang HF, Gupta N, Alshareef A, Wang Q, Huang YH, Lewis JT, Douglas DN, Kneteman
40 613 NM, Lai R: **A positive feedback loop involving the Wnt/ β -catenin/MYC/Sox2 axis defines a**
41 614 **highly tumorigenic cell subpopulation in ALK-positive anaplastic large cell lymphoma.** *J*
42 615 *Hematol Oncol* 2016, **9**:120.
- 43 616 37. Spranger S, Bao R, Gajewski TF: **Melanoma-intrinsic beta-catenin signalling prevents anti-**
44 617 **tumour immunity.** *Nature* 2015, **523**:231-235.
- 45 618 38. Luke JJ, Bao R, Sweis RF, Spranger S, Gajewski TF: **WNT/beta-catenin Pathway Activation**
46 619 **Correlates with Immune Exclusion across Human Cancers.** *Clin Cancer Res* 2019, **25**:3074-
47 620 3083.
- 48 621 39. Jung YS, Park JI: **Wnt signaling in cancer: therapeutic targeting of Wnt signaling beyond beta-**
49 622 **catenin and the destruction complex.** *Exp Mol Med* 2020, **52**:183-191.
- 50 623 40. Morioka K, Tanikawa C, Ochi K, Daigo Y, Katagiri T, Kawano H, Kawaguchi H, Myoui A,
51 624 Yoshikawa H, Naka N, et al: **Orphan receptor tyrosine kinase ROR2 as a potential therapeutic**
52 625 **target for osteosarcoma.** *Cancer Sci* 2009, **100**:1227-1233.
- 53 626 41. Menck K, Heinrichs S, Wlochowitz D, Sitte M, Noeding H, Janshoff A, Treiber H, Ruhwedel T,
54 627 Schatlo B, von der Brélie C, et al: **WNT11 is a novel ligand for ROR2 in human breast cancer.**
55 628 *bioRxiv* 2020:2020.2012.2018.423402.

- 629 42. Laird DJ, Altshuler-Keylin S, Kissner MD, Zhou X, Anderson KV: **Ror2 enhances polarity and**
1 630 **directional migration of primordial germ cells.** *PLoS Genet* 2011, **7**:e1002428.
- 2 631 43. Forrester WC, Kim C, Garriga G: **The Caenorhabditis elegans Ror RTK CAM-1 inhibits EGL-**
3 632 **20/Wnt signaling in cell migration.** *Genetics* 2004, **168**:1951-1962.
- 4 633 44. O'Connell MP, Fiori JL, Xu M, Carter AD, Frank BP, Camilli TC, French AD, Dissanayake SK,
5 634 Indig FE, Bernier M, et al: **The orphan tyrosine kinase receptor, ROR2, mediates Wnt5A**
6 635 **signaling in metastatic melanoma.** *Oncogene* 2010, **29**:34-44.
- 7 636 45. Guo M, Ma G, Zhang X, Tang W, Shi J, Wang Q, Cheng Y, Zhang B, Xu J: **ROR2 knockdown**
8 637 **suppresses breast cancer growth through PI3K/ATK signaling.** *Aging (Albany NY)* 2020,
9 638 **12**:13115-13127.
- 10 639 46. Roarty K, Pfefferle AD, Creighton CJ, Perou CM, Rosen JM: **Ror2-mediated alternative Wnt**
11 640 **signaling regulates cell fate and adhesion during mammary tumor progression.** *Oncogene*
12 641 2017, **36**:5958-5968.
- 13 642
- 14 643
- 15 644
- 16 645
- 17 646
- 18 647
- 19 648
- 20 649
- 21 650
- 22 651
- 23 652
- 24 653
- 25 654
- 26 655
- 27 656
- 28 657
- 29 658
- 30 659
- 31 660
- 32 661
- 33 662
- 34 663
- 35 664
- 36 665
- 37 666
- 38 667
- 39 668
- 40 669
- 41 670
- 42 671
- 43 672
- 44 673
- 45 674
- 46 675
- 47 676
- 48 677
- 49 678
- 50 679
- 51 680
- 52 681
- 53 682
- 54 683
- 55 684
- 56 685
- 57 686
- 58 687
- 59 688
- 60 689
- 61 690
- 62 691
- 63 692
- 64 693
- 65 694

644 **Table 1: IHC analysis of ROR2 in patient samples**

645 Tumor samples: P1 to P27 (cohort #1), P28 to P44 and controls 1 to 3 (cohort #2). The table indicates
 646 diagnosis, ALK status, the ALK fusion partner gene (when identified), percentage of tumor cells positive
 647 for ROR2, ROR2 staining intensity [from 0 (null) to 3 (high)] and cellular sublocalization of ROR2
 648 (cyto= cytoplasm; membr=membrane). CT: common type, SC: small cell ; LH: lymphohistiocytic ; HL
 649 : Hodgkin-like ; T-NOS: T lymphoma not otherwise specified, NA : non-applicable.

650 Cohort #1

PATIENTS (Fig. 6G)	Diagnostic	ALK	ALK partner	ROR2 intensity	Percentage of tumoral cells expressing ROR2	Localization
P1	ALCL CT	pos	NPM	3	100	Cyto
P2	ALCL CT+SC	pos	non NPM	1	80	Membr
P3	ALCL CT	pos	NPM	3	80	Cyto
P4	ALCL CT+SARC	pos	non NPM	3	100	Cyto
P5	ALCL CT	pos	NPM	3	100	Membr
P6	ALCL LH	pos	NPM	1	40	Cyto
P7	ALCL SC+CT	pos	NPM	2	100	Membr
P8	ALCL CT	pos	NPM	2	70	Cyto
P9	ALCL CT	pos	NPM	2	100	Cyto
P10	ALCL CT	pos	NPM	2	100	Membr
P11	ALCL CT	pos	NPM	3	100	Membr+Cyto
P12	ALCL HD+SC	pos	NPM	2	100	Membr
P13	ALCL CT	pos	NPM	1	100	Membr
P14	ALCL CT	pos	ALO17	2	100	Membr
P15	ALCL CT	pos	NPM	2	90	Cyto
P16	ALCL CT	pos	NPM	1	100	Cyto
P17	ALCL CT	pos	NPM	1	NA	
P18	ALCL SC+CT	pos	NPM	2	90	Membr
P19	ALCL SC	pos	NPM	1	50	Membr

Cohort #1

P20	ALCL CT	pos	NPM	2	NA	Cyto
P21	ALCL CT	pos	NPM	2	90	Cyto+membr
P22	ALCL CT+SC+LH	pos	NPM	1	40	Cyto
P23	ALCL CT	pos	TFG	3	100	Cyto
P24	ALCL SC	pos	NPM	0	0	NA
P25	ALCL CT	neg	0	0	0	NA
P26	ALCL CT	neg	0	3	90	Cyto
P27	T-NOS	neg	0	2	100	Cyto+membr

651

Cohort #2

PATIENTS	Diagnostic	ALK	ROR2 intensity	Percentage of tumoral cells expressing ROR2	Localization
P28	ALCL	pos	3	100	Membr+Cyto
P29	ALCL	pos	2	80	Membr
P30	ALCL	pos	1	50	Membr
P31	ALCL	pos	1to 2	90	Membr
P32	ALCL	pos	2	100	Membr+Cyto
P33	ALCL	pos	3	70	Membr+Cyto
P34	ALCL	pos	3	80	Membr
P35	ALCL	pos	1 to 3	100	Cyto
P36	ALCL	pos	3	70	Membr+Cyto
P37	ALCL	pos	3	80	Membr+Cyto
P38	ALCL	pos	2	90	Membr+Cyto
P39	ALCL	pos	3	100	Membr+Cyto
P40	ALCL	pos	3	100	Membr+Cyto
P41	ALCL	pos	2	100	Membr+Cyto

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

P42	Large cell NHL CD30+	<i>neg</i>	0	0	0
P43	ALCL	<i>neg</i>	0	0	0
P44	ALCL	<i>neg</i>	2	50	Membr

CLT samples

CTL1	Non tumoral lymph nodes	<i>neg</i>	0	0	0
CTL2	Non tumoral lymph nodes	<i>neg</i>	0	0	0
CTL3	Nnon tumoral lymph nodes	<i>neg</i>	0	0	0

652
653

Figure 1

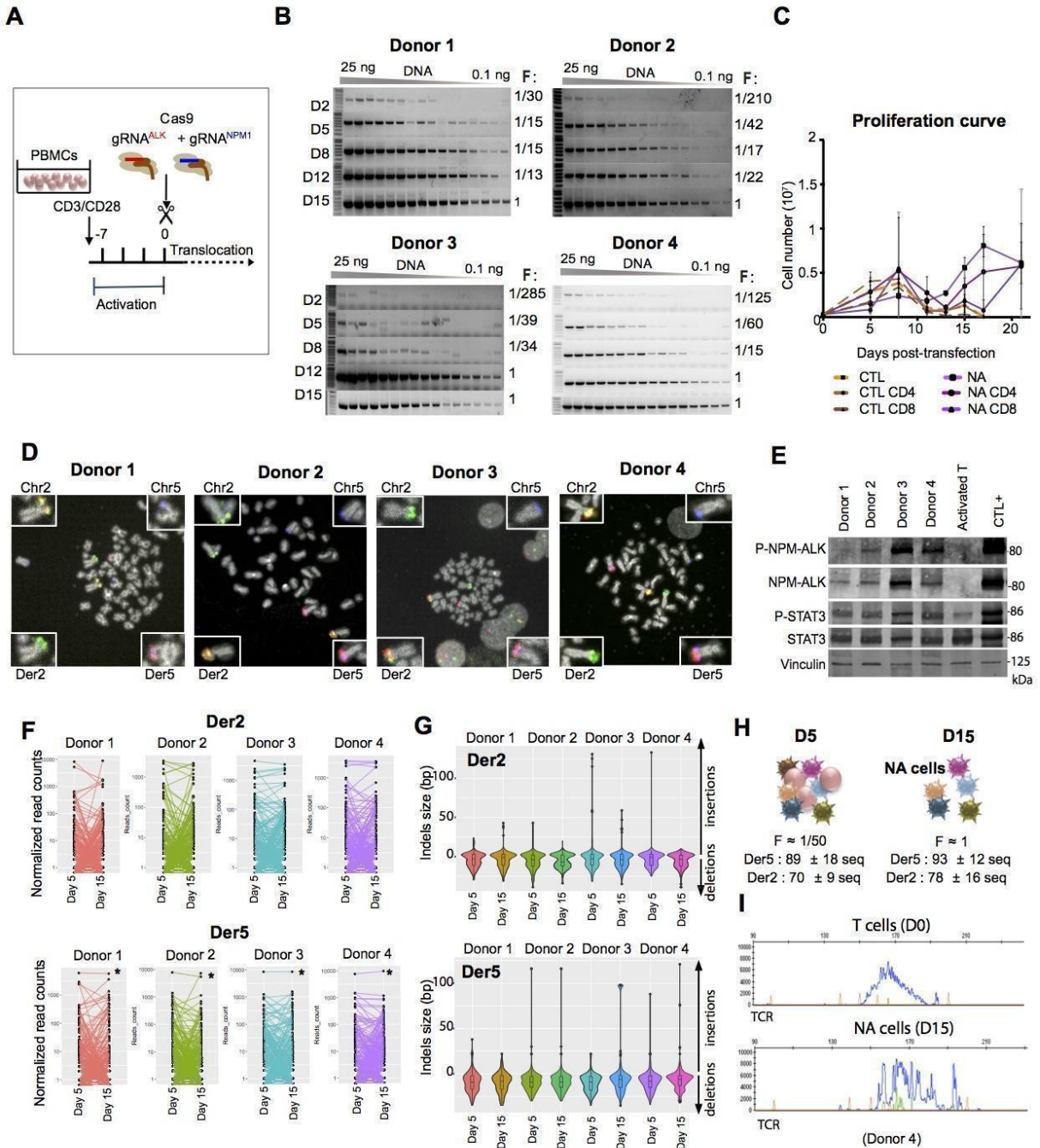


Figure 1: Generation of NA cells derived from activated T lymphocytes

- A-** CRISPR/Cas9-based strategy performed to obtain ALK+ ALCL model cells from activated T lymphocytes. PBMCs were collected from healthy donor blood and activated with anti-CD3/CD28 for 7 days. Activated cells were transfected with RNP complex (Cas9 + gRNA^{NPM1}/gRNA^{ALK}). DNA was extracted at day 0 and then every three days to estimate translocation frequency overtime.
- B-** Translocation frequency assessed by PCR amplification of derivative chromosome 5, Der5, from 2 (D2) to 15 days (D15) post-transfection (n=4 independent donors). PCR were performed in duplicate

663 on DNA dilutions (dilutions: 25 ng to 0.1 ng). The translocation frequency (F) was calculated as
1 664 described in [21] presuming that a human diploid cell contains approximately 6 pg of DNA.

2 665 **C-** Proliferation curve of control T lymphocytes (control cell groups: unsorted, CD4+CD8-, CD8+CD4-
3 666) and CRISPR/Cas9-transfected lymphocytes (NA cell groups: unsorted, CD4+CD8-, CD8+CD4-).
4
5 667 Median with standard deviation for n=3.

6 668 **D-** Representative image of metaphases (FISH analysis) obtained from NA cells, 1 month post-
7
8 669 transfection. Break-apart probe (green + red): *ALK* gene; blue probe: *NPM1* gene.

9 670 **E-** Western blot analysis of NPM-ALK, STAT3, phosphorylated NPM-ALK (P-NPM-ALK) and
10 671 phosphorylated STAT3 (P-STAT3) in NA cells from Donors 1 to 4, at 1 month post-transfection.
11
12 672 Activated T lymphocytes were used as negative controls, and an ALK+ ALCL cell line was used as
13 673 a positive control (CTL+). Vinculin was used as the loading control.

14 674 **F-** Evolution of sequencing read numbers for each type of translocation breakpoint junction sequence
15 675 obtained by targeted sequencing (for der2 and der5) at day 5 and day 15 after transfection. Each
16 676 curve represents the number of normalized reads of one single sequence. For Der5, one sequence is
17 677 overrepresented (*) (corresponding to a Δ AG deletion).

18 678 **G-** Violin plots of indel size (in bp for deletions and insertions) for Der2 and Der5 breakpoint junction
19 679 sequences, at day 5 and day 15 for the four donors.

20 680 **H-** Schematic diagram of NA cell selection showing the number of breakpoint junctions at day 5 and
21 681 15 post-transfection (normalized mean \pm SD of number of different junction sequences observed per
22 682 transfection and calculated from the sequencing of at least 900 reads). F = translocation frequency
23 683 estimated by PCR of DNA dilutions (see Figure 1B).

24 684 **I-** Analysis of TCR γ clonality via multiplex PCR of activated T lymphocytes (day of transfection: D0)
25 685 and NA cells 15 days (D15) post-transfection (corresponding to Donor 4).

26 686

Figure 2

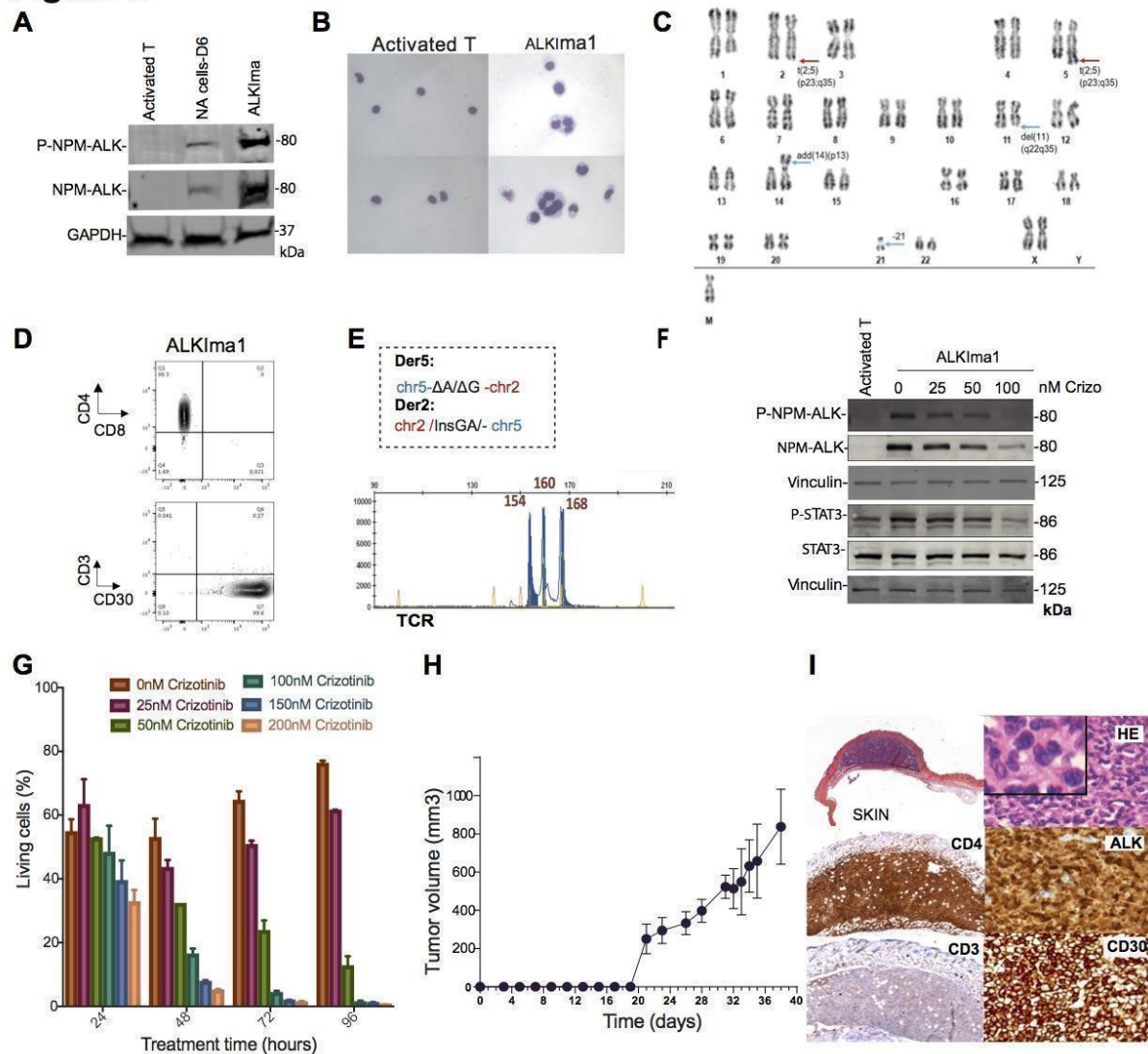


Figure 2: *In vivo* tumors derived from the ALK1ma1 cell line

- A-** Western blot analysis of NPM-ALK, STAT3, phosphorylated NPM-ALK (P-NPM-ALK) and phosphorylated STAT3 (P-STAT3) in CD4+ NA cells, 6 days post-transfection (NA cells-D6) and the ALK1ma1 cell line. GAPDH was used as a loading control.
- B-** Cytospin analysis of primary activated T cells (5 days post-activation) and ALK1ma1 cells.
- C-** Karyotype analysis of ALK1ma1 cells. Red arrows show the t(2;5)(p23;q35) translocation, blue arrows indicate additional chromosomal events.
- D-** Flow cytometry assessment of CD4, CD8 and CD3, CD30 expression levels in ALK1ma1 cells.
- E-** Der2 and Der5 sequences obtained by Sanger sequencing of ALK1ma1 cells, and analysis of TCR γ clonality via multiplex PCR of ALK1ma1 cells.
- F-** Western blot analysis of NPM-ALK, STAT3, phosphorylated NPM-ALK (P-NPM-ALK) and phosphorylated STAT3 (P-STAT3) in ALK1ma1 cells treated with crizotinib for 48 hours versus

700 untreated ALK1ma1 cells. Activated T lymphocytes were used as a negative control. Vinculin was
1 701 used as a loading control. Crizo: crizotinib.
2
3 702 **G-** Cell survival analysis of ALK1ma1 cells treated with increasing doses of crizotinib for 24, 48, 72 and
4
5 703 96 hours.
6
7 704 **H-** Temporal evolution of tumor volume in mice injected with ALK1ma1 cells (subcutaneous injection).
8 705 **I-** Histologic analysis of ALK1ma1 tumor cells in skin nodules: H&E staining, anti-ALK, anti-CD3,
9 anti-CD4 and anti-CD30.
10 706
11 707
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure 3

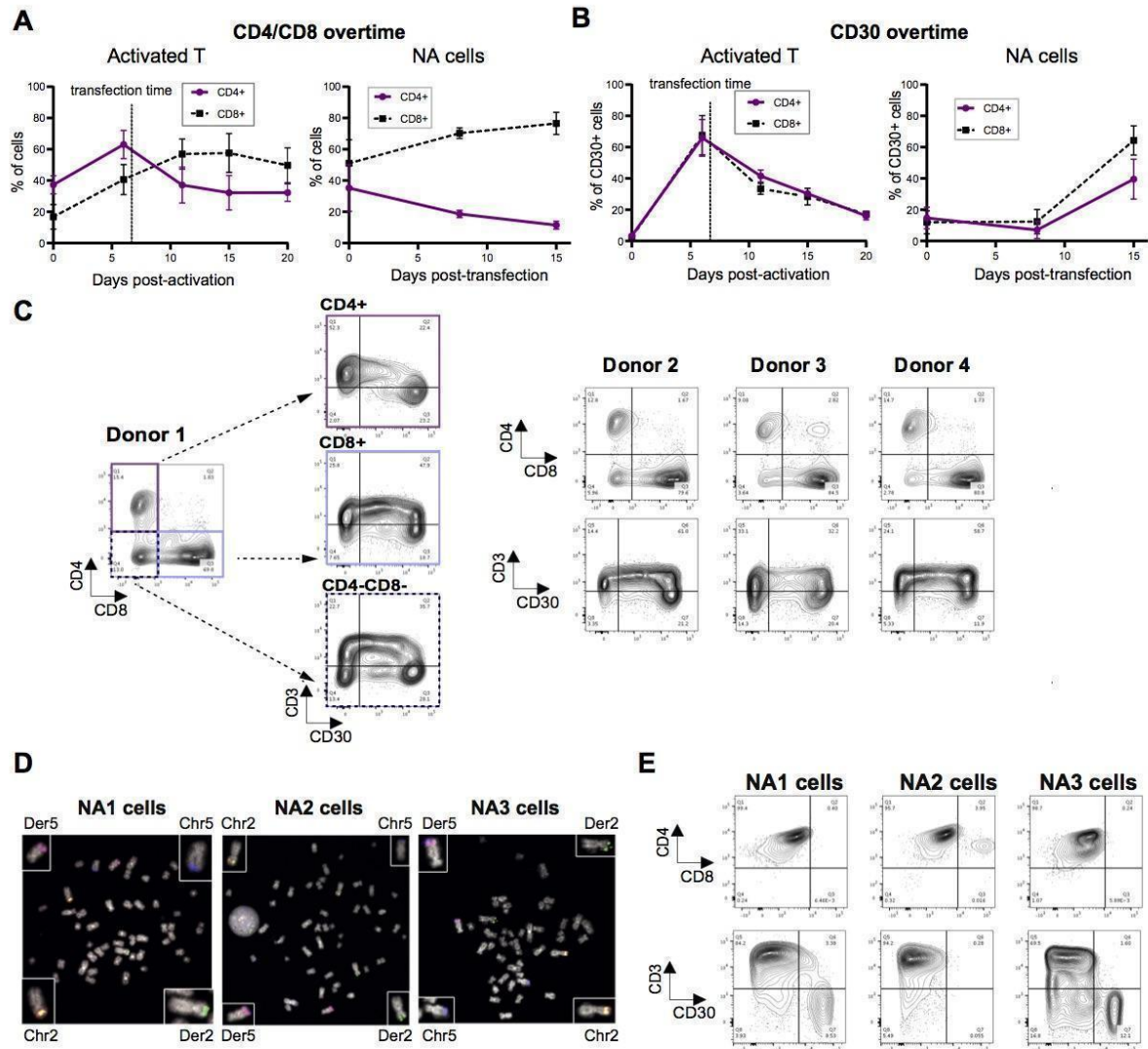


Figure 3: Immunophenotype of NA cells

A- CD4+ and CD8+ cell survival overtime (analyzed by flow cytometry) for primary activated T lymphocytes (between 0 and 20 days post-activation) and NA cells (between 0 and 15 days post-transfection). The time of transfection is indicated by a dashed line for primary activated T lymphocytes (n=3).

B- CD30 expression for each cell population (CD4+ or CD8+ cells) for primary activated T lymphocytes (between 0 and 20 days post-activation) and NA cells (between 0 and 15 days post-transfection). The transfection timepoint is indicated by a vertical dashed line for the primary activated T lymphocytes (n=3).

C- Flow cytometry assessment of the CD4, CD8, CD3 and CD30 markers. For all donors, cell populations were obtained at day 15 post-transfection. Subpopulations (CD4+CD8-, CD8+CD4- and CD4-CD8-) were also analyzed for Donor 1 (left).

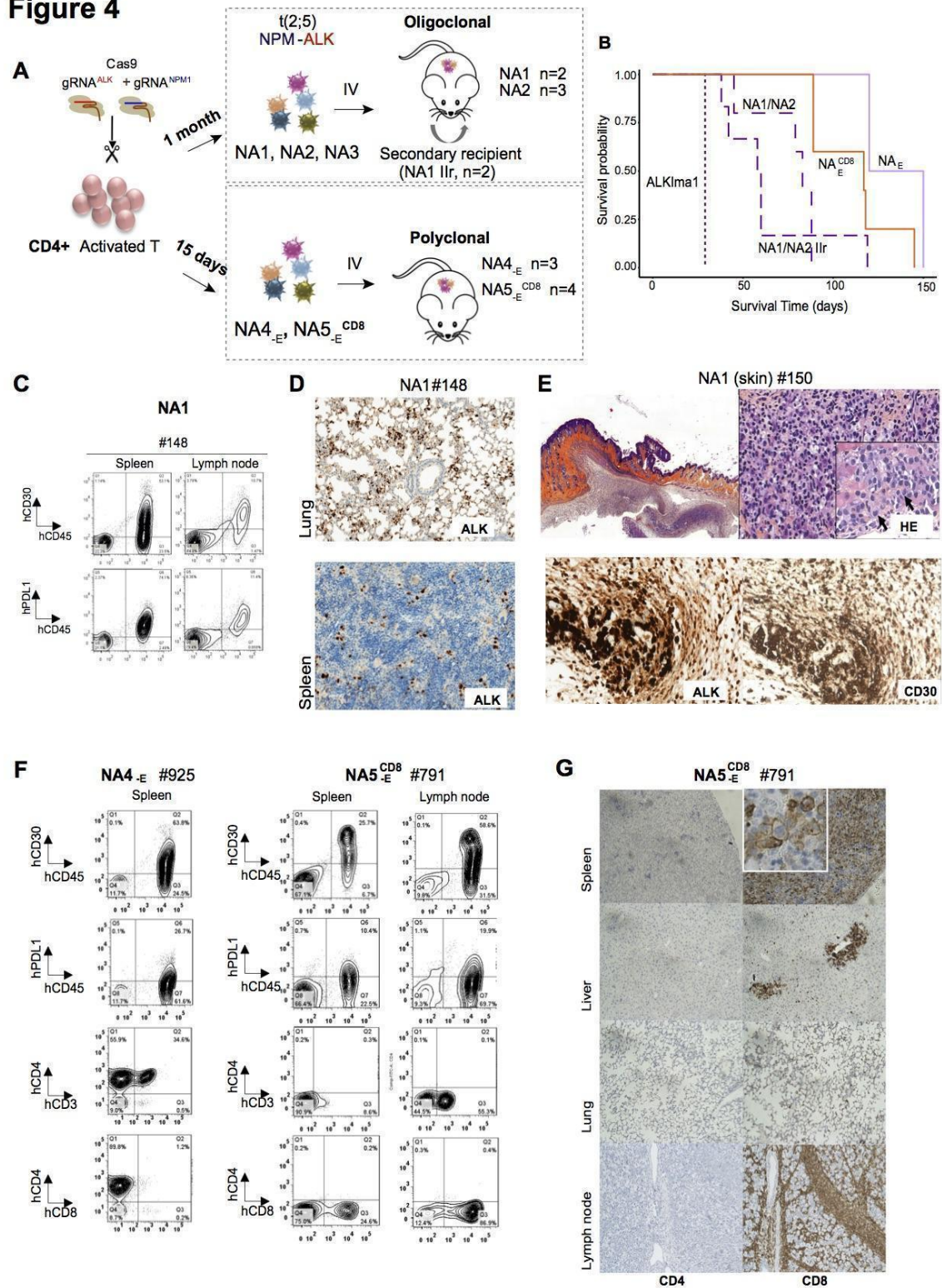
721 **D-** Representative image of metaphases (FISH analysis) of NA1, NA2 and NA3 cells, 1 month post-
1 722 transfection. Break-apart probe (green + red): *ALK* gene; blue probe: *NPM1* gene.

3 723 **E-** Flow cytometry assessment of CD4, CD8, CD3 and CD30 expression in NA1, NA2, NA3 cells (1
4 724 month post-transfection, time point corresponding to mice injections).

6 725

7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure 4



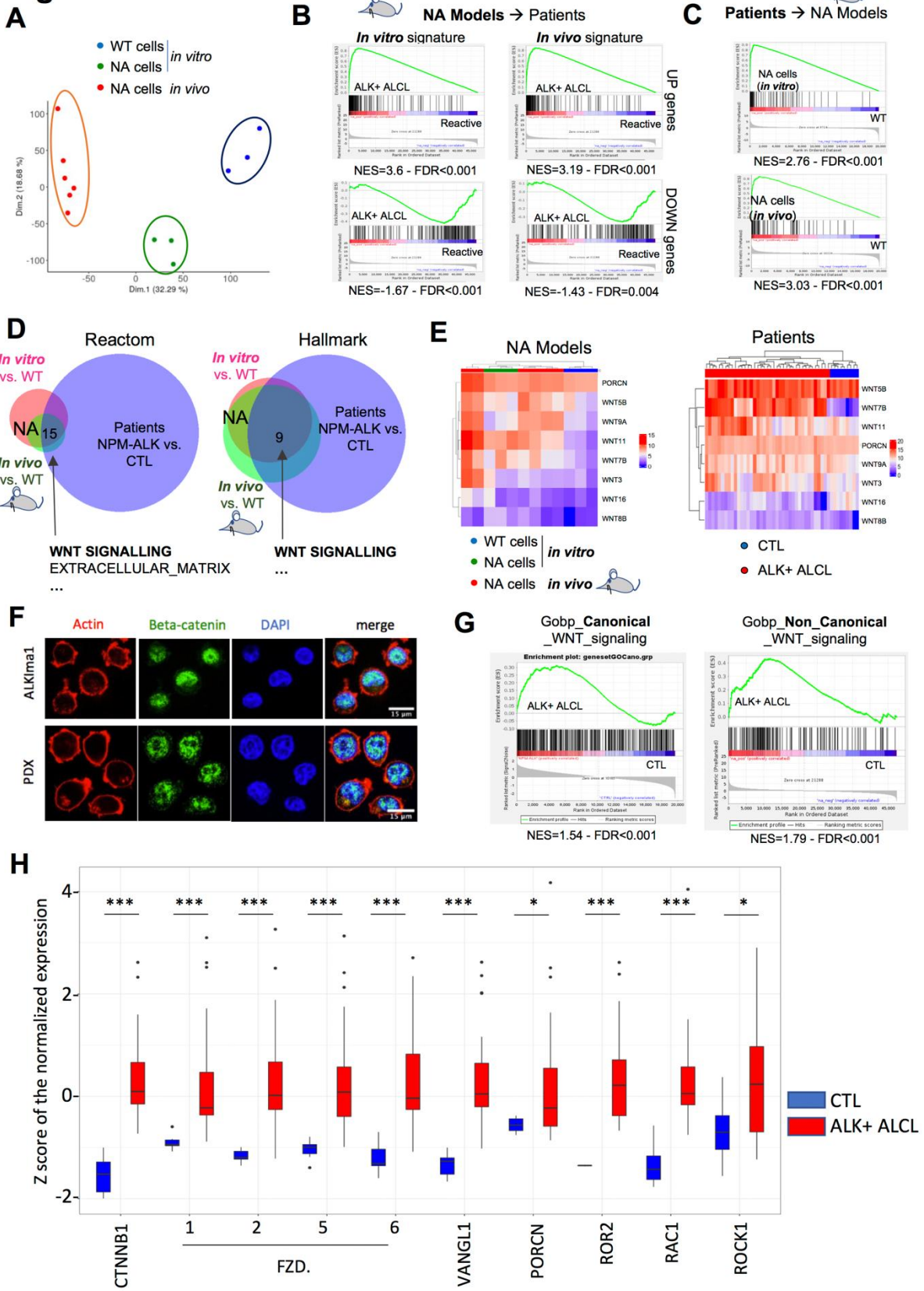
726

727

728 **Figure 4: *In vivo* ALK+ ALCL lymphomagenesis derived from NA cells**

- 1 729
2 730 **A-** Schematic illustration of the experiment: NA1, NA2 and NA3 cells were intravenously injected into
3
4 731 NSG mice at 1 month post-transfection. Tumor cells from NA1 cells were injected in secondary
5 732 recipients (NA1 Iir). In parallel, NA4_E (unsorted cells) and NA5_E^{CD8} (CD8+) cells were
6
7 733 intravenously injected in NSG mice at 15 days post-transfection. IV: intravenous injection.
8
9 734 **B-** Kaplan-Meier survival curve of first and second recipient mice intravenously injected with different
10 735 pools of NA cells obtained from various time points after transfection (15 days (NA4_E, NA5_E^{CD8}), 1
11 736 month (NA1 and NA2) or ALKIma1 cell line). Iir: secondary mouse recipients.
12
13
14 737 **C-** Flow cytometry assessment of CD45, CD30 and PDL1 expression in tumors derived from NA1 cells
15 738 (spleen and lymph node) for mouse #148.
16
17 739 **D-** Histologic analysis of tumors derived from NA1 cells: anti-ALK in lung and spleen tumors for mouse
18
19 740 #148.
20
21 741 **E-** Histologic analysis of tumors from NA1 cells in skin nodules: H&E staining, anti-CD30, anti-ALK
22 742 for mouse #150.
23
24 743 **F-** Flow cytometry assessment of CD45, CD30, PDL1, CD4, CD8 and CD3 expression in tumor cells
25 744 (spleen and lymph node) from mice #925 (injected with NA4_E) and mouse #791 (injected with NA5_E
26 745 ^{CD8} cells).
27
28
29 746 **G-** Histologic analysis of tumors in spleen, liver, lung and lymph node from mouse #791 injected with
30 747 NA5_E^{CD8} cells: anti-CD4 and anti-CD8.
31
32 748

Figure 5



749
750

751 **Figure 5: Expression analysis of NA *in vitro* and *in vivo* models reveals WNT pathway activation**
1 752 **in ALK+ ALCL patient cells**

- 3 753 **A-** Principal component analysis of the RNAseq data (Dim 1 and 2). Data were scaled to unit variance
4 before performing the representation.
5 754
- 6 755 **B-** Gene set enrichment analysis of the 200 most differentially upregulated or downregulated genes
7 obtained from the models [comparison of NPM-ALK-edited *in vitro* models vs. wild type cells (*in*
8 756 *vitro*) or NPM-ALK *in vivo* models vs. wild type cells] on expression data from ALK+ ALCL
9 757 patients vs. non tumoral reactive lymph nodes (CTL). UP genes: upregulated genes, DOWN genes:
10 758 downregulated genes, WT: wild type.
11 759
- 12 760 **C-** Gene set enrichment analysis using an ALK+ ALCL patient signature of 200 upregulated genes, on
13 expression data from our models: NPM-ALK *in vitro* vs. wild type cells (upper panel), NPM-ALK
14 761 *in vivo* models vs. wild type cells (lower panel). WT: wild type.
15 762
- 16 763 **D-** Gene set enrichment analysis was performed on the Reactom and Hallmark gene lists for the
17 following pairwise comparisons: NPM-ALK *in vitro* vs. wild type cells, NPM-ALK *in vivo* models
18 764 vs. wild type cells, and NPM-ALK+ patients and non tumoral reactive lymph nodes (CTL). The
19 765 overlap of significantly enriched genes is represented as Venn diagrams. WT: wild type.
20 766
- 21 767 **E-** Heatmap representation of expression levels of the enriched WNT genes in the model (left panel)
22 and the patient (right panel) datasets.
23 768
- 24 769 **F-** Immunofluorescence analysis of beta-catenin in ALK^{Ima1} and PDX cells. Blue: DAPI, green: beta-
25 770 catenin, red: actin.
26 771
- 27 772 **G-** Gene set enrichment analysis of the canonical and the non-canonical WNT signaling pathway gene
28 lists to compare ALK+ ALCL patients vs. non-tumoral reactive lymph node expression data (CTL).
29 773
- 30 774 **H-** Significantly upregulated WNT pathway genes in ALK+ ALCL patient cells compared with
31 reactive lymph nodes (CTL), represented as Z scores computed from Congras et al, 2020 (Journal
32 775 of Clinical Investigation) using DESeq2. p-values were adjusted for multiple comparisons with the
33 776 Benjamini-Hochberg correction (*: FDR<0.05, **: FDR<0.01, ***: FDR<0.001).
34 777

Figure 6

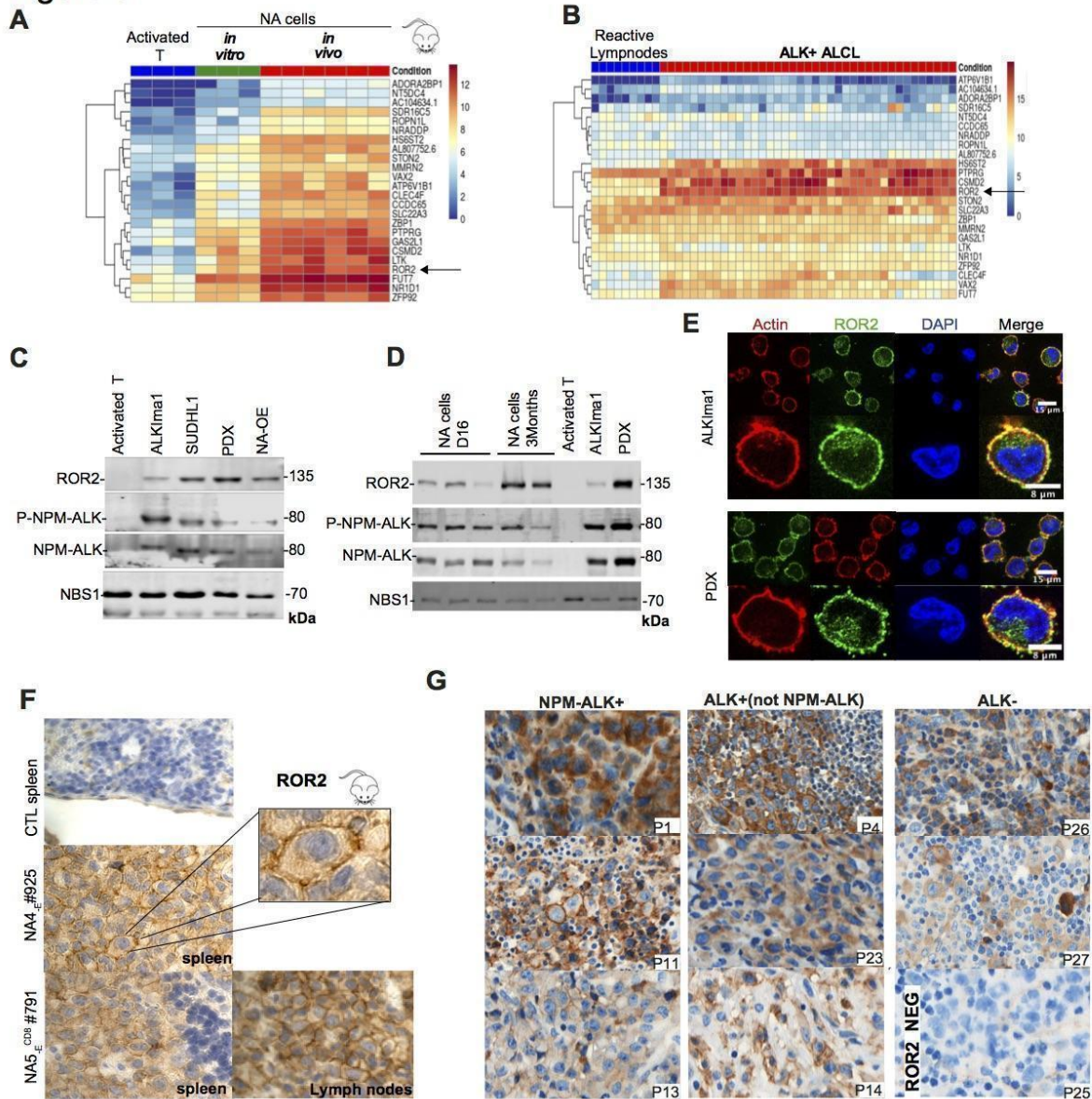
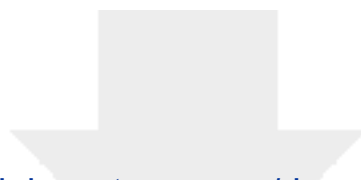


Figure 6: The ROR2 receptor is a specific ALK+ ALCL marker

- A-** Heatmap representation of genes showing progressive upregulation: from activated T lymphocytes to NA cells *in vitro*, then from NA cells *in vitro* to *in vivo* models (a threshold of twofold increased expression was applied for each assessment) in the model dataset.
- B-** Heatmap representation of the list of genes found in A in the patient dataset (n=39). Log2 transformed expression matrix was used to generate representations.
- C-** Western blot analysis of ROR2, NPM-ALK and phosphorylated NPM-ALK (P-NPM-ALK) in activated T lymphocytes, ALK1ma1 cells, an ALK+ ALCL cell line (SUDHL1), patient PDX cells and an NPM-ALK overexpression model (NA-OE). NBS1 was used as a loading control.
- D-** Western blot analysis of ROR2, NPM-ALK and phosphorylated NPM-ALK (P-NPM-ALK) in NA cells 16 days and 3 months post-transfection in activated T lymphocytes, ALK1ma1 cells, and patient PDX cells. NBS1 was used as a loading control.

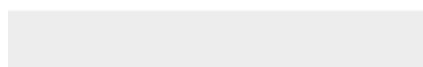
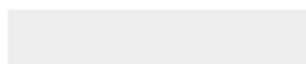
- 791 **E-** Immunofluorescence analysis of ROR2 expression in ALK1ma1 and PDX patient cells. Green:
1 792 ROR2, red: actin, blue: DAPI.
2
3 793 **F-** Histologic analysis of a CD4+ spleen tumor (corresponding to mouse #925 injected with NA4-E) and
4 a CD8+ spleen and lymph node tumors (corresponding to mouse #791 injected with NA5-E^{CD8}); anti-
5 794 ROR2 IHC. The negative control spleen (CTL) was obtained from a wild type NSG mouse.
6 795
7
8 796 **G-** Histologic analysis of ALCL patient tumors: anti-ROR2 IHC. Left: NPM-ALK+ ALCL tumors (P1,
9 P11, P13), middle: ALK+ ALCL tumors (other fusion partners) (P4, P23 and P14); right: ALK-
10 797 ALCL tumors (P26 and P27). See also **Table1** for all sample data.
11 798
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65



[Click here to access/download](#)

Supplementary Material

[SupplementaryData-MolCancer-Revised.docx](#)





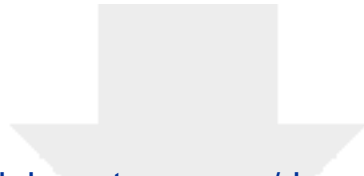
Click here to access/download
Supplementary Material
Supl-TableS1.xlsx



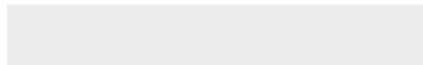


Click here to access/download
Supplementary Material
Supl-TableS2.xlsx





Click here to access/download
Supplementary Material
Supl-TableS3toS4.xlsx





Click here to access/download
Supplementary Material
Supl-TableS5toS9.xlsx





Click here to access/download
Supplementary Material
Supl-TableS10.xlsx

