SUPPLEMENTARY APPENDIX

Anaplastic lymphoma kinase-positive anaplastic large cell lymphoma with the variant RNF213-, ATIC- and TPM3-ALK fusions is characterized by copy number gain of the rearranged ALK gene

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Supplemental information

Supplemental Methods

5' Rapid Amplification of cDNA Ends Polymerase-Chain-Reaction (RACE-PCR)

Total RNA was extracted from 20µm sections of frozen tissue samples using TRIzol Reagent (Life Technologies, Merelbeke, Belgium). With random primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), one microgram of total RNA was reverse transcribed into cDNA using random hexamers and SuperScript III (Life Technologies, Merelbeke, Belgium).

5'RACE-PCR experiments were performed following a previously described protocol.[1] The used 5' RACE PCR primers are listed in the Supplemental Table 2. The final PCR products were cloned in into the pJET1.2 CloneJET vector (Thermo Fisher Scientific (Fermentas), Waltham, MA). Subsequently, Sanger sequencing of the PCR products was performed and Sanger chromatograms were analyzed using CLC Main Workbench 6 (CLC Bio Inc., Cambridge, MA).

Low coverage full genome sequencing (LCFGS)

The Illumina standard kit (Illumina® TruSeq™ DNA Sample Preparation Kit) was used for the DNA-sequencing sample preparation according to the manufacturer's protocol (Illumina, San Diego, CA). The quality of the libraries was checked by Agilent Technologies 2100 Bioanalyzer with the Agilent DNA 1000 Kit (Agilent, Santa Clara, CA). Prepared libraries were sequenced using HiSeq 2000 (Illumina) operated in paired-end 2×100 bp mode. Reads were quality-filtered using standard Illumina process. To analyze the data, the fastq files were mapped to the reference human genome (Human.B37.3) using the Ensembl gene model (Homo_sapiens.GRCh37.67). The mapping and downstream analysis were performed with the software ArrayStudio, version 6.2 (www.omicsoft.com).

Targeted Locus Amplification

Viably frozen lymphoma cells were sent to Cergentis B.V. (Utrecht, the Netherlands) for Targeted Locus Amplification (TLA) and sequencing. Sample preparation, sequencing and data analysis were performed as previously described.[2]

Nested Reverse-Transcription Polymerase-Chain-Reaction (RT-PCR)

Fusion *ALK* transcripts were validated by nested RT-PCR. First strand cDNA was prepared as described above and RT-PCR was done on a cDNA template using two rounds of conventional PCR following standard protocols that come with *Taq* DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The primers were designed to amplify 400-600 bp fragments containing the identified fusion boundary. Subsequently, Sanger sequencing of the PCR products was performed and Sanger chromatograms were analyzed using CLC Main Workbench 6 (CLC Bio Inc.). The primers used can be found in the Supplemental Table 2.

Supplemental Tables

Supplemental Table 1. List of FISH probes.

Supplemental Table 2. List of primers.

Supplemental Table 3. The guide RNA sequences.

Supplemental Figures

Supplemental Figure 1. Generation of Ba/F3 Cas9 expressing cells.

Supplemental Figure 2. Map of the plasmid pX321.

Supplemental Figure 3. Morphology and immunophenotype. Representative histopathology of case 1 with the novel EEF1G-ALK fusion and case 4 with the ATIC-ALK fusion. (A and G) Hematoxylin and eosin staining showing an anaplastic lymphoid cell proliferation with characteristic hallmark and doughnut cells (top insets). The neoplastic cells overexpress CD30 (B and H), cytoplasmic ALK1 (C and I), perforin (D and J), but no TIA1 (E and K). There is a prominent stromal infiltrate containing numerous neutrophils (see lower inset in G) and histiocytes (F and L, CD68 staining).

Supplemental Figure 4. Results of the TLA/sequencing performed in case 8. (A) The primer sets were used in individual TLA amplifications. PCR products were purified and library prepped using the Illumina NexteraXT protocol and sequenced on an Illumina Miniseq sequencer. Reads were mapped using BWA-SW, which is a Smith-Waterman alignment tool. This allows partial mapping which is optimally suited for identifying break spanning reads. The human genome version hg19 was used for mapping.

(B) TLA sequence coverage across the human genome. Data shown was generated using primer set 1 (upper panel) and set 2 (lower panel). The different chromosomes are indicated on the y-axis, the chromosomal position on the x-axis. Encircled in blue is the position of the primer-set at ALK gene locus, in red is the fusion partner. A high coverage peak in chromosome 2 was identified at the ALK locus. An additional peak at chromosome 1 at the TPM3 gene was identified. (C) IGV screenshot showing the sequence coverage across the ALK gene human locus. Coverage generated across chr2:29,446,372-29,447,630 using primer set 1 (top panel) and set 2 (bottom panel) are depicted. Blue arrow indicates the breakpoint position. Green arrows indicate primer position. Y-axis is limited to 100x. (D) IGV screenshot showing the sequence coverage across the breakpoint at the TPM3 gene human locus. Coverage generated across chr1:154,121,228-154,168,475 using primer set 1 (top panel) and primer set 2 (bottom panel) are depicted. Blue arrow indicates the breakpoint. Y-axis is limited to 100x. (E) Results of sequencing.

References

1. Cools J, Wlodarska I, Somers R, et al. Identification of novel fusion partners of ALK, the anaplastic lymphoma kinase, in anaplastic large-cell lymphoma and inflammatory myofibroblastic tumor. Genes Chromosomes Cancer. 2002;34(4):354-362.
2. de Vree PJ, de Wit E, Yilmaz M, et al. Targeted sequencing by proximity ligation for comprehensive variant detection and local haplotyping. Nat Biotechnol. 2014;32(10):1019-1025.

Supplemental Table 1. List of FISH probes

Probes*	Clones	Cytoband	Genomic localization	Reference
LSI ALK		2p23		Abbott Molecular
LSI MYC		8q24		Abbott Molecular
3'ALK	P1 1111H1	2p23		Mathew et al, 1997 (PMID: 9057650)
EEF1G BA	RP11-68P15 (SG)	11q12.3	61953810-62102465	www.ensembl.org/Homo_sapiens
	RP11-115P03 (SG)	11q12.3	62158421-62306547	www.ensembl.org/Homo_sapiens
	RP13-978C16 (SO)	11q12.3	62349950-62479328	www.ensembl.org/Homo_sapiens
	RP11-231P15 (SO)	11q12.3	62485680-62649265	www.ensembl.org/Homo_sapiens
RNF213 BA	RP11-98G11 (SO)	17q25.3	79805268-79977085	www.ensembl.org/Homo_sapiens
	RP11-712A09 (SO)	17q25.3	78003484-78151971	www.ensembl.org/Homo_sapiens
	RP11-1285L18 (SG)	17q25.3	78394068-78589748	www.ensembl.org/Homo_sapiens
	RP11-810D12 (SG)	17q25.3	78580736-78761411	www.ensembl.org/Homo_sapiens
RNF212 BA	RP11-187M02 (SG)	4p16.3	943555-1094591	www.ensembl.org/Homo_sapiens
	RP11-815M22 (SO)	4p16.3	1121971-1316061	www.ensembl.org/Homo_sapiens
ITK BA	RP11-641B02 (SO)	5q35	156336853-156485286	www.ensembl.org/Homo_sapiens
	RP11-179D10 (SO)	5q35	157049883-157214338	www.ensembl.org/Homo_sapiens
	RP11-135N24 (SG)	5q35	157214332-157385690	www.ensembl.org/Homo_sapiens
	RP11-152N09 (SG)	5q35	157325533-157527934	www.ensembl.org/Homo_sapiens
ATIC BA	RP11-501N03 (SO)	2q35	215646436-215842107	www.ensembl.org/Homo_sapiens
	RP11-352A24 (SO)	2q35	215871050-216044463	www.ensembl.org/Homo_sapiens
	RP11-458D08 (SG)	2q35	216373206-216563572	www.ensembl.org/Homo_sapiens
	RP11-798J19 (SG)	2q35	216562206-216755043	www.ensembl.org/Homo_sapiens
TPM3 BA	RP11-762F22 (SG)	1q21.3	152139606-152302465	www.ensembl.org/Homo_sapiens
	RP11-120L12 (SG)	1q21.3	152227183-152376962	www.ensembl.org/Homo_sapiens
	RP11-749D14 (SO)	1q21.3	152524737-152689783	www.ensembl.org/Homo_sapiens
	RP11-719O24 (SO)	1q21.3	152680290-152870820	www.ensembl.org/Homo_sapiens
Npm1-Alk BA	RP23-217E1 (SG)	11A4	33281545-33480470	www.ensembl.org/Mouse
	RP23-431J18 (SG)	11A4	33478471-33676790	www.ensembl.org/Mouse
	RP23-385I2 (SO)	17E1.3	71611889-71792146	www.ensembl.org/Mouse
	RP24-158M15 (SO)	17E1.3	71523478-71674524	www.ensembl.org/Mouse
Atic-Alk BA	RP24-153C14 (SG)	1C3	71084713-71327059	www.ensembl.org/Mouse
	RP23-409K18 (SG)	1C3	71084713-71327059	www.ensembl.org/Mouse
	RP23-385I2 (SO)	17E1.3	71611889-71792146	www.ensembl.org/Mouse
	RP24-158M15 (SO)	17E1.3	71523478-71674524	www.ensembl.org/Mouse

* BA, break apart

Supplemental Table 2: Primer sequences used for 5' RACE PCR, nested RT-PCR and QRT-PCR

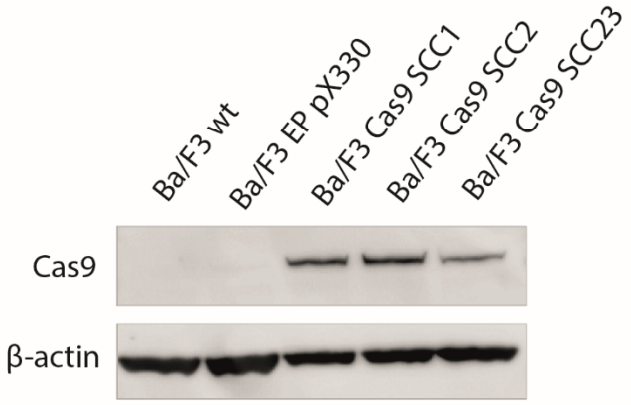
Name	Sequence	Usage
ALK-R1 (712)[18]	5'-TGATGATCAGGGCTTCCATGAGG-3'	5' RACE PCR
ALK-R2 (713)[18]	5'-AGCACACTTCAGGCAGCGTCTTC- 3'	
467[18]	5'-CCAGTGAGCAGAGTGACG-3'	
ALK-R4 (714)[18]	5'- AGCTCCATCTGCATGGCTTG-3	
468[18]	5'-GAGGACTCGAGCTCAAGC-3'	
EEF1G-F1 (771)	5'-GTGTGAGAAGATGGCCCAGT-3'	Nested RT-PCR
EEF1G-F2 (772)	5'-AAGGACCCCTTCGCTCAC-3'	
ALK-R (739)	5'-TGCCAGCAAAGCAGTAGTTG-3'	
ALK-R3 (740)[18]	5'-ATTCCGGACACCTGGCCTTCATA-3'	
Npm1-F	5'-GCACCAGTTGTCATTAAGAACG-3'	QRT-PCR
Npm1-R	5'-TTGCTTCTGCCTCTACGATG-3'	
Atic-F	5'-TCTGTGAACCAGAGGACTATGC-3'	
Atic-R	5'-TGAGCGGTATGAGTGAATGC-3'	
Hprt1-F	5'-CATTATGCCGAGGATTTGG-3'	
Hprt1-R	5'-GCAAGTCTTTCAGTCCTGT-3'	
Rpl4-F	5'-AGAAGACCAAGGAGGCTGTTCA-3'	
Rpl4-R	5'-ATCCGCCGTCGGTTTCTCAT-3'	

Supplemental Table 3: guide RNA sequences cloned into expression plasmid pX321. To optimize expression from the U6 promoter, an extra G was added at the 5' end of gRNAs Npm1-2, Atic and Alk.

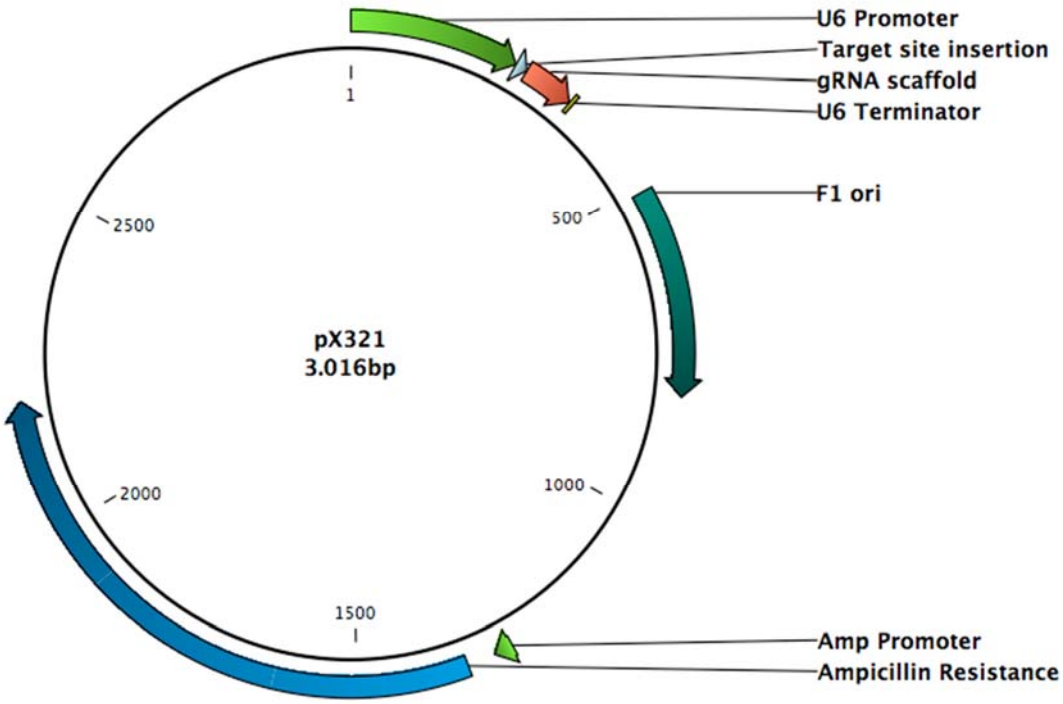
Npm1-1	5'-GGAAATTGTCTTGTCTGACC-3'
Npm1-2	5'-**G**CTGCCATGGAATGCTTGTCC-3'
Atic	5'-**G**TTCACTGTCTGGCAAACGCC-3'
Alk	5'-**G**CAGATAGGTGTCCGCTGTGA-3'

Supplemental figure 1: generation of Ba/F3 Cas9 expressing cells.

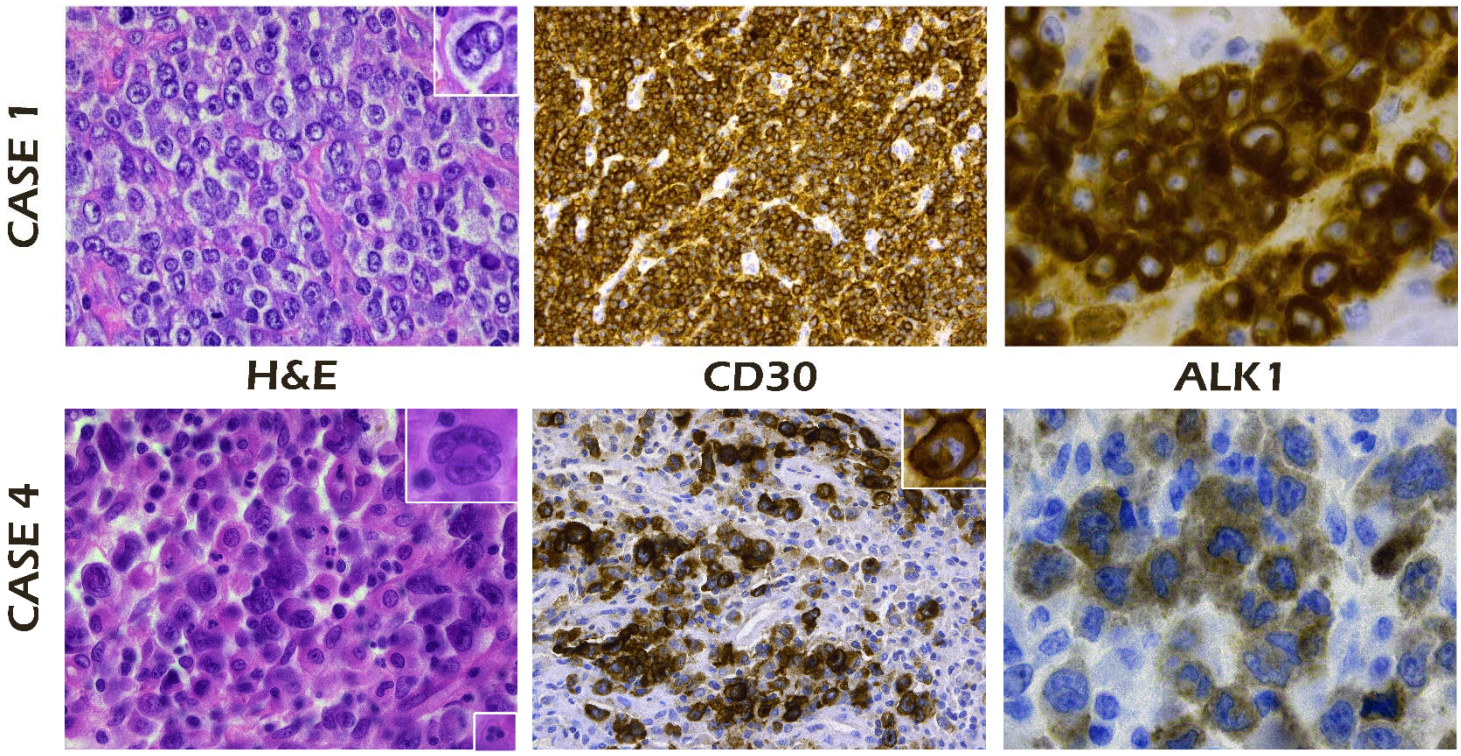
Ba/F3 cells were transduced with a retroviral vector containing a Cas9 expression cassette and a mCherry expression cassette. Transduced cells were seeded in Clonacell-TCS medium (StemCell Technologies) to generate single cells clones.
Single cell clones were assessed on mCherry expression using Flow Cytometry and Cas9 expression using western blot.



Supplemental Figure 2: Map of plasmid pX321 (derived from plasmid pX330 from the Zhang lab, by removing the Cas9 expression cassette)



Supplemental Figure 3. Morphology and immunophenotype



Supplemental Figure 4

A

Primers used in the analysis

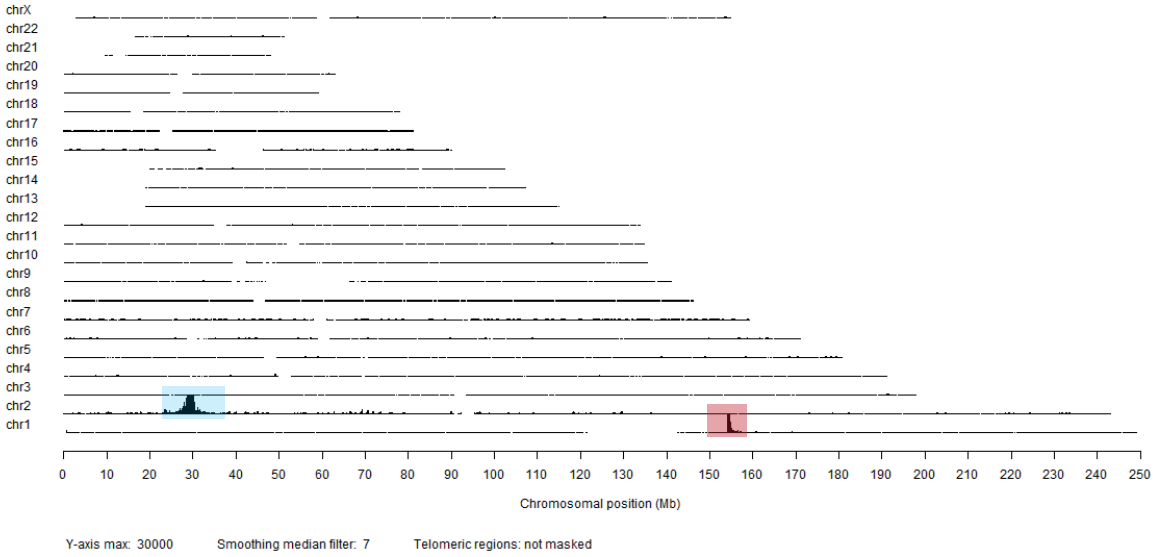
Set1

(chr2: 29450469-29450488)	AGTGGACTGATGAAGGAAAC
(chr2: 29450205-29450224)	TAATAGCCCAATACCTGCAC

Set2

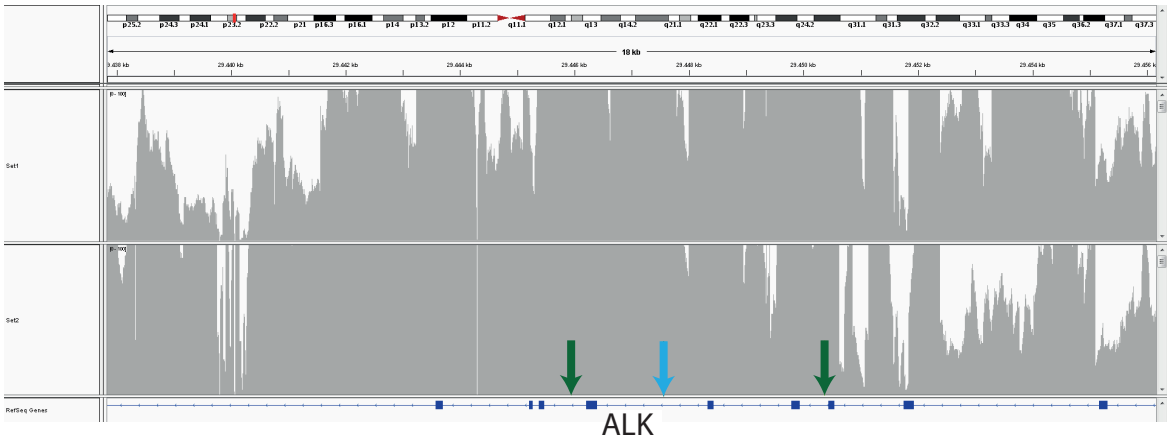
(chr2:29446023-29446042)	TGGGAGTTAATGTAAGCCAA
(chr2:29445848-29445867)	CTCTGTCACTCACTGGAAAT

B

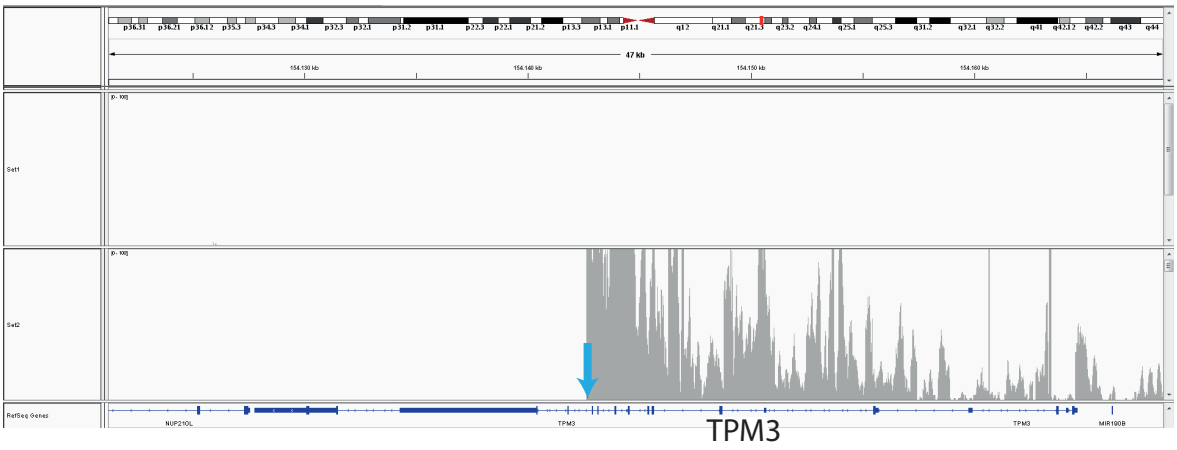


C

Locus wide coverage



D



E

The following fusion read was identified marking the fusion between ALK and TPM3:

Chr2: 29446999 (rev) fused to Chr1: 154142622 (forw)

...GTCATCAGAGGAGTCCCATGACTCCCAGGAATTGGCCTGCCTTAGTATTTCTGCTGTGCTCAGCC
ATTGGGTAGGGCAGCTTCAGTCCAATCACACATTCTCCTCAAAAGGGAAGGGTACAAATTGCAGAC
TATTTGGTAAAGTCTTCTTA...