Novel Intergenically-spliced Chimera, *NFATC3-PLA2G15*, is Associated with Aggressive T-ALL Biology and Outcome

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Abstract:

Leukemias are frequently characterized by the expression of oncogenic fusion chimeras that normally arise due to chromosomal rearrangements. Intergenically-spliced chimeric RNAs (ISCs) are transcribed in the absence of structural genomic changes, and aberrant ISC expression is now recognized as a potential driver of cancer. To better understand these potential oncogenic drivers, high-throughput RNA-sequencing (RNA-seq) was performed on T-acute lymphoblastic leukemia (T-ALL) patient specimens (n=24) and candidate T-ALL-related ISCs were identified (n=55; a median of 4 per patient). In-depth characterization of the NFATC3-PLA2G15 chimera, which was variably expressed in primary T-ALL, was performed. Functional assessment revealed that the fusion had lower activity than wild-type NFATC3 in vitro, and T-ALLs with elevated *NFATC3-PLA2G15* levels had reduced transcription of canonical NFAT pathway genes in vivo. Strikingly, high expression of the *NFATC3-PLA2G15* chimera correlated with aggressive disease biology in murine patient-derived T-ALL xenografts, and poor prognosis in human T-ALL patients.

Introduction: Gene fusion is a frequent hallmark of leukemia, and can arise due to a variety of structural chromosomal rearrangements, including translocation (e.g. *BCR-ABL1*), inversion (e.g. *CBFβ-MYH11*) and interstitial deletion (e.g. *FIP1L1-PDGFRA*) (1). Fusion products are often critical mediators of leukemogenesis, and therefore represent attractive therapeutic targets, as typified by the founder example of BCR-ABL kinase inhibition with imatinib (2).

The advent of high-throughput RNA-sequencing has provided novel insights into the transcriptional landscapes of normal and malignant cells. It is now clear that expression of fusion mRNAs in the absence of structural rearrangements is more common than previously recognized. In particular, transcriptional read-through of a single mRNA between contiguous loci, also known as *cis*-splicing of adjacent genes (*cis*-SAGe), has been estimated to occur at 4-5% of the human genome (3, 4). Expression of the resultant intergenically-spliced chimeric mRNAs (ISCs) is frequent in normal cells (5, 6). Mounting evidence suggests that multiple cancers demonstrate aberrant ISC expression, and that experimental inhibition of specific fusion transcripts can be toxic for malignant cells (7, 8).

In order to investigate whether *cis*-SAGe generates biologically important fusions in T-acute lymphoblastic leukemia (T-ALL), we performed RNA-sequencing of 12 diagnostic leukemic samples. We detected a high frequency of T-ALL-associated ISCs, and notably found that expression of the *NFATC3-PLA2G15* chimera correlated with aggressive disease biology.

Materials and Methods:

RNA-sequencing: Paired-end stranded RNA-sequencing (2 x 50 bp) of the initial series of 12 samples was performed with poly(A)-enriched RNAs using the SOLiD HQ5500XL system (Life Technologies). Mapping, coverage and fusion discovery were determined using LifeScope™ (Life Technologies) using default parameters, with reference to version hg19 of the human genome. Selection of fusion transcripts required a total of 3 reads spanning two distinct gene transcripts (including at least 1 paired-end read and 1 split read). Fusions detected in normal thymic RNA-seq samples were removed. Fusions that involved genes located within 30 kb of each other with the same transcriptional orientation were defined as candidate ISCs. Sequencing of the second series of 12 samples was performed with poly(A)-enriched RNAs using the Illumina platform (paired-end 2 x 75 bp) and mapped with the TopHat-fusion algorithm. Sequencing depth in this series ranged between 30 and 70 millions of reads.

Extra information regarding the choice of filter criteria and parameters for detection of ISCs are included in the Supplementary Methods. Details of sequencing read depth are shown in Supplementary Table S1.

PCR: RT-PCR was performed using two fusion-specific primer sets:

Set 1 5': CAACCATTGGTCTGCAGGAC 3': GGTGTGGGGACGCCAGTAC;

Set 2: CAGGGGGGTCTTTCTGCAC, 3': GGTGTCTGCACGAACACCTTC.

Transcript expression was confirmed by direct sequencing of PCR products.

NFATC3-PLA2G15 levels were quantified by a fusion-specific Taqman QPCR system:

5': GAACCAGAAGATCGAGAGCCTAAC, 3': TCCGGTTGTTGTCTCCATCA,

Probe: TTGCAACCATTGGTCTGCAGGACATC. Fusion transcript levels were normalized to

ABL expression: 5': TGGAGATAACACTCTAAGCATAACTAAAGGT,

3': GATGTAGTTGCTTGGGACCCA, Probe: CCATTTTTGGTTTGGGCTTCACACCATT.

5' RACE PCR was performed using the SMARTer™ RACE cDNA Amplification Kit (Clontech), following the manufacturer's instructions. A schematic representation of primer positions is shown in Supplementary Figure S1. The sequences were:

Primer *NFATC3-PLA2G15*: GGGATCCGGTTGTTGTCTCCATCATCTA,

Primer *NFATC3* WT: AGGCTGAAGCTGAGGAGATGGTGGCC.

Array Competitive Genomic Hybridization (CGH) was performed using the Affymetrix Genome-Wide Human SNP Array 6.0, using leukemic DNA extracted from 115 T-ALL samples at diagnosis. CGH data were analyzed using Chromosome Analysis Suite software (Affymetrix).

NFATC3 Expression Vectors: The pMSCV-IRES-GFP (pMIG)-HA-NFATC3 vector was generated by insertion of a Topo HA-NFATC3 fragment (obtained following PCR amplification from the pOTB7-NFATC3 plasmid (Biovalley)) between the XhoI and EcoRI restriction sites of the pMIG multiple cloning site. The pMIG-HA-NFATC3-PLA2G15 vector was generated following PCR amplification of a 1.3kb segment of *NFATC3-PLA2G15* cDNA from a human leukemic sample. The amplified fragment was then cloned into the pMIG-NFATC3 vector backbone, using a naturally-occurring MfeI site in the *NFATC3* cDNA.

Immunofluorescence: Cells were attached to slides using poly-L-lysine (0.01%), fixed with formaldehyde (3.5%) and permeabilized with Triton X-100 (1%). Slides were incubated with an anti-NFATC3 (R&D Systems, MAB5834) primary antibody and a goat anti-mouse 555 Alexa red (Life #A21422) secondary antibody. Images were acquired on

a Carl Zeiss LSM 700 confocal microscope with Zen 2011 software, and processed using ImageJ software (National Institutes of Health).

Luciferase Assays: The pΔODOLO-NFAT/luc vector contains three copies of the distal NFAT binding site in the IL-2 gene promoter, upstream of a Drosophila *Adh* promoter that drives luciferase expression. The pΔODOLO-Luc vector is the identical vector lacking the NFAT binding sites, providing a control for the basal activity of the *Adh* promoter. 293T cells were transfected with *NFATC3* expression vectors and luciferase vectors using Lipofectamine®2000 Reagent (Life technologies). The total amount of DNA transfected per experiment was kept constant through the addition of empty pMIG vector, where appropriate. Luciferase activity was measured in triplicate 48 hours after transfection, using the Dual Luciferase® Reporter Assay System (Promega). Values for pΔODOLO-NFAT/luc activity were corrected for both measured pΔODOLO-Luc activity and NFATC3 protein expression, quantified using the BioRad ChemiDoc™ XRS+ machine with Image Lab™ software.

Gene Set Enrichment Analysis (GSEA): GSEA was performed using a set of genes described to be regulated by calcineurin/ NFAT in normal peripheral lymphocytes (http://www.ncbi.nlm.nih.gov/biosystems/137993 and Supplementary Table S2). GSEA was run using signal-to-noise for the ranking gene metric and 1000 permutations. The analysis was performed using RNA-sequencing data from 20 T-ALL samples, which were defined as being *NFATC3-PLA2G15* high or low according to the results of *NFATC3-PLA2G15* RT-QPCR (see above). The 10 *NFATC3-PLA2G15* high cases all had expression levels in the highest quartile of results for T-ALL samples, while the 10 *NFATC3-PLA2G15* low cases all had expression levels in the lowest two quartiles.

Murine Patient-Derived Xenografts: NSG mice were maintained under specific pathogen-free conditions in the animal facilities of the Institut Curie (Orsay, France). All experimental procedures were performed in strict accordance with the recommendations of the European Commission (Directive 2010/63/UE) and French National Committee (87/848) (authorisation APAFiS #7393-20161028104744-v1). Following injection of patient T-ALL blasts obtained at leukemia diagnosis, mice were followed for tumor engraftment by regular flow cytometry analysis of peripheral blood using hCD45 and hCD7 (eBiosciences) as markers for human leukemic cells. Mice were euthanized when terminally ill, as evidenced by either severe dyspnea or weakness caused by leukemic dissemination in the thymus or vital organs (bone marrow, lung, and liver), respectively.

Survival Analyses: Statistical analyses and survival curves for patient-derived murine xenografts and for human T-ALL patients treated during the GRAALL-2003 and -2005 studies were calculated using Prism 5 (GraphPad). Kaplan-Meier survival curves were compared using the log-rank (Mantel-Cox) test. The GRAALL-2003 and GRAALL-2005 studies were registered at http://www.clinicaltrials.gov as #NCT00222027 and #NCT00327678, respectively. Informed consent was obtained from all patients at trial entry. Both studies were conducted in accordance with the Declaration of Helsinki and approved by local and multicenter research ethical committees. The sole criteria for inclusion in the current project were a diagnosis of T-ALL and the availability of diagnostic material for measurement of *NFATC3-PLA2G15* expression.

Results and Discussion: Our RNA-sequencing analysis pipeline is depicted in Figure 1A, and notably excluded fusions that were also detected in normal thymic RNA sequenced in parallel. Strikingly, we found that 55 of the 140 total candidate fusions involved genes located within 30 kb of each other, in the same transcriptional orientation. This distance is consistent with that previously observed for *cis*-SAGE (9), suggesting that ISCs are a common event in T-ALL. In total, putative ISCs were detected in 10/12 samples, with a median of 4 (range 0-15) per patient. Full details of the candidate ISCs detected in this study are shown in Supplementary Table S3.

We noted that several of the ISC gene partners have important roles in normal and leukemic T-cell development. We decided to perform further analysis on the candidate ISC *NFATC3-PLA2G15*, which was detected in 2/12 T-ALLs (Figure 1B). Nuclear factor of activated T-cells (NFAT) proteins are critical regulators of normal thymopoiesis and mature T-cell function (10), and murine Nfatc3 has specific roles in T helper cell differentiation from naive to effector states (11), and in double positive (CD4+CD8+) to single positive (CD4+/CD8- or CD4-/CD8+) thymocyte transition (12). We have also previously shown that the calcineurin/ NFAT pathway activation is essential for T-ALL leukemia-initiating cell function (13). *PLA2G15* (Phospholipase A2 Group XV) is located 16kb downstream of *NFATC3*, and encodes a lysosomal enzyme with both phospholipase and transacylace activities (14-16). We initially confirmed the presence of *NFATC3-PLA2G15* mRNA in leukemic cells by RT-PCR and direct sequencing (Figure 1C). We additionally verified that the same *NFATC3-PLA2G15* fusion transcript was detectable in an independent RNA-sequencing series of T-ALL samples that were analyzed using a different system (Supplementary Figure S1). As expression in patient samples appeared variable, we designed a fusion-specific RT-QPCR, in order to quantify the levels of

NFATC3-PLA2G15 transcription more precisely. We found that primary T-ALLs exhibited a wide range of *NFATC3-PLA2G15* expression, with low levels being found in the majority. Of note, *NFATC3-PLA2G15* levels in normal tissue samples were consistently very low or undetectable (Figure 1D). The results of 5' RACE PCR of leukemic cDNA were consistent with initiation of fusion transcription in the first exon of *NFATC3* (Supplementary Figure S2). We also performed array competitive genomic hybridization of 115 diagnostic T-ALL samples, and found no evidence of microdeletions that would result in *NFATC3-PLA2G15* transcript expression (Supplementary Figure S3), providing strong evidence that *NFATC3-PLA2G15* is a true ISC that is generated by *cis*-SAGe.

We next tested the activity of the NFATC3-PLA2G15 fusion. Immunofluorescent staining showed that NFATC3-PLA2G15 could localize to the nucleus, suggesting that the fusion might have the capacity to affect NFAT target gene transcription (Figure 2A). The results of luciferase assays showed that the fusion had NFAT reporter activity *in vitro*, but that this was considerably lower than the WT protein (Figure 2B and Supplementary Figure S4). We then performed gene set enrichment analysis (GSEA) of expression data from primary T-ALL samples, using a set of genes that are regulated by calcineurin/ NFAT in normal human lymphocytes (Supplementary Table S2). This analysis revealed that T-ALL cases with high *NFATC3-PLA2G15* levels had generally lower expression of canonical NFAT target genes than *NFATC3-PLA2G15* low cases (Figure 2C).

Finally, we tested whether *NFATC3-PLA2G15* transcription correlated with T-ALL biology *in vivo*. Strikingly, we found that higher *NFATC3-PLA2G15* levels strongly predicted shorter time to leukemia development (Figure 3A) and survival (Figure 3B) in patient-derived T-ALL xenografts in immunodeficient mice (see Supplementary Figure S5 for fusion transcript expression in individual samples used for xenofrafts). In order to

estimate the clinical relevance of *NFATC3-PLA2G15* expression, we analyzed the outcome of human T-ALL patients treated as part of the Francophone multinational GRAALL-2003 and -2005 studies. In line with the murine xenograft results, we found that patients with the highest quartile of *NFATC3-PLA2G15* expression (Figure 1D) had reduced overall and event-free survival compared with the rest of the patient cohort (Figures 3C and 3D). As shown in Supplementary Table S4, *NFATC3-PLA2G15* high patients did not differ from the *NFATC3-PLA2G15* low group with regard to classical risk factors such as age, *NOTCH1/FBXW7* mutations and initial treatment response. The prognostic effect of *NFATC3-PLA2G15* expression was however outweighed by our recently reported mutational classifier (see Supplementary Table S4), and the potential influence of *NFATC3-PLA2G15* on patient outcome requires further examination in independent studies, ideally complemented by more extensive evaluation of fusion transcription by RNA-sequencing.

Further work is necessary to determine the mechanism by which the fusion may alter T-ALL biology. We found that, unlike constitutively nuclear mutants of NFAT (17), ectopic expression of NFATC3-PLA2G15 was insufficient to induce transformation of NIH 3T3 fibroblasts *in vitro* (data not shown). This finding is not unusual for confirmed T-ALL oncogenes. For example, most *NOTCH1* gain-of-function mutants are not sufficient to induce leukemia in murine models (18). It is therefore likely that the oncogenic effects of the fusion might require additional cooperative events. It remains to be seen whether the altered NFAT activity detected *in vitro* contributes to reduced canonical NFAT target transcription in patient samples *in vivo*. The latter finding should be interpreted with caution, as we have previously found that the transcriptomic signature associated with

calcineurin activity in murine T-ALL models is distinct from that seen in normal human T-cells (13).

Interestingly, *NFATC3-PLA2G15* fusions have also been described in an isolated case of acute myeloid leukemia (19) and in colorectal cancer, where experimental inhibition of the fusion transcript was reported to cause decreased proliferation and invasion of a cell line *in vitro* (20). Unfortunately, none of the T-ALL cell lines we tested had significant *NFATC3-PLA2G15* expression (data not shown), so we were unable to investigate similar effects in a T-lymphoid context. Although the involved exons reported in the above cases differed from the T-ALL-associated chimera reported here, these data suggest that *NFATC3-PLA2G15* ISCs might have important activities in other malignancies.

Finally, our discovery of frequent ISC expression in this series identifies a novel oncogenic mechanism in T-ALL, and provides a rationale for further evaluation of this phenomenon in acute leukemia.

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Figures Legends:

Figure 1: *NFATC3-PLA2G15* is a novel T-ALL-associated intergenically spliced chimeric RNA. (A) RNA-sequencing analysis pipeline. Total and median numbers of fusions detected following the application of each filter are shown. The full list of candidate ISCs is shown in Supplementary Table S3. **(B)** Genomic visualization of chimeric *NFATC3-PLA2G15* transcripts using a Sashimi-plot representation generated using the Integrative Genomics Viewer tool. Genomic tracks display splicing junctions in two samples (T-ALLs 1 and 2) positive for *NFATC3-PLA2G15* chimeric transcripts, indicated by black arrows. Analysis of two representative samples negative for the fusion (T-ALLs 3 and 4) and human thymic RNA are shown for comparison. Chromosomal coordinates correspond to the hg19 assembly. **(C)** Expression of the *NFATC3-PLA2G15* chimeric mRNA was confirmed by RT-PCR (upper panel) and direct sequencing (lower panel). Involved exons are indicated. NTC = No Template Control. **(D)** Distribution of *NFATC3-PLA2G15* expression in primary human T-ALL and normal tissue samples (comprising bone marrow, thymic and peripheral blood lymphocyte extracts). Chimeric transcript levels were quantified by fusion-specific RT-QPCR and calculated relative to an *ABL* housekeeping gene control. The horizontal line delineates the upper quartile of expression. *NFATC3-PLA2G15* high T-ALL cases (n= 30) are shown in red, *NFATC3-PLA2G15* low T-ALL cases (n = 93) in blue, and normal samples (n = 8) in black.

Figure 2: Activity of the NFATC3-PLA2G15 chimera. (A) Immunofluorescent staining of 293T cells transfected with either NFATC3 wild-type (upper panel) or NFATC3-PLA2G15 (lower panel) expression vectors. The relevant staining is indicated. **(C)** Luciferase assay. 293T cells were transfected with NFATC3 expression vectors and an NFAT-specific luciferase reporter. Activity was calculated relative to an empty vector

luciferase control. The mean results of three experiments are shown. Verification of NFATC3 protein expression is shown in Supplementary Figure S4. Error bars represent standard error of the mean. Statistically significant differences are indicated. **(D)** Gene set enrichment analysis (GSEA) of canonical NFAT pathway transcription. *NFATC3-PLA2G15* high cases (n = 10) exhibited negative enrichment for genes involved in calcineurin-regulated NFAT-dependent transcription in normal lymphocytes, as compared with *NFATC3-PLA2G15* low cases (n = 10). See Methods and Supplementary Table S2 for gene details.

Figure 3: Biological correlates of *NFATC3-PLA2G15* expression. Correlation of *NFATC3-PLA2G15* chimeric transcript levels with time to leukemia appearance **(A)** and survival **(B)** in murine patient-derived xenografts. *NFATC3-PLA2G15* levels in patient blasts used for xenografts are shown in Supplementary Figure S5. Each point represents the median figure for 2 xenografts derived from a single patient. N = 13 patients. R^2 and p values were determined by Pearson correlation analysis.

Correlation of diagnostic *NFATC3-PLA2G15* expression levels with **(C)** Overall survival (OS) and **(D)** Event-free survival (EFS) of T-ALL patients treated as part of the GRAALL-2003 and -2005 studies (n = 123). 5 year OS was 52.6% (95% CI 33.3% - 68.7%) in *NFATC3-PLA2G15*-high cases and 69.8% (95% CI 58.8% - 78.3%) in *NFATC3-PLA2G15*-low cases. 5 year EFS was 46.2% (95% CI 27.8% - 62.7%) in *NFATC3-PLA2G15*-high cases and 63.2% (95% CI 52.5% - 72.1%) in *NFATC3-PLA2G15*-low cases. P values are indicated. Clinico-biological comparison of fusion high and low cases is shown in Supplementary Figure S4.

Figure 1

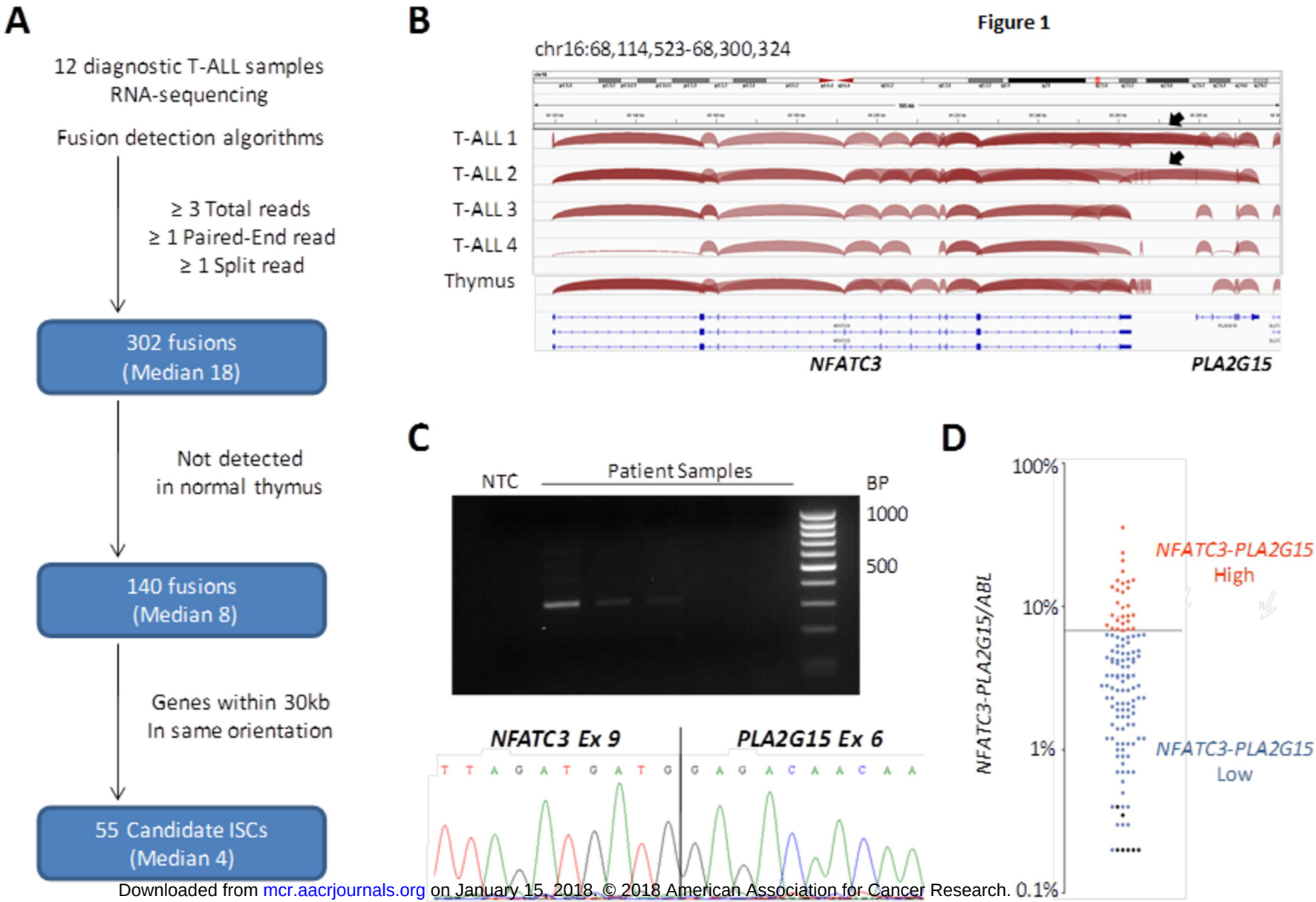
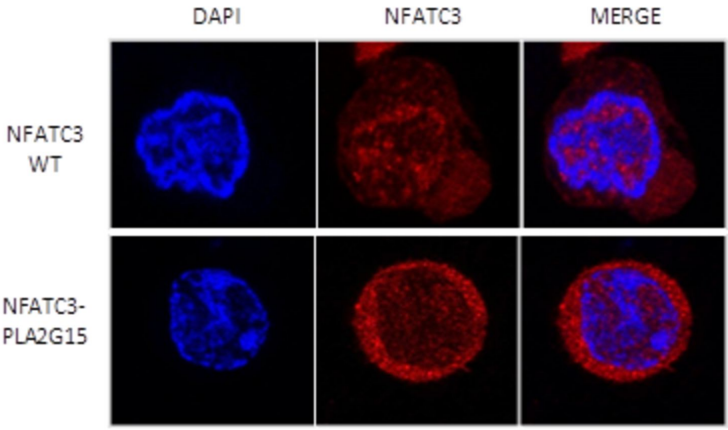


Figure 2

A



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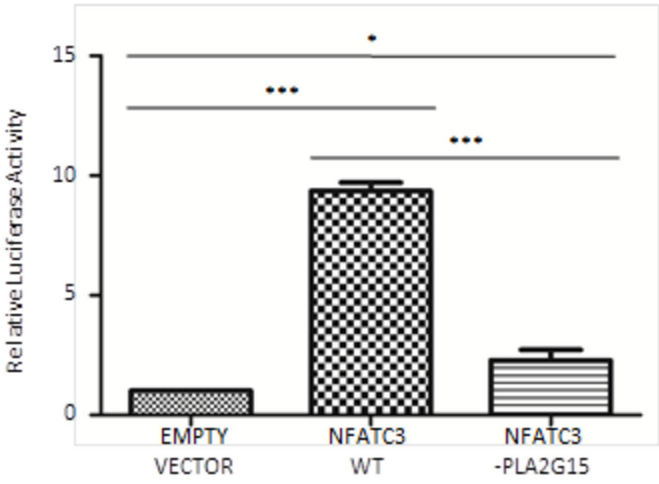

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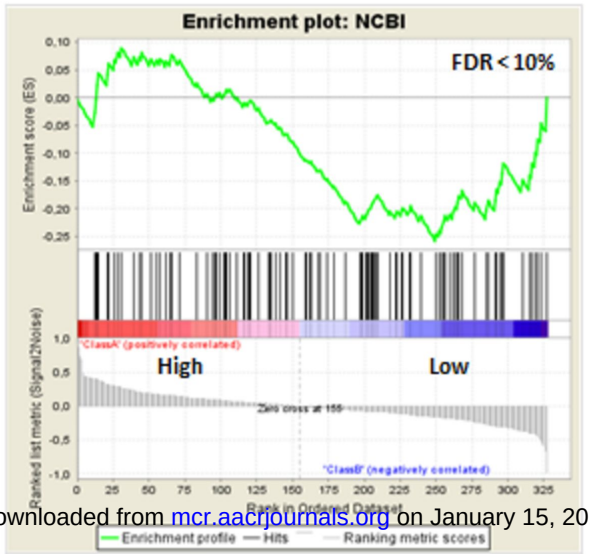
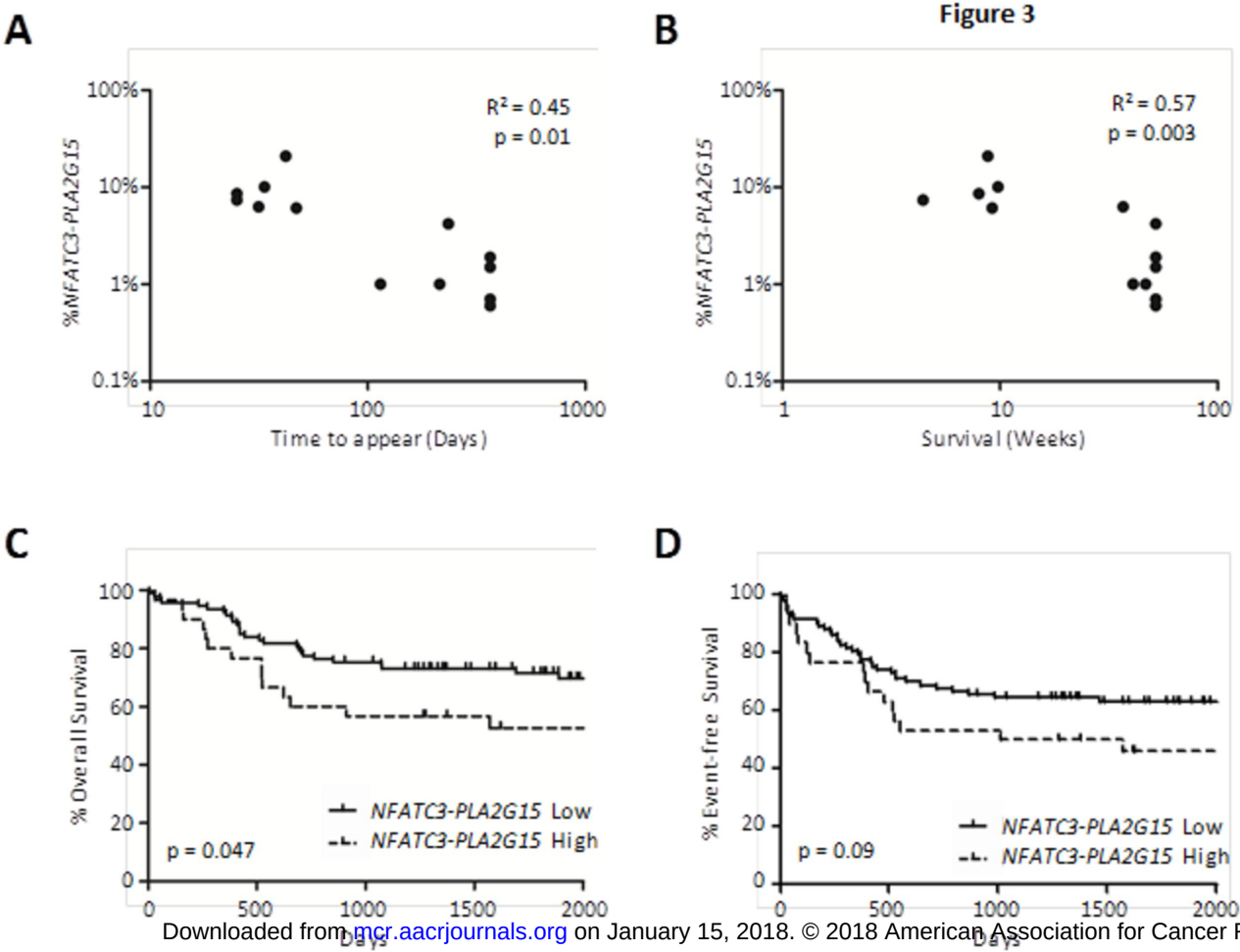


Figure 3



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