LYMPHOID NEOPLASIA

Integrated mate-pair and RNA sequencing identifies novel, targetable gene fusions in peripheral T-cell lymphoma

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Key Points

- *VAV1* rearrangements are recurrent in PTCLs, drive tumor cell growth, and can be targeted by clinically available RAC1 inhibitors.
- Integrated mate-pair/RNA sequencing of PTCLs identifies expressed fusion transcripts that represent candidate therapeutic targets.

Peripheral T-cell lymphomas (PTCLs) represent a heterogeneous group of T-cell malignancies that generally demonstrate aggressive clinical behavior, often are refractory to standard therapy, and remain significantly understudied. The most common World Health Organization subtype is PTCL, not otherwise specified (NOS), essentially a "wastebasket" category because of inadequate understanding to assign cases to a more specific diagnostic entity. Identification of novel fusion genes has contributed significantly to improving the classification, biologic understanding, and therapeutic targeting of PTCLs. Here, we integrated mate-pair DNA and RNA next-generation sequencing to identify chromosomal rearrangements encoding expressed fusion transcripts in PTCL, NOS. Two of 11 cases had novel fusions involving *VAV1*, encoding a truncated form of the VAV1 guanine nucleotide exchange factor important in T-cell receptor signaling. Fluorescence in situ hybridization studies identified *VAV1* rearrangements in 10 of 148 PTCLs (7%). These were observed exclusively in PTCL, NOS (11%) and anaplastic large cell lymphoma (11%). In vitro, ectopic expression of a *VAV1* fusion promoted cell growth and migration in a RAC1-dependent manner. This growth was inhibited by azathioprine, a clinically available RAC1 inhibitor. We also identified novel kinase gene fusions, *ITK-FER* and *IKZF2-ERBB4*, as candidate therapeutic targets that show similarities to known recurrent oncogenic *ITK-SYK* fusions and *ERBB4* transcript variants in PTCLs, respectively. Additional novel and potentially clinically relevant fusions also were discovered. Together, these findings identify *VAV1* fusions as recurrent and targetable events in PTCLs and highlight the potential for clinical sequencing to guide individualized therapy approaches for this group of aggressive malignancies. (*Blood*. 2016;128(9):1234-1245)

Introduction

Peripheral T-cell lymphomas (PTCLs) represent a heterogeneous group of non-Hodgkin lymphomas (NHLs) of mature T-cell origin with poor prognosis.[1] The most common subtype of PTCL in Western countries is PTCL, not otherwise specified (NOS), which has 5-year overall and failure-free survival rates of 32% and 20%, respectively.[2] Although less common than B-cell NHLs, the incidence of PTCL, NOS and PTCLs in general has been increasing steadily in the United States in recent years.[3] By definition, PTCL, NOS includes cases failing to meet criteria for a more specific PTCL subtype and has been referred to as a "wastebasket" category.[4,5] Therefore, PTCL, NOS is markedly heterogeneous, and a major goal of PTCL research is to identify molecular abnormalities that improve classification and identify candidate therapeutic targets.[6]

Recurrent chromosomal rearrangements giving rise to expressed fusion transcripts play a key role in the pathogenesis and clinical management of hematologic malignancies.[7] Among PTCLs, rearrangements of the tyrosine kinase gene, *ALK*, have diagnostic, prognostic, and therapeutic significance in ALK-positive anaplastic large cell lymphoma (ALCL).[8-10] In addition, rearrangements producing fusion genes involving *TP63*, *ROS1*, and *TYK2* recently have been identified in ALCLs that are negative for ALK.[11-15] However, beyond rare *ITK-SYK* fusions,[16] the contribution of gene fusions to the pathogenesis of PTCL, NOS remains poorly understood.

We previously have shown the efficacy of mate-pair DNA sequencing (MPseq) to identify chromosomal rearrangements in PTCLs

and other human cancers.[12,17,18] MPseq utilizes ligation of end-labeled DNA fragments followed by refragmentation and affinity purification to generate libraries of fragments containing 2 DNA sequences originally separated by a genomic distance of several kilobases. This technique has superb sensitivity for detecting rearrangements across the entire genome at a fraction of the cost of whole-genome sequencing; however, not all chromosomal rearrangements identified by MPseq involve named genes or give rise to expressed fusion transcripts. Conversely, RNA sequencing (RNAseq) preferentially identifies expressed fusion transcripts, but false positives remain a challenge due to difficulties mapping RNA reads to the genome.[19] Taking advantage of the strengths of both approaches and simultaneously providing orthogonal validation of the results, we integrated MPseq and RNAseq data to identify expressed fusion transcripts in 11 cases of PTCL, NOS. We then performed further validation, functional studies, and assessment in additional PTCL tissue samples to explore potential clinical implications of selected findings.

Materials and methods

Patients and clinical samples

Eleven cases of PTCL, NOS were analyzed by integrating data from MPseq (including 2 patients reported previously[12]) and RNAseq. These cases were selected based on availability of sufficient frozen material for sequencing studies as outlined below. Data on biopsy site and treatment status at time of biopsy are presented in supplemental Table 1, available on the *Blood* Web site. Additional PTCLs (n = 137) were evaluated by fluorescence in situ hybridization (FISH) for *VAV1* rearrangements. The study was approved by Mayo Clinic and University of Iowa Institutional Review Boards.

Sequencing and bioinformatics

MP libraries were prepared from genomic DNA extracted from frozen PTCL samples using the Mate Pair Library Prep Kit (Illumina) and sequenced on a HiSeq2000 (Illumina) as previously published.[12,17] Reads were mapped to the human genome (GRCh38/hg38) using BIMA-V3 as published.[18] Candidate events were eliminated if they had mapping scores that did not meet filtering criteria; were intrachromosomal events spanning <30 000 bp; were supported by ≤3 fragments; or were contained within a mask table including events occurring in noncancerous samples. RNAseq was performed on RNA extracted from frozen samples and chimeric transcripts were identified using SnowShoes-FTD as published.[12,19]

FISH

A breakapart FISH probe flanking the *VAV1* locus on 19p13.3 was developed by labeling bacterial artificial chromosomes with SpectrumOrange (telomeric/5′: RP11-134L9, RP11-479K19, RP11-828J24, RP11-114A7) or SpectrumGreen (centromeric/3′: CTD-2596O14, CTD-2564J11, RP11-876D1), and interphase FISH was performed and scored on paraffin tissue sections of PTCLs as previously described.[12,17,20] A normal intact *VAV1* gene region is indicated by a yellow signal. Disruption of the *VAV1* gene region is indicated by separation of the yellow signal into one red (5′) signal and 1 green (3′) signal. Two-sided χ^2 tests were used to evaluate subtype specificity.

Cell lines, transfection, and inhibitor treatment

Jurkat cells (ATCC) were maintained in RPMI, and NIH-3T3 and HEK-293T (ATCC) were maintained in Dulbecco's modified Eagle medium, all containing 10% fetal bovine serum (Clontech) and 1% penicillin/streptomycin (Gibco). They were transfected using electroporation (Jurkat) or Lipofectamine 3000 (Life Technologies; NIH-3T3 and HEK-293T). Functional experiments were performed after 24 to 72 hours. *RAC1* (Dharmacon) or control (AllStars; Qiagen) small interfering RNAs were transfected by electroporation, and cell growth was assessed at 72 hours. Cells were treated with azathioprine (Selleck) for 72 hours.

Expression plasmids

VAV1-GSS was cloned from TCL26 cDNA into TOPO-TA (Invitrogen) using primers 5′-GGCGACAGTTACAGGCAAAGAAG-3′ and 5′-TAGGAGAGG AATGACAAATACAGAGG-3′, sequenced verified, and cloned into pCI (Promega) using primers 5′-CTGTATGACTGCGTGGAGAATG-3′ and 5′-TAG GAGAGGAATGACAAATACAGAGG-3′. *ITK-FER* was cloned into TOPO-TA from TCL72 cDNA using primers 5′-ATGAACAACTTTATCCTCCTGG-3′ and 5′-AATAATGTTACTCTGCTGGAGG-3′ and then shuttled into pcDNA3.1(+) (Invitrogen) using *Bam*HI and *Eco*RV restriction enzyme digestion. Human pCI2-*VAV1* was from D.D.B.

Immunofluorescence and immunoblotting

NIH-3T3 cells were transfected and replated on acid-washed coverslips after 24 hours. At 48 hours after transfection, cells were fixed, permeabilized, and stained for actin (tetramethylrhodamine-phalloidin) and VAV1 (Abcam ab97574 and goat anti-rabbit Alexa-Fluor-488). Cells were mounted using Vectashield with 4′,6-diamidino-2-phenylindole (Vector Laboratories). Fluorescence micrographs (40×) were captured using iVision (BioVision) and processed using Adobe Photoshop. Circularity was calculated using ImageJ (National Institutes of Health) with a value of 1 indicating a perfect circle.

Immunoblotting was performed as previously described[21] using antibodies for pVAV1(Y174) (Abcam; EP510Y), β-actin (Novus; AC-15), VAV1 (Cell Signaling; #2502), and RAC1 (Millipore; clone 23A8). Proteins were detected using IRDye 800CW and 680RD antibodies (LI-COR) on the LI-COR Odyssey CLx. Rac activity was assessed using GST-PAK1-PBD (p21-binding domain) pulldown as described previously.[22]

Functional assays

Cell migration was quantified by fluorometric assay 48 hours after transfection. Jurkat cells were labeled with 2 μM Calcein AM (Molecular Probes) for 45 minutes at 37°C in serum-free RPMI. Cells were then washed, resuspended in serum-free RPMI, and added to 5-μm inserts (Costar) equilibrated with RPMI containing 10% serum. Cells were allowed to migrate for 3 hurs at 37°C and 5% CO_2. Migrated cells then were harvested and fluorescence was measured using a CytoFluor 4000 plate reader (Applied Biosystems). Percent of cells migrated was calculated by dividing the fluorescence of the migrated cell suspension by that of the starting cell suspension.

Colony formation was performed by seeding 200 cells per well of a 6-well plate at 24 hours after transfection and incubating for 8 days. Plates were imaged and counted with AlphaImager HP (ProteinSimple). Cell growth was measured by plating at 10 000 cells per well in a 96-well plate, treating for 48 to 72 hours, and incubating with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Promega) for 2 to 4 hours. Absorbance was measured at 490 nm. Significance was evaluated using a Student t test.

Results

Integrated MPseq/RNAseq identifies novel expressed fusion transcripts in PTCL, NOS

Eleven cases of PTCL, NOS were analyzed by integrated MP/RNA-sequation (9 men and 2 women; median age, 62 years; range, 48-75 years; Table 1). The number of genomic events identified in each case by MPseq varied from 2 to 143 (378 total events; mean/case ± standard deviation, 34 ± 43; Figures 1A-D). Seventy-two percent of events were intrachromosomal (range/case, 42-100%; Figure 1E). One case (TCL66) showed evidence of catastrophic chromosomal rearrangement, or chromothripsis,[23] involving chromosomes 16 and 19 (Figure 1C). Sixty-five percent of events involved 1 to 2 nongenic regions (Figure 1F). An additional 11% of events were intragenic. The remaining 24% of events (n = 90) each involved 2 named genes.

Table 1. Expressed fusion transcripts identified by integrated MPseq/RNAseq in PTCL, NOS

Case	Age (years)	Sex	Diagnosis	DNA breakpoints	Fusion transcript(s)
TCL26	70	Male	PTCL, NOS	19p13.3, 20q11.22	*VAV1 ↔ GSS*
TCL32	72	Male	PTCL, NOS	None	None
TCL40	50	Female	PTCL, NOS	None	None
TCL65	61	Male	PTCL, NOS	3q28, 6p22.3	*ATXN1 → TP63*
				1p36.23, 11q24.1	*CRTAM ↔ TNFRSF9*
TCL66	59	Male	PTCL, NOS	16p13.3, 19q13.33	*BAX → TCEB2*
				19q13.2, 19q13.33	*GSK3A → ZNF541*
				19q13.12, 19q13.43	*LOC100128398 → THAP8*
TCL72	48	Male	PTCL, NOS	5q33.1, 5q33.1	*ANXA6 → RBM22*
				19p13.2, 19p13.2	*CARM1 → ZNF627*
				1p33, 1p35.3	*PPP1R8 → EFCAB14*
				5q21.3, 5q33.3	*ITK → FER*
				1p35.2, 16q22.3	*PUM1 → ZFHX3*
				4p16.3, 4p16.3	*STX18 → ZNF718*
TCL75	67	Male	PTCL, NOS	2q34, 2q34	*IKZF2 → ERBB4*
				2q12.3, 2q13	*RANBP2 → LIMS1*
TCL80	56	Male	PTCL, NOS	19p13.2, 19p13.3	*VAV1 ↔ MYO1F*
TCL81	64	Female	PTCL, NOS	14q32.13, 14q32.2	*ATG2B → CLMN*
				1p13.1, 1q23.2	*COPA → CD58*
				1p33, 1p34.2	*SZT2 → EFCAB14*
TCL84	62	Male	PTCL, NOS	None	None
TCL86	75	Male	PTCL, NOS	None	None

→, gene direction of fusion transcript; ↔, reciprocal fusion transcript also present.

Among the 90 events involving 2 genes, RNAseq identified expressed fusion transcripts in 18 (mean, 1.6 fusions/case; range, 0–6 fusions/case; Table 1); 13 (72%) were derived from intra-chromosomal events, identical to the proportion among all DNA events. Three DNA events yielded reciprocal fusion transcripts. All gene pairs were unique and novel except *ATXN1→TP63* as previously reported.[12] Two genes, *VAV1* and *EFCAB14*, were involved in fusions in >1 case.

VAV1 rearrangements are recurrent events in PTCLs and show subtype specificity

We examined *VAV1* rearrangements in greater detail because they were recurrent and because *VAV1* encodes a guanine nucleotide exchange factor (GEF) critical in T-cell receptor (TCR) signaling.[24,25] The 2 cases showed rearrangements with *GSS* on 20q11.22 (Figure 2A) and *MYO1F* on 19p13.2, respectively. RNAseq identified reciprocal expressed fusion transcripts in both cases. *VAV1-GSS* is shown in Figure 2B and also was revalidated by Sanger sequencing (Figure 2C). We also confirmed the presence of the resultant VAV1-GSS fusion protein by western blot (supplemental Figure 1). *VAV1-GSS* (*GSS* exons 2-13) and *VAV1-MYO1F* (*MYO1F* exons 26-28) showed identical *VAV1* fusion sites, resulting in loss of the VAV1 C-terminal SH3 (CSH3) domain (Figure 2D). The reciprocal fusions, *GSS-VAV1* and *MYO1F-VAV1*, encoded only the CSH3 domain of VAV1.

We then investigated the frequency of *VAV1* rearrangements by interphase FISH on 148 PTCLs (Table 2; Figure 2E). FISH results were 100% concordant with MPseq/RNAseq findings for the 11 cases analyzed by both methods. *VAV1* rearrangements were present in 10 of 148 cases (7%). These rearrangements were restricted solely to PTCL, NOS (5 of 45; 11%; $P = .0153$) and ALCL (5 of 47; 11%; $P = .0175$). Because some PTCLs, NOS show similarities to ALCL and may represent a pathologic spectrum characterized by CD30 expression,[26] we examined CD30 expression in the cases with *VAV1* rearrangements. Although all ALCLs strongly expressed CD30, 5 of 5 cases of PTCL, NOS with *VAV1* rearrangement were negative for CD30 (Figure 2F).

VAV1-GSS promotes cell migration and growth

Because the VAV1 CSH3 domain mediates autoinhibition of VAV1 GEF activity,[27] we hypothesized that *VAV1* fusions, which lack this domain, promote VAV1 activation. Because VAV1 mediates cytoskeletal remodeling,[28] we first performed immunofluorescence for actin in adherent NIH-3T3 fibroblasts transfected with *VAV1-GSS*, wild-type (wt)*VAV1*, or an empty control vector. We observed cytoskeletal rearrangement characterized by increased circularity in *VAV1-GSS* transfected cells (0.834 ± 0.033, where 1 indicates a perfect circle) compared with either wt*VAV1* (0.765 ± 0.048; $P = .045$) or control (0.453 ± 0.089; $P < .0001$; Figure 3A-B).

We then examined the effect of the VAV1-GSS fusion protein on migration and growth in the neoplastic T-cell line, Jurkat. VAV1-GSS was phosphorylated at VAV1(Y174), indicative of GEF activity[29] (Figure 3C). Cells expressing VAV1-GSS also showed increased migration (124% of empty vector control, $P = .0016$; $P = .05$ vs wtVAV1; Figure 3D). Finally, VAV1-GSS strongly promoted cell growth (159% of empty vector control; $P < .0001$ vs both control and wtVAV1; Figure 3E).

VAV1-GSS drives growth through RAC1 and can be targeted by Rac inhibition

Because VAV1 GEF activity predominantly targets the Rho family GTPase RAC1 (and to a lesser extent RHOA and CDC42),[30,31] and RAC1 has been demonstrated to promote migration in an ALCL model,[32] we measured Rac activation following *VAV1-GSS* transfection in Jurkat cells. VAV1-GSS overexpression increased active Rac (GTP-Rac; 173% of control; $P = .054$; Figure 4A-B). Furthermore, *RAC1* knockdown abrogated the growth advantage conferred by *VAV1-GSS*, reducing growth from 125% to 102% of the baseline control value ($P = .0004$; Figure 4C). *RAC1* knockdown had no effect on growth of control-transfected Jurkat cells. These data indicate that VAV1-GSS function is at least in part RAC1 dependent.
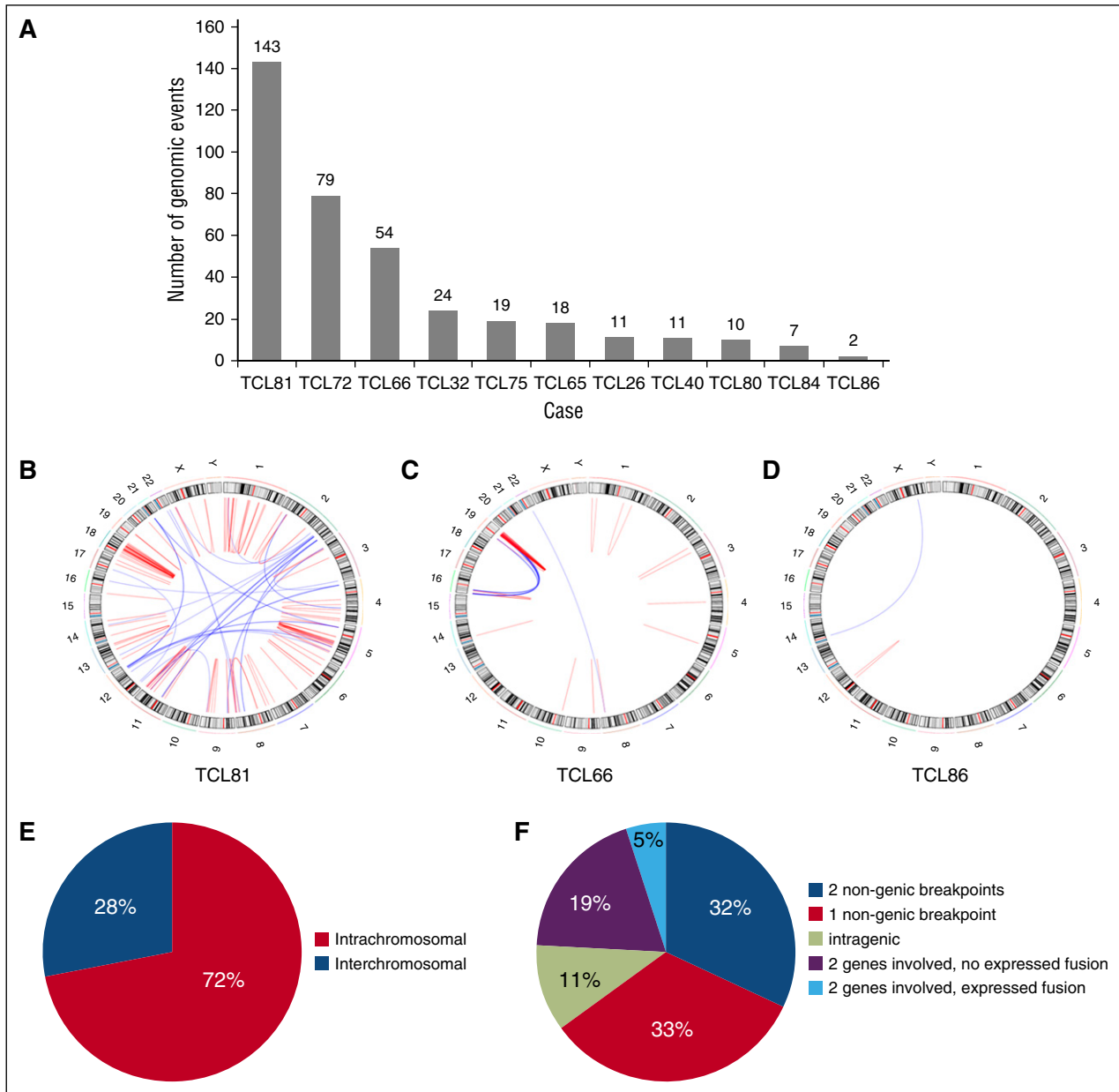
Figure 1. Distribution of 378 genomic events identified by mate-pair sequencing in 11 cases of PTCL, NOS. (A) Number of genomic events per case. (B-D) Representative Circos diagrams illustrating heterogeneity in the degree of complexity of genomic events among PTCLs: (B) high complexity; (C) focally high complexity with multiple rearrangements involving chromosomes 16 and 19 (chromothripsis); and (D) low complexity. (E) Distribution of intrachromosomal and interchromosomal events. (F) Distribution of events based on involvement of genes or nongenic regions and identification of expression fusion transcripts by RNA sequencing.

We then treated transfected Jurkat cells with azathioprine, a clinically available immunosuppressant and Rac inhibitor.[33-35] At a dose of 10 μM, azathioprine inhibited growth of *VAV1-GSS*–transfected cells by 79.4 ± 1.4% compared with 26.0 ± 2.0% in control-transfected cells (*P* < .0001), and 62.1 ± 4.5% in wt*VAV1*-transfected cells (*P* = .0001; Figure 4D). Taken together, these findings demonstrate that VAV1-GSS promotes increased GEF activity and Rac activation that drives cell migration and growth and confers enhanced sensitivity to Rac inhibition.

MPseq/RNAseq identifies novel kinase fusions in PTCL, NOS

Among the other events identified by MPseq/RNAseq (Table 1) were fusions involving kinase genes known to be involved in oncogenic events in other cancers. Case TCL72 had an in-frame *ITK-FER* fusion (Figure 5A-C). Although ITK is expressed consistently in PTCL, NOS,[36] read-count data showed *FER* expression only in TCL72 exons 11 to 20 encoded by *ITK-FER* (Figure 5D). Of note, *ITK-SYK* fusions are recurrent in PTCL, NOS and SYK inhibitors have been proposed therapeutically.[16,37] *ITK*, *SYK*, and *FER* all encode SH2 domain-containing nonreceptor tyrosine kinases.[38] Like *ITK-SYK*, *ITK-FER* retains the N-terminal plekstrin homology domain of ITK required for ITK-SYK function[39] and contains the FER C-terminal kinase domain in place of the SYK kinase domain (Figure 5E).[16] In line with this, expression of *ITK-FER* in HEK-293T cells resulted in increased colony formation (148% of control, *P* = .0001; Figure 5F). *FER* fusions have not been reported in PTCLs, although an *SSBP2-FER* fusion has been reported in T-cell acute lymphoblastic leukemia.[40] FER is sensitive to
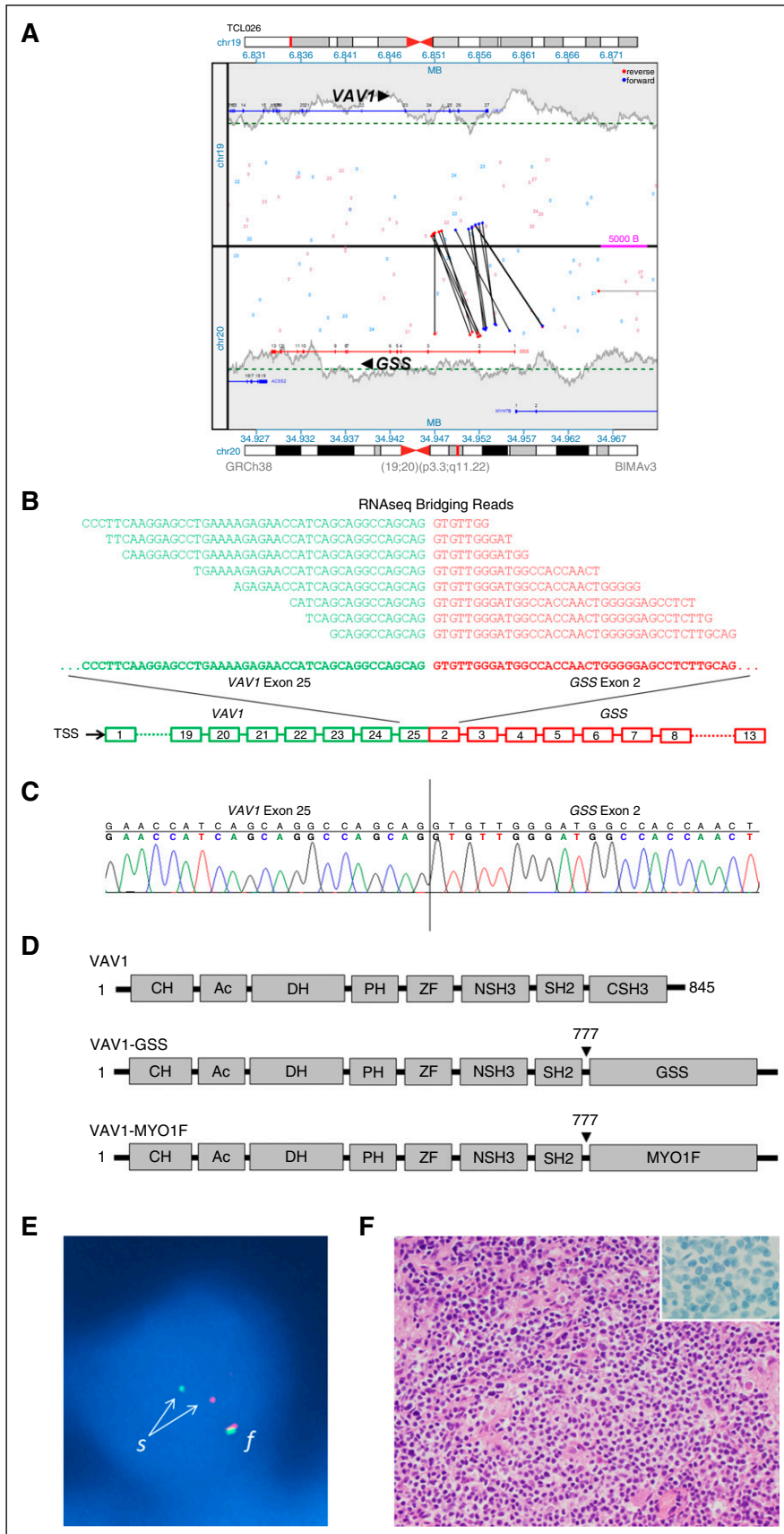
Figure 2. Identification and validation of *VAV1* fusions in PTCL, NOS. (A) Rearrangement of *VAV1* and *GSS* genes as detected in genomic DNA by MPseq. Vertical and oblique black lines represent aberrant read-pairs; blue ends indicate mapping to (+) strand, whereas red ends indicate mapping to (−) strand. (B) *VAV1-GSS* fusion transcript as detected by RNAseq. Bridging reads spanning the fusion site are shown. TSS, transcription start site. (C) Sanger sequencing of the fusion site in *VAV1-GSS* fusion. (D) Schematic diagrams of VAV1 protein domains in wild-type VAV1 and resulting from *VAV1* fusions. (E) FISH confirming a *VAV1* rearrangement in a tumor cell nucleus (blue) from a PTCL, NOS specimen. The normal intact *VAV1* allele is indicated by a red-green fusion (*f*) signal. Disruption of the *VAV1* gene region on the other allele is indicated by separation (*s*) into one red (5′) signal and 1 green (3′) signal. (F) Pathologic features of PTCL, NOS with *VAV1* fusion (hematoxylin and eosin stain; inset, CD30 immunostain). Original magnification ×400 (inset, ×1000).

Table 2. Frequency of *VAV1* rearrangements in 148 PTCLs

	MP/RNA cases*			Additional FISH cases			Total			
	n positive	n tested	%	n positive	n tested	%	n positive	n tested	%	P value†
PTCL, NOS	2	11	18	3	34	9	5	45	11	**0.0153**
AITL	0	0	—	0	36	0	0	36	0	—
ALCL	0	0	—	5	47	11	5	47	11	**0.0175**
ALK⁺	0	0	—	1	18	6	1	18	6	0.2685
ALK⁻	0	0	—	4	25	16	4	25	16	**0.0060**
Cutaneous	0	0	—	0	4	0	0	4	0	—
EATL	0	0	—	0	5	0	0	5	0	—
NKTL	0	0	—	0	7	0	0	7	0	—
CTCL	0	0	—	0	6	0	0	6	0	—
T-LGL	0	0	—	0	2	0	0	2	0	—
Total	2	11	18	8	137	6	10	148	7	—

Bold entries denote statistical significance, $P < .05$.

AITL, angioimmunoblastic T-cell lymphoma; ALK, anaplastic lymphoma kinase; CTCL, cutaneous T-cell lymphoma; EATL, enteropathy-associated T-cell lymphoma; NKTL, extranodal NK-/T-cell lymphoma, nasal type; T-LGL, T-cell large granular lymphocytic leukemia.

*All cases also tested by FISH with 100% concordance.

†Versus AITL, EATL, NKTL, CTCL, and T-LGL together.

kinase inhibitors under evaluation, including aminopyridine 8e, a drug developed to target mutant ALK.[41]

We also identified a novel *IKZF2-ERBB4* fusion in case TCL75 (supplemental Figure 2A-C). *IKZF2* encodes Helios, a T cell–restricted Ikaros-family transcription factor, and is expressed in various PTCLs.[42-45] *IKZF2* was expressed similarly in TCL75 and other cases, whereas *ERBB4* was expressed only in TCL75 exons 2 to 28 encoded by *IKZF2-ERBB4*, which includes the ERBB4 kinase domain (supplemental Figure D-E). Thus, the fusion, which contains only exons 1 to 2 of *IKZF2*, co-opts the *IKZF2* promoter for expression.[46] These findings complement a recent report of truncated *ERBB4* transcripts promoted by intronic long terminal repeats in ALCL, which retain ERBB4 kinase activity and are sensitive to ERBB family kinase inhibitors such as lapatinib.[47]

Examination of exon-level read plots for genes involved in the remaining fusions in Table 1 demonstrated 2 additional events with altered read counts in the case of interest: diminished *CD58* expression in TCL81 (*COPA-CD58* fusion; supplemental Figure 3A) and increased *ZNF541* expression (exons 11-15) in TCL66 (*GSK3A-ZNF541* fusion; supplemental Figure 3B).

Discussion

Here, we integrated MPseq and RNAseq data to identify expressed fusion transcripts in PTCL, NOS, an aggressive malignancy that is the most common form of T-cell NHL.[2] Our findings highlight the marked genomic heterogeneity underlying this disorder, both in overall structural complexity and in the distribution of specific genetic events. We identified recurrent, novel, and targetable rearrangements of the *VAV1* gene, which encodes a critical component of TCR signaling. We also discovered novel kinase fusions that represent candidate therapeutic targets. These data suggest that clinical sequencing approaches may help match PTCL patients to targeted therapeutics for personalized cancer therapy.

We identified *VAV1* rearrangements in 11% of PTCL, NOS and ALCL, but not in other PTCL subtypes, suggesting that the biologic role of the resultant fusion proteins in T-cell lymphomagenesis may be highly context dependent. Of note, knockdown of *VAV1* previously has been shown to lead to cell cycle arrest and apoptosis of ALCL cells.[48] In addition, predicted gain-of-function hotspot mutations of *VAV1*

recently have been reported in adult T-cell leukemia/lymphoma (ATL).[49] ATL was not evaluated in our study group as it is associated with human T-cell leukemia virus type-1 infection, which is non-endemic in the Midwestern United States.

We demonstrated that *VAV1* fusions increased Rac activation and promoted cell growth. We focused on RAC1 because mutation of the VAV1 CSH3 domain strongly activates RAC1, whereas its effects on the ρ GTPases CDC42 and RHOA are more modest.[27] RAC1 also has been shown to be activated by NPM-ALK in ALCL cells, and a Rac small molecule inhibitor blocked lymphoma development and dissemination.[50,51] We showed that cells expressing VAV1-GSS were exquisitely sensitive to azathioprine, which inhibits activation of RAC1 but not of CDC42 or RHOA.[33,34] Other clinically available drugs that inhibit RAC1 and have been proposed to be repurposed as anticancer agents include metformin, statins such as simvastatin, and R-ketorolac.[52-54] RAC1 small molecule inhibitors are in ongoing development.[55,56] Taken together, these data suggest RAC1 inhibition is a potential therapeutic strategy for PTCLs with *VAV1* fusions, noting, however, that these fusions are present in the minority of PTCLs.

Our data add to a growing body of evidence supporting the importance of ρ-family GTPases in PTCL, including recently reported *RHOA* mutations in PTCL, NOS and angioimmunoblastic T-cell lymphomas[57-59] and redundant and nonredundant roles for RAC1 and CDC42 in ALCL cell growth, migration, and dissemination.[32,48] More broadly, these findings suggest the importance of TCR signaling as a key pathway involved in lymphomagenesis,[60] as has been demonstrated in mice[61] and is evidenced further by other genes altered by various mechanisms in PTCLs, including rearrangements of *SYK*, copy number gains of *ITK*, and point mutations of *FYN*.[16,49,57,62] These potentially targetable molecular alterations show promise for advances analogous to those resulting from elucidation of dysregulated B-cell receptor signaling in B-cell NHLs.[63,64]

It should be noted that, in addition to the RAC1-mediated effects we demonstrated in *VAV1-GSS*-transfected cells, VAV1 has non–GEF-associated functions in T cells as well.[65] For example, loss of the VAV1 CSH3 domain also might increase activity of nuclear factor of activated T cells through loss of nuclear factor of activated T cells–inhibitory residues.[66] RAC1 also has multiple mechanisms of oncogenic activity, including its ability to enhance cytokine-mediated activation of signal transducer and activator of transcription 3 (STAT3),[67] which plays a central pathogenetic role in many PTCLs.[14,68-71] Finally, the role of the
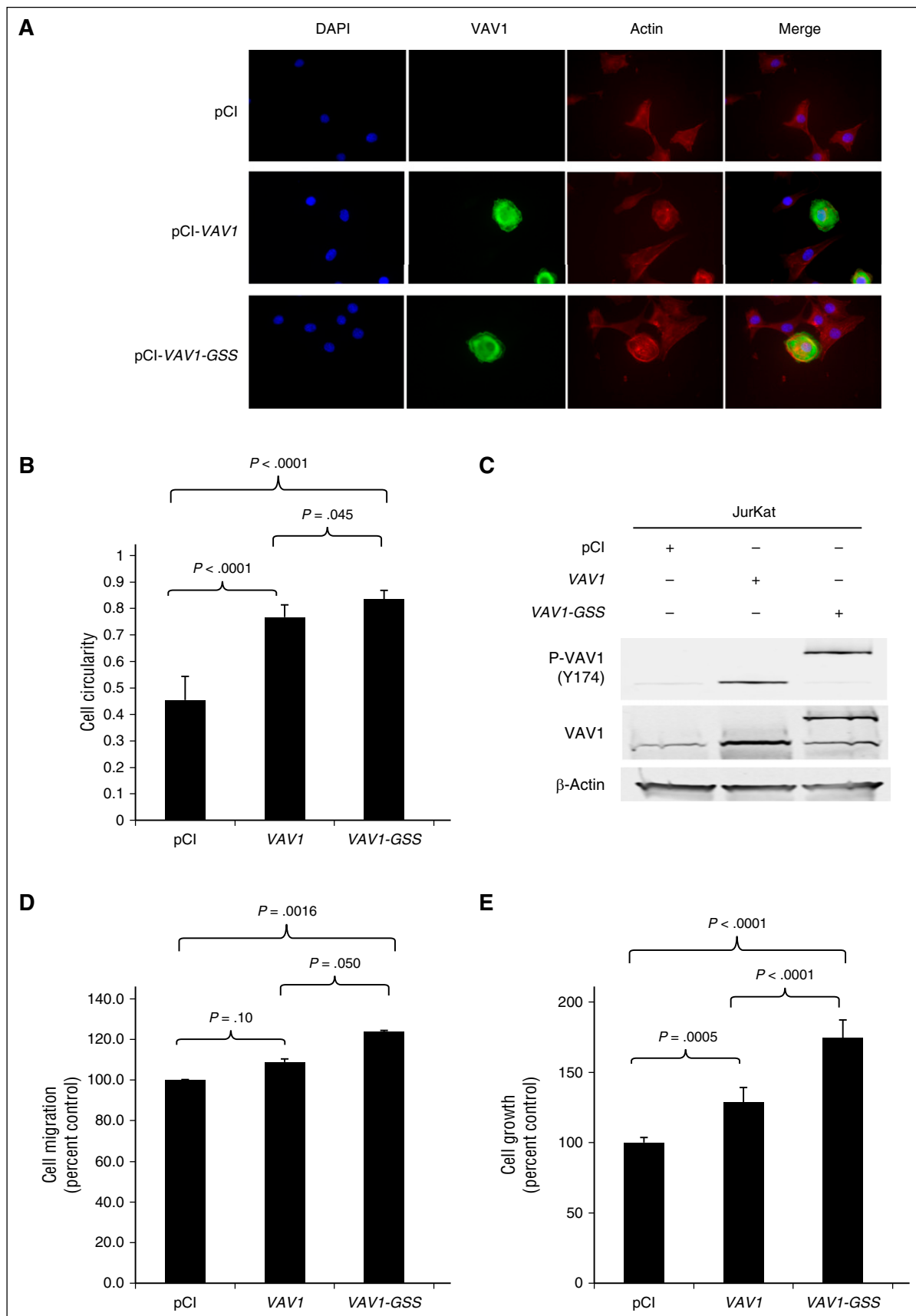
Figure 3. *VAV1-GSS* fusion drives cytoskeletal reorganization, migration, and proliferation. (A) *VAV1-GSS* fusion promotes cytoskeletal reorganization and (B) increased cell circularity in adherent NIH-3T3 cells. A circularity value of 1 indicates a perfect circle. (C) The VAV1-GSS fusion protein is phosphorylated at VAV1 Y174 in Jurkat cells. In Jurkat, VAV1-GSS drives cell (D) migration and (E) growth relative to both wild-type VAV1 and an empty vector control.
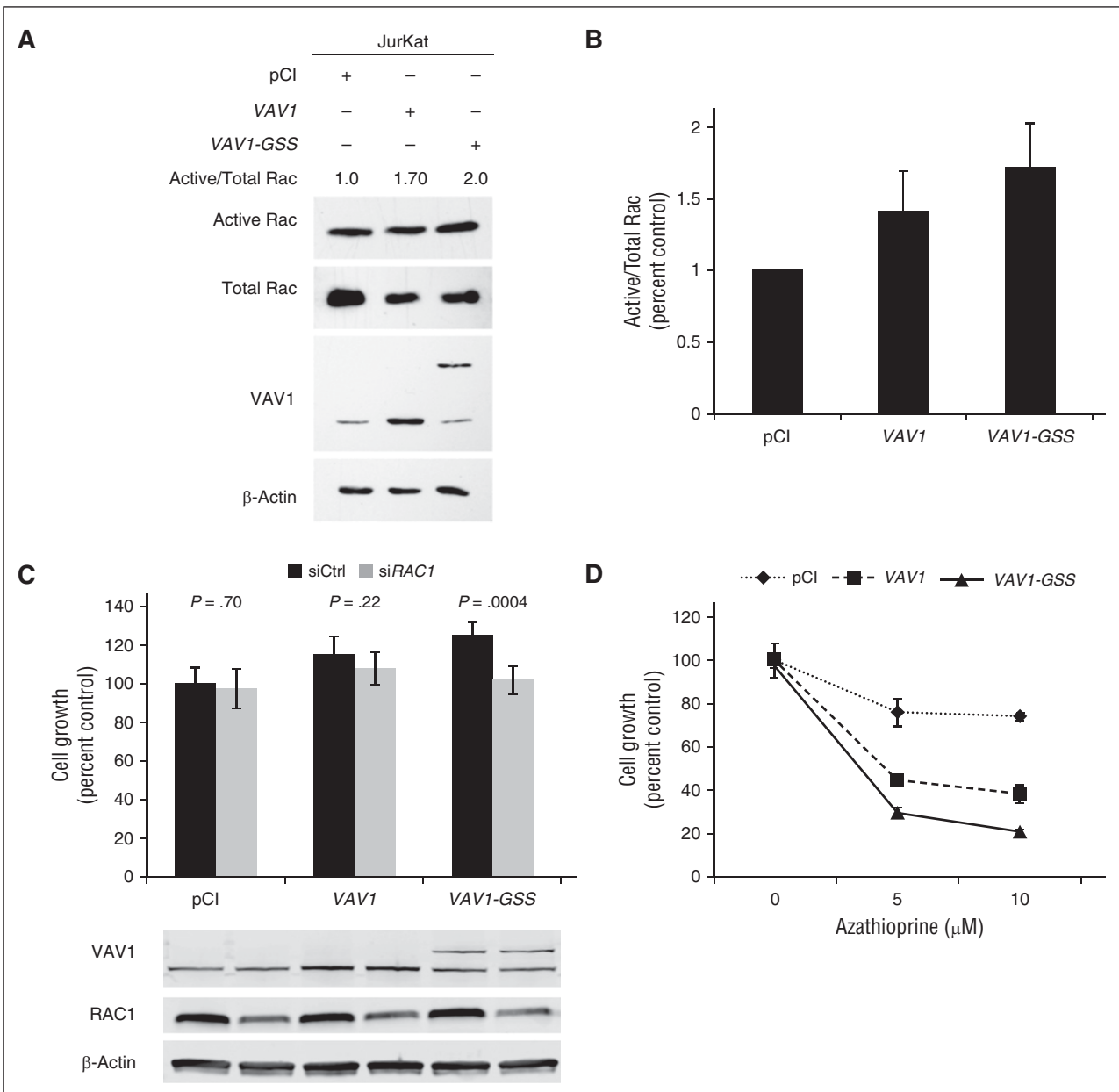
Figure 4. VAV1-GSS fusion induces targetable RAC1 dependence. (A) VAV1-GSS fusion induces Rac activation in Jurkat cells. (B) Rac activation data summarizing 3 independent experiments as shown in A. (C) *RAC1* knock-down abrogates effect of VAV1-GSS fusion on cell growth. (D) Jurkat cells transfected with *VAV1-GSS* exhibit greater sensitivity to azathioprine than cells transfected with wild-type *VAV1* or an empty vector control.

partner gene in *VAV1* fusions, as well the potential function of the reciprocal expressed transcripts, may merit further study.

Our institution and others have developed clinical infrastructure to facilitate a personalized medicine approach to malignant diseases, in which genomic testing might inform therapeutic strategies specific for each individual.[72] Although this concept is still evolving, data on PTCLs from our group and others, as well as our institutional experience with an Individualized Medicine Clinic, suggest PTCL patients might be particularly suitable for this approach.[13,73-75] PTCLs are relatively uncommon and generally are clinically aggressive. They are markedly heterogeneous, both at the genetic–biologic level and at the clinicopathologic level, with >15 subtypes recognized by the World Health Organization.[76] Current standard therapy is acknowledged to be suboptimal, but a number of targeted agents have shown clinical efficacy in

subsets of patients.[1,74] With this in mind, we examined the events identified by MPseq/RNAseq for their potential to suggest therapeutic options.

In addition to *VAV1* rearrangements, we also discovered 2 novel kinase gene fusions, *ITK-FER* and *IKZF2-ERBB4*. As previously exemplified among PTCLs by *ALK* fusions, both fusions resulted in overexpression of a kinase not normally expressed in the cell-of-origin under the control of the partner gene promoter. In keeping with its predicted oncogenic function, *ITK-FER* promoted colony formation in vitro. *ERBB4* encodes a member of the ERBB family, a group of targetable receptor tyrosine kinases that also includes epidermal growth factor receptor (ERBB1), HER2 (ERBB2), and ERBB3, and historically has been associated with the pathogenesis of epithelial and other solid tumors.[77] Recent data, however, support the relevance of this family in PTCLs, including aberrant *ERBB4* transcripts, elevated
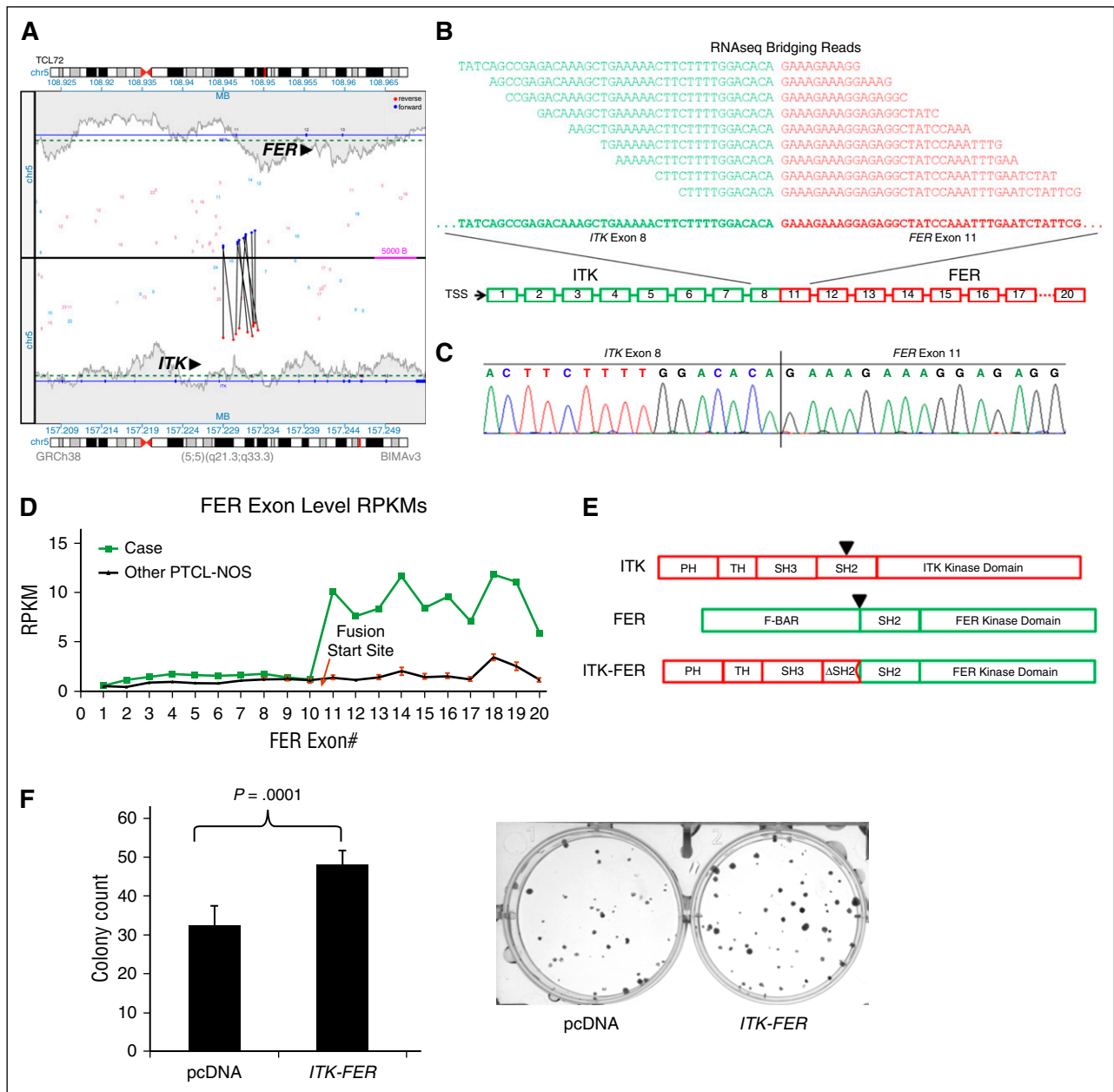
Figure 5. Novel *ITK-FER* kinase fusion in PTCL, NOS. (A) *ITK-FER* rearrangement as detected by MPseq. (B) *ITK-FER* fusion transcript as detected by RNAseq. (C) Sanger sequencing of the *ITK-FER* fusion site. (D) Exon-level RNAseq read plot for *FER* in TCL72 ("Case") compared with the remaining 10 sequenced cases of PTCL, NOS. RPKM, reads per kilobase per million. (E) Schematic diagrams of protein domains in ITK, FER, and the ITK-FER fusion protein. (F) *ITK-FER* expression promotes colony formation in HEK-293 cells. PH, pleckstrin homology; TH, Tec homology; SH, Src homology; F-BAR, FER-CIP4 homology–Bin-amphiphysin-Rvs.

circulating epidermal growth factor levels, and enrichment for somatic mutations targeting the ERBB signaling pathway.[47,78,79]

We also rediscovered a previously reported *ATXN1-TP63* fusion. *TP63* rearrangements are recurrent in PTCL, NOS and ALK-negative ALCL and are characterized by high proliferation indices and poor prognosis.[12,13] In fact, this patient (TCL65) had aggressive disease refractory to multiple regimens, did not achieve a response sufficient to be transplant eligible, and died 13 months after diagnosis. Poor outcomes with standard therapy suggest that patients with *TP63*-rearranged PTCLs might be considered for more intensive or experimental upfront treatment approaches.[12,13]

We identified 2 other genes, *CD58* and *ZNF541*, whose expression differed between rearranged and nonrearranged cases. TCL81 carried a *COPA-CD58* rearrangement associated with decreased *CD58* expression. *CD58* encodes a member of the immunoglobulin superfamily, CD58/LFA-3, an adhesion molecule known to be genetically inactivated by deletions and mutations in B-cell NHLs as a mechanism of immune escape.[80] Deletions and mutations, but not rearrangements, of *CD58* recently have been reported in PTCLs as well.[57,58,81] Rearrangements are a less common structural cause of gene inactivation than deletions but have been reported. For example, we have shown that rearrangements disrupting the *DUSP22* phosphatase gene are associated with decreased expression of the putative tumor suppressor gene, *DUSP22*, in ALCL.[17] We also identified marked expression of *ZNF541* only in TCL66, which carried a *GSK3A-ZNF541* fusion. *GSK3A*, which was ubiquitously expressed in all sequenced samples (data not shown), encodes glycogen

synthase kinase-3a, which itself has been proposed as a therapeutic target in cancer.[82] ZNF541 normally is expressed in germ cells, where it mediates chromatin remodeling and is associated with histone hypoacetylation.[83] Notably, treatment with histone deacetylase inhibitors, a class of drugs US Food and Drug Administration approved for PTCL,[74] dramatically reduced ZNF541 protein levels.

EFCAB14 was involved in fusions in 2 of 11 sequenced cases. This gene encodes a member of the EF-hand superfamily of calcium-binding proteins, which play a role in TCR signaling.[84] In 1 of these cases (TCL72), *EFCAB14* was fused to *PPP1R8*, which encodes a phosphatase inhibitor critical for the activity of EZH2, a promising therapeutic target in PTCL.[85,86] We also identified a reciprocal fusion of *TNFRSF9* (TCL65) encoding CD137/4-1BB, a costimulatory molecule proposed as a therapeutic target in PTCL.[87] Of note, fusions and mutations affecting the costimulatory molecules CD28 and ICOS recently have been observed in PTCL.[49,88] We also identified a fusion involving the proapoptotic gene, *BAX* (TCL66), which has been reported to undergo loss-of-function mutations, but not rearrangements, in hematologic malignancies.[89] Sequencing of additional PTCLs is likely to help prioritize these events for further study and identify additional candidates.

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Authorship

Contribution: R.L.B. and A.L.F. designed the study and wrote the manuscript; D.D.B., M.A.M., and G.V. contributed to study design; R.L.B., G.L.R., Y.Z., G.H., J.C.P., and B.W.E. conducted experiments; R.L.B., S.D., J.I.D., S.H.J., J.B.S., Y.W.A., G.V., and A.L.F. analyzed data; R.A.K. and P.T.G. conducted cytogenetic studies; and P.J.K., B.K.L., S.M.A., and J.R.C. contributed clinical specimens.

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