Title: Frequent and persistent *PLCG1* mutations in Sézary cells directly enhance PLCγ1 activity and stimulate NFκB, AP-1 and NFAT signaling.

Short title: Gain-of-function *PLCG1* mutations in Sézary Syndrome.

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Abbreviations: ATLL (Adult T-cell leukemia/lymphoma), CTCL (Cutaneous T-cell lymphoma), Inositol phosphate (IP), MF (Mycosis Fungoides), NGS (Next Generation Sequencing), *PLCG1* (Phospholipase C Gamma 1 gene), PLCγ1 (Phospholipase C Gamma 1 protein), SS (Sézary Syndrome), TCR (T-cell receptor).

ABSTRACT

Phospholipase C Gamma 1 (*PLCG1*) is frequently mutated in primary cutaneous T-cell lymphoma (CTCL). This study functionally interrogated 9 *PLCG1* mutations (p.R48W, p.S312L, p.D342N, p.S345F, p.S520F, p.R1158H, p.E1163K, p.D1165H and the in-frame indel p.VYEEDM1161V) identified in Sézary Syndrome, the leukemic variant of CTCL. The mutations were demonstrated in diagnostic samples and persisted in multiple tumor compartments over time, except in patients who achieved a complete clinical remission. In basal conditions, the majority of the mutations confer PLCγ1 gain-of-function activity through increased inositol phosphate production and downstream activation of NFκB, AP-1 and NFAT transcriptional activity. Phosphorylation of the p.Y783 residue is essential for the proximal activity of wild-type PLCγ1, but we provide evidence that activating mutations do not require p.Y783 phosphorylation to stimulate downstream NFκB, NFAT and AP-1 transcriptional activity. Finally, the gain-of-function effects associated with the p.VYEEDM1161V indel suggests that the C2 domain may have a novel role in regulating PLCγ1 activity. These data provide compelling evidence to support the development of therapeutic strategies targeting mutant PLCγ1.

INTRODUCTION

Cutaneous T-cell lymphoma (CTCL) is an extra-nodal mature T-cell lymphoma derived from skin-homing memory CD4$^+$ T-cells (Kim et al., 2005). Sézary Syndrome (SS) is a rare leukemic subtype of CTCL, closely related to Mycosis Fungoides (MF), and is associated with a dismal prognosis and an urgent need for effective therapies (Swerdlow et al., 2017).

Recent next generation sequencing (NGS) studies including our own work have identified dysregulation of T-cell signaling and differentiation pathways as a defining feature of mature T-cell lymphomas including CTCL (Choi et al., 2015, da Silva Almeida et al., 2015, Kataoka et al., 2015, Kiel et al., 2015, Kiel et al., 2014, McGirt et al., 2015, McKinney et al., 2017, Palomero et al., 2014, Prasad et al., 2016, Sakata-Yanagimoto et al., 2014, Simpson et al., 2015, Ungewickell et al., 2015, Vallois et al., 2016, Vaqué et al., 2014, Wang et al., 2015, Wang et al., 2017, Woollard et al., 2016, Yoo et al., 2014). After *TP53*, the Phospholipase C Gamma 1 (PLCγ1) gene *PLCG1,* has been identified as the second most commonly mutated gene in SS (Chang et al., 2018, Park et al., 2017) and is also frequently mutated in advanced stage MF (McGirt et al., 2015, Ungewickell et al., 2015, Vaqué et al., 2014) and adult T-cell leukemia/lymphoma (ATLL)(Kataoka et al., 2015). PLCγ1 plays a pivotal role in TCR signaling and is activated by receptor and non-receptor tyrosine kinases, which trigger signaling cascades to activate the transcription factors NFκB, NFAT and AP-1 (Smith-Garvin et al., 2009). These transcription factors regulate the expression of genes involved in cell proliferation, survival, differentiation and death (Smith-Garvin et al., 2009). Nuclear accumulation and constitutive activation of NFκB signaling has been consistently reported in Sézary cells and CTCL cell lines but the underlying mechanism remains unexplained (Giri and Aggarwal, 1998, O'Connell et al., 1995, Sors et al., 2006). Increased NFκB activity is also a key feature of other mature T-cell lymphomas including ATLL and sub-types of peripheral T-cell lymphomas (Martinez-Delgado et al., 2005, Yamagishi and Watanabe, 2012). Elevated

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NFκB activation induces expression of anti-apoptotic genes including *BCL-2*, leading to apoptosis resistance, another hallmark of CTCL (Juvekar et al., 2011).

This study investigates the functional effects of 9 *PLCG1* mutations reported in SS patients on PLCγ1 activity. We show that 5/9 variants confer constitutive activation of PLCγ1 as demonstrated by significant induction of downstream NFκB, NFAT and AP-1 transcriptional activity and increased proximal activity in basal conditions as well as confirming previous findings showing induction of NFAT transcriptional activity. Furthermore, we provide evidence that these gain-of-function mutations increase downstream signaling without phosphorylation of the PLCγ1 p.Y783 residue. We also propose a novel role for C2 domain residues regulating PLCγ1 activity and our findings suggest that mutant PLCγ1 proteins represent potential therapeutic targets in CTCL.

RESULTS

Persistence of *PLCG1* mutations in multiple SS tumor compartments.

Our recent NGS study identified seven coding *PLCG1* mutations in tumor cells from 11 SS patients, including four recurrent aberrations (p.R48W, p.D342N, p.S345F and p.E1163K)(Woollard et al., 2016). In this study, Sanger sequencing confirmed all 11 mutations in DNA from diagnostic blood samples and demonstrated persistence in different tumor compartments including serial blood samples, lesional skin, involved lymph nodes and RNA extracted from tumor-enriched peripheral blood CD4$^+$ T-cells. Using four representative cases, we demonstrate that *PLCG1* mutations persist several years after diagnosis in multiple tumor compartments, strongly implicating their role as driver mutations (Figure 1). In Sézary cells from patient 1, the p.E1163K mutation was detected in all four tumor compartments at diagnosis. In patient 2, the p.S345F mutation persisted in the blood for 17 months after diagnosis, but was not detected after triple therapy (Interferon-α, Photopheresis and

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Bexarotene) led to resolution of the leukemic disease. Subsequently, the mutation was detected in an involved lymph node when the patient developed nodal progression at 72 months. In patient 3, the p.S345F mutation was detected in the blood and Sézary cell-derived RNA up to 51 months after diagnosis, but was absent in the blood 78 months post diagnosis at the time of a complete clinical remission following chemotherapy. Finally, in patient 4, the p.R48W mutation was detected in the blood and at the transcriptional level at diagnosis but not in lesional skin, which is likely attributed to lack of detection sensitivity associated with a sparse cutaneous tumor cell infiltrate. The mutant allele and clonal TCR rearrangement were absent in the blood after the patient achieved complete remission following allogeneic hematopoietic stem cell transplantation.

PLCG1 mutations are frequent in CTCL and other mature T-cell lymphomas.

To identify the most frequent *PLCG1* mutations and those to prioritize for functional studies, we performed a comprehensive analysis of previously published NGS studies (whole-exome and targeted gene sequencing) on CTCL and other mature T-cell lymphomas (see Supplementary Table S1). *PLCG1* mutations were found in 36.2% of ATLLs, 18.2% of peripheral T-cell lymphomas-(nos), 15% hepatosplenic T-cell lymphomas, 12% angioimmunoblastic T-cell lymphomas, 11.8% of CTCLs (MF and SS) and 5.6% T-cell-prolymphocytic leukemias but not identified in anaplastic large cell lymphomas, enteropathy-associated T-cell lymphomas and natural killer/T-cell lymphomas. Collating all mutations reported by NGS studies demonstrated that *PLCG1* harbors 5 hotspot mutations (p.R48W, p.S345F, p.S520F, p.E1163K and p.D1165H)(Figure 2a) with p.S345F being the most frequently reported in 74 individual tumors. All 5 mutations were detected in the SS tumors in our NGS study (Woollard et al., 2016). The seven *PLCG1* mutations (p.R48W, p.S345F, p.S520F, p.E1163K, p.D1165H, p.S312L and p.D342N) confirmed by Sanger sequencing and two additional variants reported in SS (p.R1158H and the p.VYEEDM1161V indel)(Kiel et

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al., 2015) were selected for further analyses (Figure 2b). Pathogenicity predictions suggested that all but one of these mutations (p.S312L) are predicted to be damaging by a consensus drawn from 5/6 algorithms for missense mutations and 3/4 algorithms for the indel (see Supplementary Table S2). PLCγ2 is an isozyme of PLCγ1 and both have high protein sequence identity in all critical functional domains (Koss et al., 2014). PLCγ2 has an analogous role to PLCγ1 and is a critical mediator of B-cell receptor signaling (Koss et al., 2014). Aligning the isozyme sequences revealed that the PLCγ1 p.D342N, p.D1165H and p.M1166 (deleted in p.VYEEDM1161V) mutant residues correspond to the PLCγ2 p.D334H, p.D1140E/G/N/Y and p.M1141K/R mutations somatically acquired in B-cell Ibrutinib-resistant chronic lymphocytic leukemia (Figure 2c)(Burger et al., 2016, Jones et al., 2017, Maddocks et al., 2015).

Gain-of-function PLCγ1 mutations drive NFκB transcriptional activation.

We generated mutant constructs for the 9 PLCγ1 mutations and compared activity and expression to wild-type PLCγ1 using an NFκB luciferase reporter assay and western blotting in HEK293 cells. In basal conditions, 5/9 mutant PLCγ1 proteins (p.S345F, p.S520F, p.E1163K, p.D1165H and p.VYEEDM1161V) significantly increased NFκB transcriptional activity 3.3 – 6.1-fold relative to the wild-type protein (p≤0.03), suggesting they represent gain-of-function mutations (Figure 3b).

Next, we sought to confirm previous reports showing that PLCγ1 mutations can activate NFAT transcriptional activity in basal conditions. Our data is entirely consistent with published findings (Vallois et al., 2016, Vaqué et al., 2014) and confirms that the p.S345F, p.S520F, p.E1163K and p.D1165H mutations significantly elevate NFAT transcriptional activity 3.3 – 4.8-fold relative to wild-type (p≤0.0013)(see Supplementary Figure S1). However in addition we found that the novel p.VYEEDM1161V indel, was the most potent

activator (10-fold; p<0.0001) of NFAT transcriptional activity compared to the other mutant proteins. Interestingly and consistent with a published report (Vallois et al., 2016), in HEK293T cells p.R48W had no significant effect on NFAT transcriptional activity compared to wild-type protein.

Western blotting detected reduced total mutant PLCγ1 expression associated with the p.VYEEDM1161V indel, whist all other mutant proteins had comparable expression to wild-type protein (Figure 3c). However, a PLCγ1 p.Y783 phosphorylation-specific (p-PLCγ1) antibody revealed that the p.VYEEDM1161V indel is associated with increased protein phosphorylation relative to total PLCγ1 expression, whilst the other mutations showed similar levels of phosphorylation to the wild-type protein (Figure 3d).

PLCγ1 mutations activate proximal signaling in basal conditions through a direct increase in inositol phosphate production.

To confirm the observed gain-of-function in transcriptional activity associated with the 5 PLCγ1 mutations in basal conditions, proximal PLCγ1 signaling was investigated. PLCγ1 hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases intracellular calcium to activate calmodulin/calcineurin signaling and drive NFAT activation (Hogan et al., 2003). We used our previously published method (Everett et al., 2009) to assay inositol phosphate (IP) production in COS-7 cells transfected with PLCγ1 gain-of-function mutants (p.S345F, p.S520F, p.E1163K, p.D1165H and p.VYEEDM1161V) and wild-type constructs. Consistent with the transcriptional activation data all 5 mutations were associated with 3.4 − 6.6-fold increase in IP production compared to wild-type PLCγ1 in basal conditions (Figure 4).

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PLCγ1 p.R48W is activating in a T-cell line

Given the high incidence rate of p.R48W in SS (Figure 2a) it was surprising that this variant did not have gain-of-function properties. Therefore, to exclude the possibility that our findings were cell line-specific, we tested the two commonest variants reported in CTCL (p.R48W and p.S345F) in T-cell lines. Overexpression of PLCγ1 in the SS derived cell line SeAx and Jurkat cell lines was associated with high cytotoxicity (data not shown), which has also been reported in other lymphoid cell lines (Vaqué et al., 2014). We therefore used the J.gamma1 T-cell lymphoma line (Irvin et al., 2000), which is a *PLCG1* null line derived from Jurkat cells. Absence of PLCγ1 expression in J.gamma1 cells compared to the parent Jurkat cell line was confirmed by western blotting (Figure 5a). In basal conditions, p.R48W significantly increased NFAT and AP-1 transcriptional activity 2.47 and 2.35-fold respectively relative to wild-type (p≤0.001)(Figure 5b-5c). NFκB activity was also increased (Figure 5d) however this did not reach statistical significance, which may be attributed to the high levels of endogenous NFκB activity in this cell line (data not shown). In addition p.S345F significantly activated NFκB, AP-1 (both p≤0.05) and NFAT (p≤0.001) consistent with the data in HEK293 cells.

Phosphorylation of p.Y783 is not essential for transcriptional activation by gain-of-function PLCγ1 mutations

In normal physiological conditions, activation of wild-type PLCγ enzymes is associated with phosphorylation of specific tyrosine residues in the highly conserved PLCγ-specific array (γSA) domain by receptor and non-receptor tyrosine kinases (Koss et al., 2014). Moreover, studies have shown that mutating the PLCγ1 p.Y783 residue (to p.Y783F) drastically reduces but does not obliterate IP production *in vitro* (Bunney et al., 2012, Poulin et al., 2005). To determine whether the activating mutations require p.Y783 phosphorylation to increase

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downstream transcriptional activity, the phosphorylation residue was mutated to p.Y783F in plasmids harboring the p.S345F, p.S520F and p.E1163K mutations. These mutations were chosen to represent *bona fide* gain-of-function mutations in specific PLCγ1 domains, namely the catalytic X-box domain (p.S345F), the γSA (p.S520F) and the C2 domain (p.E1163K). The absence of p-PLCγ1 was shown in cells transfected with vectors harboring the mutated p.Y783F residue (Figure 6a). In cells transfected with either the p.S345F, p.S520F, p.E1163K plasmids or the p.S345F-Y783F, p.S520F-Y783F, p.E1163K-Y783F vectors, there was no significant difference between the single and double mutants in NFAT, NFκB and AP-1 transcriptional activity (Figure 6b-6d). To exclude the possibility that these observations were cell type-specific, we also tested p.S345F and the double mutant p.S345F-Y783F in the J.gamma1 T-cell lymphoma line. These data confirm the findings in HEK293 cells and suggest that phosphorylation of the PLCγ1 p.Y783 residue is not essential for the PLCγ1 activity associated with gain-of-function mutations (Figure 6e-6g).

DISCUSSION

This study has demonstrated for the first time that the majority of PLCγ1 variants identified in SS, act as gain-of-function mutations by significant induction of both NFκB and AP-1 transcriptional activity and increased proximal activity in basal conditions. In addition, we have confirmed previous studies showing that PLCγ1 mutations activate NFAT transcriptional activity. Importantly, our results indicate that these gain-of-function mutations can increase downstream signaling without phosphorylation of the PLCγ1 p.Y783 residue.

We have also shown that identical *PLCG1* mutations are present in multiple tumor compartments and persist several years after diagnosis, suggesting that these are likely driver gene mutations, which are positively selected. Our review of published literature also confirms a high prevalence of *PLCG1* mutations in CTCL (MF 15% and SS 11%) and other mature T-cell malignancies notably ATLL.

In CTCL, several genes involved in TCR signaling harbor mutations including *CARD11, CD28* and *TNFRSF1B* as well as *PLCG1* (Chang et al., 2018, Choi et al., 2015, da Silva Almeida et al., 2015, Park et al., 2017, Ungewickell et al., 2015, Vaqué et al., 2014, Wang et al., 2015, Woollard et al., 2016). Although one study reported that *PLCG1* mutations were uncommon in CTCL occurring in . 3-5% of cases (Caumont et al., 2015), our data and other reports suggest that *PLCG1* mutations are amongst the most common mutations in CTCL occurring in approximately 10% of tumors (Chang et al., 2018, Park et al., 2017, Woollard et al., 2016). This apparent inconsistency is likely attributable to the selective sequencing of . two *PLCG1* exons harboring hotspot mutations by Caumont *et al.* (2015), whereas *PLCG1* mutations occur throughout the gene. Importantly, a recent study analyzing integrated NGS datasets from 139 CTCL (MF and SS) cases identified *PLCG1* as the most frequently mutated gene involved in the NFκB pathway (Chang et al., 2018). Furthermore, analysis of mutations within the NFκB pathway revealed that mutations in *PLCG1*, *CARD11* and *TNFRSF1B* were mutually exclusive in SS. We believe our study to be the first to demonstrate that gain-of-function PLCγ1 mutations in SS can activate both proximal IP and distal NFκB pathway signaling in the absence p.Y783 phosphorylation and stimulation. This is a potentially crucial mechanism driving hyperactivation of TCR signaling. Interestingly, in B-cell malignancies, approved therapies such as Ibrutinib target B-cell receptor activation but resistance can emerge due to acquisition of downstream activating *PLCG2* mutations (Liu et al., 2015).

Our data also extends on previous reports showing that recurrent PLCγ1 mutations in CTCL and angioimmunoblastic T-cell lymphoma increase NFAT activation (Vallois et al., 2016, Vaqué et al., 2014). These studies used the canonical human embryonic kidney cell line HEK293T and consistent with our findings show that p.R48W is not activating in basal conditions. However, given the high frequency of p.R48W in SS, suggesting this mutation is

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positively selected, it seemed unlikely that it would not be associated with PLCγ1 gain-of-function. Reassuringly, our data demonstrates that in a T-cell line, p.R48W is associated with increased NFAT and AP-1 transcriptional activity and it could be argued that this is more biologically relevant to CTCL than the findings in HEK293 cells.

Interestingly, functional studies of *CARD11* mutations reported in CTCL have also demonstrated activation of NFκB signaling (da Silva Almeida et al., 2015). Enhanced NFκB, NFAT and AP-1 activity may contribute to increased Th2 cytokine expression in CTCL, inhibiting the anti-tumor effects of the Th1 cytokines IL-2 and IFNγ, thereby providing an advantageous microenvironment for tumor cells (Miyagaki and Sugaya, 2014). Further work is required to determine the effect of specific *PLCG1* mutations on key parameters such as T-cell survival and proliferation of tumor cells.

PLCγ1 is maintained in an inactive state by an auto-inhibitory interaction between the catalytic X-Y domains and the cSH2 domain (see Supplementary Figure S2)(Gresset et al., 2010, Koss et al., 2014). The phosphorylated p.Y783 residue interacts with the cSH2 domain to disrupt the auto-inhibitory interface and makes the active site available for substrate hydrolysis (Poulin et al., 2005) and subsequent activation of wild-type PLCγ1 to produce IP (Bunney et al., 2012, Poulin et al., 2005). Germline deletions of the PLCγ2 cSH2 and cSH2-SH3 domains disrupt auto-inhibition and elevate protein activity in rare inherited conditions such as Phospholipase Cγ2–Associated Antibody Deficiency and Immune Dysregulation (Ombrello et al., 2012). The p.S345F and p.S520F mutations have also been proposed to act via disruption of auto-inhibition (Koss et al., 2014). The p.S345F and p.E1163K mutations are predicted to be located at the protein surface that faces the plasma membrane, based on a 3D PLCβ2/3 model (Choi et al., 2015, Vaqué et al., 2014). Similarly, using our PLCγ2 model, we suggest that p.R48W, p.S345F, p.E1163K, p.D1165H and p.VYEEDM1161V mutations are also likely to interact with the plasma membrane where the PLCγ substrate, PIP$_2$, resides (see

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Supplementary Figure S3). We hypothesize, that these mutant proteins may have increased affinity for the plasma membrane and therefore could enhance PIP_2 hydrolysis, leading to amplified downstream signaling. Furthermore, this may also explain why these gain-of-function mutations appear to act independently of p.Y783 phosphorylation. However the hotspot p.S345F mutation is also predicted to disrupt auto-inhibition (Koss et al., 2014) and may therefore act by a dual mechanism, as suggested for the PLCγ2 Ali5 (p.D993G) mutation (Everett et al., 2009).

Importantly, we have shown that the p.VYEEDM1161V indel in the PLCγ1 C2 domain is also a potent mediator of gain-of-function and therefore conclude that in addition to the cSH2 domain, the C2 domain is fundamental for regulating PLCγ1 activity. The C2 domains in PLCγ1 and PLCγ2 harbor somatic hotspot mutations in mature T-cell lymphomas and Ibrutinib-resistant chronic lymphocytic leukemia, respectively (Choi et al., 2015, da Silva Almeida et al., 2015, Jones et al., 2017, Kataoka et al., 2015, Kiel et al., 2015, Vallois et al., 2016, Wang et al., 2015, Woollard et al., 2016) and based on protein sequence homology and functional analogy of PLCγ1 and PLCγ2, it is likely that the PLCγ2 C2 domain also has regulatory functions. Notably, the p.VYEEDM1161V indel reduced total PLCγ1 protein expression, suggesting that it affects protein stability and may be subjected to degradation faster than wild-type PLCγ1. Further functional work is now required to determine if the p.VYEEDM1161V indel harbors key regulatory residues controlling PLCγ1 activity. U73122, is an inhibitor of PLC, which has been shown to reduce elevated NFAT activity in HEK293T cells transfected with wild-type and mutant PLCγ1 (Vaqué et al., 2014) but U73122 paradoxically activates the PLCβ3 isozyme, highlighting the lack of specific PLCγ1 inhibitors (Klein et al., 2011).

In summary, this comprehensive study has shown that PLCγ1 mutations reported in CTCL represent *bona fide* gain-of-function mutations that drive constitutive activation of proximal

and distal PLCγ1 signaling cascades. These data provide further evidence that PLCγ enzymes are potentially important therapeutic targets.

MATERIALS AND METHODS

Patient samples

All patients fulfilled the WHO-EORTCC diagnostic criteria for SS and were classified as blood stage B2 (Swerdlow et al., 2017). An identical clonal TCR rearrangement was demonstrated in peripheral blood and lesional skin. Tumor samples were obtained with informed consent from a nationally approved research tissue bank (IRAS Project ID: 238203).

Polymerase chain reaction and Sanger sequencing

PCR, RT-PCR and Sanger sequencing (Source Bioscience, Cambridge, U.K.) were used to validate the mutations in tumor-derived DNA and RNA and to confirm the introduction of mutant bases into the PLCγ1-pTriEx-4 vectors. Sequencing chromatograms were analyzed using FinchTV (Geospiza Incorporation, Seattle, Washington).

Mutation mapping to protein domains.

PLCγ1 mutations were mapped to the functional protein domains based on the amino acid numbering and protein domain schematic presented by Bunney *et al.* (2012).

Protein sequence alignment

Human PLCγ1 and PLCγ2 protein sequences were obtained from the Genome browser Ensembl (Zerbino et al., 2018) and aligned using multiple sequence alignment software Clustal Omega (Sievers et al., 2011).

Site-directed mutagenesis

The PLCγ1-pTriEx-4 vector, was used to generate mutant constructs with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, California).

Cell lines

HEK293 and COS-7 cells were obtained from the ECACC (Salisbury, England), Jurkat cells were obtained from Public Health England (Salisbury, England), J.gamma1 cells were obtained from the ATCC (Manassas, Virginia) and SeAx cells were a gift from Professor M. Vermeer (Leiden University Medical Centre, Leiden, Netherlands). Adherent cells were cultured in DMEM, non-adherent cells were maintained in RPMI 1640 Medium (both Life Technologies, Carlsbad, California). Cultures were supplemented with 10% (v/v) FBS (Biosera, Nuaille, France), 100 Units/mL Penicillin and 100 μg/mL Streptomycin (Life Technologies). SeAx cells were supplemented with 3 ng/mL rhIL-2 (R&D systems, Minneapolis, Minnesota). Cultures were routinely tested for Mycoplasma contamination using PCR (van Kuppeveld et al., 1994).

Dual-luciferase reporter assays

HEK293 cells were co-transfected with 1 μg PLCγ1-pTriEx-4 vectors, 0.05 μg pRL-TK Renilla luciferase construct and 0.1 μg of NFκB-firefly-luciferase, pGL3-NFAT-firefly-luciferase or pGL3-AP-1-luciferase reporter plasmids as described by Vaqué *et al*. (2014). J.gamma1 cells were co-transfected with 0.5-5 μg PLCγ1-pTriEx-4 vectors, 0.5-1 μg pRL-TK Renilla luciferase construct and 1 μg of NFκB-firefly-luciferase or 5 μg pGL3-NFAT-firefly luciferase/pGL3-AP-1-luciferase reporter plasmids. Transfections were performed by electroporation as per manufacturer's instructions (Lonza, Basel, Switzerland). Lysates were

analyzed using the dual-luciferase reporter assay system (Promega, Madison, Wisconsin). Assays were repeated at least three times.

Western blotting

HEK293 cells were transfected with 2 μg PLCγ1-pTriEx-4 plasmids and 6 μL FuGene HD Transfection Reagent (Promega). After 23 h, cells were treated with 100 μM pervanadate (protein-tyrosine phosphatase inhibitor) for 1 h to stabilize PLCγ1 phosphorylation (Kunze et al., 2014) and then harvested. HEK293, Jurkat and J.gamma1 whole cell lysates were probed with antibodies listed in Supplementary Table S3. Experiments were repeated twice.

Inositol phosphate quantification assay

IP production was quantified in counts per minute in COS-7 cells transfected with 2.5 μg PLCγ1-pTriEx-4 vectors (Everett et al., 2009). Assays were repeated at least twice. IP levels were normalized to wild-type PLCγ1 activity.

DATA AVAILABILITY

No data sets were generated or analyzed during the current study.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: VMP, CEF, SJW, TJM; Data Curation: VMP, CEF, CLJ, WJW; Formal Analysis: VMP, CEF, CLJ, WJW, SJW, TJM; Funding Acquisition: SJW, TJM; Investigation: VMP, CEF, MM, CLJ, RMB, WJW, FSB, AY; Methodology: VMP, CEF, MM, CLJ, SJW, TJM; Project Administration: SJW, TJM; Resources: SJW, TJM; Software: VMP, CEF, CLJ, WJW, SJW, TJM; Supervision: MK, SJW, TJM; Validation: VMP, CLJ, WJW, NB; Visualization: VMP, CEF, CLJ, TJM; Writing - Original Draft Preparation: VMP, CEF, CLJ, SJW, TJM; Writing - Review and Editing: VMP, CEF, TJM.

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FIGURE LEGENDS

Figure 1. Persistence of mutant *PLCG1* alleles in multiple tumor compartments in SS patients. Sanger sequencing of DNA extracted from PBMCs, lesional skin biopsies and involved lymph nodes. RNA was isolated from enriched CD4$^+$ tumor cells and reverse transcribed into cDNA. Chromatograms were analyzed using the FinchTV software. CD3$^+$/CD4$^+$ counts/µL are shown as a proxy for tumor burden. N/A = CD3$^+$/CD4$^+$ unavailable, but no diagnostic TCR clonal gene rearrangement was detected in the post-transplant sample.

Figure 2. *PLCG1* is frequently mutated in mature T-cell lymphomas. (a) PLCγ1 mutations identified by next-generation sequencing of different mature T-cell lymphomas were plotted to determine the incidence of each mutation per disease type. MF: Mycosis Fungoides, SS: Sézary Syndrome, PTCL-nos: peripheral T-cell lymphomas-not otherwise specified, AITL: angioimmunoblastic T-cell lymphomas, TFH-derived PTCL: follicular helper T-cell-derived lymphomas, ATLL: adult T-cell leukemia/lymphoma, HSTL: hepatosplenic T-cell lymphoma and TPLL: T-cell-prolymphocytic leukemia. (b) Schematic representation of the eight missense mutations and one in-frame indel (p.VYEEDM1161V) examined in this study mapped to the PLCγ1 functional protein domains. (c) PLCγ1 and PLCγ2 residues were aligned using the Clustal Omega software. PLCγ1 mutations analyzed in this study and corresponding PLCγ2 mutations reported in B-cell Ibrutinib-resistant chronic lymphocytic leukemia are shown.

Figure 3. PLCγ1 mutations increase NFκB transcriptional activity (a) Schematic representation of PLCγ1 mediating TCR signaling via IP$_3$ and DAG to the NFκB, AP-1 and NFAT transcription factors. (b) HEK293 cells were co-transfected with PLCγ1-pTriEx-4, pRL-TK Renilla-luciferase and NFκB-firefly-luciferase plasmids. Cells were starved overnight and transcriptional activity was analyzed. Activation fold changes were normalized

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to cells overexpressing wild-type PLCγ1. Data is represented as Mean ± SEM; n=4. Student's t-test, *p<0.05, **p<0.01, ***p<0.001. (c,d) HEK293 cells were transfected with PLCγ1-pTriEx-4 vectors and treated with 100 μM pervanadate before harvesting. Lysates were probed with (c) an anti-PLCγ1 antibody and (d) an anti-p-PLCγ1 antibody to analyze total and phosphorylated protein expression, respectively relative to β-actin expression as a loading control.

Figure 4. PLCγ1 mutations elevate proximal signaling. COS-7 cells were transfected with PLCγ1-pTriEx-4 vectors and IP production was quantified by liquid scintillation counting. IP production was normalized to cells overexpressing wild-type PLCγ1. Data is represented as Mean ± SD and is representative of two independent experiments.

Figure 5. PLCγ1 p.R48W is activating in a T-cell lymphoma cell line. (a) Whole cell lysates from J.gamma1 and Jurkat cells were probed with an anti-PLCγ1 antibody to analyze total protein expression using GAPDH expression as a loading control. (b-d) J.gamma1 cells were co-transfected with PLCγ1-pTriEx-4, pRL-TK Renilla-luciferase and pGL3-NFAT-firefly-luciferase, pGL3-AP-1-luciferase or NFκB-firefly-luciferase vectors. Cells were starved overnight and then lysed; n=3 for each assay. Activation fold changes were normalized to cells overexpressing wild-type PLCγ1. Data is represented as Mean ± SEM; n=3. Student's t-test, *p<0.05, **p<0.01, ***p<0.001.

Figure 6. Mutant proteins mediate gain-of-function effects without PLCγ1 phosphorylation. (a) HEK293 cells transfected with PLCγ1-pTriEx-4 vectors were treated with 100 μM pervanadate before harvesting. Lysates were probed with anti-PLCγ1 and anti-p-PLCγ1 antibodies to analyze total and phosphorylated protein expression using β-actin expression as a loading control. (b-d) HEK293 and (e-g) J.gamma1 cells were co-transfected with PLCγ1-pTriEx-4, pRL-TK Renilla-luciferase and pGL3-NFAT-firefly-luciferase, NFκB-firefly-luciferase or pGL3-AP-1-luciferase vectors. Cells were starved overnight and then

lysed; n=3 for each assay. Activation fold changes were normalized to cells overexpressing wild-type PLCγ1. Data is represented as Mean ± SEM. Student's t-test, ns=not significant.
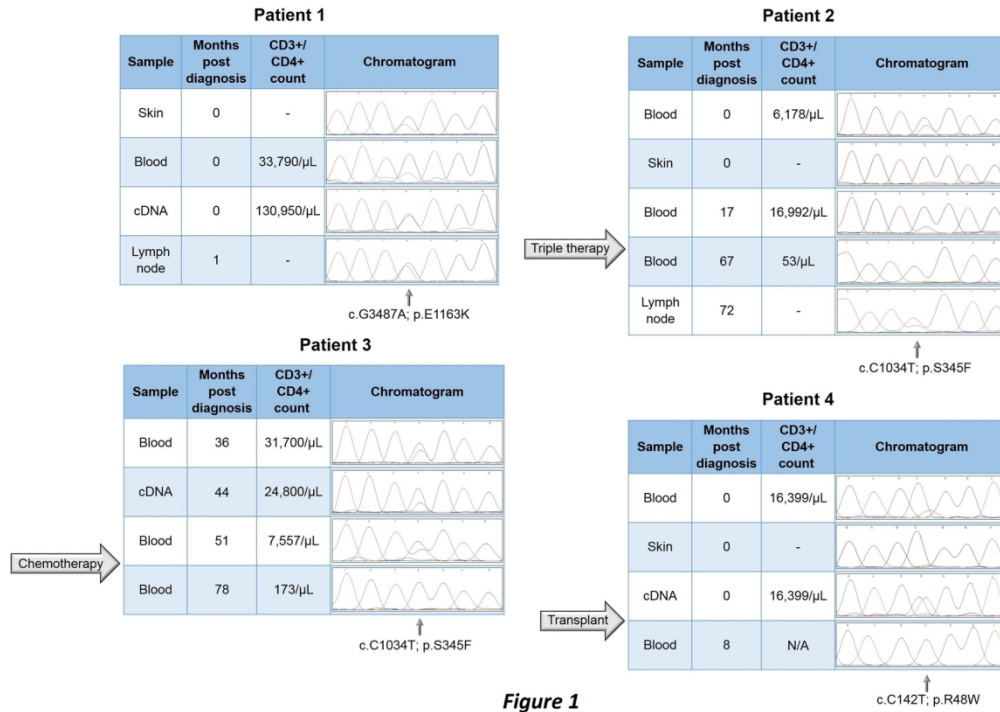
Figure 1 Persistence of mutant PLCG1 alleles in multiple tumor compartments in SS patients.
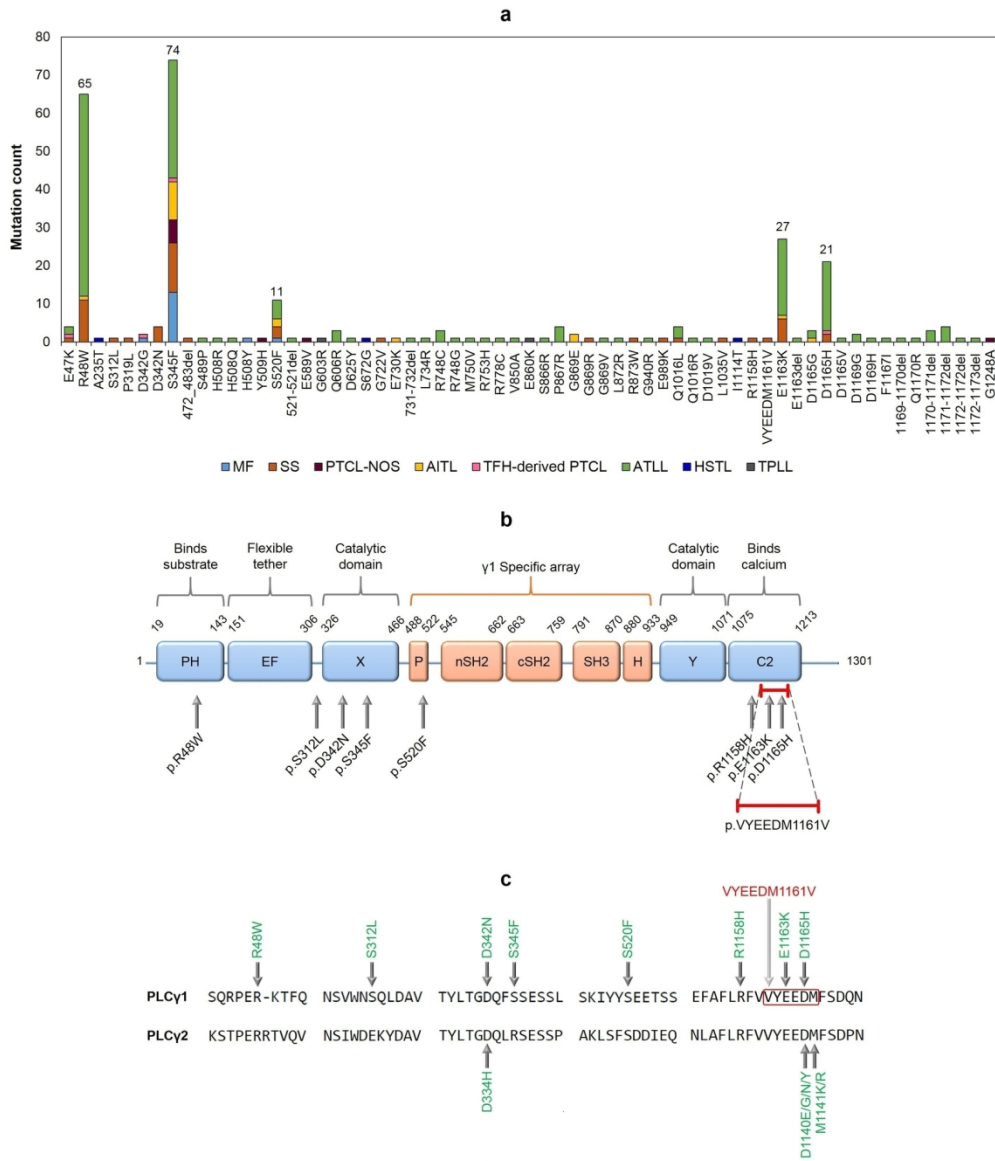
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Figure 2. PLCG1 is frequently mutated in mature T-cell lymphomas.
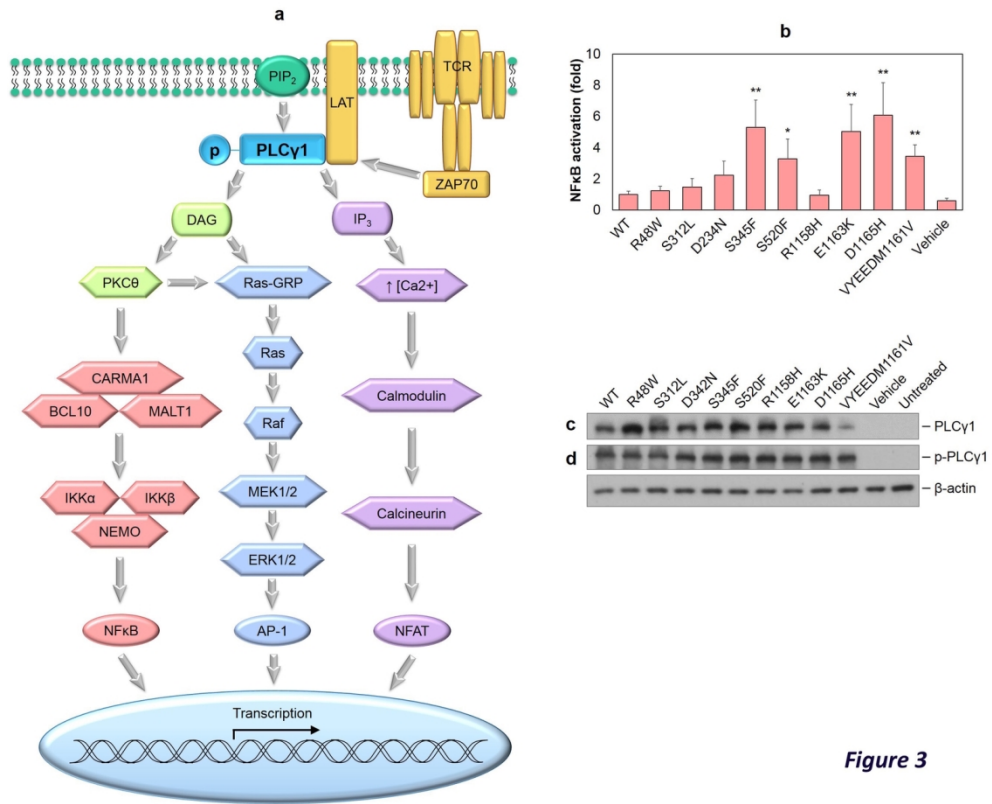
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Figure 3. PLCγ1 mutations increase NFκB transcriptional activity
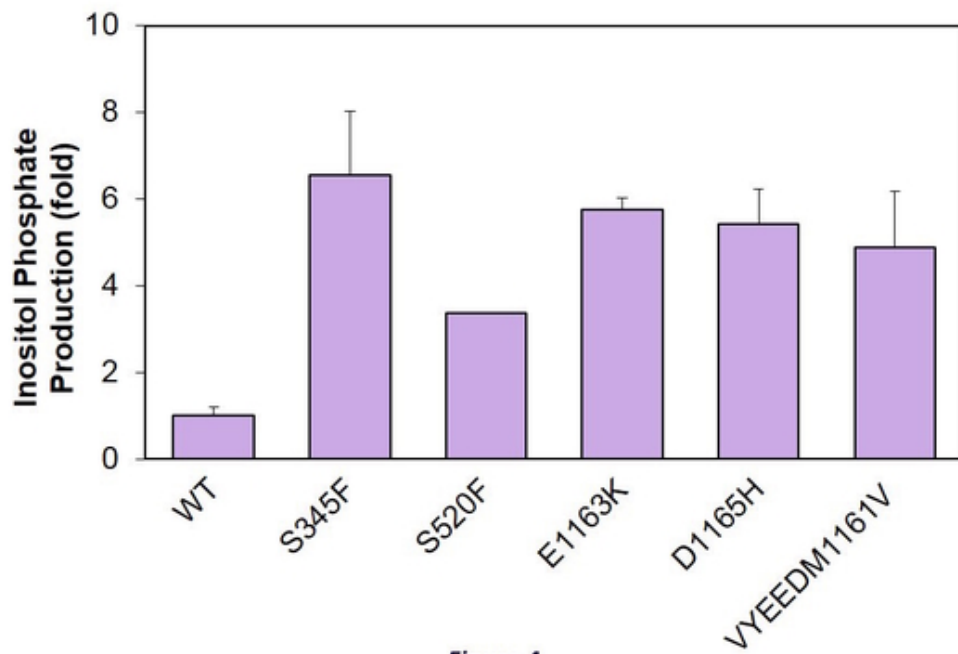
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Figure 4

Figure 4. PLCγ1 mutations elevate proximal signaling.
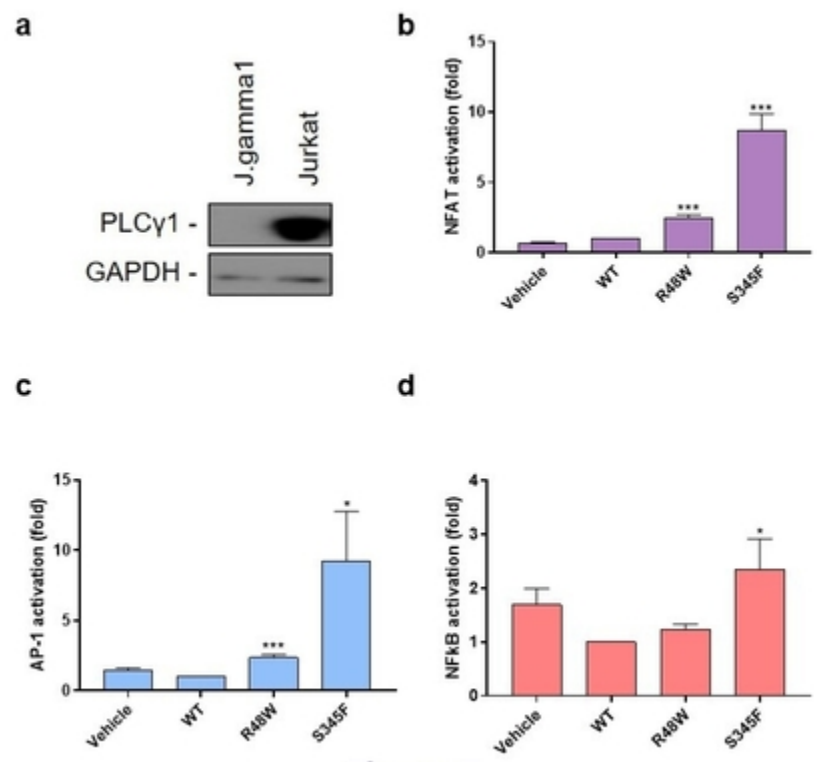
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Figure 5. PLCγ1 p.R48W is activating in a T-cell lymphoma cell line.
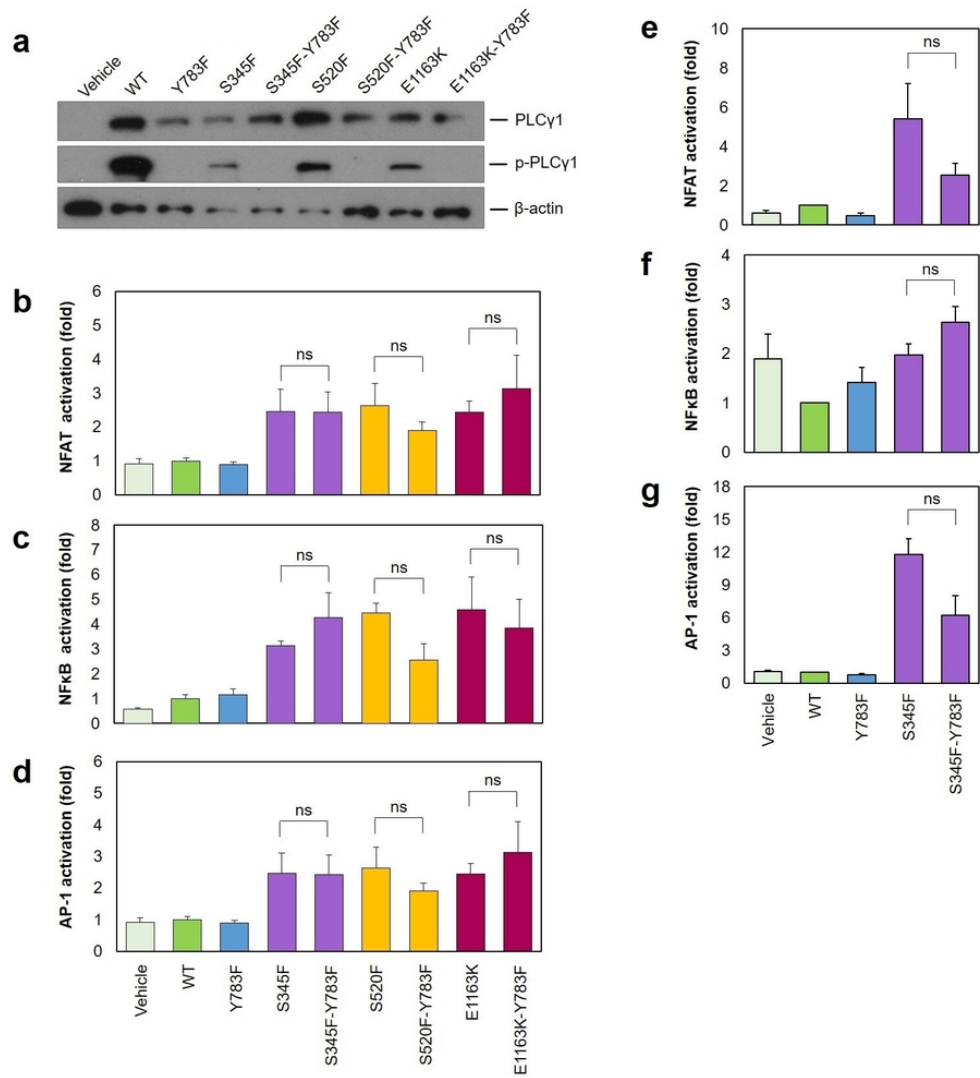
17x17mm (600 x 600 DPI)

Figure 6

Figure 6. Mutant proteins mediate gain-of-function effects without PLCγ1 phosphorylation.

38x46mm (600 x 600 DPI)

Supplementary materials for:

Frequent and persistent *PLCG1* mutations in Sézary cells directly enhance PLCγ1 activity and stimulate NFκB, AP-1 and NFAT signaling.

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Supplementary methods:

Comprehensive literature analysis

The PubMed database was used to identify publications incorporating the World Health Organization classification of T-cell lymphomas (T-cell prolymphocytic leukemia, T-cell large granular lymphocyte leukemia, adult T-cell leukemia/lymphoma, extranodal NK/T-cell lymphoma (nasal type), enteropathy-associated T-cell lymphoma, hepatosplenic T-cell lymphoma, Mycosis Fungoides / Sézary Syndrome, primary cutaneous CD30-positive T cell lymphoproliferative disorders, primary cutaneous anaplastic large cell lymphoma, lymphomatoid papulosis, peripheral T-cell lymphoma-not otherwise specified, angioimmunoblastic T-cell lymphoma and anaplastic large cell lymphoma) in combination with either the term "whole-exome sequencing" or "next-generation sequencing". The resulting publications and the corresponding supplementary data were interrogated for *PLCG1* mutations. Mutations identified by subsequent targeted gene sequencing were also included to obtain the overall frequency of *PLCG1* mutations per disease subtype.

Pathogenicity prediction

SIFT, Provean, PolyPhen2, Mutation Taster2, MutPred2 and CADD were used to predict the potential effect of the *PLCG1* variants on the protein level (Adzhubei et al., 2013, Choi and Chan, 2015, Kircher et al., 2014, Kumar et al., 2009, Li et al., 2009, Schwarz et al., 2014). A consensus prediction was determined using the criteria of 5/6 algorithms having the same result for missense mutations. The PolyPhen2 and MutPred2 algorithms only predict the effect of point mutations, therefore the criteria of 3/4 algorithms having the same result was used to predict the effect of the indel.

3D protein modelling

The 3D model of the full length PLCγ protein was generated by using the structure of PLCγ2 to represent the PLCγ core. PLCγ2 structures from the Protein Data Bank were used for modelling; spPH (PDB: 2K2J), nSH2 (PDB: 2DX0), cSH2 (PDB: 2EOB) and SH3 (PDB: 2EQI). Firstly, the structure of the tandem SH2 domains from PLCγ1 (PDB: 3GQI) was used to arrange these domains relative to each other. Docking of the domains to the triosephosphate isomerase barrel was carried out using the ClusPro web-server (Comeau et al., 2004a, 2004b). The two SH2 domains were docked with the catalytic domain then the spPH domain was docked to this complex, followed by the SH3 domain. At each stage, the most likely conformation was chosen based on biochemical data. The models were analyzed using DeepView-Swiss-PDBViewer and Pymol (Guex and Peitsch, 1997, Schrodinger, 2015). *PLCG1* mutations were subsequently mapped to this model.

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Supplementary tables:

Table S1. Whole-exome and targeted gene sequencing studies reporting *PLCG1* mutations in mature T-cell lymphomas. ATLL: adult T-cell leukemia/lymphoma, PTCL-nos: peripheral T-cell lymphoma-not otherwise specified, MF: Mycosis Fungoides, HSTL: hepatosplenic T-cell lymphoma, AITL: angioimmunoblastic T-cell lymphoma, SS: Sézary Syndrome, TPLL: T-cell-prolymphocytic leukemia, ALCL: anaplastic large cell lymphoma, EATL: enteropathy-associated T-cell lymphoma, NKTCL: natural killer/T-cell lymphoma. Refer to main manuscript for references.

Malignancy	Mutation frequency	References
ATLL	**36.2%** (134/370)	(Kataoka et al., 2015)
PTCL-nos	**18.2%** (4/22)	(Palomero et al., 2014, Sakata-Yanagimoto et al., 2014, Vallois et al., 2016)
MF	**15.1%** (8/53)	(McGirt et al., 2015, Ungewickell et al., 2015, Vaqué et al., 2014)
HSTL	**15.0%** (3/20)	(McKinney et al., 2017)
AITL	**12.0%** (11/92)	(Palomero et al., 2014, Sakata-Yanagimoto et al., 2014, Vallois et al., 2016, Wang et al., 2017, Yoo et al., 2014)
SS	**11.3%** (44/388)	(Choi and Goh, 2015, da Silva Almeida et al., 2015, Kiel et al., 2015, Prasad et al., 2016, Ungewickell et al., 2015, Vaqué et al., 2014, Wang et al., 2015, Woollard et al., 2016)
TPLL	**5.6** (2/36)	(Kiel et al., 2014)
ALCL	**0%** (0/23)	(Crescenzo et al., 2015)
EATL	**0%** (0/89)	(Moffitt and Ondrejka, 2017, Nairismagi et al., 2016, Palomero et al., 2014, Roberti et al., 2016)
NKTCL	**0%** (0/27)	(Jiang et al., 2015, Palomero et al., 2014)

Table S2. Pathogenicity predictions performed using multiple algorithms for 9 SS-associated PLCγ1 variants. Consensus criteria for missense mutations: 5/6 algorithms. Consensus criteria for indel: 3/4 algorithms.

Nucleotide substitution	Amino acid change	SIFT	Provean	PolyPhen2	Mutation Taster	MutPred2	CADD	Consensus
c.C142T	p.R48W	Damaging	Deleterious	Probably damaging	Disease causing	Damaging	Deleterious	**Damaging**
c.C935T	p.S312L	Tolerated	Deleterious	Benign	Disease causing	Neutral	Deleterious	Inconclusive
c.G1024A	p.D342N	Tolerated	Deleterious	Probably damaging	Disease causing	Damaging	Deleterious	**Damaging**
c.C1034T	p.S345F	Damaging	Deleterious	Probably damaging	Disease causing	Damaging	Deleterious	**Damaging**
c.C1559T	p.S520F	Damaging	Deleterious	Probably damaging	Disease causing	Damaging	Deleterious	**Damaging**
c.G3473A	p.R1158H	Damaging	Deleterious	Probably damaging	Disease causing	Damaging	Deleterious	**Damaging**
c.G3487A	p.E1163K	Damaging	Deleterious	Probably damaging	Disease causing	Damaging	Deleterious	**Damaging**
c.G3493C	p.D1165H	Damaging	Deleterious	Probably damaging	Disease causing	Damaging	Deleterious	**Damaging**
c.GTGTATGAGG AAGACAdelG	p.VYEEDM1161V	Damaging	Deleterious	N/A	Disease causing	N/A	Deleterious	**Damaging**

Table S3. Antibodies used for western blotting.

Antibody	Product code	Dilution	Manufacturer
Rabbit monoclonal anti-PLCγ1	5690	1:20,000	
Rabbit polyclonal anti-p-PLCγ1	2821	1:50,000	Cell Signaling Technology, Danver, Massachusetts, U.S.A
Rabbit polyclonal anti-β-Actin	4967	1:10,000	
Rabbit monoclonal anti-GAPDH	2118	1:2,000	

Supplementary figures:
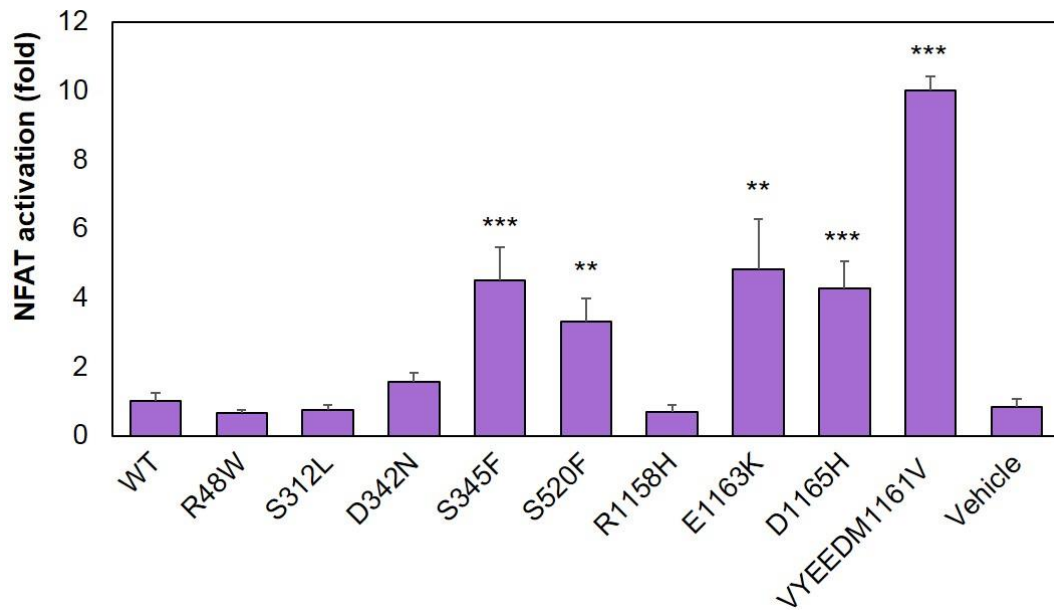
Figure S1. PLCγ1 mutations significantly increase NFAT transcriptional activity. Five mutations significantly enhance NFAT activity in basal conditions in HEK293 cells. Transcriptional activity was analyzed using dual-luciferase reporter assays. Data is represented as Mean ± SEM; n=3 Student's t-test, *p<0.05, **p<0.01, ***p<0.001.
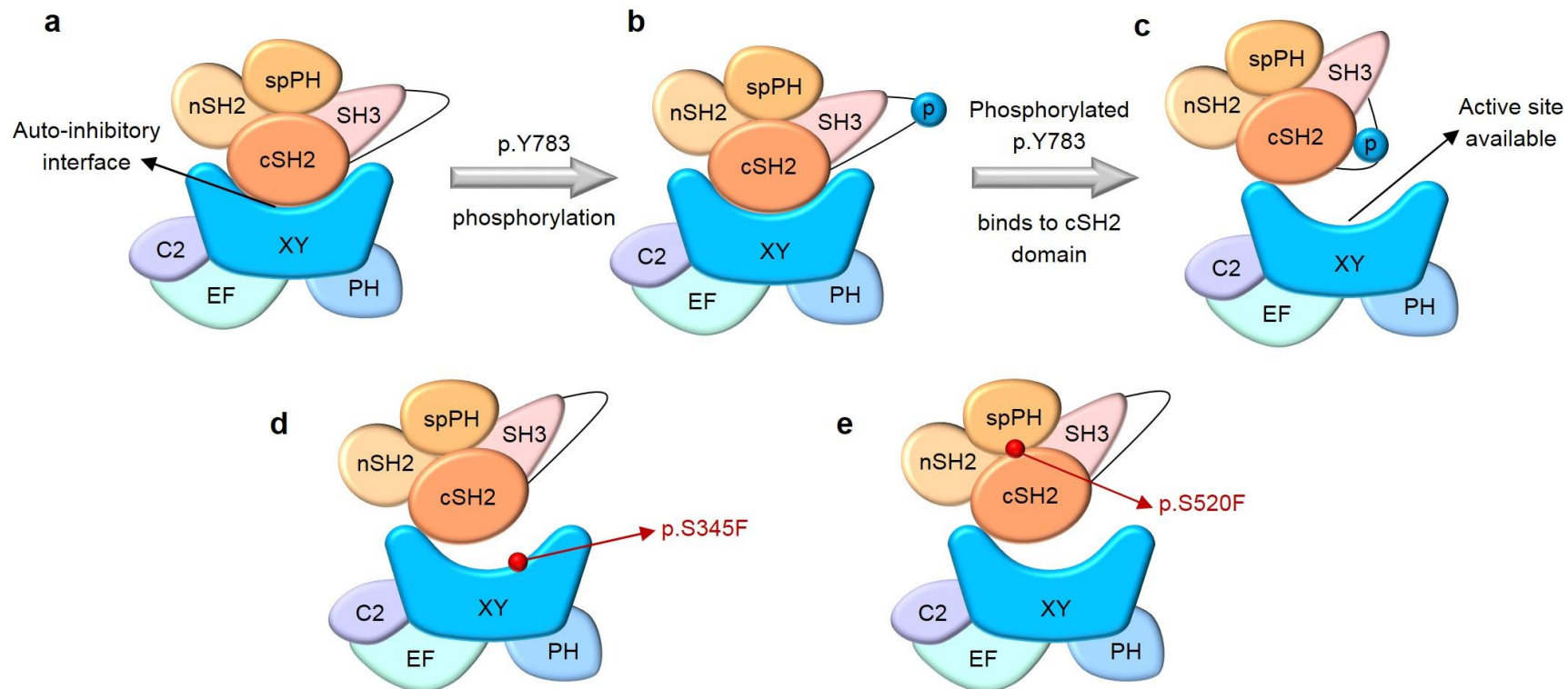
Figure S2. Gain-of-function PLCγ1 mutations disrupt auto-inhibition, leading to constitutively active mutant proteins. (a) PLCγ1 is maintained in an inactive state by an auto-inhibitory interaction between the X-Y catalytic domains and the cSH2 domain, which blocks substrate accessing the active site. (b, c) Receptor and non-receptor tyrosine kinases phosphorylate the p.Y783 residue, which interacts with the cSH2 domain and disrupts the auto-inhibitory interface, making the active site available for substrate hydrolysis. (d,e) The activating mutations p.S345F and p.S520F are predicted to locate on the surface of the catalytic X domain and at the cSH2-split PH domain interaction surface, respectively and are likely to disrupt auto-inhibition.
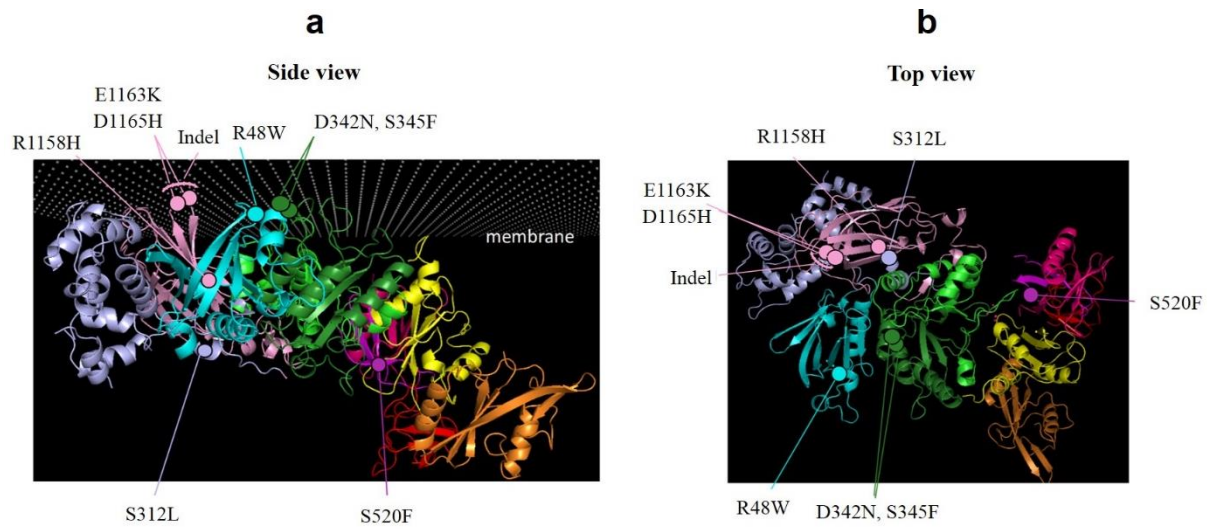
Figure S3. *Bona fide* **activating PLCγ1 mutations p.R48W, p.S345F, p.E1163K, p.D1165H and the indel p.VYEEDM1161V map to the surface of the protein that is predicted to interact with the plasma membrane.** (a) Top and (b) side view of the PLCγ1 mutations mapped to the PLCγ2 protein structure.