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PSTPIP1 controls immune synapse stability in human T-cells

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Abstract

Background: PSTPIP1 is a cytosolic adaptor protein involved with T-cell activation, differentiation, and migration. Upon cognate T-cell contact, PSTPIP1 is recruited to surface-expressed CD2, where it regulates f-actin remodeling. An immune synapse (IS) is thereby rapidly formed, consisting of TCR clusters surrounded by a ring of adhesion molecules including CD2.

Objective: From genetic screening of primary immunodeficiency patients, we identified two mutations in PSTPIP1, R228C and T274M which we further characterized in primary patient T-cells.

Methods: F-actin dynamics were assessed in patient and healthy control primary T-cells by use of FACS. HEK293T and Jurkat cells were transfected with R228C, T274M and WT PSTPIP1 in order to visualize f-actin in immune synapse formation. CD2-PSTPIP1 association was quantified through immunoprecipitation assays.

Results: The patients presented with immunodeficiency without signs of auto-inflammation. The R228C patient had expansion of mostly naive phenotype T-cells and few memory T-cells; the T274M patient had 75% reduction in CD4 T-cells that were predominantly of memory subset. We observed f-actin polymerization defects in both PSTPIP1 patient T-cells, most notably T274M. Capping of CD2-containing membrane microdomains was disrupted. Analysis of IS formation using Jurkat T-cell transfectants revealed a reduction in f-actin accumulation at the IS, again especially in T274M PSTPIP1 cells. Patient T274M T-cells migrated spontaneously at increased speed as assessed in a 3D collagen matrix, while TCR crosslinking induced a significantly diminished calcium flux.

Conclusions: We propose that PSTPIP1 T-cell differentiation defects are caused by defective control of f-actin polymerization. A pre-activated polymerized f-actin status, as seen in PSTPIP1-T274M T-cells, appears particularly damaging. PSTPIP1 controls IS formation and cell adhesion, through its function as orchestrator of the f-actin cytoskeleton.

Clinical Implications: Pathogenic PSTPIP1 mutations can be found in CVID patients and likely contribute to altered T-cell phenotypes in these patients.

Capsule Summary: We identified two novel PSTPIP1 mutations in two CVID patients. We propose that PSTPIP1 mutations may cause T-cell differentiation defects by defective control of f-actin polymerization and diminished cell adhesion.

Keywords:

CVID; CD2; PSTPIP1; T cells; F-actin; Immune synapse

Abbreviations:

CVID: Common Variable Immunodeficiency

HC: Healthy Control

Ig: Immunoglobulin

IP: Immunoprecipitation

IS: Immune synapse

PSTPIP1: Proline-Serine-Threonine Phosphatase Interacting *Protein* 1

WASp: Wiskott–Aldrich Syndrome *protein*

WB: Western Blot

Introduction

Common Variable Immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency (PID) in adults and children. Patients encounter recurrent viral and bacterial infections of the respiratory and gastrointestinal tract, due to ineffective pathogen clearance through strongly diminished immunoglobulin levels¹⁻⁴. Novel PID associated genetic defects are now identified more rapidly through next generation sequencing (NGS) techniques^{5, 6}. However in case of CVID, monogenetic causes of disease are not identified in the vast majority of patients^{7, 8}. Besides B-cell differentiation and maturation disturbances (low class switched memory B-cell numbers) CVID patients may also exhibit aberrations within the T-cell compartment^{4, 7-9}. Typically these patients display low circulating T-cells numbers, relative CD8 T-cell expansion due to diminished CD4 T-cell numbers and low numbers of CD4 naive T-cells. Several early T-cell signaling gene mutations have been described in PID patients, which can be (in)directly linked to f-actin remodeling, essential for immune synapse (IS) formation¹⁰. Protein defects in Vav1 and ZAP70 are examples of T-cell early signaling genes that associate with CVID disorders, where the hampered TCR activation and strongly diminished f-actin remodeling results in defective IS formation^{9, 11}.

We identified two novel Proline Serine Threonine Phosphatase Interacting Protein 1 (PSTPIP1) mutations, a threonine to methionine substitution at aa274 (T274M) and arginine to cysteine at aa228 (R228C), in two patients with CVID and T-cell differentiation disturbances. The R228C patient expressed defective memory CD4 T-cell differentiation and the T274M patient had few CD4 T-cells overall but a relative expansion of memory CD4 T-cells. Both patients had decreased memory B-cells. Upon T-cell activation via antigen-specific T-cell receptor (TCR) interaction with cognate peptide/HLA complexes, cytosolic PSTPIP1 protein is recruited to the IS-associated adhesion molecule CD2. PSTPIP1 thereafter acts as a scaffolding protein for signal transduction towards the nucleus. Accordingly, PSTPIP1 is dephosphorylated by PTP-PEST thereby controlling CD2-mediated cell adhesion and f-actin polymerization through the coupling of PTP-PEST to WASP, towards formation of a stable IS¹²⁻¹⁶. Clinically, PSTPIP1 mutations are known to cause pyogenic arthritis, pyoderma gangrenosum and acne (PAPA)-syndrome and are associated with Familial Mediterranean Fever (FMF) syndromes¹⁷⁻²¹. PSTPIP1 might relate to IS stability and thereby immune function.

In this study we addressed the role of PSTPIP1 in f-actin remodeling and IS stability, by study of the two CVID patients in which the T274M and R228C PSTPIP1 variants were found. To this end,

we performed functional experiments with primary T-cells and cell lines transfected with PSTPIP1 WT, T274M and R228C gene variants. We demonstrate in primary patient CD4 T-cells that the T274M PSTPIP1 variant derails activation-induced f-actin polymerization, contributed by pre-existing nucleation for f-actin filaments. Furthermore the T274M patient CD4 T-cells were hypo-responsive to TCR activation, as shown by reduced induction of cytosolic calcium mobilization and f-actin recruitment to the IS. Overall our findings support that PSTPIP1 mutations can underlie T-cell defects in CVID patients, expanding the phenotype beyond PAPA- and FMF-syndrome

Methods

Patient samples

Patients gave written informed consent to participate in this study, which was approved by the Medical Ethical Committee of the UMC Utrecht. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using a standardized ficol procedure. CD4⁺ T cells were enriched by use of a CD4⁺ T lymphocyte enrichment cocktail (BD) and streptavidin beads, according to manufacturers protocol.

Flow Cytometry

For f-actin quantification, primary CD4 T-cells were fixed and permeabilized (FixPerm BD) at several time points (0,2,5, 10 min) after anti-CD2 stimulation with CD2 TII2 and TII3 antibodies 1:100 dilution (kindly provided by dr E. Reinherz Dana Farber Institute Boston) and anti-CD3 stimulation (OKT3 clone eBioscience). Upon fixation cells were stained with Phalloidin-FITC 50ug/ml dilution (Enzo Life sciences) in permeabilization buffer (BD) for 20 min 4C, washed and immediately acquired by flow cytometry (Canto II BD).

Cytosolic calcium mobilization

PBMC of the patient were stained with anti-CD3, anti-CD4 and anti-CD45RA/RO antibodies in RPMI 1640 medium without phenol red. To visualize cytosolic calcium increase Fluo-3 am (5umol/l) and Fura-Red (10umol/l)(Thermo Fisher Scientific) were used as calcium indicators. Calcium dyes were incubated at 37 C degrees for 30 min the last 10 minutes specific antibodies were added. Cells were washed and resuspended in calcium containing Hanks Balanced Salt Solution (HBSS) (Thermo Fisher Scientific) with 10% FCS added. Calcium mobilization was acquired with flow cytometry, to induce calcium fluxes anti-CD3 was crosslinked with a goat anti-mouse IgG antibody 20 mg/ml (Jackson ImmunoResearch).

Site-directed mutagenesis

Mutated PSTPIP1 was generated by site-specific mutagenesis of wild type PSTPIP1. The vector EX-T2966-M68 (GeneCopoeia) was grown in DH5a in order to obtain methylated DNA and cDNA was purified by maxiprep according to manufacturer's instruction (Macherey-nagel). PSTPIP1 variants were entered by use of PCR reaction according to the guidelines of Phusion DNA polymerase (Finnzymes, F-5302S) using primers. Template (methylated) DNA was degraded by adding 1 μ L DpnI (NEB, R0176s) and incubated overnight at 37 °C. Wild type and mutant vector were transformed in commercial DH5a (NEB, C2987I), and cDNA was purified. The PSTPIP1 variants were confirmed by sequencing.

Transfection

Jurkat cells were electroporated at 250 V and 975 mF (Genepulser II from Bio-Rad Laboratories, Hercules, Calif) with an EX-T2966-M68 vector (GeneCopoeia) containing human HA tagged (N-terminus) WT-PSTPIP1, T274M- or R228C- mutated PSTPIP1 and flag tagged CD2 (C terminus). HEK293T cells were transfected with the same vectors, by use of FuGENE HD Transfection Reagent (Promega), according to manufacturer's instructions.

Immunoprecipitation

HEK293T cells transfected with WT-, or T274M-, or R228C PSTPIP1-HA and CD2-flag and cultured in DMEM supplemented with 10% FCS. Approximately one and a half days after transfection cells were harvested, resuspended in PBS and stimulated with anti-CD2 antibodies and kept on ice directly after 2 min stimulation. Subsequently cells were lysed in a standardized immunoprecipitation buffer with 1% Triton and 1% protease inhibitor (HALT Thermo Fisher Scientific) and CD2-flag pulldown was performed with FLAG conjugated beads (Sigma). Lysates were subjected to SDS-PAGE and Western blotting for CD2-flag (monoclonal anti-FLAG M2-peroxidase Sigma Aldrich) and PSTPIP1-HA (mouse IgG2bk anti-HA 12CA5) and Donkey anti-Mouse IgG light chain specific antibody-HRP conjugated (Jackson ImmunoResearch).

Confocal imaging

Jurkat cells were transfected WT-, or T274M, or R228C PSTPIP1-HA and kept in RPMI 1640 medium supplemented with 10% FCS. After two days cells were adhered to poly-l-lysine coated confocal chambers and stimulated with anti-CD3/CD28 beads for 10 minutes. Upon stimulation cells were kept on ice and fixed/permeabilized with fix/perm (BD) for 30 min, after fixation cells were stained with phalloidin-FITC 5mg/ml (1:200 dilution) (Enzo Life Sciences) and washed with permeabilization buffer (BD).

For imaging of HEK 293T cells transfected with WT-, or T274M, or R228C PSTPIP1-HA and CD2-flag, cells were permeabilized in triton 0.1%, blocked with 5% BSA and washed in PBS with Tween 0.5%. Cells were stained with monoclonal FLAG M2-FITC (1:200) and mouse IgG2bk anti-HA 12CA5 (both Sigma Aldrich) and after block with goat serum with goat anti-mouse Alexa Fluor 647. Acquisition was performed on a Zeiss LSM710 confocal microscope. Images were obtained with a 1.3× optical zoom using Plan-Apochromat 63× 1.40 oil differential interference contrast M27 objective (Zeiss) and processed using Zen 2009 software (Zeiss Enhanced Navigation)

Results

Two CVID patients with novel PSTPIP1 mutations

Patient 1, R228C, presented with recurrent respiratory tract infections complicated with bronchiectasis, severe enteropathy and an unclassified skin rash. Laboratory screening revealed low IgG, IgA and IgM levels ($<-2SD$), absent specific anti-pneumococcal serotype IgG responses and initially diminished memory B-cell percentages, however normalizing in time (Fig 1A). Patient 2, T274M, presented with recurrent viral and bacterial infections of the respiratory tract, low IgG and IgA levels ($<-2SD$) and absent specific responses to pneumococcal serotypes. The memory B-cell percentages were consistently disturbed (no detected IgA memory B-cells, IgG memory B-cells 0.2%) (Fig 1A). Both patients exhibited severe T-cell deficiencies. Patient 1 presented with very few memory CD4⁺ T-cells and expansion of naive T-cells (2286/mm³ N 700-900). Patient 2 presented with a diminished number of T-cells (305 /mm³ N 700-900), composed of very low CD4 T-cell numbers (157/mm³ N 560-1067) and low naive CD4⁺ T-cell percentages ($<-2SD$) (Fig1A), while over representing the central memory T cell subset (Fig 1B).

Based on the clinical and laboratory findings both patients were diagnosed as common variable immunodeficiency (CVID) and were treated with immunoglobulin substitution therapy²². Through next generation sequencing⁵, patient 1 was found to have a heterozygous mutation c.682C>T in the *PSTPIP1* gene, resulting in a R228C amino-acid substitution in the coiled coil region of the PSTPIP1 protein. Patient 2 had a heterozygous mutation at c.821C>T resulting in a T274M amino-acid substitution (Fig 1D,E). Both mutations were predicted to be pathogenic; not or only three times detected in control populations (ExAC database, Broad institute), highly conserved amino acid residues, substantial physiochemical differences between the amino acid residues. For both patients also the fathers were carrying the mutations (Fig 1C). In case of the R228C variant, the father had diminished immunoglobulin levels and acne. For the T274M variant the father was not further screened due to unavailability. The sister of the T274M patient is currently experiencing immunodeficiency symptoms.

PSTPIP1 mutations are shown to be causative of Pyogenic Arthritis, Pyoderma gangrenosum and Acne (PAPA)-syndrome^{20, 23}. Our patients did not have any PAPA syndrome characteristics, even more, serum IL1-beta levels were not elevated (data not shown), LPS stimulation of PBMC did not trigger increased IL1-beta levels¹⁷ (Fig 1F). Finally, considering that PSTPIP1 binds WASP (12), we

stained for WASP protein in T-cells of the R228C patient and HC (patient cells from the T274M PSTPIP1, cells were unfortunately unavailable). No significant changes were seen (Fig. 1G), which is expected as PSTPIP1 binds to WASP via its SH3 domain (12) which was unscathed by the T274M mutation. We thus concluded that the PSTPIP1 mutations as found in our patients did not result in a typical PAPA syndrome, but might contribute to immunodeficiency.

Defective activation-induced f-actin polymerization of T274M PSTPIP1 patient CD4 T-cells

Upon TCR and CD2 ligation, PSTPIP1 recruitment serves to scaffold the interaction between CD2, protein tyrosine phosphatase (PTP-PEST) and the Wiskott Aldrich Syndrome Protein (WASP), the latter protein driving f-actin polymerization. PTP-PEST down-regulates TCR/CD2 signaling and WASP activation through dephosphorylation of PSTPIP1, thus keeping the f-actin modulation and IS formation under tight control^{14, 16, 21, 23}. We first addressed whether PSTPIP1 may control T-cell activation.

We stimulated primary CD4 T-cells of both patients using anti-CD2 and anti-CD3 antibodies and crosslinking with goat anti-mouse IgG fragments, for 0, 2, 5 and 10 minutes. Subsequently f-actin polymerization was visualized through staining with phalloidin-FITC and quantified using flow cytometry¹². We found that unstimulated T274M already displayed polymerized f-actin, whereas healthy control CD4 T-cells did not (Fig 2A,B). The CD4 T-cells of the T274M patient were hyporesponsive to anti-CD2 stimulation, with a significantly lower area under the curve (AUC) when compared to healthy age matched controls (Fig 2C). Stimulation of CD4+ T274M patient T-cells with anti-CD3 resulted in a steady increase of f-actin content over time (Fig 2A,B), as opposed to the healthy controls and the R228C patient which showed a decrease of f-actin after 10 min. Thus, especially the CD4+ T274M T-cells show a pre-activated status of f-actin, which derails T-cell activation-induced cytoskeletal remodeling. Corresponding to the pre-existing hyperactivated state of the T274M patient T-cells, also the f-actin content of the T274M resting B-cells was also increased, when compared to healthy controls, while R228C B-cells had less baseline levels of f-actin (Fig 2D).

We continued our PSTPIP1 primary T-cell studies comparing T274M with healthy control and CVID patient T-cells because of the more striking phenotype observed in T274M. We addressed cytosolic calcium mobilization of primary patient and healthy control CD4 memory and naive T-cells, as triggered by crosslinking of CD3 with anti-CD3 and goat anti-mouse IgG fragments. Baseline

calcium levels were much increased in the T274M patient CD4 T-cells, compared to CVID patients with normal or altered CD4 T-cell differentiation (Fig 3A), again suggestive of a pre-activated state. Activation-induced calcium mobilization of T274M T-cells was low when compared to either healthy control T-cells or CVID patient T-cells with T-cell differentiation defects (low CD4 naive T-cell percentages) (Fig 3B). Separate analysis of naive and memory T-cell calcium fluxes (CD4CD45RA+ and CD4CD45RO+) revealed that especially the memory subset of CD4 T274M T-cells were less responsive than their cellular counterparts of CVID patients with altered T-cell differentiation (Fig 3B). The TCR CDR3 repertoire however did not differ significantly from healthy control in contrast to what has been described in other T-cell CVID patients^{24, 25} (our unpublished data).

Considering that controlled f-actin polymerization is pivotal to cell motility^{26, 27}, we investigated T-cell migration of primary CD4 T-cells of the T274M patient and healthy controls. To this end, we enriched T274M patient CD4 T-cells (n=2) and healthy control CD4 T-cells (n=5). We tracked the motility of CD4 T-cells through collagen matrix using time-lapse light microscopy (2 images per minute, 4 hours). We found that in comparison to healthy control CD4 T-cells, the T274M CD4 T-cells displayed faster motility without prior stimulation (Fig 3C). Thus, the increased level of ready-made f-actin polymers in resting T274M PSTPIP1 CD4 T-cells translates to increased f-actin mediated motility. The pre-existence of f-actin filament nucleation may contribute to the observed shortfall of T274M CD4 T-cells to respond to TCR and anti-CD2 triggering-induced activation.

Loss of CD2 foci in activated T274M PSTPIP1 transfected HEK293T cells

PSTPIP1 in resting cells is distributed in the cytosol, available to be recruited to surface-expressed CD2 upon triggering of the TCR and co-receptors¹³. We observed especially for the unstimulated T274M PSTPIP1 T-cells, ready-made f-actin polymers and defective control of anti-CD2 activation-induced f-actin polymerization. These data prompted us to address the binding between CD2 and PSTPIP1. We therefore transfected HEK293T cells with expression plasmids encoding HA-tagged versions of WT, R228C and T274M PSTPIP1 together with a flag-tagged CD2 expression plasmid; transfection efficiencies were always 60-80%. We generated cell lysates using 1% triton X-100 detergent, and from these lysates we performed pull-down of CD2-flag with anti-flag conjugated agarose beads. In the CD2-flag pull-down fractions, we measured the amount of CD2-associated PSTPIP1 variants, both under unstimulated and anti-CD2 stimulated (2 min) conditions. We found that

CD2 stimulation stimulates PSTPIP1 binding to CD2, although pre-bound PSTPIP1 is already present at least in our cell lysate-based experimental setup. Particularly in the T274M T-cells, more PSTPIP1 appeared pre-bound to CD2, although data did not reach significance (Fig 4A, n=3).

We next addressed membrane remodeling by live cell confocal microscopy. CD2 stimulation caused CD2 clustering in WT PSTPIP1 confirming earlier data¹³. In T274M PSTPIP1 transfectants, CD2 remained distributed in scattered fashion throughout cellular membranes, and in R228C PSTPIP1 transfectants, an intermediate phenotype was seen (Fig 4B). Cross-sectional analyses supports these notions, with higher PSTPIP1 signals colocalizing with the CD2 signal in the T274M PSTPIP1 variant. These data support a role for PSTPIP1 as scaffold protein that connects CD2 to f-actin polymerization, as mediated by WASP^{12, 28}.

Defective immune-synapse formation in T274M PSTPIP1 T-cells

We finally investigated a possible role for PSTPIP1 in IS formation. Jurkat T-cells were transfected with plasmids encoding the WT, T274M and R228C PSTPIP1 variants, incubated with anti-CD3/CD28 beads (10 min) and subsequently fixed, permeabilized and stained with phalloidin-FITC. Confirming the elevated baseline f-actin content of the patient, without stimulation, the T274M PSTPIP1 transfected Jurkat cells already expressed increased phalloidin-FITC fluorescence (Fig 5A,B). We next visualized the specific recruitment of f-actin towards the bead contact site, and quantified images using ImageJ. Jurkat T-cells overexpressing the R228C variant showed intermediately reduced f-actin recruitment to the anti-CD3/CD28 bead, while the T274M PSTPIP1-overexpressing Jurkat T-cells much reduced f-actin recruited to the beads (Fig 5B,C). Thus, PSTPIP1 controls IS formation, which already had been shown to be an f-actin-dependent process^{29, 30}. Considering that the two patients expressing R228C and T274M PSTPIP1 demonstrated T and B-cell immunodeficiency, the f-actin polymerization defects observed may contribute to the clinical phenotypes observed.

Discussion

We here describe two novel PSTPIP1 mutations, R228C and T274M in two CVID patients presenting with aberrant T-cell compartments, but with no classical PAPA or Familial Mediterranean Fever syndrome characteristics. Specifically the T274M patient resting CD4 T-cells, we found were in a pre-activated state and subsequently less responsive to T-cell activation. The latter we argue may relate to the pre-existing nucleation for f-actin filaments.

In this study we primarily focused on the T-cell compartments of the patients, elaborating onto earlier T-cell model-based PSTPIP1 research^{12, 13}. To our knowledge, we are the first to report how PSTPIP1 mutations may contribute to PID, by showing how PSTPIP1 mutations modulate the early signaling events in primary CD4 T-cells. T-cells showed defects in stimulation-induced f-actin polymerization and failure to create sustained calcium fluxes. T-cell unresponsiveness was especially evident in CD4 memory T-cells, displaying diminished cytosolic calcium mobilization upon TCR triggering, onward from an elevated baseline calcium level. Of note, in healthy conditions, naive T-cells mobilize relatively more calcium upon TCR specific triggering than memory T-cells³¹ (and own unpublished data). The severe reduction in calcium mobilization of CD4 memory T-cells of the T274M patient might relate to the altered composition of the T-cell memory compartment as consequence to lymphopenia-induced homeostatic memory cell proliferation, although further investigation falls beyond the scope of this study.

We next looked into cell migration of the primary CD4 T-cells from patient and healthy donor controls, without prior stimulation. CD4 T-cells of healthy controls (n=5) migrated at a median velocity of 3 $\mu\text{m}/\text{min}$, as earlier reported for similar collagen densities (1.7 mg/ml)³². T274M patient T-cells velocity approached 4 $\mu\text{m}/\text{min}$ instead. Since T-cells rely on intact actin dynamics for their motility, the increase in f-actin polymers in resting T274M patient T-cells likely contributes to increased motility. In contrary, increase in f-actin polymers in T274M PSTPIP1 expressing cells was associated with defective immune synapse formation. Thus the higher f-actin content at baseline facilitates enhanced T-cell motility but probably ineffective adhesion once an antigen would be encountered, since CD2 mediated f-actin polymerization is hampered.

A limitation of our study is the paucity of functional B-cell experiments. Both patients were incapable to generate normal memory class switched B-cell numbers (combined <2% of total B-cell population), limiting our ability for functional B-cell assays. The patients had absent anti-pneumococcal

serotype responses. We propose that the aberrant f-actin polymerization and signaling of CD4 T-cells in the T274M PSTPIP1 patient may similarly cause unstable immune synapses between CD4 T-follicular helper cells and B-cells, for abolishment of T-cell help as needed for B-cell differentiation and memory class switching. Of note, also the T274M PSTPIP1 B-cells expressed increased baseline f-actin levels, supporting that PSTPIP1 might also control B-cell activation and function. However, PSTPIP1 is expressed in B-cells at lower levels than in T-cells¹³. Lack of sufficient patient B-cells, absence of pre-existing research on PSTPIP1-associated surface proteins in B-cells and a good model to study the PSTPIP1 functioning in B-cells precluded further research at this time.

In conclusion, our data supports a role for PSTPIP1 mutations in immunodeficiency, expanding the PSTPIP1 phenotype beyond inflammasome-mediated disorders such as PAPA syndrome. We here show a role for PSTPIP1 that revealed itself in CVID patients, by contribution to T-cell deficiency through altered f-actin polymerization. Further studies on PSTPIP1 to elucidate the mechanisms of PSTPIP1 in antibody deficiencies are warranted.

Figure 1

A) Total and CD4⁺ T-cell numbers of R228C and T274M PSTPIP1 patients as compared to HC (n=80)
 B) CD4⁺ memory T-cells of T274M and HC (representative from n=5), down left effector memory, up
 left central memory, up right naive CD4 T-cells C) Family tree of the R228C and T274M patients D) in
 silico model of the PSTPIP1 coiled coil domain with identified mutations E) DNA sequencing of the
 R228C (up) and T274M (down) PSTPIP1 patients F) LPS stimulation of R228C and T274M PBMC in
 comparison to HC (n=3) G) Peripheral blood CD3⁺ T-cells from R228C (red line) and HC (black line)
 were stained for WASP, and compared to isotype control-stained HC T-cells (solid grey).
 HC: Healthy control; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cells

Figure 2

A) Representative histograms of f-actin MFI's (as stained with phalloidin-FITC) of R228C, T74M, and
 HC CD4⁺ T-cells stimulated with anti-CD2 and anti-CD3 antibodies B) Baseline f-actin percentages of
 R228C and T274M PSTPIP1 patients relative to HC baseline f-actin C) f-actin increase of R228C
 (n=2), T274M (n=2) and HC (n=8) upon anti-CD2 and anti-CD3 stimulation relative to the baseline f-
 actin levels (as depicted in 2B) of the individual patients and HC, AUC's were calculated as the sum of
 the f-actin increases over the time points 0,2,5, 10 min per individual subject (HC and patients).
 D) Baseline f-actin percentages of R228C and T274M PSTPIP1 patient B-cells (n=1) relative to HC
 (n=3)
 AUC: Area Under the Curve, CVID: Common Variable Immunodeficiency; HC: Healthy control; MFI:
 mean fluorescence intensity

Figure 3

A) Baseline cytosolic calcium levels of CVID patients with normal CD4⁺ T-cell compartments as
 compared to CVID patients with low CD4⁺ naive T-cells and HC with normal CD4⁺ naive T-cells, the
 T274M patient is highlighted in pink B) Cytosolic calcium increase upon anti-CD3 stimulation of CD4⁺
 T-cells, naive CD4⁺CD45RA⁺ T-cells and memory CD4⁺CD45RA⁻ T-cells of CVID patients with
 normal CD4⁺ T-cell compartments (n=6) as compared to CVID patients with low CD4⁺ naive T-cells

(n=6) and HC's (n=6) C) Unstimulated CD4+ T-cell migration of T274M CD4+ T-cells (n=2) and HC (n=4)

CVID: Common Variable Immunodeficiency; HC: Healthy control; MFI: mean fluorescence intensity

Figure 4

A) Representative Western blot of CD2-flag pull down lysates upon anti-CD2 stimulation (2 min) of HEK293T cells transfected with WT, R228C and T274M PSTPIP1-HA and CD2-flag (n=3); Representative Western blot of total lysates of CD2-flag and PSTPIP1-HA before pull down and densitometry data of co-IP PSTPIP1-HA relative to CD2-flag pulldown (n=3) B) Localization of CD2-flag and WT, R228C and T274M PSTPIP1-HA transfected HEK293T cells (2 min anti-CD2 stimulation) as visualized using confocal imaging. Right: histograms of anti-flag FITC and anti-HA AF647 intensities

WT: wild type

Figure 5

A) Representative confocal images of f-actin recruitment in unstimulated and anti-CD3/CD28 bead stimulated (10 min) WT, R228C and T274M PSTPIP1-HA transfected Jurkat cells B) Baseline f-actin MFI's of R228C PSTPIP1-HA and T274M PSTPIP1-HA relative to WT PSTPIP1-HA transfected Jurkat cells (n=5) as quantified with flow cytometry C) Quantification of f-actin recruitment around anti-CD3/CD28 beads as visualized by confocal imaging

MFI: mean fluorescence intensity; WT: wild type

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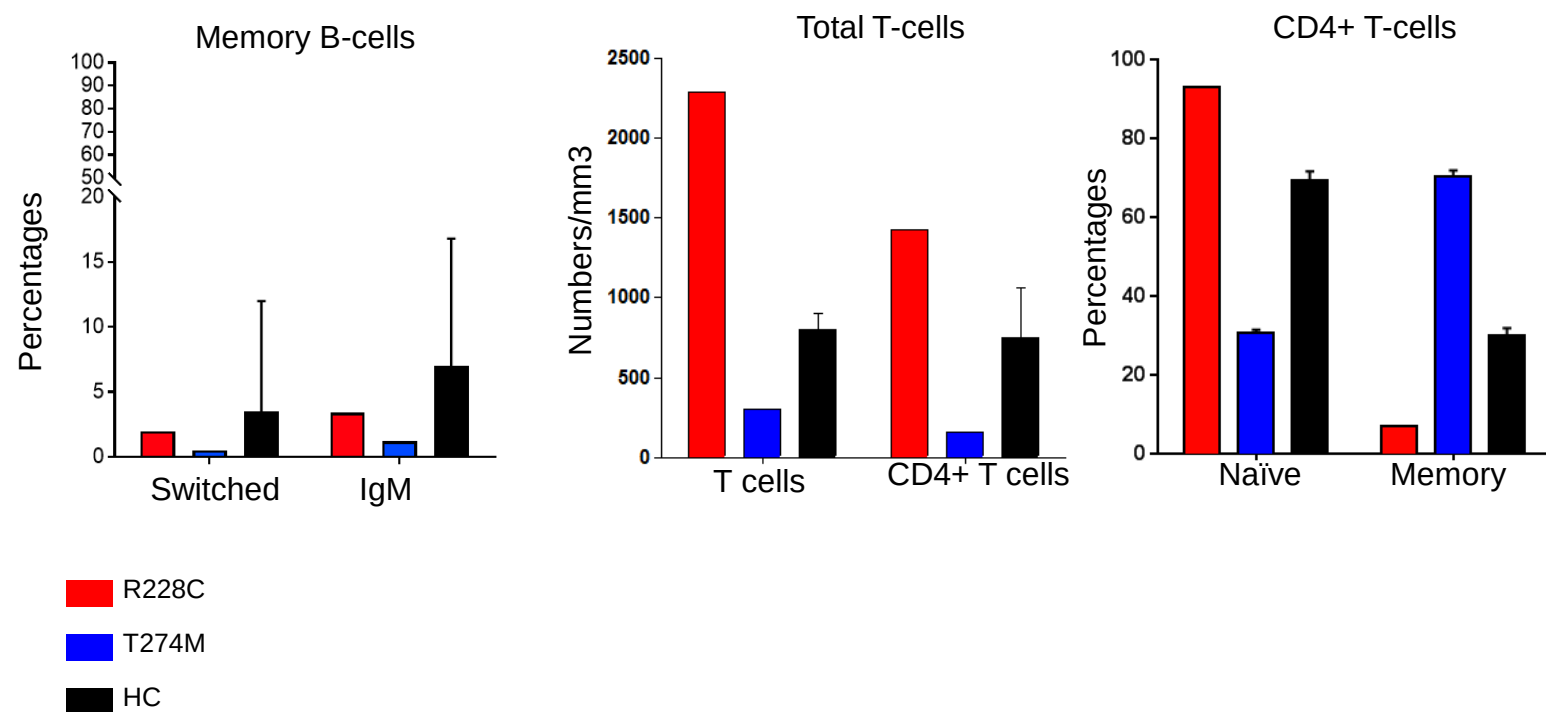
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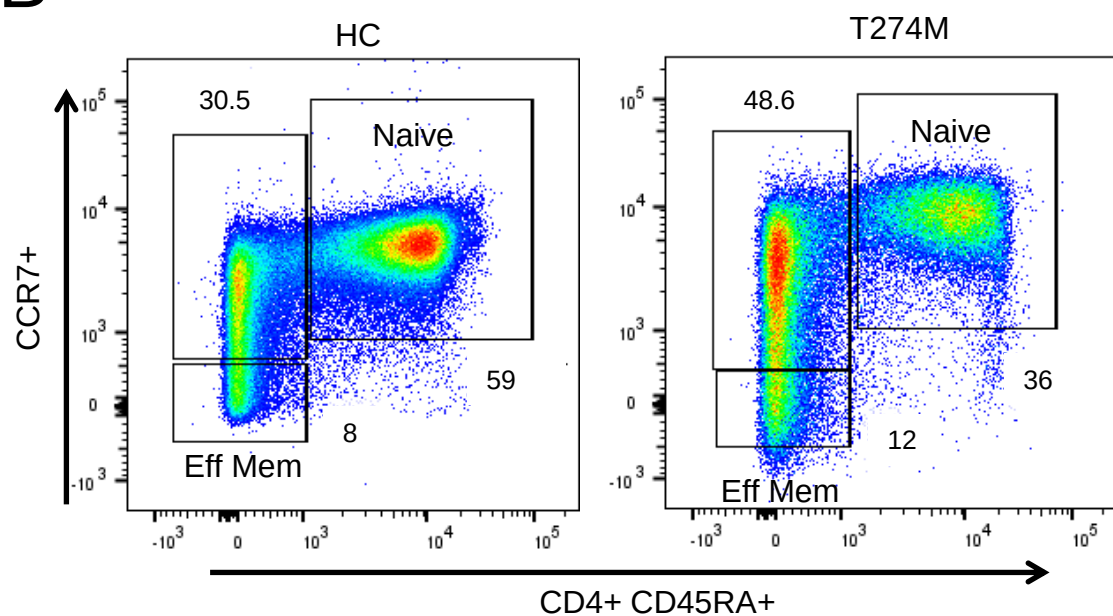
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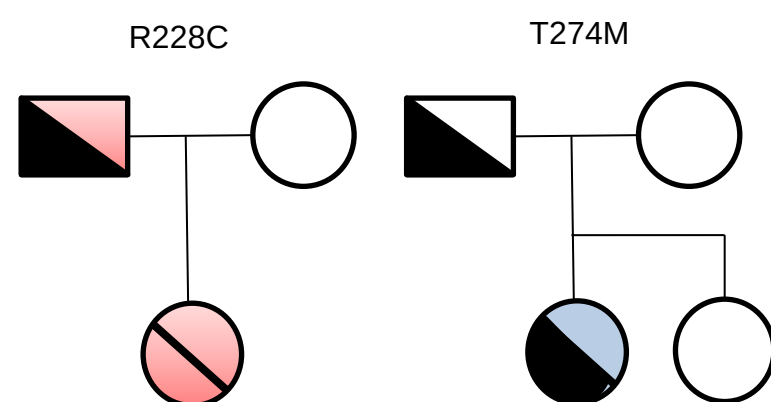
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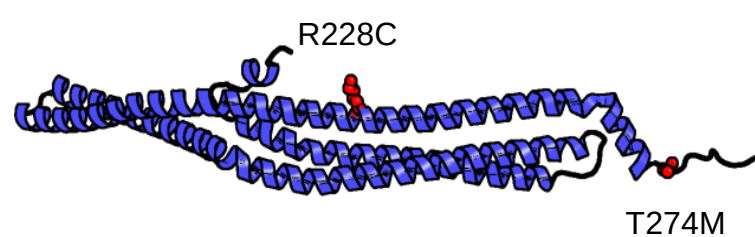
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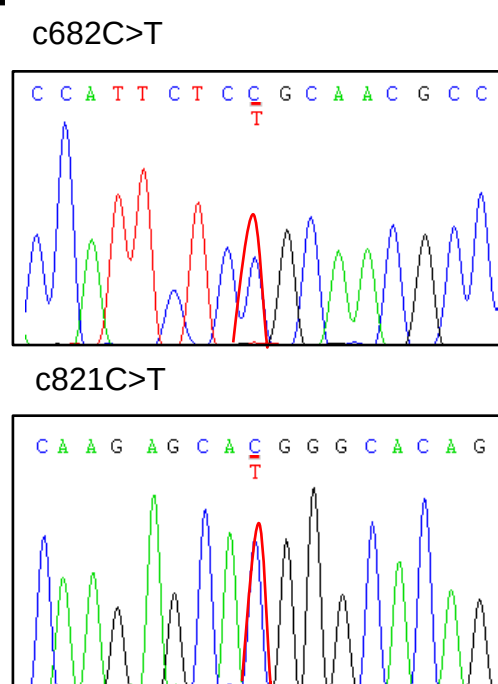
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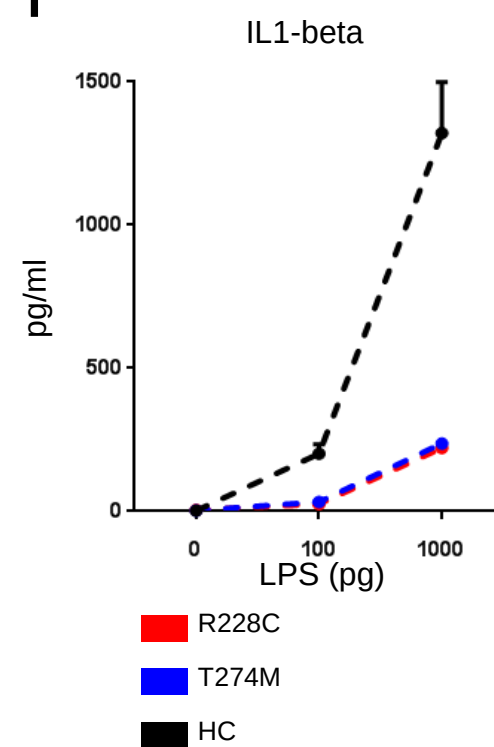
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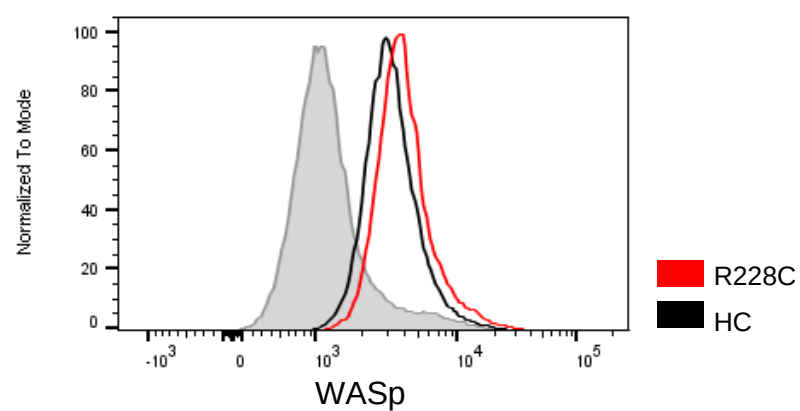
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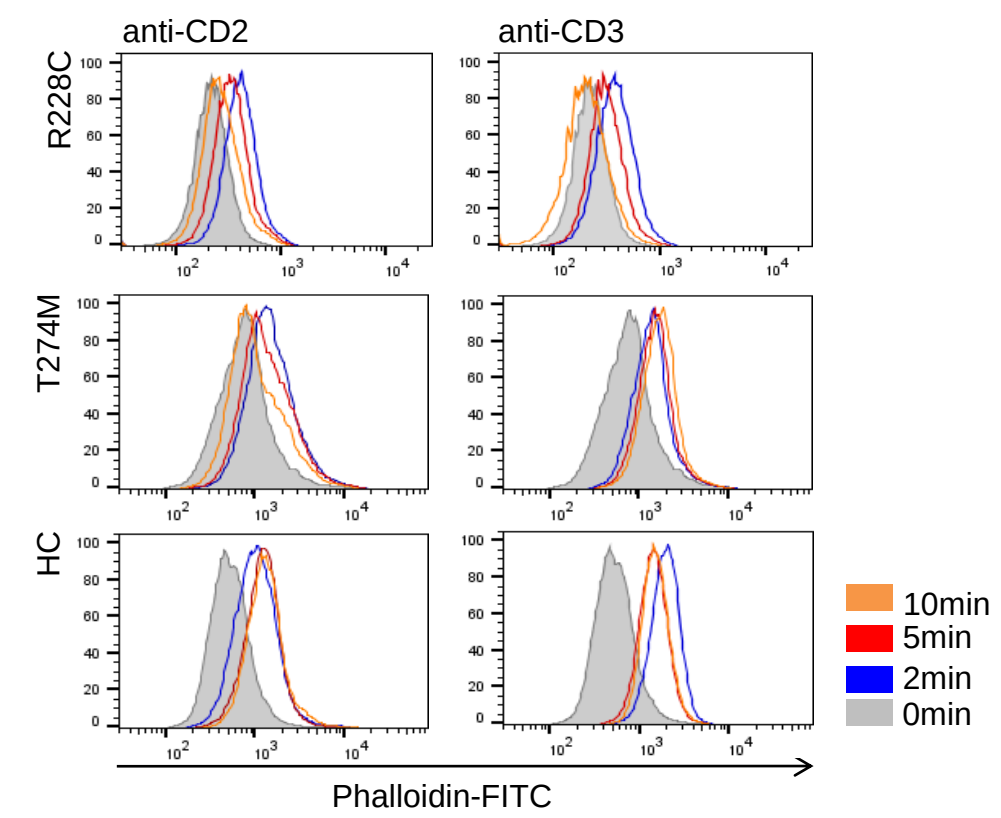
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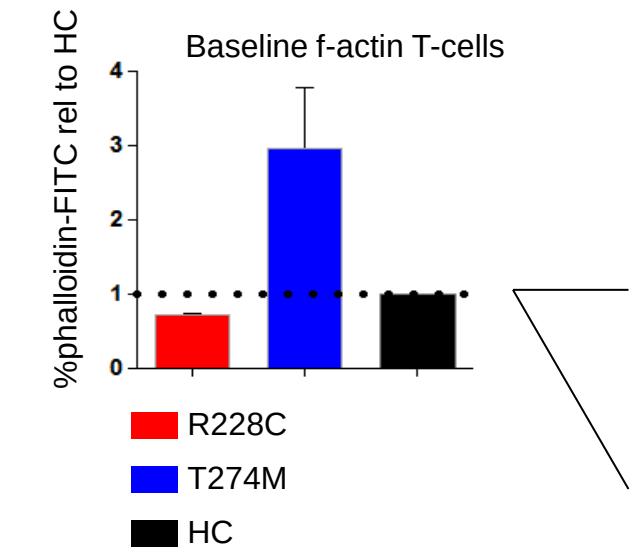
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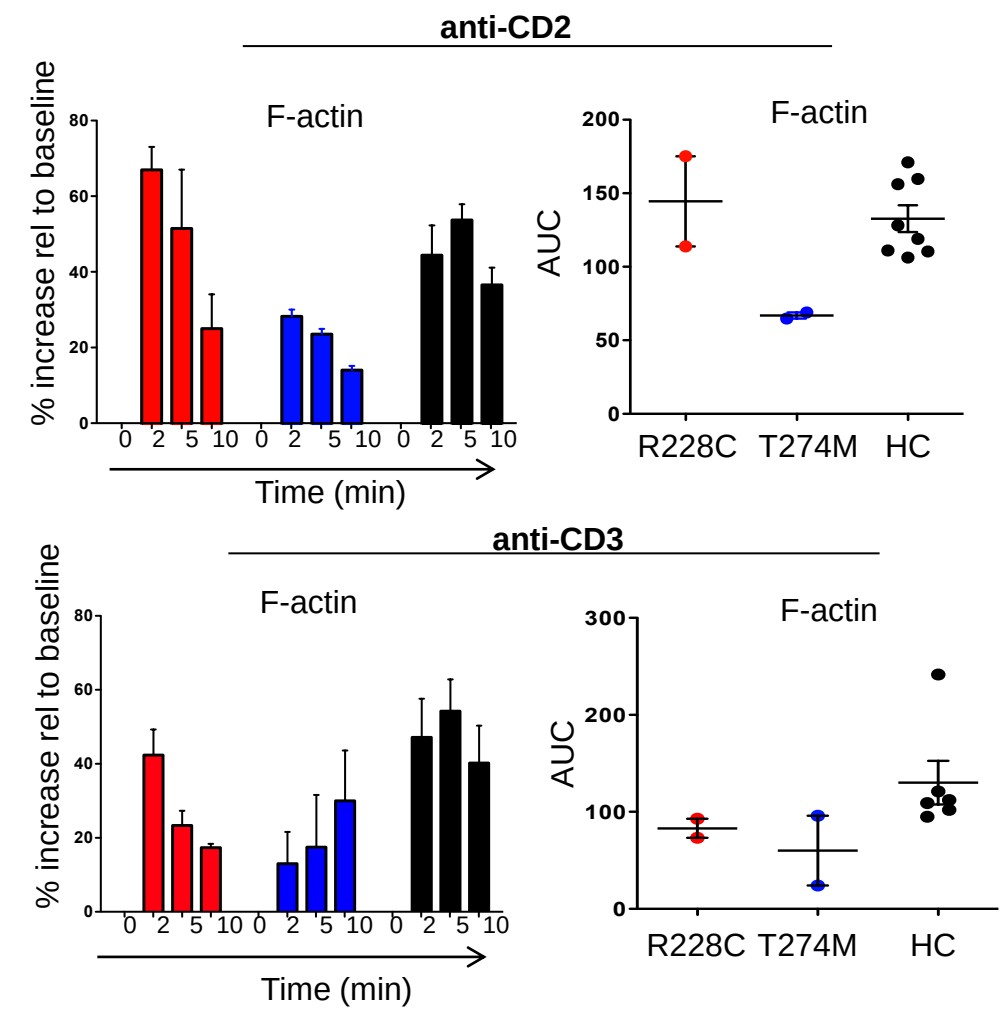
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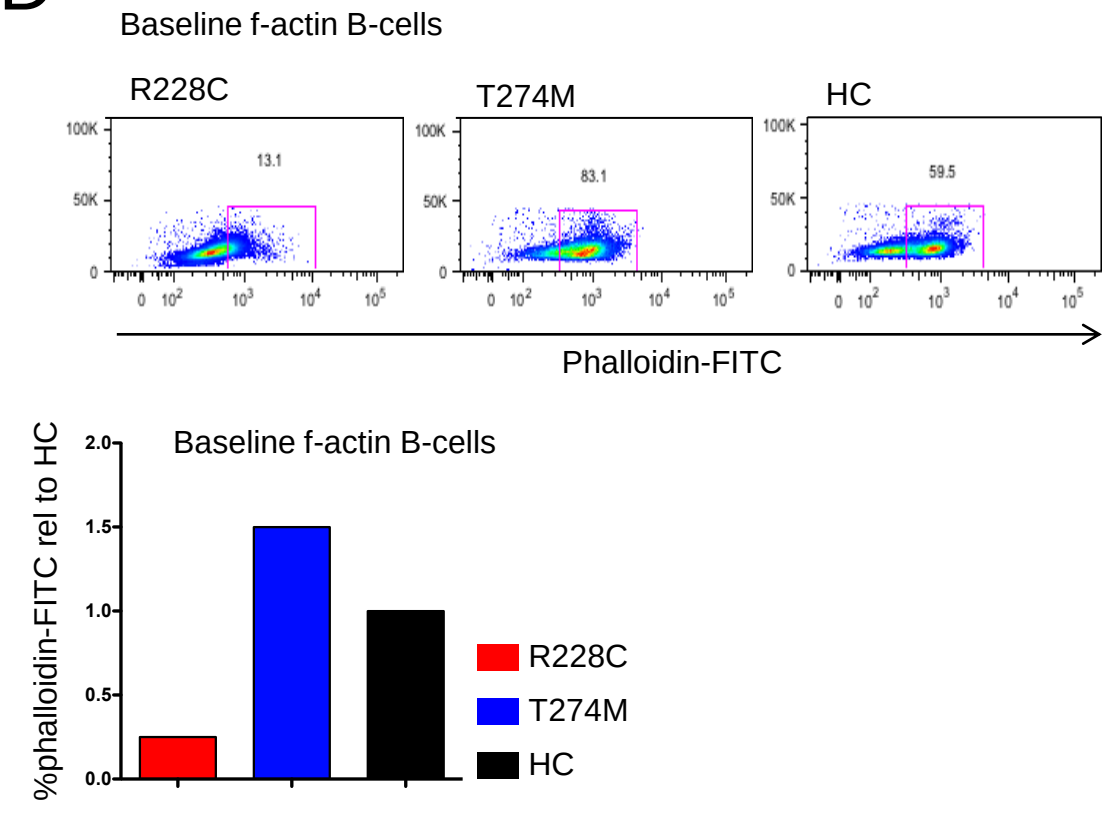
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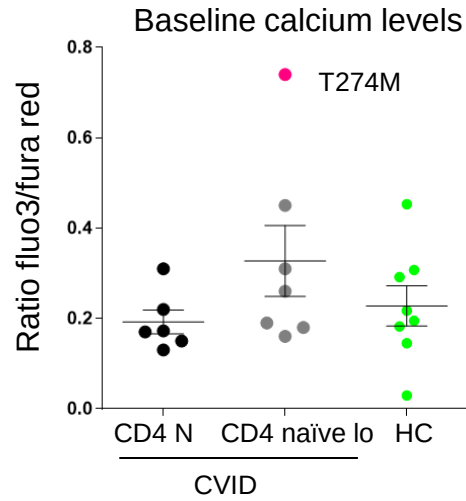
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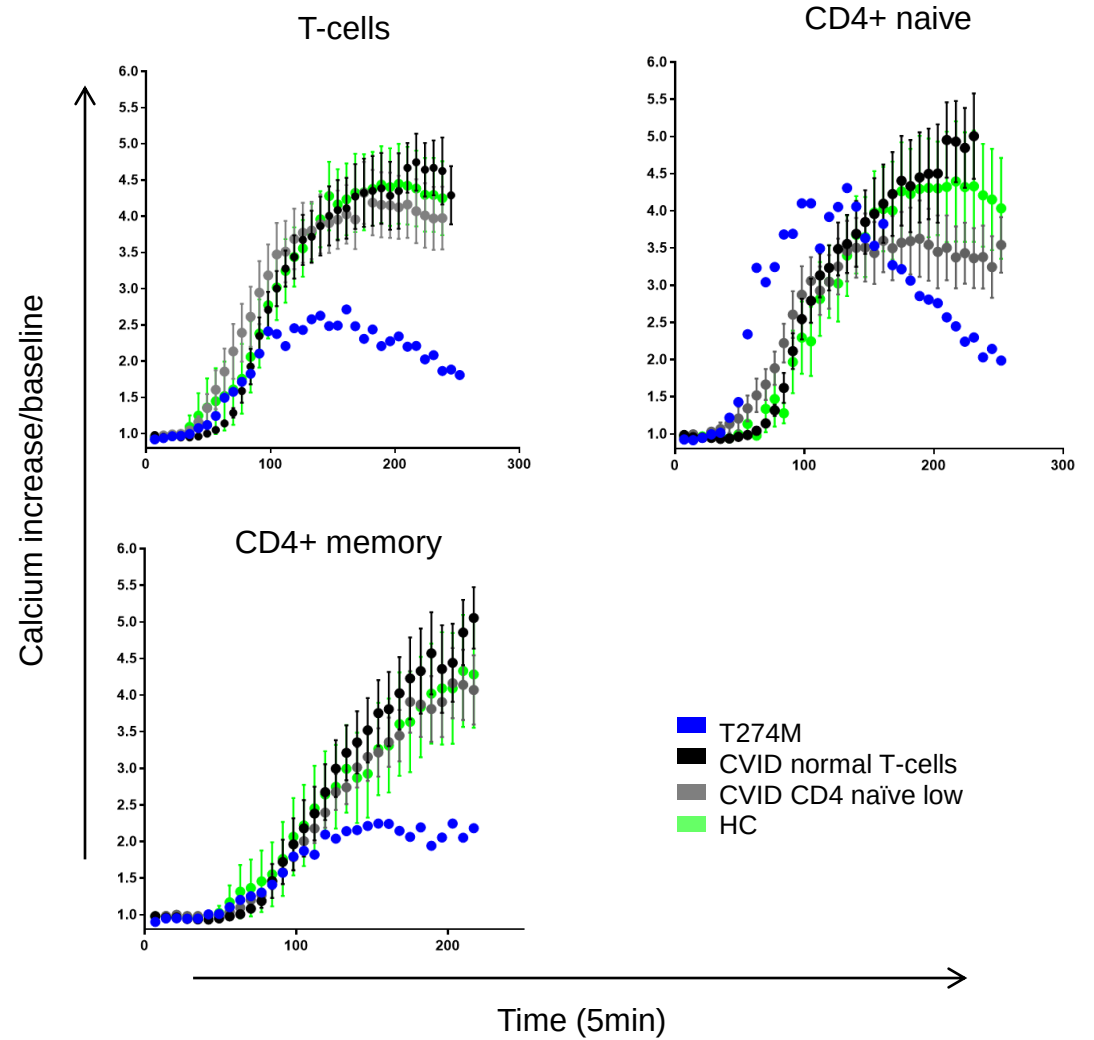
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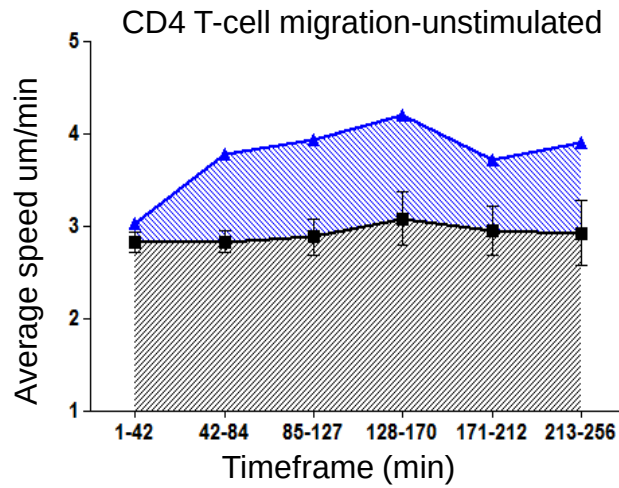
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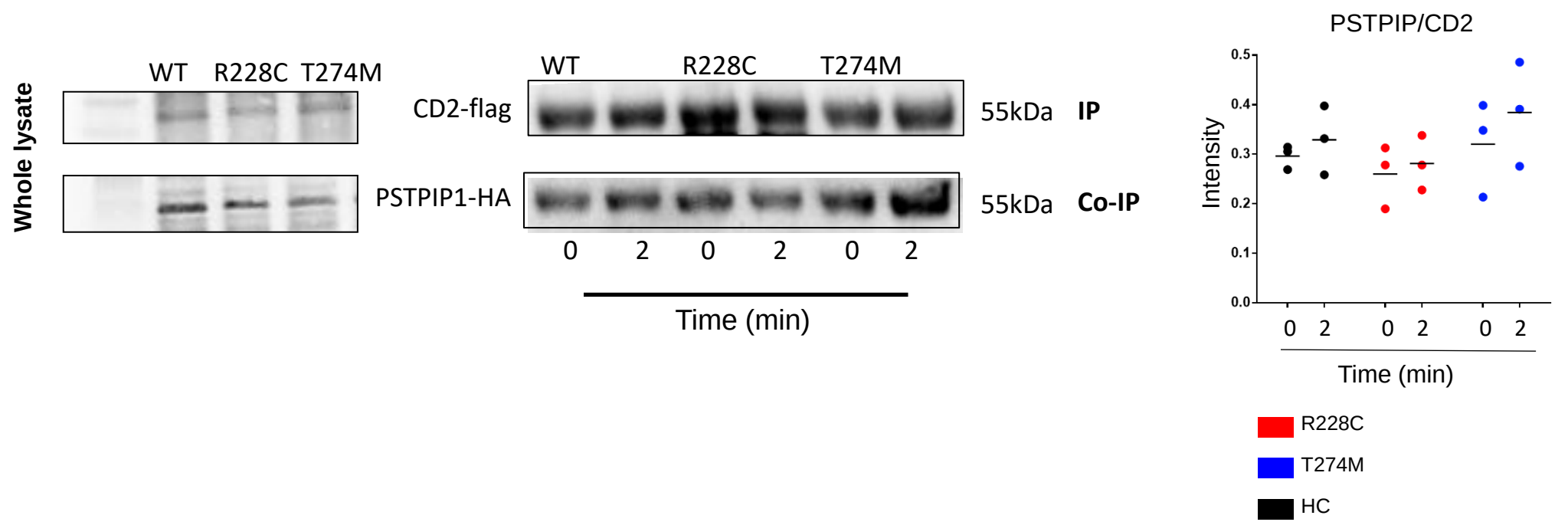
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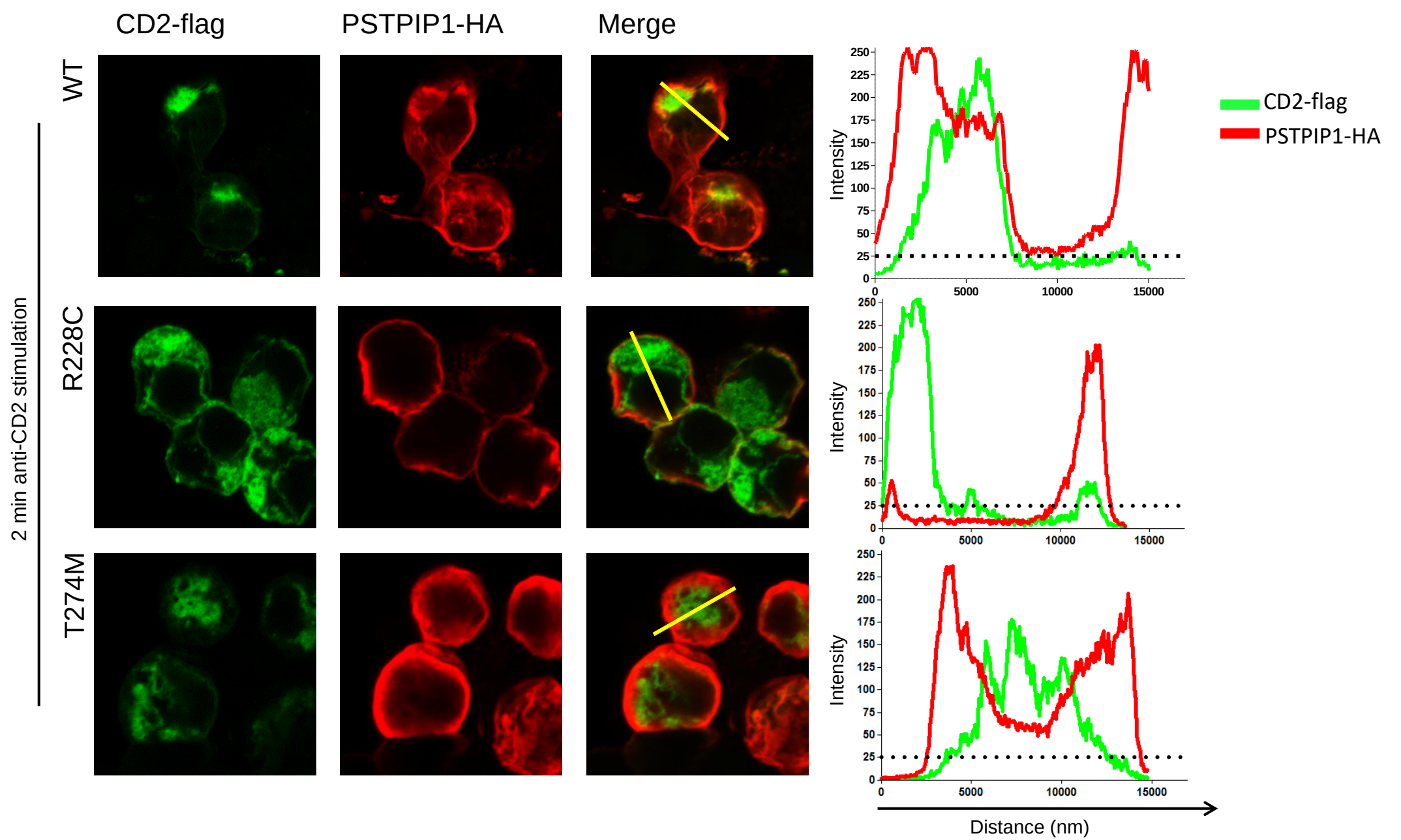
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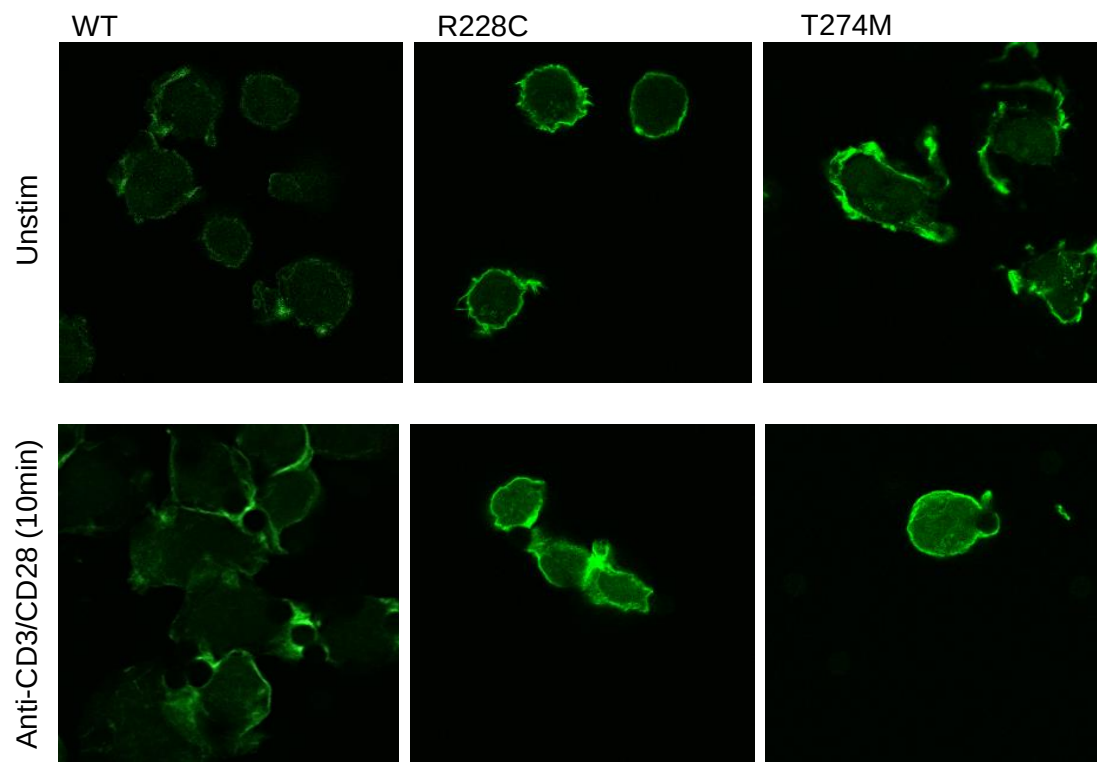
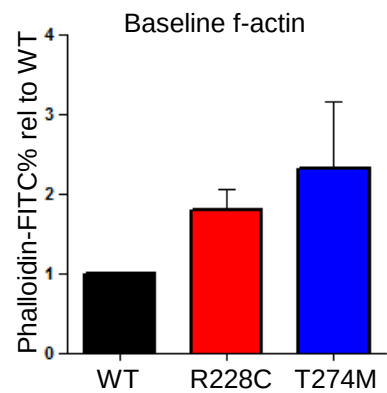


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