# Cyclophilin A associates with and regulates the activity of ZAP70 in TCR/CD3 stimulated T cells

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# Abstract

The ZAP70 protein tyrosine kinase (PTK) couples stimulated T cell antigen receptors (TCRs) to their downstream signal transduction pathways and is *sine qua non* for T cell activation and differentiation. TCR engagement leads to activation-induced post-translational modifications of the ZAP70 protein tyrosine kinase, predominantly by kinases, which modulate its conformation, leading to activation of its catalytic domain. Here we demonstrate that ZAP70 in activated T cells is regulated by the cyclophilin A (CypA) peptidyl-prolyl *cis-trans* isomerase (PPIase), and that this regulation is blocked by the CypA inhibitor, cyclosporin A (CsA). We found that TCR crosslinking promoted the rapid and transient formation of cyclophilin A (CypA)-ZAP70 complexes, which were dependent on Lck-mediated phosphorylation of ZAP70. Cyclosporin A (CsA) inhibited CypA binding to ZAP70 and prevented CypA-ZAP70 colocalization at the cell membrane. In addition, fluorescent cell staining and imaging analyses revealed that TCR/CD3 triggering led to the association of the ZAP70-bound CypA with the TCR/CD3 complex within the immunological synapse. Enzymatically active CypA downregulated the catalytic activity of ZAP70 *in vitro*, an effect that was reversed by CsA and confirmed *in vivo* by FRET-based studies. We suggest that CypA plays a role in determining the conformational regulation and activity of ZAP70 in TCR-engaged T cells and impact on T cell activation by intervening with the activity of multiple downstream effector molecules.


# Introduction

The Syk family protein tyrosine kinase, ζ-chain-associated protein of 70kDa (ZAP70), plays a critical role in the induction of T cell activation following T cell antigen receptor (TCR) engagement [1]. The crucial role of ZAP70 in the initiation of TCR signaling was observed in ZAP70-deficient humans that suffered from severe combined immunodeficiency (SCID) due to lack of $CD8^+$ T cells and defective activation of their $CD4^+$ T cells [2–4]. In addition, knock-out of the mouse ZAP70 resulted in an early developmental arrest of thymic T cells at the $CD4^+/CD8^+$ double-positive stage [5, 6]. In resting T cells, ZAP70 resides within the cytoplasm as a non-phosphorylated and autoinhibited protein. Its activation occurs following TCR stimulation-induced recruitment of ZAP70 to the immunological synapse (IS) where it undergoes a series of post-translational modifications.

Activation of T cells is initiated by TCR engagement with agonistic peptide-bound MHC complex molecules on the surface of antigen-presenting cells (APC). The simultaneous interaction of the T cell coreceptor molecules, CD4 or CD8, with the MHC class I or class II receptors, respectively, positions the CD4/CD8-associated, lymphocyte-specific kinase (Lck) in close proximity with the immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of the CD3 chains [7]. Lck-mediated phosphorylation of the ITAMs' tyrosine residues promote the interaction of the ZAP70 tandem SH2 domains with doubly phosphorylated ITAMs, leading to a conformational change in ZAP70 that makes it more accessible to phosphorylation by Lck [8]. The phosphorylation of ZAP70 by Lck [9–11] and its autophosphorylation [12–14] on tyrosine residues located in critical positions for secondary structure determination and for interaction with binding partners introduces further modifications in its overall

conformation in order to unleash its catalytic activity. Activated ZAP70 then phosphorylates TCR downstream effector molecules, including the linker for the activation of T cells (LAT) [15], the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) [16], and the p38 mitogen-activated protein kinase (MAPK) [17], which promote signal propagation leading to cell activation and proliferation [18].

In general, the correlation between the extent of phosphorylation of ZAP70 and the increase of its catalytic activity suggests that tyrosine phosphorylation serves to positively regulate ZAP70 enzymatic activity. However, depending on the T cell subtype and its mode of activation, ZAP70 may undergo phosphorylation at distinct sites which might impose different effects on the conformation and activity of ZAP70. For example, Lck mediated phosphorylation of ZAP70-Tyr493, at the activation loop of the catalytic domain, promotes a conformational change which increases ZAP70 activity [13, 19]. In contrast, phosphorylation of ZAP70 on Tyr 292, has a negative regulatory role, as demonstrated by Y292F knock-in studies in primary mouse T cells (where phenylalanine at position 292 serves as a non-phosphorable tyrosine mimetic) which led to enhanced TCR signaling and increased T cell proliferation [14, 20, 21]. Two additional tyrosines that are frequently phosphorylated in TCR engaged T cells, Tyr315 and Tyr319, are located in the interdomain B region, and appear to play a role in stabilizing the active conformation of ZAP70 [22].

Independently of its enzymatic activity, tyrosine-phosphorylated ZAP70 also serves as an adaptor protein that recruits SH2-containing effector molecules to the IS. These proteins may contribute to the regulation of ZAP70 activity and play significant roles in the propagation of signals downstream of activated TCRs [11, 23–27]. For example, ZAP70-phospho-Tyr319 functions as a binding site for the Lck-SH2 domain, and upon docking, Lck can phosphorylate additional tyrosines on ZAP70 and neighboring proteins that participate in the TCR-linked signaling cascades [10, 11, 28]. In addition, ZAP70 phospho-Tyr315 serves as a binding site for the SH2 domain of the guanine nucleotide exchange factor, Vav [29], and the CT10 regulator of kinase II (CrkII) adaptor protein [30, 31] in TCR-stimulated T cells. These proteins possess additional protein-protein interaction domains through which they can associate with other effector molecules and recruit them to the IS. Furthermore, binding of these proteins to ZAP70 may impact on the conformation, subcellular location, and ability of ZAP70 to interact with substrates or other regulatory molecules. Recent studies demonstrated that ZAP70-Tyr126, which undergoes phosphorylation in TCR-stimulated T cells, serves as a binding site for the protein kinase C theta (PKCθ) phosphotyrosine-binding domain and that this interaction is essential for the promotion of proximal TCR signaling events [32]. Phospho-Tyr315 and phospho-Tyr319 of ZAP70 were required for the above interaction, apparently because of their positive role in stabilizing the “open” conformation of ZAP70 which exposes phospho-Tyr126 for the interaction with PKCθ [32]. ZAP70 phospho-Tyr292 also serves as a binding motif for the ubiquitin ligase, c-Cbl (Casitas B-lineage lymphoma), raising the possibility that c-Cbl-mediated ubiquitination of ZAP70 downregulates proximal TCR signals by promoting ZAP70 degradation [33, 34].

Additional studies demonstrated the existence of phosphorylation-independent mechanisms of regulation of ZAP70, which are mediated by ubiquitinating and deubiquitinating enzymes. For example, the ring finger-type E3 ligase, Nrdp1, can negatively regulate ZAP70 activity by ubiquitinating ZAP70-

Lys578, generating a binding site for the suppressor of TCR signaling 1 (Sts1) and Sts2 protein phosphatases which dephosphorylate ZAP70 and downregulate its activity [35]. Abrogation of a distinct ZAP70 ubiquitination site at Lys217 resulted in increased activity of ZAP70 and TCR-downstream events, suggesting a negative regulatory role of this motif in ZAP70-dependent functions [36]. Furthermore, deubiquitinating carboxyl-terminal hydrolases, such as Usp9X [37] and Otud7b [38], can catalyze the deubiquitination of ZAP70 and facilitate T cells activation, while oxidation of ZAP70-Cys575 has an impact on ZAP70 stability and activity [39]. The above studies and additional information substantiate the assumption that the conformation of ZAP70, which is critical for its function, is subjected to regulation by multiple post-translational mechanisms that may potentially act in synergism or antagonism.

In the present work, we provide data supporting the existence of an additional mechanism of regulation of ZAP70 in TCR activated T cells, which is mediated by the peptidyl-prolyl *cis-trans* isomerase, cyclophilin A (CypA), the predominant cyclophilin in T cells. We found that ZAP70 interacts with CypA in a TCR activation-dependent manner, which requires Lck, and is inhibited by the CypA inhibitor, cyclosporin A (CsA). TCR crosslinking promotes the recruitment of the ZAP70-associated CypA to the CD3/TCR receptor complex within the IS. Functional studies revealed that CypA, but not the Pin1 PPIase, downregulated the catalytic activity of ZAP70, a phenomenon that was reversed by CsA. We suggest that CypA regulates ZAP70 activity by isomerization or physical interaction with ZAP70, that CypA-mediated regulation of ZAP70 can be reversed by CsA, and that the CsA-regulated CypA-mediated effect on ZAP70 may synergize with the effect of CypA/CsA on the calcineurin/NF-AT signaling cascade which promotes T cell immunosuppression.

# Results

### T lymphocyte TCR crosslinking promotes the association of CypA with ZAP70

To identify CypA-binding partners in TCR-engaged T cells, which might play a role in T cell activation and serve as targets for regulation by CypA we immunoprecipitated CypA from lysates of resting and OKT3-stimulated Jurkat T cells and immunoblotted the samples with phospho-Tyr-specific mAbs. A prominent protein band of 70 kDa was observed in lysates of activated but not resting Jurkat T cells (Fig. 1A). Reblotting of the membrane with anti-ZAP70 mAbs indicated that this protein band corresponds to ZAP70 (Fig. 1B). To ascertain that the CypA-associated 70 kDa protein band represents ZAP70, we repeated the experiment using a ZAP70-deficient Jurkat T cell subline, P116. Anti-CypA mAbs were found to coimmunoprecipitate a ZAP70 immunoreactive protein from OKT3-stimulated Jurkat, but not P116 T cell lysates (Fig. S1A-C). In addition, heterologous expression of GST-CypA and Myc-ZAP70 in Jurkat T cells followed by anti-GST immunoprecipitation and anti-Myc immunoblotting reconfirmed the association between CypA and ZAP70 in OKT3-treated, but not resting T cells (Fig. 1D-G). An immunoprecipitation study in Jurkat T cells involving early and late time points of T cell activation demonstrated that binding of CypA to ZAP70 is transient and peaks at ~60 sec post TCR crosslinking (Fig. 1H-L). A reverse coimmunoprecipitation study revealed the ability of ZAP70 to coimmunoprecipitate

CypA from OKT3-stimulated Jurkat cells. (Fig. S1D-F). Finally, coimmunoprecipitation of ZAP70 with CypA was also observed in lysates of TCR-triggered C57BL/6J mouse spleen and thymus lymphocytes (Fig. S1G-L), suggesting that CypA binding to ZAP70 is a physiological phenomenon. To define the subcellular location of CypA-bound ZAP70 in TCR-engaged T cells, we stained the Jurkat T cells with CypA- and ZAP70-specific Abs followed by confocal microscope analysis. Cell staining with p-Tyr-specific mAbs verified the efficiency of the TCR stimulation by showing tyrosine-phosphorylated proteins at the membrane of the stimulated, but not resting T cells (Fig. 1M). We found that in resting T cells, CypA and ZAP70 reside predominantly in the cytosol. In contrast, TCR crosslinking led to translocation and partial colocalization of the two proteins at the plasma membrane (Fig. 1N, O).

### The transient binding of CypA to ZAP70 is dependent on the presence of active Lck

TCR ligation triggers a rapid phosphorylation of multiple effector proteins on tyrosine residues, hence we suspected that CypA-ZAP70 interaction might be regulated by one or more protein tyrosine kinases (PTKs). One of the major candidates for this phosphorylation is the Lck PTK which associates with the cytoplasmic tails of the CD4 and CD8 coreceptors. Lck recruits to the TCR/CD3, upon coreceptor binding to MHC, and phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on the TCR/CD3 chains, as well as ZAP70 [47]. To test the involvement of Lck in CypA-ZAP70 interaction, we compared the ability of CypA to coimmunoprecipitate ZAP70 from wild type Jurkat *vs*. the Lck-deficient Jurkat subline, termed JCaM.1. We observed that CypA coimmunoprecipitated ZAP70 from lysates of OKT3-treated Jurkat, but not JCaM.1 T cells (Fig. S2A-E). The results suggest that the *in vivo* interaction between CypA and ZAP70 is dependent on Lck, which is known to phosphorylate ZAP70 in TCR-triggered T cells [47]. Furthermore, we immunoprecipitated CypA from anti-CD3ε (2C11)-stimulated C57BL/6J mouse spleen cells, and incubated the bead-immune complexes in the presence or absence of calf-intestinal alkaline phosphatase (CIP) for 1 hr. As observed above, CypA coimmunoprecipitated ZAP70 from lysates of 2C11-treated spleen cells. However, CIP treatment of the immune complexes resulted in ZAP70 dissociation from the immune complexes (Fig. S2F-I) suggesting that CypA-ZAP70 interaction is dependent on the phosphorylation of ZAP70, CypA, or both proteins.

### Association of CypA with activated ZAP70 enables its recruitment to the TCR within the IS

The ZAP70 PTK resides in the cytoplasm of resting T cells. Following TCR stimulation it translocates to the cell membrane and undergoes phosphorylation which peaks at ~60 sec [1]. Since CypA association with ZAP70 occurs in a similar time kinetic post TCR stimulation we tested whether the membrane-translocating ZAP70 pulls with it the CypA protein. Jurkat T cells were stimulated with OKT3 for 60 sec and their lysates were subjected to immunoprecipitation using phospho-CD3ζ (pCD3ζ)-specific Abs. As previously reported [1, 48], ZAP70 and Lck coimmunoprecipitated with the pCD3ζ-containing CD3/TCR complex of TCR activated T cells (Fig. 2A, B). In addition, CypA was found to coimmunoprecipitate with pCD3ζ (Fig. 2E). CypA recruitment to the cell membrane was dependent on the presence of ZAP70 and did not occur in the ZAP70-deficient Jurkat subline, P116. Immunofluorescence studies utilizing similar cells and activation conditions confirmed the colocalization of CypA and pCD3ζ at the plasma membrane

of activated Jurkat T cells (Fig. 2J, L, M). To test whether the localization of CypA at the membrane of TCR-activated T cells occurs via its association with ZAP70 or perhaps by its direct interaction with pCD3ζ, the experiment was repeated in the ZAP70-deficient P116 Jurkat T cells. We found that lack of ZAP70 disrupts the ability of CypA to coimmunoprecipitate with pCD3ζ (Fig. 2E) or colocalize with it at the plasma membrane (Fig. 2K, L, M), suggesting the requirement of ZAP70 for the association of CypA with the activated TCR.

We further speculated a tripartite complexing of CD3 receptor-ZAP70-CypA upon TCR crosslinking. In order to detect CD3 receptor in both resting and activated cells, we utilized an antibody against total CD3 for the immunofluorescence studies. Since ZAP70 interacts with CD3ζ as well as CD3ε ITAM motifs [49] we used a combination of anti-CD3ε, -ZAP70 and -CypA Abs to immunostain OKT3-stimulated Jurkat T cells. While all three proteins were found to be distributed in the same subcellular compartments, stain overlapping analysis revealed a striking colocalization of CypA-ZAP70, ZAP70-CD3ε, and CypA-CD3ε -in OKT3 stimulated, in comparison to the resting Jurkat T cells (Fig. 3A-D). To test whether CypA recruits to the IS of T cells triggered by a specific peptide antigen presented by APC, by virtue of its interaction with ZAP70, we utilized CH7C17 Jurkat T cells, which express the influenza hemagglutinin (HA) peptide-specific TCR, and co-cultured them with antigen-fed LG2 cells, as APC. LG2 cells fed with the HA 307-319 peptide formed conjugates with the CH7C17 T cells in which CypA and ZAP70 colocalized at the T cell-APC contact area (Fig. 3E). The two proteins also colocalized with the F-actin binding protein, phalloidin, which serves as an IS-specific marker. These results suggest a potential regulatory role for CypA at the vicinity of the ZAP70-TCR complex within the IS which might contribute to the regulation of IS-residing effector molecules that impact on the TCR-downstream signaling cascades.

**CypA inhibits ZAP70 catalytic activity** in vitro

To test the direct effect of CypA on ZAP70 activity we performed an *in vitro* radioactive kinase assay on ZAP70 immunoprecipitates from 1 min OKT3-treated Jurkat T cells and 2C11-treated C57BL/6J mice spleen and thymus T cells. Preincubation of ZAP70 from all three sources with enzymatically active recombinant CypA (rCypA) inhibited ZAP70 autophosphorylation activity in a concentration- (Fig. 4A-L) and time-dependent manner (Fig. 4M-X). The *in vitro* data demonstrate a linear inhibition of ZAP70 catalytic activity by CypA in Jurkat T cells, and splenic/thymic cells derived *ex vivo* from C57BL/6J mice (Fig. 4Y, Z). To test whether ZAP70 is sensitive to isomerases in general, or whether its regulation is selective to CypA, we repeated the assay comparing the effects CypA *vs*. Pin1 PPIase on the catalytic activity of ZAP70.

We found that CypA, but not Pin1, downregulated the autophosphorylation activity of ZAP70 (compare Fig. S3A-E *vs*. S3F-J). A control experiment validated that rPin1 is catalytically active by showing its ability to modulate the activity of PKCα, a known Pin1 substrate [50] (Fig. S3K-O). Inclusion of a ZAP70 substrate, the cytoplasmic fragment of human erythrocyte band 3 (cfb3) [51] in the *in vitro* kinase assay further demonstrated that rCypA inhibited the ability of ZAP70 to phosphorylate cfb3 in a concentration- (Fig. S4A-D, I) and time-dependent manner (Fig. S4E-H, J). We also noticed that inclusion of recombinant

CypA whole protein in the ZAP70 kinase assay system did not result in ZAP70-mediated phosphorylation of CypA, negating a possibility of reciprocal regulation of CypA by ZAP70 *in vitro* (Fig. 4A, E, I, M Q, U). While the above studies suggest that CypA inhibits the phosphorylation activity of ZAP70, they do not rule out the possibility that the reduction in ZAP70 phosphorylation is due to CypA-mediated dephosphorylation of ZAP70. This hypothesis was tested by co-incubation of enzymatically active rCypA with an inactive phospholabeled ZAP70. We found (Fig. S4K-O) that rCypA was unable to dephosphorylate ZAP70, supporting the assumption that CypA is a conformational regulator of ZAP70.

### CsA reverses the regulatory effect of CypA on ZAP70

CsA is a potent immunosuppressive drug that mediates high-affinity binding to CypA and inhibits its enzymatic activity [52, 53]. To test whether CypA binding to ZAP70 is affected by CsA, we treated Jurkat T cells with CsA and subsequently stimulated the cells with OKT3 followed by immunoprecipitation of CypA. We found that cell treatment with CsA inhibited the ability of CypA to associate with ZAP70 (Fig. S5A-D). The effect of CsA on the ability of CypA to associate with ZAP70 was also tested by cell staining and immunofluorescent imaging using confocal microscopy. The results demonstrated a strong CsA-mediated inhibition of CypA colocalization with ZAP70 (Fig. SA, B, Fig. S5E). A major mechanism by which CsA-CypA complexes contribute to immunosuppression is by binding to and inhibition of calcineurin and its downstream signaling pathway that controls IL-2 gene transcription. However, the above findings also suggest the involvement of CypA in the regulation of ZAP70. We tested the effect of CsA on the ability of rCypA to attenuate ZAP70 catalytic activity. The inclusion of CsA at the rCypA-ZAP70 preincubation step reversed the inhibitory effect of CypA on ZAP70 autophosphorylation in a concentration-dependent manner (Fig. 5C-G). The results suggest a potential role for CsA in activated T cells in the regulation of CypA activity and CypA-dependent effects on TCR-downstream effector molecules, including ZAP70.

Previous studies demonstrated that CypA is a negative regulator of the IL-2-inducible T-cell kinase (ITK) in T cells [54] and that CsA can augment the tyrosine phosphorylation of PLCγ1, the primary substrate of Itk. In analogous to this system, we tested the effect of CsA on ZAP70-mediated phosphorylation of LAT, the immediate physiological substrate of ZAP70 in TCR-stimulated T cells [15]. Immunoprecipitation of LAT from OKT3-treated Jurkat T cells revealed its rapid phosphorylation at 60 sec post CD3/TCR stimulation. Pretreatment of the cells with CsA led to increased tyrosine phosphorylation of LAT (Fig. 5H-K). CsA had not effect on the phosphorylation level of LAT at a longer time point post TCR stimulation (10 min), when negligible amounts of CypA associate with ZAP70. The results suggest that the *in vivo* ZAP70-mediated phosphorylation of LAT, which occurs at an early time-point post TCR stimulation, is subjected to a regulation by CypA, which by itself can be blocked by CsA.

To substantiate the latter findings showing the *in vivo* effects of CypA/CsA on ZAP70-mediated LAT phosphorylation, we transfected Jurkat T cells with the ROZA-XL plasmid which functions as a biosensor of ZAP70 activity (see Fig. 5L). FACS analysis of ROZA-XL-expressing cells revealed that their stimulation with OKT3 led to a decreased in their FRET values which reflect increased ZAP70 activity. No change in FRET was observed in OKT3-stimulated cells that express the ROZA-XL-YF plasmid which encodes a

ZAP70 activation-insensitive protein [43]. To ensure the accuracy of FRET readings, the transfection efficiency of ROZA-XL/YF was validated by FACS analysis and equal numbers of ROZA-XL/YF positive cells were used in each experimental group. Interestingly, pretreatment of the ROZA-XL-expressing cells with CsA further reduced the OKT3 stimulation-induced FRET values, suggesting that inhibition of CypA at an early time-point post TCR stimulation increases the activity of ZAP70 (Fig. 5M). The results suggest the involvement of CypA in the regulation of the early T cell activation response which downregulates ZAP70 catalytic activity, and that the effect of CypA on ZAP70-mediated T cell activation can be abrogated by CsA.

# Discussion

CypA is a member of the immunophilins that catalyzes the reversible *cis-trans* conversion of peptide bonds containing the amino acid proline. The two major groups of mammalian immunophilins, the cyclophilins and FK506-binding proteins (FKBPs), function as chaperons and assist newly synthesized proteins to undergo proper folding and acquire a conformation that is essential for their stability, localization, and biological function. The T lymphocyte immunophilins, CypA and FK506, are of particular interest because of their ability to bind the CsA and FK506 (tacrolimus) compounds, respectively, and promote immunosuppression by inhibition of T cell activation [53, 55]. Triggering of the TCR stimulates a rapid phospholipase C (PLC)-mediated breakdown of inositol phospholipids, resulting in the production of second messengers, including inositol trisphosphate ($IP_3$), which promotes the rise in intracellular free $Ca^{2+}$ concentration and the activation of calcineurin [56]. Once activated, the calcineurin associates with and dephosphorylate the nuclear factor of activated T cells (NFAT) [57], which can then translocate to the nucleus, bind to selected DNA promoter regions, and initiate the transcription of interleukin-2 (IL-2) [58] and other proinflammatory cytokines [59].

Inhibition of calcineurin by immunophilin-drug complexes [60, 61] hinders NFAT translocation to the nucleus and inhibits the formation of IL-2 and other cytokines which are crucial for the maintenance, survival, differentiation, and activation of distinct T cell subtypes [62]. While inhibition of calcineurin activity is a major mechanism by which immunophilin-drug complexes induce immunosuppression, immunophilins were found to impair T cell functions by intervening with the activity of several additional effector molecules. Functional studies demonstrated that immunophilin-drug complexes block the activation of Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs) in TCR-stimulated T cells, via a calcineurin-independent mechanism [63]. p38 is a direct substrate for activated ZAP70 in TCR-engaged T cells, and phosphorylation of p38 initiates a negative feedback loop that promotes the dissociation of ZAP70 from CD3ζ and negatively regulates TCR proximal signals [64]. Based on the above data, the effect of immunophilin-drug complexes on p38 may be indirect, reflecting the effect of the immunophilin-drug complexes on ZAP70, which is an upstream regulator of p38.

Studies by Brazin et al., [54] revealed that CypA can form a stable complex with Itk, an essential kinase for signal transduction downstream of activated TCR, which plays a key role in T cell activation, proliferation, and differentiation [65–67]. CypA binding to Itk induces a proline-dependent conformational

switch within the Itk SH2 domain leading to inhibition of Itk enzymatic activity and modulation of the Itk ligand recognition, a mechanism that can be disrupted by the presence CsA [54]. Further studies revealed that irrespective of CsA, CypA can downregulate TCR signal strength in CD4+ T cells [68]. CT10 regulator of kinase II (CrkII) adaptor protein is an additional TCR-coupled signaling protein that is regulated by CypA. *In vitro* studies of a recombinant protein consisting of the SH3N-linker-SH3C of the chicken CrkII showed that this peptide can undergo CypA-mediated *cis-trans* isomerization at the linker region Pro238 residue [69, 70]. The *cis* conformer of CrkII is autoinhibited due to the intramolecular interaction between its two SH3 domains. In contrast, the trans conformer of CrkII acquires an extended conformation in which its SH2 and SH3 domains are available for interaction with binding partners. TCR stimulation promotes a direct physical interaction between CrkII and ZAP70, which is mediated by the Crk-SH2 domain and phospho-Tyr315 in the ZAP70, an interaction that is dependent on the presence of an active Lck [27, 30]. Both CypA and FKBP were found to associate with CrkII in resting Jurkat T cells and regulate its conformation [46]. In addition, CypA increased the ability of CrkII to interact with the guanine-nucleotide releasing factor (C3G), which promotes integrin-mediated cell adhesion and migration [46]. As a result, CsA/FK506-mediated inhibition of PPIase decreased the ability of T cells to adhere to fibronectin-coated surfaces and migrate toward the stromal cell-derived factor 1α, suggesting that CsA/FK506 interferes with the PPIase-mediated, CrkII-dependent mechanisms that regulate selected effector T cell functions [71].

In the present study, we found that CypA association with ZAP70 peaks at about 1 min post TCR stimulation, when Lck-mediated phosphorylation of ZAP70 is near maximum, suggesting that CypA regulates predominantly the phosphorylated and not the non-phosphorylated ZAP70. The results also suggest that CypA-mediated regulation of ZAP70 *in vivo* occurs in TCR-stimulated, and not in resting T cells, and that CsA prevents the TCR engagement-dependent formation of CypA-ZAP70 complexes. The early event which promotes ZAP70 association with activated TCR is phosphorylation of the CD3 chain ITAMs, predominantly those of the CD3ζ. We found that CypA association with ZAP70 enables its recruitment to the vicinity of the activated TCR within the IS. In agreement, the time kinetic of CypA-ZAP70 association paralleled that of the ZAP70 recruitment to the IS of TCR-stimulated T cells. Furthermore, CsA was found to inhibit CypA association with ZAP70 and prevent the colocalization of CypA with ZAP70 at the cell membrane. The results suggest that catalytically active CypA plays a role in the conformational regulation of ZAP70, perhaps by isomerization of ZAP70, although conformational constraints imposed on ZAP70 due to its association with CypA might also affect ZAP70 activity by modulating its ability to undergo phosphorylation and/or interaction with binding partners and substrate proteins.

The finding showing that cell treatment with CsA augment the phosphorylation of LAT, an immediate substrate of ZAP70 in TCR-stimulated T cells, is a reminiscent of the observation made in studies of the regulation of another CypA-interacting T cell PTK, the Itk [54]. In both cases, inclusion of CsA at a time point in which CypA binds its target PTK (either Itk or ZAP70) augments the PTK-induced phosphorylation of its respective primary substrates (PLCγ1 and LAT, respectively). These observations highlight the notion that CypA functions as a negative regulator of PTKs in the early phases of T cell activation.

Studies in Jurkat T cells that express the ZAP70 activity-biosensor, ROZA-XL, revealed that CsA increases ZAP70 activity at 1 min post TCR-stimulation of T cells, indirectly implying that CypA is a negative regulator of the ZAP70 catalytic activity and that CsA can reverse the effect of CypA on ZAP70. This *in vivo* assay is based on the ability of active ZAP70 to phosphorylate a Tyr-containing LAT epitope in the biosensor protein, an epitope that its phosphorylation directly correlates with ZAP70 activity. Recent studies demonstrated that Lck-mediated phosphorylation of ZAP70 is a crucial step for ZAP70 bridging to LAT [72] and the present studies further demonstrates that ZAP70 phosphorylation by Lck is required for the ZAP70 interaction with CypA.

Notably, inclusion of CsA in the assay system at a late time point of cell activation (10 min), when CypA association with ZAP70 is negligible, CsA had no effect on ZAP70-mediated substrate phosphorylation. Complexes of CsA-CypA are known to interact with and inhibit the cytoplasmic phosphatase, calcineurin, and thereby its primary target, the NFAT transcription factors [61, 73]. More recent studies reported the recruitment of calcineurin to the TCR signaling complex, where it reverses inhibitory phosphorylation on Lck and indirectly promote the activation of ZAP70 [74]. It is possible therefore that some of the *in vivo* effects of CsA on ZAP70 that were observed in the present studies reflect the CsA-CypA-mediated inhibition of calcineurin, which prevent the activation of Lck, and its downstream protein, ZAP70. *In vitro* kinase assay of ZAP70 performed in the absence or presence of enzymatically active human recombinant CypA demonstrated that CypA induces a time- and concentration-dependent reduction in the extent of ZAP70 autophosphorylation as well as substrate phosphorylation (cfb3). Inclusion of CsA in the preincubation step reversed the effect of CypA on ZAP70. These results suggest that CypA, which associates with ZAP70 in TCR-engaged T cells, can impose its effect on ZAP70 activity via a direct mechanism. We hypothesize that CypA modulates ZAP70 activity by isomerization of ZAP70 and/or physical interaction with a ZAP70 motif that alters the catalytic activity of ZAP70 and/or accessibility to substrates or ATP. The observation that Pin1, in contrast to CypA, does not affect ZAP70 catalytic activity suggests a selectivity in PPIases towards ZAP70.

Our data support the hypothesis that maximal phosphorylation of ZAP70 at ~60 sec post TCR stimulation involves the concomitant association of ZAP70 with CypA which can modulate the catalytic activity of ZAP70 (Fig. 6). Under the assays conditions we have used, CypA was found to inhibit ZAP70 activity. However, CypA can interconvert both *cis* and *trans* isomers and the preferred direction of ZAP70 isomerization under physiological conditions might be determined by the phosphorylation status of ZAP70 or its association with selected binding proteins. The results suggest that CypA functions as a physiological regulator of ZAP70 that might contribute to the amplitude, duration, and fine-tuning of the T cell activation response.

## Materials And Methods

# Reagents and antibodies

Sandimmune (CsA; 50 mg/ml) was from Novartis Pharma AG (Basel, Switzerland). Aprotinin, leupeptin, Triton X-100, and TPA were from Sigma-Aldrich (St. Louis, MO, USA). Protein A-Sepharose beads, electrochemiluminescence (ECL), and nitrocellulose membranes were from Amersham Pharmacia Biotech (Uppsala, Sweden). RPMI-1640, Pen/Strep, and FBS were from Biological Industries (Beit Haemek, Israel). *A*EBSF was from ICN Biomedicals (Aurora, OH). A mouse anti-ZAP70 (clone 1E7.2 ) was from BioLegend (San Diego, CA) and a mouse anti-phosphotyrosine (p-Tyr) mAb (4G10) was from Upstate Biotechnology (Lake Placid, NY). Mouse anti-GST, mouse anti-cyclophilin A (CypA), mouse anti-phospho-CD3ζ (pCD3ζ; clone C145.9A), rabbit anti-Lck, rabbit anti-CypA, and goat anti-CD3ε (clone M-20 ) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-β actin (clone AC-15) and mouse anti-MBP (clone M-1891) mAbs were from Sigma-Aldrich. Mouse anti-human CD3ε (OKT3) and c-Myc mAbs (clone 9E10) [40] were prepared as previously described. Rabbit anti-CypA, rabbit anti-LAT, rabbit anti-Pin1, goat polyclonal anti-mouse IgG and anti-hamster IgG Abs were from Abcam (Cambridge, UK). Mouse anti-PKC alpha (PKCα) mAbs were from BD Transduction Laboratories (Lexington, KY). FITC-conjugated goat Abs directed against mouse IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit immunoglobulin Abs were from Amersham Pharmacia Biotech. Alexa Fluor™ 488-, 546- and 633-conjugated rabbit and goat anti-mouse Abs, and Alexa Fluor™ 633-conjugated phalloidin were from Thermo Scientific Inc. (Waltham, MA). Human recombinant Cyclophilin A (rCypA), human recombinant Pin1 (rPin1) and CytoPainter Cell Staining Reagent were from Abcam (Cambridge, UK). [γ-$^{32}$P] ATP was from Perkin Elmer (Waltham, MA).

# Animals, cell lines, and culture conditions

Spleen and thymus cells were obtained from 8-wk-old C57BL/6J female mice that were housed under controlled conditions with 12 h light/dark cycle and free access to food and water. This study was approved in advance by the Ben-Gurion University Institutional Animal Care and Use Committee and conducted in accordance with the Israeli Animal Welfare Act following the guidelines of the Guide for Care and Use of Laboratory Animals (National Research Council 1996). The Animal ethical clearance protocol used for this research is IL-44-08-2017.

Cell lines used include the Jurkat human T cell line (clone E6-1; ATCC® TIB-152$^{TM}$), Jurkat TAg T cells which express the Simian Vacuolating Virus 40 (SV40) T antigen, ZAP70-deficient Jurkat T cell line, P116, Lck-deficient Jurkat T cell line, JCaM1.6, TCR-deficient Jurkat T cell line, CH7C17, which was transfected with the influenza hemagglutinin (HA$^{307-319}$) peptide-specific, DR1-restricted HA1.7 TCR, and the EBV-transformed human B cell line, LG2, which expresses the HLA-DR1 and B7.1 surface receptors. Cells were maintained at a logarithmic growth phase in complete RPMI (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (all from Biological Industries, Beit Haemek, Israel), and 0.5 μM β-mercaptoethanol (Sigma-Aldrich)). Cells were grown in 75-cm$^2$ growth-area tissue culture flasks (Cellstar, Greiner, Germany) in a humidified atmosphere of 95% air and 5% $CO_2$, at 37°C. Jurkat and Jurkat-derived subline cells were stimulated with anti-CD3ε mAbs (OKT3; 1:1000 dilution of ascites, 30-min incubation on ice) plus crosslinking with a

secondary Ab (goat anti-mouse IgG, 1:200) for the indicated time intervals at 37°C. Mouse T cells were stimulated with anti-CD3ε mAbs (2C11 mAbs by 30-min incubation on ice plus crosslinking with a secondary Ab (goat anti-hamster IgG) for the indicated time intervals at 37°C.

# DNA constructs

A Myc-tagged full-length ZAP70 in pSXSR expression vector was prepared and its sequence was verified, as described [30, 41]. GST-tagged human cyclophilin A (hCypA) in pEF expression vector was a gift of Michael Emerman (Fred Hutchinson Cancer Research Center, Seattle, WA) [42]. The fluorescence resonance energy transfer (FRET)-based biosensor plasmids which are designed to detect ZAP70 activity (ROZA)-XL and ROZA-XL-YF (ROZA-XL in which LAT Tyr191 was replaced by Phe) were gifts of Annemarie Lellouch (INSERM U1067, Marseille, France) [43] (Addgene plasmid # 64194; http://n2t.net/addgene:64194; RRID: Addgene_64194).

# Nucleofection of Jurkat T cells

For FRET analysis, Jurkat T cells ($15x10^6$/group) were nucleofected with ROZA-XL or ROZA-XL-YF plasmids using Bio-Rad Gene Pulser Xcell electroporation system. Briefly, cells were suspended in pre-warmed (37°C) Mirus Ingenio® electroporation solution, mixed with indicated plasmids (10μg) in sterile Bio-Rad cuvettes (0.4 cm gap). The cuvettes were then placed in the electroporator and pulsed using the exponential decay pulsing protocol. After electroporation, cells were carefully transferred to a culture medium-containing tissue culture dish and were incubated at 37°C for 48 hr prior to CsA treatment followed by cell activation and FACS analysis. For protein binding studies, Jurkat T cells ($20x10^6$/group) were nucleofected with the indicated plasmids (10μg/cuvette) using the above-mentioned electroporation protocol. At 48 hr post-nucleofection, cells were either stimulated or left untreated and subsequently subjected to immunoprecipitation. Unless otherwise indicated, all nucleofection experiments were carried out in triplicates using 3 separate dishes for each point.

# Preparation of cell lysates and Immunoprecipitation

Cell lysates were prepared by resuspension of cells in lysis buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM $Na_3VO_4$, 50 mM NaF, 10 mg/ml each of leupeptin and aprotinin, 2 mM AEBSF, and 1% Triton X-100), followed by a 30-min incubation on ice. Lysates were spun down at 13,000xg for 30 min at 4°C and the nuclear-free supernatants were subjected to immunoprecipitation.

Immunoprecipitation was performed by pre-adsorption of primary Abs to protein A/G-agarose beads for 2 hr at 4°C. Excess Abs were removed by 3 washes in lysis buffer and Ab-coated beads were incubated with cell lysates for 16 hr at 4°C. Immune complexes were precipitated by centrifugation followed by extensive washing in lysis buffer. Equal volumes of 2xSDS sample buffer were added to immunoprecipitates or whole-cell lysates (WCL), which were vortexed, boiled for 5 min, and fractionated by SDS-PAGE.

# Electrophoresis and Immunoblotting

Whole-cell lysates and Ab immunoprecipitates were resolved by electrophoresis either on 8%, 10% or 12.5% polyacrylamide gels using Bio-Rad Mini-PROTEAN II cells. Proteins from the gel were electroblotted onto nitrocellulose membranes (Schleicher and Schuell) at 100V for 1 hr, using BioRad Mini Trans-Blot transfer cells. After 1 hr of blocking with 3% BSA in TBST at 37°C, the nitrocellulose membranes were incubated in the presence of the indicated primary Abs, followed by incubation with HRP-conjugated secondary Abs. Immunoreactive protein bands were visualized using an ECL reagent and autoradiography. Whenever required, nitrocellulose membranes were stripped by incubation in stripping buffer (100 mM 2-ME, 2% SDS, and 62.5 mM Tris/HCl, pH 6.8) for 30 min at 50°C, followed by 1-h incubation with blocking buffer (3% BSA in TBST).

# Cell treatment with Cyclosporin A (CsA)

CsA (Sandimmun, 50 mg/ml in oil solution) was diluted in RPMI 1640 culture medium before each experiment. For immunoprecipitation assays, Jurkat T cells were cultured in 75-$cm^2$ growth-area tissue culture flasks at a concentration of $50x10^6$ cells/group in the presence or absence of CsA (5 µg/ml ), or as otherwise indicated in the figure legend, and tested on the following day. For immunofluorescent cell staining, Jurkat T cells ($2x10^6$/group) were cultured in the presence or absence of 5 µg/ml CsA for 24 hr. For FRET analysis, ROZA-XL/ROZA-XL-YF-transfected Jurkat T cells ($15x10^6$/group) were cultured for 48 hr and then treated in the presence or absence of CsA (5 µg/ml) for an additional 24 hr.

# Fluorescent cell staining and confocal microscopy

Jurkat T cells ($2x10^6$/group) were left unstimulated or stimulated by anti-CD3ε mAbs (OKT3; 30-min incubation on ice) plus crosslinking with a secondary Ab (goat anti-mouse IgG, 1:200) for 1 min at 37°C. Cells were plated on poly-L-lysine-coated chamber slides and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT). Cells were washed twice, permeabilized with 0.1% triton-X-100 for 10 min, and blocked with Ab Diluent blocking solution (GBI Labs) for 1 hr at RT. Cells were reacted with mouse anti-ZAP70 mAbs and rabbit anti-CypA mAbs diluted in blocking buffer overnight at 4°C. After washing with PBS, the cells were incubated with Alexa Fluor 546-conjugated anti-mouse Ig and Alexa Fluor 488-conjugated anti-rabbit Ig secondary Abs for 1 hr in the dark at RT. Cells were then counterstained with the nuclear stain DAPI diluted in Tris-HCl (pH 7.5) at RT for 5 min. Cells were also stained for tyrosine-phosphorylated proteins using mouse anti-pTyr mAbs (4G10) and a secondary FITC-conjugated anti-mouse Ig mAbs plus counterstain with DAPI. Similarly, in Jurkat T cells and in ZAP70-deficient P116 cells, after the fixation and permeabilization, cells were immunoreacted with mouse anti-pCD3ζ and rabbit anti-CypA followed by the incubation with Alexa Fluor 546-conjugated anti-mouse Ig and Alexa Fluor 488-conjugated anti-rabbit Ig secondary Abs and counterstaining with DAPI. For the triple staining procedure in Jurkat T cells which involved CD3 receptor, the cells were stained using the primary Abs: mouse anti-ZAP70, rabbit anti-CypA and goat anti-CD3ε, followed by incubation with the respective secondary antibodies: Alexa Fluor 633-conjugated anti-mouse Ig, Alexa Fluor 488-conjugated anti-rabbit Ig, and Alexa Fluor 546-conjugated anti-goat Ig, plus counterstaining with DAPI. The coverslips were mounted on slides using DAKO mounting medium and imaged by Olympus FluoView FV1000 laser-

scanning confocal microscope. The extent of colocalization of CypA and ZAP70 was quantified using the JACoP ImageJ plugin [44].

# Conjugate formation assay and immunofluorescence

Immunological synapse studies were performed as previously described [45]. Briefly, Jurkat- CH7C17 T cells, which express the influenza hemagglutinin ($HA^{307-319}$) specific TCR, were incubated with peptide-loaded antigen-presenting cells (APCs), LG2, at a ratio of 1:2 at 37°C for 5 min. APCs were pre-loaded with 200 µg/ml of $HA^{307-319}$ peptide, or an inactive HA peptide, in which Lys316 was replaced by Glu (K316E), for 3 hours at 37°C. To enable the discrimination between the two cell types, LG2 cells were prestained using CytoPainter Cell Staining Reagent prior to the cell mixing. Following 5 min of co-incubation, the cells were fixed, permeabilized, and analyzed by confocal microscopy. Immunofluorescence staining was performed using the primary Abs: mouse anti-ZAP70 and rabbit anti-CypA, followed by incubation with Alexa Fluor 633-conjugated phalloidin and: Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 546-conjugated anti-rabbit IgG. CytoPainter was detected at 405nm (Olympus FluoView FV1000 laser-scanning confocal microscope).

# Fluorescence resonance energy transfer analysis by FACS

Fluorescence resonance energy transfer (FRET) analysis of live cells was performed by flow cytometry as previously described [46]. Briefly, Jurkat T cells ($15x10^6$/group) transfected with ROZA-XL or ROZA-XL-YF plasmids were cultured for 48 hr and then split into two identical groups that were cultured for an additional 24 hr in the presence or absence of CsA (5 µg/ml). After washing with PBS (without $Ca^{2+}$ and $Mg^{2+}$), cells ($2x10^6$/group) were placed in Eppendorf tubes and resuspended in phenol red-free RPMI 1640 plus 1 mM HEPES buffer. Following the addition of anti-CD3ε mAbs (OKT3) and crosslinking with a goat anti-mouse IgG secondary Ab for the specified time intervals at 37°C, the cell activation was immediately terminated by the addition of cold PBS (without $Ca^{2+}$ and $Mg^{2+}$), and cell pellets were resuspended in FACS buffer (2% FBS, 1 mM EDTA, and PBS (without $Ca^{2+}$ and $Mg^{2+}$)) followed by live data acquisition on a FACS Canto II device (BD Biosciences). Excitation of CFP was at 405 nm and emission was detected simultaneously in the CFP and YFP emission windows. A shift in the ratio of YFP/CFP emission intensities reflects the change in FRET efficiency. Due to the unique structure of ROZA-XL, higher values of FRET efficiency were observed in resting cells, where ZAP70 was inactive, while ZAP70 activation resulted in reduced FRET efficiency values (see ref. [43]. The data were analyzed using FlowJo10.7 software.

# In vitro kinase assays

ZAP70 kinase assay: Jurkat T cells were activated using OKT3 mAbs for 1 min at 37°C. ZAP70 was immunoprecipitated from whole-cell lysates using protein A/G-agarose bead-immobilized mouse anti-ZAP70 Abs (Biolegend), and the immunoprecipitates were washed four times with Triton X-100-containing lysis buffer and once with a ZAP70 kinase buffer (1 M Tris, 3M NaCl, 100 mM $MnCl_2$). Samples were pre-incubated in the absence or presence of the indicated amounts of catalytically active

recombinant human CypA in kinase buffer for indicated time intervals at 37°C. The samples were resuspended in 100 µl kinase reaction mixture containing kinase buffer plus [γ-$^{32}$P]-ATP (5 µCi) and incubated for 10 min at 37°C with gentle shaking. Reactions were terminated by the addition of 5x sample buffer and boiling for 5 min, followed by SDS-PAGE on 10% acrylamide gels under reducing conditions. Samples were then transferred to nitrocellulose membranes that were developed by autoradiography.

PKCα kinase assay: Jurkat T cells were activated using PMA for 30 min at 37°C. PKCα was immunoprecipitated from whole-cell lysates using protein A-agarose bead-immobilized mouse anti-PKCα Abs (BD Transduction Laboratories), and the immunoprecipitates were washed four times with Triton X-100-containing lysis buffer and once with a PKC kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 0.1 mM EGTA). Samples were pre-incubated in the absence or presence of the indicated amounts of catalytically active recombinant human Pin1 in kinase buffer for indicated time intervals at 37°C. The samples were then resuspended in 100 µl kinase reaction mixture containing kinase buffer and 50 µg/µl phosphatidylserine, 0.3 mM $CaCl_2$, 100 nM PMA, [γ-$^{32}$P]-ATP (5 µCi) and MBP (5µg) as a substrate and incubated for 30 min at 32°C with gentle shaking. Reactions were terminated by the addition of 5x sample buffer and boiling for 5 min, followed by SDS-PAGE on 10% acrylamide gels under reducing conditions. Samples were then transferred to nitrocellulose membranes that were developed by immunoblot and autoradiography.

# Statistical Analysis

Statistical analyses were carried out using either MS office Excel 365 software Version 2107 or GraphPad Prism software Version 7.00. Microscopy data was quantified using ImageJ plugin, JACoP and FRET analysis with FlowJo Version 10.7. Statistical significance of differences between groups of averaged data points were assessed using Student's unpaired *t*-test.

# Abbreviations

C3G, CrkII binding of guanine-nucleotide releasing factor

c-Cbl, Casitas B-lineage lymphoma

cfb3- cytoplasmic fragment of human erythrocyte band 3

CrkII, CT10 regulator of kinase II

CypA, cyclophilin A

CsA, cyclosporin A

*FRET, fluorescence* resonance energy transfer

*HRP,* horseradish peroxidase

*$IP_3$, inositol trisphosphate*

*IS, immunological synapse*

*ITAM, immunoreceptor tyrosine-based activation motif*s

Itk, interleukin-2-inducible T cell kinase

*JNK,* Jun N-terminal kinase

LAT, linker of activated T cells

*Lck, lymphocyte*-specific protein tyrosine *kinase*

MAPK, mitogen-*activated* protein *kinase*

NFAT, nuclear factor of activated T cells

Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1

PKCα, protein kinase C α

PLC, phospholipase C

PPIase, peptidyl-prolyl *cis-trans* isomerases

SCID, severe combined immunodeficiency

Sts1, suppressor of TCR signaling 1

TCR, T cell antigen receptor

WCL, whole cell lysates

ZAP70, ζ-chain-associated protein of 70kDa

# Declarations


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### Competing interest

The authors have no relevant financial or non-financial interests to disclose.

### Author contributions

N.I. conceived the study, designed experiments, and supervised the project; N.P.A., A.W.A., A.M., J.S., and P.R.N. carried out the experiments; Z.S., A.B., and N.I. analyzed the data; E.L. provided essential tools for the study; N.P.A., A.W.A., and N.I. wrote the manuscript. All authors commented on the previous versions of the manuscript. All authors have read and approved the final manuscript and have given consent for publishing the work.

### Data Availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

### Ethics approval

This study was approved in advance by the Ben-Gurion University Institutional Animal Care and Use Committee and conducted in accordance with the Israeli Animal Welfare Act following the guidelines of the Guide for Care and Use of Laboratory Animals (National Research Council 1996). The Animal ethical clearance protocol used for this research is IL-44-08-2017.

### Consent to participate

Not Applicable as human subjects were not involved in the study.

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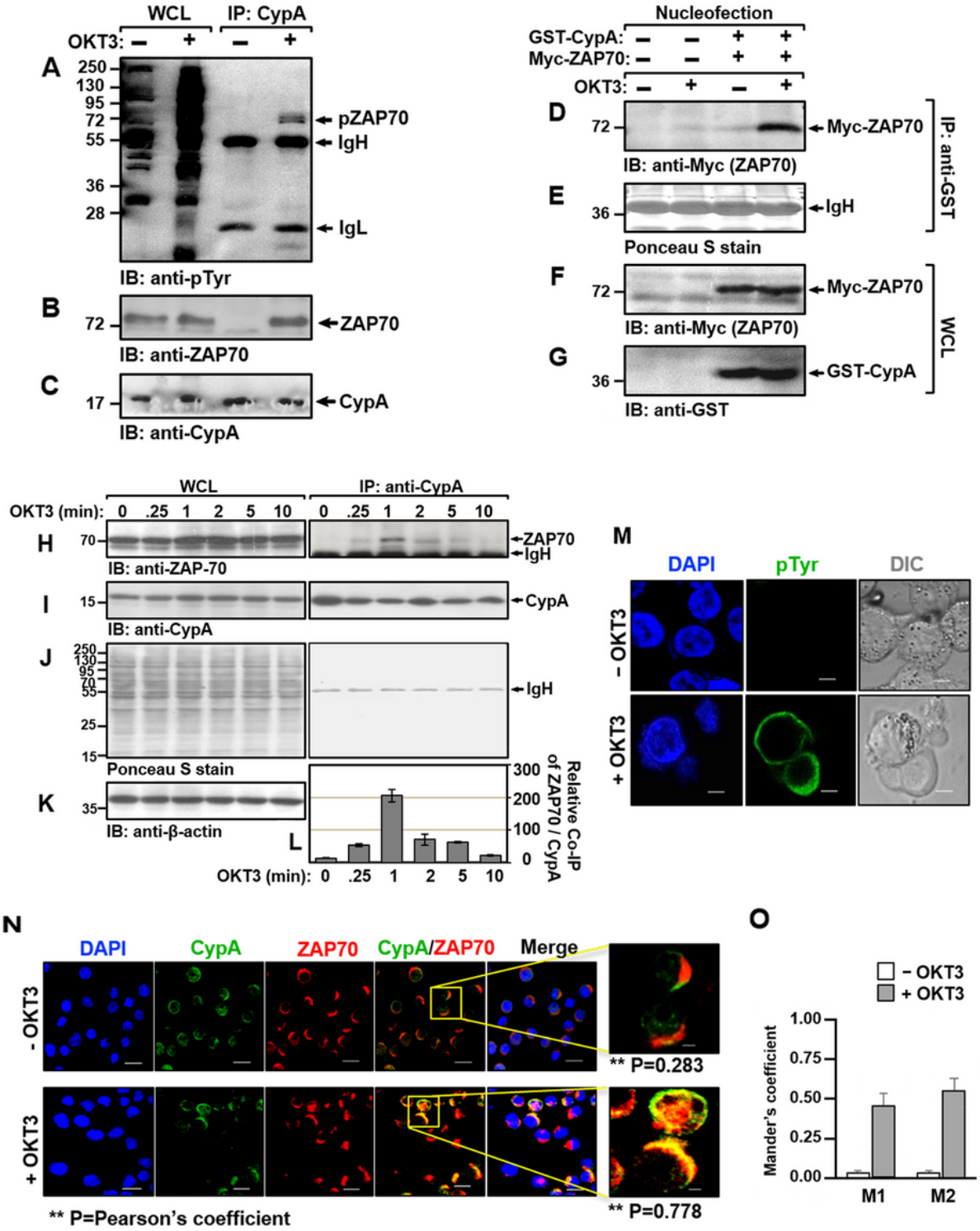
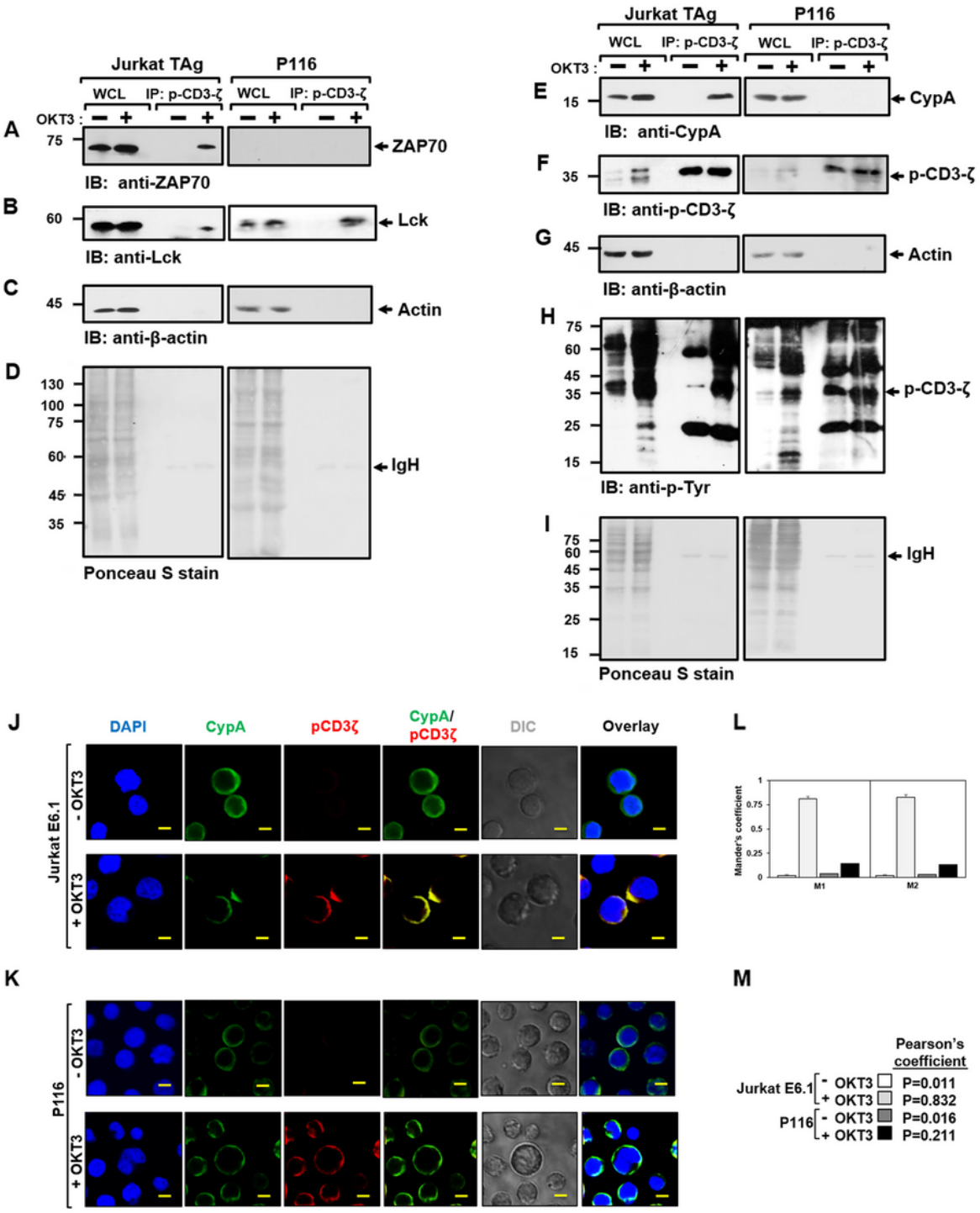
# Figures




## Figure 1

**Crosslinking of the TCR/CD3 on Jurkat T cells promotes the association of cyclophilin A with ZAP70.** A-C. Jurkat T cells were stimulated by TCR/CD3 crosslinking with OKT3 mAbs for 1 min, followed by cell lysis and immunoprecipitation, as described in Materials and Methods. Whole-cell lysates (WCL) and CypA immunoprecipitates (IP) were then subjected to SDS-PAGE under reducing conditions and immunoblotting (IB) with anti-pTyr mAbs (A). Presence of ZAP70 (B) and CypA (C) on the same

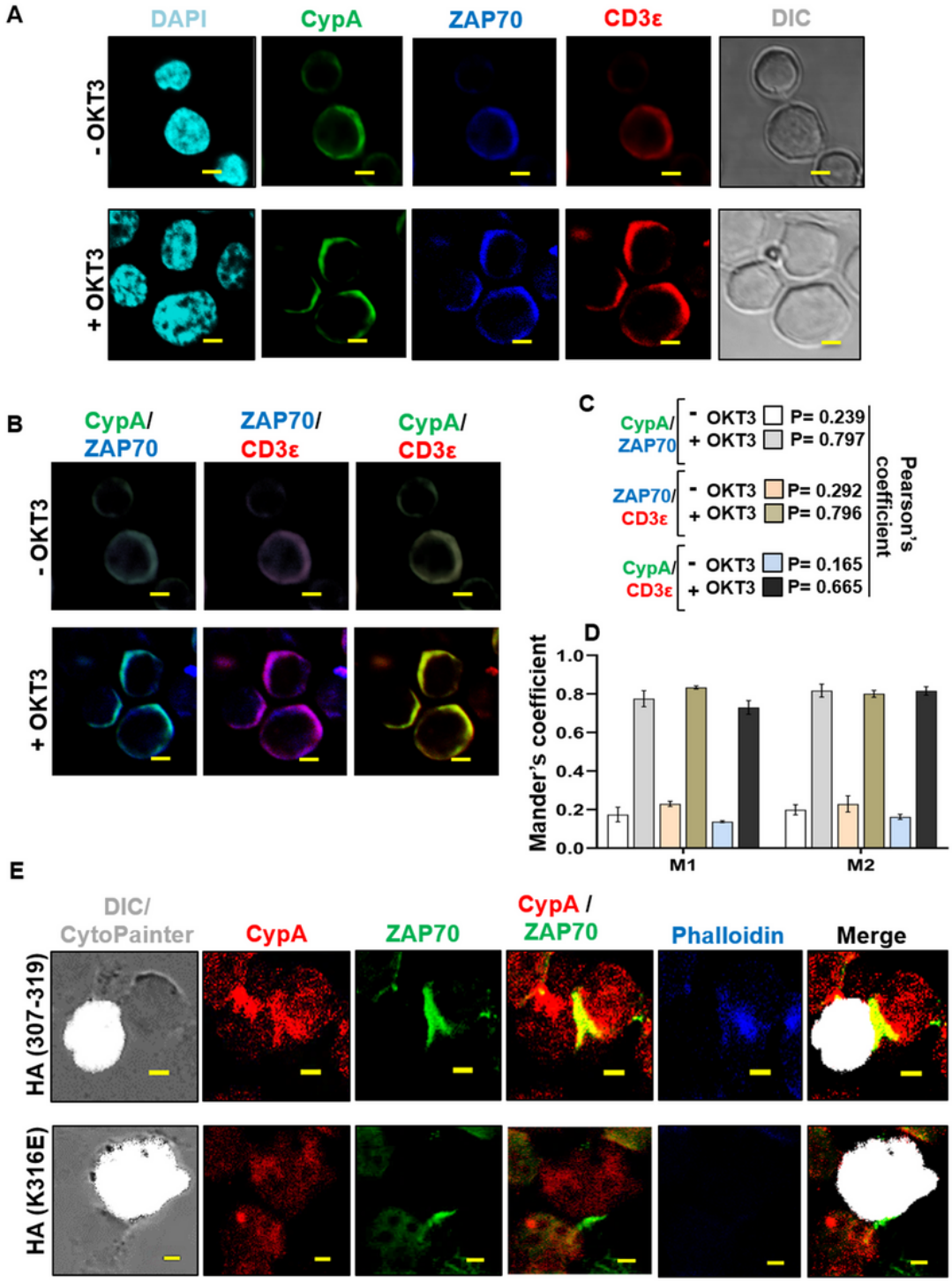
membrane was determined by membrane stripping and reblotting with the indicated Abs. D-G. Jurkat T cells co-transfected with GST-CypA- and Myc-ZAP70-encoding eukaryotic expression vectors were stimulated with OKT3 mAbs for 1 min and cell lysates were subjected to immunoprecipitation using anti-GST mAbs. Immunoprecipitates (D, E) and WCL (F, G) were subjected to SDS-PAGE and sequential immunoblotting with anti-Myc mAbs (D, F) and anti-GST mAbs (G) and membrane staining with Ponceau S (E). H-L. Jurkat T cells were stimulated with OKT3 mAbs for the indicated time intervals. Cell lysates were then subjected to CypA immunoprecipitation and samples of whole-cell lysates (WCL) and immunoprecipitates (IP) were subjected to SDS-PAGE on 10% acrylamide gels under reducing conditions followed by sequential immunoblotting (IB) using anti-ZAP70 (H), anti-CypA (I), and anti-β-actin (K) mAbs. Membrane staining with Ponceau S (J) determined the equal loading of proteins in all lanes, and densitometry analysis (L) determined the relative amount of ZAP70 that coimmunoprecipitated with CypA. Molecular weight markers (in kDa) are indicated on the left and arrows mark the position of the indicated protein bands. IgH, Ig heavy chain, IgL, Ig light chain. H-J. (M) Confocal imaging of OKT3-stimulated and resting cells stained with anti-pTyr mAbs followed by FITC-conjugated anti-mouse Ig Abs and counterstained with DAPI. Jurkat T cells were stimulated with OKT3 mAbs as before, fixed, permeabilized and incubated with rabbit anti-CypA and mouse anti-ZAP70 mAbs. After washing, the cells were immunostained with Alexa Fluor 488-conjugated anti-rabbit- and Alexa Fluor 546-conjugated anti-mouse-IgG and counterstained with DAPI. The cells were analyzed using a confocal laser microscope (N). Scale bar equals 10 μm; inset, 2 μm. CypA-ZAP70 colocalization (inset) was quantified using the ImageJ plugin, and Pearson's coefficient values (**P) for resting and stimulated cells were indicated below each panel. O. A graph representing Mander's coefficient values, M1 (red overlap with green) and M2 (green overlap with red). Scale bar equals 2 μm. Results are representative of three independent experiments.




## Figure 2

**The ZAP70-associated CypA interacts with phospho-CD3ζ and recruits to the TCR/CD3 complex in activated Jurkat T cells.** A-I. Jurkat T cells and ZAP70-deficient P116 T cells were stimulated with OKT3 mAbs for 1 min followed by cell lysis and immunoprecipitation using anti-pCD3ζ mAbs. Whole-cell lysates (WCL) and pCD3ζ immunoprecipitates (IP) were then divided into two groups and subsequently subjected to SDS-PAGE under reducing conditions on an 8% and 12% polyacrylamide gels. The 8% gel-

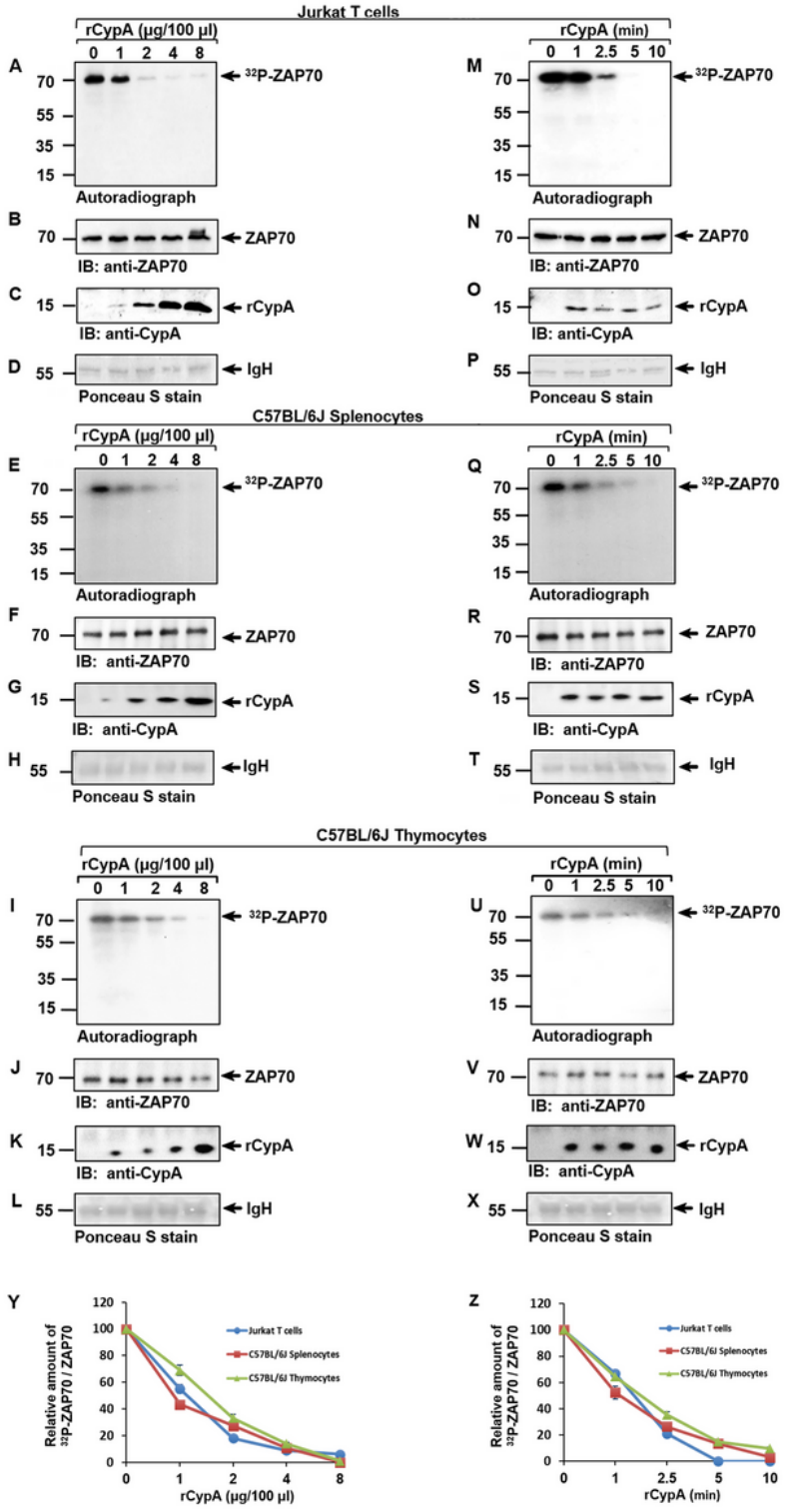
transferred nitrocellulose paper was sequential immunoblotting (IB) with anti-ZAP70 (A), anti-Lck (B), anti-β-actin (C) mAbs, and the 12% gel-transferred nitrocellulose paper was sequential immunoblotting (IB) with anti-CypA (E), anti-pCD3ζ (F), anti-β-actin (G), and anti-pTyr mAbs. (D and I) Ponceau S stain of the membranes. Molecular weight markers (in kDa) are indicated on the left and arrows mark the position of the indicated protein bands. IgH, Ig heavy chain. J-M. Jurkat T cells and ZAP70-deficient Jurkat cells, P116, were stimulated with OKT3 mAbs for 1 min. After fixation and permeabilization, the cells were incubated with rabbit anti-CypA and mouse anti-pCD3ζ mAbs, followed by immunostaining with Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 546-conjugated anti-mouse Ig Abs, and counterstained with DAPI. The staining of CypA/pCD3ζ in Jurkat T cells (J) and P116 cells (K) were analyzed using a confocal laser microscope. A comparative colocalization of CypA and pCD3ζ in Jurkat *vs*. P116 cells was quantified using the ImageJ plugin, *JACoP.* Mander's coefficient values for M1 (red overlap with green) and M2 (green overlap with red) (L) and Pearson's coefficient values (M) are indicated. Scale bar equals 2 μm. Results are representative of three independent experiments.




## Figure 3

**ZAP70-bound CypA recruits to the TCR and stimulation by peptide-loaded MHC antigens on the surface of APC induces CypA-ZAP70 colocalization at the IS.** A-D. Jurkat T cells were stimulated with OKT3 mAbs for 1 min at 37˚C or left untreated. Fixed and permeabilized cells were then incubated with rabbit anti-CypA, mouse anti-ZAP70, and goat anti-CD3ε Abs, followed by immunostaining with Alexa Fluor 488-conjugated anti-rabbit-, Alexa Fluor 633-conjugated anti-mouse-, and Alexa Fluor 546-conjugated anti-

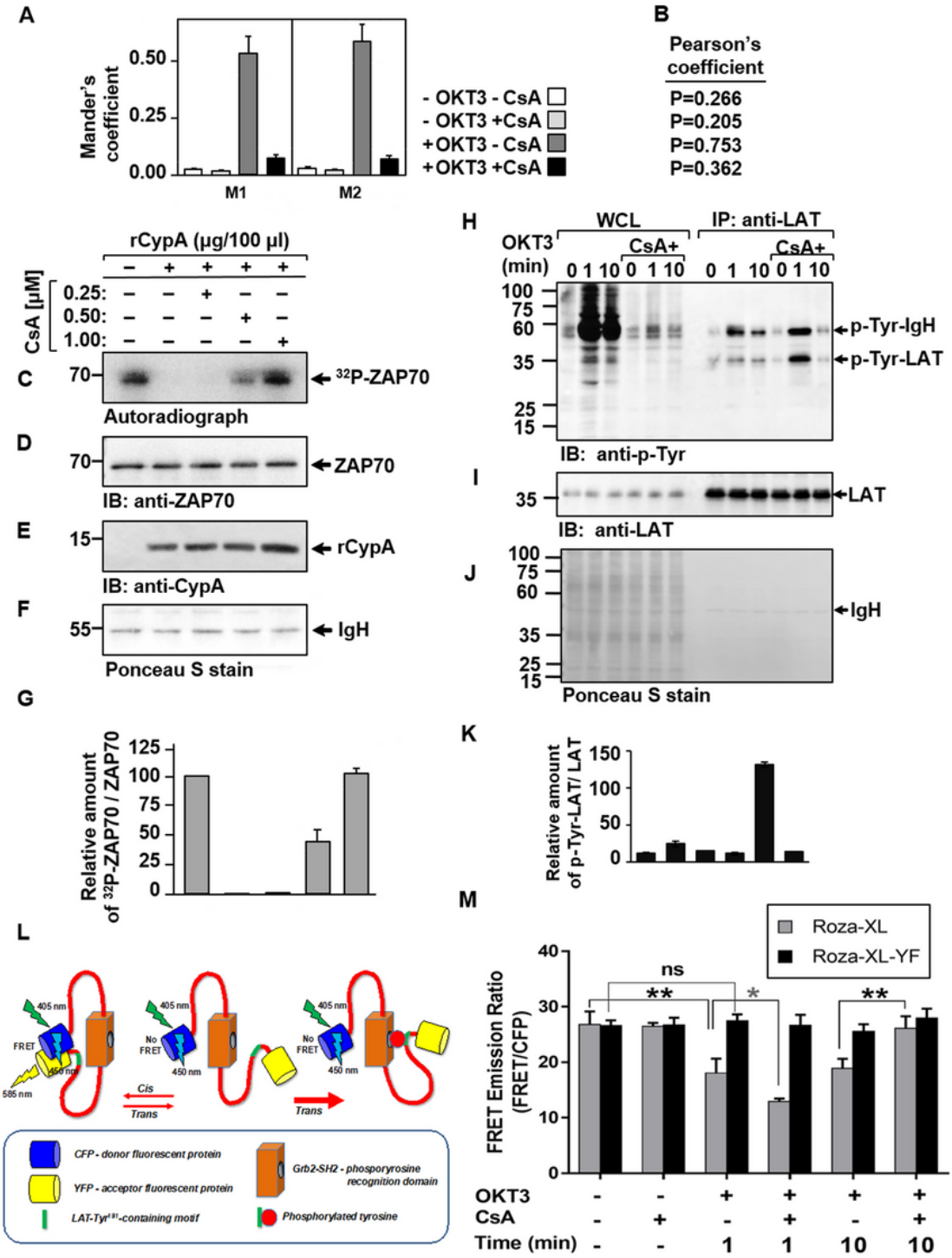
goat-Ig Abs and counterstained with DAPI. The cells were analyzed using a confocal laser microscope (A, B). The extent of colocalization of CypA and either ZAP70 or CD3ε was determined using the ImageJ plugin, *JACoP.* Pearson's coefficient values (C) and Mander's coefficient values M1 and M2 for the respective protein colocalization (D) are indicated. Scale bar equals 2 μm. Results are representative of three independent experiments. E. Conjugate formation between Jurkat T cells, clone CH7C17, and APCs (B cell line, LG2) was performed as described in Materials and Methods. Following antigen (HA peptide 307-319, or an inactive HA peptide, HA K316E) pre-pulsing and coincubation for 5 min, T cells and APCs were fixed, permeabilized, and analyzed by confocal microscopy. Immunofluorescence staining was performed using primary Abs; mouse anti-ZAP70, and rabbit anti-CypA, followed by Alexa Fluor 633-conjugated phalloidin and secondary Abs, Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 546-conjugated anti-rabbit IgG. CytoPainter stain was detected at 405nm wavelength. Scale bar equals 2 μm. Results are representative of three independent experiments.




## Figure 4

**CypA downregulates the catalytic activity of ZAP70.** C57BL/6J mice-derived spleen and thymus cells were stimulated with 2C11 mAbs and Jurkat T cells were stimulated with OKT3 mAbs for 1 min. Subsequently the cells were lysed, and lysates were subjected to ZAP70 immunoprecipitation. The ZAP70-containing beads were incubated in the presence of the indicated concentrations of enzymatically active recombinant human CypA (rCypA) for 10 min at 37°C (A-L), or with 4 μg/ml of rCypA for indicated

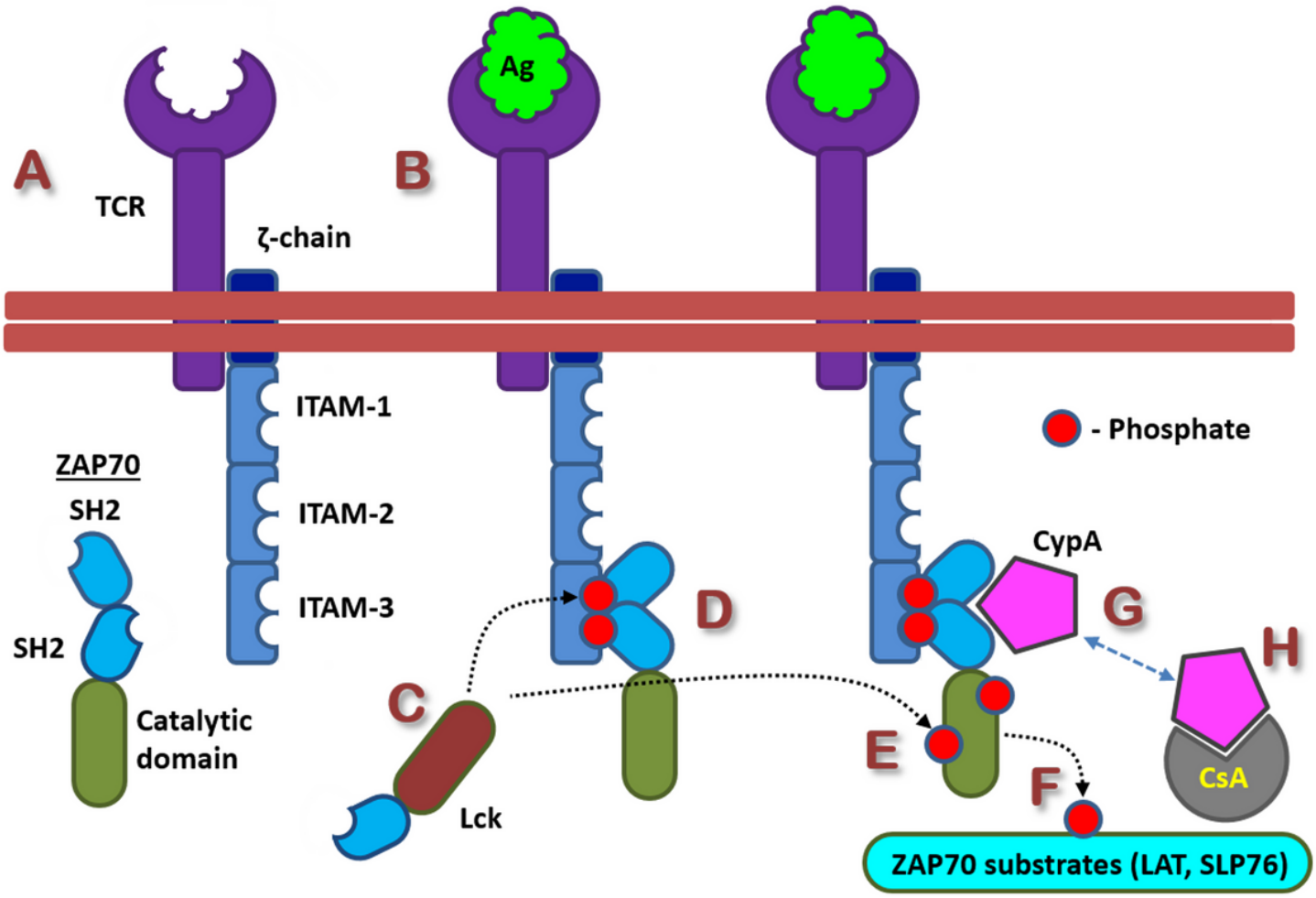
time intervals (M-X). Samples were then subjected to a ZAP70 kinase assay in the presence of [γ-$^{32}$P]-ATP, as described in Materials and Methods. Sample proteins were then resolved by SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and developed by autoradiography (A, E, I, M, Q, U) and immunoblotting (IB) using anti-ZAP70 (B, F, J, N, R, V) and anti-CypA (C, G, K, O, S, W) mAbs. Ponceau S staining of the nitrocellulose membrane (D, H, L, P, T, X). pZAP70 and ZAP70 protein band signals were quantified using the ImageJ software, and the relative amounts of pZAP70/ZAP70 in Jurkat T cells, splenic T cells, and thymic T cells were presented in a line graph (Y, Z). Molecular weight markers (in kDa) are indicated on the left and arrows mark the positions of the indicated protein bands. Results are representative of three independent experiments. IgH, Ig heavy chain.




## Figure 5

**Cyclosporin A reverses the effect of CypA on ZAP70 activity**. A-B. Quantification of CypA-ZAP70 colocalization (from Figure. S 8, inset) was performed using the ImageJ plugin, *JACoP*. Mander's coefficient values for M1 (red overlap with green) and M2 (green overlap with red) (A) and Pearson's coefficient values (B) are indicated. C-G. Jurkat T cells were stimulated with OKT3 mAbs for 1 min. Cell lysates were then subjected to ZAP70 immunoprecipitation (IP) followed by incubation of ZAP70-

containing beads in the presence or absence of enzymatically active recombinant human CypA (rCypA; 4 µg/100 µl) plus the indicated concentrations of CsA, for 10 min at 37°C. Samples were then subjected to a radioactive ZAP70 kinase assay in the presence of [γ-$^{32}$P]-ATP followed by SDS-PAGE under reducing conditions and protein electroblotting onto nitrocellulose membranes. The membranes were developed by autoradiography (C) and immunoblotting (IB) using anti-ZAP70 (D) and anti-CypA (E) mAbs. Ponceau S staining of the nitrocellulose membrane monitored the equal usage of ZAP70 mAbs (F). $^{32}$P-ZAP70 (pZAP70) and ZAP70 protein band signals were quantified using the ImageJ software and the relative amounts of pZAP70/ZAP70 were presented in a bar graph (G). H-K. Jurkat T cells were left untreated or treated with CsA (5 µg/ml) for 24 h, and subsequently stimulated with OKT3 mAbs for 1 min or left unstimulated. Cell lysates were then subjected to LAT immunoprecipitation and samples of whole-cell lysates (WCL) and immunoprecipitates (IP) were subjected to SDS-PAGE on 10% acrylamide gels under reducing conditions followed by sequential immunoblotting using anti-p-Tyr (H), and anti-LAT (I) mAbs. Membrane staining with Ponceau S (J) determined the equal loading of proteins in all lanes. pTyr-LAT and LAT protein band signals were quantified using the ImageJ software and the relative amounts of pTyr-LAT / LAT were presented in a bar graph (K). Molecular weight markers (in kDa) are indicated on the left and arrows mark the position of the indicated protein bands. IgH, Ig heavy chain. L. Schematic representation of the ROZA-XL ZAP70 biosensor and its mode of operation. The core region of ROZA-XL possesses a LAT-derived sequence motif containing LAT-Tyr191 and a cognate phosphopeptide-binding domain derived from Grb2-SH2 which are flanked by CFP donor fluorescent protein and YFP acceptor fluorescent protein. When ZAP70 is not active, the ROZA-XL flexible core region can adopt several different conformations, some of which allow FRET to occur. Following phosphorylation of LAT-Tyr191 by ZAP70, the Grb2-SH2 domain interacts with phospho-LAT-Tyr191, allowing ROZA-XL to adopt a constrained conformation which is incompatible with FRET. The control construct, ROZA-XL-YF is insensitive to ZAP70 activity due to replacement of LAT-Tyr191 by phenylalanine. Jurkat T cells were nucleofected with ROZA-XL or ROZA-XL-YF plasmids. 48 hr post-transfection, the cells were left untreated or treated with CsA (5 µg/ml) for 24 hr, and subsequently stimulated with OKT3 mAbs for indicated time points or left unstimulated. Cells were then analyzed by FACS at CFP excitation wavelength (405 nm) and simultaneous emission was detected in the CFP and YFP emission spectra. FRET data analysis was performed using FlowJo software and data were presented in a bar graph (M). Data are representative of three independent experiments. *$p$<0.05 and **$p$<0.01, using Student's unpaired $t$-test.




## Figure 6

**A schematic model of ZAP70 regulation by CypA.** Resting T lymphocytes (A) can be activated by the engagement of their antigen receptors (TCR) with a peptide antigen (Ag) presented on MHC molecules on the surface of an antigen-presenting cell (B). CD4- or CD8-associated Lck is then recruited to the liganded TCR (C) and phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of the CD3 chains, predominantly CD3ζ. The phosphorylated ITAMs serve as docking sites for the SH2 domains of the cytosolic, inactive, ZAP70 tyrosine kinase (D). Lck-mediated phosphorylation of ZAP70 (E) upregulates the catalytic activity of ZAP70 which then undergoes autophosphorylation and transphosphorylate TCR downstream effector molecules, including LAT and SLP76. Cyclophilin A (CypA) binding to phosphorylated ZAP70 (G) inhibits ZAP70 catalytic activity, either by steric hindrance of ATP and/or substrate binding, or by modulation of its conformation, leading to downregulation of phosphorylation of downstream substrates (F). Cyclosporin A (CsA) interaction with CypA reverses the effect of CypA on ZAP70 (H), either by reducing the affinity of CypA to ZAP70 and outcompeting CypA binding to ZAP70 or by inhibition of the CypA catalytic activity and reversing the effect of CypA on ZAP70 enzymatic activity.

## Supplementary Files

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- SupplementaryInformationCypAZAP70.pdf