## Phylogeny

Tyrosine-protein kinase ZAP-70 is a member of the Syk family of protein tyrosine kinases (PTKs), as classified within the human kinome by Manning et al., 2002 (deindl2007structuralbasisfor pages 1-3, chen2020zap70shapesthe pages 7-8, bene2006whatiszap‐70? pages 4-5). It belongs to the tyrosine kinase group and is evolutionarily related to Syk, which is considered its B-cell homologue (yan2013structuralbasisfor pages 1-2, unknownauthors2024targetingzap70protein pages 74-79). The protein is conserved across species, with orthologs documented from fish to mammals, including in mouse (Mus musculus) and rat (Rattus norvegicus) (shah2016anelectrostaticselection pages 21-23, fischer2010zap70amaster pages 1-2, fernandezaguilar2023astoryof pages 5-7).

## Reaction Catalyzed

The enzyme catalyzes the phosphorylation of tyrosine residues on substrate proteins (bene2006whatiszap‐70? pages 4-5, deindl2007structuralbasisfor pages 12-12). The reaction is: ATP + a protein-L-tyrosine = ADP + a protein-L-tyrosine phosphate (huber2015thestructuralbasis pages 15-16, yan2013structuralbasisfor pages 14-14).

## Cofactor Requirements

Catalytic activity is dependent on the presence of divalent cations, specifically Mg2+ or Mn2+ (bene2006whatiszap‐70? pages 4-5, chen2020zap70shapesthe pages 7-8, deindl2007structuralbasisfor pages 1-3).

## Substrate Specificity

The substrate specificity of the ZAP-70 kinase domain is governed by an electrostatic selection mechanism, where it preferentially binds and phosphorylates tyrosine residues located within a sequence of high local negative charge (shah2016anelectrostaticselection pages 4-6, shah2016anelectrostaticselection pages 6-7). This is characterized by a preference for multiple acidic residues (aspartate or glutamate) surrounding the phosphorylation site and a strict disfavoring or exclusion of positively charged residues like lysine and arginine (shah2016anelectrostaticselection pages 9-10, shah2016anelectrostaticselection pages 2-4). This electrostatic complementarity is mediated by a positively charged substrate-binding region on the ZAP-70 kinase domain (shah2016anelectrostaticselection pages 2-4, shah2016anelectrostaticselection pages 21-23).

Positional scanning peptide array analysis has been used to define the intrinsic substrate consensus motif from positions P-5 to P+5, though the provided sources present contradictory findings regarding specific amino acid preferences. \* One source describes the consensus motif as having a strong preference for acidic residues (D/E) at the P-1 position and a moderate preference for polar residues (S/T) at P-3 and P-2. Downstream, small nonpolar residues (G/A) are favored at P+3 and P+4, while proline and basic residues are disfavored at P+1 and P+2. This leads to a representative consensus sequence of [X]-[D/E]-[S/T]-Y-[G/A]-[G/A]-X (unknownauthors2003regulationofprotein pages 27-31). \* In contrast, a second source reports enrichment for acidic residues (D/E) at positions P-2 and P-3, but a strong preference for isoleucine (I) and valine (V) at the P+1 position (yaronbarir2024theintrinsicsubstrate pages 2-2). \* A third source presents a conflicting view, stating that ZAP-70 disfavors acidic residues near the phosphorylation site and instead prefers aliphatic hydrophobic residues, such as isoleucine, at positions P-1 and P+3 (yaronbarir2024theintrinsicsubstrate pages 3-3).

This kinase substrate specificity is distinct from the recognition motif for the tandem SH2 domains, which bind to doubly phosphorylated Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) with the consensus sequence (D/E)xxYxxL(x)6–8YxxL (bene2006whatiszap‐70? pages 1-2, deindl2007structuralbasisfor pages 1-3).

## Structure

ZAP-70 is a 70 kDa protein composed of two tandem N-terminal Src homology 2 (SH2) domains and a C-terminal kinase domain (SH1) (fischer2010zap70amaster pages 1-2, bene2006whatiszap‐70? pages 4-5). The kinase domain possesses a bilobal fold, with a smaller N-terminal lobe containing five antiparallel beta strands and a C-helix (αC helix) that anchors ATP, and a larger C-terminal lobe that contains substrate-binding sites (unknownauthors2003regulationofprotein pages 27-31, huber2015thestructuralbasis pages 1-2). Critical regulatory elements, including the C-helix and the regulatory and hydrophobic spines, stabilize the active conformation (fischer2010zap70amaster pages 1-2, huber2015thestructuralbasis pages 1-2). In the inactive state, the αC helix is stabilized by a salt bridge between K369 and E386; this helix repositions upon activation (huber2015thestructuralbasis pages 13-15, huber2015thestructuralbasis pages 10-12). The kinase domain contains the conserved HRDLAARN motif, characteristic of tyrosine kinases, and a critical activation loop situated between conserved DFG and APE motifs (bene2006whatiszap‐70? pages 1-2, unknownauthors2003regulationofprotein pages 27-31). The crystal structure of autoinhibited human ZAP-70 (PDB ID 2OZO) reveals how intramolecular interactions maintain the inactive state (deindl2007structuralbasisfor pages 12-12).

## Regulation

ZAP-70 activity is regulated by autoinhibition and a series of phosphorylation events (deindl2007structuralbasisfor pages 1-3, yan2013structuralbasisfor pages 1-2). In its basal state, ZAP-70 is autoinhibited (deindl2007structuralbasisfor pages 1-3). Upon T-cell receptor (TCR) engagement, the Src family kinase Lck phosphorylates ITAMs on the TCR complex, creating docking sites for the ZAP-70 tandem SH2 domains (unknownauthors2024targetingzap70protein pages 79-83). This binding induces a conformational change that partially relieves autoinhibition (chen2020zap70shapesthe pages 7-8, deindl2007structuralbasisfor pages 1-3).

Full activation requires phosphorylation by Lck at several key tyrosine residues (yan2013structuralbasisfor pages 1-2, bene2006whatiszap‐70? pages 4-5): \* **Y492 and Y493**: Located in the activation loop, their phosphorylation is critical for full catalytic activity and stabilization of the active conformation. Y493 phosphorylation is particularly important for positive regulation, while phosphorylation at Y492 has been described as a negative regulatory site (unknownauthors2024targetingzap70protein pages 79-83, fischer2010zap70amaster pages 2-3). \* **Y315 and Y319**: Located in the interdomain B region (SH2-kinase linker), their phosphorylation destabilizes the autoinhibited state. Y319 phosphorylation is essential for downstream signaling, recruiting effectors like Lck and PLCγ1, while Y315 phosphorylation is involved in actin remodeling (deindl2007structuralbasisfor pages 1-3, fischer2010zap70amaster pages 1-2, williams1999phosphorylationoftyr319 pages 1-2). \* **Y292**: Phosphorylation at this site creates a binding site for the E3 ubiquitin ligase c-Cbl, which mediates negative regulation of TCR signaling (unknownauthors2024targetingzap70protein pages 74-79, kong1996distincttyrosinephosphorylation pages 6-9).

## Function

ZAP-70 is essential for adaptive immunity and is predominantly expressed in T cells (thymocytes and peripheral T cells) and natural killer (NK) cells (fischer2010zap70amaster pages 1-2, fernandezaguilar2023astoryof pages 5-7). In TCR signaling, ZAP-70 functions downstream of the upstream kinase Lck (fernandezaguilar2023astoryof pages 5-7). Upon activation, ZAP-70 phosphorylates downstream adaptor proteins, primarily LAT and LCP2 (SLP-76) (bene2006whatiszap‐70? pages 4-5, deindl2007structuralbasisfor pages 1-3). This initiates the assembly of a multi-protein signaling complex that propagates signals leading to T-cell activation, including calcium mobilization, MAPK pathway activation, cytoskeletal reorganization, and cytokine production such as IL-2 (fischer2010zap70amaster pages 2-3). This signaling cascade is critical for thymocyte development as well as the motility, adhesion, and function of mature T-cells (chen2020zap70shapesthe pages 7-8).

## Inhibitors

Experimental small-molecule inhibitors targeting ZAP-70 have been developed (deindl2007structuralbasisfor pages 11-12). These include the ATP-competitive inhibitor staurosporine, which has been structurally characterized in complex with the ZAP-70 kinase domain (huber2015thestructuralbasis pages 1-2, deindl2007structuralbasisfor pages 11-12). Other experimental inhibitors explored for T-cell malignancies include CPI-818 (an ITK-SYK family inhibitor) and cerdulatinib (a dual SYK/JAK inhibitor) (unknownauthors2024targetingzap70protein pages 74-79). Fragment screening has also identified a novel cryptic pocket adjacent to the activation loop as a potential allosteric drug target (huber2015thestructuralbasis pages 1-2, huber2015thestructuralbasis pages 13-15).

## Other Comments

Mutations in the *ZAP70* gene cause an autosomal recessive form of severe combined immunodeficiency (SCID) (bene2006whatiszap‐70? pages 4-5, deindl2007structuralbasisfor pages 1-3). This immunodeficiency is characterized by a selective T-cell defect, with an absence of peripheral CD8+ T cells and dysfunctional CD4+ T cells, resulting in impaired TCR signaling and severe recurrent infections (fischer2010zap70amaster pages 6-7, fischer2010zap70amaster pages 1-2). Partial loss-of-function mutations have also been linked to autoimmunity (fischer2010zap70amaster pages 1-2). Furthermore, aberrant expression of ZAP-70 in B-cell chronic lymphocytic leukemia (B-CLL) is a well-established prognostic marker associated with poor prognosis and increased antigen receptor activation status (bene2006whatiszap‐70? pages 4-5, unknownauthors2024targetingzap70protein pages 79-83).

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