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Experimental approaches for investigation of aminoacyl tRNA synthetase phosphorylation

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Abstract

Phosphorylation of many aminoacyl tRNA synthetases (AARSs) has been recognized for decades, but the contribution of post-translational modification to their primary role in tRNA charging and decryption of genetic code remains unclear. In contrast, phosphorylation is essential for performance of diverse noncanonical functions of AARSs unrelated to protein synthesis. Phosphorylation of glutamyl-prolyl tRNA synthetase (EPRS) has been investigated extensively in our laboratory for more than a decade, and has served as an archetype for studies of other AARSs. EPRS is a constituent of the IFN- γ -activated inhibitor of translation (GAIT) complex that directs transcript-selective translational control in myeloid cells. Stimulus-dependent phosphorylation of EPRS is essential for its release from the parental multi-aminoacyl tRNA synthetase complex (MSC), for binding to other GAIT complex proteins, and for regulating the binding to target mRNAs. Importantly, phosphorylation is the common driving force for the context- and stimulus-dependent release, and non-canonical activity, of other AARSs residing in the MSC, for example, lysyl tRNA synthetase (KARS). Here, we describe the concepts and experimental methodologies we have used to investigate the influence of phosphorylation on the structure and function of EPRS. We suggest that application of these approaches will help to identify new functional phosphorylation event(s) in other AARSs and elucidate their possible roles in noncanonical activities.

Graphical Abstract

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1. Introduction

The 20 aminoacyl-tRNA synthetases (AARSs) are ubiquitous and evolutionarily conserved enzymes that catalyze the highly specific acylation of amino acids to cognate tRNAs [1]. Thus, AARSs are essential for accurate decoding of the triplet genetic code and for sustenance of all domains of life, including bacteria, archaea, and eukarya [2, 3]. However, AARSs also perform critical cellular activities unrelated to their primary function in aminoacylation. These noncanonical activities include regulation of gene transcription, mRNA translational control, angiogenesis, apoptosis, amino acid sensing, and cell signaling, among others [4–6]. The evolutionary emergence of noncanonical activities approximately coincided with the incorporation of a subset of AARSs into a macromolecular complex, namely, the multi-aminoacyl tRNA synthetase complex (MSC) [4, 7]. The 1.5 mDa mammalian MSC comprises nine AARS activities in eight polypeptides, EPRS, MARS, DARS, KARS, IARS, LARS, QARS, and RARS, bridged by three scaffolding proteins, AIMP (aminoacyl tRNA synthetase complex interacting multifunctional protein) 1, 2, and 3 [5, 8]. EPRS is unique among the mammalian synthetases as a bifunctional enzyme containing two AARSs, EARS and PARS, in a single polypeptide chain [9]. The physiological function of the MSC is not clear. MSC binding to ribosomes might permit “channeling” of charged tRNAs directly to the A-site to improve efficiency of protein synthesis [10–12]. Alternatively, the MSC can serve as a cytoplasmic “depot” for context- and stimulus-dependent release of AARSs for performance of noncanonical activities [13, 14]. Phosphorylation of eukaryotic AARS has been recognized for decades, but most studies show little influence on synthetase activity [15]. However, recent studies suggest that post-translational modifications, particularly phosphorylation, are key drivers of the noncanonical activities of AARSs. In the earliest, and perhaps the most extensively studied example, interferon (IFN)- γ induced phosphorylation of EPRS was shown to induce transcript-selective translation inhibition in myeloid cells [16, 17]. In subsequent studies, phosphorylation of KARS was observed upon immunologic challenge in mast cells to

activate a noncanonical transcriptional activity; and by laminin in cancer cells to control cell migration [18, 19]. For both, EPRS and KARS, phosphorylation is the signal-dependent event that drives their release from the MSC to permit interaction with other binding partners, intracellular relocation (for example to the nucleus and plasma membrane in the case of KARS), and ultimately, execution of the AARS-specific noncanonical activity [19–21]. Based on these examples, future studies are likely to reveal additional AARSs, on or off the MSC, which are activated by phosphorylation to perform novel noncanonical functions.

In human myeloid cells, IFN- γ induces phosphorylation of EPRS at Ser⁸⁸⁶ and Ser⁹⁹⁹ in the noncatalytic linker domain that joins the two synthetase cores [20]. The two phosphorylation events co-ordinate multiple activities in the GAIT complex-mediated translational control system. These events include (i) release of EPRS from the MSC, (ii) assembly of the active heterotetrameric GAIT complex by interaction with ribosomal protein L13a, NS1-associated protein-1 (NSAP1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and (iii) repression of translation via direct binding to a bipartite, stem-loop GAIT element in the 3' untranslated region (UTR) of a family of inflammation-related transcripts, including ceruloplasmin (Cp), vascular endothelial growth factor (VEGF)-A, and several chemokines and their receptors [22–24]. Ser⁸⁸⁶ phosphorylation is required for interaction of EPRS with NSAP1 to form an intermediate “pre-GAIT” complex, whereas Ser⁹⁹⁹ phosphorylation is critical for binding with L13a and GAPDH to form the functional, four-component GAIT complex that represses translation of GAIT element-bearing mRNAs by blocking ribosome binding to eIF4G in the translation-initiation complex [20]. Interestingly, the heterotrimeric GAIT complex in mice lacks NSAP1, and the EPRS linker does not contain the NSAP1-binding phosphorylation site at Ser⁸⁸⁶ attesting to the critical importance of Ser⁹⁹⁹ phosphorylation [25]. Recently, viral infection has been shown to induce phosphorylation of EPRS at Ser⁹⁹⁰ in the linker domain, and subsequent release from the MSC for viral clearance [14].

Identification and elucidation of the signaling pathway that regulates stimulus-dependent phosphorylation, and particularly the upstream and proximal kinases, can provide important clues to the physiological function of the phosphorylation event. Cyclin-dependent kinase-5 (Cdk5), in conjunction with its activator protein p35, is the proximal kinase that phosphorylates Ser⁸⁸⁶ in human EPRS, and is upstream of an unidentified AGC kinase group that mediates EPRS Ser⁹⁹⁹ phosphorylation [26]. This finding was unexpected because Cdk5 has been considered a central nervous system-specific kinase [27]. However, recent studies have shown its induction in lipopolysaccharide-stimulated macrophages [28], and its regulation of PPAR γ function in adipocytes [29], implicating the kinase in inflammation and metabolism, respectively. In mast cells and cardiomyocytes, KARS, another integral constituent of MSC, is phosphorylated at Ser²⁰⁷ by mitogen-activated protein kinase (MAPK) phosphorylation inducing a conformational alteration that releases it from the MSC [21, 30]. Released cytosolic KARS translocates to the nucleus, enhances production of the 2nd messenger, diadenosine tetraphosphate (A_p4_A), which activates microphthalmia-associated transcription factor (MITF) by inhibiting the Hint-1 repressor. Thus, KARS phosphorylation induces positive regulation of MITF target gene transcription. Thr⁵² phosphorylation of KARS by p38MAPK also induces its release from MSC and

translocation to the plasma membrane for laminin-dependent cell migration with metastatic implications [19].

Although phosphorylation of several other AARSs displaying noncanonical activities has been reported, little is known about their specific role in controlling these activities [31–34]. We specifically describe the role characterized phospho-sites play in EPRS noncanonical activities *i.e.*, release from MSC, interaction with binding partners, and effect on gene expression. Taking advantage of the information derived from our investigation of the mechanisms underlying phosphorylation of EPRS, and the consequent influence on its function, here we provide a technical and conceptual tool kit to facilitate identification by others of novel phosphorylated AARSs, their phosphorylation sites, upstream signaling pathway, proximal kinases, and their noncanonical activities.

2. Overview

The following description of approaches and methodologies for investigation of AARS phosphorylation are approximately in the temporal sequence in our investigation of EPRS phosphorylation, and its role in the GAIT system. EPRS phosphorylation events have been studied primarily in myeloid cells, including monocytes and macrophages, taking advantage of established methods that have been used by other laboratories for detailed investigation of the phosphorylation of a myriad of proteins. Therefore, instead of detailing step-by-step protocols for each method, we provide succinct descriptions of the concepts and approaches for an orderly application of the existing techniques as applicable for rigorous and extensive characterization of phosphorylation of AARSs (as well as other proteins). An overview of the workflow is illustrated in Fig. 1.

3. Cell culture, treatments, lysates, and general considerations

All buffers and solutions must be prepared with double-distilled (e.g., Milli-Q) water using high-grade (e.g., analytical or molecular biology) chemicals and other reagents. For live cells, cell culture-approved reagents should be used. Careful consideration must be given to avoid phosphatase contamination by using sterile pipettes, glass, and plastic wares. For treatment with growth factors or cytokines, e.g., IFN- γ , pre-incubate $\sim 1 \times 10^7$ cells in 10 ml RPMI 1640 media supplemented with 2% heat-inactivated fetal bovine serum (FBS) for 1 hr at 37 °C in a 5% CO₂ incubator. Treat cells with 500 units/ml of IFN- γ (R&D Systems) or with vehicle control consisting of phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) for up to 24 h. Cell lysates should be prepared using Phosphosafe extraction buffer (Novagen) supplemented with EDTA-free protease inhibitor cocktail (Roche). Alternatively, other lysis buffers (such as RIPA) can be used. Protease and phosphatase inhibitor cocktail (e.g., Halt Protease or phosphatase inhibitor cocktail) must be included during preparation of cell lysates to maintain protein integrity and phosphorylation state.

We have used the following cells for investigation of EPRS phosphorylation.

- i. Human U937 monocytes (ATCC) in RPMI 1640 medium and maintained in 10% FBS.

- ii. Peripheral blood mononuclear cells (PBMCs) or peripheral blood monocytes (PBMs) isolated from human blood by leukapheresis and elutriation, and maintained in RPMI 1640 medium with 10% FBS.
- iii. Mouse Raw 264.7 macrophages (ATCC) maintained in DMEM with 10% FBS.
- iv. Bone marrow-derived macrophages (BMDMs) from combined marrows of mouse femurs and tibias, and maintained in RPMI 1640 with 10% FBS and 20% L929 cell-conditioned medium.

4. Detection of phosphorylation

4.1. ^{32}P -labeling for detection of endogenous protein phosphorylation in vivo

^{32}P -labeling of cells using radioisotope-labeled inorganic phosphate, followed by immunoprecipitation (IP) with a specific antibody against the target protein, is a classic method for detection of endogenous protein phosphorylation [35]. This biochemical method remains widely used despite the requirement for a high level of radioactivity, e.g., 100 μCi or more. The method can be applied to both adherent and non-adherent cells, has high sensitivity, and can yield informative results even with low efficiency of IP typical for most antibodies. To detect phosphorylation of EPRS, $\sim 1 \times 10^7$ U937 or RAW 264.7 cells can be incubated in phosphate-free RPMI 1640 medium with 2% FBS for 2 h. Subsequently, cells are incubated with IFN- γ (500 U/ml/ 10^6 cells) and 250–500 μCi of ^{32}P -labeled, aqueous H_3PO_4 (NEX053H005MC; PerkinElmer) for 2 to 24 h as needed. Radiolabel can be added simultaneously with agonist or after initiation of the signaling cascade. In the latter case, prior knowledge of the kinetics of the signaling events or activation is important. Prolonged incubation with ^{32}P -orthophosphate risks labeling nucleic acids, but this is generally not an issue due to subsequent affinity purification by IP [36, 37]. At the end of incubation, cells are lysed in Phospho-safe extraction or RIPA buffer, followed by direct IP (or co-IP), SDS-PAGE, gel drying, and imaging by autoradiography or phosphorimager.

4.2. Detection of phosphorylation using anti-phospho-Ser/Thr/Tyr antibodies

Eukaryotic protein phosphorylation predominates on Ser, Thr, and Tyr amino acids [38]. Phospho-antibodies that specifically detect phosphorylation at these residues are available from multiple commercial sources, including EMD Millipore, Cell Signaling, and Meridian Life Sciences, among others. Although these antibodies do not reveal the specific phosphorylation site (unless the protein contains a single target amino acid), they are extremely helpful for initial identification of a phosphorylated protein, the kinetics of phosphorylation, and the nature of the amino acid target, i.e., Ser, Thr, or Tyr. Since about one-third of the proteome is thought to undergo phosphorylation [39], application of these antibodies to whole cell lysates without a subsequent purification step is likely to be uninformative. Therefore these antibodies are generally used subsequent to IP or other protein purification methods. In our early experiments, IFN- γ treated U937 cells were subjected to IP with anti-EPRS antibody, and analyzed by SDS-PAGE followed by

immunoblotting with anti-phospho-Ser, -Thr and -Tyr antibodies [16]. The approach revealed IFN- γ stimulated phosphorylation of EPRS exclusively at Ser residues. Specific cross-reactivity was verified by protein phosphatase treatment, which prevented antibody recognition.

4.3 Detection of protein phosphorylation by mobility shift

Protein phosphorylation often alters electrophoretic mobility causing a shift in apparent molecular weight relative to the unphosphorylated protein on a polyacrylamide gel [40, 41]. Addition of a phospho-moiety increases the mass of phosphorylated protein only by ~80 Da, and is insufficient to explain the observed mobility shift even following multisite phosphorylation. Although the mechanism remains largely undefined, it is thought that phosphorylation disrupts uniform binding of SDS to protein (aided by local charge of surrounding residues), possibly inducing a charge-based conformational change [42–44]. Indeed, phosphorylation has been shown to alter the effective Stokes radius of phospholamban and phenylalanine hydroxylase [45, 46]. Despite the uncertain mechanism, the observed mobility shift due to phosphorylation provides an extremely simple and sensitive method for detection of phosphorylation. Moreover, the ratio of signal strengths quantitatively reflects the stoichiometry, i.e., the amount of shifted protein reveals the fraction of protein phosphorylated. Because other post-translational modifications, e.g., ubiquitination, glycosylation, sumoylation, lipidation, might also generate a mobility shift, reversal of the mobility shift by sample pre-treatment with protein phosphatases is essential for verification of phosphorylation [41, 47]. In our experiments, phosphorylation-induced mobility shift was assessed in lysates from IFN- γ treated U937 cells following resolution on SDS-PAGE (4.5% for EPRS and 12–16% acrylamide for ribosomal protein L13a) and immunoblotting with anti-EPRS and -L13a antibodies [16, 20, 48]. In both cases, the shift was validated by pre-incubation of cell lysates with shrimp alkaline phosphatase (5–10 U) for 1 h at 37 °C that reversed the mobility shift.

4.4 Detection of protein phosphorylation by alternative methods

Phosphorylation-induced mobility shift can be detected using polyacrylamide-bound $\text{Mn}^{2+}/\text{Zn}^{2+}$ -Phos-tag ligand that selectively retards the migration of phosphorylated proteins and not the non-phosphorylated counterpart. A detailed step-by-step protocol for Phos-Tag staining is outlined elsewhere [42]. Fluorescent stains such as ProQ Diamond (Molecular Probes) or quercetin-aluminum (III)-appended complex can be used to selectively visualize phosphorylated proteins (total or following IP) following resolution on polyacrylamide gel [49, 50]. These staining methods eliminate the need for immunoblotting with phospho-specific antibodies, but are rarely used due to lack of specificity.

5. Identification of specific phosphorylation sites

Key to successful molecular and functional characterization of protein phosphorylation is identification of the specific Ser, Thr, or Tyr residue(s) modified. Here, we discuss the approaches used for identification of IFN- γ -induced sites of phosphorylation in EPRS.

5.1 Mass spectrometry (MS) for phospho-site identification

Two-dimensional phospho-peptide mapping coupled with phospho-amino acid analysis has been used to identify specific phospho-sites [51]. However, these traditional biochemical methods have been nearly rendered obsolete by the advent of MS approaches that have revolutionized proteomics in general, and phospho-proteomics in specific [52]. MS is highly sensitive, and often identifies the sites of modification, and the modifying moiety, with near certainty. MS-based identification of phosphorylation can also reveal information on the relative stoichiometry of phosphorylation, which is generally helpful for understanding the physiological role of the modified protein. Methods for mapping protein phosphorylation sites by MS have been described in detail elsewhere [53], so we will focus specifically on sample preparation and liquid chromatography tandem-MS (LC-MS) as applied by us to EPRS [20]. Cell lysates from 24-h IFN- γ -treated U937 cells were first enriched for phosphorylated proteins using a phospho-protein enrichment kit (Clontech) that employs phosphate metal affinity chromatography, and significantly enhances the probability of detecting sites of phosphorylation. The enriched fraction was affinity-purified with anti-EPRS antibody, and following SDS-PAGE, stained with Coomassie blue. Phosphorylation state and specificity of EPRS pull-down were confirmed by immunoblot analysis with anti-EPRS and phospho-Ser antibodies. Subsequently, the EPRS band was in-gel digested with trypsin and analyzed by capillary column LC-MS analysis to map peptides, and identify and characterize specific phospho-peptides. To map peptides, collision-induced dissociation (CID) spectra were compared to the EPRS protein sequence using Sequest software. To identify phospho-peptides and specific phosphorylation sites, CID spectra were searched for peptides that contain modification(s) at Ser, Thr, or Tyr by a combination of database searches, analysis of neutral loss chromatograms for characteristic loss of phosphate group, and selected reaction-monitoring (SRM). Using these approaches, we identified Ser⁸⁸⁶ as well as Ser⁹⁹⁹ or Ser¹⁰⁰⁰ as IFN- γ -induced phosphorylation sites in EPRS.

5.2 Verification of phospho-sites by site-directed mutagenesis

Following identification of phosphorylation sites by MS, candidate phospho-sites should be validated, for example by site-directed mutagenesis [52]. Generally, one or more specific point mutations are introduced into eukaryotic expression plasmids containing the coding sequence of the phospho-protein under investigation. Mutagenesis of Ser/Thr/Tyr to Ala, often referred to as phospho-defective or loss-of-function mutants, abrogates phosphorylation, and is an excellent way to conclusively verify phosphorylation sites. Because of structural similarity, mutation to Phe is a generally preferred loss-of-function mutation for Tyr. Alternatively, mutation of Ser or Thr to Asp or Glu, due to the negative charge of the latter and structural similarities to the phosphorylated form of Ser and Thr, are commonly used as phospho-site mimetics, thus leading to a gain-of-function mutation [54]. In contrast, phospho-Tyr has a negative charge but bears little structural similarity to Asp or Glu. Nonetheless, investigators have Asp/Glu substitutions for Tyr have been successfully used as phospho-site mimetics [55, 56]. Specific mutations in the target coding sequence (CDS) can be generated using commercially available site-directed mutagenesis kits (e.g., QuikChange, Agilent or GeneArt, Invitrogen). We have used this approach to validate two IFN- γ -induced Ser phosphorylation sites in EPRS (Fig. 2) [20]. A similar approach using

tandem mass spectrometry coupled with mutagenesis revealed EPRS Ser⁹⁹⁰ phosphorylation induced by viral infection [14].

MS analysis revealed two stimulus-inducible EPRS phospho-peptides - one containing Ser⁸⁸⁶ and a second containing Ser⁹⁹⁹ or Ser¹⁰⁰⁰. All combinations of single, double, and triple mutations of Ser⁸⁸⁶, Ser⁹⁹⁹, and Ser¹⁰⁰⁰ were generated in full-length EPRS and the linker domain, and in both phospho-defective and -mimetic forms. The mutants were cloned in the mammalian expression vector pcDNA3 with an N-terminus Flag tag. The constructs were transiently transfected in U937 cells using Amaxa nucleofector kit V (Lonza) according to the manufacturer's protocol. Loss of IFN- γ -induced phosphorylation of the Ser⁸⁸⁶- and Ser⁹⁹⁹-to-Ala mutants was detected by ³²P-labeling and by phospho-Ser antibodies as described above. In addition to mutations providing precise information on phosphorylation sites, the same constructs are extraordinarily helpful in providing insights into the functional consequence of loss- and gain-of function mutations, for example, by analysis of the association of EPRS with either the MSC or GAIT complex (see section below).

6. Generation of site-specific phospho-antibodies

Phospho-specific antibodies that recognize specific phospho-sites can facilitate elucidation of the signaling pathway inducing phosphorylation as well as the physiological significance. A monoclonal or polyclonal phospho-specific antibody can be generated using a synthetic peptide immunogen bearing a phospho-moiety at the desired site(s) [57, 58]. Synthetic phospho-peptide immunogens direct the immune response specifically towards the phospho-site of interest [59]. However, such short peptides can be insufficiently immunogenic, and lack the native conformation state of the full-length protein. Low immunogenicity of the phospho-peptide can be countered by increasing the molecular mass via conjugation to a carrier protein, e.g., keyhole limpet hemocyanin. In many instances, phospho-sites lie in flexible or unstructured regions (e.g., EPRS phospho-sites Ser⁸⁸⁶, Ser⁹⁹⁰, and Ser⁹⁹⁹), reducing the importance of the structural limitation of the short phospho-peptide [14, 20]. We successfully generated affinity-purified polyclonal phospho-specific antibodies against Ser⁸⁸⁶ and Ser⁹⁹⁹ using as antigens ⁸⁸⁰SQSSDS(pS)PTRNSE and ⁹⁹¹KNQGGGLS(pS)SGAGE peptides, respectively (Open Biosystems). These antibodies not only confirmed the induced phosphorylation of the Ser⁸⁸⁶ and Ser⁹⁹⁹ sites, but also helped to elucidate the function of phospho-EPRS in the GAIT system.

7. Elucidation of upstream signaling pathways and proximal kinases

Identification of upstream signaling pathways and proximal kinases regulating target substrate phosphorylation usually provide critical information about the physiological role of the phosphorylation event [60]. The human kinome contains more than 500 kinases, with many sharing common mechanisms for substrate recognition and catalysis [61]. Furthermore, kinase pathways often function hierarchically i.e., activation of a kinase depends on the sequential activation of a series of upstream kinases or regulators. Thus, conclusive identification of proximal kinases, i.e., the immediate upstream kinase that binds and phosphorylates the target protein, is possibly the most difficult challenge in the

characterization of any phosphorylation event. Despite the accumulation of substantial information about protein phosphorylation mechanisms, and the availability of excellent screening tools, e.g., genetic screening with knockout models, peptide libraries, or kinase arrays, multiple parallel approaches are generally necessary to confidently pinpoint the proximal kinase directly responsible for substrate phosphorylation [52]. These screening tools are generally successful for identifying novel substrates for a given kinase, but relatively less so for the converse, i.e., identifying proximal kinases for a given target. To complicate the investigation more, a single phosphorylation site can be targeted by multiple kinases. Here, we describe the combination of approaches used to identify Cdk5 as the proximal EPRS Ser⁸⁸⁶ kinase.

7.1. Identification of kinase recognition motif (KRM) and candidate kinase or group

A KRM is a consensus sequences, generally 2–8 amino acids in length, on either flank or surrounding the phospho-site, and considered to be an essential prerequisite for substrate recognition by a kinase and site-specific phosphorylation [60]. However, notable exceptions to this rule have been reported, and thus the absence of any known consensus KRM is insufficient to eliminate a specific proximal kinase or conclude that a potential phospho-site is not an authentic kinase target [62]. Decades of research has established specific KRMs for a host of kinases, forming the basis of several bioinformatic tools, e.g., Scansite and PhosphoMotif Finder, that predict substrates for a kinase, or the putative kinase targeting a specific site in a given target [63, 64]. Lists of known KRMs are available in multiple excellent reviews [65, 66]. KRM predictor tools can be exceptionally helpful for searching KRMs and candidate kinase(s). Nonetheless, these tools failed to identify any known KRM for the EPRS Ser⁹⁹⁹ phospho-site. However, a well established KRM “-SPXR-” (where X = any amino acid) was identified for the EPRS Ser⁸⁸⁶ phospho-site. Employing the site-directed mutagenesis approach we verified that the amino acids in “-SPXR-” are indeed critical for Ser⁸⁸⁶ phosphorylation [20]. The “-SPXR-” motif is primarily a target of Pro-directed Ser/Thr kinases (PDKs) such as several cyclin-dependent kinases (Cdk), extracellular signal-regulated kinase (Erk), and glycogen synthase kinase (Gsk) [67]. These and other candidate kinases were screened for specific Ser⁸⁸⁶ phosphorylation activity by pharmacological inhibitors [20]. Since most kinase inhibitors have limited specificity, treatment of cells with inhibitors near their IC₅₀ (i.e., the dose at which activity is inhibited by 50%) is recommended and less likely to yield non-specific inhibition. Taking advantage of these approaches, we shortlisted PDKs as the likely Ser⁸⁸⁶ kinase group.

7.2. Immune-complex kinase assay for determination of proximal kinase

The immune-complex kinase assay can provide evidence for the proximal kinase directly responsible for target phosphorylation. In this approach, the endogenous active kinase or kinase complex is immunoprecipitated from the cell lysate, and used for *in vitro* phosphorylation of an oligopeptide, polypeptide, or full-length protein substrate in the presence of [γ -³²P]ATP. ³²P-phosphorylated protein can be detected by resolution on SDS-PAGE and autoradiography [68, 69]. ³²P-phosphorylated peptides are preferentially detected by spotting on P81 phospho-cellulose strip (that permits binding of basic residues) and scintillation counting [70]. Alternate, non-radioactive methods such as the luminescence-based ADP-Glo kinase assay (Promega) are available as well. To apply the immune-complex

approach to EPRS Ser⁸⁸⁶ phosphorylation, lysates (~500 µg) from IFN-γ-treated cells were pre-cleared and incubated with antibodies targeting specific PDKs. The complex was captured with protein A-Sepharose beads, washed, suspended in kinase assay buffer (50 mM Tris-HCl, pH 7.6, 1 mM DTT, 10 mM MgCl₂, 1 mM CaCl₂, and phosphatase inhibitor mixture), and used for *in vitro* phosphorylation of an EPRS linker target containing a Ser⁹⁹⁹-to-Ala (permitting site-specific Ser⁸⁸⁶ phosphorylation only) in the presence of [γ ³²P]ATP. These experiments revealed robust and highly specific Ser⁸⁸⁶ phosphorylation by Cdk5 [26].

7.3. Verification of putative kinase by gene silencing

Gene silencing or RNAi-based screening of the human kinome has elucidated signaling pathways regulating kinase activity despite the limitation that it does not establish a direct kinase-substrate relationship [52]. However, rigorous application of the siRNA approach can reliably validate the requirement of the specific kinase in phosphorylation of substrate(s) under investigation. Abrogation of target phosphorylation following knockdown by transient or stable siRNA transfection is strong evidence for upstream (or proximal) involvement of the kinase. Using siRNA duplexes, followed by detection with phospho-specific antibodies and ³²P-labeling, we identified the requirement of Erk2 and Cdk5 (and its activator, p35) for EPRS Ser⁸⁸⁶ phosphorylation [26].

7.4. Direct interaction of kinase and substrate provides evidence for proximal nature

The proximal kinase directly binds the substrate to catalyze phospho-group donor transfer to the amino acid target [60]. Thus, demonstration of direct physical interaction between kinase and substrate is an exceptionally strong indicator of a proximal kinase. However, interaction of the endogenous active kinase with substrates, particularly in cells, is frequently transient and difficult to capture. Information on the kinetics of substrate phosphorylation can be helpful for optimizing the detection of the interaction by co-IP. The likelihood of successful capture of a kinase-substrate interaction can be significantly enhanced by ectopic expression of the kinase in the appropriate cells. Alternatively, kinase-substrate interaction can be captured by stabilization with a bifunctional cross-linker to covalently link the substrate and the kinase catalytic domains, followed by co-IP and detection with immunoblotting or by mass spectrometry [52, 71]. Using co-IP (described below) of lysates from cells treated with IFN-γ for 4 h, a time when EPRS is maximally phosphorylated, we identified a specific interaction of Cdk5 with EPRS supporting it as the proximal kinase for Ser⁸⁸⁶ phosphorylation [26].

7.5. Phosphorylation by purified or recombinant kinase supports proximal nature

To help establish a kinase as proximal, *in vitro* reconstitution by direct phosphorylation of the substrate by purified (usually recombinant), catalytically active kinase and donor phospho-group is critical [52, 60]. Failure of direct *in vitro* phosphorylation provides evidence that the identified kinase is not the proximal kinase, but rather an upstream regulator. It is important to show activity of the purified kinase by determining phosphorylation of known substrate. For EPRS Ser⁸⁸⁶ phosphorylation, incubation with purified, active Cdk5 (complexed with its non-kinase activator, p35), but not with Erk2, stimulated ³²P incorporation into Ser⁸⁸⁶-containing EPRS-linker in the *in vitro* phosphorylation assay, thus supporting Cdk5/p35 as the proximal kinase [26].

8. Methods for investigation of phospho-AARS function

8.1. AARS release from the MSC

Stimulus- and phosphorylation-dependent release of EPRS (as well as KARS) from the parental MSC is the initial and essential trigger of noncanonical AARS activity. The following biochemical assays have been used in our investigations of EPRS, and can be extended for analysis of the status of AARS association with the MSC, and their release upon phosphorylation.

8.1a. Determination of AARS residence in MSC by size-exclusion

chromatography—Size-exclusion chromatography has been applied to investigate AARS association with the MSC [16, 18, 19]. The MSC is a large, relatively stable macromolecular complex, and thus is suitable for investigation by this approach. Its non-compact and elongated nature increases the surface area for contact with tRNA and regulatory proteins including kinases [72]. Phosphorylation directs AARS structural alteration and/or facilitates binding to protein partners, thereby inducing AARS dissociation from the MSC. Constituents released from the MSC can be resolved from the parental complex by size-exclusion chromatography. The specificity of AARS release can be detected by immunoblotting using antibodies targeting both the released AARS as well as AARSs or scaffolding proteins that remain MSC-bound. For size-exclusion chromatography, the selection of appropriate matrix depends on two criteria: (i) a low molecular weight (LMW) cut-off matrix can be used to separate MSC in the void volume and released AARS in eluates, alternatively, (ii) a high molecular weight (HMW) cut-off matrix can separate both MSC-contained and MSC-released AARSs within eluates. For example LMW Superdex 200 (600-10 kDa cut-off) or Sephacryl S-300 (1500-10 kDa cut-off) will resolve the MSC-released components in eluates, and retain MSC-associated constituents in the void volume (Fig. 3). LMW cut-off matrix has been effectively utilized to resolve release of KARS, MARS, and QARS as well as to define the function of the MSC scaffolding components, AIMP1-3 [18, 73]. A caveat of LMW cut-off matrices is that they cannot reveal altered MSC mass following constituent release. Furthermore, if a released component joins another large complex, for example EPRS incorporation into the large, heterotetrameric GAIT complex, both complexes will co-elute in the void volume and thus be indistinguishable. In this case, a HMW cut-off matrix such as Superose 6 with a resolving range spanning 5,000 to 5 kDa is preferable. Using a HMW matrix, we demonstrated release of EPRS from the MSC, and incorporation first into the pre-GAIT complex (consisting of EPRS and NSAP1 only), and finally into the functional, heterotetrameric GAIT complex [16]. For determination of the release of a specific AARS from the parental MSC, using either LMW or HMW matrices, the column matrix must be pre-equilibrated with non-denaturing running buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, and 10% glycerol). Both void volume and resolution range of the matrix should be determined by pre-running the column with dextran blue (Sigma) and then with gel-filtration molecular weight protein standards (Bio-Rad). For assay of released and MSC-associated AARSs, S100 cytosolic lysates (prepared by ultracentrifugation at 100,000 g for 1 h) can be loaded, and 0.5 to 1 ml fractions collected at a low flow rate (0.25 to 0.5 ml/min). Finally, equal volume (100 µl) from each fraction is analyzed by SDS-PAGE and immunoblot with appropriate anti-AARS antibodies.

8.1b. Determination of AARS residence in MSC by co-immunoprecipitation

(co-IP)—Co-IP is possibly the most widely used method to investigate protein-protein interactions in cells and tissues. Multiple co-IP methods have been described with detailed step-by-step protocols [74, 75]. Although this approach can be used to investigate interactions *in vitro* using purified or semi-purified samples, a distinct advantage is that co-IPs can assess formation of protein complexes *in vivo*. We have used the method extensively to explore the GAIT complex assembly, and dissect the mechanism of activation [17]. For example, by co-IP we have elucidated the role of phosphorylation in EPRS release from MSC, and subsequent association with other GAIT complex constituents, i.e., NSAP1, phospho-L13a and GAPDH. Co-IPs can be done using an antibody cross-linked (i.e., immobilized) on a solid support (e.g., protein A/G agarose or Sepharose beads), followed by incubating with the sample (e.g., cell or cytosolic extract) and elution [20]. Alternatively, direct incubation of the antibody and sample, followed by addition of affinity beads to capture the antigen-antibody complex, and precipitation of the target antibody. Co-IP with cross-linked antibody has the advantage of substantially reducing co-elution of antibody heavy and light chains (i.e., 50 and 25 kDa respectively), which is often critical if the target protein is in the size range of the antibody chains. Despite ongoing improvement of the method (e.g., magnetic bead-based methods), the specificity and titer of the antibody against the target protein is the single most important determinant of successful co-IP. High cellular abundance of the target protein is also very helpful. In our own studies, we benefited greatly from the very high cellular abundance of all GAIT complex constituents and the commercial availability of high-specificity antibodies. We used these reagents and co-IP methods to investigate EPRS dissociation from the MSC and subsequent association with NSAP1, RP L13a, and GAPDH. Briefly, cell or cytosolic extracts are incubated with antibodies cross-linked to protein A-Sepharose or agarose in detergent-free IP buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and EDTA-free protease and phosphatase inhibitor cocktails for 12 to 16 h. Antibody-bound protein or protein complexes are washed at least 3 times with the same buffer. Bound protein is eluted with 0.1 M glycine (pH 3.0) followed by neutralization with an equal volume of 1 M Tris (not pH-adjusted), or by directly boiling in SDS-loading buffer. Subsequently, the complex formation is interrogated by SDS-PAGE followed by immunoblotting with GAIT constituent-specific antibodies.

8.2. Methods to assess formation of complexes with AARSs, including RNA-protein and protein-protein interactions

Following release of EPRS from the MSC, it binds both other GAIT complex constituents and also specific mRNAs bearing defined GAIT elements [16, 17, 20]. Here we describe the methodologies used to elucidate the interaction of EPRS with both RNA and proteins as summarized in Fig. 2.

8.2a. RNA electrophoretic mobility shift assay (EMSA) for RNA-AARS

interaction—To avoid RNA degradation by RNases, all reagents must be prepared with either diethylpyrocarbonate (DEPC)-treated or nuclease-free water. GAIT-element RNA probes are prepared from a synthetic oligonucleotide template following the instructions of the T7 transcription kit (Megashortscript, Ambion). Subsequently, RNA probes are labeled with α -³²P-UTP through *in vitro* transcription reaction, and purified by gel filtration to

reduce background signal of free α - ^{32}P -UTP. Following *in vitro* transcription, the reaction mix must be incubated with DNase to eliminate residual DNA contamination. For binding, ^{32}P -labeled RNA is incubated with a 10-fold molar excess of recombinant EPRS-linker protein in binding buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.005% surfactant P20, 125 $\mu\text{g}/\text{ml}$ g/ml tRNA, 1 mM DTT, and 5% glycerol) at 37 °C for 30 min. To analyze binding, the protein-RNA mixture is subjected to electrophoresis on a 5% native-PAGE (5% polyacrylamide in 0.5X Tris-buffered EDTA), and visualized by autoradiography or phosphorimaging.

8.2b. Surface plasmon resonance (SPR) for analysis of AARS-RNA interaction

—SPR is a label-free method to determine real-time macromolecular interactions by measuring the binding-induced refractive index [76]. SPR analyzes ligand-analyte interaction where the ligands are first immobilized on a sensor chip and the analyte is subsequently flown through in the running buffer on the chip. (Fig. 4, top). The real-time binding of the analyte to ligand will induce a change in the refractive index that can be recorded and analyzed by BIAevaluation software of the SPR Biacore 3000 system (or equivalent). SPR is a powerful tool for kinetic analysis of both protein-protein and protein-RNA interactions. The RNA ligand is generally biotinylated to permit immobilization on a streptavidin (SA)-coated sensor chip (Biacore). The magnitude of the SPR response directly reflects the mass of the mobile molecule that binds the sensor. Sensitivity is significantly improved by immobilizing the smaller molecule on the sensor chip, e.g., the *in vitro*-synthesized biotinylated 29-nt Cp GAIT element oligonucleotide (Dharmacon) rather than the larger binding protein. SPR accuracy depends on the purity of the reagents, in our case, recombinant EPRS-linker, phospho-EPRS-linker, and NSAP1 protein [77]. It is also essential that all reagents, and the sensor chip, be at room temperature prior to the binding experiment. Using SPR, we have demonstrated high-affinity binding of EPRS to a 29-nt Cp GAIT element, as well as the specific requirement of EPRS phosphorylation for EPRS-protein interactions (see below) for GAIT complex assembly [20, 77]. Briefly, for RNA-protein binding, biotinylated RNA is injected on the SA chip at a flow rate of 10 $\mu\text{l}/\text{min}$ to a level of 40-50 response units (RU). To detect protein binding to immobilized RNA, protein is injected for 3 min at a flow rate 10 $\mu\text{l}/\text{min}$. Following each injection, running buffer is flowed for 2 min to facilitate RNA-protein dissociation. After the experiment, the chip is regenerated with 2 M NaCl for 30 sec to remove all proteins before analysis of other analytes. For binding affinity determination, varying concentrations of recombinant EPRS linker (e.g., 3 to 200 nM) is used. Binding kinetics is determined using BIAevaluation software at default 1:1 binding model.

8.2c. SPR for analysis of AARS-protein interactions and functional reconstitution

—Using SPR we elucidated the role of EPRS phosphorylation in the assembly of the active GAIT complex, i.e., interaction of EPRS with GAIT complex constituents and binding to biotinylated 29-nt Cp GAIT element (Jia 2008, Arif 2009). For functional reconstitution, biotinylated RNA was immobilized on a SA sensor as above, followed by stepwise injection of purified proteins. GAIT element RNA-EPRS linker (or P-EPRS linker) binding was detected via injection-dissociation-regeneration steps. To test the effect of other GAIT proteins, i.e., NSAP1, Ser⁷⁷-phosphorylated RP L13a (P-L13a) in

RNA-EPRS linker binding, unmodified and P-EPRS linker proteins were pre-incubated for 30 min with a 10-fold molar excess of NSAP1, or the combination of NSAP1, P-L13a and GAPDH (Fig. 4, bottom). The protein mix was injected for analysis of association and dissociation as above. Using this approach, we demonstrated that P-EPRS can binds the GAIT element with high affinity, that presence of NSAP1 inhibits this binding, and finally, addition of the GAPDH and P-L13a, restores RNA binding activity of P-EPRS, thereby reconstituting the functional GAIT complex [20, 26, 77].

8.2d. Förster resonance energy transfer (FRET) for analysis of protein-protein interactions

Protein-protein interactions usually induce a conformational change in one or both of the proteins. FRET can be used to monitor the interatomic distances of fluorescence-labeled proteins by measuring the energy transfer between appropriate pairs of fluorescent probes covalently linked to the proteins. We investigated conformational changes during GAIT complex assembly using this approach [77]. Briefly, NSAP1 and EPRS linker fused to C-terminus of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), respectively, were co-expressed in U937 cells by nucleofection (Lonza). After recovery, cells were treated with IFN- γ and lysates prepared in CellLytic M buffer. Subsequently, 100 μ l aliquots in a 96-well plate format were used to record CFP and YFP spectra at 460 to 600 nm with 447 nm excitation and 510 to 600 nm with 490 nm excitation, respectively, in a plate reader (SpectraMax). For analysis, signals from control were subtracted, and spectra normalized to CFP emission ⁶) was applied to of a reference spectrum. Finally, the Förster equation, $E = 1/(1+[R/R_0]^6)$ was applied to convert FRET efficiency (E) into apparent distance (R), where R_0 for the CFP/YFP couple is 49 Å. Since CFP and YFP from pAmCyan and pZsYellow are ~25 KDa proteins, their presence at either terminus may influence folding and conformation of the chimeric proteins. To minimize interference, a short spacer peptide is added between CFP/YFP and the fused protein. Alternatively, the influence of CFP/YFP on protein interactions can be determined by other biochemical approaches, e.g., by co-IP and SPR.

8.2e. In vitro translation and functional reconstitution

To investigate the role of P-EPRS linker in assembly of a functional GAIT complex that inhibits translation, we took advantage of *in vitro* translation of a GAIT element-bearing RNA reporter RNA consisting of capped, luciferase (Luc) ORF, followed by 29-nt GAIT element, and a 30-nt poly(A) tail (Luc-Cp-GAIT); T7 gene 10 was used as control RNA. Both RNAs were prepared using mMessage mMachine SP6 and T7 Kits (Ambion), and gel-purified. The GAIT complex was reconstituted *in vitro* by incubating 5 to 40 pmol each of EPRS linker (wild-type, phosphoform, or mutant), NSAP1, GAPDH, and P-L13a. For *in vitro* translation assays, purified reporter Luc-Cp GAIT and T7 gene 10 reporter RNAs (~200 ng of each) were incubated with cytosolic extracts from U937 cells (500 ng protein) or purified GAIT complex constituents (5–40 pmol) in the presence of 1 μ Ci of [³⁵S]methionine and wheat germ extract (Promega), or if necessary, rabbit reticulocyte lysate (RRL, Promega) [16, 77]. The translation reaction was terminated by adding 2X SDS-gel loading dye (Biorad). The stability of the reporter RNAs were assessed by RT-PCR before and after the *in vitro* translation reaction using Luc- and T7 gene 10-specific primers. Following this approach, we successfully, demonstrated the critical requirement of phosphorylation of EPRS (at

Ser⁹⁹) as well as L13a (at Ser⁷⁷) for translational repression of GAIT element-bearing RNA [20, 77, 78].

9. Conclusions

Phosphorylation is emerging as an essential regulatory event during for transformation of EPRS and multiple other AARSs for their execution of noncanonical functions. As examples, phospho-EPRS promotes translation repression for control of inflammatory gene expression; the nuclear pool of phospho-KARS modulates transcription of key genes; and phospho-MARS contributes to adaptive methionylation during oxidative stress [18, 20, 79]. Besides being kinase targets, AARSs can also act as important signaling molecules. For example, LARS is a sensor for amino acid activation of mammalian target of rapamycin (mTOR) signaling, and others are secreted to act as physiocrines [80–84]. The role of AARS phosphorylation in canonical aminoacylation cannot be completely ruled out as UV irradiation-induced Ser⁶⁶² phosphorylation in MARS, and subsequent release of AIMP3, influences global translation inhibition [85]. Nonetheless, these examples underscore the potential of phosphorylation in controlling AARSs noncanonical activities. Moreover, the number of known human AARSs potentially subject to phosphorylation is greater than the 19 principal proteins if one considers more than 60 newly discovered splice variants and the alternatively polyadenylated truncated isoform of EPRS [86, 87]. The experimental methods and approaches described here outline a comprehensive framework for discovery of new functions of AARSs lacking known noncanonical activities, as well as for elucidation of novel AARS phosphorylation events and signaling pathways.

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References

1. Ribas de Pouplana L, Schimmel P. Aminoacyl-tRNA synthetases: potential markers of genetic code development. *Trends Biochem Sci.* 2001; 26:591–596. [PubMed: 11590011]
2. Ibba M, Soll D. Aminoacyl-tRNA synthesis. *Annu Rev Biochem.* 2000; 69:617–650. [PubMed: 10966471]
3. Schimmel P. Aminoacyl tRNA synthetases: general scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. *Annu Rev Biochem.* 1987; 56:125–158. [PubMed: 3304131]
4. Guo M, Schimmel P. Essential nontranslational functions of tRNA synthetases. *Nat Chem Biol.* 2013; 9:145–153. [PubMed: 23416400]
5. Kim S, You S, Hwang D. Aminoacyl-tRNA synthetases and tumorigenesis: more than housekeeping. *Nat Rev Cancer.* 2011; 11:708–718. [PubMed: 21941282]
6. Yao P, Fox PL. Aminoacyl-tRNA synthetases in medicine and disease. *EMBO Mol Med.* 2013; 5:332–343. [PubMed: 23427196]
7. Guo M, Yang XL, Schimmel P. New functions of aminoacyl-tRNA synthetases beyond translation. *Nat Rev Mol Cell Biol.* 2010; 11:668–674. [PubMed: 20700144]
8. Havrylenko S, Mirande M. Aminoacyl-tRNA synthetase complexes in evolution. *Int J Mol Sci.* 2015; 16:6571–6594. [PubMed: 25807264]

9. Rho SB, Lee JS, Jeong EJ, Kim KS, Kim YG, Kim S. A multifunctional repeated motif is present in human bifunctional tRNA synthetase. *J Biol Chem.* 1998; 273:11267–11273. [PubMed: 9556618]
10. Kyriacou SV, Deutscher MP. An important role for the multienzyme aminoacyl-tRNA synthetase complex in mammalian translation and cell growth. *Mol Cell.* 2008; 29:419–427. [PubMed: 18313381]
11. Mirande M. Processivity of translation in the eukaryote cell: role of aminoacyl-tRNA synthetases. *FEBS Lett.* 2010; 584:443–447. [PubMed: 19914240]
12. David A, Netzer N, Strader MB, Das SR, Chen CY, Gibbs J, Pierre P, Bennink JR, Yewdell JW. RNA binding targets aminoacyl-tRNA synthetases to translating ribosomes. *J Biol Chem.* 2011; 286:20688–20700. [PubMed: 21460219]
13. Ray PS, Arif A, Fox PL. Macromolecular complexes as depots for releasable regulatory proteins. *Trends Biochem Sci.* 2007; 32:158–164. [PubMed: 17321138]
14. Lee EY, Lee HC, Kim HK, Jang SY, Park SJ, Kim YH, Kim JH, Hwang J, Kim JH, Kim TH, Arif A, Kim SY, Choi YK, Lee C, Lee CH, Jung JU, Fox PL, Kim S, Lee JS, Kim MH. Infection-specific phosphorylation of glutamyl-prolyl tRNA synthetase induces antiviral immunity. *Nat Immunol.* 2016
15. Clemens MJ. Does protein phosphorylation play a role in translational control by eukaryotic aminoacyl-tRNA synthetases? *Trends Biochem Sci.* 1990; 15:172–175. [PubMed: 2193433]
16. Sampath P, Mazumder B, Seshadri V, Gerber CA, Chavatte L, Kinter M, Ting SM, Dignam JD, Kim S, Driscoll DM, Fox PL. Noncanonical function of glutamyl-prolyl-tRNA synthetase: gene-specific silencing of translation. *Cell.* 2004; 119:195–208. [PubMed: 15479637]
17. Mukhopadhyay R, Jia J, Arif A, Ray PS, Fox PL. The GAIT system: a gatekeeper of inflammatory gene expression. *Trends Biochem Sci.* 2009; 34:324–331. [PubMed: 19535251]
18. Yannay-Cohen N, Carmi-Levy I, Kay G, Yang CM, Han JM, Kemeny DM, Kim S, Nechushtan H, Razin E. LysRS serves as a key signaling molecule in the immune response by regulating gene expression. *Mol Cell.* 2009; 34:603–611. [PubMed: 19524539]
19. Kim DG, Choi JW, Lee JY, Kim H, Oh YS, Lee JW, Tak YK, Song JM, Razin E, Yun SH, Kim S. Interaction of two translational components, lysyl-tRNA synthetase and p40/37LRP, in plasma membrane promotes laminin-dependent cell migration. *FASEB J.* 2012; 26:4142–4159. [PubMed: 22751010]
20. Arif A, Jia J, Mukhopadhyay R, Willard B, Kinter M, Fox PL. Two-site phosphorylation of EPRS coordinates multimodal regulation of noncanonical translational control activity. *Mol Cell.* 2009; 35:164–180. [PubMed: 19647514]
21. Ofir-Birin Y, Fang P, Bennett SP, Zhang HM, Wang J, Rachmin I, Shapiro R, Song J, Dagan A, Pozo J, Kim S, Marshall AG, Schimmel P, Yang XL, Nechushtan H, Razin E, Guo M. Structural switch of lysyl-tRNA synthetase between translation and transcription. *Mol Cell.* 2013; 49:30–42. [PubMed: 23159739]
22. Ray PS, Fox PL. A post-transcriptional pathway represses monocyte VEGF-A expression and angiogenic activity. *EMBO J.* 2007; 26:3360–3372. [PubMed: 17611605]
23. Vyas K, Chaudhuri S, Leaman DW, Komar AA, Musiyenko A, Barik S, Mazumder B. Genome-wide polysome profiling reveals an inflammation-responsive posttranscriptional operon in gamma interferon-activated monocytes. *Mol Cell Biol.* 2009; 29:458–470. [PubMed: 19001086]
24. Jia J, Yao P, Arif A, Fox PL. Regulation and dysregulation of 3'UTR-mediated translational control. *Curr Opin Genet Dev.* 2013; 23:29–34. [PubMed: 23312843]
25. Arif A, Chatterjee P, Moodt RA, Fox PL. Heterotrimeric GAIT complex drives transcript-selective translation inhibition in murine macrophages. *Mol Cell Biol.* 2012; 32:5046–5055. [PubMed: 23071094]
26. Arif A, Jia J, Moodt RA, DiCorleto PE, Fox PL. Phosphorylation of glutamyl-prolyl tRNA synthetase by cyclin-dependent kinase 5 dictates transcript-selective translational control. *Proc Natl Acad Sci USA.* 2011; 108:1415–1420. [PubMed: 21220307]
27. Arif A. Extraneuronal activities and regulatory mechanisms of the atypical cyclin-dependent kinase Cdk5. *Biochem Pharmacol.* 2012; 84:985–993. [PubMed: 22795893]

28. Na YR, Jung D, Gu GJ, Jang AR, Suh YH, Seok SH. The early synthesis of p35 and activation of CDK5 in LPS-stimulated macrophages suppresses interleukin-10 production. *Sci Signal*. 2015; 8:ra121. [PubMed: 26602020]
29. Banks AS, McAllister FE, Camporez JP, Zushin PJ, Jurczak MJ, Laznik-Bogoslavski D, Shulman GI, Gygi SP, Spiegelman BM. An ERK/Cdk5 axis controls the diabetogenic actions of PPARgamma. *Nature*. 2015; 517:391–395. [PubMed: 25409143]
30. Motzik A, Nechushtan H, Foo SY, Razin E. Non-canonical roles of lysyl-tRNA synthetase in health and disease. *Trends Mol Med*. 2013; 19:726–731. [PubMed: 23972532]
31. Dang CV, Traugh JA. Phosphorylation of threonyl- and seryl-tRNA synthetase by cAMP-dependent protein kinase. A possible role in the regulation of P1, P4-bis(5'-adenosyl)-tetraphosphate (Ap4A) synthesis. *J Biol Chem*. 1989; 264:5861–5865. [PubMed: 2925638]
32. Venema RC, Peters HI, Traugh JA. Phosphorylation of elongation factor 1 (EF-1) and valyl-tRNA synthetase by protein kinase C and stimulation of EF-1 activity. *J Biol Chem*. 1991; 266:12574–12580. [PubMed: 2061327]
33. Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci USA*. 2004; 101:12130–12135. [PubMed: 15302935]
34. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, Nardone J, Lee K, Reeves C, Li Y, Hu Y, Tan Z, Stokes M, Sullivan L, Mitchell J, Wetzel R, Macneill J, Ren JM, Yuan J, Bakalarski CE, Villen J, Kornhauser JM, Smith B, Li D, Zhou X, Gygi SP, Gu TL, Polakiewicz RD, Rush J, Comb MJ. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell*. 2007; 131:1190–1203. [PubMed: 18083107]
35. de Graauw M, Hensbergen P, van de Water B. Phospho-proteomic analysis of cellular signaling. *Electrophoresis*. 2006; 27:2676–2686. [PubMed: 16739229]
36. Garrison JC, Wagner JD. Glucagon and the Ca²⁺-linked hormones angiotensin II, norepinephrine, and vasopressin stimulate the phosphorylation of distinct substrates in intact hepatocytes. *J Biol Chem*. 1982; 257:13135–13143. [PubMed: 6290494]
37. Avruch J, Witters LA, Alexander MC, Bush MA. Effects of glucagon and insulin on cytoplasmic protein phosphorylation in hepatocytes. *J Biol Chem*. 1978; 253:4754–4761. [PubMed: 659446]
38. Moorhead GB, Trinkle-Mulcahy L, Ulke-Lemee A. Emerging roles of nuclear protein phosphatases. *Nat Rev Mol Cell Biol*. 2007; 8:234–244. [PubMed: 17318227]
39. Cohen P. The regulation of protein function by multisite phosphorylation--a 25 year update. *Trends Biochem Sci*. 2000; 25:596–601. [PubMed: 11116185]
40. Peck SC. Analysis of protein phosphorylation: methods and strategies for studying kinases and substrates. *Plant J*. 2006; 45:512–522. [PubMed: 16441346]
41. Shi Y, Mowery RA, Ashley J, Hentz M, Ramirez AJ, Bilgic B, Slunt-Brown H, Borchelt DR, Shaw BF. Abnormal SDS-PAGE migration of cytosolic proteins can identify domains and mechanisms that control surfactant binding. *Protein Sci*. 2012; 21:1197–1209. [PubMed: 22692797]
42. Kinoshita E, Kinoshita-Kikuta E, Koike T. Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. *Nat Protoc*. 2009; 4:1513–1521. [PubMed: 19798084]
43. Dephoure N, Gould KL, Gygi SP, Kellogg DR. Mapping and analysis of phosphorylation sites: a quick guide for cell biologists. *Mol Biol Cell*. 2013; 24:535–542. [PubMed: 23447708]
44. Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc Natl Acad Sci USA*. 2009; 106:1760–1765. [PubMed: 19181854]
45. Li M, Cornea RL, Autry JM, Jones LR, Thomas DD. Phosphorylation-induced structural change in phospholamban and its mutants, detected by intrinsic fluorescence. *Biochemistry*. 1998; 37:7869–7877. [PubMed: 9601048]
46. Miranda FF, Teigen K, Thorolfsson M, Svebak RM, Knappskog PM, Flatmark T, Martinez A. Phosphorylation and mutations of Ser(16) in human phenylalanine hydroxylase. Kinetic and structural effects. *J Biol Chem*. 2002; 277:40937–40943. [PubMed: 12185072]

47. Kim YS, Nagy K, Keyser S, Schneekloth JS Jr. An electrophoretic mobility shift assay identifies a mechanistically unique inhibitor of protein sumoylation. *Chem Biol.* 2013; 20:604–613. [PubMed: 23601649]
48. Mazumder B, Sampath P, Seshadri V, Maitra RK, DiCorleto PE, Fox PL. Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. *Cell.* 2003; 115:187–198. [PubMed: 14567916]
49. Agrawal GK, Thelen JJ. A high-resolution two dimensional Gel- and Pro-Q DPS-based proteomics workflow for phosphoprotein identification and quantitative profiling. *Methods Mol Biol.* 2009; 527:3–19. ix. [PubMed: 19241001]
50. Meisrimler CN, Schwendke A, Luthje S. Two-dimensional phos-tag zymograms for tracing phosphoproteins by activity in-gel staining. *Front Plant Sci.* 2015; 6:230. [PubMed: 25926840]
51. van der Geer P, Hunter T. Phosphopeptide mapping and phosphoamino acid analysis by electrophoresis and chromatography on thin-layer cellulose plates. *Electrophoresis.* 1994; 15:544–554. [PubMed: 8055882]
52. Johnson SA, Hunter T. Kinomics: methods for deciphering the kinome. *Nat Methods.* 2005; 2:17–25. [PubMed: 15789031]
53. Witze ES, Old WM, Resing KA, Ahn NG. Mapping protein post-translational modifications with mass spectrometry. *Nat Methods.* 2007; 4:798–806. [PubMed: 17901869]
54. Pearlman SM, Serber Z, Ferrell JE Jr. A mechanism for the evolution of phosphorylation sites. *Cell.* 2011; 147:934–946. [PubMed: 22078888]
55. Stateva SR, Salas V, Benaim G, Menendez M, Solis D, Villalobo A. Characterization of phospho-(tyrosine)-mimetic calmodulin mutants. *PLoS One.* 2015; 10:e0120798. [PubMed: 25830911]
56. Rescher U, Ludwig C, Konietzko V, Kharitonov A, Gerke V. Tyrosine phosphorylation of annexin A2 regulates Rho-mediated actin rearrangement and cell adhesion. *J Cell Sci.* 2008; 121:2177–2185. [PubMed: 18565825]
57. Archuleta AJ, Stutzke CA, Nixon KM, Browning MD. Optimized protocol to make phospho-specific antibodies that work. *Methods Mol Biol.* 2011; 717:69–88. [PubMed: 21370025]
58. Taya Y, Nakajima K, Yoshizawa-Kumagaye K, Tamai K. Generation and application of phospho-specific antibodies for p53 and pRB. *Methods Mol Biol.* 2003; 223:17–26. [PubMed: 12777717]
59. Brumbaugh K, Johnson W, Liao WC, Lin MS, Houchins JP, Cooper J, Stoesz S, Campos-Gonzalez R. Overview of the generation, validation, and application of phosphosite-specific antibodies. *Methods Mol Biol.* 2011; 717:3–43. [PubMed: 21370022]
60. Ubersax JA, Ferrell JE Jr. Mechanisms of specificity in protein phosphorylation. *Nat Rev Mol Cell Biol.* 2007; 8:530–541. [PubMed: 17585314]
61. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science.* 2002; 298:1912–1934. [PubMed: 12471243]
62. Ruvinsky I, Meyuhas O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem Sci.* 2006; 31:342–348. [PubMed: 16679021]
63. Amanchy R, Periaswamy B, Mathivanan S, Reddy R, Tattikota SG, Pandey A. A curated compendium of phosphorylation motifs. *Nat Biotechnol.* 2007; 25:285–286. [PubMed: 17344875]
64. Obenaus JC, Cantley LC, Yaffe MB. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic acids Res.* 2003; 31:3635–3641. [PubMed: 12824383]
65. Rust HL, Thompson PR. Kinase consensus sequences: a breeding ground for crosstalk. *ACS Chem Biol.* 2011; 6:881–892. [PubMed: 21721511]
66. Safaei J, Manuch J, Gupta A, Stacho L, Pelech S. Prediction of 492 human protein kinase substrate specificities. *Proteome Sci.* 2011; 9(Suppl 1):S6. [PubMed: 22165948]
67. Songyang Z, Lu KP, Kwon YT, Tsai LH, Filhol O, Cochet C, Brickey DA, Soderling TR, Bartleson C, Graves DJ, DeMaggio AJ, Hoekstra MF, Blenis J, Hunter T, Cantley LC. A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. *Mol Cell Biol.* 1996; 16:6486–6493. [PubMed: 8887677]
68. Burkhardt AL, Bolen JB. Immune-complex assays for tyrosine protein kinases. *Curr Protoc Immunol.* 2001; Chapter 11(Unit 11):14.

69. Cherkasova VA. Measuring MAP kinase activity in immune complex assays. *Methods*. 2006; 40:234–242. [PubMed: 16890454]
70. Casnellie JE. Assay of protein kinases using peptides with basic residues for phosphocellulose binding. *Methods Enzymol*. 1991; 200:115–120. [PubMed: 1956315]
71. Maly DJ, Allen JA, Shokat KM. A mechanism-based cross-linker for the identification of kinase-substrate pairs. *J Am Chem Soc*. 2004; 126:9160–9161. [PubMed: 15281787]
72. Dias J, Renault L, Perez J, Mirande M. Small-angle X-ray solution scattering study of the multi-aminoacyl-tRNA synthetase complex reveals an elongated and multi-armed particle. *J Biol Chem*. 2013; 288:23979–23989. [PubMed: 23836901]
73. Han JM, Lee MJ, Park SG, Lee SH, Razin E, Choi EC, Kim S. Hierarchical network between the components of the multi-tRNA synthetase complex: implications for complex formation. *J Biol Chem*. 2006; 281:38663–38667. [PubMed: 17062567]
74. Phizicky EM, Fields S. Protein-protein interactions: methods for detection and analysis. *Microbiol Rev*. 1995; 59:94–123. [PubMed: 7708014]
75. Sambrook J, Russell DW. Identification of associated proteins by coimmunoprecipitation. *CSH Protoc*. 2006; 2006
76. Jason-Moller L, Murphy M, Bruno J. Overview of Biacore systems and their applications. *Curr Protoc Protein Sci*. 2006; Chapter 19(Unit 19):13.
77. Jia J, Arif A, Ray PS, Fox PL. WHEP domains direct noncanonical function of glutamyl-Prolyl tRNA synthetase in translational control of gene expression. *Mol Cell*. 2008; 29:679–690. [PubMed: 18374644]
78. Mukhopadhyay R, Ray PS, Arif A, Brady AK, Kinter M, Fox PL. DAPK-ZIPK-L13a axis constitutes a negative-feedback module regulating inflammatory gene expression. *Mol Cell*. 2008; 32:371–382. [PubMed: 18995835]
79. Lee JY, Kim DG, Kim BG, Yang WS, Hong J, Kang T, Oh YS, Kim KR, Han BW, Hwang BJ, Kang BS, Kang MS, Kim MH, Kwon NH, Kim S. Promiscuous methionyl-tRNA synthetase mediates adaptive mistranslation to protect cells against oxidative stress. *J Cell Sci*. 2014; 127:4234–4245. [PubMed: 25097229]
80. Park MC, Kang T, Jin D, Han JM, Kim SB, Park YJ, Cho K, Park YW, Guo M, He W, Yang XL, Schimmel P, Kim S. Secreted human glycyl-tRNA synthetase implicated in defense against ERK-activated tumorigenesis. *Proc Natl Acad Sci USA*. 2012; 109:E640–647. [PubMed: 22345558]
81. Park SG, Kim HJ, Min YH, Choi EC, Shin YK, Park BJ, Lee SW, Kim S. Human lysyl-tRNA synthetase is secreted to trigger proinflammatory response. *Proc Natl Acad Sci USA*. 2005; 102:6356–6361. [PubMed: 15851690]
82. Han JM, Jeong SJ, Park MC, Kim G, Kwon NH, Kim HK, Ha SH, Ryu SH, Kim S. Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. *Cell*. 2012; 149:410–424. [PubMed: 22424946]
83. Bonfils G, Jaquenoud M, Bontron S, Ostrowicz C, Ungermann C, De Virgilio C. Leucyl-tRNA synthetase controls TORC1 via the EGO complex. *Mol Cell*. 2012; 46:105–110. [PubMed: 22424774]
84. McCormick ME, Rojas M, Moser-Katz T, Tzima E, Reader JS. Natural aminoacyl tRNA synthetase fragment enhances cardiac function after myocardial infarction. *PLoS One*. 2014; 9:e109325. [PubMed: 25296172]
85. Kwon NH, Kang T, Lee JY, Kim HH, Kim HR, Hong J, Oh YS, Han JM, Ku MJ, Lee SY, Kim S. Dual role of methionyl-tRNA synthetase in the regulation of translation and tumor suppressor activity of aminoacyl-tRNA synthetase-interacting multifunctional protein-3. *Proc Natl Acad Sci USA*. 2011; 108:19635–19640. [PubMed: 22106287]
86. Yao P, Potdar AA, Arif A, Ray PS, Mukhopadhyay R, Willard B, Xu Y, Yan J, Saidel GM, Fox PL. Coding region polyadenylation generates a truncated tRNA synthetase that counters translation repression. *Cell*. 2012; 149:88–100. [PubMed: 22386318]
87. Lo WS, Gardiner E, Xu Z, Lau CF, Wang F, Zhou JJ, Mendlein JD, Nangle LA, Chiang KP, Yang XL, Au KF, Wong WH, Guo M, Zhang M, Schimmel P. Human tRNA synthetase catalytic nulls with diverse functions. *Science*. 2014; 345:328–332. [PubMed: 25035493]

Highlights

- Phosphorylation is a critical regulator of noncanonical activities of tRNA synthetases.
- Framework of approaches for identification of signaling pathways and phosphorylation events in tRNA synthetases.
- Isotope- and non-isotope-based approaches for detection of phosphorylation events.
- Biochemical and genetic approaches for identification of phospho-sites, signaling mechanisms, proximal kinases, and functional relevance.

Fig. 1.

Workflow used for investigation of AARS phosphorylation sites, the upstream and proximal kinases. Flow-chart illustrates a generic step-by-step approach to identify stimulus-dependent phosphorylation sites and kinases. In parallel we show the specific steps and results used for elucidation of IFN-γ-induced EPRS phosphorylation in myeloid cells, and identification of Cdk5 as Ser⁸⁸⁶ kinase (shaded rounded rectangles). Dashed arrows indicate future unidentified approaches to elucidate unidentified proximal kinase for EPRS Ser⁹⁹⁹ phosphorylation.

Fig. 2. Approaches employed for the identification of IFN- γ and infection-induced phosphorylation events in EPRS, and for the determination of specific role of Ser⁸⁸⁶ and Ser⁹⁹⁹ phosphorylations in the GAIT pathway.

Fig. 3.

Investigation of AARS release from MSC by size fractionation. Theoretical profile shows elution positions of intact MSC and AARSs (or non-AARS constituents) released from MSC. The profile is based on resolution of molecular weight standards on a low-molecular weight cut-off matrix Superdex 200 (approximately 600-10 kDa).

Fig. 4.

Determination of AARS-RNA and AARS-protein interactions by SPR. (Top) Generic diagram showing binding, dissociation, and regeneration phases of analyte binding to immobilized ligand. (Bottom) Biotinylated Cp GAIT element RNA is immobilized on streptavidin-coated SPR sensor. P-EPRS, P-EPRS plus NSAP1, and P-EPRS plus NSAP1 plus P-L13a plus GAPDH are streamed over the sensor in that order, separated by dissociation and chip re-generation phases. Binding is measured as resonance units (RU).