**STK40 Is a Pseudokinase that Binds the E3 Ubiquitin Ligase COP1**

**Highlights**
- STK40 binds the COP1 WD40 domain using a VPD/E motif in its C-terminal tail
- X-ray structure reported for the kinase homology domain of STK40
- STK40 has a partially occluded active site and lacks a DFG motif
- The STK40 kinase homology domain does not bind ATP

**In Brief**
STK40 is a distant homolog of Tribbles proteins required for normal lung maturation. Here, Durzynska et al. show that STK40 binds to the WD40-repeat domain of the E3 ubiquitin ligase COP1 using its C-terminal tail, and report the structure of the STK40 kinase homology domain, which, together with ATP-binding assay results, show why STK40 is a pseudokinase.

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STK40 Is a Pseudokinase that Binds the E3 Ubiquitin Ligase COP1

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SUMMARY

Serine/threonine kinase 40 (STK40) was originally identified as a distant homolog of Tribbles-family proteins. Despite accumulating data attesting to the importance of STK40 in a variety of different physiological processes, little is known about its biological activity or mechanism of action. Here, we show that STK40 interacts with Constitutive Photomorphogenic Protein 1 (COP1), relying primarily on a C-terminal sequence analogous to the motif found in Tribbles proteins. In order to further elucidate structure-function relationships in STK40, we determined the crystal structure of the STK40 kinase homology domain at 2.5 Å resolution. The structure, together with ATP-binding assay results, show that STK40 is a pseudokinase, in which substitutions of conserved residues within the kinase domain prevent ATP binding. Although the structure of the kinase homology domain diverges from the analogous region of Trib1, the results reported here suggest functional parallels between STK40 and Tribbles-family proteins as COP1 adaptors.

INTRODUCTION

Protein kinases regulate complex biological processes, including proliferation, differentiation, and cell death. Approximately 10% of proteins containing kinase homology domains are predicted to be enzymatically inactive (pseudokinases), owing to the absence of at least one of the conserved motifs required for efficient ATP binding or catalysis. Nearly half of these pseudokinases are reported to bind ATP, but the functional role of nucleotide binding in such cases remains unknown (Hammaren et al., 2016; Manning et al., 2002).

STK40 (serine/threonine kinase 40), also known as SgK495 (Sugen kinase 495) or SHIK (SINK-homologous inhibitory kinase), was originally identified as a homolog of Trib3 (SINK) (Huang et al., 2003). Although Tribbles-family (Trib) pseudokinases are the closest relatives of STK40, they share only 20% (Trib1), 19% (Trib2), and 21% (Trib3) sequence identity.

The precise function of STK40 is unknown, but current evidence points toward a role in promoting cell differentiation. For example, studies in knockout mice show that loss of STK40 results in respiratory failure and perinatal lethality due to the failure of maturation in lung epithelial cells, a phenotype similar to that of mice lacking the differentiation factor C/EBPα in respiratory epithelial cells (Martis et al., 2006; Yu et al., 2013). Forced expression of STK40 in embryonic stem cells induces extraembryonic endoderm differentiation, a process that may be mediated through the Erk/MAPK pathway (Li et al., 2010; Zhang et al., 2014). Conversely, knockdown of STK40 in cultured mouse embryonic fibroblasts promotes differentiation into adipocytes, attributed in part to translational control of the levels of C/EBPα and C/EBPδ (Yu et al., 2015). A growing number of studies also suggest roles for STK40 in other cellular contexts. STK40 may be a regulatory target of miR-31, and suppression of STK40 expression correlates with the development of psoriasis and esophageal squamous cell carcinoma in a Zn-deficient rat model (Taccioli et al., 2015; Xu et al., 2013).

Despite this growing evidence attesting to the importance of STK40 in a variety of biological processes, there is no structural and little biochemical evidence to provide a molecular mechanistic understanding of STK40 function. Here, we show that STK40 binds to the COP1 E3-ubiquitin ligase using a C-terminal COP1-binding sequence analogous to motifs present in Tribbles proteins and Jun and ETS transcription factors, and report the crystal structure of the STK40 kinase homology domain to 2.5 Å resolution, which, together with biochemical studies, reveals that STK40 is a pseudokinase. Despite structural divergence of the kinase homology domain from the analogous region of Trib1, the similar modular configuration of a pseudokinase preceding a COP1-binding motif hints at the potential for functional parallels between STK40 and Trib-family proteins.

RESULTS

STK40 Binds to COP1 in Human Cells

In order to begin deciphering the function of STK40, we performed a proteome-wide search for STK40-binding proteins in human cells. We generated HeLa cells stably expressing a full-length STK40 containing N-terminal FLAG and hemagglutinin (HA) tags (Figures 1A and 1B), and then purified STK40...
Flag-GFP-fused peptide derived from STK40 (PEP, amino acids 339–351) was also recovered using anti-FLAG resin. The cleared lysates (Input) and the im-
munoprecipitations carried out in 293T cells. N-terminal Flag-tagged FL, KHD, and STK40 proteins were recovered using anti-Flag resin. A cytoplasmic fraction is listed for each of three independent experiments. Endogenous COP1 peptides recovered from the nuclear fraction are in parentheses.

**Structure**

The putative COP1 binding motif in full-length (FL) STK40 (SSLSGPLOVWPDI) is aligned with that of Trib1 with the critical VP colored in cyan. KHD: kinase homology domain.

The number of unique endogenous COP1 peptides recovered from the inputs of parental HeLa cells and Flag-HA-STK40 transfected HeLa cells is indicated in Table S1. The change in fluorescence polarization is plotted as a function of COP1 protein concentration. Trib1 peptide (FITC-SEIGTSGQIVPEY) is included for comparison.

**Figure 1. STK40 Binds to COP1 in Human Cells**

(A) Schematic diagrams of human STK40 constructs used in the accompanying binding studies. The putative COP1 binding motif in full-length (FL) STK40 (SSLSGPLOVWPDI) is aligned with that of Trib1 with the critical VP colored in cyan. KHD: kinase homology domain.

(B) Results of tandem immunoprecipitation experiments in HeLa cells using STK40 as bait. The number of unique endogenous COP1 peptides recovered from the cytoplasmic fraction is listed for each of three independent experiments. Endogenous COP1 peptides recovered from the nuclear fraction are in parentheses.

(C) Flag-STK40 immunoprecipitations carried out in 293T cells. N-terminal Flag-tagged FL, KHD, and STK40 proteins were recovered using anti-Flag resin. A Flag-GFP-fused peptide derived from STK40 (PEP, amino acids 339–351) was also recovered using anti-FLAG resin. The cleared lysates (input) and the immunoprecipitates (Flag IP) were western blotted and probed with anti-Flag (top) and anti-COP1 antibodies (bottom). The position of endogenous COP1 is indicated by a black arrow.

(D) Fluorescence polarization assay measuring the binding affinity of the human COP1 WD40 (propeller (386–731) for the STK40 peptide FITC-SSLSGPLOVWPDI. The change in fluorescence polarization is plotted as a function of COP1 protein concentration. Trib1 peptide (FITC-SEIGTSGQIVPEY) is included for comparison.

(E) Competition assay comparing binding affinities of STK40 proteins, including the KHD, KHD+20 and STK40 peptides (wild-type PEP and the two STK40 VP mutants labeled m1, m2) to the COP1 WD40 domain. Displacement of the consensus STK40 peptide FITC-SSLSGPLOVWPDI was monitored using fluorescence polarization. The change in polarization is plotted for the indicated competitor as a function of unlabeled competitor concentration. Error bars represent the SEM.

**STK40 Interacts with the WD40 Domain of COP1 via a C-Terminal Binding Motif**

In order to assess further the interaction between STK40 and COP1, we performed Flag immunoprecipitations on HEK293T cells co-transfected with Flag-STK40 and COP1 (Figure 1C). Full-length STK40 indeed associates with full-length COP1 (Figure 1C, lane 5). Domain mapping studies show that the WD40 domain of COP1 is sufficient for binding full-length STK40 (Figure 1C, lane 6). Reassuringly, full-length STK40 also immunoprecipitates with endogenous COP1 (Figure 1C, black arrow).
Alignment of the STK40 and Tribbles sequences reveals a potential COP1-binding motif in STK40 immediately C-terminal to the kinase homology domain (amino acids [aa] 339–351, SSSLGPLQVVPDI) (Figure 1A). This motif aligns well to the COP1-binding sequence of Trib1 and contains the signature "SS" motif. This domain is critical for peptide binding in the kinase homology domain (KHD, 22–339), and C terminus (340–435). The STK40 N lobe (green, residues 35–71) superimposes well with the N lobe of Trib1 (light pink, residues 94–123). The N lobe of STK40 (green, residues 35–71) is in magenta (215–243), analogous to the Lys72-Glu91 interaction in PKA (wheat, residues 42–75) and on the C terminus (340–435). The crystal structure in Figure 2B, blue) includes the entire KHD. The C lobe of STK40 superimposes well with the C lobes of the related pseudokinase Trib1 (green, residues 35–71) on the N lobe of PKA (wheat, residues 42–75) and on the N lobe of Trib1 (light pink, residues 94–123). Coot least squares alignment (LSQ) was used for superpositions. RMSD values are listed.

Structure of STK40

To gain insight into the mechanism of action of STK40, we solved the crystal structure of a portion of the STK40 protein that includes the complete KHD (Figure 2A) to 2.5 Å resolution (Table 1). STK40 adopts the overall architecture of a protein kinase with canonical N- and C-terminal lobes (Figure 2B). The asymmetric unit (ASU) contains four molecules, which superimpose with a backbone root-mean-square deviation (RMSD) of <1.1 Å for the core regions (aa 37–116 and 136–335, using the SSM [secondary structure matching] algorithm) (Krivenski and Henrick, 2004). Note that the extent of disorder varies among the four copies in some regions due to crystal packing interactions (Figure S2); the coordinates from chain A, which exhibits the lowest overall B factors, are used for all figures except one (Figure 3A). The N lobe (Figure 2B, green) consists of a five-stranded anti-parallel β sheet and a single conserved helix, the canonical kinase C helix (Figure 2B, cyan). The helical C lobe (Figure 2B, blue) includes a well-structured activation loop (Figure 2B, pink).

The C lobe of STK40 superimposes well with the C lobes of prototypical kinase domains, represented here by the enzymatically active protein kinase A (PKA) (PDB: 1ATP) (Zheng et al., 1993) and the pseudokinase Trib1 (PDB: SCEM) (Murphy et al., 2015), with backbone RMSD values of 1.4 Å and 1.2 Å, respectively, over 170 residues (SSM, STK40, aa 159–333; PKA, aa 128–298; Trib1, aa 166–339). Surprisingly, the N lobe of STK40 more closely resembles that of the active kinase PKA (N lobe LSQ [least squares superposition] RMSD, 3.6 Å over 37 Cα atoms; Figure 2C) than that of the related pseudokinase Trib1 (N lobe LSQ RMSD, 11.4 Å over 35 Cα atoms; Figure 2D). The αC helix of STK40 is rotated inward, an orientation typically associated with the active state of a true kinase like PKA. This conformation of the C helix is stabilized by a salt bridge between Lys66 on β3 and Glu93 on αC, analogous to the Lys72-Glu91 interaction in PKA (Figure 2C, wheat). In contrast, the STK40 N lobe superimposes poorly onto that of Trib1, primarily because Trib1 contains a truncated, bent αC helix, incapable of forming a
STK40 possesses two atypical sequence insertions when compared with the canonical active kinase PKA. First, there is an extended loop between β4 and β5 (aa 116–135, Figure 3A). This loop, which contains a predicted nuclear localization signal (NLS) (black bracket, Figure 3A), is likely disordered in the protein but is visible in two of the four copies (chains C and D) within the asymmetric unit because of crystal packing interactions as part of an extended β hairpin. A DALI search using the STK40 structure reveals one other distantly related pseudokinase, ROP2 (PDB: 2W1Z), that contains a similar β4 and β5 strand extension (Figure 3B) (Holm and Rosenstrom, 2010; Labesse et al., 2009). In addition, STK40 contains a unique 10 amino acid insert in the hinge region (aa 145–155, Figure 3B). When the structures of PKA and STK40 are superimposed, the unique extended hinge region of STK40 does not align with the hinge region of PKA (Figure 3C, red). Rather, the extended hinge region of STK40 more closely resembles a portion of the C-terminal extension of PKA (residues 316–327, cyan, Figure 3C). This C tail is present in the AGC-family kinases and is important for correct positioning of the C helix in the active conformation (Kannan et al., 2007; Taylor et al., 2013). In STK40, however, it appears to be a structurally analogous element buttressing the N lobe, rather than a modulator of kinase activity (see below).

### STK40 Contains Sequence Substitutions of Kinase Residues Critical for Catalytic Activity

The STK40 KHD maintains the overall fold of an active kinase and the catalytic loop contains the HRD sequence found in active kinases (HRDxKxxN) (Figure 4A). Hydrophobic residues corresponding to the regulatory spine positions of active kinases make packing interactions that stabilize the conformational relationship between the N and C lobes of STK40. However, STK40 bears mutations that degrade key motifs important for kinase activity, consistent with its designation as a pseudokinase. First, the glycine-rich loop (or P loop) of STK40 is highly divergent from canonical kinases, with two of the canonical glycine residues replaced by a serine and a proline (LG to NPS) (Figure 4A). Second, whereas active kinases envelope the adenine ring of ATP using hydrophobic residues (Figure 4B), STK40 contains substitutions in the β2 (V) and β3 (AxK) loops, as the alanine residue in β3 is replaced by glutamine (Q64). Substitution of this crucial position is a sensitive indicator of loss of ATP binding. When ATP is modeled into our STK40 structure, it is evident that Q64 projects into the site normally occupied by ATP in an active kinase (Figures 4B and S3). In ROP2, the analogous E280 also partially occludes the nucleotide binding site (Labesse et al., 2009). In the case of VRK3, the alanine is mutated to a small polar residue, serine (S201), that forms a hydrogen bond with the backbone amide of L262, also resulting in partial occlusion of the ATP-binding site (Scheeff et al., 2009). By contrast, Trib1 has no steric hindrance to ATP binding (Figure 4B). Rather, the C helix is so degraded that the ATP pocket can scarcely be said to exist at all.

Another striking change in the putative ATP-binding pocket in STK40 is the modification of the highly conserved magnesium binding motif (DFG) important for the catalytic function of active kinases. In place of the DFG motif, STK40 has an NFC binding site (Labesse et al., 2009). In ROP2, the analogous E280 also partially occludes the nucleotide binding site (Labesse et al., 2009). In the case of VRK3, the alanine is mutated to a small polar residue, serine (S201), that forms a hydrogen bond with the backbone amide of L262, also resulting in partial occlusion of the ATP-binding site (Scheeff et al., 2009). By contrast, Trib1 has no steric hindrance to ATP binding (Figure 4B). Rather, the C helix is so degraded that the ATP pocket can scarcely be said to exist at all.

Another striking change in the putative ATP-binding pocket in STK40 is the modification of the highly conserved magnesium binding motif (DFG) important for the catalytic function of active kinases. In place of the DFG motif, STK40 has an NFC sequence, eliminating the charged aspartate participating in magnesium coordination. In addition, the glycine is replaced by a cysteine (C218), contributing to further degradation of the site for ATP binding and phosphate transfer (Figures 4A and S3). The related Trib1 KHD harbors an unusual SLE sequence in this position, which also disrupts the magnesium ion-coordination site.

To determine whether the purified STK40 pseudokinase domain binds ATP or the promiscuous ATP competitor staurosporine at various concentrations, we performed a fluorescent thermal shift assay using the crystallized construct (residues 22–339).
The serine/threonine maternal embryonic leucine zipper kinase (MELK), used as a positive control, binds ATP in a magnesium-dependent manner with a thermal shift (ΔTm) of 2.0–5.5°C, and exhibits a thermal shift of 15–16°C upon binding staurosporine (Figures 4C and 4D, white bars). In contrast, neither Trib1 pseudo-kinase (gray bars, used here as a negative control) nor STK40 (black bars) exhibits a significant thermal shift in the presence of magnesium-ATP or staurosporine. These results are consistent with a prior report showing no ATP binding by a partially purified full-length STK40 at a single ATP concentration (0.2 mM) (Murphy et al., 2014).

DISCUSSION

Here, we demonstrate an interaction in human cells between the pseudokinase STK40 and the E3 ubiquitin ligase COP1. We have determined that the STK40 C-terminal peptide (339–351) recapitulates binding in a purified system and contributes to binding in cells, and that the COP1 WD40 domain is necessary and sufficient for binding. These results echo our recent findings for the binding of the pseudokinase Trib1 to the WD40 domain of COP1 (Uljon et al., 2016), although the functional significance of the STK40-COP1 interaction is still unknown. Intriguingly, the observation that both STK40 and Tribbles pseudokinases bind to the same site on the COP1 WD40 domain, together with the lack of evidence that either is a target of COP1-mediated degradation, raises the possibility that STK40 and Tribbles may act as adaptors for COP1 substrate selection or as regulators of COP1 ligase activity.

The structure of the STK40 KHD reveals the position of key kinase motifs otherwise not readily identifiable by multiple sequence alignment due to its sequence divergence. Even though substitutions of key residues conserved in active kinases degrade the ATP-binding site and render the protein incapable of ATP binding, it is remarkable that the fold of STK40 nevertheless closely resembles that of an active kinase and differs notably from its closest structural pseudokinase relative Trib1, especially in the conformation of the αC helix in the N lobe (Figures 2C and 2D). The substitutions responsible for inactivating the ATP-binding site are evolutionarily conserved in the furthest identifiable STK40 ortholog in frogs, indicating that loss of kinase activity occurred early in evolution.

Figure 3. Atypical Sequence Insertions in STK40 Structure
(A) Structural and sequence alignment of the β4-β5 loop extension. Left: STK40 N lobe (chain D) β4-β5 loop (green) is aligned to the PKA short connecting loop (wheat), with the predicted NLS indicated. Right: sequence insertion of STK40 aligned to selected active kinases: PKA (PDB: 1ATP), EGFR (PDB: 1M14), Chk1 (PDB: 1A18), CaMKII (PDB: 2V26), Aurora-A (PDB: 2WTV), CASK (PDB: 3MFS); and pseudokinases: Trib1 (PDB: 5CEK), VRK3 (PDB: 2JII), TYK2 (PDB: 3ZON), HER3 (PDB: 4RIW), ILK (PDB: 3REP), STRAD (PDB: 3GNI), ROP2 (PDB: 2W1Z). Brackets indicate predicted nuclear localization signal (NLS).
(B) Sequence alignment of hinge loop insertion. Residues flanking the unique hinge insertion in STK40 are aligned to analogous residues in known structures as in (A).
(C) Structure of the hinge region of STK40 (red arrow) compared with the hinge region (red arrow on PKA) and the C-terminal tail of PKA (cyan). Coot secondary structure alignment was used for superpositions.
STK40 contains two unusual sequence insertions, one in the N lobe between β4 and β5 and another in the hinge loop. Within the β4 and β5 insertion, we identify a potential NLS. The loop is highly solvent exposed and two serine residues present upstream of the NLS could function as a switch for cellular localization of STK40 by undergoing phosphorylation to neutralize the positive change of the putative NLS, although it remains to be determined whether this predicted NLS motif is active in cells and whether the phosphorylation of the upstream serines plays a role in nuclear localization. The hinge insertion of STK40 is highly unusual and no other structures containing a hinge insertion were found in a DALI search. We speculate that it serves a purely structural role in stabilizing the conformation of the N lobe.

Overall, the resemblance of the STK40 global fold to that of the active kinase PKA in the closed state is remarkable, but belies its inability to bind ATP. Because our structure contains only the KHD, and our ATP-binding experiments employ the crystallized construct, we cannot rule out the possibility that full-length STK40, with or without cellular partners, may in some cases function as an active kinase. However, the most parsimonious interpretation is that STK40 belongs to the growing list of inactive pseudokinases that act as scaffolds to alter the activity of their binding partners.
was subsequently used as a search model for molecular replacement after re-processing the same dataset with Xia2 (Winter et al., 2013), which improved data processing and scoring over iMosflm processing. During refinement of the molecular replacement model, a dataset from a crystal grown using SeMet-labeled STK40 was collected at APS beamline 24-ID-E, which was used to validate the molecular replacement solution we initially obtained for the native dataset. Selenium atoms were located by generating a difference map using the CAD and FFT modules in ccp4, where all structure factors and model phases were obtained from the native set-based solution and only anomalous difference factors were taken from the SeMet data (Pottorff et al., 2003). This step identified the positions of 15 of the 24 SeMet sites (the remaining sites lie in unstructured regions of the four molecules in the asymmetric unit). Manual building and refinement of the model from the native dataset were carried out in Coot using 2Fo − Fc, Fo − Fc, and composite-omit maps. Refinement (xyz coordinates, individual B factors, NCS restraints, TLS parameters) was carried out using Phenix refine (Papini et al., 2009). Search parameters included trypsin specificity with up to two missed cleavages, fixed carbamidomethylation (C, +57 Da), and variable oxidation (M, +16 Da). Precursor and product ion mass tolerances were set to 15 ppm and 0.02 Da, respectively. The search databases consisted of human protein sequences (downloaded from RefSeq on 11 July 2011) and protein sequences of factors that are common lab contaminants both appended to their own decoy database. Sequence matches to the decoy databases were used to implement a global 1% false discovery rate filter for the resulting peptide identifications. A fast peptide matching algorithm was used to map peptide sequences to all possible human genes (Askenazi et al., 2010). We discarded candidate proteins that were detected in a large compendium of negative TAP controls with a frequency greater than 1% (Rozenblatt-Rossi et al., 2012). Three sets of negative control and STK40 samples were independently purified from cytoplasmic and nuclear extracts and analyzed by liquid chromatography (LC)-MS/MS. Tryptic peptides for one pair of negative control and STK40 samples purified from cytoplasmic extracts were analyzed in two replicate LC-MS/MS runs.

Fluorescence Polarization Assay
Fluorescence polarization assays were performed as previously described (Uijiton et al., 2016). Briefly, for the initial binding experiment, 25 nM of fluorescein isothiocyanate (FITC)-STK40 or Trib1 peptide was mixed with increasing concentrations (0–10.5 μM) of purified COP1 WD40 domain (386–731) in HBS-P buffer (GE Healthcare Life Sciences; catalog no. BR100368) supplemented with 1 mM TCEP and 0.02% Tween 20. FITC-labeled and unlabeled competing peptides were obtained from LifeTein at a purity of 96%, as assessed by reverse-phase high-performance liquid chromatography (HPLC), and used without further purification. KHD and KHD+20 STK40 constructs used in the competition assay were purified to homogeneity. In the competition assay concentrations of 25 nM FITC-peptide and 1,500 nM COP1 WD40 (386–731) were used. Experiments were conducted in a 30 μL well volume in 384-well plates. Polarization values were read at 538 nM on a Spectramax M plate reader (Molecular Devices). Plots present data from three independent experiments. Error bars represent SEM.

Tandem-IP Experiments
HeLa cell lines were transduced with retroviruses expressing the STK40 full-length protein, and a stable cell line was generated by sorting for co-expressed interleukin-2 receptor (IL2R). For each proteomic experiment, the STK40 stable cell line, together with HeLa controls, was grown in 15 cm plates to confluence and harvested. After Dounce homogenization and separation of the nuclear and cytoplasmic fractions by centrifugation, Flag immunoprecipitations were performed using Flag-conjugated agarose beads (Sigma; catalog no. A2220) in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP40, and 10% glycerol supplemented with EDTA-free protease inhibitor tablets (Roche) and phosphatase inhibitor cocktail (Cell Signaling Technology). The beads were washed three times and the immunoprecipitated protein was eluted with Flag peptide (Sigma; catalog no. F3290). The eluate was then subjected to a second HA pull-down using similar conditions, and HA-bound protein and its interactors were eluted with HA peptide.

Tandem affinity purified (TAP) samples were reduced with 10 mM DTT for 30 min at 56°C in the presence of 0.1% RapiGest SF (Waters). Cysteines were alkylated with 22.5 mM iodoacetamide for 20 min at room temperature in the dark. Samples were digested overnight at 37°C with 4 μg trypsin (Promega). Tryptic peptides were acidified and purified by batch mode reversed phase and strong cation-exchange chromatography (Advion et al., 2012). Purified peptides were loaded onto a precolumn (4 cm POROS 10F2; Applied Biosystems) and eluted with an HPLC gradient (NanoAcuity UPLC system, Waters; 2%–35% B in 45 min; A = 0.2 M acetic acid in water, B = 0.2 M acetic acid in acetonitrile) (Ficarro et al., 2009). Peptides were resolved on a self-packed analytical column (50 cm Monitor C18; Column Engineering) and introduced in the mass spectrometer (Q-Exactive HF; Thermo Scientific) equipped with a Digital PicoView electrospray source platform (New Objective; electrospray ionization spray voltage = 2.8 kV). The mass spectrometer was programmed to perform data-dependent tandem mass spectrometry (MS/MS) on the ten most abundant precursors in each MS1 scan using higher energy dissociation (HCD with 30% normalized collision energy). MS spectra were converted into a Mascot generic file format (.mgf) using multiplexier scripts (Parik et al., 2009). Search parameters included tryptic specificity with up to two missed cleavages, fixed carbamidomethylation (C, +57 Da), and variable oxidation (M, +16 Da). Precursor and product ion mass tolerances were set to 15 ppm and 0.02 Da, respectively. The search databases consisted of human protein sequences (downloaded from RefSeq on 11 July 2011) and protein sequences of factors that are common lab contaminants both appended to their own decoy database. Sequence matches to the decoy databases were used to implement a global 1% false discovery rate filter for the resulting peptide identifications. A fast peptide matching algorithm was used to map peptide sequences to all possible human genes (Askenazi et al., 2010). We discarded candidate proteins that were detected in a large compendium of negative TAP controls with a frequency greater than 1% (Rozenblatt-Rossi et al., 2012). Three sets of negative control and STK40 samples were independently purified from cytoplasmic and nuclear extracts and analyzed by liquid chromatography (LC)-MS/MS. Tryptic peptides for one pair of negative control and STK40 samples purified from cytoplasmic extracts were analyzed in two replicate LC-MS/MS runs.
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REFERENCES


