JLP (JNK-associated leucine zipper protein) is a scaffolding protein that interacts with various signaling proteins associated with coordinated regulation of cellular process such as endocytosis, motility, neurite outgrowth, cell proliferation, and apoptosis. Here we identified PLK1 (Polo-like kinase 1) as a novel interaction partner of JLP through mass spectrometric approaches. Our results indicate that JLP is phospho-primed by PLK1 on Thr-351, which is recognized by the Polo box domain of PLK1 leading to phosphorylation of JLP at additional sites. Stable isotope labeling by amino acids in cell culture and quantitative LC-MS/MS analysis was performed to identify PLK1-dependent JLP-interacting proteins. Treatment of cells with the PLK1 kinase inhibitor BI2536 suppressed binding of the Forkhead box transcriptional repressor to JLP. JLP was found to interact with PLK1 and FOXK1 during mitosis. Moreover, knockdown of PLK1 affected the interaction between JLP and FOXK1. FOXK1 is a known transcriptional repressor of the CDK inhibitor p21/WAF1, and knockdown of JLP resulted in increased FOXK1 protein levels and a reduction of p21 transcript levels. Our results suggest a novel mechanism by which FOXK1 protein levels and activity are regulated by associating with JLP and PLK1.

Assembly of protein complexes that mediate responses to extracellular stimuli is critical to ensuring the execution of specific signaling pathways. Scaffolding proteins play key roles in this process by recruiting and tethering the proteins that convey these messages within the cell (1). One such well studied scaffolding protein family is the family of JNK-interacting proteins (JIPs). The JIP family consists of four members: JIP1, JIP2, JIP3, and JLP (2). Of all the members characterized in the JIP family, JLP is characterized by the presence of two leucine zipper domains and a JNK binding domain in its N terminus and a conserved C-terminal domain, all of which functions in binding to and forming specific cell signaling complexes (3). Although JLP is ubiquitously expressed, previous studies have shown that the protein plays a role in regulating neurite outgrowth by interacting with SCG10 (4), activating JNK/p38 MAPK signaling (3), regulating cell migration by interacting with Gα12 and Gα13 (5, 6), and functioning in vesicle transport by binding to kinesin light chain (KLC1) and dynein microtubule motor proteins (7). Recent studies have identified that JLP and JIP3 can interact with ADP-ribosylation factor 6 (ARF6), a small G protein that function in the regulating cytokinesis by transporting recycling endosomes to the cleavage furrow (8). This study showed that binding of ARF6 to JLP or JIP3 causes a switch in the binding to dynein as opposed to kinesin and that this pathway functions in the transport of dynein cargo proteins out of the midbody during cytokinesis (8). Studies following this using JIP3/JLP double knock-out mouse embryonic fibroblasts have shown that these cells, when cultured in vitro, have an increased frequency of binucleate cells (9). In addition, this study demonstrated the importance of JLP in ARF6 localization to the midbody and efficient cytokinesis (9). These studies uncovered a role for JLP in the cell cycle, which was not previously reported for other JIP proteins.

In this study, we used a mass spectrometry-based approach to identify JLP-associated proteins and identified the mitotic kinase, PLK1 (Polo-like kinase 1), as a novel interaction partner of JLP. PLK1 belongs to the Polo-like kinase family of Ser/Thr kinases and functions as a proto-oncogene, with increased regulation of FOXK1 by associating with JLP-PLK1 complex.
expression in various cancer types (10, 11). The protein has an N-terminal conserved kinase domain that functions in phosphorylating substrates by promoting a transient interaction with the protein (12, 13). In addition, the C-terminal Polo box domain (PBD), which recognizes and binds phospho-primed substrates, regulates substrate specificity and localization of the protein during mitosis (14–18). PLK1 has been characterized to phosphorylate and regulate the function of many proteins during the G2/M phase, ensuring smooth progression of mitosis (19). Here we show that JLP interacts with the Polo box domain of PLK1 in a phosphorylation-dependent manner. In addition, we provide evidence that the interaction of JLP with PLK1 results in the recruitment of the transcription factor FOXK1 (Forkhead box protein K1) during mitosis, and this JLP-regulated function is required for down-regulation of FOXK1 protein expression and activity.

Experimental Procedures

Plasmids—The expression vectors encoding murine S-tagged full-length JLP, JLP domains, and shJLP have been described previously (3, 7). Expression vectors for FLAG-hJLP were constructed by subcloning PCR fragments into the EcoRI/XhoI sites of the pCMV-Tag2B (Agilent Technologies) vector. PLK1 expression vectors were constructed by subcloning PCR fragments of the PLK1 cDNA IMAGE clone into the pHM6-HA vector. Site-directed mutagenesis of JLP (T351A, T334A, and 2TA) and PLK1 (H538A/K540M) was performed using QuikChange II XL kit according to the manufacturer’s instructions (Agilent Technologies). Wild-type FLAG-FOXK1, R127A, H355A, and the wild-type FLAG-FOXK2 plasmids have been described previously (20, 21). The FOXK1 domain mutants (I, II, and III) were constructed by subcloning PCR-amplified fragments of the FLAG-FOXK1 cDNA into the parental vector.

Antibodies and Reagents—The JLP-specific antibody has been described previously (3). Antibodies against PLK1, GAPDH, HA tag, FLAG tag, Cyclin E, S tag, Myc tag, and p21 were obtained from Santa Cruz Biotechnology. Antibodies against FOXK1, FOXK2, BubR1, and T7 tag were obtained from Cell Signaling Technology and Abcam. On-Target plus Smart pool siPLk1, siFOXK1, siFOXK2, and siScramble were obtained from Cell Signaling Technology and Abcam. Stock solutions of BI2536 (Selleckchem), RO3306 (Tocris), and nocodazole (Sigma) were purchased from GE Dharmacon. Stock solutions of DUO92101 (Sigma). Coverslips were mounted on the slide using DUOLINK in situ mounting medium with DAPI. Images were obtained using a Carl Zeiss Axiolmager Z1 and processed with Zen pro 2012 imaging software (Zeiss).

Kinase Assays—Kinase assays were performed as described previously (22). Briefly, 10 ng of His-PLK1 (PV3501; Invitrogen) or GST-PLK2 (PV4204; Invitrogen) was incubated with 2 μg of purified substrate, and ATP mix (10 μM cold ATP, 30 μCi of [γ-32P]ATP) for 30 min at 30 °C. Where required, kinase was preincubated with indicated concentration of inhibitor for 30 min at room temperature before the addition of substrate and ATP mix. The reactions were terminated by the addition of sample buffer and resolved by SDS-PAGE. The gels were Coomassie-stained, dried, and subjected to autoradiography.

Cell Synchronization and Flow Cytometry—To synchronize cells in the G1 phase, cells grown to 60% confluence were washed with PBS and released in medium containing 0.1% serum for 24 h. Where indicated, the cells were treated with 2 μg/ml aphidicolin (A0781; Sigma) or 1 μM nocodazole overnight to synchronize cells in the S and G2/M phases, respectively. For double thymidine block, HeLa cells were treated with 2 mM thymidine for 18 h and released into complete medium for 9 h followed by a second round of treatment with 2 mM thymidine for 18 h. Cells were released into complete medium and harvested at the indicated time points. For flow cytometric analysis, cells were harvested by trypsinization and fixed for 15 min using 0.5% paraformaldehyde at room temperature. Fixed cells were resuspended in ice-cold 90% methanol added drop-
wise. Cells were then blocked in 2% BSA in PBS and stained with anti-pHis H3 antibody (Millipore) overnight at room temperature. The cells were then stained for 2 h using an Alexa Fluor 488-conjugated secondary antibody prior to staining with propidium iodide to quantitate DNA. DNA content was measured using a BD-FACSCalibur (BD Biosciences). Cell cycle distribution was analyzed using FlowJo (Treestar).

SILAC Labeling—HEK293T cells were grown for six generations in heavy and light amino acid medium prior to transfection with FLAG-JLP plasmid. Cells were subjected to treatment as indicated and lysed in a maitoside-based lysis buffer. 2 mg of cell lysate was incubated with FLAG-conjugated beads for 1 h at 4 °C, and bound proteins were eluted through treatment with 10 μM urea. The eluted protein was digested for 4 h with 0.2 μg of Lys-C (Wako) and then for 14 h with 0.2 μg of Trypsin (Promega). The samples were desalted using Vivapure C18 microspin columns and lyophilized. Mass spectrometric analysis of the lyophilized peptides was performed by the Proteome Exploration Laboratory, California Institute of Technology as described previously (23).

Results

Polo-like Kinase 1 (PLK1) Is a Novel Interaction Partner of JLP—We undertook a mass spectrometry-based approach to identify novel JLP interaction partners. To that end, pulldown assays were performed from cells transfected with an expression vector encoding S-tagged JLP or a parental control plasmid. The protein complexes were resolved by SDS-PAGE, stained with Coomassie Blue. As shown in Fig. 1A, there were a number of bands specifically detected in the JLP-S-tagged precipitates. These bands were excised, and protein constituents were determined by LC-MS/MS (Fig. 1A). The predominant peptides identified in bands labeled 1, 2, and 4 were JLP itself, likely because of proteolytic cleavage of the full-length protein. However, band 3 was made up of peptides derived from the

mitotic serine/threonine kinase PLK1. To validate the novel JLP-PLK1 interaction, we subjected the S tag pulldown precipitates to immunoblotting using a PLK1-specific antibody. As shown in Fig. 1B, PLK1 was specifically precipitated by JLP but not in the vector control sample. To determine whether endogenous JLP associates with PLK1, HeLa cell extracts were incubated with either JLP or PLK1-specific antisera, and the resulting immunoprecipitates were subjected to Western blot analysis. The results of this study (Fig. 1C) show that both proteins associate in reciprocal co-immunoprecipitation assays, thereby confirming that endogenous JLP and PLK1 associate in cells. The JLP family of scaffolding proteins contains four members, JIP1, JIP2, JIP3, and JLP. Of these, JIP3 is highly homologous to JLP in its sequence and domain structure (3), which suggests that it might interact with PLK1. We therefore performed FLAG pulldown assays from cells overexpressing either FLAG-tagged JIP3 or JLP and subjected the precipitates to Western blot analysis using PLK1-specific antisera. Although JLP readily precipitated PLK1, we failed to detect any associated PLK1 protein in the JIP3 sample (Fig. 1D).

To determine whether JLP interacts with PLK1 during mitosis, HeLa cells were synchronized in S phase using a double thymidine block and released into the cell cycle in the presence of DMSO or nocodazole. Cells were then harvested at the indicated time points to determine the level of JLP-PLK1 complexes as a function of cell cycle progression. The results of this study showed that interaction of JLP with PLK1 increases significantly as cells enter the mitotic phase (Fig. 2, A and B). To further demonstrate that JLP and PLK1 associate during mitosis, we also performed an in situ proximity ligation assay, which detects protein-protein interactions with high degrees of specificity and sensitivity. HeLa cells incubated with JLP and PLK1 antisera that recognize the endogenous proteins emitted a strong proximity ligation assay signal only in mitotic cells (Fig. 2C). Background signals observed in interphase cells were comparable with the negative control (Fig. 2C), suggesting that JLP and PLK1 proteins interact predominantly during mitosis.

The JLP protein has several functional domains, including two leucine zipper domains, a JNK binding domain, and a C-terminal domain, which mediate protein-protein interactions (3, 27). To map the region of JLP that interacts with PLK1, HEK293T cells were transfected with S-tagged WT and domain mutants of JLP (Fig. 1E). Pulldown experiments showed that in addition to the full-length (WT) protein, PLK1 interacts with the N-terminal domain of JLP, which spans two leucine zipper domains and the JNK binding domain (amino acids 1–463), but not with any of the individual domains within the N-terminal region of JLP or with the C terminus of the protein (domain III and CTD) (Fig. 1, E and F). To map the region of PLK1 that interacts with JLP, cells expressing HA-tagged wild-type and domain mutants of PLK1 (Fig. 1G) were subjected to pulldown assays using HA-agarose and subsequent immunoblotting with JLP-specific antisera. Our results show that JLP interacts with the full-length (WT) PLK1 protein and the domain mutant expressing the PLK1 Polo box domain (PBD amino acids 306–603) (Fig. 1, G and H), but not with the kinase domain (protein kinase domain amino acids 1–316). To confirm that the JLP N-terminal domain and the PLK1 Polo box domain interact, we
performed additional immunoprecipitation assays using extracts derived from cells overexpressing a S-tagged version of the JLP N-terminal domain (amino acids 1–463) and a HA-tagged PLK1 Polo box domain mutant (amino acids 306–603). As shown in Fig. 1 (I and J), reciprocal pulldown experiments followed by Western blot analyses confirmed the association between the two domains.

The Interaction between JLP and PLK1 Is Phosphorylation-dependent—It has previously been established that the PBD of PLK1 is involved in recognizing and interacting with phospho-primed substrates (15). Because we determined that JLP binds to the PLK1 PBD (Fig. 1H), we performed experiments to determine whether the interaction is phosphorylation-dependent. JLP was immunoprecipitated from asynchronous cell lysates and the immunocomplex was treated with λ-phosphatase to dephosphorylate the proteins. Following treatment with phosphatase, the precipitate was washed rigorously to remove any unbound proteins, and the level of associated PLK1 was determined by Western blot analysis. As can be seen in Fig. 3A, treatment with λ-phosphatase greatly reduced the interaction between JLP and PLK1, suggesting that the interaction is dependent on phosphorylation. We next performed additional interaction studies using HA-tagged WT PLK1 and a PBD mutant in which two residues that are critical for recognizing phospho-primed substrates, His-538 and Lys-540, were mutated to Ala and Met, respectively. Fig. 3B shows that although WT PLK1 readily associated with JLP, the H538A/K540M PBD mutant (H/K) failed to do so. These experiments provide additional evidence that the JLP-PLK1 interaction is phosphorylation-dependent.
The Polo box domain of PLK1 is known to initiate interactions by recognizing phosho-primed sites on its substrates with a consensus binding motif of Ser-[Ser(P)/Thr(P)]-Pro (15, 16). The protein sequence of JLP was analyzed for the presence of this consensus site, and a corresponding motif was identified at amino acid residues Thr-334 and Thr-351 within the JLP N-terminal region. To test whether this motif functions as a PLK1 docking site, these two residues were mutated to Ala either individually (T334A and T351A) or in combination (2TA) in the JLP N-terminal domain plasmid and tested for their ability to bind to PLK1 in pulldown assays. The results of this study showed that although mutation of JLP at residue Thr-334 did not affect binding to PLK1, mutation at Thr-351 (T351A), either alone or in the 2TA mutant, abolished this association (Fig. 3C). Similar results were obtained using cells expressing FLAG-tagged, full-length versions of the wild-type or T351A JLP proteins (Fig. 3D). In addition, the reciprocal experiment, whereby endogenous PLK1 was immunoprecipitated from cells expressing FLAG-tagged WT and JLP-T351A mutant plasmids, also confirmed that wild-type JLP, but not the T351A mutant, associated with PLK1 (Fig. 3E). Taken together, these studies suggest that phosho-priming of JLP at Thr-351 is required for its interaction with PLK1.

Previous studies have shown that CDK1 serves as a priming kinase for PLK1 (15). There have also been several reports suggesting that PLK1 can also serve as a priming kinase that stabilizes its interaction with other proteins (28–30). To determine whether these kinases play a role in priming JLP for PLK1 binding, HeLa cells synchronized in mitosis were treated with BI2536 (PLK inhibitor), RO3306 (CDK1 inhibitor), CX4945 (CK2 inhibitor), or DMSO for 2 h. Nocodazole was used to synchronize cells in the mitotic phase of the cell cycle, where PLK1 and CDK1 are expressed in their active state (31–33). Whole cell extracts were prepared and subjected to immunoprecipitation using a PLK1 antibody. Treatment with BI2536, but not the other kinase inhibitors, inhibited the association of JLP with PLK1 (Fig. 3F), suggesting that PLK1 creates its own docking site on JLP to initiate the interaction. PLK1, when bound to substrates through its PBD, catalyzes their phosphorylation on the (D/E)X[S/T]/H9021X(DE) (where X indicates any amino acid, and Φ indicates a hydrophobic amino acid) consensus sequence (13). We performed in vitro kinase assays using recombinant Hist-PLK1 and GST-PLK2 to determine whether JLP is a substrate of one or both kinases. Kinase assays showed that PLK1, but not PLK2, can specifically phosphorylate JLP (Fig. 3G). Furthermore, this phosphorylation is inhibited by
Because these studies indicated that JLP is a substrate of PLK1, we mapped the phosphorylation sites on JLP using a series of GST-tagged JLP deletion mutants as substrates in *in vitro* kinase assays. As shown in Fig. 3H, JLP fragments, amino acids 355–559 and 763–966, were phosphorylated by PLK1, suggesting that JLP contains multiple PLK1 phosphorylation sites.

**Interaction of JLP with PLK1 Recruits FOXK1 to the Complex**—Having determined that JLP interacts with and undergoes phosphorylation by PLK1, we next sought to determine whether PLK1-promoted phosphorylation of JLP regulates the association of this scaffolding protein with other binding factors. To that end, SILAC and a quantitative solution-based LC-MS/MS interaction proteomics analysis (34, 35) were performed in cells grown in media containing heavy or light isotope amino acids (Fig. 4A). HEK293T cells transfected with WT HA-PLK1 or the PLK1 H538A/K540M (H/K) mutant. HA-PLK1 was precipitated using HA beads and immunoblotted with JLP-specific antiserum. C, HEK293T cells were co-transfected with plasmids encoding HA-tagged PLK1 PBD and the indicated S-tagged JLP N-terminal domain plasmids. Lysates were subjected to pulldown assay using HA beads, and the level of associated S-tagged JLP N-terminal domain was determined by immunoblotting. D and E, lysates from HEK293T cells transfected with FLAG-tagged wild-type JLP and the T351A mutant were subjected to pulldown assays using FLAG beads (D) or immunoprecipitation using a FLAG antibody (E) or a control IgG antibody. The immunocomplexes were analyzed by Western blotting using the indicated antiserum. F, HEK293T cells were treated overnight with 0.5 μM nocodazole followed by a 2-h treatment with 2 μM BI2536, 9 μM RO3306, 2 μM CX4945, or DMSO prior to harvesting. PLK1 was immunoprecipitated from the cell lysates, and the level of associated JLP was analyzed by Western blotting. G, purified His-tagged JLP protein was subjected to *in vitro* kinase assay using recombinant PLK1 or PLK2 in the presence of 0.1 μM BI2536 or DMSO. The reactions were resolved by SDS-PAGE, fixed in Coomassie stain, and subjected to autoradiography. H, individual regions of JLP protein (200 amino acids in length) were expressed as bacterial GST fusion proteins. The purified GST-tagged JLP protein domains were subjected to *in vitro* kinase assays using recombinant PLK1. The reactions were resolved on a SDS-PAGE and subjected to autoradiography. IB, immunoblot; IP, immunoprecipitation.
identified two transcription factors, FOXK1 and FOXK2, that specifically co-precipitated with JLP in mock treated cells (Table 1; 22 and 5 spectra, respectively) but not in cells that were treated with PLK inhibitor, BI2536 (0 spectra for both FOXK1 and FOXK2). To validate association of JLP with FOXK1 and FOXK2, we performed pulldown assays from cell lysates derived from asynchronous and G2/M-arrested HEK293T cells expressing FLAG-tagged FOXK1 and FOXK2 proteins. Immunoblotting showed that FLAG-FOXK1 and FOXK2 interact with both JLP and PLK1, predominantly in cells arrested in the G2/M phase (Fig. 4B), which express the highest levels of PLK1. Similar results were obtained using
nocodazole-treated cells that overexpressed Myc-tagged JLP and FOXK1 or FOXK2 (Fig. 4, C and D), which confirmed that JLP and PLK1 interact with FOXK1 or FOXK2 in G2/M phase cells.

To determine whether the interaction between JLP and FOXK1 is a cell cycle phase-specific event, HeLa cells overexpressing FLAG-tagged FOXK1 were synchronized in the G1, S, or G2/M phase as described under "Experimental Procedures." Cell lysates were subjected to pulldown assays using FLAG-agarose, and the precipitates were analyzed by immunoblotting. FOXK1 has a conserved DNA-binding domain (DBD 305–400 amino acids) and an N-terminal Forkhead association (FHA) domain (123–175 amino acids), the latter of which functions in recognizing and binding Thr(P) residues (37, 38). To map the region(s) with which JLP and PLK1 interact, we overexpressed WT and domain mutants of FOXK1 (I, II, and III) and subjected the cell lysates to pulldown assays using FLAG beads. Our results show that although JLP strongly interacts with the domain mutant I containing the FHA domain and to a lesser extent with the DBD-containing domain mutant II (Fig. 5, C and D), PLK1 associates with the FOXK1 domain II, which contains the DBD (Fig. 5, C and D). PLK1 has been shown to interact with and phosphorylate several FOX transcription factors, including FOXO1, FOXO3, and FOXM1 (39–41). To determine whether FOXM1 can also form a ternary complex with JLP and PLK1, we performed pulldown assays using cell

FOXK1 forms a ternary complex with JLP and PLK1 during mitosis. A, HeLa cells transfected with FLAG-tagged FOXK1 were synchronized in the G, S, or G2/M phase as described under "Experimental Procedures." Cell lysates were subjected to pulldown assays using FLAG-agarose, and the precipitates were analyzed by immunoblotting. * indicates non-specific band. B, schematic representation of the FOXK1 protein domains used in the pulldown assays. Amino acid numbers are indicated. D, whole cell extracts isolated from HEK293T cells transfected with Myc-tagged JLP, wild-type FLAG-tagged FOXK1 and the indicated domain mutants were subjected to pulldown assays using FLAG beads. Protein complexes were resolved by SDS-PAGE and subjected to immunoblotting using the indicated antisera. V, vector; IB, immunoblot.
extracts derived from cells expressing FOXM1, PLK1, and JLP. The results of this study showed that PLK1, but not JLP, could interact with FOXM1 (Fig. 5E), suggesting that the interaction of FOXK1 with the JLP-PLK1 complex is highly specific.

Interaction of JLP with FOXK1 Is PLK1-dependent—Mass spectrometric data from the FLAG-JLP pulldown assay showed a loss of FOXK1 in the BI2536-treated sample (Table 1). To further investigate this observation, mitotic cells overexpressing FLAG-tagged FOXK1 were either mock treated or treated with BI2536 for 2 h prior to being subjected to pulldown assays using FLAG beads. The resulting precipitates were subjected to immunoblotting using the indicated antibodies. D, HeLa cells expressing shScramble (shS) and shJLP were transfected with a FLAG-FOXK1 expression vector, arrested in mitosis, and subjected to pulldown assay using FLAG beads. The levels of associated proteins were determined by Western blot analysis using the indicated antibodies. E, siControl (siCnt) and siPlk1 cells were transfected with a FLAG-FOXK1 expression plasmid, treated with nocodazole for 16 h prior to harvesting, and subjected to pulldown assay using FLAG beads. Associated proteins were examined by immunoblot (IB) analysis.

Within the FOX family of transcription factors, FOXK1 and FOXK2 are the only two proteins that have an N-terminal FHA domain in addition to the highly conserved winged helix DNA-binding domain. Because the FHA domain functions in recognizing and binding Thr(P) residues (42) and because our results show that the FHA domains interact with JLP (Fig. 5, C–D), we sought to determine whether disrupting function of the FHA domain would result in changes in its ability to form the ternary complex. To that end, Arg-127 in the FOXK1 FHA domain, which mediates the recognition and association with Thr(P) residues, was mutated to Ala (R127A). As a control, His-355 in the winged helix domain of FOXK1, which is important for the DNA binding function of the protein, was mutated to Ala (H355A) (Fig. 6C). Pulldown assays from cells overexpressing wild-type and mutant FLAG-FOXK1 proteins showed that although the WT and H355A-FOXK1 proteins retained the ability to form a complex with PLK1 and JLP, the R127A mutant failed to do so (Fig. 6C). This suggests that the FOXK1 FHA domain recognizes a phosphorylation site(s) on JLP when interacting with these proteins. Taken together, our results suggest that interaction of JLP with FOXK1 is dependent on phos-
JLP Forms a Ternary Complex with PLK1 and FOXK1

To test the hypothesis that the formation of the ternary complex is PLK1-dependent, we performed interaction studies in cells where the expression of JLP or PLK1 was knocked down using shRNA or siRNA technology. HeLa cells expressing shJLP or scramble control (shS) were transfected with FLAG-tagged FOXK1, synchronized in mitosis using nocodazole, and subjected to pulldown assay using FLAG beads. Knockdown of JLP did not affect the ability of PLK1 to interact with FOXK1 (Fig. 6D). However, a parallel experiment whereby HeLa cells that overexpressed FLAG-tagged FOXK1 were transfected with a PLK1 siRNA or control siRNA for 24 h showed that the level of associated JLP with FLAG-FOXK1 was significantly reduced in the siPLK1 cells (Fig. 6E), confirming that PLK1 is required for the formation of a JLP-PLK1-FOXK1 ternary complex.

Knockdown of JLP Leads to an Increase in FOXK1 Protein Levels—FOXK1 has been shown to promote myocyte proliferation by recruiting transcriptional co-repressor complexes to regulate gene transcription (43), as well as by binding to and inhibiting transcription factors involved in differentiation (44). To determine whether JLP affects FOXK1 activity, we knocked down JLP expression in U2OS osteosarcoma cells using shRNA and examined the effects on FOXK1 activity. Loss of JLP expression (shJLP) resulted in an increase in and a reduction in FOXK1 and p21 protein levels, respectively (Fig. 7A). qPCR analysis showed that there is a significant reduction in p21 mRNA levels ($p < 0.02$) in shJLP cells compared with the control cells (Fig. 7B). Furthermore, no significant differences in FOXK1 mRNA levels were observed (Fig. 7B), suggesting that knockdown of JLP affects FOXK1 protein levels. We also performed qPCR analysis in siControl and siFOXK1 cells, which showed a significant increase in p21 relative expression levels ($p = 0.0005$) upon knockdown of FOXK1 (Fig. 7C). These results are consistent with previous reports that demonstrated FOXK1-mediated repression of p21 gene expression and increased proliferation in the C2C12 myoblast cell line (45). Furthermore, we performed immunoblotting of cell lysates derived from asynchronous and mitotic cells expressing control

![Image](https://example.com/image.png)

**FIGURE 7. Knockdown of JLP results in an increase in FOXK1 protein levels.** A, U2OS cells were transduced with shScramble (shS) or shJLP lentiviruses. Protein lysates from these cells were immunobotted (IB) using the indicated antisera. B and C, quantitative mRNA expression (qPCR) of differentially expressed genes in control (shS, B) and shJLP cells and in siControl and siFOXK1 cells (C). All results were normalized to $\beta$-actin expression and graphed as average fold change ($\pm$ S.E.) for two independent experiments performed in triplicate. *, $p < 0.02$; **, $p = 0.0005$. D, shS and shJLP U2OS cells were treated overnight with 1 $\mu$M nocodazole (Noc) or DMSO as a control. Nocodazole-treated cells were subjected to mitotic shake-off, and cell lysates from the mitotic cells and DMSO-treated cells were analyzed by immunoblotting using the indicated antisera. E, model depicting the interaction of JLP with PLK1 and FOXK1. PLK1 serves as the priming kinase of JLP and interacts with the N-terminal domain of JLP, resulting in phosphorylation of JLP at additional sites. FOXK1 is recruited to bind JLP in a PLK1-dependent manner, which regulates FOXK1 protein levels and the transcript levels of its downstream targets.
(shS) and shJLP. As shown in Fig. 7D, knockdown of JLP resulted in increased levels of FOXK1 and a reduction in p21 levels in both cell types. Because our results show an increase in FOXK1 protein levels in shJLP cells (Fig. 7, A and D), they suggest that JLP can potentially regulate the stability and/or degradation of FOXK1 protein, in turn regulating the levels of its transcriptional targets.

Discussion

JLP is a JNK scaffolding protein that binds to signaling proteins and regulates various developmental processes such as neurite outgrowth, myogenesis, TGN transport, and cytokinesis (2). In this study, using a mass spectrometric approach, we have identified the mitotic Ser/Thr kinase, PLK1 as a JLP-associated protein. Our results presented here show that the interaction of JLP with PLK1 is phosphorylation-dependent (Figs. 3, A and B, and 7E) and that phosphorylation of JLP Thr-351 is required for this association (Figs. 3, C–E, and 7E). This interaction of PLK1 with JLP appears to be unique among the JIP family of scaffolding proteins, because JIP3, a member of this family that is highly homologous to JLP (3) fails to interact with PLK1 (Fig. 1D). Furthermore, our results suggest that PLK1 serves as the priming kinase that phosphorylates JLP (Fig. 3F) and uses this phosphorylated site for binding and phosphorylating additional sites in JLP (Figs. 3, G and H, and 7E). Because JLP knockdown by shRNA did not result in an interruption of cell cycle progression (data not shown), we reasoned that the interaction between JLP and PLK1 might result in the recruitment of additional protein partners to the JLP-PLK1 complex. SILAC and quantitative LC-MS/MS analysis was performed to identify PLK1-dependent JLP interacting proteins (Table 1), and we identified that the FOXK1 transcription factor interacts with the JLP-PLK1 complex in a phosphorylation-dependent manner during mitosis. Our pulldown assays show that PLK1 interacts with the FOXK1 DNA-binding domain, whereas JLP interacts with the FHA domain (Fig. 5D), suggesting that PLK1 phosphorylates JLP, and this phosphorylation site is recognized by the FOXK1 FHA domain. Our observation that inhibition of PLK1 kinase activity by BI2536 (Fig. 6A) or knockdown by siRNA (Fig. 6E) affects the interaction between JLP and FOXK1 highlights the importance of PLK1 kinase activity in the formation of the JLP, PLK1, and FOXK1 ternary complex (Fig. 7E). Recent studies have shown that the FOXK transcription factors can interact with different protein complexes when they are “on” or “off” the chromatin (46, 47). This study suggests that the recruitment of co-regulatory proteins to the transcription factors determines their transcriptionally dependent and independent functions. Furthermore, FOXK1 has been shown to interact with and function in the nuclear translocation of the WNT scaffolding protein DVL (Dishevelled), resulting in the activation of WNT/β-catenin pathway (48). Our results show that JLP interacts with FOXK1 predominantly during mitosis, and knockdown of JLP results in an increase in FOXK1 protein levels and a reduction in p21 mRNA and protein levels (Fig. 7, A and B). This suggests that the increase in the FOXK1 protein levels could be the result of changes in the stability or degradation of the protein, which is mediated by JLP, but the detailed mechanisms remain to be elucidated.

PLK1 has been shown to phosphorylate and regulate the localization and transcriptional function of various FOX transcription factors, FOXM1 (39), FOXO1 (40), and FOXO3 (41). Although FOXK1 is implicated as a regulator of myogenic proliferation (21, 43–45), little is known about its function during mitosis. Previous studies have shown that CYCLIN-CDK complex phosphorylates FOXK2, a protein that is highly homologous to FOXK1, during mitosis and that such phosphorylation regulates its stability and transcriptional function (20). Because our studies show the mitotic kinase, PLK1 to interact with FOXK1 and FOXK2, and we observed potential phosphorylation of FOXK1 during mitosis (data not shown), a similar mechanism of regulation might exist.

In summary, we report for the first time that JLP can interact with a mitotic kinase, PLK1, and that this interaction results in recruitment of a transcription factor, FOXK1, to form a ternary complex during mitosis (Fig. 7E). Although there are many reports suggesting a role for FOXK1 in proliferation and maintenance of myogenic progenitor cells (21, 43, 44), the mechanism by which FOXK1 functions during this process is not well defined. Our study describes a potentially novel mechanism by which FOXK1 regulates the cell cycle-dependent gene expression via association with a scaffolding protein, JLP.

Author Contributions—P. R. designed the study, conducted most of the experiments, and wrote the paper. E. P. R. designed the study, helped in the interpretation of results and wrote the paper. C. M. L. constructed the JLP-S plasmds and contributed to Figs. 3, 5, and 7. A. M., M. J. S., S. H., and D. S. H. performed SILAC labeling, mass spectrometry, data analysis, and writing of the paper. A. D. S. provided the FOXK vectors and helped in the interpretation of FOXK1 results, drafting, and critical review of the manuscript. All authors reviewed the results and approved final version of the manuscript.

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