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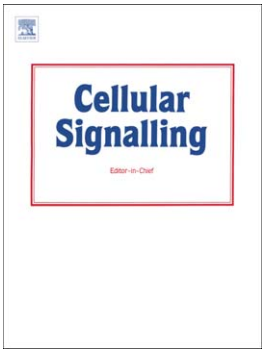

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JIP4 is a PLK1 binding protein that regulates p38MAPK activity in G2 phase

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Abstract

Cell cycle progression from G2 phase into mitosis is regulated by a complex network of mechanisms, all of which finally control the timing of Cyclin B/CDK1 activation. PLK1 regulates a network of events that contribute to regulating G2/M phase progression. Here we have used a proteomics approach to identify proteins that specifically bind to the Polobox domain of PLK1. This identified a panel of proteins that were either associated with PLK1 in G2 phase and/or mitosis, the strongest interaction being with the MAPK scaffold protein JIP4. PLK1 binding to JIP4 was found in G2 phase and mitosis, and PLK1 binding was self-primed by PLK1 phosphorylation of JIP4. PLK1 binding is required for JIP4-dependent p38MAPK activation in G2 phase during normal cell cycle progression, but not in either G2 phase or mitotic stress response. Finally, JIP4 is a target for caspase-dependent cleavage in mitotically arrested cells. The role for the PLK1-JIP4 regulated p38MAPK activation in G2 phase is unclear, but it does not affect either progression into or through mitosis.

Keywords: JIP4; PLK1; p38MAPK; G2 phase; mitosis

1. Introduction

Entry into mitosis is at its most basic level controlled by the activation of Cyclin B/CDK1. Cyclin B/CDK1 is regulated in a complex manner, but the final step in its regulation is the removal of the inhibitory phosphorylation of Thr14 and Tyr15 on the CDK1 subunit by the dual specificity CDC25 phosphatases. The three members of this family CDC25A,B,C have all been demonstrated to have roles in progression into mitosis, although CDC25B appears to be most responsible for the timing of Cyclin B/CDK1 activation and progression into mitosis [1-4]. The inhibitory CDK1 Tyr15 phosphorylation is primarily regulated by the Wee1 kinase, and inhibition of WEE1 is sufficient to prematurely drive cells in mitosis [5]. Multiple mechanisms operate upstream of WEE1 and CDC25 to regulate this final step in response to a range of different stimuli and stresses. Many of the mechanisms identified appear to directly or indirectly regulate CDC25B activity. DNA damage can utilise ATM/ATR checkpoint signaling to block entry into mitosis, in part through inhibition of CDC25-dependent dephosphorylation [1]. The p38MAPK pathway acting through MAPKAPK2 (MK2) is also a critical regulator of sustained G2 phase arrest in response to DNA damage, acting through cytoplasmic factors [6, 7], and in response to stresses such as osmotic shock can also directly phosphorylate and regulate CDC25B activity and stability [8, 9]. Acute activation of MEK/ERK signaling in G2 phase similarly delays entry into mitosis by destabilizing CDC25B [10]. Cyclin A/CDK2 is a regulator of the timing of Cyclin B/CDK1 activation in both normal progression into mitosis and recovery from a G2 phase checkpoint arrest, and is the target of CDC25B [2, 11].

PLK1 is required for normal entry into mitosis and recovery from the checkpoint arrest [12, 13]. It regulates G2 phase progression via a number of mechanisms. It regulates WEE1 stability [14], Cyclin B1 nuclear localization [15] and FoxM1 transcriptional activity that regulates the expression of a large number of mitotic regulators [16]. Much of the

function and substrate specificity of PLK1 is conferred by its Polobox domain (PBD) which binds primarily to mitotic phospho-Ser/Thr residues [17] and confers functional specificity [18, 19]. A number of studies have taken a proteomics approach to identify mitotic PLK1 PBD-dependent binding partners and substrates [20, 21]. However, these studies have identified few novel regulators of G2/M phase progression. This suggests that the PLK1-dependent regulation of progression into mitosis involves binding of specific targets during G2 phase. Here we have used a proteomics approach to identify proteins that bind to the PLK1 PBD during G2 phase that may contribute to the PLK1-regualted G2 phase progression.

2. Materials and Methods

2.1 Cell culture and synchrony

HeLa, U2OS, HEK293T and HaCaT cell lines were cultured in DMEM (Gibco/Sigma) supplemented with 10% FBS (Bovogen), 2 mM L-glutamine (Gibco/Sigma), 1mM sodium pyruvate (Gibco/Sigma), and 20 mM Hepes (Gibco/Sigma). Asynchronously growing cells were synchronized to G2 phase by single thymidine block (2.5 mM) for 16-18 hours, released into DMEM further supplemented with 24 mM Thymidine and 24 mM 2-deoxycytidine and harvested 8 hours post release. For mitotic block 0.5 mg/ml nocodazole (Sigma) was added to asynchronously growing cells for 24 hours and harvested by shake off. For osmotic shock experiments, D-sorbitol (Sigma) was added to cells for a final concentration of 0.5M 30 minutes prior to harvesting. For flow cytometery, cells were fixed in 70% EtOH at -20°C overnight then stained with propidium iodide (2ug/ml propidium iodide, 0.5mg/ml RNase A

in PBS). DNA profiles of cells were analysed by FACS (BD FACSCanto) with G1, S, and G2/M populations quantified by analysis of histograms generated by FlowJo data analysis software (Ashland, Oregan, USA).

2.2 Immunoblotting

Cells were harvested and lysed on ice for 30 min with NETN lysis buffer (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.5% IGEPAL CA-630, 0.5 mM DTT, 1mM Na_3VO_4, 1 mM PMSF, 10 mM NaF, 25 mM β-glycerophosphate, and protease inhibitor cocktail (Sigma)) with protein concentration determined by Bradford assay. Protein concentrations were equalized with addition of NETN lysis buffer, 3x SDS sample buffer (187.5mM Tris-HCl pH6.8, 6% SDS, 30% glycerol, 120mM DTT) for a final concentration of 1x SDS sample buffer. Protein samples were separated by SDS-PAGE and semi-dry transferred onto PVDF membranes (Millipore/Pierce). Membranes were blocked with 5% non-fat milk/TBST and probed with antibodies against Plk3, Phospho-cdc2 (Tyr15), JIP4/SPAG9, p38MAPK, phospho-p38MAPK, phospho-MAPKAPK-2 (Thr222), PARP, Wee1, phospho-MEK1 (Thr286) (all from Cell Signaling Technologies), GFP (Roche), Plk1 (Calbiochem), phospho-Plk1 (T210) and 53BP1 (BD Pharmingen), PCNA (DAKO), a-tubulin (Sigma). Horseradish Peroxidase-conjugated secondary antibodies were applied to detect bound primary antibody and visualized using enhanced chemiluminescence.

2.3 Immunofluorescence

Asynchronously growing HeLa cells were seeded onto glass coverslips, fixed with 4% PFA and permeabilised with 0.25% Triton X-100 followed by blocking with 3% BSA/0.1%

Saponin. Coverslips were incubated with primary antibodies JIP4/SPAG9 (Cell Signaling Technologies) and Plk1 (Calbiochem), diluted in blocking buffer and incubated overnight at 4°C. Fluorescently conjugated secondary antibodies Alexa Fluor 488 (Invitrogen) and DyLight 649 (Abcam) were incubated together with DAPI for 1 hour at room temperature and cells imaged by fluorescence microscopy. Time lapse microscopy was performed as reported previously [22].

2.4 Affinity pull down and Immunoprecipitation

Asynchronously growing HeLa cells were transfected with pEGFP-C1 (Clontech) and pEGFP-C1-Plk1-PBD expression vectors using Lipofectamine 2000 (Invitrogen) according to manufactures instructions. Transfected cells were harvested alongside G2 synchronised HeLa cells, lysed with NETN lysis buffer (250 mM NaCl) and protein concentration determined by Bradford Assay. Samples were pre-cleared with Protein G Sepharose (Sigma) for 1 hour at 4°C with the EGFP and EGFP-PBD samples subsequently applied to GFP-CAP agarose beads for 1hour at 4°C. The GFP-CAP agarose beads were washed with NETN lysis buffer yielding GFP and GFP-PBD bound agarose beads. These were used to bind proteins from a pre-cleared G2 phase or mitosis synchronised HeLa cell lysates by incubating with the beads overnight at 4°C and beads washed in NETN lysis buffer with the final wash containing 500mM NaCl. Bound proteins were eluted by boiling in SDS sample buffer and analysed by Western blot and Mass Spectrometry.

For reciprocal JIP4/Plk1 immunoprecipitations, G2 synchronised HeLa cell lysates (1mg) pre-cleared Protein G-sepharose then JIP4/SPAG9 or Plk1 antibodies, and the corresponding species non-immune IgG antibodies were incubated with the samples overnight at 4°C. Protein G-sepharose was then added to each IP, incubated overnight at 4°C followed by 5

washes in NETN lysis buffer. Bound proteins were eluted by boiling in SDS sample buffer and analysed by Western blot.

2.5 Transient Transfections

For over expression experiments vectors pRcCMV-Plk1-wt and pRcCMV-Plk1 T210D, pEGFP-C1 (Clontech) and pEGFP-C1-Plk1-PBD were transfected into asynchronous HEK293T and HeLa cells using Lipofectamine 2000 (Invitrogen) according to manufactures instructions. The following day, cells were synchronised using a single thymidine block-release and harvested as the cells entered G2 phase. For siRNA knockdown, JIP4 ON-TARGETplus SMARTpool and Plk1 siGENOME SMARTpool (Dharmacon) and a non-targeting siRNA 5'- ACUCUUAACAGGUAGAUCAUU were transfected into asynchronous U2OS and HeLa cells using Lipofectamine 2000 (Invitrogen) according to manufactures instructions, then synchonised as above. In some experiments 1 mM Plk1 inhibitor BI 2536 was added to cells 2 hours prior to harvest for G2 phase.

2. 6 Mass Spectrometry analysis

SDS PAGE protein bands were excised and subjected to robot assisted in-gel digest. Gel slices were destained (90 mins, 50% acetonitrile, 25 mM NH_4HCO_3), dehydrated in speed vac, rehydrated in reducing buffer (30 min, 20 mM DTT 50mM NH_4HCO_3 at 37^{o}C), then alkylation buffer (20 min, 50 mM iodoacetamide) and overnight digest with 0.4ug trypsin in 10% acetonitrile, 25 mM NH_4HCO_3. Trypsin was inactivated with 5% Formic acid and peptides eluted with 1% formic acid, 60% acetonitrile. The extracted peptides were dried using a speed vac and resuspend in 5% formic acid for mass spec analysis. Samples were

analysed on an Agilent HPLC CHIP QTOF 6520. Peptides were loaded onto an Agilent G4240-62010 Large Capacity Chip with 3% buffer B at 4 μl/min. Buffer A and B were 0.1% formic acid and 0.1% formic acid in 90% acetonitrile respectively. The peptides were separated at 0.3 μl/min over a 45 min gradient 5% to 50% Solvent B, the column was washed with 95% B for 6mins and equilibrated at 5% B for 7 mins. Ms1 acquired ions from 100m/z to 3200 m/z at a rate of 8 spectra/sec. MS2 acquired ions from 59m/z to 3200m/z at a rate of 4spectra/s. The mass spectrum data was process with Spectrum Mill Rev B.04.00.127 (Agilent). Data were extracted as per standard settings and searched against Swiss Prot Human (version 05/14/2014). Search parameters were set to digest with Trypsin with a maximum of 2 miscleavages. Peptide modifications were set to fix carbamidomethylation C and variable oxidized M. Tolerance was set to 50% minimum matched peak intensity, with 20 ppm and 50 ppm precursor and product mass tolerance respectively. Proteins were summarized from IDs with the following cut-off settings: protein score of >11, peptide score>10, 60% scored peak intensity and false discovery rate of >0.5%.

3. Results

3.1 JIP4 binds to PLK1 in G2 phase

PLK1 has roles in progression into and through mitosis, readily observed using the small molecule PLK inhibitor BI-2536 (Figure 1A, B). Over-expression of the Polobox domain (PBD) of PLK1 as a GFP fusion protein was sufficient to promote the delay (Figure 1B), suggesting that PBD binding to cellular regulators was likely to be a significant contributor to the G2 phase delay observed.

In an effort to indentify G2 phase PLK1 binding proteins, mass spectrometry analysis of proteins bound to the GFP-PBD was performed. GFP or GFP-PBD was expressed in

HeLa cells, the GFP affinity purified using GFP-Trap beads (Supplementary Figure S1). Lysates of synchronised G2 phase or mitotic HeLa cells were then incubated with the GFP loaded beads to affinity purify PBD binding proteins. The beads were washed and eluted using SDS sample buffer then resolved by SDS-PAGE and visualized by colloidal Coomassie stain. Gel slices were excised, digested with trypsin and the release peptides subjected to tandem mass spectrometry analysis to identify proteins specifically associated with GFP-PBD. A number of proteins were consistently found specifically associated with the GFP-PBD (Table 1). The MAPK scaffold protein JIP4/SPAG9 was consistently the most abundantly associated protein in both G2 phase and mitotic samples (Table 1; Supplementary Table S1). A number of proteins previously identified in screens for mitotic interactors with PLK1 were also identified. These include 53BP1, RANBP2 [20, 23], and PRKDC/DNA-PK catalytic subunit [24], although these were found only in the G2 phase samples. No other high confidence G2 phase PLK1 interactors were identified. Several previously identified interactors were also identified that were mitosis-specific, including SPAG5/Astrin [25], ERCC-6L/PICH and NEDD1[23, 26, 27], BICD2 [28], and BORA [23]. In addition, several novel PLK1-interacting proteins were identified; ATP5A1, ANKRD28/PP6-ARS-A, PPP6R2, TuFM/EF-Tu, HSPA9 and HNRNPF, although several of these have been previously identified as be PLK1 substrates (Table 1), but were found associated with the GFP-PBD in both G2 phase and mitotic samples.

JIP4 was of particular interest as it is a MAPK scaffold protein, a member of the JNK-interacting protein (JIP) family that is specifically responsible for p38MAPK activation [29], suggesting it may link PLK1 and p38MAPK-dependent regulation of G2 phase progression. PBD binding of a number of these proteins was validated by immunoblotting. JIP4 and 53BP1 bound to GFP-PBD beads but not control GFP beads, validating the mass spectrometry results (Figure 2a). Wee1 was used as a further control for PLK1 binding as it

has previously been reported to be phosphorylated by PLK1[14]. . interaction between JIP4 and PLK1 was confirmed by by co-immunoprecipitation experiments using both antibodies (Figure 2b). Next we evaluated the distribution of JIP4 relative to PLK1 during cell cycle. Immunofluorescent staining showed JIP4 to be predominantly cytoplasmic in interphase cells, whereas PLK1 showed stronger nuclear staining, although some cytoplasmic staining was evident (Figure 2c). As cells progressed into mitosis, PLK1 displayed a strong accumulation on the mitotic spindle and on the midbody in cytokinesis (Figure 2c-e). JIP4 showed diffuse staining that surrounded the spindle structures and was excluded from the DNA, but failed to strongly localise on the spindle or midbody as seen with PLK1.

The interaction of PLK1 and JIP4 in G2 phase suggested that JIP4 may have a cell cycle specific function, and be responsible for G2 phase p38MAPK activity. Hence we examined the level of JIP4 protein during cell cycle by immunoblotting and found JIP4 was relatively unchanged through the cell cycle in synchronised HeLa cells (Figure 3a). This paralleled the relatively unchanged level of activated p38MAPK across the cell cycle. Mitosis was identified by the accumulation of phospho-Mek1 Thr286, a Cyclin B/CDK1 substrate and marker of mitosis [30]. The binding of PLK1 to its substrates can be regulated by phosphorylation of the PBD binding site on the substrate protein by either PLK1 itself or another Pro-directed kinase such as CDK1/2, termed self and non-self priming, respectively [31]. JIP4 immunoprecipitates from G2 phase HeLa cells treated with inhibitors of either Cdk1, Cdk2 or PLK1 revealed a loss of associated PLK1 only with PLK1 inhibitor treatment (Figure 3B). The binding of PLK1 appeared to be specific as the closely related Plk3 was not found associated with JIP4. As previously reported, although JIP4 acts as an activation scaffold for p38MAPK, it does not stably associate with the kinase and no p38MAPK was detected in immunoprecipitated JIP4 complexes (Figure 3b).

3.2 JIP4-PLK1 regulates G2 phase p38MAPK activation

The potential role for JIP4 in regulating G2 phase p38MAPK was assessed by depleting JIP4 using specific siRNA in several cell lines. SiRNA depletion of >85% of JIP4 in G2 phase HeLa cells revealed a >50% reduction in activated phospho-p38MAPK (pp38MAPK) levels (Figure 4a). The reduction in p38MAPK activation was also found in U2OS cells, the extent of reduction reflecting the degree of JIP4 depletion in this cell line (Figure 4a). The contribution of PLK1 to JIP4-dependent p38MAPK activation in G2 phase was examined by over-expressing either wild type or the constitutively activated Thr210Asp (T210D) mutant of full length PLK1, or the polobox binding domain (PBD) GFP fusion protein in HEK293T and HeLa cells. These were synchronised into G2 phase using thymidine block release then harvested and immunoblotted for p38 activation. Over-expression of PLK1 wild type and T210D mutant increased pp38MAPK levels modestly, but PBD-GFP expression reduced pp38 levels by 50% in both cell lines (Figure 4b; Supplementary Figure S2a). The effect of PLK1 T210D was always larger than the wild type PLK1, although over-expression of the wild type PLK1 was sufficient to drive its phosphorylation on the activating Thr210 site. SiRNA depletion of PLK1 similarly reduced G2 phase pp38MAPK levels (Supplementary Figure S2b), and treatment with the PLK1 inhibitor BI-2536 had no extra effect over JIP4 depletion on G2 phase p38MAPK activation (Supplementary Figure S2c). These data suggests that the JIP4 scaffold function for p38MAPK activation was regulated by PLK1in G2 phase. PLK1 phosphorylation of a number of proteins targets them for destruction via $SCF^{\beta TrcP}$ [32-34], but neither PLK1 inhibitor nor siRNA depletion of PLK1 had any noticeable effect on G2 phase JIP4 levels (Supplementary Figure S2d).

Inhibition of p38MAPK has been reported to delay G2/M phase progression in unstressed conditions [35, 36], as does inhibition of PLK1 (Figure 1). Our data demonstrates

that PLK1-JIP4 is required for p38MAPK activation in G2 phase, suggesting that JIP4 scold function may be required for normal G2/M progression and mitosis. The effect of JIP4 depletion on cell cycle progression into and through mitosis was assessed by siRNA depletion of JIP4 in synchronised HeLa cells, and following these cells into mitosis. JIP4 depletion had no effect on progression into mitosis whereas inhibition of PLK1 delayed entry significantly (Figure 4c), and was no effect of JIP4 depletion on either the duration of mitosis or the structure of the mitotic apparatus (data not shown). However, that pharmacological inhibition of p38MAPK activity using concentration of inhibitor sufficient to inhibit >90% of its kinase activity (Supplementary Figure S3a) had no effect on mitotic entry (Figure 4c), suggesting that complete inhibition/depletion of p38MAPK activity may be required to observe the reported effects on progression into mitosis. This indicates that although PLK1 regulated G2 phase p38 MAPK activity through JIP4, this did not contribute significantly to the normal progression into or through mitosis.

3.3 JIP4 does not contribute to stress activated p38MAPK

P38MAPK is activated in response to a range of stresses including osmotic shock [37]. While osmotic shock of G2 phase cells promoted a very strong increase in p38MAPK activation, JIP4 depletion had only a small effect on this activation (Figure 5a). Likewise over expression of either wild type or constitutively activated T210D PLK1, or PBD-GFP had little effect on p38MAPK activation in either G2 phase osmotically shocked HEK293 (Figure 5b) or HeLa cells (Supplementary Figure S3b).

P38MAPK is also reported to have a role in the mitotic arrest imposed through activation of spindle assembly checkpoint that detects spindle defects [36]. SiRNA depletion of JIP4 had no effect on either the stability of mitotic arrest imposed by nocodazole in either

HeLa or U2OS cells, indicated by the constant pMEK Thr286 levels in the nocodazole treated samples (Figure 6a). JIP4 depletion also had no effect on p38MAPK activation in mitotic arrested cells. There was a reduction in the level of JIP4 protein in nocodazole treated samples, with the emergence of lower molecular weight bands. These were reduced in the JIP4 siRNA depleted cells indicating they were JIP4 truncation products (Figure 6a). The reduction of full length JIP4 and increase in the lower molecular weight bands was also found with PLK1 inhibitor BI2536 in HeLa and HaCaT cells (Supplementary Figure S4). The appearance of the shorter JIP4 species was associated with PARP cleavage, a Caspase 3-dependent event in apoptosis (Figure 6a). A time course of thymidine-synchronised cells arrested in mitosis with nocodazole showed that the shorter JIP4 species was temporally correlated with PARP cleavage (Figure 6b). The shorter forms of JIP4 appeared to be a product of caspase action as the pan-caspase inhibitor zVAD at concentrations that partially inhibit caspase-dependent PARP cleavage also increased the level of full length JIP4 in the nocodazole arrested cells, and decreased the level of the shortest JIP4 species (Figure 6c).

4. Discussion

PLK1 has been demonstrated to regulate an increasing number of molecular pathways. In many cases this appears to be through changing the stability of the protein, marking it for destruction. In progression into mitosis, PLK1 regulates the stability of negative regulators of G2/M progression Wee1 [34], USP37 [38] and Claspin [39, 40]. In all these cases PLK1 phosphorylation produces a phosphodegron recognized by the β-TrCP E3 ubiquitin ligase that promotes the degradation of the target proteins. Other G2 phase regulators such as MDC1 a negative regulator of G2/M progression [41], and G2/M phase drivers the G2/M transcriptional regulator FoxM1 [16] and CDK1/2 activator CDC25B [42] are also targets for

PLK1 phosphorylation, in these cases regulating the activity rather than stability of these proteins. The total effect of these changes in the stability and activity is to promote mitotic entry. In each of these cases, PLK1 binds these proteins either as a consequence of prior CDK1-dependent phosphorylation to produce a Polobox binding site as for Wee1, USP37, and FoxM1 [16, 34, 38]. Here we have investigated the Polobox domain-binding proteins in G2 phase cells, with the aim of identifying further PLK1 interacting proteins that contribute to the G2 phase arrest imposed by and blocking PLK1 binding to its substrates by over-expression of the Polobox domain. This appears to be equivalent to inhibition of PK1 activity in delaying progression into mitosis. We have identified a number of proteins previously identified as PLK1 binding proteins, including JIP4, as well as a number of novel interactors. The Polobox binding site identified in JIP4 was phospho-Thr217, but a phosphoproteomics screen for PLK1 phosphorylation sites found this site was not responsive to the PLK1 inhibitor BI-4834 [21]. Two other sites were identified as PLK1 phosphorylation sites on JIP4, Ser183/185 and Ser 311 [23], although neither of these match consensus Polobox binding sites [20]. The lack of sensitivity of the putative PLK1 binding site to the PLK1 is surprising in view of our finding that PLK1 binding to JIP4 was only sensitive to PLK1 inhibitor treatment, although this may reflect a difference between G2 phase and mitotic PLK1 binding to JIP4.

A number of mitotic spindle associated proteins were also identified as PLK1 binding proteins, including SPAG5/Astrin which is phosphorylated by PLK1 in mitosis [25], ERCC-6L/PICH and NEDD1 [23, 26, 27]. BICD2 is an adaptor between microtubule motors and their cargo, and binds to RanBP2 in G2 phase where they are involved in the regulation of centrosome position relative to nucleus [28].RANBP2 is phosphorylated by PLK1 in mitosis [21]. In our experiments, RANBP2 and BICD2 were found exclusively in G2 phase and mitotic samples only, suggesting that the interaction with PLK1 is a dynamic process,

possibly regulating the interaction of these two proteins during the G2/M transition. It was unsurprising that the mitotic spindle associated PLK1-inding proteins were only found in the mitotic samples, although the DNA-PK associated being exclusively found in the G2 phase samples was unexpected. DNA-PK has been shown to be phosphorylated by and bind PLK1 in mitosis [24]. PRKDC/DNA-PKcs, the catalytic subunit of DNA-PK is phosphorylated and binds PLK1, and PRKDC/ DNA-PKcs forms a complex with Protein Phosphatase 6 (PPP6)/PPP6R2 [24, 43]. ANKRD28/PP6-ARS-A binds to PPP6 complex through a SAPS domain PPP6R regulatory domains [44], thus these latter proteins are likely to be part of the DNA-PK complex. However, as with the RANBP2-BICD2 interactions, PRKDC/DNA-PKcs binding appears to be restricted to G2 phase, whereas PPP6R2 and ANKRD28/PP6-ARS-A were found associated with the PBD in both G2 phase and mitosis, suggesting that PLK1 interaction with these complex components may be dynamic and possible regulate the activity or even interaction of PRKDC/DNA-PKcs with the PPP6 complex during G2/M phase transition. It is likely that some of the novel interactors identified here where through their interactions with PLK1 bound proteins. For example, ATP5A1 is a mitochondrial ATPase that associates with ATPH which has been reported to be a PLK1 binding protein [23]. The interaction between PLK1 and EFTU, HSPA9/mortalin and HNRNPF require further validation, although HNRNPF has been identified as PLK1 substrate [21].

The role of PLK1 in regulating p38MAPK is novel, although it is at present unclear as to the physiological role of this mechanism. We have observed no cell cycle defects with depletion of JIP4 in either entry into or through mitosis. The effects of depletion or inhibition of p38MAPK on progression into and through mitosis were subtle [35, 36], and it may be that the incomplete depletion of JIP4 which reduced G2 phase p38MAPK activation by only 60% was insufficient to detect these subtle effects. However, the regulation of G2 phase p38MAPK activation through JIP4-PLK1 provides a mechanism by which the p38MAPK

pathway can be directly linked to the cell cycle machinery, specific to regulate normal progression into and through mitosis. The lack of effect of either PLK1 or JIP4 depletion on G2 phase osmotic shock, ultraviolet radiation exposure (data not shown) or mitotic defect initiated p38MAPK activation is evidence that stress initiated p38MAPK activation is not regulated through this JIP4-PLK1 mechanism. Thus the pathway to p38MAPK activation appears dependent on the context of the signal triggering this activation.

5. Conclusions

We have identified JIP4 as a PLK1 substrate and binding protein, PLK1 self priming its binding by G2 phase phosphorylation of JIP4. This interaction is required for activation of p38MAPK during normal G2 phase progression, but not in either G2 phase or mitotic stress response. Finally, JIP4 is a target for caspase-dependent cleavage in mitotically arrested cells. The role for the PLK1-JIP4 regulated p38MAPK activation in G2 phase is unclear, but it does not affect either progression into or through mitosis.

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Figure legends

Figure 1: Inhibiting PLK1 activity delays progress into mitosis.

A: HeLa cells were synchronised with a thymidine block, released and at 6 h after release treated with either 1 or 5 μM PLK1 inhibitor BI-2536 and followed by time lapse microscopy for the timing of entry into mitosis. The data is expressed as a percentage of the total cell number. At least 200 cells were counted for each condition.

B: HeLa cells were transfected with an expression plasmid containing either GFP or GFP-polobox domain (PBD), then synchronised using thymidine arrest, released from the block and followed from 6 h after release by time lapse microscopy. The GFP-expressing cells were score for the timing of entry into mitosis. The data is expressed as a percentage of the total GFP expressing population.

Figure 2: PLK1 binds to JIP4 in G2 phase

A: GFP-PBD and control GFP bound beads were used to affinity purify proteins from G2 phase HeLa cell lysates. The bound proteins were eluted and immunoblotted for JIP4, 53BP1 and Wee1 using lysate as the Input.

B: JIP4 and PLK1 antibodies were used to immunoprecipitate their respective protein complexes from G2 phase HeLa lysates, then immunoblotted with each antibody using an appropriate non-immune control antibody (NI).

C: Asynchronously growing HeLa cells were fixed and stained for JIP4 (green), PLK1 (red) and DNA (blue).

Figure 3: PLK1 binding to JIP is self-primed

A: HeLa cells were synchronised by double thymidine block-release then samples analysed as cells progressed into mitosis. Cell lysates were immunoblotted for the indicated proteins. pMEKThr286 is a marker of Cyclin B/CDK1 activity and mitosis. The percentage of cells in each cell cycle phase determined by DNA FACS analysis is shown.

B: G2 phase synchronised HeLa cells were treated with either CDK1 (9 μM RO-3306), CDK2 (5 μM PHA533533), PLK1 (1 μM BI-2539) inhibitors or DMSO (Con) for 2 h then JIP4 complexes immunoprecipitated. The immunoprecipitates were immunoblotted for the indicated proteins. Input was 5 % cell lysate prior to immunoprecipitation, IP; JIP4 immunoprecipitate, NI is non-immune serum immunoprecipitate from a parallel lysate.

Figure 4: JIP4-PLK1 regulates G2 phase p38MAPK activity.

A: HeLa and U2OS cells were transfected with siRNA to depleted JIP4 the thymidine synchronised and harvested when cells were in G2 phase. The levels of JIP4 and activated p38MAPK (pp38) were analysed. The levels of pp38 relative to non-targeting siRNA (NT) from at least three experiments are shown.

B: HEK293T cells were transfected with vectors expressing either GFP, or wild type (WT), T210D mutant (Mut), or GFP-Polobox domain (PBD) proteins. Cells were then synchronised by thymidine block-release and harvested as they progressed into G2 phase. Cell lysates were immunoblotted for the indicated proteins including pPLK1 T210. PCNA was used as a loading control. The levels of pp38 relative to the GFP expressing control from at least three experiments are shown.

C: HeLa cells were transfected with either non-targeting (NT) or JIP4 siRNA, then synchronised to progress into mitosis. At 6 hours after synchrony release, cells were treated with either DMSO or p38 inhibitor (p38i) and then imaged every 20 min for 15 h. Cells were scored for entry into mitosis. This data is representative of three independent experiments. * $p<0.05$, *** $p<0.001$.

Figure 5:

HeLa cells were transfected with either non-targeting or JIP4 siRNA then synchronised. As cells progressed in G2 phase cultures were treated with or without D-sorbitol to induce osmotic shock and harvested. Lysates were immunoblotted for the indicated proteins. The level of pp38MAPK relative to non-targeting control from at least three experiments are reported.

B: HEK293T cells were transfected with vectors expressing either GFP, or wild type (WT), T210D mutant (Mut), or GFP-Polobox domain (PBD) proteins. Cells were then synchronised by thymidine block-release, treated without and with osmotic shock as in A, and harvested as they progressed into G2 phase. Cell lysates were immunoblotted for the indicated proteins including pPLK1 T219. PCNA was used as a loading control.

Figure 6:

A: HeLa and U2OS cells were transfected without or with either non-targeting or JIP4 siRNA, treated without or with nocodazole for 28 h. Cells lysates were immunoblotted for the indicated proteins.

B: HeLa cells were synchronised then released to progress to mitosis. At 6 hours after release cells were treated with nocodazole then harvested at the indicated times after release. Cells not treated with nocodazole were harvested in G2 phase (G2).

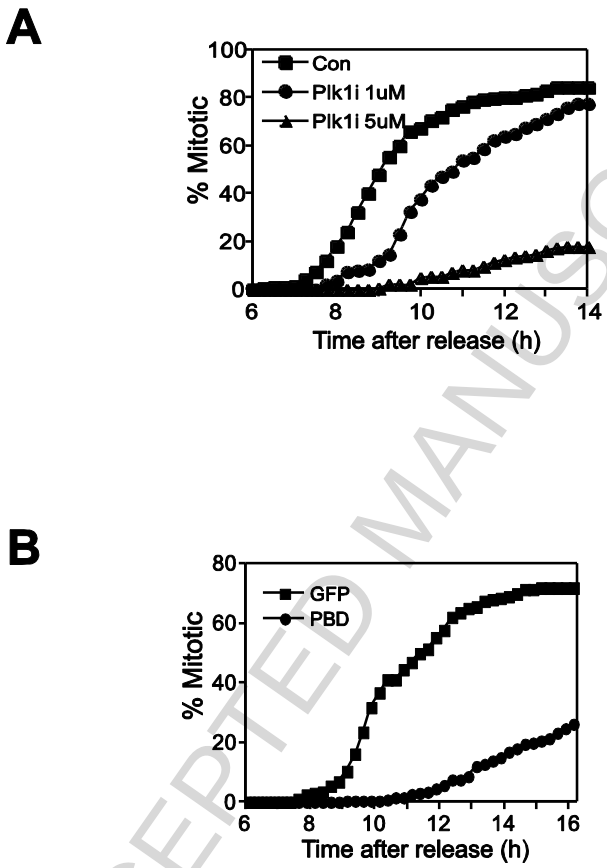
C: HeLa cells, either untreated (AS) or treated with nocodazole without (N) and with 20 μM zVAD-Fmk (N+zV) were harvested at 28 h. Lysates were immunoblotted for the indicated proteins.

Table 1

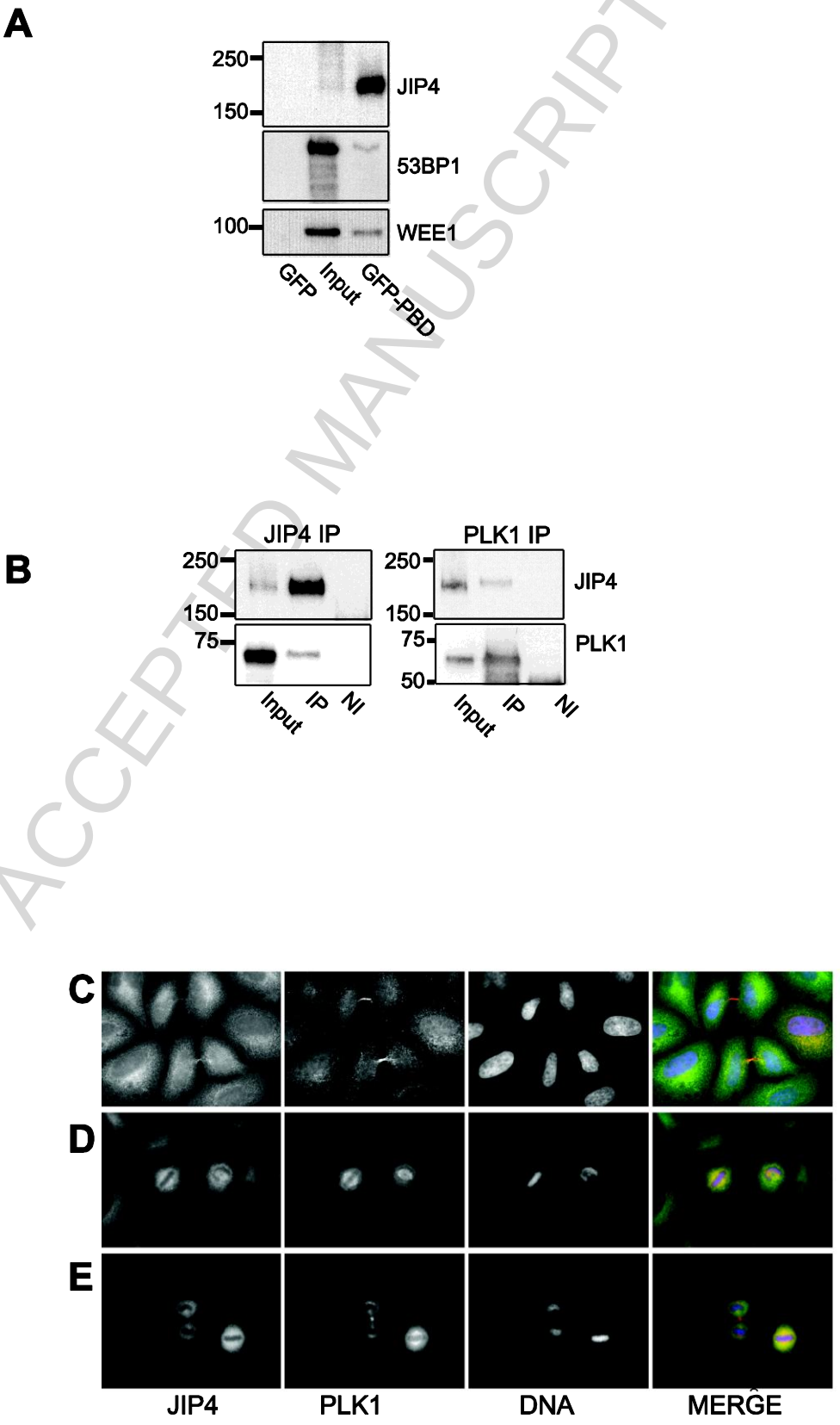
		G2 exp1		G2 exp2		Mitosis exp3		Mitosis exp4	
Accession number	Protein	GFP	PBD	GFP	PBD	GFP	PBD	GFP	PBD
O60271	**JIP4*#**	**0**	**38**	**0**	**20**	**0**	**7**	**0**	**15**
P53350	**PLK1**	**0**	**53**	**0**	**38**	**0**	**39**	**0**	**64**
P25705	ATP5A1	0	11	0	15	0	2	0	6
P78527	PRKDC (DNA-PKcs) *#	0	5	0	14	0	0	0	0
Q8TD16	BICD2*#	0	0	0	0	0	5	0	14
O15084	ANKRD28 (PP6-ARS-A)#	0	2	0	11	0	4	0	5
P49792	RANBP2#	0	6	0	3	0	0	0	0
O75170	PPP6R2	0	0	0	5	0	0	0	3
P49411	TUFM (EF-Tu)	0	3	0	0	0	2	0	3
Q12888	53BP1 *#	0	3	0	4	0	0	0	0
Q96R06	SPAG5 (Astrin) *#	0	0	0	0	0	4	0	2
P38646	HSPA9 (GRP-75, Mortalin)	0	0	0	1	0	6	0	4
Q8NHV4	NEDD1 *	0	0	0	0	0	3	0	2
Q2NKX8	ERCC-6L (PICH) *#	0	0	0	0	0	3	0	1
P52597	HNRNPF#	0	2	0	0	0	3	0	1
Q6PGQ7	BORA *#	0	0	0	0	0	0	0	3

Spectrum count for high probability peptides (global peptide FDR <0.05) identifying the indicated protein from two independent G2 phase and two independent mitotic (nocodazole arrested) mass spectrometry analyses of control GFP and GFP-Polobox domain (PBD) bound proteins. The asterix indicates proteins previously reported as PLK1 binding proteins, the hash marked proteins are reported phosphorylated by PLK1, the light grey shaded proteins were identified in only G2 phase samples, and dark grey proteins only the mitotic samples.

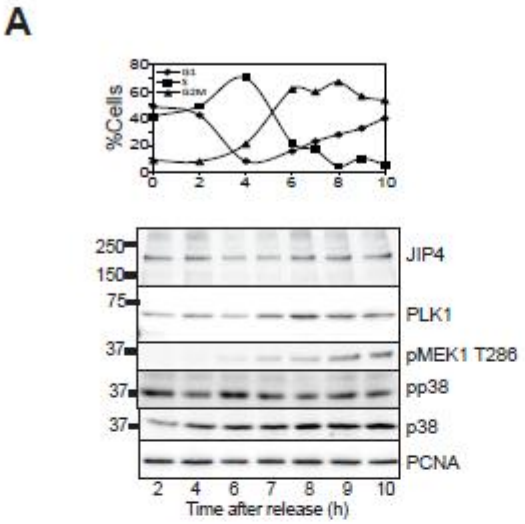
Pinder et al., Figure 1



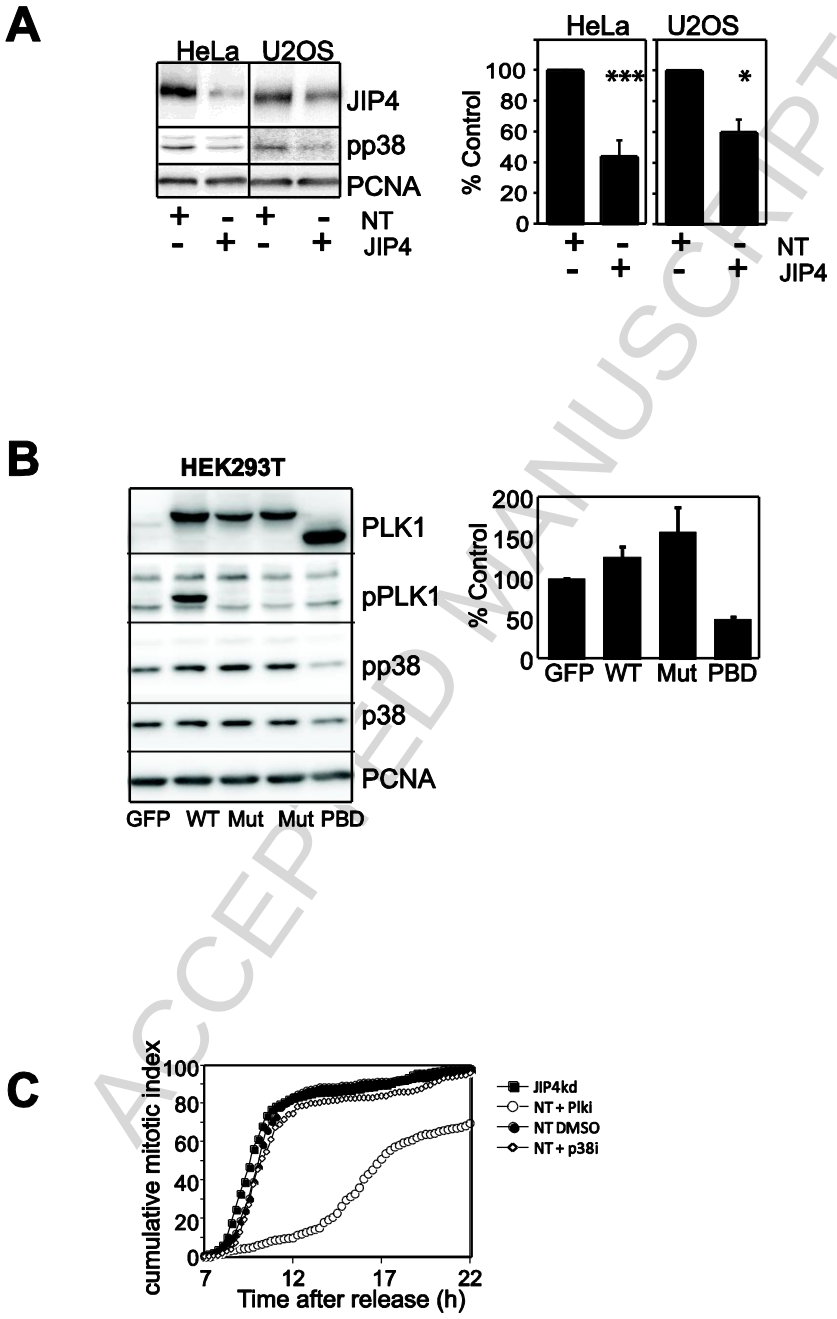
Pinder et al., Figure 2



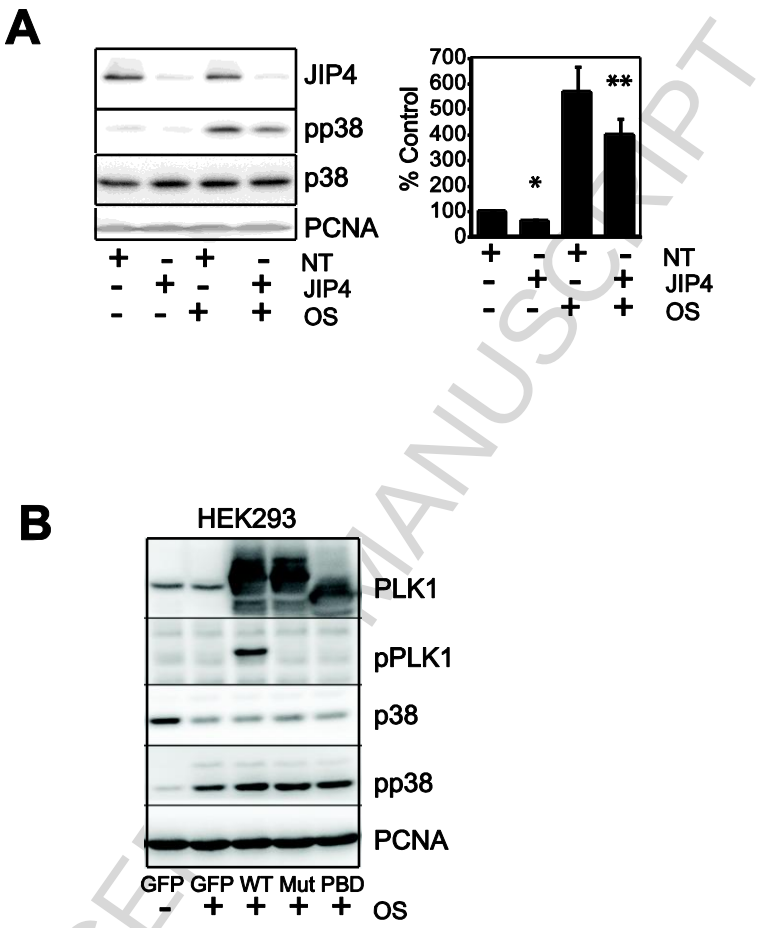
Pinder et al., Figure 3



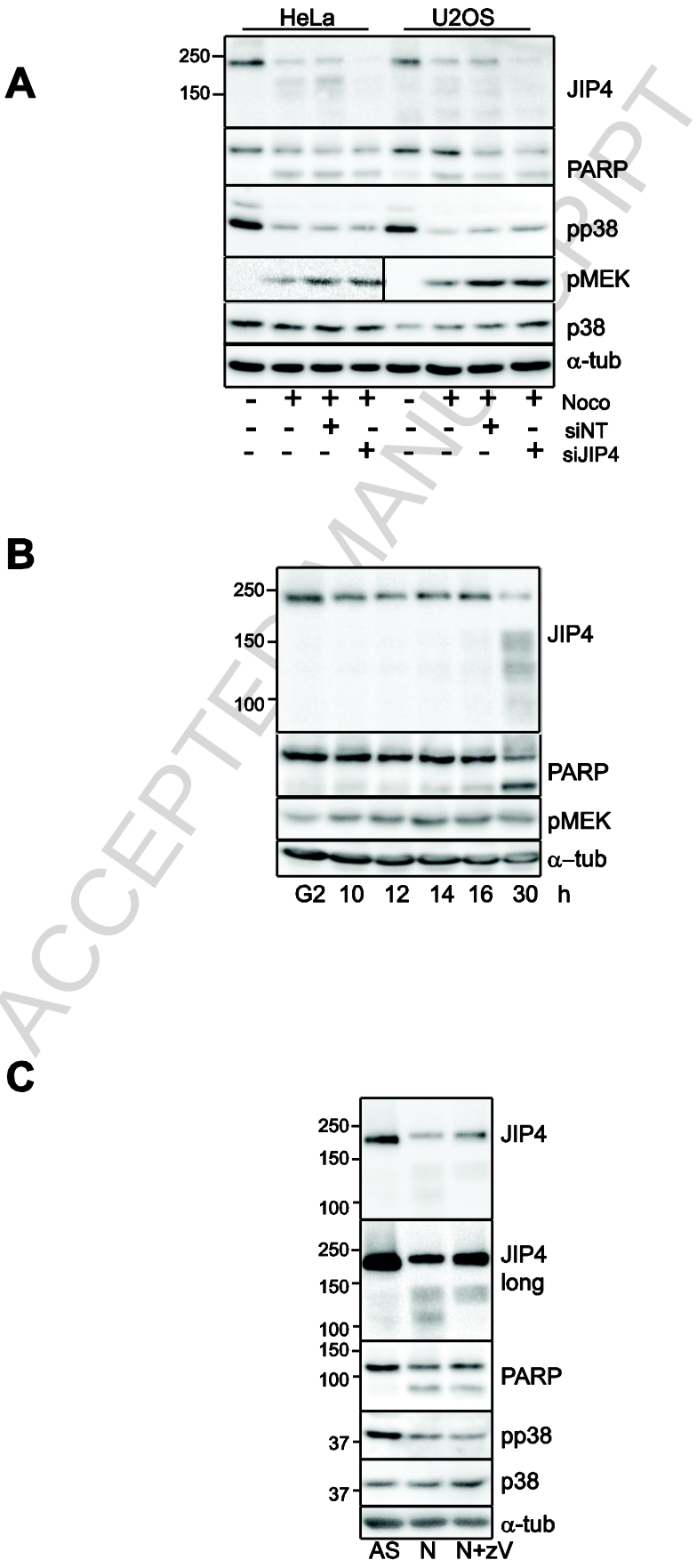
Pinder et al., Figure 4



Pinder et al., Figure 5



Pinder et al., Figure 6



Highlights

- PLK1 binds to the MAPK scaffold protein JIP4 in G2 phase of the cell cycle via its Polobox domain
- PLK1 binding to self-priming mechanism
- PLK1 binding is necessary for JIP4 dependent activation of p38MAPK in G2 phase
- JIP4 is not required for stress activated p38MAPK in either G2 phase or mitosis