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Phosphoregulation of human Mps1 kinase

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SYNOPSIS

The dual-specificity protein kinase Mps1 is a phosphoprotein required for error-free mitotic progression in eukaryotes. In this study, we have investigated human Mps1 phosphorylation using combined mass spectrometric, mutational and phosphospecific antibody approaches. We identify sixteen sites of Mps1 autophosphorylation *in vitro*, several of which are required for catalytic activity after expression in bacteria or in cultured human cells. Using novel phosphospecific antibodies, we show that endogenous Mps1 is phosphorylated on Thr⁶⁸⁶ and Ser⁸²¹ during mitosis, and demonstrate that phosphorylated Mps1 localises to the centrosomes of metaphase cells. Taken together, these data reveal the complexity of Mps1 regulation by multi-site phosphorylation, and demonstrate conclusively that phosphorylated Mps1 associates with centrosomes in mitotic human cells.

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Introduction

Monopolar spindle 1 (Mps1) is a cell cycle regulated protein kinase with an important catalytic role in maintaining the spindle assembly checkpoint (SAC), which prevents premature anaphase in eukaryotes [1]. Experimental manipulation of Mps1 function perturbs the SAC [2, 3] and induces chromosome segregation defects [4]. Since aneuploidy is a common trait in cancer cells, dissecting the Mps1 regulatory mechanism is important for understanding cancer biology.

As cells enter mitosis Mps1 becomes hyperphosphorylated and activated and is then dephosphorylated around the time of mitotic exit [3, 5]. Several sites of Mps1 autophosphorylation have been reported, including Thr⁶⁷⁶ in the Mps1 activation loop, which regulates Mps1 catalytic activity and is important for normal mitotic progression in eukaryotic cells [6-8]. Mps1 has also been demonstrated to be a target for the proline-directed kinases Cdk2 and MAPK in several model eukaryotes [9, 10], where Mps1 phosphorylation might control subcellular distribution during the cell cycle.

Centrosomal Mps1 localisation has been demonstrated in human cells, suggesting that a centrosome-dependent function might be conserved between fungi and metazoans [5, 9, 11]. However, independent studies have failed to find evidence for centrosomal Mps1 [3, 12] and this proposed localisation is therefore controversial.

In this study, we have employed a powerful combination of collision-induced dissociation (CID) and electron transfer dissociation (ETD) based tandem mass spectrometry (MS/MS) to show that the Mps1 catalytic domain autophosphorylates on at least sixteen residues *in vitro*, many of which represent novel phosphoacceptor sites. Using a mutagenic approach, we have analysed the importance of these residues for Mps1 activation by autophosphorylation after expression in both bacteria and mitotic human cells. Additionally, we have evaluated several novel Mps1 phosphospecific antibodies, two of which we exploit to demonstrate for the first time that endogenous phosphorylated Mps1 localises to the centrosomes during mitosis.

Results and Discussion

Phosphorylation site analysis by mass spectrometry

Human Mps1 was initially characterised as a phosphoprotein after expression and isolation from bacteria [13, 14]. However, the extent of Mps1 catalytic domain phosphorylation *in vitro* has remained unclear. We focused our initial studies on bacterially expressed human Mps1 containing the kinase domain and its C-terminal extension, which is soluble and catalytically active after affinity purification [15]. Bacteria represent an attractive model organism for investigating Mps1 autophosphorylation, since the active enzyme is generated in the absence of human protein phosphatases, permitting the isolation of large amounts of kinase phosphorylated to high stoichiometry. Consequently, we exploited a combination of CID and ETD-based MS/MS to identify the sites of Mps1 phosphorylation after expression in bacteria (Table 1). In contrast to CID, ETD fragmentation preserves the phosphate group on the modified amino acid, aiding identification of the site(s) of phosphorylation. We obtained overall Mps1 sequence coverage of 87%, and definitive evidence for sixteen phosphorylation sites, the majority of which were validated internally using the two complementary methods of peptide fragmentation (Table 1). The fully annotated tandem MS spectra of the Mps1 phosphopeptides obtained after fragmentation are presented in Supplementary Figures S1-S3. The documented Mps1 phosphorylation sites exhibit a high degree of conservation between model vertebrate species (Figure 1A), although they map to different regions of the human kinase domain, including several residues in the activation segment located between Asp⁶⁶⁴ and Glu⁶⁹³ (Figures 1B and 1C). We also identified a cluster of Mps1 autophosphorylation sites located outside the catalytic domain, including Ser⁸²¹, the human equivalent of the previously reported *Xenopus* MAPK site Ser⁸⁴⁴ [10]. A D664A kinase dead Mps1 catalytic domain mutant was analysed in an identical fashion, but no phosphopeptides were detected, demonstrating that all the identified sites were due to Mps1 autophosphorylation (data not shown).

Biochemical analysis of Mps1 kinase activity *in vitro*

We mutated eleven of the most conserved Mps1 phosphorylation sites to Ala, to prevent phosphorylation, or Asp, to attempt to mimic phosphorylation, and assayed their activity after affinity purification from bacteria. Wild type (WT) Mps1 catalytic domain was active towards MBP, and this served as a standard for comparative analysis of each Mps1 mutant. The bacterially expressed WT catalytic domain did not autophosphorylate to any great extent in the presence of [γ -³²P]Mg-ATP, presumably because it had already become highly autophosphorylated in bacteria (Figure 1); we were therefore unable to use this parameter to assess the activity of Mps1 in a kinase assay. Kinase dead D664A Mps1 was catalytically inactive, and exhibited an enhanced electrophoretic mobility by SDS-PAGE compared to WT, indicating that autophosphorylation of the kinase in bacteria is responsible for the reduced mobility of the WT enzyme. As shown in Figure 2A, mutation of Thr⁶⁷⁵ to Ala increased Mps1 catalytic domain activity, and this was reduced to WT levels by mutation to Asp, suggesting that a negative charge at this position might be important to maintain wild type Mps1 levels of activity. The mobility of T675A was reduced when compared with the WT enzyme, and this shift was reversed in the T675D mutant, verifying that activity correlates positively with decreased gel mobility. The

mutation of Thr⁶⁷⁶ to Ala reduced catalytic activity markedly, and this mutant exhibited high gel mobility. Interestingly, neither Mps1 activity, nor the associated increased gel mobility, could be rescued by mutation to Asp, suggesting either that autophosphorylation on this T-loop site is not mimicked by aspartic acid, or that Thr⁶⁷⁶ might have a structural role in catalysis. Given the importance of phosphorylation at the equivalent T-loop site for full kinase activity in the protein kinase superfamily [16], we favour the former model, although a high-resolution crystal structure of Mps1 phosphorylated on Thr⁶⁷⁶ will be needed to prove that the phosphorylation of this amino acid does indeed regulate activity. Interestingly, even amongst protein kinases that have been verified by structural means to be regulated by phosphorylation at the T-loop residue, such as Aurora A and B, introduction of negatively charged amino acids at this position does not always rescue catalytic activity *in vitro* [17, 18]. As shown in Figure 2A, Thr⁶⁸⁶ was indispensable for kinase activity, because the fast migrating, completely inactive Ala mutant could not be rescued by mutation to either Asp or Glu (data not shown). This suggests that this residue is either critically required for the structural integrity of the kinase, or is an important phosphoacceptor site that cannot be mimicked by negatively charged amino acids. Analysis of the dephosphorylated Mps1 crystal structure reveals that this amino acid lies in the P+1 loop of the kinase (Figure 1C), pointing towards a dominant structural role for this residue in either substrate binding or ATP positioning during catalysis [15]. This does not, however, rule out an additional regulatory role for the phosphorylation of this residue.

Interestingly, autophosphorylation outside the activation segment was also important for activity *in vitro*, since S582A/D and Y811F mutants exhibited decreased activity, and a S742A mutation led to Mps1 inactivation, which was completely restored by mutation to Asp (Figure 2A). T806D Mps1 was significantly less active than T806A, demonstrating a potential negative correlation between phosphorylation and activity at this site. The mutation of Thr⁵⁶⁴, Ser⁵⁸², Ser⁶⁸², Thr⁷⁹⁵ or Ser⁸²¹ to Ala did not alter Mps1 activity significantly (Figure 2A), suggesting that phosphorylation at these sites is not rate-limiting for Mps1 catalytic activity.

Biochemical analysis of Mps1 kinase activity in HeLa cells

To investigate the activity requirements for full length Mps1 phosphorylation in cells, we transfected GFP-tagged Mps1 constructs containing selected Ala or Asp mutations, or WT or D664A plasmids as controls. As shown in Figure 2B, WT GFP-Mps1 was active after immunoprecipitation from HeLa cell extracts, because it readily phosphorylated both itself and MBP. In contrast, D664A GFP-Mps1 was inactive, and served as a negative control to account for assay background. WT GFP-Mps1, but not D664A GFP-Mps1, was detected as a doublet after immunoblotting of the kinase reaction, suggesting that the more slowly migrating band might be due to Mps1-dependent autophosphorylation, representing an independent marker for kinase activity. Thr⁶⁷⁶ and Thr⁶⁸⁶ were important for Mps1 activity in cells, because mutation of Thr⁶⁷⁶ to Ala partially blocked activity, and mutation of Thr⁶⁸⁶ to Ala completely inactivated the kinase. A triple T-loop Ala mutant was largely inactive, demonstrating conclusively that phosphorylation of one or more of these amino acids is required for full enzymatic function in cells. In addition, the Thr⁶⁷⁶, Thr⁶⁸⁶ and triple T-loop Ala mutants all exhibited increased electrophoretic mobility compared to WT, indicating low levels of autophosphorylation and activity. While mutation of Thr⁶⁷⁶ to Asp partially rescued

both Mps1 activity and the associated electrophoretic shift, Thr⁶⁸⁶ Asp or Glu (not shown) mutants were completely inactive in human cells, mirroring our finding that mutation of this residue abolishes Mps1 activity *in vitro*. Interestingly, both S582A and S582D mutants appeared to be less active than the WT enzyme, whilst S742D and S742E (data not shown) mutants were hyperactive and electrophoretically retarded, suggesting that these two sites might be involved in modulating enzyme activity in cells. None of the other identified phosphoacceptor sites tested, including Thr⁶⁷⁵, appeared to be critical for Mps1 activity under these conditions, since their gel mobility and activity were similar to that of WT Mps1. As previously reported for *Xenopus* Mps1 [10], Ser⁸²¹ phosphorylation was not essential for the activity of human Mps1 in cells, since S821A and S821D mutants both retained WT activity and reduced gel mobility.

We consistently noted a difference in the activity of some Mps1 mutants, depending upon whether they were expressed in bacteria, or immunoprecipitated from human cells, prior to assay under identical reaction conditions with the non-specific substrate MBP. For example, mutation of Thr⁸⁰⁶ to Asp markedly decreased Mps1 catalytic domain activity *in vitro*, suggesting that phosphorylation at this site might decrease activity, however the same mutation did not negatively influence Mps1 activity after isolation from mitotic cells. In addition, a T675A mutant was hyperactive *in vitro*, but exhibited similar activity to WT Mps1 after isolation from cells. In contrast, a S742A mutant was inactive when isolated from bacteria, but exhibited WT activity after expression in cells, which was enhanced several fold by mutation to Asp or Glu. We also demonstrated that a Y811F mutant isolated from HeLa cells exhibited kinase activity and a decreased electrophoretic mobility, despite being expressed at much lower levels than other Mps1 proteins (Figure 2B). This suggests, but does not prove, that Tyr⁸¹¹ phosphorylation might be associated with Mps1 stability and/or decreased activity in cells. This is in contrast with the inhibitory effect of abolishing phosphorylation at this site in a Y811F mutation in bacteria (Figure 1A). To explain these differences, we speculate that the folding of the Mps1 catalytic domain mutants might be particularly susceptible to certain amino acid mutations in bacteria, but that these may be counterbalanced or masked by other factors, such as protein phosphatases or molecular chaperones in human cells, leading to different results when full length Mps1 is assayed after immunoprecipitation.

The significance of Thr⁶⁷⁶ and Thr⁶⁸⁶ phosphorylation for Mps1 activity in human cells has also recently been recognised by others [6-8], and given the importance of Mps1 kinase activity for the SAC and accurate chromosome segregation in human cells [6-8, 19], it will be important to assess the effects of all our phosphorylation site mutants on physiological Mps1 function in cells. Based on our activity assays, we hypothesise that several of the newly identified phosphorylation sites, including those positioned outside the catalytic domain like Ser⁸²¹, might be important for non-enzymatic functions in human cells, such as substrate docking interactions or subcellular targeting.

Analysis of phosphospecific Mps1 antibodies and mechanism of autophosphorylation

To analyse Mps1 autophosphorylation further, we generated four phosphospecific antibodies, designated pThr⁶⁷⁵, pThr⁶⁷⁶, pThr⁶⁸⁶ and pSer⁸²¹ respectively. The specificity of these antibodies was demonstrated *in vitro* using WT (active) and D664A (inactive) Mps1 catalytic domain mutants. WT Mps1, but not

D664A catalytic domain, was recognised by all four purified phosphospecific antibodies (Figure 3A), whereas the D664A and WT proteins were recognised equally well by a pan-Mps1 antibody. These data validate some of our MS findings and help prove phosphospecificity for each antibody. Phosphospecificity was also demonstrated independently by incubation of the Mps1 catalytic domains with λ phosphatase (Figure 3A), by specific peptide neutralisation experiments (Figure 3B) and by immunoblotting a panel of Mps1 point mutants (Figure 3C).

We also exploited these phosphospecific antibodies to investigate the mechanism of Mps1 autoactivation. Interestingly, the reactivation of Mps1 catalytic activity after phosphatase-treatment was accompanied by autophosphorylation of Thr⁶⁷⁶ and Thr⁶⁸⁶, but not Thr⁶⁷⁵ or Ser⁸²¹ (Figure 3A), reinforcing the strong positive correlation previously identified between phosphate occupancy at Thr⁶⁷⁶ and Thr⁶⁸⁶ and Mps1 activity (Figures 2A and B). Reactivated Mps1 was only 20% as active towards MBP as the untreated enzyme, and only partially phosphorylated at Thr⁶⁷⁶ and Thr⁶⁸⁶ under these conditions (Figure 3A). This suggests that sub-stoichiometric phosphorylation at these sites within the population might limit activity, or that additional phosphorylated amino acids could be required to generate the Mps1 conformation exhibiting full catalytic activity.

The analysis of Mps1 point mutants with phosphospecific antibodies also revealed additional insights into the mechanism of Mps1 activation *in vitro*. As shown in Figure 3C, the pThr⁶⁷⁵ antibody failed to recognise phosphorylated Thr⁶⁷⁵ in the T686A, but not T686D Mps1 mutant, suggesting that an aspartate at this position might generate sufficient Mps1 activity in bacteria to permit autophosphorylation at Thr⁶⁷⁵. Both T686A and T686D Mps1 mutants contained phosphorylated residues in addition to Thr⁶⁷⁵, including Thr⁶⁷⁶ and Ser⁸²¹. This demonstrates that although these mutants exhibit no detectable activity when assayed under rate-limiting assay conditions with MBP *in vitro* (Figures 2A and B), and the same T686A mutant adopts an “inactive” conformation when crystallised [15], the high enzyme and ATP concentrations in bacteria might promote autophosphorylation by this mutant when overexpressed. Importantly, the D664A mutant was not detected by any of the phosphospecific antibodies, either after phosphatase treatment and ATP incubation, or after bacterial synthesis and purification using identical conditions as the WT enzyme.

In addition, these data also provide some evidence for the hierarchical nature of Mps1 autophosphorylation. For example, the finding that Thr⁶⁷⁶ and Thr⁶⁸⁶, but not Thr⁶⁷⁵ or Ser⁸²¹, became re-phosphorylated in an ATP-dependent manner after phosphatase treatment, suggests that Thr⁶⁷⁶ and Thr⁶⁸⁶ are preferential sites of autophosphorylation (Figure 3A). Moreover, the finding that Thr⁶⁷⁶ and Ser⁸²¹ were occupied in the T686A mutant, and that Thr⁶⁷⁵ and Ser⁸²¹ phosphate occupancy was enhanced many fold in the T686D mutant, (Figure 3C), further suggests that the putative phosphomimetic Asp mutant possesses an increased ability to autophosphorylate in bacteria, explaining why autophosphorylation of Thr⁶⁷⁵ and Ser⁸²¹ approaches levels found in the WT enzyme. A mixture of MS and high resolution crystallography of the active, phosphorylated enzyme, will be required to determine how phosphorylation controls Mps1 activity, and to define sites that are necessary and sufficient for Mps1 activity. Such an analysis might also permit some insight into any consensus sequences utilised by Mps1 for directing substrate phosphorylation, which remain unclear despite the emergence of cellular targets in addition to Mps1 [4].

A surprising observation was that the pSer⁸²¹ antibody was able to detect the bacterially expressed S821D mutant after electrophoresis. This suggests that the

aspartate side chain might be sufficiently phosphomimetic to allow the pSer⁸²¹ antibody to recognise the Asp mutant by Western blotting. Phosphospecific antibodies would not normally be expected to recognise side chains such as Asp, whose structure would presumably be significantly different to that presented by the phosphorylated amino acid. However, the finding that mutation of Ser⁸⁴⁴ of *Xenopus* Mps1 to Asp, but not Ala, induces Mps1 localisation at kinetochores in egg extracts [10], supports the hypothesis that imitating the phosphate charge at this site with Asp might also provide some structural mimicry in this region of the protein.

Mps1 phosphorylation in eukaryotic cells

To investigate the phosphorylation status of Mps1 after expression in a eukaryotic cell type, we immunoblotted Mps1 and D664A Mps1 purified from Sf9 insect cells. Although WT and D664A Mps1 were both detected by pan-Mps1 antibody, the pThr⁶⁸⁶ antibody only recognised the WT Mps1 protein. In contrast the pSer⁸²¹ antibody recognised both the WT and D664A mutants (Figure 3D), suggesting that in contrast to Thr⁶⁸⁶, Ser⁸²¹ might not be an autophosphorylation site in cells. To further investigate Mps1 autophosphorylation, we transfected HeLa cells with either GFP-Mps1 or D664A GFP-Mps1, and blotted nocodazole-arrested cells with each phosphospecific antibody. As shown in Figure 3E, GFP-Mps1 was phosphorylated on Thr⁶⁷⁵, Thr⁶⁷⁶, Thr⁶⁸⁶ and Ser⁸²¹ in nocodazole-exposed cells. Thr⁶⁷⁵, Thr⁶⁷⁶ and Thr⁶⁸⁶ phosphorylation was due to Mps1 autophosphorylation, since these sites were absent from the inactive D664A GFP-Mps1 mutant. In contrast, Ser⁸²¹ phosphorylation is unlikely to be due to autophosphorylation in human cells, since the pSer⁸²¹ antibody also detected phosphorylation on the D664A GFP-Mps1 mutant. These data demonstrate that all four phosphospecific Mps1 antibodies recognise overexpressed Mps1 in human mitotic cell extracts, further validating our *in vitro* data.

Mps1 is phosphorylated during mitosis in human cells

We next examined the phosphorylation of endogenous Mps1 in HeLa cells. We found that Mps1 was phosphorylated at Ser⁸²¹ at low levels in asynchronous extracts, but at high levels in nocodazole-treated extracts, suggesting mitosis-specific occupancy of this site. As shown in Figure 4A, treatment of extracts with λ phosphatase abolished the pSer⁸²¹ signal, demonstrating phosphospecificity against endogenous protein. To prove that this protein was Mps1, and not another protein of similar electrophoretic mobility, we repressed Mps1 by siRNA. Mps1 levels in asynchronous cells were markedly decreased after Mps1, but not Lamin A/C siRNA, and this correlated with loss of the pSer⁸²¹ Mps1 signal (Figure 4A).

To assess Ser⁸²¹ phosphorylation throughout the cell cycle, synchronised HeLa cells were released from a double thymidine block and cell extracts prepared at different time points. Flow cytometric analysis demonstrated that the cells synchronously cycled between S phase, mitosis and G1 (data not shown). The mitotic window was between 10 and 13 hrs after release, as shown by the phosphorylation of Aurora A at Thr²⁸⁸ (Figure 4B). As a positive control, we also released cells into nocodazole, which promotes Mps1 activity and arrests cells in mitosis [3]. Total Mps1 protein was detected at all stages of the cell cycle, and it underwent a modest decrease in electrophoretic mobility during mitosis, or after nocodazole exposure,

both of which have been shown to correlate with Mps1 activation [3, 5]. We next exploited the pSer⁸²¹ antibody to demonstrate that Mps1 was only weakly phosphorylated in S phase, but became electrophoretically retarded and phosphorylated in mitosis (Figure 4B). To prove that the pSer⁸²¹ antibody was only recognising Mps1 phosphorylated at Ser⁸²¹, we performed antibody-blocking experiments with phosphorylated or dephosphorylated immunogenic peptides prior to Western blotting (Figure 4B). Together, these data show that endogenous Mps1 phosphorylation increases markedly at Ser⁸²¹ during mitosis. Although Ser⁸²¹ phosphorylation does not appear to be required for activity (Figures 1 and 2), this modification coincides precisely with Mps1 activation [3], and it therefore represents a novel marker for mitotic Mps1 in cell extracts.

Phosphorylated Mps1 localises to centrosomes in mitosis

There is agreement that a fraction of Mps1 is targeted to the kinetochores during mitosis in human cells [3, 11]. In contrast, the centrosomal targeting of endogenous Mps1 in human cells is controversial. To investigate this issue further, we exploited two phosphospecific Mps1 antibodies to study Mps1 localisation in fixed mitotic HeLa cells, where both endogenous and overexpressed Mps1 have been reported to localise to mitotic centrosomes [5, 11]. Consistently, we found that endogenous Mps1 phosphorylated at Ser⁸²¹ co-localised with Aurora A, a known centrosomal protein in mitotic cells (Figure 5A). Co-localisation was not detected if pre-immune rabbit serum was used for analysis, but was evident with the pSer⁸²¹ antibodies if the cells were fixed in methanol or formaldehyde. Furthermore, strong centrosome reactivity was detected in other human cancer cell lines, including U2OS (data not shown) and DLD1 cells (see later). Surprisingly, given the inferred targeting of Mps1 to kinetochores through Ser⁸⁴⁴ phosphorylation in *Xenopus* [10], we did not find any evidence for Ser⁸²¹ phosphorylation at kinetochores in human cells, although these mitotic structures were readily detected with a Bub1 antibody (Figure 5A).

To prove that the phosphospecific Mps1 antibodies were recognising Mps1, we employed siRNA to show that the loss of total Mps1, as detected by Western blotting of the same transfected cell population (Figure 4A), also led to the loss of centrosome reactivity to the pSer⁸²¹ antibodies by immunofluorescence (Figure 5B), together with impairment of metaphase chromosome alignment, as previously described [8, 11]. We next demonstrated that a pThr⁶⁸⁶ Mps1 antibody, which we previously validated using both recombinant and exogenously expressed Mps1 (Figure 3), also specifically stained the centrosome, where it co-localised with the marker Aurora A (Figure 5C). To prove that this antibody was specifically recognising centrosomal Mps1, we showed that pre-immune rabbit serum did not stain the centrosome (data not shown), and validated that the staining pattern disappeared when Mps1 levels were specifically repressed using siRNA-mediated depletion (Figure 5C). To confirm that both these antibodies were recognising Mps1 in a phosphospecific manner, we also demonstrated neutralisation of both antibodies by the appropriate phosphorylated immunising peptides (Figures 5D and E).

To investigate intracellular Mps1 localisation further, we next demonstrated an interaction between endogenous Mps1 and the well characterised centrosomal protein γ -tubulin in both fixed cells and cell extracts. Centrosomal γ -tubulin co-localised with pSer⁸²¹ and pThr⁶⁸⁶ Mps1 antibodies in both HeLa and DLD1 cells (Figures 6A and B). Furthermore, γ -tubulin antibodies immunoprecipitated Mps1

from nocodazole-treated HeLa cell extracts (Figure 6C). In complementary experiments, Mps1 antibody also immunoprecipitated γ -tubulin from HeLa cell extracts (data not shown). These data clearly demonstrate that Mps1 localises to the centrosome in human cells.

Conclusions

The affinity of some polyclonal Mps1 antibodies has been demonstrated to be sensitive to the phosphorylation status of Mps1 [11], providing a potential reason for their reported failure to detect Mps1 at the centrosome [3]. In agreement with this explanation, the pan-Mps1 antibody used in this study did not detect endogenous Mps1 at kinetochores or centrosomes by immunofluorescence, although we used siRNA to validate that it recognised Mps1 by Western blotting (Figure 3A). In contrast, the pThr⁶⁸⁶ antibody did not detect endogenous Mps1 by Western blotting, but recognised exogenous GFP-Mps1 (Figure 3D) by immunoblotting and endogenous centrosomal Mps1 by immunofluorescence (Figures 6 A and B). Taken together, these data suggest that antibodies raised against *bona fide* sites of Mps1 phosphorylation such as Thr⁶⁸⁶ and Ser⁸²¹ might be superior reagents with which to investigate Mps1 function by immunofluorescence. Indeed, given the strong correlation between Mps1 activity and the phosphorylation of Thr⁶⁸⁶ (Figure 2A), our discovery that Mps1 is phosphorylated at Thr⁶⁸⁶ on the centrosome indicates that this might represent a catalytically active subcellular pool of the enzyme.

In conclusion, our analysis of the phosphorylation of human Mps1 demonstrates a highly complex pattern of *in vitro* regulation that is closely mirrored in cells. Phosphate occupancy at Thr⁶⁷⁵, Thr⁶⁷⁶ and Thr⁶⁸⁶ appears to be dependent on Mps1 activity under all conditions studied, and Thr⁶⁷⁶ and Thr⁶⁸⁶ phosphorylation appear to be critically important for Mps1 catalytic activity. In contrast, Ser⁸²¹ phosphorylation is not required for activity, and this modification might be mediated by a distinct proline-directed kinase in eukaryotic systems [10]. We have identified a series of novel Mps1 autophosphorylation sites, several of which map to regions of the catalytic domain outside the activation segment (Figure 1). Although the function of several of these phosphorylation events is not yet known, we speculate that they may be important in regulating Mps1 autophosphorylation and activity (Figure 2), or subcellular targeting in cells. Our finding that endogenous centrosomal Mps1 is phosphorylated on Thr⁶⁸⁶ and Ser⁸²¹ opens up several avenues for investigating the regulation and function of these specific modifications in cells. These include identification of the human Mps1 Ser⁸²¹ kinase and dissection of the regulatory requirements for Thr⁶⁸⁶ and Ser⁸²¹ phosphorylation for Mps1 activity, targeting and stability at the centrosome. In addition, validated Mps1 phosphospecific antibodies will be invaluable for investigating the intricate regulation of Mps1 by multi-site phosphorylation under a variety of experimental and pathological conditions.

EXPERIMENTAL

Molecular biology and protein expression

Human Mps1 catalytic domain (encoding residues 510-857), was amplified by PCR and inserted into the plasmid pET-30 Ek/LIC (Novagen) for bacterial expression. Full length Mps1 (encoding residues 1-857) was cloned into the vector pBAC-2cp (Novagen) for production in Sf9 cells or into pcDNA5/FRT/TO (Invitrogen) that had been engineered to include GFP. Mps1 was mutagenised by PCR to generate kinase dead (D664A) or phosphorylation site mutations. All constructs were sequenced over the entire coding region. Protein was produced in *Escherichia coli* strain BL21 (DE3) pLysS and purified as described [15]. The sequence of DNA primers and siRNA duplexes are available on request.

Phosphorylation site mapping by mass spectrometry

His-tagged Mps1 (510-857) was purified by affinity chromatography, dialysed into 50 mM ammonium bicarbonate pH 8.0, then reduced, alkylated and trypsinised prior to LC-MS/MS analysis on a Q-ToF I instrument (CID) or a HCTultra instrument (ETD). The centroided peak lists of the CID and ETD spectra were searched against the Mps1 protein sequence using MASCOT, with carbamidomethyl Cys set as a fixed modification, and Met oxidation and phosphorylation on Ser, Thr, and Tyr as variable modifications. The CID and ETD spectra were subjected to manual inspection to confirm phosphorylation sites.

Protein kinase and phosphatase assays

500 ng purified bacterially expressed Mps1 catalytic domain proteins or GFP-Mps1 protein immunoprecipitated from 200 µg HeLa cell lysate were assayed by autophosphorylation (GFP-Mps1), together with the exogenous substrate MBP (20 µg) or using MBP alone for 30 mins at 30°C, essentially as described [15]. Phosphatase experiments were performed as previously reported [15].

Antibodies

Sheep Aurora A and Bub1 antibodies were provided by Stephen Taylor (University of Manchester). Monoclonal GFP antibody was from Cancer Research UK. Anti-γ-tubulin (clone GTU-88) was from Sigma. A monoclonal C-terminal pan-Mps1 antibody that recognises phosphorylated and dephosphorylated Mps1 equally well (Figure 3A) was purchased from Millipore. Fluorescent secondary antibodies were from Jackson Immunochemicals. Phosphospecific Mps1 antibodies were generated in rabbits (Eurogentec) and purified and neutralised with immunizing peptides using standard procedures.

Cell culture, synchronisation and transfection.

GFP-Mps1 plasmids or Mps1 siGENOME ON-TARGET plus, or Lamin A/C siRNA duplexes (Dharmacon) were transiently transfected into HeLa cells using Lipofectamine 2000™ according to the manufacturers instructions (Invitrogen, UK), prior to fixation, or incubated with nocodazole for 12 h prior to lysis. Cleared supernatants were prepared prior to Western blotting as previously described [20]. HeLa cells were fixed in methanol at -20°C, and stained using standard immunofluorescence protocols. For synchronisation, HeLa cells were cultured and arrested using a standard double thymidine block and release protocol [20].

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Figure Legends

Figure 1 Mps1 structure comparison

(A) Conservation of Mps1 autophosphorylation sites determined in this study. Amino acid usage at the equivalent site is detailed for three model eukaryotic species. (B) Mps1 activation segment sequences (human numbering) from selected species are aligned between kinase subdomains VII and VIII. Also detailed is part of the Mps1 C-terminal extension, which only appears to be conserved in vertebrate genomes. This segment contains several *in vitro* Mps1 autophosphorylation sites, including Ser⁸²¹. Hs = Homo sapiens, Mm = Mus musculus, Xl = Xenopus laevis, Dr = Danionerio, Dm = Drosophila melanogaster, Sc = Saccharomyces cerevisiae, Sp = Schizosaccharomyces pombe. (C) Location of autophosphorylation sites mapped on a ribbon diagram determined from the crystal structure of the Mps1 catalytic domain. Selected autophosphorylation sites are shown in ball and stick representation. Thr⁵⁶⁴, Ser⁵⁸² and Ser⁷⁴² are located outside the activation segment. The published Mps1 structure does not extend to phosphorylation sites identified in the C-terminus.

Figure 2 Analysis of Mps1 phosphorylation site mutants

(A) The indicated bacterially expressed Mps1 catalytic domains were purified, dialysed, and 500 ng of each protein was separated by SDS-PAGE and immunoblotted with pan-Mps1 antibody. Kinase activity of 500 ng of the indicated protein was assessed using 20 µg MBP in the presence of [γ -³²P]Mg-ATP, and phosphate incorporation was visualised by autoradiography after SDS-PAGE. Incorporation of ATP into MBP was linear over the course of the assay. (B) N-terminally GFP-tagged Mps1, or the indicated Mps1 mutants (all full length), were transfected and assayed after immunoprecipitation from nocodazole-treated HeLa cells. A GFP immunoblot demonstrates similar recovery of GFP-Mps1 in the immunoprecipitates (top panel). Immunoprecipitates were assayed with MBP and [γ -³²P]Mg-ATP and ³²P incorporation into Mps1 (middle panel) or MBP (bottom panel) was visualised after SDS-PAGE and autoradiography. Mock-transfected lysates (-) were used as assay controls to account for background. AAA refers to an Mps1 mutant in which Thr⁶⁷⁵, Thr⁶⁷⁶ and Ser⁶⁷⁷ were all mutated to Ala. Similar results were seen in three independent experiments.

Figure 3 Characterisation of phosphospecific antibodies

(A) 500 ng of bacterially expressed WT or D664A Mps1 catalytic domain proteins (amino acids 510-857) were either mock-treated or incubated with λ phosphatase. The phosphatase was inactivated with orthovanadate prior to assay in the presence or absence of [γ -³²P]Mg-ATP or the substrate MBP. After SDS-PAGE, the proteins were immunoblotted with the indicated Mps1 antibodies and radiolabelled MBP was identified by autoradiography. (B) WT or D664A Mps1 catalytic domain proteins (500 ng) were detected with the indicated phosphospecific Mps1 antibodies that had either been untreated (No peptide), or pre-incubated with either the immunising phosphopeptide (+ phosphopeptide) or the immunising dephosphopeptide (+ peptide) prior to immunoblotting. (C) The indicated purified bacterially expressed Mps1 catalytic domain mutants were affinity purified, separated by SDS-PAGE and then immunoblotted with the indicated Mps1 antibodies. (D) His-tagged Mps1 or D664A Mps1 (both full length) were expressed in Sf9 cells and purified by affinity chromatography. Purified protein (500 ng) was separated by SDS-PAGE and blotted with the indicated Mps1 antibody. (E) GFP-Mps1, or

GFP-D664A Mps1 (both full length), were transfected into HeLa cells and then incubated with nocodazole for 12 h prior to lysis. Soluble extracts (20 μ g) were separated by SDS-PAGE and immunoblotted with the indicated Mps1 antibodies. Untransfected cells are indicated by (-) and equal Mps1 expression in the lysates was demonstrated by blotting with a GFP antibody.

Figure 4 Endogenous Mps1 is phosphorylated on Ser⁸²¹

(A) Upper panels, 200 μ g asynchronous or nocodazole-exposed HeLa cell lysate was incubated with 400 U λ phosphatase, or buffer, at 30°C. Lysates were blotted with the indicated Mps1 antibodies. Lower panels, Mps1 or Lamin A/C were repressed in HeLa cells using siRNA. To demonstrate protein amounts, lysates were immunoblotted with the indicated antibodies. (B) HeLa cells were released from a double thymidine block into medium or medium containing nocodazole, and extracts were made at the indicated times. 30 μ g of extract was immunoblotted with pThr²⁸⁸ Aurora A, γ -tubulin, pan-Mps1, pSer⁸²¹Mps1, or pre-neutralised pSer⁸²¹ Mps1 antibodies.

Figure 5 Centrosomal Mps1 is phosphorylated on Ser⁸²¹ and Thr⁶⁸⁶ in HeLa cells

(A) Localisation of pSer⁸²¹ Mps1 and Aurora A (top panels) and pSer⁸²¹ Mps1 and Bub1 (bottom panels) in HeLa cells in metaphase of mitosis. Colour images are merged in the right panels. (B) HeLa cells were transfected with the indicated siRNA duplexes, fixed and co-stained with pSer⁸²¹ and Aurora A antibodies. (C) As in B, except cells were co-stained with pThr⁶⁸⁶ and Aurora A antibodies. (D, E) Metaphase HeLa cells were stained with pSer⁸²¹ or pThr⁶⁸⁶ Mps1 antibodies that had been pre-incubated with the indicated immunising peptides prior to analysis. Scale bars = 10 μ m.

Figure 6 Centrosomal Mps1 interacts with γ -tubulin in human cells

(A) Localisation of pThr⁶⁸⁶ and pSer⁸²¹ Mps1 in HeLa cells. Fixed cells were co-stained with the indicated antibodies and individual colour images merged in the right panels. (B) Localisation of pSer⁶⁸⁶ and pSer⁸²¹ Mps1 in DLD1 cells. Fixed cells were co-stained with the indicated antibodies and images merged. (C) γ -tubulin antibody immunoprecipitates Mps1. HeLa extracts were immunoprecipitated with beads alone, pan-Mps1 antibody or γ -tubulin antibody and then blotted with pan-Mps1 antibody. Scale bars = 10 μ m.

Table 1 Summary of Mps1 autophosphorylation sites identified by CID and ETD tandem mass spectrometry

Phosphopeptide Sequence	Phosphorylation Site(s)	Theoretical Mass (Da)	Observed Mass (Da)	CID	ETD
YVNLEEADNQ(pT)LDSYR	T564	2008.84	2008.77	✓	✓
LQQH(pS)DKIIR	S582	1316.67	1316.66	✓	X
LIDFGIANQMCPD(pT)(pT)SVVK	T675, T676	2235.99	2235.90	✓	✓
LIDFGIANQMCPDT(pT)SVVK	T676	2156.02	2155.92	✓	✓
D(pS)QVG(pT)VNYMPPEAIK	T682, T686	1907.79	1907.80	✓	X
DSQVG(pT)VNYMPPEAIK	T686	1827.91	1827.79	✓	✓
TPFQQIINQI(pS)K	S742	1495.80	1495.72	✓	✓
ISIPPELLAHPYVQIQ(pT)HPVNQMAK	T795	2806.43	2806.34	✓	X
G(pT)(pT)EEMKYVLGQLVGLNSPNSILK	T805, T806	2751.31	2750.26	X	✓
GT(pT)EEMK(pY)VLGQLVGLNSPNSILK	T806, Y811	2751.31	2750.75	✓	✓
(pY)VLGQLVGLNSPNSILK	Y811	1894.00	1893.92	✓	✓
YVLGQLVGLN(pS)PNSILK	S821	1894.07	1893.96	✓	✓
YVLGQLVGLNSPN(pS)ILK	S824	1894.07	1893.95	✓	✓
TLYEHYSGGESHNS(pS)SSK	S845	2048.81	2049.55	X	✓
TLYEHYSGGESHNSSS(pS)KTFEK	S847	2554.06	2555.06	X	✓
TLYEHYSGGESHNSSS(pS)K(pT)FEK	S847, T849	2634.03	2635.52	X	✓

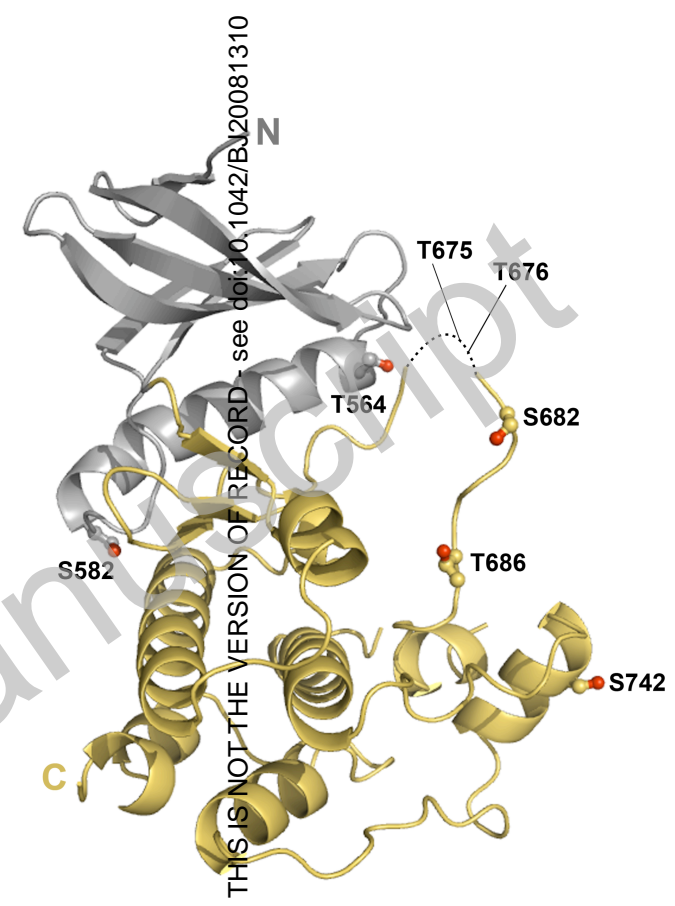
Da, Dalton; CID, Collision Induced Dissociation; ETD, Electron Transfer Dissociation

Table 1 Tyler et al

A

Amino Acid (Human Numbering)	<i>H. sapiens</i>	<i>M. musculus</i>	<i>X. laevis</i>
564	Thr	Thr	Thr
582	Thr	Thr	Cys
675	Thr	Thr	Val
676	Thr	Thr	Thr
682	Ser	Ser	Ser
686	Thr	Thr	Thr
742	Ser	Ser	Ser
795	Thr	Pro	Pro
805	Thrr	Ala	Thr
806	Thr	Thr	Thr
811	Tyr	Tyr	Arg
821	Ser	Ser	Ser
824	Ser	Ser	Ser
845/849	Ser/Thr	Ser/Thr	Ser/Thr
S847	Ser	Ser	Leu

C



B

	VII				VIII		
	604	675	676	682	686	693	821
Hs Mps1	DFGIANQM	QPD	TSVVKDS	QVGT	VN	YMP	PE--GLNSPN
Mm Mps1	DFGIANQM	QPD	TT SVVKDS	QVGT	VN	YMA	PE--GLNSPN
Xl Mps1	DFGIANQI	QPD	VSIVKDS	QVGT	IN	YMP	PE--GLNSPN
Dr Mps1	DFGIANQI	QPD	VSIMKDS	QVGT	LN	YMP	PE--ALQSPN
Dm Mps1	DFGIASNI	AVD	ST SI	IKFS	QAG	TFNY	ISPE--
Sc Mps1	DFGIANAV	PEHT	VNIYRE	TQ IG	TPNY	MA	PE--
Sp Mps1	DFGIAKA	IGND	TT NI	HRD	SH IG	TINY	MAPE--

Figure 1 Tyler et al

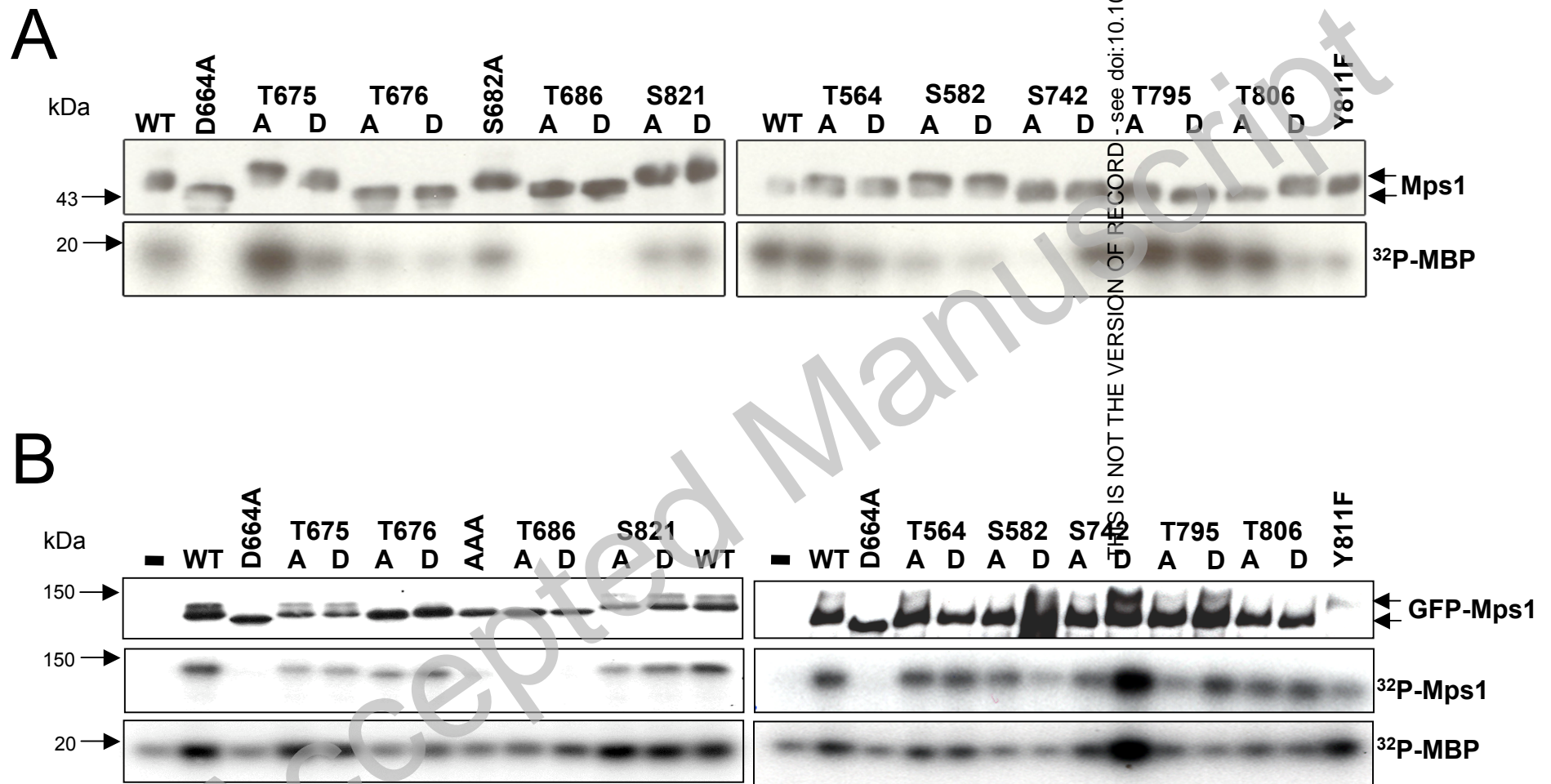
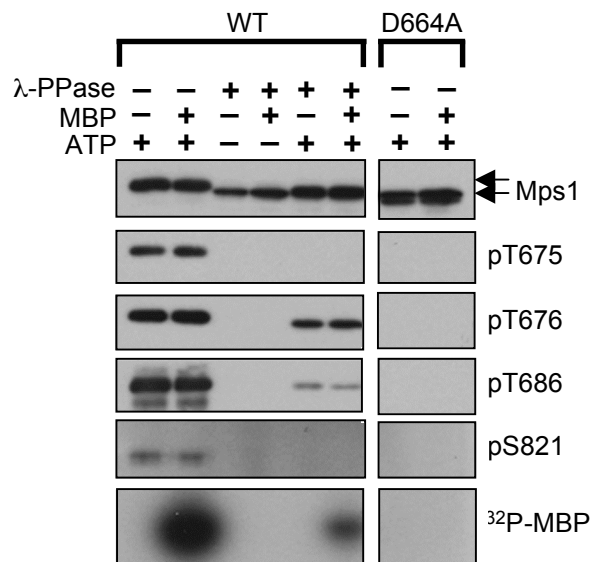
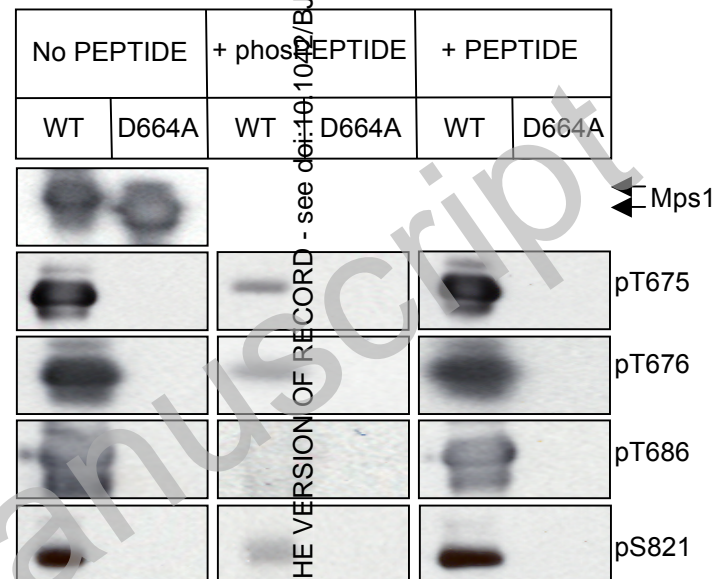


Figure 2 Tyler et al.

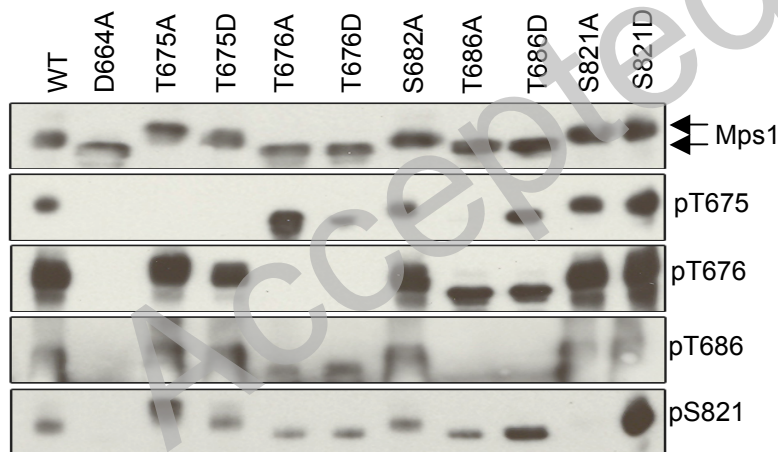
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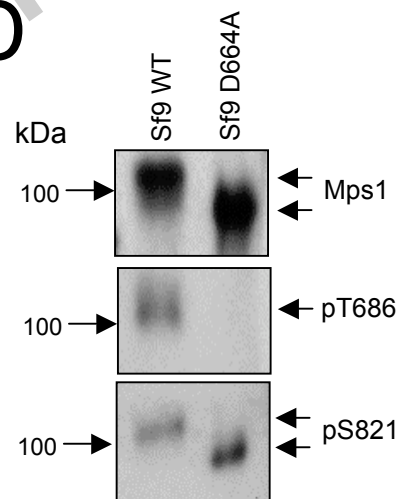
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D



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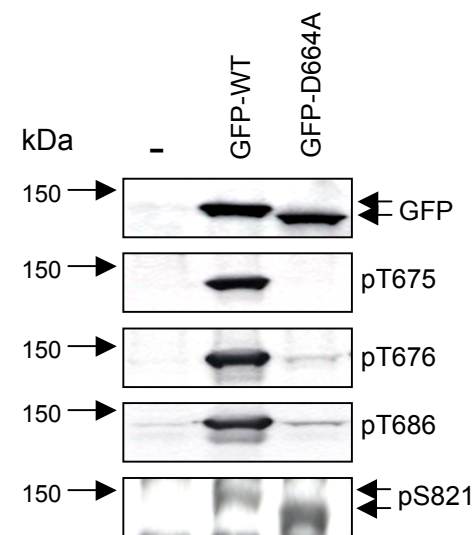
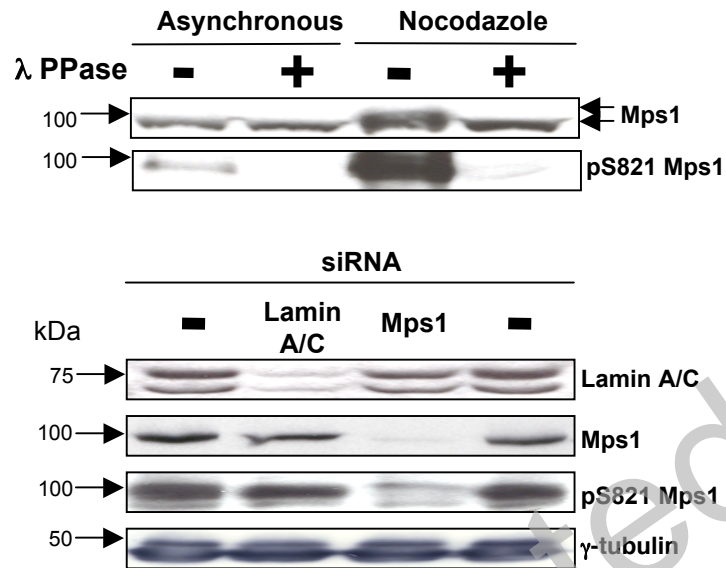


Figure 3 Tyler et al

A



B

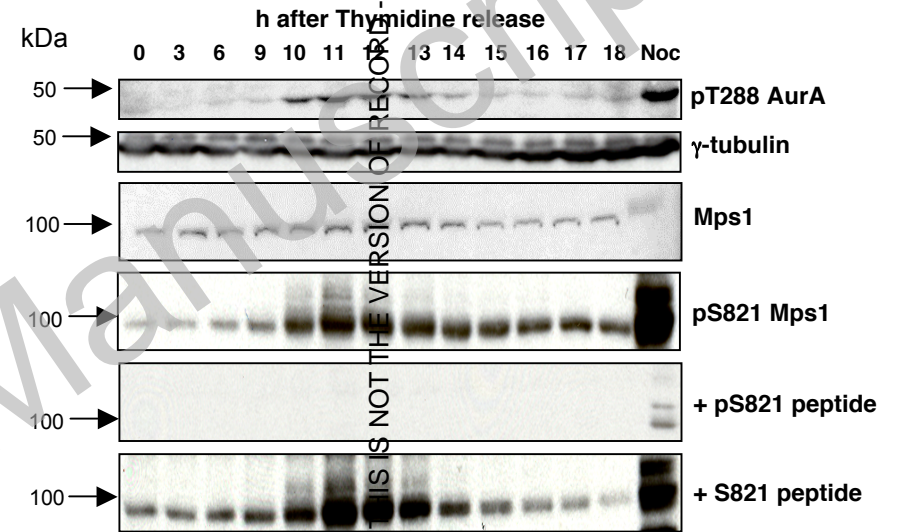


Figure 4 Tyler et al

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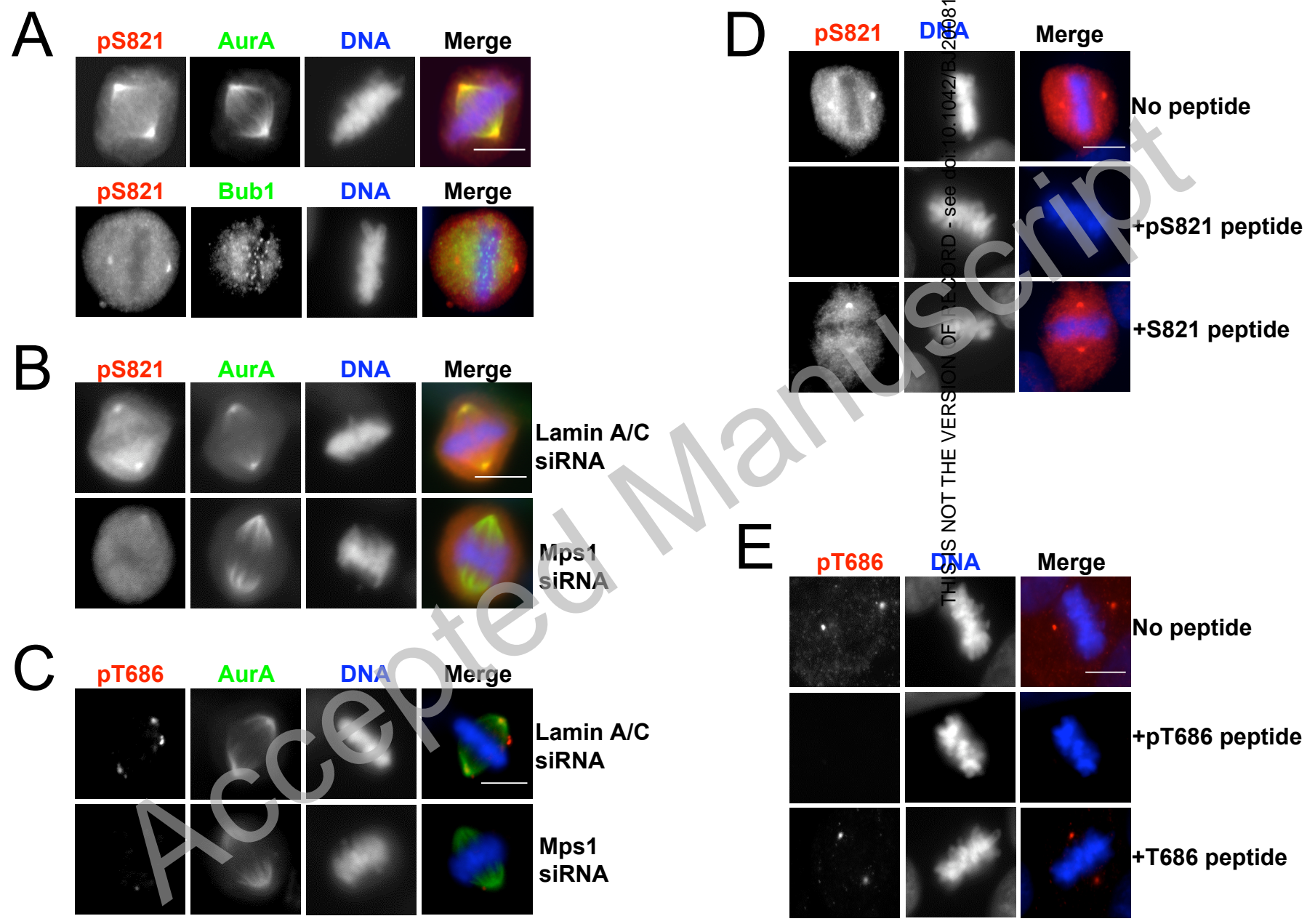
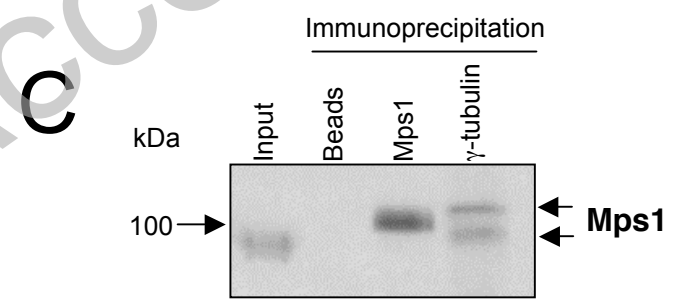
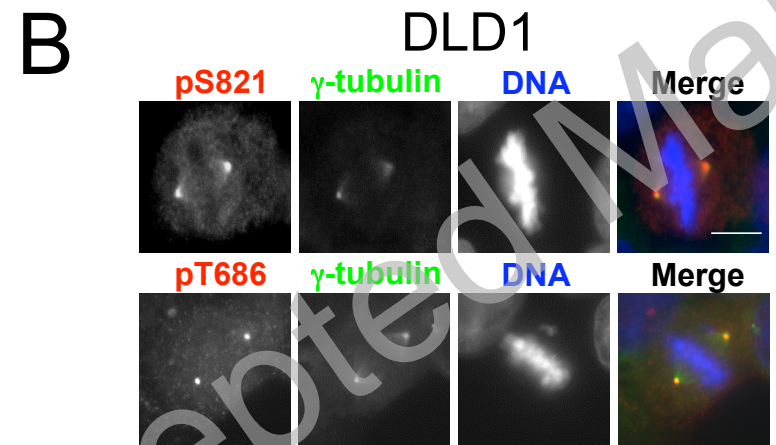
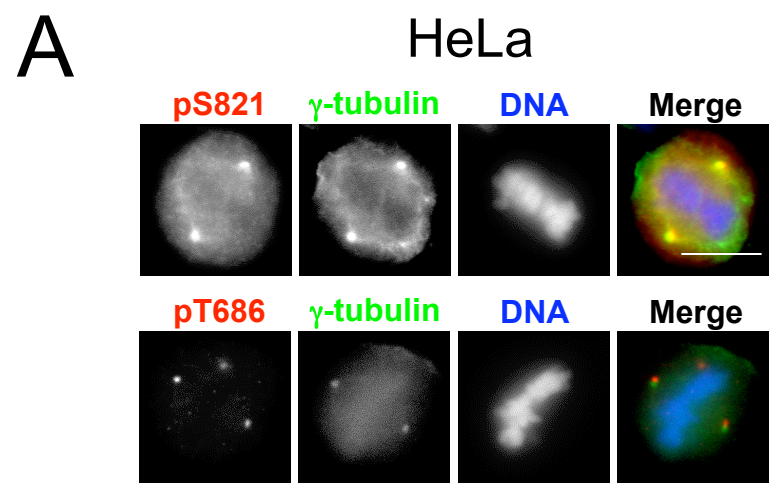


Figure 5 Tyler et al



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Figure 6 Tyler et al