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## The C-terminal domain of Mnk1a plays a dual role in tightly regulating its activity

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## Abstract

The human family of MAP kinase signal-integrating kinases (Mnks) comprises four related proteins derived from two genes by alternative splicing. The Mnk1 gene gives rise to two proteins, Mnk1a and Mnk1b, which possess distinct C-termini and properties.

Despite lacking the C-terminal MAP kinase-binding site, Mnk1b shows higher basal activity than Mnk1a. In contrast, the activity of Mnk1a is tightly regulated by signalling through ERK and p38 MAP kinase.

We show that the short C-terminus of Mnk1b confers on it a 'default' behaviour of substantial, but unregulated, activity. In contrast, the longer C-terminus of Mnk1a represses the basal activity and T (activation)-loop phosphorylation of this isozyme while allowing both properties to be stimulated by upstream MAP kinase signalling.

Two features of the C-terminus of Mnk1a appear to account for this behaviour: the known MAP kinase-binding site and a region (predicted to be  $\alpha$ -helical) which occludes access to the catalytic domain and the T-loop. The activation of Mnk1a results in a marked conformational change leading to a more 'open' structure. We also identify a conserved phenylalanine in an Mnk-specific insert as playing a key role in governing the ease with which Mnk1a can be phosphorylated.

These studies help to identify the features that give rise to the diverse properties of human Mnk isoforms.

**Keywords:** protein kinase; Mnk; ERK; p38 MAP kinase; eIF4E

**Abbreviations used:** eIF, eukaryotic initiation factor; ERK, extracellular ligand-regulated kinase; HEK, human embryonic kidney; hMnk, human Mnk; MAP kinase, mitogen-activated protein kinase; mMnk, mouse Mnk; Mnk, MAP kinase signal-integrating kinase (or MAP kinase-interacting kinase); NES, nuclear export sequence; NLS, nuclear localisation sequence.

## Introduction

The MAP kinase signal-integrating kinases (also termed 'MAP kinase-interacting' kinases; Mnks) were first discovered by virtue of their abilities to be phosphorylated by [1] or to bind to [2] ERK and/or p38 MAP kinase. There are two Mnk genes in mice and humans, Mnk1 and Mnk2. The Mnk proteins that were originally reported (here termed Mnk1a and Mnk2a) each contain, within their C-terminal regions, a motif for binding to MAP kinases (Fig. 1A). Mnk1a binds well to both ERK and p38 MAP kinases ( $\alpha/\beta$ ) while Mnk2a binds better to ERK and only weakly to p38 MAP kinases  $\alpha/\beta$  [2;3]. ERK and p38 MAP kinases phosphorylate two threonine residues in Mnk1/2 which are located in the activation or 'T'-loop. In many protein kinases phosphorylation in this region leads to their activation.

Subsequently it was shown that the human Mnk1 and Mnk2 genes each give rise to two mRNAs, and thus to two distinct polypeptides, as a consequence of alternative mRNA splicing [4-7] (Fig. 1A). In each case, the second form of each polypeptide (Mnk1b or Mnk2b) contains a distinct C-terminal region which is shorter than the corresponding part of Mnk1a/Mnk2a and lacks the MAP kinase-binding site.

The C-terminal region of Mnk1a contains a functional nuclear-export signal (NES) [5] (Fig. 1A). The N-termini of all Mnk isoforms contains a polybasic sequence that binds both to the nuclear transport protein importin- $\alpha$  [5;8] and to eukaryotic initiation factor eIF4G, a scaffold protein [9;10]. Although they possess nuclear localisation signals (NLS), Mnk2a and Mnk1a are either primarily or exclusively cytoplasmic [5;11]. In the case of Mnk1a, this reflects the function of its C-terminal NES. For Mnk2a, which lacks the NES, it seems that the C-terminal region impedes access to the NLS/eIF4G-binding site [5]. Both Mnk1b and Mnk2b localise to the nucleus as well as to the cytoplasm [5;6].

The different Mnk isoforms also display striking differences in basal activity and in regulation (summarised in Table 1). Mnk1b shows higher basal activity than Mnk1a, and this is not stimulated e.g., by treating cells with agents that activate MAPK signalling [6;7].

Mnk2a has high basal activity, which is at least partly due to its ability to remain bound to ERK even after ERK has been activated [3;5]. Treatment of cells with agents that activate ERK or p38 MAP kinases  $\alpha/\beta$ , or with inhibitors of these pathways, has little effect on the activity of Mnk2a. In contrast, Mnk1a has very low basal activity, which can be greatly enhanced by activation of ERK or p38 MAP kinases [9;12]. Mnk1a does not bind stably to phospho-ERK. However, other features of Mnk2a, in addition to the C-terminal domain containing the MAP kinase binding site, are important for its high basal activity: for example, simply replacing the C-terminus of Mnk1a with that of Mnk2a does not confer high basal activity [3]. Features of the catalytic domain also therefore appear to be important.

A striking feature of both Mnk1 and Mnk2 is that their catalytic domains show two highly unusual features: first, their catalytic domain contains a DFD motif (Fig. 1B), which replaces the DFG motif found in subdomain VII of other members of the main protein serine/threonine/tyrosine kinase superfamily [13;14]. Second, the catalytic domain also contains two short inserts relative to other protein kinases (Fig. 1B). Recent determination of the three-dimensional structure of the catalytic domains of Mnk1 and Mnk2 [13;14] indicates that this may cause the Mnks to have a lower ability to bind their substrate ATP than other protein kinases. These studies also revealed features that may be important for the high basal activity of Mnk2a vs. Mnk1a (which may allow Mnk2a more easily to adopt the active conformation; Fig. 1B).

Recent structural studies of the catalytic domains of the Mnks revealed residues which may play a role in determining their properties, including the differences between Mnk1 and Mnk2 [13;14], thereby prompting questions about the role of the specific conserved residues which differ between these domains of Mnk1 and Mnk2.

In this study, we have sought to achieve a better understanding of the properties of the activity of the Mnk1a and Mnk1b isoforms. In particular, we have elucidated the basis of the marked differences in basal activity and regulation of these isoforms. Furthermore, we have examined the roles of specific features of the Mnk catalytic domains in determining the characteristics of the Mnk isoforms.

## Experimental procedures

### *Expression vectors*

For expression in human embryonic kidney (HEK) 293 cells, Mnk cDNAs were cloned into the BamHI and SpeI sites of vector pEBG-6P [15] which allows their expression as fusion proteins with a N-terminal glutathione S-transferase (GST) tag cleavable by PreScission Protease (Amersham Biosciences). Point mutations were introduced using QuikChange site-directed mutagenesis (Stratagene). Truncations were generated by introducing stop codons via QuikChange site-directed mutagenesis. For bacterial expression, Mnk constructs were subcloned from the pEBG-6P vector into the BamHI and NotI sites of pGEX-6P1 (Amersham Biosciences).

### *Cell culture, transfection, treatment and lysis*

Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% (v/v) foetal bovine serum (both Gibco BRL). Transient transfections were carried out using the calcium phosphate precipitation method in *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-buffered saline with cells at a density of approximately  $6 \times 10^5$  to  $8 \times 10^5$  cells per 6-cm plate or  $1.5 \times 10^6$  to  $2 \times 10^6$  cells per 10-cm plate. After 24h, the transfection medium was removed and cells were serum-starved for 16h. Treatment with PMA (phorbol 12-myristate 13-acetate) was for 30 min at a final concentration of  $1 \mu\text{M}$ . Where indicated, PD098059, SB203580 (both Calbiochem) or SP600125 (Biomol) were added 30min prior to addition of PMA, at final concentrations of  $50 \mu\text{M}$ ,  $10 \mu\text{M}$  and  $5 \mu\text{M}$  respectively. Details of other treatments are given in the figure legends. Following treatment, cells were washed once with phosphate-buffered saline (PBS) and harvested in  $400 \mu\text{l}$  (per 10 cm plate) of lysis buffer (20 mM HEPES-KOH [pH 7.5], 50mM  $\beta$ -glycerophosphate, 0.2mM EDTA, 10% glycerol, 1% (v/v) Triton X-100, 1mM dithiothreitol, 0.5mM sodium orthovanadate, plus the protease inhibitors 1mM phenylmethanesulfonyl fluoride, 1 mM benzamidine-HCl,  $1 \mu\text{g/ml}$  leupeptin,  $1 \mu\text{g/ml}$  antipain and  $1 \mu\text{g/ml}$  pepstatin). Cell debris and nuclei were spun down for 5 min at  $16,000 \times g$  and the supernatants transferred to new tubes.

### *SDS-PAGE and immunoblotting*

These procedures were carried out as described earlier [16]. Antibodies used included commercially available anti-phospho-Mnk1 (Cell Signaling Technology) and anti-Mnk1 (M-20) (Santa Cruz) which was raised against an epitope in the N-terminus of Mnk1. We also generated a new antiserum against human Mnk1a by immunising a rabbit with recombinant human Mnk1. Human Mnk1a was expressed as a GST fusion protein in *E. coli*, then cleaved with PreScission protease and purified by SDS-PAGE. The antiserum was suitable for use in western blots without further purification.

### *Mnk1 activity assays*

For the purification of Mnks from HEK 293 lysates,  $50 \mu\text{l}$  of glutathione-Sepharose beads (washed 3 times with lysis buffer), were added to cleared lysate from one 10cm plate and then rotated for 3 hr at  $4^\circ\text{C}$ . Samples were centrifuged 3min at  $1000 \times g$ , the supernatant removed, and the beads washed twice with lysis buffer, once with 0.5M LiCl, and twice with



PreScission Protease cleavage buffer (50mM HEPES-KOH pH 7.5, 150mM NaCl, 0.5mM EDTA, 1mM dithiothreitol). Beads were then resuspended in 50µl of cleavage buffer and digested with PreScission Protease overnight at 4°C. Following digestion, the beads were pelleted at 1000xg for 3min and the supernatant (containing the Mnk protein) was transferred to new tube.

For kinase assays: as below, except used 100ng purified Mnk per assay. Aliquots (5µl) of the beads with bound fusion proteins were incubated in a total volume of 30µl in 20mM HEPES-KOH (pH 7.5), 50mM KCl, 2mM MgCl<sub>2</sub>, 200µM ATP, 2µCi of [ $\gamma$ -<sup>32</sup>P]ATP, 200ng of recombinant eIF4E for 15min at 30°C. The reaction was stopped by adding SDS-PAGE sample buffer and heating the sample for 5 min at 95°C. Samples were analyzed by SDS-PAGE and autoradiography or using a phosphorimager, as indicated.

#### *Analysis of Mnk proteins by limited proteolysis*

Human embryonic kidney (HEK) 293 cells were transfected with plasmids encoding WT or various mutants of Mnk1a with Myc tag. After 16 h, the cells were serum starved for 16 h followed by various treatments as indicated in the Results. The cells were lysed and the lysates were incubated with 0.2 µg of anti-Myc monoclonal antibody for 1 h. An aliquot (20 µl) of protein G agarose beads were added to each sample. After an extra 1 h shaking, the beads were washed twice with a buffer containing 0.5 M LiCl and twice with a buffer containing 50 mM β-glycerophosphate, 1 mM EDTA, 1.5 mM EGTA, and 1 mM dithiothreitol. The beads were resuspended with 30 µl of the same buffer with 1 ng/µl trypsin. The samples were shaken at room temperature for different periods of time and reactions were terminated by adding phenylmethylsulfonyl fluoride (final concentration, 3mM) and sample buffer. The samples were analysed with Western blot probed with antibody against Myc or Mnk1.

#### *In vitro phosphorylation of Mnk proteins by recombinant ERK*

Recombinant Mnk proteins (500ng/assay) were incubated with activated glutathione *S*-transferase-ERK (500ng/assay) produced in *E. coli* [17] for the times indicated at 30°C. Conditions were as described above for assays of Mnk activity except that unlabelled ATP was used. Reaction products were analyzed by SDS-PAGE followed by western blot with an anti-(P)Mnk antibody.

#### *Reproducibility of data*

All data shown are typical of those from at least three independent experiments.

## **Results**

### *Mnk1b displays constitutive activity*

Human Mnk1a and Mnk1b were expressed as GST-tagged fusions in HEK293 cells, using a system where the GST tag can be removed by cleavage (with the PreScission protease). The cells were starved of serum overnight and, in some cases, then treated with the phorbol ester PMA, which activates the ERK pathway. They were then isolated from the resulting cell lysates using glutathione (GSH)-Sephacrose beads. The Mnks were cleaved from GST using the PreScission® protease and the activities of the released Mnks were measured using eIF4E as substrate, taking care that the activity lay within the linear range of the assay. Levels of the Mnk proteins were assessed by blotting with anti-Mnk1 (Fig. 2A; bottom section). As expected from earlier studies [9;12], Mnk1a from serum-starved cells showed low activity while Mnk1a from PMA-treated cells was more active (Fig. 2A; top section). In contrast, Mnk1b showed higher basal activity than Mnk1a in serum-starved cells and this was not further increased by PMA treatment (Fig. 2A; top). Treatment of cells with the MAP

kinase signalling inhibitors PD098059 and SB203580 decreased the basal and PMA-stimulated activities of Mnk1a, but had no discernible effect on Mnk1b activity (Fig. 2A). These findings are broadly in accordance with other recent data [6;7]. The extent of radiolabelling of the Mnk1a polypeptide that occurred during the kinase assay (which is likely due to autophosphorylation) mirrored the activity against eIF4E (Fig. 2A; top). The level of radiolabelling of Mnk1b was lower than that of Mnk1a, when compared either to Mnk levels or to the phosphorylation of eIF4E (Fig. 2A). This lower extent of autophosphorylation could arise if Mnk1b had higher affinity than Mnk1a for eIF4E and thus 'preferentially' phosphorylated eIF4E. Alternatively, some of the phosphorylation sites within Mnk1a may lie in the C-terminal region that is missing from Mnk1b: it is interesting to note that a number of the phosphorylation sites identified in Mnk2a [20] lie within this region and are conserved within Mnk1a.

To study the phosphorylation state of the activation (T-) loop in the Mnks, we used a commercially-available anti-phospho-Mnk (p-Mnk) antibody, which recognises both Mnk1 and Mnk2 (see, e.g., [18]). However, it is not known whether this antibody recognises one or both of the phosphorylated threonines in the activation (T-)loop. Mutation of T209 to alanine or glutamate resulted in the complete loss of reactivity with the phosphospecific anti-Mnk antibody, while in contrast mutation of T214 had only a small effect on reactivity with this antibody (Fig. 2B). These data indicate that the phosphospecific antibody mainly recognises the more N-terminal phosphothreonine (T209) in Mnk1.

For Mnk1a, the levels of T-loop phosphorylation (assessed by western blot) changed roughly in parallel with the observed effects on activity, being increased by PMA and decreased by PD098059 plus SB203580 (Fig. 2A; middle section). The level of T-loop phosphorylation of Mnk1b was essentially the same under all conditions, consistent with its unchanging level of activity. Samples were run in parallel on the same gel to facilitate comparison of the levels of T-loop phosphorylation (see, e.g., Fig. 2A). From the combined data for six independent experiments, it is clear that the level of T-loop phosphorylation of Mnk1b is slightly higher than that of Mnk1a from serum-starved cells (ratio of T-loop phosphorylation of Mnk1b to that of Mnk1a, normalised to total Mnk levels) was  $1.15 \pm 0.06$  (mean  $\pm$  SEM).

The signal for T-loop phosphorylation of Mnk1a after PMA treatment is stronger than that for Mnk1b (taking into account the signals for the anti-Mnk 'N-terminal' antibody (which recognises both Mnk1a and Mnk1b)). This observation implies that T209 in Mnk1b is not fully phosphorylated under the conditions tested here. Nonetheless, Mnk1b tends to show higher phosphorylation than Mnk1a under basal conditions. These findings are broadly consistent with other recent work [6;7] and suggest that a feature of Mnk1a impairs its phosphorylation under serum-starved conditions: this is presumably the region that differs from Mnk1b, i.e., the C-terminus. Furthermore, although the T-loop phosphorylation of Mnk1b was only slightly higher than that of Mnk1a from serum-starved cells, its activity against eIF4E was much higher (Fig. 2A). This suggests that the C-terminus of Mnk1a also impairs its activity against eIF4E, a point which is examined further below.

The above observations raise a number of important questions. Firstly, do MAP kinase family members phosphorylate the T-loop residues in Mnk1b? Secondly, why is the basal phosphorylation of Mnk1a lower than that of Mnk1b, even though the latter lacks the MAP kinase-binding motif found in Mnk1a? Thirdly, why is the activity of Mnk1a from PMA-treated cells lower than that of Mnk1b even though its level of T-loop phosphorylation is generally quite similar to that of Mnk1b?

### *Phosphorylation of Mnk1b*

Nothing is known about the kinases that are responsible for the rather high basal phosphorylation of the T-loop residues in Mnk1b. Certain stimuli, e.g., sorbitol and arsenite, stimulate stress-activated MAP kinases such as the p38 MAP kinases and JNK (Fig. 2C). Treatment of cells with either of these agents only elicited a very small increase, if any, in Mnk1b activity or T-loop phosphorylation (Fig. 2C). Similarly, treatment with pervanadate, which activates a number of MAP kinase signalling pathways (Fig. 2C), also had only a very small effect on the activity or T-loop phosphorylation of Mnk1b (Fig. 2C).

To assess the potential role of JNKs in regulating Mnk1b, we made use of the selective JNK inhibitor SP600125. As shown in Fig. 2D, anisomycin strongly activated JNK isoforms (as revealed using an antibody that recognises phosphorylated [active] JNKs). However, anisomycin did not increase the T-loop phosphorylation or activity of Mnk1b, and treatment of cells with SP600125 did not affect these parameters either (Fig. 2D). These data indicate that Mnk1b is not activated by ERK signalling but may respond slightly to other MAP kinase signalling pathways. However, in view of the small magnitude of these effects, we have not explored this further.

#### *The C-terminal region of Mnk1a governs T-loop phosphorylation and kinase activity*

One possible reason why the basal activity (and to a limited extent, the T-loop phosphorylation) of Mnk1b are generally higher than those of Mnk1a is that the twelve C-terminal residues that are specific to Mnk1b confer these properties. To test this, we created a truncation of Mnk1b, by removing these C-terminal residues and thereby generating a polypeptide that contains the 'core' that is common to Mnk1a and Mnk1b. The behaviour of this protein (Mnk1 $\Delta$ CT) was identical to that of Mnk1b itself, in terms of activity and T-loop phosphorylation, under all conditions tested (Fig. 3A). This indicates that the C-terminal region that is specific to Mnk1b does not exert a positive effect on its T-loop phosphorylation or activity. Indeed, it is consistent with the idea that the difference between Mnk1a and Mnk1b is actually due to the C-terminus of Mnk1a. To test this, we then created a truncation of Mnk1a in which the final 77 residues are removed, to produce a protein which also has 12 residues in addition to the common region (although in this case those 12 residues are different from those in Mnk1b; termed Mnk1a $\Delta$ 77). Its levels of phosphorylation and activity are almost identical to those of Mnk1b and Mnk1 $\Delta$ CT (Fig. 3A). Thus, it apparently the final C-terminal 77 residues of Mnk1a which confer upon this isoform its low basal phosphorylation and activity and conversely allow these parameters to be stimulated, e.g., by PMA in an ERK-dependent manner.

#### *Multiple features in the C-terminal region of Mnk1a modulate its phosphorylation and activity*

To test these ideas, we created a series of deletions of the C-terminus of Mnk1a (depicted schematically in Fig. 3B). As shown in Fig. 3C, the removal of the final 25 residues resulted in a form of Mnk1a that showed low activity under both basal and PMA-stimulated conditions. This is consistent with the dual role for the C-terminus proposed above, because the final 25 residues contain the MAP kinase binding motif (Figs. 1, 3B) and the remaining 64-residue region may act to repress basal T-loop phosphorylation.

To test which parts of the C-terminus are important for modulating the activity of Mnk1a, we created further truncations. Bioinformatic analysis of the C-terminal part of Mnk1a indicates that it may contain several regions of helical structure (as noted in Fig. 3B). As is the case for MK2, another MAP kinase-activated protein kinase, C-terminal  $\alpha$ -helical regions could be important in autoinhibition of the catalytic domain [19]. The  $\Delta$ 25 truncation already removes the most C-terminal  $\alpha$ -helical region. To disrupt the next most C-terminal of the predicted helices, we created a  $\Delta$ 41 truncation mutant (Fig. 3B). This did not increase the



basal phosphorylation or activity of the catalytic domain (Fig. 3C), indicating that the region that was removed does not repress basal Mnk1a phosphorylation or activity.

The remaining region between residues 354 and 372 is strongly predicted to contain a helical feature (Fig. 3B). To dissect further the features that are important for the control of Mnk1a activity, we created a further truncation mutant ( $\Delta 54$ ) which retains the predicted  $\alpha$ -helical feature but lacks the adjacent sequence. As shown in Fig. 3D, the  $\Delta 54$  truncation showed low activity that was not stimulated by PMA, indicating that the remaining C-terminal  $\alpha$ -helical region suffices to block access to the catalytic domain and to the T-loop. To disrupt this region, we introduced a proline residue (A362P) into the middle of this part of full-length Mnk1a, since proline residues cannot be tolerated in  $\alpha$ -helices and therefore 'disrupt' them. Strikingly, this mutant showed very high basal activity that was hardly increased by treating cells with PMA (Fig. 3D). This increase could reflect an effect such as that seen for Mnk1b, where basal activity is high and is apparently not affected by activation or inhibition of MAP kinase signalling (Fig. 2A,C).

To test whether the phosphorylation and activity of the Mnk1a(A362P) variant still depends upon MAP kinases, we treated cells with PD098059 and SB203580 to inhibit ERK1/2 and p38 MAP kinase  $\alpha/\beta$  signalling. This decreased the phosphorylation and activity of Mnk1a(A362P) (Fig. 3D), implying that these pathways maintain the high basal activity of this mutant. This contrasts with the situation for Mnk1b (Fig. 2A). To test this further, we mutated the highly conserved leucyl residue (Leu403) in the MAP kinase-binding site of Mnk1a(A362). This resulted in a dramatic decrease in both T-loop phosphorylation and kinase activity (Fig. 3D; extreme right-hand four lanes). This demonstrates that the MAP kinase-binding site plays an important role in the phosphorylation of the Mnk1a(A362P) mutant, as it does in wildtype Mnk1a [3]. This implies that the A362P mutation allows the T-loop sites to be more readily phosphorylated, but in contrast to Mnk1b, this is mediated by MAP kinases.

The data described above show that the distinct properties of Mnk1a are thus largely determined by two features of its C-terminus, i.e., its MAP kinase-binding domain (required for its activation) and a predicted  $\alpha$ -helical feature (which 'governs' access to the catalytic domain, serving to suppress basal T-loop phosphorylation and activity).

#### *Activation is associated with a marked conformational change in Mnk1a*

The idea that the C-terminus of Mnk1a impedes access for the activating kinases to the T-loop implies that it would also block access for protein substrates such as eIF4E. This suggests that following activation, Mnk1a must undergo conformational changes that allow access to its substrate. To test whether Mnk1a does indeed undergo a conformational change upon activation, we isolated Mnk1a from control and PMA-treated cells and subjected it to limited tryptic proteolysis (Fig. 4A). Samples were then analysed by SDS-PAGE followed by western blotting either with anti-Mnk1 (raised against the whole protein) or an antibody to the N-terminal myc tag. The data (Fig. 4A) reveal that Mnk1a becomes markedly more susceptible to tryptic digestion following its activation, as indicated by its faster cleavage to smaller fragments, which is evident after detection with either antiserum.

#### *Role of T344*

It has previously been reported that mutation of T332 to aspartate in mouse Mnk1 (similar to Mnk1a) led to increased kinase activity [9] although the mechanisms underlying this effect remained unclear. The constructs used in that study encoded a murine protein that is shorter than the originally-identified human Mnk1 proteins due to the absence of twelve N-terminal residues (MVSSQKLEKPIE, in human Mnk1), so that in human Mnk1a this residue is actually T344. T344 lies in the first part of the unique C-terminus of Mnk1a (Fig. 3B) and

could serve to modulate the conformation of this region and its ability to control access to the T-loop and the catalytic domain. The corresponding residue in mouse Mnk2 is known to be phosphorylated [20]. Like T332 in mouse Mnk1, this residue in Mnk1 is followed by a proline, so it could be a substrate for MAP kinases.

Mutation of T344 to Glu (potentially mimics phosphothreonine) increased the activity of Mnk1a after PMA treatment and, more strikingly, both enhanced its basal activity in serum-starved cells (Fig. 4B) and led to a modest increase in the level of T-loop phosphorylation. Interestingly, mutation of T344 to alanine also tended to enhance Mnk1a's basal and PMA-stimulated activities (Fig. 4B). This observation indicates that, although T344 is a potential phosphorylation site, its phosphorylation is not required for activation of Mnk1a.

The T344E mutation clearly also affects the conformation of Mnk1a as assessed by the limited proteolysis approach described above: this mutant yielded a different (smaller) major cleavage product than the wildtype protein indicating an altered conformation (Fig. 4C). The T344A mutant also yielded a higher proportion of the smaller product than wildtype Mnk1a, although the difference was less marked than for the T344E mutant (Fig. 4C). This suggests that mutation of T344 alters the structure of Mnk1a in a way that makes it more accessible for protease digestion. The observation that this mutation also tends to make Mnk1a more susceptible to proteolysis suggests that it may lead to the loss of an interaction (such as an H-bond) in which threonine, but not alanine or glutamate, can participate.

The effects of the T344E mutation on the conformation of Mnk1a are independent of the phosphorylation of the T-loop as shown by the observation that a T344E,T2A2 mutant (in which both T-loop threonines are replaced by alanines) still shows enhanced susceptibility to cleavage by trypsin (Fig. 4D). In contrast, this mutant is catalytically inactive indicating that the activation of Mnk1a induced by the T344E mutation still depends upon T-loop phosphorylation (Fig. 4E).

Although Mnk1b displays higher activity than Mnk1a under basal conditions, wildtype Mnk1b and the Mnk1 $\Delta$ CT mutant show similar levels of T-loop phosphorylation to Mnk1a (Fig. 3A). One possible explanation for this is Mnk1b adopts an "active" conformation, even when it is not phosphorylated at T209. This would be consistent with the observations in Fig. 4B,D which reveal that the change in conformation associated with high basal activity is independent of T-loop phosphorylation. To test this hypothesis we compared the sensitivity to tryptic digestion of wildtype Mnk1b and the T2A2 mutant. As shown in Fig. 4F, the tryptic cleavage patterns, including time courses, observed for the two proteins are almost indistinguishable. This suggests that the conformation of Mnk1b is not greatly affected by the state of phosphorylation of its T-loop.

#### *Residues in the catalytic domain of Mnk1 influence its activity and T-loop phosphorylation*

The above data suggest that features of the C-terminus of Mnk1a strongly influence its activation state and/or activity, likely by affecting access for MAP kinases to the catalytic domain and the activation loop. It has been suggested, on the basis of the available crystal structures [13;14], that specific residues that differ in the catalytic domains of Mnk1 and Mnk2 themselves may cause the activation loop of the latter to be less stringently auto-inhibited by the conformation of the activation loop than that of Mnk1 [14]. This could help explain both the higher activity of Mnk2 [3;5] and its better binding to an ATP analog [14]. In particular, Y60 and T97 in Mnk1 may form stronger interactions with certain backbone amides and carbonyls than the corresponding residues in Mnk2 (H95 and M132; numbering based on the human proteins).

To study the roles of these residues, we created the single Y60H and T97M mutants of Mnk1, as well as the corresponding double mutant. In addition, since we showed earlier, using 'chimeric' proteins, that the C-terminal region of Mnk2a plays a critical role in its

higher activity [3], we also created the same mutations in the previously-described chimera (“Mnk1-C2”), in which the C-terminal tail of mouse Mnk2 replaces murine Mnk1’s own C-terminal region [3]. Strikingly, the level of phosphorylation of the T-loop was also increased in these mutants, under serum-starved conditions, both for the variants based on wildtype Mnk1 and for those based on the chimera (Fig. 5A,B).

In Mnk1, the T97M mutation reproducibly increased the basal levels of activity and T-loop phosphorylation almost to those seen in wildtype Mnk1a after PMA stimulation (Fig. 5A). In contrast, the Y60H mutation itself had essentially no effect and did not enhance that of the T97M mutation. In the Mnk1-C2 chimera, which is based on mouse Mnk1, the corresponding mutations (Y48H and T85M) had qualitatively similar effects to those they had in Mnk1 itself (Fig. 5B), but the magnitude of the effect of the T85M mutation on T-loop phosphorylation was much greater, and the double mutation had a larger effect than the T→M mutation alone. Thus, the activity of Mnk1-C2[Y48H/T85M] was similar to that of the mMnk2-C1 chimera although it was lower than that of mMnk2 itself (Fig. 5B). The double mutant (Y48H/T85M) showed greatly enhanced T-loop phosphorylation (Fig. 5B).

Two unusual features of the catalytic domains of the Mnks are the presence of (i) a DFD rather than the ‘canonical’ DFG motif in sub-domain VII and (ii) a short insert (EVFTD in Mnk1) between sub-domains VIII and IX [14]. The crystal structure suggested that the phenylalanine residue of the insert (F230 in hMnk1a) prevents the DFD motif from forming the ‘DFG/D-in’ conformation which allows ATP binding [13;14]. Other features of the DFD motif stabilize the resulting ‘DFG/DFD’-out conformation.

We mutated the DFD motif to DFG. In Mnk1a (D193G mutant), this resulted in, at most, a very small increase in basal activity and T-loop phosphorylation (Fig. 5A). In Mnk1-C2 (D181G mutant), there was a discernible increase in both T-loop phosphorylation and catalytic activity (Fig. 5B). The D→G mutation is predicted to make it easier for ATP to bind to the Mnk proteins, which should decrease the  $K_m$  for ATP. However, in multiple experiments, we failed to observe any difference in apparent  $K_m$ (ATP) when the D→G mutant was compared with the wildtype protein (data not shown), suggesting that the DFD motif does not normally impair ATP binding to full-length Mnk1a. Indeed, the properties of the DFG mutant were very similar to those of wildtype Mnk1a.

To test the role of the phenylalanine residue within the insert (EVFTD), we mutated it to alanine in Mnk1a (F230A) and in the Mnk1-C2 chimera (where the corresponding mutation is F218A). In both cases, this strikingly and very substantially increased T-loop phosphorylation (Fig. 5A,B). It also slightly enhanced activity against eIF4E, although the magnitude of this effect was much smaller than might have been expected based on the change in T-loop phosphorylation. This suggests that the F→A mutation may increase access to the T-loop for activating kinases but may actually impair the binding of protein substrates or interfere with catalytic activity in another way. This distinction between T-loop phosphorylation and activity is of particular interest and again suggests that phosphorylation in the T-loop does not by itself suffice to activate Mnk1a.

To try to gain insight into the mechanism by which the F230A mutation affects the Mnk protein, we tested its susceptibility to tryptic proteolysis in parallel with WT Mnk1a: as shown in Fig. 5C, no discernible difference was observed. It therefore appears that this point mutation within the catalytic domain does not open up the structure in an analogous way to the T344E mutation in the tail.

Given the high basal level of T-loop phosphorylation of the F230A mutant, it seemed possible that it was phosphorylated by MAP kinases with greater efficiency than wildtype Mnk1a. To study this, we expressed WT Mnk1a and the F230A, T344E and (as a control)  $\Delta 71$  mutants in *E. coli*, and then incubated them with active ERK and ATP. Samples were analysed by western blot using the T-loop P-specific antibody. To establish suitable



conditions for assessing the phosphorylation of Mnks by ERK, we first incubated wildtype Mnk1a with ERK for various times (Fig. 6A). The untreated recombinant Mnk1a showed no reaction with the P-specific antibody, while the signal seen with this antibody increased with increasing time of incubation with ERK. The data suggested that 10-15 min was within the linear range of the assay, and these times were therefore used to test the susceptibility of different Mnk proteins to phosphorylation by ERK. The F230A mutant underwent phosphorylation by ERK much more readily than WT Mnk1a (Fig. 6B). This appears not to be due to a gross conformational change of the kind observed upon activation of Mnk1a or for the T344E mutant (cf. Figs. 4A,C and 5C). In contrast, the T344E mutant showed lower T-loop phosphorylation after incubation under identical conditions (Fig. 6B). The  $\Delta 71$  mutant was not detectably phosphorylated, and neither was Mnk1b, tested in parallel (Fig. 6B). This is consistent with earlier studies [6]. The Mnk1a( $\Delta 71$ ) protein was a very poor substrate for ERK *in vitro*, consistent with the absence of the known ERK-binding site (Fig. 3B). Mnk1b was also not detectably phosphorylated by ERK in this type of assay (Fig. 6B).

## Discussion

The present study helps to explain the differences in the properties between Mnk isoforms. We show that the C-terminal part of Mnk1a acts in two main ways to affect the properties of this protein: first, it ensures a low basal level of activity and phosphorylation, and second, it allows activation by signals which activate ERK (or p38 MAP kinase  $\alpha/\beta$ , see, e.g., [12]). The latter property appears to be conferred by the C-terminal MAP kinase-binding site ([1;3], this report). For example, deletion of the final 25 residues which contain this feature, or mutation of the leucine in the motif, abolishes T-loop phosphorylation of Mnk1a. Interestingly, deletion of further stretches of the C-terminal part of Mnk1a actually enhanced its T-loop phosphorylation and *in vitro* activity (e.g.,  $\Delta 77$  mutant, Fig. 3). This suggests that the region between residues 348 and 400 (between the  $\Delta 77$  and  $\Delta 25$  truncations) represses T-loop phosphorylation and thus activity (as depicted schematically in Fig. 7). This section contains two predicted  $\alpha$ -helical regions. Removing the more C-terminal one did not affect basal activity or T-loop phosphorylation ( $\Delta 41$ ,  $\Delta 54$  mutants), indicating that the N-terminal one plays the dominant role in affecting the properties of Mnk1a (see Fig. 7). This is confirmed by the finding that mutating a residue within that  $\alpha$ -helical region to proline (A362P) dramatically enhanced basal phosphorylation and activity. However, the behaviour of this mutant still differs strikingly from that of Mnk1b, in that its basal activity/phosphorylation are inhibited by PD098059/SB203580 and increased by PMA: i.e., they still require MAP kinase signalling. This is consistent with the fact that this mutant still contains the MAP kinase-recruitment motif. The ability of Mnk1a's C-terminus to obstruct access to the catalytic domain may also explain why, for a given level of T-loop phosphorylation, Mnk1b shows higher activity than Mnk1a against the protein substrate eIF4E. The fact that the tryptic sensitivities of wildtype Mnk1b and the T-loop (T2A2) mutant are almost identical suggests that the conformation of this enzyme is not greatly affected by T-loop phosphorylation. Thus it may adopt an active conformation even when the T-loop is not phosphorylated, perhaps explaining why, for a given apparent level of T-loop phosphorylation, Mnk1b shows appreciably higher activity than Mnk1a. It is possible that Mnk1b is able to adopt this "active" state because it lacks the inhibitory C-terminal tail that is present in Mnk1a.

Unlike Mnk2a, Mnk2b does not show high basal activity. Indeed, its intrinsic activity is very low and is not increased by agents that activate ERK, p38 MAP kinases or a number of other signalling pathways [5]. This likely reflects the absence of the MAP kinase-binding motif. Mnk1b also lacks the MAP kinase-binding site, but, surprisingly, is reported to display higher activity than Mnk1a [6;7].

Like Mnk1a, MK2 is a MAP kinase-activated protein kinase [21]. Its regulation shares some features in common with Mnk1a. The C-terminal region of MK2 is also important in its control and apparently also contains a helical feature, which likely packs against the catalytic domain [19]. Molecular modeling suggests that this involves hydrophobic residues from the catalytic core of MK2. Activation of MK2 is accompanied by a major conformational change which appears to depend upon T-loop phosphorylation [22]. PMA-induced activation of Mnk1a was accompanied by a marked increase in its sensitivity to tryptic digestion, again indicating a probable 'opening' of its structure.

Mutation of T344 (T332 in mouse Mnk1) has previously been shown to affect Mnk1 activity [6;7;23]. Our data show that mutating T344 to glutamate both activates Mnk1a and induces a conformational change making it more prone to tryptic digestion. This residue lies in a suitable context for phosphorylation by MAP kinases and is indeed phosphorylated in mouse Mnk2 [20]. However, we show that mutating T344 to a non-phosphorylatable (alanine) residue did not affect basal or PMA-stimulated Mnk1a activity. In fact, the T344A mutation actually tended to induce a small increase in activity (see also [7]). Thus, T344 appears to help keep Mnk1a in a less active, relatively closed conformation under basal conditions, perhaps by participating in the interactions involving the C-terminal region which suppress its activity. Mutation to glutamate (and perhaps alanine) may weaken this interaction, thereby opening up the conformation and activating Mnk1a. Phosphorylation of T344 may exert a similar effect but the fact that mutation to a non-phosphorylatable residue (alanine) also leads to slight activation precludes us from testing this directly.

Structural studies on their catalytic domains have suggested that the Mnks are controlled by an 'auto-inhibition' mechanism; this would presumably operate in concert with the effects of the C-terminal region described above. It was suggested that specific residues (Y60 and T97 in Mnk1; corresponding to a histidine and a methionine, respectively, in Mnk2) are involved in the differential stringency of the autoinhibition of these proteins. We have now studied the roles of these residues in modulating Mnk1a activity. Converting Y60 in Mnk1a to histidine had little effect on Mnk1a activity or phosphorylation, while the T97M mutation slightly increased both, as did the double mutation. Nonetheless, both activity and T-loop phosphorylation remained well below the levels seen for human Mnk2a. Thus, these residues do not by themselves determine the differing activities of Mnk1a/2a, consistent with our earlier work, which showed their C-termini play major roles in determining their properties [3].

In view of these earlier observations, we analysed the effect of the point mutations in the context of a chimera comprising the N-terminal and catalytic domains of Mnk1 and the C-terminus of Mnk2 ("mMnk1-C2") using these domains of the mouse Mnks to facilitate comparison with our earlier work [3]. The T85M, alone or in combination with the Y48H mutation, had a much greater effect on the chimera, increasing its T-loop phosphorylation and (probably as a consequence) activity markedly. Thus, these two residues do markedly affect the properties of the Mnks but (i) this is only observed in combination with the C-terminal domain of Mnk2 and (ii), rather than affecting activity *per se*, they strongly influence T-loop phosphorylation, probably by increasing access for the activating kinases to the T-loop. These observations support the idea that communication between the N-terminal/catalytic and C-terminal regions of the Mnks is important in modulating their functions, as exemplified by other data from this study and earlier findings [3].

All Mnk isoforms contain two other unusual features within their catalytic domains: (i) a DFD rather than DFG motif (in sub-domain VII) and (ii) an Mnk-specific insert (EVFTD in Mnk1) between sub-domains VIII and IX. The roles of these features in affecting the activity and/or regulation of the Mnks have not previously been tested. It has been suggested that the DFD motif makes it more difficult for ATP to bind to the catalytic domain [13;14].



However, this mutation had little effect on Mnk activity, even at sub-saturating concentrations of ATP (data not shown). Surprisingly, mutation of the DFD motif to the canonical DFG sequence in the Mnk1-C2 chimera (but not in Mnk1a itself) actually increased T209 phosphorylation, suggesting enhanced access to the T-loop.

Structural studies show that the conserved phenylalanine residue in the insert lies within the DFG/D pocket [13;14]. We therefore also tested the effect of mutating it to alanine: in both Mnk1a and the mMnk1-C2 chimera, this mutation markedly increased T-loop phosphorylation, suggesting that the phenylalanine residue may place the activation (T-)loop in a less-favourable conformation for phosphorylation by the activating kinases. This is borne out by the observation that the F230A mutant of human Mnk1 is phosphorylated much more readily by ERK (*in vitro*) than the wildtype protein (Fig. 6B). This finding contrasts with the interpretation of the structural studies, which suggested that the positioning of the T-loop in wildtype Mnks actually represented a favourable conformation for phosphorylation: the F230A mutation apparently results in an even more favourable configuration. The observation that the DFD-to-DFG mutation in Mnk1a did not have this effect indicates that the variant DFD motif itself is not an important factor here, while the phenylalanine residue in the EVFTD insert does play a major role. In contrast to the effects of changes in the C-terminus, the F230A mutation did not increase the susceptibility of Mnk1a to tryptic digestion, suggesting it has different and/or more limited, effects on the overall conformation of the protein. This is likely because it does not actually lie within the C-terminal region.

Structural studies on the (activated) full-length Mnk proteins are required to understand more fully how their activities are controlled (the available crystal structures are of fragments that lack the C-terminal regions which play a key role in regulating Mnk activity ([3]; this study) and the activation loop was disordered in these structures [13;14]).

In summary, our data extend earlier functional and structural studies on the Mnks, reveal that the tight regulation of Mnk1a activity involves interplay between the catalytic and C-terminal domains (Fig. 7) and provide an explanation for the markedly different properties of the Mnk1a and Mnk1b isoforms. The C-terminal region of Mnk1a plays multiple roles in its control, including recruiting the activating kinases and modulating the accessibility of the T-loop. In contrast, the short C-terminus of Mnk1b seems to allow easy access of the T-loop to phosphorylation by kinases that are basally active and to allow Mnk1b to display a relatively higher activity than Mnk1a for a given level of T-loop phosphorylation. This may reflect easier access for the protein substrate eIF4E to the catalytic domain of Mnk1b than for Mnk1a (Fig. 7).

Unlike Mnk2a, Mnk2b does not show high basal activity. Indeed, its intrinsic activity is very low and is not increased by agents that activate ERK, p38 MAP kinases or a number of other signalling pathways [5]. This likely reflects the absence of the MAP kinase-binding motif. Mnk1b also lacks the MAP kinase-binding site, but, surprisingly, is reported to display higher activity than Mnk1a [6;7].

These data extend understanding of the molecular basis of the human Mnks, a family of protein kinases with a broad repertoire of regulatory and other characteristics (summarised in Table 1).

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### Footnotes

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**Table 1. Characteristics of human Mnk isoforms.**

All isoforms contain N-terminal polybasic region (PBR; Fig. 1A) which acts as an NES and is involved in binding to eIF4G. The N-termini of each pair are identical, but differ between Mnk1a/b and Mnk2a/b.

<b>Isoform</b>	<b>Basal activity</b>	<b>Regulation of activity</b>	<b>Subcellular localisation</b>	<b>Comments (see also Fig. 1A)</b>
Mnk1a	Low	Markedly increased by ERK/p38 MAP kinases	Mainly cytoplasmic	Long C-terminus contains NES (nuclear export signal), and MAP kinase-binding site
Mnk1b	High	No marked regulation	Partly nuclear	Very short C-terminus lacks NES and MAP kinase-binding site
Mnk2a	Very high	Slightly increased by ERK/p38 MAP kinases	Mainly cytoplasmic	Long C-terminus contains no NES; C-terminus impairs access to NLS; can bind activated ERK
Mnk2b	Very low	Not known	Partly nuclear	Shorter C-terminus lacks NES or MAP kinase binding site

### Figure legends

**Fig. 1.** Schematic diagram of the overall structures of the Mnks. **(A)** overall layout of the four known human Mnk isoforms (hMnks) and mouse (m) Mnk1 and Mnk2. The catalytic domains, polybasic regions (PBR), nuclear export sequences (NES; in hMnk1a, mMnk1) and MAP kinase (MAPK) binding sites (black box) are indicated. **(B)** Comparison of sections of the sequences of the catalytic domains of human Mnk1 and Mnk2. Residues or features that are mutated in this study are highlighted: from the N-terminal end, these are: Y60 and T97 (white on black background); the DFD motif (shaded box); T209/T214 in the T-loop (indicated by (P)), and the kinase inserts (MKLNNSCT and EVFTD in Mnk1; shaded boxes).

**Fig. 2.** Comparison of the regulation of the activities of Mnk1a, Mnk1b, and certain mutants. **(A)** HEK293 cells were transfected with vectors encoding Mnk1a or Mnk1b. 24h later, cells were starved of serum overnight, and then treated with PMA. Where indicated, PD098059 (PD) and SB203580 (SB) were added 30 min prior to PMA. Cells were lysed and GST-pull downs were performed. After cleaving off the GST, samples of the supernatant were analysed for Mnk activity, using eIF4E as substrate (top section of each panel; phosphorimage) or by immunoblot using anti-Mnk1, anti-phospho-Mnk or other antisera as indicated. The vertical line denotes that the signals are from non-adjacent lanes from the same gel. **(B)** HEK293 cells were transfected with vectors encoding the indicated forms of myc-tagged human Mnk1a. 24h later, cells were starved of serum overnight, and then treated with PMA. Samples of cell lysates were analysed by western blot with the anti-phospho-Mnk and anti-myc antibodies. **(C,D)** HEK293 cells were transfected with a vector encoding Mnk1b. Serum-starved cells were treated with sodium pervanadate (15 min; prepared by mixing orthovanadate and H<sub>2</sub>O<sub>2</sub>, such that the final concentrations are 0.1 mM vanadate and 0.25mM H<sub>2</sub>O<sub>2</sub>), PMA, sodium arsenite (0.4 M, 30 min) or sorbitol (0.5mM, 30 min). Samples of lysates were analysed by western blot using the indicated antisera (top four images) or for eIF4E kinase activity (autoradiograph) or by anti-Mnk1 western blot (lower two panels). In (D), where indicated, cells were transfected with a vectors encoding Mnk1b. The cells were treated with SP600125 prior to addition of anisomyacin or pervanadate. [vec] indicates cells were transfected with the empty vector.

**Fig. 3.** Effects of truncation or mutation of the C-terminal region on the phosphorylation and activity of Mnk1a/b. **(A)** HEK 293 cells were transfected with vectors encoding the indicated Mnk polypeptides. Cells were starved of serum and subjected to treatment with PMA or signalling inhibitors as described for Fig. 1C-E. Cell lysates were processed for Mnk activity assays as described in the Experimental Procedures, and samples were also analysed for T-loop phosphorylation and total Mnk protein by western blot (middle and bottom sections). The upper section shows phosphorimager data for the fixed gel: the positions of the labelled eIF4E and autophosphorylated Mnk proteins are indicated. The vertical lines denote that the signals are from non-adjacent lanes. **(B)** Schematic depiction of the C-terminal region of Mnk1a, showing the predicted  $\alpha$ -helical sections, the truncations tested here, T344 and the A362P mutant. **(C,D)** as (A), using the indicated variants of Mnk1a. <sup>32</sup>P-labelled proteins were visualised using a phosphorimager.

**Fig. 4.** Limited proteolytic digestion of Mnk proteins. **(A)** Mnk1a (myc-tagged) was expressed in HEK293 cells. Cells were starved of serum overnight and, where indicated, treated with PMA. After immunoprecipitation, samples were incubated with trypsin (1  $\mu$ g/ml) for the indicated times and the reaction products were analysed by SDS-PAGE and western blot using anti-Mnk1. **(B)** Cells were transfected with vectors for myc-tagged Mnk1a or the T344A or T344E mutants. Cells were starved of serum overnight and, where indicated,



treated with PMA. Mnk proteins were immunoprecipitated and analysed by western blot using the anti-(P)Mnk or myc antibodies (indicated) or by kinase assay using eIF4E as the substrate ('eIF4E<sup>32P</sup>']; phosphorimager analysis). Shown are data from five independent experiments (mean  $\pm$  SEM). (C) WT Mnk1a and the T344E and T344A mutants were expressed in HEK293 cells, immunoprecipitated and subjected to limited proteolysis as in panel A. (D) As A and C, but using the T2A2 and T2A2/T344E mutants. (E) The indicated forms of Mnk1a were expressed in HEK293 cells, which were starved of serum overnight and, where indicated, treated with PMA. After myc-IP, samples were analysed by SDS-PAGE/western blot (anti-myc as 'loading control) and by kinase assay against eIF4E (bottom section; phosphorimager data). (F) As A, C and D but using wildtype Mnk1b or the T2A2 mutant as indicated.

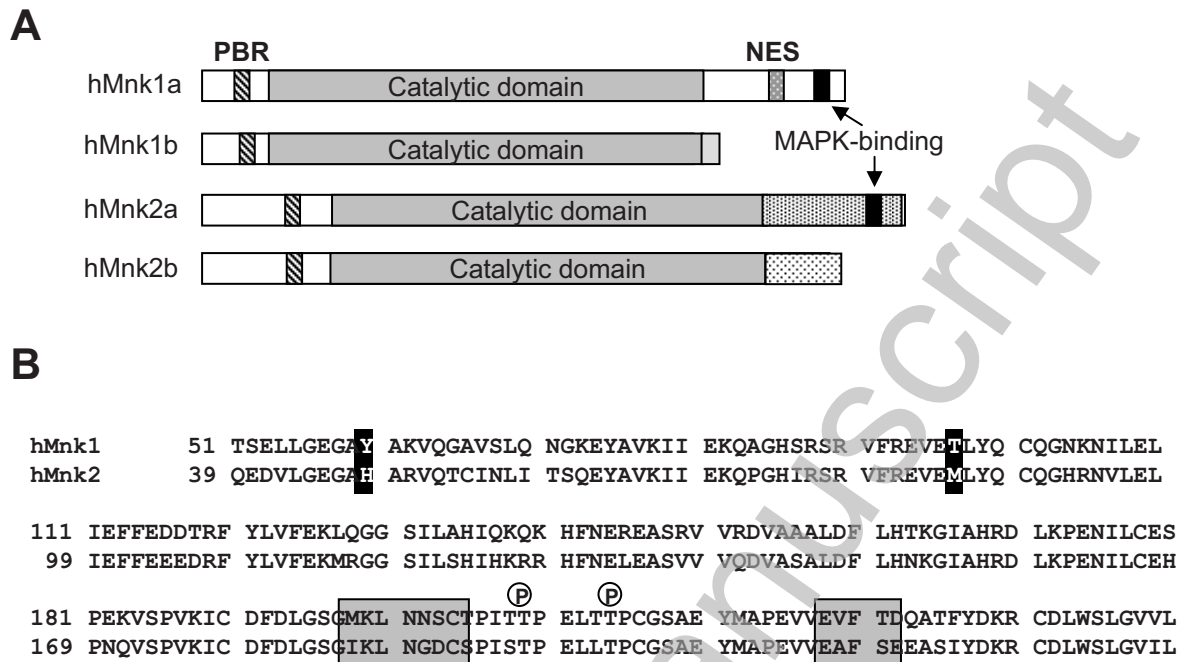
**Fig. 5.** Effects of mutations in the kinase domain on the properties of Mnk proteins. (A,B) HEK293 cells were transfected with vectors encoding GST-tagged wildtype human (h)Mnk1a, the mouse (m)Mnk1 fusion with the C-terminal part of mouse Mnk2 (termed mMnk1-C2), or the indicated mutants (including the DFD-to-DFG variants). After isolation on glutathione-Sepharose and cleavage by the PreScission protease, samples were subjected to activity assay using eIF4E as substrate; to western blot using the antiphospho-Mnk antibody or SDS-PAGE followed by visualisation using Coomassie blue. In each case, the top section shows an autoradiograph of the stained gel, the middle a western blot and the bottom one the stained gel. (C) The wildtype Mnk1a protein and the F230A mutant were expressed in *E. coli*, isolated and cleaved as in Panels A/B, and then subjected to limited tryptic proteolysis. The products were analysed by SDS-PAGE and immunoblots using the anti-Mnk antibody.

**Fig. 6.** Phosphorylation of selected Mnk1 polypeptides by ERK *in vitro*. (A,B) The indicated Mnk proteins or mutants were expressed as GST fusions in *E. coli* and then purified as described in the Experimental Procedures. They were then incubated for the indicated times with active ERK and unlabeled ATP. Samples were subjected to SDS-PAGE followed by western blot using anti-phospho-Mnk (top), anti-Mnk1 (middle) or anti-phosphoERK (bottom). In (A), '0' denotes a sample of recombinant Mnk1a that was not treated with ERK.

**Fig. 7.** Schematic summary of conclusions from these studies. Please see the text for a detailed discussion. PBR, poly-basic region; MAPK, MAP kinase-binding site. The 'T's on the catalytic domain represent the T-loop phosphorylation sites; 'T344'; threonine 344 in the C-terminus of Mnk1a.

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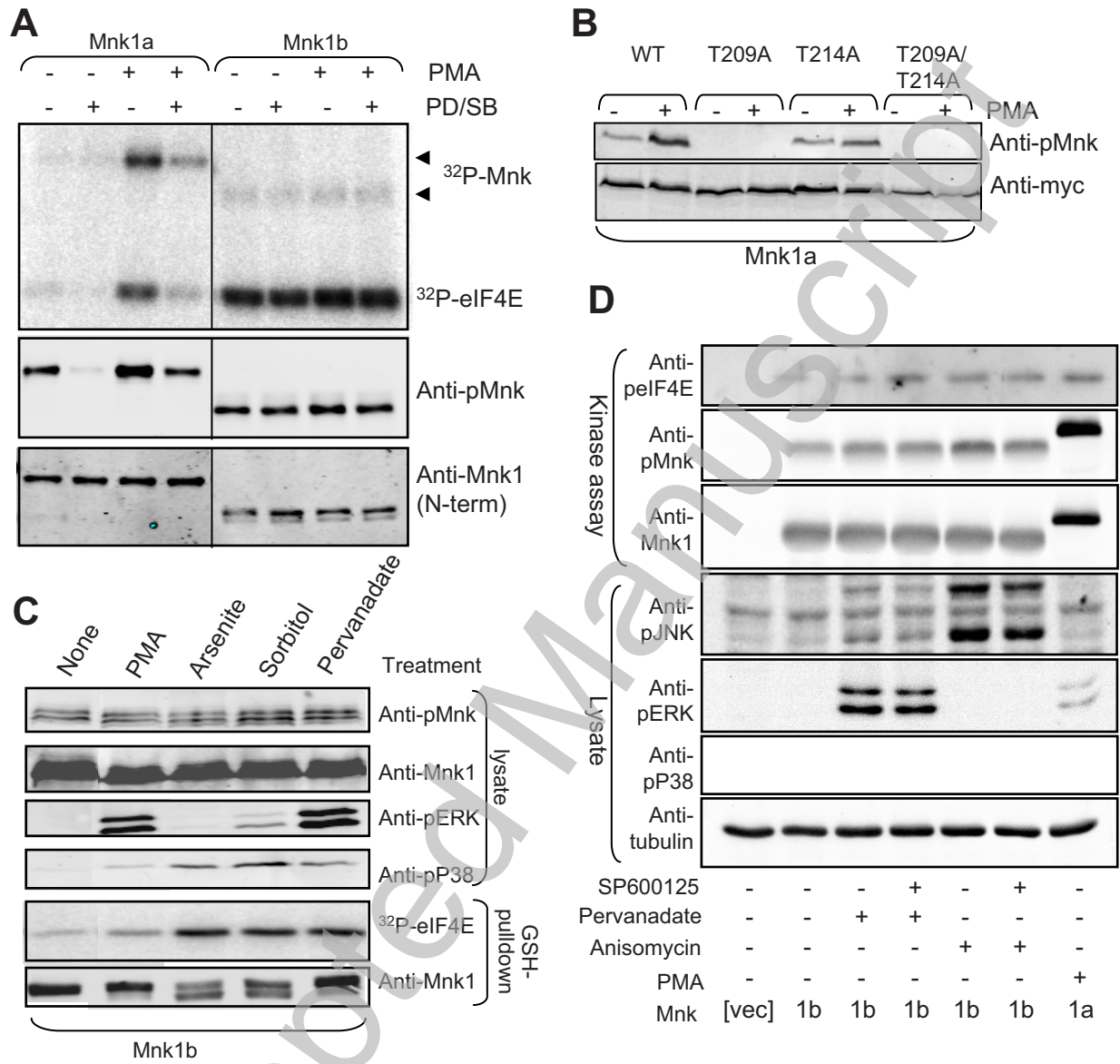
Fig. 1



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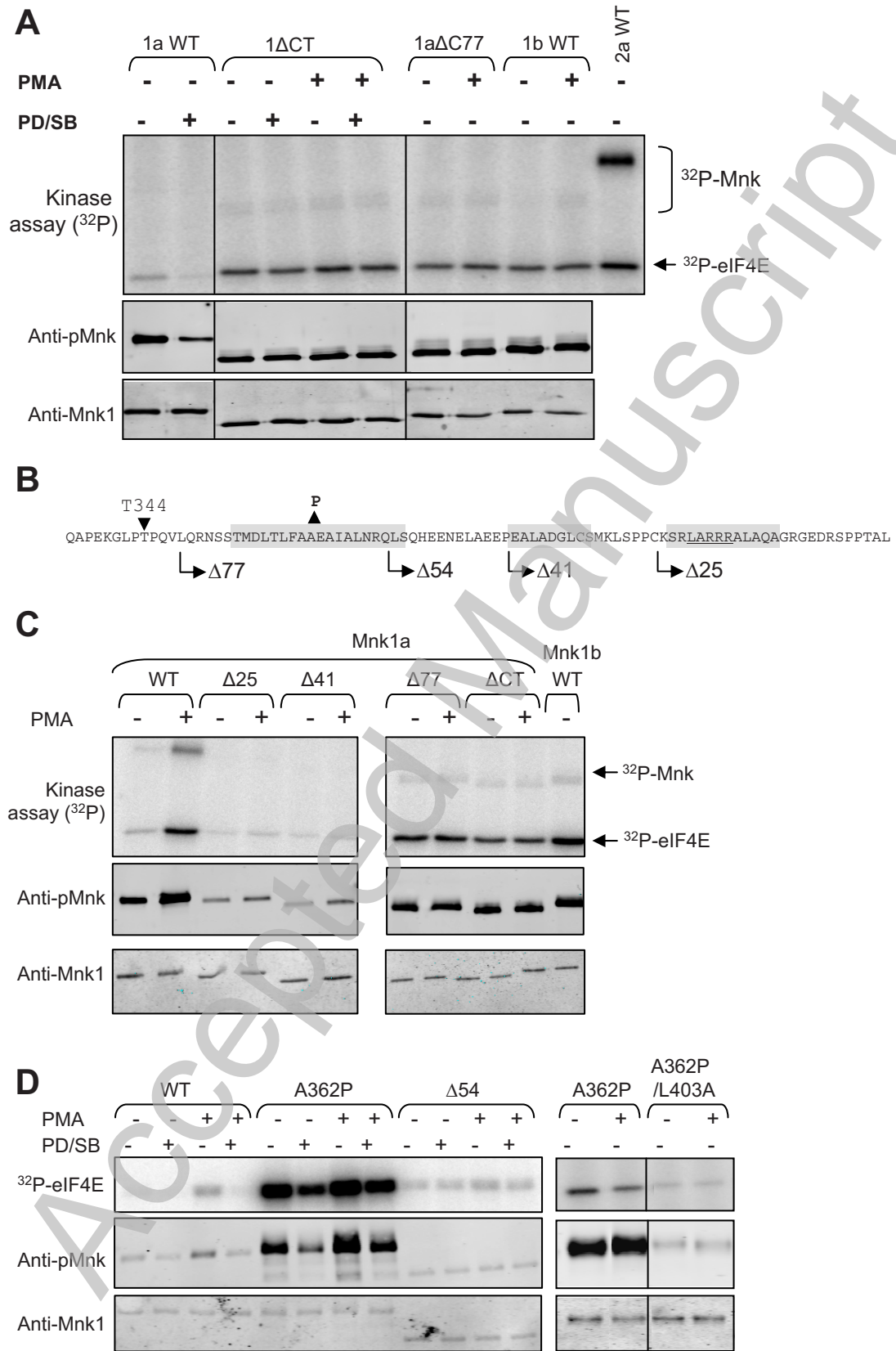
Fig. 2



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20090228

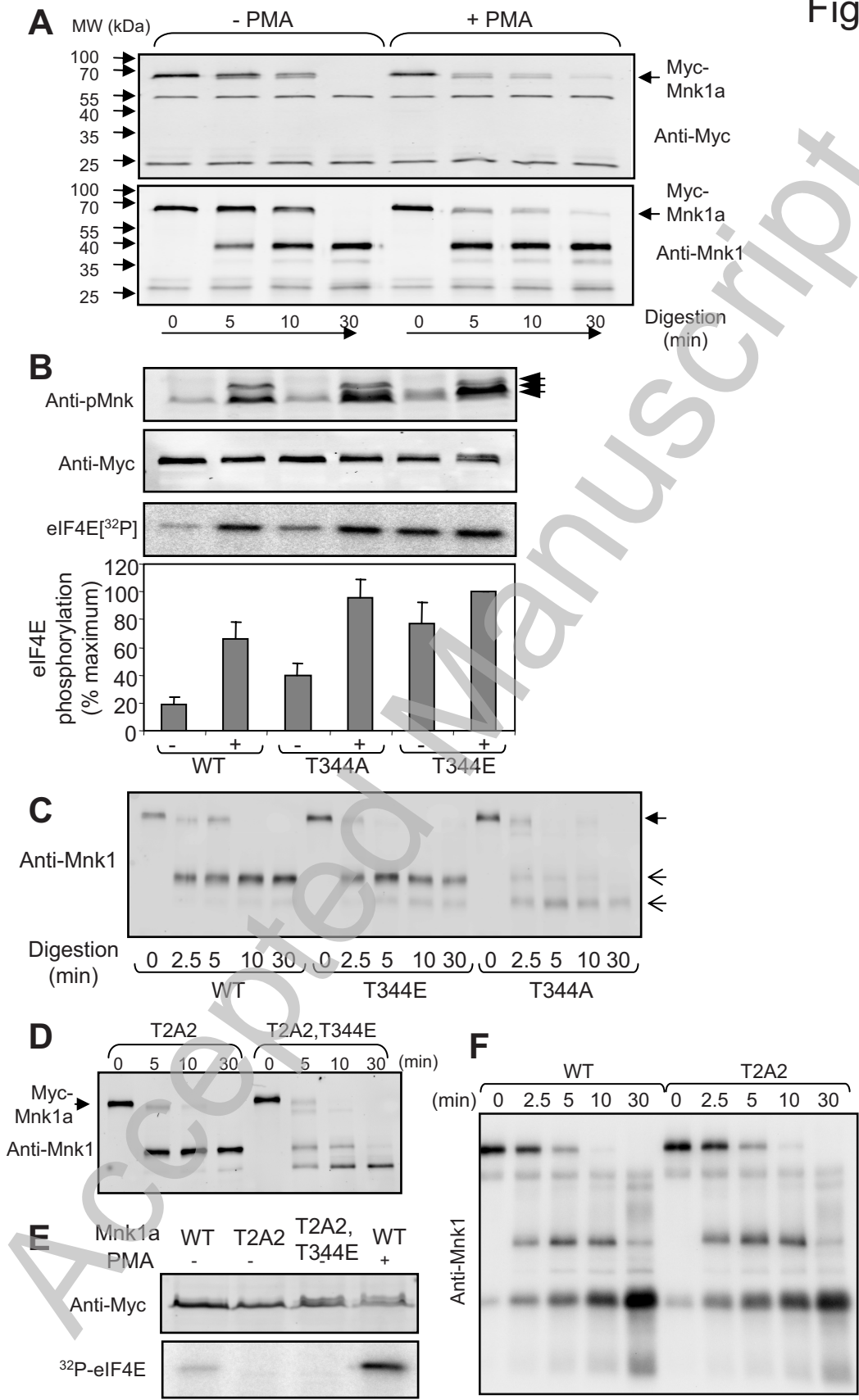
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Fig. 3



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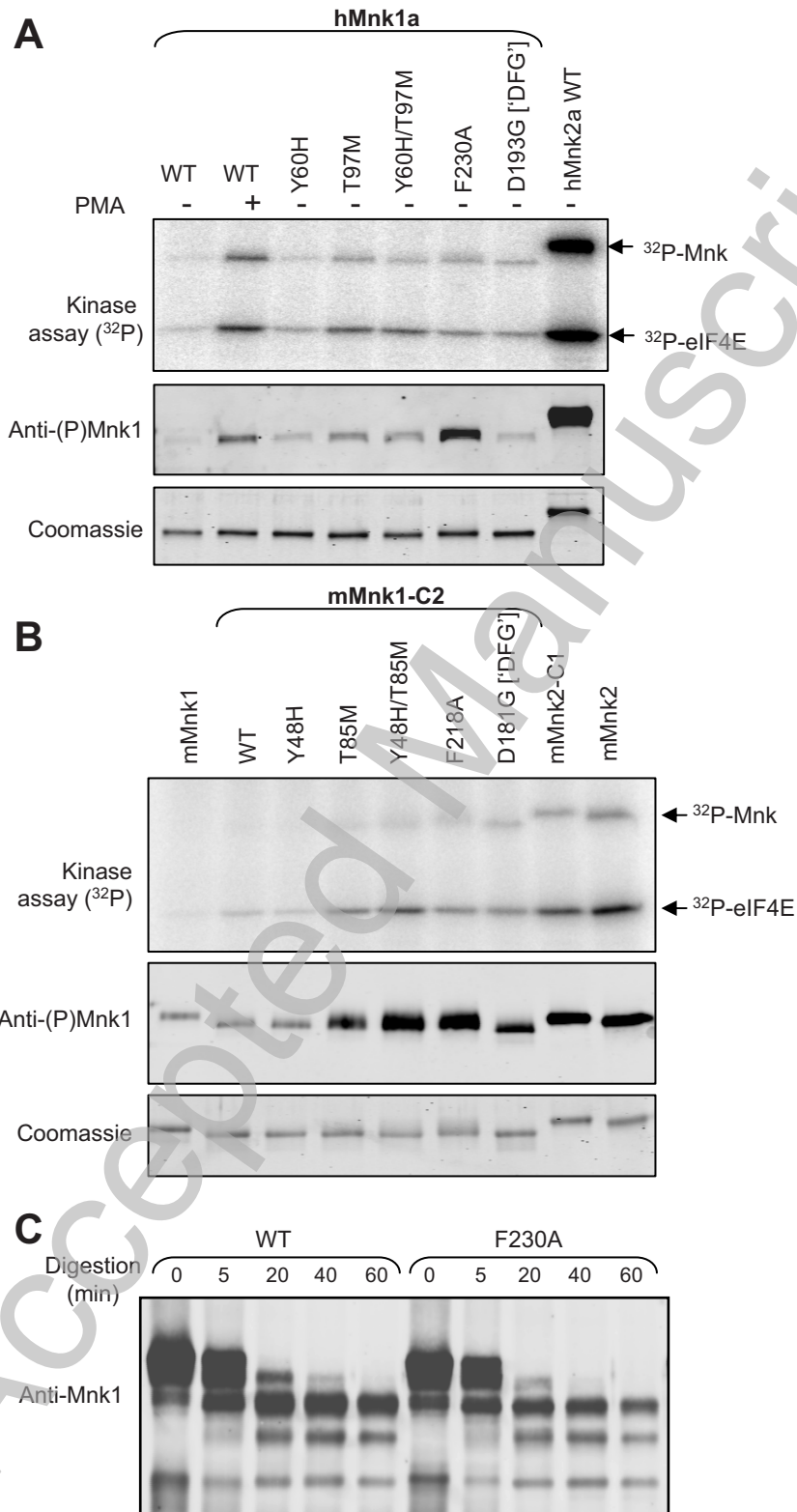
**Fig. 4**



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Fig. 5



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Fig. 6

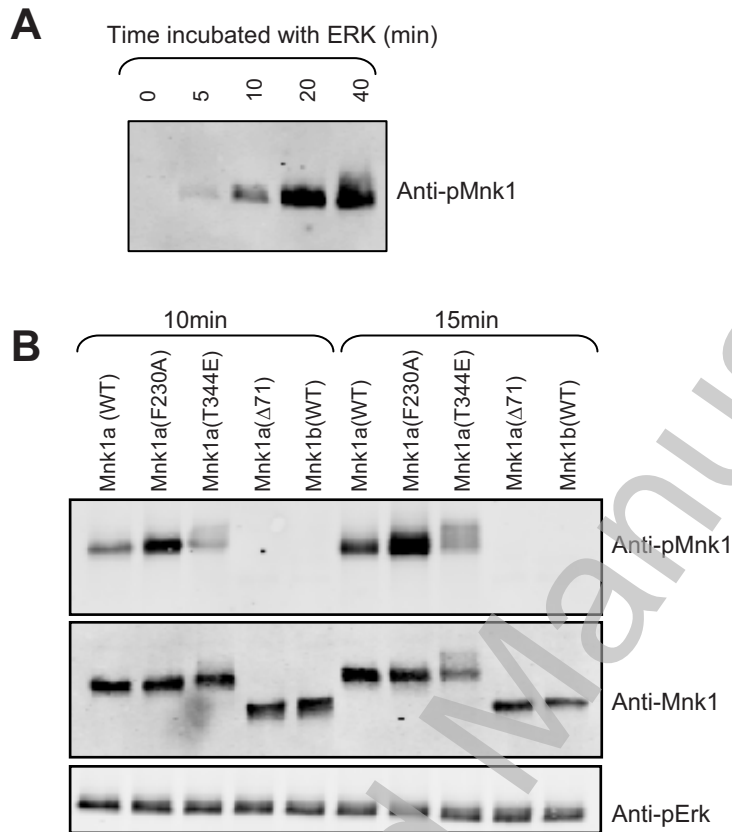


Fig. 7

