

# *In Cellulo* Kinase Screen Identifies Potential MASTL Substrates

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## Article

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# Abstract

MASTL (microtubule-associated serine/threonine kinase-like) has emerged as a critical regulator of mitosis and as a potential oncogene in a variety of cancer types. To date, Arpp-19/ENSA are the only known substrates of MASTL. However, with the roles of MASTL expanding and increased interest in development of MASTL inhibitors, it has become critical to determine if there are additional substrates and what the optimal consensus motif for MASTL is. Here we utilized a whole cell lysate (*in cellulo*) kinase screen approach combined with stable isotope labelling of amino acids in cell culture to identify potential substrates and a consensus motif of MASTL. Using the related AGC kinase family members AKT1/2, the *in cellulo* assay identified several known and new substrates highly enriched with the validated consensus motif for AKT. Applying this method to MASTL identified 59 phospho-sites on 26 novel proteins significantly increased in the presence of active MASTL. Subsequent *in vitro* kinase assays confirmed that MASTL was capable of phosphorylating hnRNPM, YB1, RPS6, TUBA1C, and RPL36A under some conditions. Taken together, these data suggest that MASTL may phosphorylate several additional substrates, providing insight into the ever-increasing biological functions and roles MASTL plays in driving cancer progression and therapy resistance.

## Introduction

MASTL (microtubule-associated serine/threonine kinase-like) is the human orthologue of *Drosophila* Greatwall (Gwl) kinase<sup>1,2</sup>, a protein essential for mitosis<sup>3</sup>. Since its discovery, MASTL has become known as a master regulator of mitotic entry and integrity<sup>4-7</sup>. More recently, its roles have expanded to include meiosis<sup>8-10</sup>, recovery from DNA damage<sup>11-13</sup>, DNA replication<sup>14</sup>, platelet maturation<sup>15,16</sup>, cell migration and actomyosin contraction<sup>17</sup>. Unsurprisingly, these roles have led to MASTL being identified as a potential oncogenic kinase<sup>18</sup> with upregulation and over-expression correlating with poor patient outcomes in a variety of cancer types, including breast<sup>19-21</sup>, colon<sup>22,23</sup> uterine<sup>22</sup>, head and neck<sup>24</sup> and pancreatic cancer<sup>25</sup>. Conversely, knockdown of MASTL has also been shown to sensitize cancer cells to a variety of DNA damaging therapies including radiotherapy<sup>26</sup>, cisplatin<sup>24,27</sup> and gemcitabine<sup>25</sup>. Understanding the functions of MASTL and how these might promote cancer development and therapy resistance are of considerable importance and have driven recent effort towards the development of specific MASTL inhibitors<sup>28-31</sup>.

Currently the only validated substrate for MASTL is alpha-endosulfine (ENSA) and the highly related cAMP-regulated phosphoprotein 19 (Arpp-19). MASTL phosphorylates these proteins on a single site (S-67 and S-62 respectively)<sup>32,33</sup>, converting ENSA/Arpp-19 into an unfair competitive inhibitor of the phosphatase PP2A-B55<sup>34</sup>. This inhibition is essential for correct mitotic entry and progression<sup>1,35</sup>, as it prevents PP2A-B55 from removing CDK1 phosphorylation events that drive correct mitotic entry and progression<sup>36,37</sup>. The MASTL/ENSA/PP2A axis is well conserved, with yeast (*S. cerevisiae*) Rim15 (MASTL) phosphorylating endosulfines Igo1 and Igo2, which in turn inhibit PP2A-Cdc55<sup>38,39</sup>. Interestingly, during nutrient starvation, Rim15 phosphorylates additional substrates including the transcription factors

Msn2p/4p and Hsf1p<sup>40</sup>. This raises the possibility that there are also additional substrates beyond ENSA/Arpp-19 for MASTL which might help explain the expanding array of functions attributed to MASTL. Several recent efforts have been made to identify potential MASTL substrates with conflicting results. Work by Bisteau *et al*<sup>7</sup>, identified 101 mitotic proteins that were dephosphorylated upon MASTL knockout in immortalized mouse embryonic fibroblasts (MEFs). Of these 6 were weakly phosphorylated *in vitro* by purified MASTL, but no decrease was observed using kinase dead MASTL, indicating that none are MASTL substrates<sup>7</sup>. However, work by Hermida *et al*<sup>41</sup> identified 56 potential substrates phosphorylated by MASTL using a Stable Isotope Labeled Kinase Assay-Linked Phosphoproteomics approach (siKALIP), suggesting that MASTL may phosphorylate a wide variety of targets. Consequently, additional research on the potential substrates of MASTL is needed.

Of note MASTL is a member of the AGC kinase family that includes AKT and PKA, which each phosphorylate more than 130 and 370 substrates respectively<sup>42</sup>. A notable characteristic of the AGC kinase family is that its members often phosphorylate the same substrates and residues. This is in part due to a shared consensus motif for AGC kinases, with a strong preference for basic residues Arginine (R) and Lysine (K) N-terminal of a Serine/Threonine (S/T) phospho-site<sup>42</sup>. Currently, the consensus motif for MASTL substrates remains unknown, however, basic Lysine (K) residues are present N-terminal of the Serine phosphorylation site (-4 and -7 positions) in Arpp-19/ENSA, suggesting that MASTL may contain the same AGC family preference for basic residues.

Here we describe an unbiased whole cell lysate kinase screen approach that combines SILAC (*in cellulo*) to identify novel substrates of MASTL kinase. We validated the utility of the *in cellulo* approach using the related AGC family member AKT2, identifying known substrates and the kinase motif for AKT2. Using active recombinant MASTL we demonstrated its significant activity towards additional substrates using a peptide library array. Furthermore, using our *in cellulo* screen, we identified 26 novel substrates potentially phosphorylated by MASTL along with its preferred kinase motif. Subsequent *in vitro* kinase assay demonstrated that MASTL can phosphorylate YB1, RPS6, TUBA1C, RPL36A and hnRNPM, under certain conditions. Collectively, these data suggest that MASTL may phosphorylate additional substrates, which likely support its expanding functional repertoire in cancer progression and therapy resistance.

## Results

### Validation of whole-cell lysate (*in cellulo*) kinase screen using AKT kinase

Identification of *in vivo* kinase-substrates has been a major stumbling block that has hampered our understanding of these important cellular enzymes. To help overcome this, we utilized a modified version of the previously reported whole-cell lysate (*in cellulo*) approach combined with stable isotope labelling with amino acids in cell culture (SILAC)<sup>43,44</sup>. This assay attempts to reconstitute the cellular environment by mixing an active recombinant kinase with a concentrated cellular lysate that has had its endogenous

kinases irreversibly inhibited, allowing phosphorylation events on any potential cellular substrate to be enriched and identified by mass spectrometry. To validate the assay, we took advantage of the well characterized and related AGC kinase family member AKT, for which active kinase is commercially available. First, *in vitro* kinase assays were performed (Fig. 1A) without (Control) or with active (AK) or heat-deactivated (DK) AKT1 kinase. These were incubated with purified recombinant YB1, a known AKT1 substrate<sup>45,46</sup>. Reactions were stopped after 30- or 60-min by the addition of Laemmli buffer, and samples were then processed for western blot and LC-MS/MS. Importantly, western blots confirmed that AKT1 was phosphorylated on T-308, indicating the kinase was active, while heat-deactivation (DK) markedly reduced the levels of phosphorylation (Fig. 1B). Furthermore, phosphorylation of YB1 on S-102, was only detected by western blot when incubated with active kinase (AK), with no bands detected in DK or control lanes (Fig. 1C). Notably, peak phosphorylation occurred after a 60 min incubation, while ATP concentration did not notably impact phosphorylation levels. Mass spectrometry confirmed these results and identified additional phosphorylation events on T-80 and S-209 of YB1 (Fig. 1C, Supplementary Table S1), which have not previously been associated with AKT1 activity. These results confirmed that commercially purchased active AKT kinase could successfully phosphorylate a known AKT substrate *in vitro*.

We therefore utilized active recombinant AKT2 kinase to validate the ability of the *in cellulo* kinase assay to identify known and novel phosphorylation events. SILAC labelled MCF10A cells were cultured as previously described<sup>19</sup>. Briefly, both heavy and light labelled cells were lysed and treated with the pan-kinase inhibitor fluorosulphonylbenzoadenosine (FSBA). Lysates were concentrated and supplemented with ATP, with or without recombinant active AKT2 kinase (Fig. 1D). Following protease digestion, samples were phospho-enriched using the EasyPhos method<sup>47</sup> and analyzed on a Q Exactive HF mass spectrometer.

The *in cellulo* screen identified a total of 3035 phospho-sites on 1085 proteins of which 89 phospho-sites on 65 proteins (including AKT2) showed a  $\text{Log}_2$  fold-change greater than 0.5, indicating potential phosphorylation by AKT2 (Fig. 1E and Supplementary Table S3). Of note there were an additional 54 known protein substrates of AKT that fell below the  $\text{Log}_2$  fold-change significance threshold. Within the 65 high confidence proteins, 9 have previously been reported as AKT substrates of which 8 are validated AKT phosphorylation sites<sup>48</sup>. These included AKT1S1 (T-246), EDC3 (S-161), RPS6 (S-236, S-240), LMNA (S-407), TBC1D4 (S-341, S-588), and IRS2 (S-306). STRING and KEGG pathway analysis found that 51 of the 65 proteins were directly or indirectly linked to AKT1/2 (Fig. 1F) and were strongly enriched for proteins involved in ribosome and AMPK and mTOR signaling (Fig. 1G), in line with the reported downstream targets of the AKT pathway<sup>49</sup>. WebLogo alignment<sup>50</sup> of the phosphorylated peptide sequences centered around the phosphorylated residue showed highly significant enrichment for serine phosphorylation with downstream enrichment of basophilic residues matching the published AKT phosphorylation motif (Fig. 1H). Taken together, these results indicate that the *in cellulo* kinase screen can identify known and potentially novel substrates along with the consensus phosphorylation motif for a well characterized AGC kinase family member.

# MASTL in cellulo kinase screen

The above results indicated that our modified *in cellulo* kinase screen could be used to identify novel substrates of MASTL kinase. However, the screen requires high quality commercially available amounts of active MASTL kinase. We therefore partnered with Reaction Biology (previously ProKinase, Freiburg, Germany) to manufacture active human MASTL kinase. Briefly, GST-tagged full length wild-type human MASTL was expressed in Sf9 insect cells. Confluent cultures were treated with or without Okadaic Acid for 3 h and the soluble, active MASTL kinase purified by affinity chromatography (Supplementary Figure S1A). The activity and ability of MASTL kinase to phosphorylate substrates was then determined using a S/T/Y-Physiological Kinase Substrate Finder Assay (Reaction Biology). Briefly, *in vitro* radioactive ATP ( $P^{33}$ ) based MASTL kinase assays were performed using a library of 720 peptides (13 amino acids long) derived from human protein sequences known to be phosphorylated *in vivo*. Over 150 peptides showed significant phosphorylation above autophosphorylation and background levels following incubation with active MASTL (Supplementary Figure S1B-C, Supplementary Table S3). This included MBP (myelin basic protein), which has previously been used as an *in vitro* substrate for MASTL<sup>51,52</sup>. Pathway analysis identified strong enrichment for proteins involved in MAPK and PI3K signaling (Supplementary Figure S1D-E), including EGFR and MYC, for which MASTL has been linked as an upstream regulator<sup>23,25</sup>. Taken together these results confirm that recombinant MASTL kinase possesses sufficient activity to be successfully used *in vitro* assays. In addition, MASTL is capable of phosphorylating peptide sequences beyond ENSA/Arpp-19.

Therefore, we next repeated the *in cellulo* kinase screen (Fig. 1D), substituting MASTL for AKT2. MCF10A cell lysates were again used as they express low levels of endogenous MASTL<sup>19</sup>. A total of 1626 phospho-peptides were identified with 59 peptides showing a  $\text{Log}_2$  fold increase greater than 0.5 (Fig. 2A, Supplementary Table S4). These 59 phospho-peptides mapped to 67 potential proteins, including exogenous active MASTL (Fig. 2B). The higher number of proteins was due to the homology of some peptides matching with multiple closely related protein variants (e.g., HIST1H1E, HIST1H1A, HIST1H1T etc.). Importantly, the top phospho-site identified was S-62/67 on ENSA/Arpp-19, providing an internal control for the *in cellulo* assay. STRING analysis of all potential phospho-peptides showed strong enrichment for proteins involved in regulation of mRNA translation, splicing, nuclear pore function cytoskeleton/migration and the expected PP2A phosphatase regulation. Alignment of phosphorylated peptide sequences, which showed a  $> 1.0 \text{Log}_2$  fold increase, revealed an enrichment for serine phosphorylation with downstream enrichment of basophilic residues (K, R) from the - 3, to -7 positions, along with upstream polar residues from + 1 to + 4 and basophilic (K) residues from + 7 to + 9 (Fig. 2C). Notably, the top phosphorylated peptide (CACNA1C) from the S/T/Y-Physiological Kinase Substrate Finder Assay, showed some similarity to the MASTL consensus motif with enrichment of basic residues downstream and polar residues upstream of the potential serine phosphorylation site (Fig. 2D). In support, Hermida *et al* also identified a strong enrichment of polar and hydrophobic residues at the + 1 to + 4 positions and a basic residue at + 5<sup>41</sup>. Taken together, these data indicate a possible MASTL consensus motif with a basic residue at the - 6 and + 5 positions, hydrophobic at -2, and

polar/hydrophobic from +1 to +4. In summary, the *in cellulo* assay results demonstrate that MASTL may phosphorylate several potential novel substrates and define a consensus kinase motif for MASTL.

## In vitro kinase validation of MASTL substrates

Our next goal was to functionally characterize the candidate substrate proteins from the *in cellulo* screen. To further refine the list of potential substrates, we cross-referenced the phosphorylated proteins with our previously published list of proteins that co-immunoprecipitated (Co-IP) with MASTL from mitotic HeLa cell extracts<sup>53</sup> and MASTL substrates predicted by Hermida *et al*<sup>41</sup>. There were 11 proteins that were present in all three datasets, an additional 11 common to our *in cellulo* assay and Hermida's dataset, and a further 15 common to our *in cellulo* assay and our previous Co-IP (Fig. 3A, Supplementary Table S4). These 3 groups of 37 proteins were clustered around the regulation of cell migration, RNA splicing, RNA translation and chromatin condensation (Fig. 3B). We selected 6 proteins spanning these 3 groups (LMNA, YBX1, TUBA1C, NPM1, HNRNPM and ENSA) and 2 found only our *in cellulo* assay (LYRA, RPS6), for further validation by *in vitro* kinase assay. These were selected based on previously reported functions that correlated with any of the known roles for MASTL (e.g., mitosis, EMT, cancer), the availability of commercial recombinant purified protein and those with the highest Log<sub>2</sub> fold-change from our *in cellulo* assay. Before beginning more detailed functional analysis we confirmed the activity of MASTL towards ENSA under the same *in vitro* assay conditions used for AKT1 (Fig. 1A). We compared ENSA phosphopeptide intensities between ATP-only, active MASTL and heat-deactivated MASTL at different timepoints as a marker for specific kinase activity. Significant phosphorylation of ENSA on S-67 was only detected in presence of active kinase (AK), with peak phosphorylation observed in the 60 min reaction (Fig. 3C). Western blot analysis confirmed that active MASTL kinase (AK), significantly and specifically phosphorylated ENSA on S-67, with a 60 min incubation showing consistently higher levels of phosphorylation (Fig. 3D). Interestingly, multiple phospho-peptides from MASTL were also detectable in our mass spectrometry analysis. Of these, phosphorylation on T-194, which is essential for MASTL activity<sup>52</sup>, was significantly more abundant in the active kinase sample compared to control, and importantly heat deactivation of MASTL (DK) reduced the abundance of T-194 and other phospho-sites (Fig. 3E). This, combined with specific phosphorylation on ENSA, confirmed that recombinant MASTL possessed sufficient activity under *in vitro* assay conditions.

The 7 remaining substrates were subjected to a single-shot *in vitro* kinase assay at 0.5- and 1.0-mM ATP for 60 min, and where sufficient substrate was available, 30 min reactions were also performed (Fig. 4, Supplementary Table S5). Strong and specific phosphorylation of ENSA was again observed (Fig. 4A), however no phosphorylation of LYRA was seen in any reaction. Similarly, while PTGES3 (p23) was phosphorylated, this was not specific and with phosphorylated residues detected under control (CTL) and dead kinase conditions (DK), indicating that neither LYRA nor p23 are likely substrates of MASTL. In contrast, a slight increased phosphorylation in the presence of the active kinase (AK) reactions was observed under some conditions for RPL36A and RPS6, while for hnRNPM, TUBA1C and YB1 more prominent increases in AK samples were present under multiple conditions (Fig. 4, blue rectangles).

Notably, phosphorylation of hnRNPM on S-633 and S-48 on TUBA1C were also identified in the MASTL *in cellulo* screen (Fig. 2). While phosphorylation of YB1 on S-209 showed the strongest specificity, this site was not seen in our MASTL *in cellulo* assay but was found in our AKT1 screen (Fig. 1E). Based on these results, we selected hnRNPM as a potential novel substrate for further validation using more detailed *in vitro* kinase assays. Triplicate analysis identified 13 unique phospho-peptides that were present in at least 2 out of 3 replicates. Increased phosphorylation of Y-64 was observed at both 30 and 60 min, however, this is likely non-specific as MASTL is a serine/threonine directed kinase. Of the remaining sites only S-633/637 was significantly and specifically more abundant in the active kinase (AK) reaction (Fig. 5A, Supplementary Table S6). Interestingly, S-633 is located just downstream from the C-terminal RNA recognition motif (RRM) of hnRNPM (Fig. 5B) and shows some homology with our predicted MASTL consensus motif (Fig. 5B), with hydrophobic/polar residues enriched in +1 to +4 positions, and a basic residue at -6. Taken together, these results indicate that MASTL is capable of phosphorylating hnRNPM both *in vitro* and *in cellulo*.

## Discussion

Here we have presented a comprehensive analysis of MASTL substrates using a reconstituted and adapted cellular protein environment for use as a novel discovery tool. Our *in cellulo* kinase assay approach has successfully identified known and novel substrates for AKT2 and MASTL kinase. Analysis of substrate phosphorylation sites showed enrichment for kinase motifs that could aid in identifying targets for either kinase in future experiments. Our evidence suggests that MASTL may phosphorylate hnRNPM, TUBA1C, YB-1, and to a lesser extent RPS6 and RPL36A, in addition to ENSA/Arpp19. These findings may provide a greater understanding of the mechanistic links between MASTL and its biological and oncogenic functions.

MASTL has previously been shown to associate with microtubules during mitosis, decorating the mitotic spindle<sup>1</sup>. It co-precipitates with tubulin from mitotic cell extracts<sup>19</sup>, which has previously been implicated as a potential MASTL substrate using a siKALIP approach<sup>41</sup>. Combined with our observation here that MASTL can phosphorylate the core microtubule protein  $\alpha$ -tubulin (TUBA1C) on multiple sites (T-41, S-48, and T-292) *in cellulo*, suggests that MASTL may regulate microtubule function. Notably, knockdown of MASTL results in multiple mitotic spindle and chromosome alignment defects<sup>1,2</sup>. Of note, the related AGC kinase, PKC, can also phosphorylate  $\alpha$ -tubulin on S-165, stimulating microtubule dynamics<sup>54,55</sup>, and supporting the potential of AGC kinases as regulators of  $\alpha$ -tubulin. Consequently, our results raise the possibility that MASTL could regulate spindle assembly by both maintaining CDK1 substrate phosphorylation, and by regulation of tubulin dynamics.

Our previous work identified several links between MASTL and epithelial mesenchymal transition (EMT), with MASTL over-expression overriding contact inhibition and disrupting cell migration in MCF10A breast epithelial cells. This corresponded with increased phosphorylation of several members of the  $\beta$ -catenin pathway and RPS6<sup>19</sup>. Interestingly, hnRNPM has been associated with increased activation of  $\beta$ -catenin

signaling in breast cancer<sup>56</sup>, and increased EMT in breast<sup>57</sup> and prostate cancer<sup>58</sup>. Both our results here and that of Hermida *et al*<sup>41</sup> identified the same S-633 phosphorylation site on hnRNPM, as a potential site phosphorylated by MASTL. Notably, the S-633 site on hnRNPM has been found in breast cancer and ischemic tumor tissues by mass spectrometry<sup>59,60</sup>, suggesting that this site is phosphorylated under certain conditions *in vivo*. However, no upstream kinase has been assigned for this phosphorylation site, nor has any functional significance been attributed. Of note, the phosphorylation site is located just next to the C-terminal RNA recognition motif, hence phosphorylation of S-633 could potentially regulate the binding of target RNA sequences, hnRNPM's splicing activity, or impact binding of upstream regulatory proteins, such as AKAP8<sup>57</sup>. Elucidating the links between hnRNPM and MASTL may give a greater understanding to the role MASTL plays in regulating EMT in breast cancer.

MASTL over-expression has also been linked with hyper-activation of the PI3K/AKT/mTOR pathway in humans<sup>61</sup>, a function that may be related to its role in regulating nutrient response in yeast<sup>40,62</sup>. Interestingly, RPS6 plays an important role in regulating nutrient-mTORC1 signaling in human cells and is phosphorylated by multiple AGC kinases including AKT and p90RSK<sup>61,63</sup>. Similarly, we found that YB1, which also plays several key roles in the AKT pathway<sup>46</sup>, could be phosphorylated by both AKT1 and MASTL on S-209 in our *in cellulo* and *in vitro* screens and immunoprecipitates with MASTL<sup>53</sup>, suggesting this site may be phosphorylated by multiple AGC family members including MASTL. It will be of future interest to determine if phosphorylation by MASTL on either RPS6 and YB1 plays any role in their regulation and if this impacts nutrient response within mammalian cells.

In summary, here we show that MASTL has the potential to act like other AGC kinases and may phosphorylate several additional substrates *in vitro* in addition to Arpp-19/ENSA. Many of these new potential substrates, such as tubulin and hnRNPM, may help explain the growing list of MASTL functions including how MASTL promotes cancer, EMT and chemoresistance. Given MASTL's rising role in cancer, there has been growing interest in the development of specific MASTL inhibitors<sup>28,30,31</sup>. We hope that our characterization of novel cellular targets and identification of a potential consensus motif for MASTL will aid their development and help improve next-generation inhibitors. Consequently, it will be of critical importance to further validate and confirm the physiological importance of these novel MASTL substrates in future research.

## Materials And Methods

A full list of reagents used along with catalogue number and suppliers can be found in Supplementary Table S7.

## Cell lines

MCF-10A breast cells were purchased from Sigma (#CLL1040) (38). MCF-10A cells were grown in DMEM/F12 (1:1) supplemented with 5% horse serum, 20 ng/mL recombinant human EGF, 0.5 ug/mL

hydrocortisone, 100 ng/mL cholera toxin, and 10 ug/mL bovine insulin as previously described <sup>19</sup>.

## Proteins, antibodies and reagents

Active MASTL kinase (#1593-0000-2) and the Ser/Thr/Tyr-Physiological Kinase Substrate Finder Assay Service was purchased from and performed by Reaction Biology (previously ProQinase, Freiburg, Germany). Purified recombinant AKT2 was purchased from Active Motif (#81146). Purified recombinant active AKT1 (#ab62279) and recombinant proteins ENSA (#ab92932), YB1 (#ab187443), hnRNPM (#ab226407), p23 (#ab113183), LYAR (#ab163160), RPL36A (#ab159415), TUBA1C (#ab164660) were purchased from Abcam, and RPS6 (#H00006194-P01) was purchased from Abnova. Kinase reaction reagents included Tris-base, hydrochloric acid, magnesium chloride hexahydrate, DL-dithiothreitol and were purchased from Sigma, and adenosine 5'-triphosphate from New England Biolabs. Antibodies used for western blotting included anti-MASTL, phospho-YB1 (S-102), pan-AKT, phospho-AKT1 (T-308), phospho-Arpp-19/ENSA (S-62/67) (Cell Signaling Technology), as well as anti-YB1, anti-rabbit IgG HRP and anti-mouse IgG HRP (Abcam), and anti-His-ENSA <sup>14</sup>. Reagents for mass spectrometry sample preparation including LC-MS grade water and formic acid were purchased from Pierce, HPLC grade trifluoroacetic acid and LiChrosolv acetonitrile from Sigma. Further details can be found in Supplementary Table S7.

## Whole cell lysate (in cellulo) Kinase Assay: Sample Preparation

MCF-10A cells were labelled by stable isotope labelling of amino acids in cell culture (SILAC) as previously described <sup>19</sup>. Briefly, cells were cultured for 6 population doublings in SILAC F12:DMEM (1:1) (Thermo Scientific) supplemented with either "heavy" lysine-<sup>13</sup>C<sub>6</sub> <sup>14</sup>N<sub>2</sub> (Lys8) and arginine-<sup>13</sup>C<sub>6</sub> <sup>14</sup>N<sub>4</sub> (Arg10), or the respective "light" counterparts (Lys0, Arg0). Labelled amino acid incorporation was checked routinely. Protein was harvested by scraping in 1 mL of ice-cold NP-40 buffer (1% NP-40, 150 mM NaCl, 50 mM Tris HCl pH 7.4, 10% Glycerol) plus protease inhibitor cocktail (Sigma) and PhosStop (Roche) and stored at -80 °C. 100 mM 5'-4-fluorosulphonylbenzoyl-adenosine (FSBA) in DMSO was slowly diluted 1:5 in pre-warmed NP-40 buffer at 37 °C. To irreversibly inhibit endogenous kinase activity, 1 mL of the FSBA solution was added to thawed lysates and shaken at 30 °C for 30 min at 1,200 rpm. Precipitated FSBA was removed by centrifugation and the supernatant was concentrated to 2.5 mL in a 10,000 MW centrifugal filter (Amicon). 1 mg of concentrated lysate was diluted 1:1 in 2x kinase assay buffer (50 mM Tris HCl pH 7.4, 10 mM MgCl<sub>2</sub>, 3 mM MnCl<sub>2</sub>, 3 μM Na<sub>3</sub>VO<sub>4</sub>, 2 mM DTT, and 500 μM ATP). 50 μg of recombinant purified MASTL kinase (Reaction Biology) or 10 μg AKT2 (Active Motif) was added to the heavy sample and incubated at 32 °C for 3 h under gentle agitation. The reaction was stopped by adding GdmCl buffer (6 M GdmCl, 100 mM Tris pH 8.5, 20 mM TCEP, 100 mM IAM) to each sample. Kinase reactions were heated to 95 °C for 5 min, cooled on ice, then kept at RT in the dark for 30 min to allow for peptide reduction and alkylation. Heavy and light samples were mixed 1:1 and 4x volumes of cold acetone were added to each mix and incubated at -20 °C overnight. Precipitated proteins were digested in Lys-C, and trypsin at 100:1 (w:w) overnight at 37 °C. The resultant mixture was then enriched

for phosphorylated peptides using the EasyPhos method as described <sup>47</sup>. Briefly, phospho-peptides were bound to TiO<sub>2</sub> beads (5 µm Titansphere, GL Sciences) at a ratio of 10:1 (protein:beads) at 40 °C for 5 min. After washing (80% ACN, 0.5% 5% trifluoroacetic acid), phospho-peptides were eluted (40% ACN, 15% Ammonium Hydroxide), dried, and loaded onto in-house packed dual layer SDB-RPS stage tips (3M empore). The stage-tips were desalted and phospho-peptides eluted (80% ACN, 5% ammonium hydroxide), dried and stored at -80 °C until mass spectrometry analysis.

## **MASTL whole cell lysate (in cellulo) Kinase Assay: Mass Spectrometry and Data Analysis**

Digested phospho-peptides were analysed as previously described. Briefly, a 50 cm x 75 µm fused silica column, packed in-house using 1.9 µm C18AQ particles using an Easy nLC-1200. Peptides were separated using a 195 min gradient using a binary buffer system of Buffer A (0.1% formic acid) and Buffer B (80% ACN, 0.1% formic acid) at a flow rate of 300 nL/min. Peptides were eluted with a gradient of 5–30% buffer B over 150 min, followed by 30–60% buffer B over 5 min, and 60–95% buffer B over 5 min. Peptides were analyzed on a Q-Exactive HF mass spectrometer operated in positive-ion DDA mode, with one full scan (300-1,650 m/z,  $R = 35,000$  at 200 m/z), at a target of  $3e^6$ , selecting top 20 most abundant precursor ions (isolation window = 1.4 m/z, ion target  $3e^5$ ) for HCD fragmentation (NCE = 27%) and MS2 scan ( $R = 35,000$  at 200 m/z). Thermo RAW files were generated in centroid mode and analyzed using MaxQuant (v.1.5.3.30) with integrated Andromeda search engine, searching against the human whole proteome, with additions (Uniprot release 07.2018; 21,050 entries) Default MaxQuant settings were used, with 1% peptide spectral match (PSM) false discovery rate (FDR) followed by further filtering to 1% protein FDR. Precursor mass tolerance was set to 20 ppm for a first search followed by mass recalibration and 7 ppm tolerance for second search. Fragment ion tolerance was set to 7 ppm. The searches were conducted with carbamidomethylation of Cys set as a fixed modification. Phosphorylation of Serine, Threonine and Tyrosine, oxidation of Met and “light” Lysine and Arginine were replaced by “heavy” isotope-labelled amino acids 13C615N4-I-Arginine (Arg 10) and 13C615N2-I-Lysine (Lys 8) with Arginine and Lysine set as variable modifications. The match between runs and re-quantify options were enabled with matching elution time of 1.0 min. Bioinformatics were performed in Perseus (1.5), and Microsoft Excel (2010). Briefly, SILAC H/L ratios determined by MaxQuant were inverted for label-swap experiments, and Log<sub>2</sub> transformed prior to statistical analysis. Non-significant Log<sub>2</sub>H/L ratios were eliminated using the Student’s t-test with Benjamini-Hochberg adjustment for multiple comparisons, with  $P < 0.05$  as a significance cut off, ratios  $> 0.5$  were considered significantly altered. KEGG pathway enrichments were determined from the parent protein identified from the individual phospho-peptides <sup>64</sup> and interaction networks were produced using STRING v11.5 <sup>64</sup>.

## **In vitro kinase assays**

*In vitro* kinase assay reaction mixtures (40 µl total) contained 500 ng of AKT1 or MASTL kinase and 100 ng of substrate protein in kinase reaction buffer (50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, pH

7.4) in the presence of 0.5 mM or 1 mM ATP. Reactions were performed at 30 °C for 30 min and 60 min, then quenched with 2X SDS-polyacrylamide gel electrophoresis sample buffer (Bolt, Invitrogen).

## **SDS-PAGE and western blotting**

Kinase reaction samples were heated for 10 min at 70 °C, then cooled on ice for 2 min. The full sample volume (less a small amount used for western blotting) was loaded onto Bolt 4–12% Bis-Tris Plus gels and run under reducing conditions with Bolt MES Running Buffer (Invitrogen) at 180–200 V. Prestained molecular weight markers were also included (SeeBlue Plus2, Invitrogen; GangNam-STAIN, iNtRON Biotechnology). Once kinase and substrate molecular weights were sufficiently separated, gels were washed and stained according to manufacturer's instructions using SimplyBlue SafeStain (Invitrogen) before proceeding to in-gel digestion and mass spectrometry. For western blotting, gels were washed in water and proteins transferred onto PVDF membranes using the iBlot2 system (Invitrogen). Membranes to be probed for phospho-proteins were blocked in 5% bovine serum albumin (BSA; Sigma) in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T), or otherwise in 5% low fat milk in TBS-T. Primary antibodies were diluted in 3% BSA/TBS-T and incubation was carried out overnight at 4 °C. Secondary antibodies were diluted in 5% LFM/TBS-T or 3% BSA/TBS-T (for phospho-proteins) and incubation was carried out for 1–2 h at room temperature. All membrane washes were carried out with TBS-T, except for the final wash before imaging, which used TBS. Protein bands were detected by chemiluminescence using Clarity Western ECL Substrate (Bio-Rad) and the ChemiDoc imaging system (Bio-Rad). Densitometry was analysed with ImageLab software.

## **In-gel digestion**

Stained protein bands were excised with a clean scalpel and underwent destaining, reduction-alkylation and digestion using an In-Gel Tryptic Digestion Kit (Thermo Scientific). Manufacturer's instructions were followed. Briefly, one volume of reagent was used per protein band, and trypsin digestion was carried out on a heat block overnight (16–18 h) at 37 °C.

## **Mass spectrometry of in vitro kinase assay samples**

Digested samples were desalted, purified, and concentrated using C18 ZipTips (Z720070 (discontinued), Millipore), then dried by vacuum centrifugation and reconstituted in loading buffer (3% acetonitrile, 0.1% formic acid). Samples were injected onto a 50 cm x 75 µm internal diameter column (packed in-house with 1.9 µm Pur 120 C18 particles) using a Thermo Ultimate-3000 UHPLC. Peptides were separated using a 110 min gradient with a binary buffer system (buffer A = 0.1% formic acid; buffer B = 80% acetonitrile, 0.1% formic acid) at a constant flow rate of 0.250 µl/min. Peptides were eluted with a gradient of 5% buffer B over 30 min, then 5–10% buffer B over 1 min, then 10–35% buffer B over 59 min, then 35–95% buffer B over 3 min, then maintained at 95% buffer B for 4 min, then 95 – 5% buffer B over 1 min, then 5% buffer B maintained for a further 12 min. The column was maintained at 30 °C. Data-dependent acquisition (DDA) was performed with a Q Exactive Plus Orbitrap mass spectrometer operating in positive-ion mode, with a full-scan MS1 measured at 35,000 resolution (350 to 1550 m/z scan range; 50 ms injection time;  $3 \times 10^6$  automated gain control (AGC) target) followed by isolation of up to 20 most

abundant precursor ions for MS/MS (1.2 m/z isolation; 26 normalized collision energy; 17,500 resolution; 60 ms injection time;  $3 \times 10^5$  AGC target). Thermo RAW files were generated in centroid mode. Raw data were processed with Proteome Discoverer v2.3 and v2.4 and searched with Sequest HT against the human UniProt database (07.2018; 21,050 entries). Full trypsin was set as the enzyme used with a maximum of 2 missed cleavages permitted. The mass tolerance for precursor ions was set to 10 ppm. The searches were conducted with carbamidomethylation on cysteine set as a static modification. Carbamidomethylation on histidine and lysine, phosphorylation of serine, threonine and tyrosine, deamidation of asparagine and glutamine, and oxidation of methionine were set as variable modifications. An automatic target FDR of 1–5% was set. Relative abundance levels of phosphorylation were found with label-free quantitation (LFQ) using the Minora Feature Detector.

## Experimental Design and Statistical Rationale

For *in cellulo* kinase screening, two independent ( $n = 2$ ) biological replicates were performed. Average  $\text{Log}_2\text{H/L}$  ratios from the 2 replicates greater than  $> 0.5$  were taken as potentially increased (phosphorylated) substrates for AKT and MASTL. Further stringency was applied using a Student's t-test, with  $P < 0.05$  taken as significant. For MASTL, substrates were further refined by cross-referencing with previously identified proteins that co-immunoprecipitated with MASTL from mitotic HeLa cell extracts<sup>53</sup>. For the AKT1 + YB1 *in vitro* kinase assay, two ( $n = 2$ ) biological replicates were performed, and for MASTL + ENSA and MASTL + hnRNPM *in vitro* kinase assays, three ( $n = 3$ ) independent biological replicates were performed. Statistical significance for western blot was determined using 2-way ANOVA with Tukey's multiple comparison's test, and significance for mass spectrometry results was determined using an unpaired 1-way ANOVA with Tukey's multiple comparisons test using GraphPad Prism software (v9.1.0).

## Abbreviations

Arpp-19 – cAMP-regulated phosphoprotein 19

CDK1 – cyclin dependent kinase 1

EMT – epithelial mesenchymal transition

ENSA – alpha-endosulfine

FSBA – 5'-4-fluorosulphonylbenzoyladenosine

hnRNPM – heterogeneous nuclear ribonucleoprotein M

IAM - iodoacetamide

K - Lysine

MASTL – microtubule-associated serine/threonine kinase-like

PP2A – protein phosphatase 2A

PKA – protein kinase A

PKC – protein kinase C

R - Arginine

siKALIP - Stable Isotope Labeled Kinase Assay-Linked Phosphoproteomics

SILAC- Stable Isotope Labelling with Amino acids in Cell culture

S/T/Y – serine/threonine/tyrosine

TCEP-HCl – tris(2-carboxyethyl)phosphine hydrochloride

## Declarations

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**Data Availability Statement:** All raw mass spectrometry data files, and MaxQuant and Proteome Discoverer output files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository <sup>65</sup> with the identifier PXD028678 (<http://proteomecentral.proteomexchange.org>).

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## Figures

### Figure 1

**AKT *In Cellulo* Kinase Assay.** (A) In-vitro kinase reaction steps between either no kinase (control, CTL), recombinant active kinase (active kinase, AK) or heat-treated kinase (denatured kinase, DK) with recombinant protein substrates. Up to four reaction conditions were tested (30 min and 60 min incubations, each in the presence of 0.5 mM ATP or 1 mM ATP). The resulting reaction mixtures were separated by SDS-PAGE and analyzed by western blot or further processed with in-gel digestion followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). (B) The reaction between AKT1/YB1 was validated by immunoblot of known phospho-sites on active AKT1 (T-308), and the AKT1-mediated YB1 phospho-site (S-102). Pan-AKT was used to show the absence, presence and denaturing effect on active AKT-1, while Total YB1 was used to show the presence of YB1 substrate in each reaction. (C) Phospho-peptides identified by LC-MS/MS were analyzed by label-free quantitation. The abundance of phospho-peptides resulting from the AKT1/YB1 reaction (0.5 mM ATP, 60 min) are represented as a heatmap (mean of two independent reactions). Square brackets indicate peptide locations within YB1 on which each phosphorylation was found. Hashtags denote a co-modification within that peptide, e.g. carbamidomethylation, deamidation, and/or oxidation. D) Graphical summary explaining the SILAC based whole cell lysate (*in cellulo*) kinase assay. E) Volcano plot of all identified phospho-peptides identified from the AKT2 *in cellulo* kinase screen. A minimal Log<sub>2</sub> Fold change cut-off <0.5 (red shading) was taken as potential increased phosphorylation events. (F) STRING cluster analysis of proteins corresponding to increased phospho-peptides identified in E. (G) KEGG pathway analysis of proteins identified in F. (H) Phospho-peptides with a Log<sub>2</sub> Fold change >1.0 were aligned using WebLogo v3.7.4. Residue colors indicate chemical properties of amino acids.

## Figure 2

**MASTL *In Cellulo* Kinase Assay.** (A) Volcano plot of all identified phospho-peptides identified from the MASTL *in cellulo* kinase screen. A minimal Log<sub>2</sub> Fold change cut-off <0.5 (red shading) was taken as potential increased phosphorylation events. (B) STRING cluster analysis of proteins corresponding to increased phospho-peptides identified in A. (C) Phospho-peptides with a Log<sub>2</sub> Fold change >1.0 were aligned using WebLogo v3.7.4. Residue colors indicate chemical properties of amino acids. (D) Alignment of top phosphorylated peptide (CACNA1C) from the S/T/Y Physiological Kinase Substrate Finder Assay (Reaction Biology), with the MASTL consensus motif identified in C.

## Figure 3

***In vitro* kinase assay validation of ENSA phosphorylation by MASTL.** (A) Venn diagram comparing previously identified MASTL interacting proteins by co-immunoprecipitation<sup>53</sup> with those identified from A, and (B) subsequent STRING cluster analysis of common proteins. (C) The abundance of each ENSA phospho-site activated in reactions between MASTL kinase and ENSA. The ion abundance for the #Ser67

[64-71] phospho-peptide is normalized to the 60 min control (CTL) reaction. Error bars display the standard error of the mean from three independent reactions. Square brackets indicate peptide locations within the protein on which each phospho-site was found. Hashtags denote a co-modification within that peptide, e.g. carbamidomethylation, deamidation, and/or oxidation (detailed in Supplementary Table S6). (D) Western blot confirmation of ENSA phosphorylation at S-67 in the presence of active MASTL kinase. Total ENSA was used as the loading control and normalization factor for densitometry analysis. P-ENSA/Total was further normalized to the 60 min control (CTL) reaction. Error bars display the standard error of the mean from three independent reactions. (E) The abundance of MASTL phospho-sites present in reactions with ENSA are represented here as a heat map. Two reaction conditions were tested (60 min incubation in presence of 0.5 mM ATP or 1 mM ATP). Phospho-site abundances were calculated using label-free quantitation.

## Figure 4

**Single-shot *in vitro* kinase screen of potential MASTL substrates.** The abundance of each phospho-site activated in reactions between either no kinase (control, CTL), recombinant active MASTL kinase (active kinase, AK) or heat-treated MASTL kinase (denatured kinase, DK) with recombinant proteins, ENSA, hnRNPM, p23, RPL36A, RPS6, TUBA1C, and YB1. Four reaction conditions were tested (30 min and 60 min incubations, each in the presence of 0.5 mM ATP or 1 mM ATP) for all protein substrates, except LYAR and RPS6 (0.5 mM and 1 mM ATP, 60 min only – absence of 30 min reactions for RPS6 indicated by crossed-out boxes) due to limited protein availability. No phospho-sites were detected on LYAR under any of these conditions. Phospho-site abundances were calculated using label-free quantitation. Square brackets indicate peptide locations within the protein on which each phospho-site was found. Single hashtags denote a co-modification within that peptide, e.g., carbamidomethylation, deamidation, and/or oxidation. Double and triple hashtags denote different combinations of co-modifications within the same peptide (detailed in Supplementary Table S6).

## Figure 5

***In Vitro* kinase assay validation of hnRNPM phosphorylation by MASTL** (A) The ion abundance of each phospho-site activated in triplicate reactions between MASTL kinase and hnRNPM (as detailed in Figure 4 legend). Phospho-site abundances were calculated using label-free quantitation. Square brackets indicate peptide locations within the protein on which each phospho-site is found. Single hashtags denote a co-modification within that peptide, e.g., carbamidomethylation, deamidation, and/or oxidation. Double hashtags denote a different combination of co-modifications within the same peptide (detailed in Supplementary Table S7). Error bars display the standard error of the mean from three (n=3) independent reactions. B) Schematic of hnRNPM showing nuclear localisation domain (NLS) RNA recognition motifs (RRM) and phospho-sites currently annotated in PhosphoSitePlus (Chk1 and PRP4) along with the novel

MASTL sites identified here. Sequence alignments of the MASTL phosphorylation sites within hnRNPM (S-633 and S-637), compared to validated substrates Arpp19/ENSA and the MASTL consensus motif identified from our *in cellulo* screen (Figure 2D).

## Supplementary Files

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