

The tRNA methylase METTL1 is phosphorylated and inactivated by PKB and RSK in vitro and in cells

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Supplementary Information

Results

Generation of antibodies that recognise METTL1.

To examine whether METTL1 became phosphorylated in cells, we generated polyclonal antibodies capable of immunoprecipitating the human protein and a phospho-specific antibody that recognised METTL1 only when it was phosphorylated at Ser27. The phospho-specific antibody did not recognise unphosphorylated METTL1 provided that it was incubated with the unphosphorylated form of the peptide immunogen to neutralise antibodies that recognise epitopes that do not contain pSer27 (Supplementary Figure 1). All subsequent experiments with this antibody were therefore carried out in the presence of the unphosphorylated form of the peptide immunogen.

The phospho-specific antibody did not recognise a mutant of METTL1 in which Ser27 had been mutated to Ala, and recognition was prevented by incubating the antibody with the phosphopeptide immunogen (Supplementary Figure 1).

Lack of effect of METTL1 phosphorylation on its interaction with WDR4.

Vectors expressing HA-tagged WDR4 and FLAG-tagged METTL1 were co-transfected into 293 cells, and the expressed proteins immunoprecipitated with anti-HA or anti-

FLAG antibodies. The anti-HA immunoprecipitated FLAG-tagged METTL1, while anti-FLAG immunoprecipitated HA-tagged WDR4. Thus these two proteins can form a complex when overexpressed in 293 cells. When the transfected cells were stimulated with IGF-1, FLAG-METTL1 phosphorylated at Ser27 was immunoprecipitated by the anti-HA antibody, demonstrating that phosphorylation does not disrupt the interaction between METTL1 and WDR4 (Fig 9B). This is supported by the finding that the mutation of Ser27 to Asp or Glu does not disrupt the interaction between METTL1 and WDR4 (Fig 9A).

Subcellular localisation of METTL1 and WDR4

Using GFP-METTL1 and HA-WDR4 we showed that both proteins co-localise in the nucleus of cells and that stimulation with IGF-1 does not affect the localisation of either protein (Supplementary Figure 3A). In parallel experiments, the transcription factor FOXO1, an established physiological substrate of PKB, exited the nucleus in response to IGF-1 (Supplementary Figure 3B), as expected (Rena et al 2001).

Materials and Methods

Materials. [γ - 32 P]ATP, [α - 32 P] GTP and materials for protein purification were obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks, U.K.), unlabelled ATP, dithiothreitol (DTT) and isopropyl-1-thio- β -D-galactopyranoside (IPTG) from Roche Molecular Biochemicals (Lewes, U.K.), precast polyacrylamide gels, running buffer, and transfer buffer from Invitrogen (Paisley, U.K.), Immobilon P membranes from Millipore (Bedford, U.K.) and cell culture media from Biowhittaker (Wokingham, U.K.). PD184352 was obtained by chemical synthesis, and wortmannin and rapamycin were

purchased from Calbiochem (Nottingham, U.K.). Microcystin-LR was purchased from Dr Linda Lawton, School of Life Sciences, Robert Gordon University, Aberdeen, UK, complete proteinase inhibitor cocktail tablets from Roche (East Sussex, U.K.), P1 nuclease from Europa Bioproducts Ltd (Cambridge, U.K.) and thin layer chromatography (TLC) plates from Merck (Poole, U.K.). Peptides were synthesised by Dr. G. Bloomberg (University of Bristol, U.K.) Other chemicals were purchased from Merck (Poole, U.K.) or Sigma-Aldrich (Poole, U.K.).

Phosphospecific antibodies that recognise PKB phosphorylated at Thr308 and ERK1/ERK2 phosphorylated at their Thr-Gly-Tyr motif were purchased from Cell Signalling Technologies (Hitchin, U.K.). The anti-FLAG monoclonal antibody M2 was obtained from Sigma-Aldrich (Poole, U.K.), the anti-HA monoclonal antibody 12CA5 from Roche (East Sussex, U.K.), and rabbit anti-sheep IgG, goat anti-rabbit IgG, and rabbit anti-mouse IgG peroxidase conjugated antibodies from Perbio Science UK (Tattenhall, U.K.).

Cloning of WDR4. DNA constructs encoding *the 266 amino acid form of WDR4 (Michaud et al, 2000)* were generated in a similar manner to METTL1 (see main text) with the ORF (NCBI BC006341) amplified from IMAGE clone 4080041.

Localisation of GFP-METTL1 and HA-WDR4. Wild-type METTL1 from pGEX6P-1 constructs was sub-cloned into pEGFPC-1 as a BamHI fragment for localisation studies and GFP-METTL1 and HA-WDR4 transfected with FuGene6 (Roche) according to manufacturer's instructions. HEK293 cells were grown on 22mm diameter cover slips previously coated with 10 mg/ml poly-L-Lysine. Transient transfection with pEGFPC-1-GFP-METTL1 and pCMV5-HA-WDR4 vectors were performed using FuGene according

to the manufacturer's instructions. After stimulation as described above, the cells were washed in PBS and fixed for 10 min at 21°C in PBS plus 4% (v/v) paraformaldehyde. The cells were then permeabilised in PBS containing 0.1% Triton-X100, blocked for 60 min at 21°C in PBS containing 2% (w/v) bovine serum albumin (BSA)/1% (v/v) goat serum / 50 mM NH₄Cl, and incubated with PBS containing the anti-HA antibody (20 mg/ml) and 2% (w/v) BSA/1% goat serum. An anti-mouse Cy5-coupled secondary antibody (Molecular Probes, Eugene, USA) was applied for 45 min in PBS containing 2% BSA/1% goat serum at the dilution recommended by the supplier. To check for specific recognition of HA-WDR4, control experiments were carried out in which transfected cells were incubated with primary antibody alone, secondary antibody alone or both antibodies in the presence of the HA peptide immunogen. All the cells were DAPI stained, and epifluorescence microscopy carried out using a Zeiss Axiovert 200M inverted microscope equipped with a PlanApo x63 1.32NA oil immersion objective. Images were captured using Improvision Openlab 3.07 software and assembled using Adobe Photoshop 4.0.

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Reference

Rena G., Prescott A.R., Guo S., Cohen P. and Unterman T.G. (2001) Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targetting. *Biochem J.* **354**, 605-12.