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Protein kinase DYRK2 is an E3-ligase specific molecular assembler

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Summary

Protein kinases play central roles in diverse cellular signal transduction pathways via their substrate phosphorylation. In this study, we discover that a protein kinase DYRK2 has unexpected role as a molecular assembler for an E3-ubiquitin ligase complex. DYRK2 associates with an E3 ligase complex containing EDD, DDB1 and VPRBP proteins (EDVP complex). Strikingly, DYRK2 serves as a molecular assembler for the EDVP complex as siRNA mediated depletion of DYRK2 disrupts the EDD-DDB1/VPRBP complex formation. Although the kinase activity of DYRK2 is dispensable for its ability to mediate EDVP complex formation, it is required for the phosphorylation and subsequent degradation of its downstream substrate, Katanin p60. Collectively, our results reveal a new type of E3-ubiquitin ligase complex that depends on a protein kinase for complex formation as well as for the subsequent phosphorylation, ubiquitination and degradation of their substrates.

DYRK2 [Dual-specificity tyrosine (Y) - phosphorylation regulated kinase 2] is a member of an evolutionarily conserved family of dual-specificity tyrosine phosphorylation regulated kinases (DYRKs) that belongs to the CMGC group of protein kinases1,2. During protein synthesis, DYRK2 autophosphorylates a tyrosine residue in its own activation loop. Once it is autophosphorylated at this tyrosine residue, DYRK2 loses its tyrosine kinase activity and functions only as a serine/threonine kinase3. DYRK2 phosphorylates a very limited number of substrates such as NFAT4, eIFB5, Glycogen synthase6, Oma-17, MEI-18, and chromatin remodeling factors SNR1 and TRX9, thus regulating calcium signaling, protein synthesis, glucose metabolism, developmental processes and gene expression. Recently, DYRK2 has also been suggested to function in the DNA damage signaling pathway via phosphorylating p53 at serine 46 in the nucleus and promoting cellular apoptosis upon genotoxic stress10. In addition to its role in cellular responses and developmental processes, DYRK2 is a potential oncogene11, since DYRK2 amplification and overexpression have been reported in adenocarcinomas of the esophagus and lung12. However, the exact mechanism of DYRK2 in tumorigenesis remains to be clarified.

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Author contributions:

S.M. performed all the experiments. S.M. and J.C. designed the experiments, analyzed the data and wrote the manuscript.

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Results

DYRK2 associates with EDVP E3-ligase complex

In an attempt to further elucidate DYRK2 function, we established 293T derivative cell line stably expressing a triple-epitope (S-protein, FLAG and streptavidin binding peptide) tagged version of DYRK2 (SFB-DYRK2). Tandem affinity purification using streptavadin agarose beads and S-protein agarose beads followed by mass spectrometry analysis allowed us to discover several DYRK2 interacting proteins (Fig.1a and supplementary table 1). Among them, we repeatedly identified EDD, DDB1 and VPRBP as major DYRK2-associated proteins (Fig. 1a). EDD (also known as UBR5, hHYD or KIAA0896) is an E3-ligase with a distinct N-terminal UBA domain, UBR box and a C-terminal HECT domain that mediates ubiquitin-dependent protein degradation13,14 . EDD is likely to be involved in tumorigenesis since an allelic imbalance at the EDD locus has been reported in several cancers15–16. DDB117 (DNA-damage binding protein 1) is an adaptor subunit of the Cul4-Roc1 E3 ligase complex18 that mediates the ubiquitin dependent degradation of various substrates including Cdt1, $p21^{cip1/waf1}$ and c-Jun. VPRBP (also known as DCAF1)19,20, a WD40 domain containing protein, is a substrate recognition subunit of the DDB1-Cul4A-Roc1 complex.

By transient overexpression of SFB-DYRK2 in 293T cells, we confirmed the *in vivo* interaction of DYRK2 with EDD, DDB1 and VPRBP (Fig. 1b). Though DDB1 and VPRBP have been discovered recently as key components in the Cul4-Roc1 E3 ligase complex18,21,22, surprisingly we did not identify either Cul4 or Roc1 in our purification. Indeed, we could not detect any interaction of overexpressed DYRK2 with either Cul4A or Roc1 (Fig. 1b), validating that Cul4-Roc1 are not components of this novel complex that contains DYRK2, EDD, DDB1 and VPRBP. We further confirmed the existence of this complex *in vivo* by demonstrating that endogenous DYRK2 co-immunoprecipitated with EDD, DDB1 and VPRBP (Fig. 1c). In contrast, Cul4A-Roc1 components were not seen in EDD immunoprecipitates (Fig. 1c). On the other hand, neither EDD nor DYRK2 was seen in Cul4A immunoprecipitates thus supporting the presence of EDVP complex independent of Cul4A-Roc1 complex (Fig. 1c). The interactions between EDD, DDB1 and VPRBP with DYRK2 are specific as we could only observe these associations in cells transfected with control siRNA, but not in cells following transfection with DYRK2 specific siRNA (Supplementary Fig. 1a). Also, exogenously expressed Myc-EDD interacted only with FLAG-DYRK2 but not with another DYRK family member, DYRK1B (Supplementary Fig. 1b and Fig. 1c), underlining the specificity of the interaction between DYRK2 and EDD. In addition, bacterially expressed GST-DYRK2 pulled down EDD, DDB1 and VPRBP, but not Cul4A from cell extracts (Fig. 1d), again arguing that DYRK2 forms a distinct complex with EDD, DDB1 and VPRBP. The formation of DYRK2-EDD-DDB1-VPRBP complex might not be strictly cell cycle regulated as we observed the interaction of DYRK2 with the other components of the complex independent of cell cycle phases, although the levels of EDD and VPRBP interacted with DYRK2 vary and are proportional to their protein levels at specific cell cycle phases (Supplementary Fig. 2c).

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EDD is a known HECT domain-containing E3 ligase that regulates ubiquitin dependent degradation of its substrates14. We named this E3 ligase complex containing EDD, DDB1 and VPRBP proteins as EDVP complex to distinguish it from the previously identified Cul4-Roc1-DDB1-VPRBP E3 ligase complex. To assess the significance of the interaction between DYRK2 and this new EDVP E3 ligase complex, we checked DYRK2 protein levels in EDD, DDB1 or VPRBP depleted cells following siRNA transfection. We found no difference in the DYRK2 protein levels in any of the knockdown cells compared to control siRNA transfected cells (Supplementary Fig. 2a). Also, we checked the protein levels of DYRK2, EDD and VPRBP at different phases of the cell cycle. While, the levels of EDD and VPRBP fluctuate during the cell cycle, DYRK2 levels remain constant (Supplementary Fig. 2b), thus ruling out the possibility that DYRK2 may be a target for proteosomal degradation mediated by EDVP ligase complex.

DYRK2 acts as an adaptor in the EDVP complex

Several Kelch motif-containing proteins were shown previously to act as E3 ligase adaptors for specific substrates23,24,25. A preliminary analysis of DYRK2 protein sequence revealed the presence of a Kelch motif (amino acids 390–433) within its protein kinase catalytic domain (Supplementary Fig. 3), so we next investigated whether DYRK2 functions as a molecular adaptor in the EDVP ligase complex. We depleted DYRK2 using siRNA and checked for complex formation of EDD, DDB1 and VPRBP. The interaction of EDD with DDB1 and VPRBP was seen only in the presence of intact DYRK2, whereas the knockdown of DYRK2 led to the loss of the interaction between EDD and DDB1 or VPRBP (Fig. 2a). Neither the interaction of DDB1 with VPRBP nor the association of Cul4A with DDB1-VPRBP was affected by the absence of DYRK2 (Fig. 2a). These experiments suggest that DYRK2 functions as a molecular assembler required for the specific recruitment of EDD to DDB1-VPRBP, thus forming a novel EDVP E3 ligase complex. Surprisingly, DYRK2 kinase activity is dispensable for its role as an assembler of this E3 ligase complex, since the transfection of either siRNA resistant wild type DYRK2 or kinase inactive DYRK2, which were validated by both expression and kinase activity using a synthetic peptide (Fig. 2b), into DYRK2-depleted cells was able to restore the association of EDD with DDB1-VPRBP complex (Fig. 2c).

EDVP-DYRK2 complex regulates Katanin p60 ubiquitination

We next examined the likely substrates of this new E3 ligase complex. Previously, MBK-2, the C. elegans homolog of mammalian DYRK2, was shown to phosphorylate and regulate MEI-1 during C. elegans meiotic maturation8. MEI-1 (a C. elegans homolog of Katanin p60), is an AAA-ATPase that associates with MEI-2 and functions as a microtubule severing enzyme. When the C. elegans embryo enters the first mitotic division after exiting the meiosis, MBK2 phosphorylates MEI-1, which is then degraded through an ubiquitin-dependent mechanism via binding to MEL-26, a BTB-domain containing substrate adaptor protein complexed with Cul324. The ubiquitin mediated degradation of MEI-1/Katanin was further regulated by a series of neddylation and deneddylation of Cul3 mediated by COP9/signalosme26. In higher eukaryotes, it is not yet known whether DYRK2 would regulate Katanin p60. Thus we first tested and showed that Katanin p60 readily associated with the DYRK2-EDVP complex *in vivo* (Fig. 3a). To identify the direct Katanin binding subunit of

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EDD-DDB1-VPRBP complex, we performed an in vitro binding assay using bacterially expressed recombinant MBP-tagged EDD, DDB1 and VPRBP along with GST-tagged Katanin. Recombinant Katanin directly binds VPRBP but not EDD and DDB1 *in vitro* (Fig. 3b). In addition, Katanin interacts with EDD and DDB1 only in the presence of intact VPRBP but not in VPRBP knock down cells thus confirming VPRBP as the substrate binding receptor subunit in EDVP complex (Fig. 3c). We further examined whether the associated Katanin p60 is a substrate of the DYRK2-EDVP E3 ligase complex. We evaluated endogenous Katanin ubiquitination in cells transfected with either control siRNA or siRNAs specific for different components of the DYRK2-EDVP complex in the presence of MG132, a proteosomal inhibitor. Katanin p60 was polyubiquitinated in the presence of the intact DYRK2-EDVP, but its ubiquitination was severely reduced by the depletion of DYRK2, EDD, DDB1 or VPRBP (Fig. 3d). In contrast, Katanin polyubiquitination was unaffected in cells transfected with siRNAs against Cul4A and Cul4B. Previously, it was shown that Cul3/MEL26 also plays a role in MEI-1 (a C. elegans homolog of Katanin p60) degradation during C. elegans meiotic maturation24,26. Thus, we tested whether a similar mechanism for Katanin regulation occurs in humans. Interestingly, knocking down Cul3 does lead to a modest reduction of Katanin polyubiquitination, although the severity of this reduction is not comparable with those observed in cells with knockdown of EDVP complex subunits (Fig. 3d). Thus, it is likely that the EDVP complex plays a primary role where as Cul3 is of secondary importance in promoting Katanin polyubiquitination in human cells. We also investigated whether EDD is the functional E3 ligase in the EDVP complex. Knock down of EDD using siRNA severely affected katanin polyubiquitination. This defect in katanin ubiquitination was fully rescued by the expression of siRNA resistant wild-type EDD, but not by the expression of a catalytically inactive HECT domain mutant of EDD (Fig. 3e). Thus, EDD is the catalytic subunit in this E3 ligase complex.

Polyubiquitination of Katanin by the DYRK2-EDVP complex is likely to be required for Katanin degradation, as the knockdown of DYRK2, EDD, DDB1 or VPRBP but not Cul4A and Cul4B increased the steady-state levels of Katanin protein (Fig. 4a). Knock down of Cul3 also resulted in a small increase in Katanin protein levels, again suggesting a secondary role of Cul3 complex in Katanin degradation. Also in a cyclohexamide chase experiment, Myc-tagged Katanin was stabilized in DYRK2, EDD or VPRBP depleted cells when compared to cells transfected with control siRNAs. In sharp contrast, overexpression of DYRK2, DDB1 or VPRBP along with Katanin led to diminished Katanin stability (Fig. 4b). Together these data suggest that Katanin is a substrate of the DYRK2-EDVP E3 ligase complex. Since Anaphase promoting complex was also shown to work with MBK-2 and regulate C. elegans Katanin p60 degradation in a Cul3 redundant pathway8, we knocked down APC2, a critical subunit of the anaphase promoting complex, in human cells. However, we did not observe any change in Katanin protein levels (Fig. 4c), and thus concluded that APC may not be involved in Katanin degradation in humans.

DYRK2 mediated phosphorylation is required for Katanin p60 degradation

MBK2/DYRK2 is known to phosphorylate Katanin homolog MEI-1 in *C. elegans*27. We next investigated whether DYRK2 would phosphorylate Katanin and be required for Katanin ubiquitination by EDVP complex. *In vitro* kinase assays revealed that

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immunoprecipitated wild-type DYRK2 but not kinase-inactive DYRK2 could phosphorylate bacterially expressed GST-Katanin (Fig. 5a). In addition, we further tested whether DYRK2 in EDVP complex can phosphorylate Katanin by performing immunoprecipitation of EDVP complex followed by *in vitro* kinase assay. Immunoprecipitates of EDD, DDB1 and VPRBP but not Cul4A showed intrinsic kinase activity towards Katanin (Fig. 5b), suggesting that DYRK2 in the EDVP complex is still capable of phosphorylating its substrate. Katanin contains several consensus DYRK2 phosphorylation sites28 (Fig. 5c). We mutated Katanin at these serine or threonine residues individually or in combination and examined whether any of these residues are potential DYRK2 phosphorylation sites *in vitro*. Serine 42, serine 109 and threonine 133 are likely to be the major DYRK2 phosphorylation sites as single mutations for these sites showed reduced phosphorylation by DYRK2 and the triple mutant showed almost no DYRK2 mediated phosphorylation (Fig. 5d). Furthermore, we detected the presence of phosphoserine and phosphothreonine residues in the immunoprecipitated WT-Katanin (Fig. 5e), indicating that Katanin is phosphorylated *in vivo*. However these phosphorylations were greatly diminished in the triple phospho-mutant (AAA mutant) of Katanin (Fig. 5f), suggesting that these residues are indeed major *in vivo* phosphorylation sites.

We further investigated whether the DYRK2-mediated Katanin phosphorylation is required for its ubiquitination *in vivo*. Ubiquitination of Myc-tagged wild-type Katanin was easily detected, whereas the ubiquitination of Katanin AAA-mutant was severely diminished (Fig. 6a). To further support the idea that the intact DYRK2-EDVP complex mediates ubiquitination of phosphorylated Katanin, we performed *in vitro* reconstitution assays using GST-Katanin as ubiquitination substrate. As shown in Figure 6b, only intact EDVP complex containing wild type EDD (but not mutant EDD) resulted in robust Katanin polyubiquitination. The prior phosphorylation of Katanin is essential for Katanin ubiquitination as only wild type Katanin but not triple phospho mutant of Katanin (Katanin AAA) could be readily ubiquitinated by EDVP complex *in vitro* (Fig. 6c). This data suggests that the DYRK2-dependent Katanin phosphorylation is a prerequisite for Katanin ubiquitination, indicating that DYRK2 kinase activity is critical for the function of the DYRK2-EDVP E3 ligase complex. The phosphorylation-dependent degradation of Katanin is further substantiated by co-transfection experiments. Co-transfection of wild-type DYRK2 but not kinase-inactive DYRK2 along with Katanin reduced the steady-state levels of Katanin protein (Fig. 6d). In contrast, the protein levels of Katanin AAA-mutant remained largely unaffected (Fig. 6d), suggesting that DYRK2-mediated phosphorylation is a priming event required for Katanin ubiquination and degradation.

EDVP-DYRK2 complex controls mitotic transition

Previous studies have reported Katanin as a microtubule AAA-ATPase that plays an important role in mitosis29,30. Katanin is required for severing microtubules at the mitotic spindles when disassembly of microtubules is required to segregate sister chromatids during anaphase. Both DYRK27 and EDD31 have also been suggested to function during mitosis. To establish a functional link between DYRK2-EDVP ligase complex and Katanin degradation, we checked the cell cycle profile of HeLa cells transiently transfected with Katanin. Overexpression of Katanin led to the accumulation of a 4N population and

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polyploidy (>4N) cells (Fig. 7a). Similarly, siRNA-mediated downregulation of either DYRK2 or EDD, which led to an upregulation of Katanin (Fig. 7b), also resulted in accumulation of cells with 4N DNA content (Fig. 7a). This abnormal accumulation of 4N cells following DRYK2 or EDD depletion could be rescued upon simultaneous depletion of Katanin by siRNA (Fig. 7a). In addition, DYRK2-mediated Katanin phosphorylation is required for proper cell cycle progression as the co-expression of DYRK2 with the phospho-mutant but not wild-type Katanin led to an increase in cells with 4N DNA content (Fig. 7c). This increase in the 4N population is attributed to defective mitotic progression as we observed an increased number of phospho-H3 positive cells when wild type or non-phosphorylatable Katanin was overexpressed (Fig. 7d). Co-expression of wild-type DYRK2 but not kinase-dead version with wild-type Katanin reduced the number of mitotic cells to normal levels, whereas co-expression of DYRK2 with Katanin AAA mutant failed to rescue this mitotic defect (Fig. 7d). Collectively these results suggest that an active DYRK2-EDVP ligase complex regulates mitotic transition through modulating Katanin protein levels.

Discussion

Protein kinases regulate a variety of biological processes including cell proliferation, apoptosis, development and tumorigenesis via phosphorylating their respective downstream substrates32. In this study, we uncover a novel role for protein kinase, e.g. functions as an assembly factor for an E3 ubiquitin ligase complex. In particular, we have shown that DYRK2 has dual roles in this E3 ligase complex. It not only is required for the assembly of the complex, but also phosphorylates its substrate and primes the substrate for degradation. Phosphorylation dependent protein degradation is a common mechanism for regulating protein stability in cell cycle- or stimulus-dependent manner. This often occurs in a two-step process33, where initially a kinase phosphorylates the substrate. Once phosphorylated, the substrate is recognized by F-box containing or BC-box substrate receptor proteins and targeted to E3 ligase complexes for degradation. Here, we provide evidence for the integral presence of a kinase, DYRK2, within the EDD-DDB1-VPRBP (EDVP) E3 ligase complex, which merges the functional properties of a protein kinase and an E3 ligase into a single unit that can recognize, phosphorylate and degrade substrates in concert. It is currently unclear how DYRK2 mediated phosphorylation of Katanin promotes its ubiquitination but it is possible that phosphorylation of Katanin leads to a conformational change which might expose some of the substrate residues for efficient ubiquitination by EDD. Our functional studies further suggest that the DYRK2-EDVP E3 ligase complex plays a crucial role in regulating normal mitotic progression. Since overexpression of both DYRK211,12 and EDD15,16 are frequently reported in cancers, it is tempting to speculate that aberrant mitosis and altered cell cycle progression via the hyperactivation of DYRK2-EDVP E3 ligase complex might be a key mechanism in promoting neoplastic transformation. Future studies using animal models will reveal the role of this DYRK2-EDVP complex in cancer development and progression.

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Materials and Methods

Plasmids

Full length DYRK2, DYRK1B, EDD, DDB1, VPRBP, Cul4A, Katanin and DYRK2-KD were cloned into S-protein/FLAG/SBP (streptavidin binding protein) - triple tagged destination vector using Gateway cloning system (Invitrogen). Full length EDD, Katanin and Katanin AAA mutant were also cloned to Myc-tagged destination vector. GST-tagged DYRK2, MBP-tagged EDD, EDDC/A, DDB1 and VPRBP bacterial expression vectors were generated by transferring their coding sequences into destination vectors using Gateway system. Various deletion and point mutants for Katanin p60 and the kinase dead version of DYRK2 were generated by PCR-based site-directed mutagenesis. Wild-type and mutants of Katanin were also cloned to GST-tagged vector. Constructs of Myc-tagged ubiquitin and HA-tagged ubiquitin were used in *in vivo* ubiquitination assays. siRNA resistant DYRK2 WT, DYRK2 KD, EDD WT and EDD C/A mutant constructs were generated by introducing silent mutations in their respective triple-tagged vectors using site-directed mutagenesis and verified by sequencing.

Antibodies

Rabbit anti-Katanin antibodies were raised by immunizing rabbits with GST-Katanin p60 fusion protein (amino acids 30–240). Antisera were affinity-purified using AminoLink plus Immobilization and purification kit (Pierce). Anti-DYRK2 (Abcam), anti-EDD, anti-DDB1, anti-VPRBP, anti-Cul4A, anti-Cul3 (all from Bethyl laboratories), anti-FBX22 (Novus biologicals), anti-PBK (Cell Signaling technology), anti-FLAG, anti-Maltose binding protein; Clone 17, anti-Actin, anti-Cul4B (Sigma), anti-Roc1 (Invitrogen), anti-GST, anti-Myc; Clone 9E10 (Santa-cruz biotechnologies), anti-phospho serine H3, anti-phosphothreonine (Cell signaling technology) and Anti-phosphoserine, anti-ubiquitin (Millipore) antibodies were used in this study.

Tandem affinity purification

293T cells were transfected with S-protein/FLAG/SBP (streptavidin binding protein) – triple tagged DYRK2 and then three weeks later puromycin resistant colonies were selected and screened for DYRK2 expression. The DYRK2 positive stable cells were then maintained in RPMI-supplemented with FBS and 2 μg/ml puromycin. The SFB-DYRK2 stable cells were lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40) containing 50 mM β-glycerophosphate, 10 mM NaF, 1 μg/ml of each pepstatin A and aprotinin on ice for 20 minutes. After removal of cell debris by centrifugation, crude cell lysates were incubated with streptavidin sepharose beads (Amersham Biosciences) for 1 hour at 4°C. The bound proteins were washed three times with NETN and then eluted twice with 2 mg/ml biotin (Sigma) for 30 minutes at 4 °C. The eluates were incubated with S-protein agarose beads (Novagen) for 1 hour at 4 °C and then washed three times with NETN. The proteins bound to S-protein agarose beads were resolved by SDS-PAGE and visualized by Coommasie Blue staining. The identities of eluted proteins were revealed by mass spectrometry analysis performed by the Taplin Biological Mass Spectrometry Facility at Harvard.

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Cell transfections, Immunoprecipitation and Immunoblotting

293T cells or HeLa cells were transfected with various plasmids using Lipofectamine (Invitrogen) according to the manufacturer's protocol. For immunoprecipitation assays, cells were lysed with NETN buffer as described above. The whole cell lysates obtained by centrifugation were incubated with 2 μg of specified antibody bound to either protein A or protein G sepharose beads (Amersham Biosciences) for 1 hour at 4 °C. The immunocomplexes were then washed with NETN buffer four times and applied to SDS-PAGE. Immunoblotting was performed following standard protocols.

GST pull-down and In vitro binding assays

Bacterial expressed GST-DYRK2 or control GST bound to glutathione-Sepharose beads (Amersham) were incubated with 293T cell lysates for 1 hour at 4°C, and the washed complexes were eluted by boiling in SDS sample buffer, separated by SDS-PAGE, and the interactions were analyzed by Western blotting. For In vitro binding assays, bacterial expressed MBP-EDD, MBP-DDB1 or MBP-VPRBP bound to Amylose sepharose beads were incubated with bacterially purified GST-Katanin for 1 hour at 4°C, and the washed complexes were eluted by boiling in SDS sample buffer and separated by SDS-PAGE, and the interactions were analyzed by Western blotting using indicated antibodies.

RNA interference

Control siRNA and the smart pool small interfering RNAs (siRNAs) against DDB1, Cul4A, Cul4B, Cul3, APC2 and Katanin and the on-target plus individual siRNAs against DYRK2, EDD and VPRBP were purchased from Dharmacon Inc. Transfection was performed twice 30 hours apart with 200 nM of siRNA using Oligofectamine reagent according to the manufacturer's protocol (Invitrogen). Following target sequences were used.

Individual siRNA sequences:

DYRK2 siRNA: (5'-GGUGCUAUCACAUCUAUAU -3')

EDD siRNA: (5'-CAACUUAGAUCUCCUGAAA-3')

DDB1 siRNA: (5'-ACACUUUGGUGCUCUCUU -3')

VPRBP siRNA: (5'-GAUGGCGGAUGCUUUGAUA -3')

Pooled siRNA sequences:

Cul4A siRNA: (5'-GCACAGAUCCUUCCGUUUA -3'; 5'-GAACAGCGAUCGUAAUCAA -3'; 5'-GCAUGUGGAUUCAAAGUUA -3'; 5'-GCGAGUACAUCAAGACUUU -3')

Cul4B siRNA: (5'-GCUAUUGGCCGACAUAUGU -3'; 5'-CAGAAGUCAUUAAUUGCUA -3'; 5'-CAAACGGCCUAGCCAAAUC -3'; 5'-CGGAAAGAGUGCAUCUGUA -3')

Cul3 siRNA: (5'-CCGAACAUCUCAUAAAUAA -3'; 5'-GAGAAGAUGUACUAAAUUC -3'; 5'-GAGAUCAAGUUGUACGUUA -3'; 5'-GCGGAAAGGAGAAGUCGUA -3')

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APC2 siRNA: (5'-GAGAUGAUCCAGCGUCUGU -3'; 5'-GACAUCAUCACCCUCUAUA -3'; 5'-GAUCGUAUCUACAACAUGC -3'; 5'-GAGAAGAAGUCCACACUAU -3')

Katanin siRNA: (5'-GGGAGGAGCUAUUACGAAU -3'; 5'-GCUGUUCGUUGUCGUGAAA -3'; 5'-GGAUCAUGCUAACUCGAGA -3'; 5'-CAUUGAAAGAUACGAGAAA -3')

In vitro kinase assay

Wild type DYRK2 and kinase inactive DYRK2, which were expressed in 293T cells were immunoprecipitated using FLAG-agarose beads and used as a kinase source. GST-tagged Katanin and its mutant proteins, expressed in *Escherichia coli* BL21 strain, were purified using Glutathione sepharose beads and used as substrates. The kinase (DYRK2) and substrates (Katanin) were incubated in kinase assay buffer (10 mM Hepes, pH 7.5, 50 mM NaCl, 10 nM $MgCl_2$, 10mM MnCl2, 1 mM EGTA, 1 mM dithiothreitol, 5 μM ATP, 10 mM NaF and 50mM glycerophosphate) along with 10 μCi [γ-^{32}P]) for 30 minutes at 30°C. Reactions were stopped by the addition of SDS sample buffer. Then samples were boiled for 5 minutes to 95°C followed by SDS-PAGE and autoradiography. The validation of siRNA resistant wild type DYRK2 and kinase dead DYRK2 was performed by DYRK2 immunoprecipitation followed by kinase assay using a synthetic woodtide peptide (KKISGRLSPIMTEQ) as a substrate (purchased from Millipore).

In vivo ubiquitination assay

HeLa Cells were transfected with various combinations of plasmids as indicated in figure 2D and figure 3F along with either Myc-tagged Ubiquitin or HA-tagged ubiquitin. At 24 hour post-transfection, cells were treated with MG132 (4 μM) for 6 hours and the whole cell extracts prepared by NETN lysis were subjected to immunoprecipitation of the substrate protein. The analysis of ubiquition was performed by immunoblotting with either anti-Myc or anti-HA antibodies.

In vitro reconstitution assay

The reactions were carried out at 30°C for 15 minutes in 25 μl of ubiquitination reaction buffer (40mM Tris-HCl, pH 7.6, 2mM DTT, 5mM $MgCl_2$, 0.1M NaCl, 2mM ATP) containing the following components: 100 μM of ubiquitin, 20 nM E1 (UBE1), 100 nM UbcH5b (all from Boston Biochem). Various combinations of EDVP E3 ligase components (25ng EDD or EDD C/A, DDB1, VPRBP and DYRK2) as indicated were added to the reaction. Either wild type GST-Katanin or AAA-mutant bound to Glutathione sepharose beads was used as a substrate in the reaction. After ubiquitination reaction, the glutathione beads were washed five times with NETN buffer, boiled with SDS-PAGE loading buffer and the ubiquitination of Katanin was monitored by Western blotting with anti-ubiquitin antibody.

Cell cycle analysis

HeLa cells transfected with desired expression vectors and siRNA were harvested, washed with PBS, and fixed with ice-cold 70% ethanol for at least 1 hour. Cells were washed twice

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in PBS and treated for 30 minutes at 37°C with RNase A (5 μg/mL) and propidium iodide (50 μg/mL), and analyzed on a FACScan flow cytometer (Becton Dickinson). The percentage of cells in different cell cycle phases was calculated using Flowjo analysis software.

Immunoflouroscence staining

Cells grown on coverslips were fixed with 3 % paraformaldehyde solution in PBS containing 50 mM sucrose at room temperature for 15 minutes. After permeabilization with 0.5 % Triton X-100 buffer containing 20 mM HEPES pH7.4, 50 mM NaCl, 3mM $MgCl_2$, and 300 mM sucrose at room temperature for 5 minutes, cells were incubated with a primary phosphor-serine H3 antibody at 37 °C for 20 minutes. After washing with PBS, cells were incubated with rhodamine-conjugated secondary antibody at 37 °C for 20 minutes. Nuclei were counterstained with 4'6-diamidino-2-phenylindole (DAPI). After a final wash with PBS, coverslips were mounted with glycerin containing paraphenylenediamine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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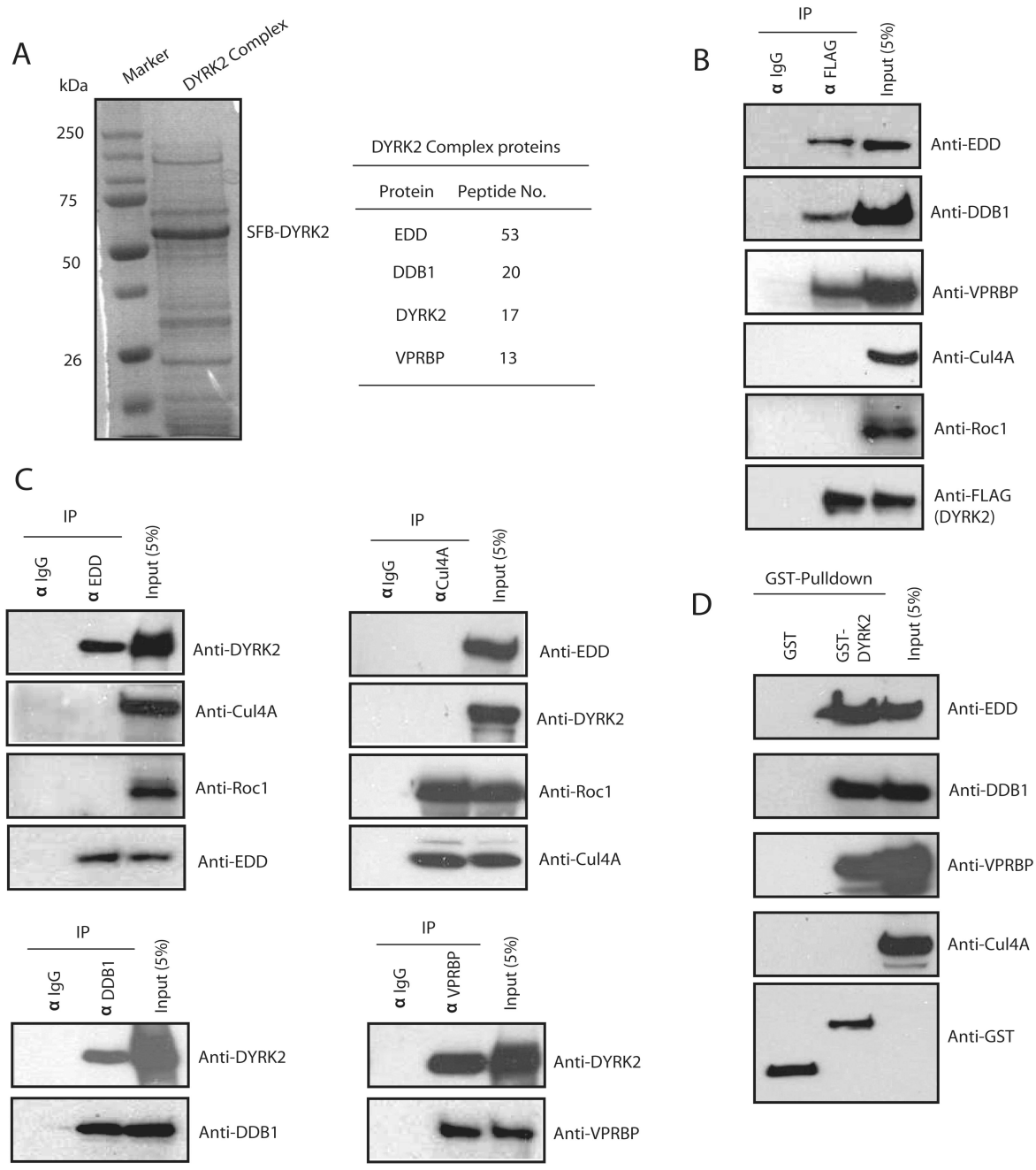

Figure 1. Identification of EDD-DDB1-VPRBP as DYRK2 associated proteins

(a) Tandem affinity purification of DYRK2-containg protein complexes was conducted using 293T cells stably expressing triple tagged DYRK2. Associated proteins were separated by SDS-PAGE and visualized by Coomassie staining. The proteins and the number of peptides identified by mass spectrometry analysis are shown in the table on the right and also in supplementary data (Supplemental Table 1) **(b)** Immunoprecipitation using control IgG or anti-FLAG (DYRK2) antibody were performed using extracts prepared from 293T derivative cells stably expressing FLAG-tagged DYRK2. The presence of EDD,

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DDB1, VPRBP, Cul4A or Roc1 in these immunoprecipitates was evaluated by immunoblotting with their respective antibodies. **(c)** Reverse co-immunoprecipitation experiments were performed using anti-EDD, anti-Cul4A, anti-DDB1 and anti-VPRBP antibodies and the associated endogenous DYRK2 and other indicated proteins was identified by Western blotting using their respective antibodies. **(d)** GST pull down assay was performed using immobilized control GST or GST-DYRK2 fusion proteins on agarose beads and incubated with extracts prepared from 293T cells. The interaction of EDD, DDB1, VPRBP or Cul4A with DYRK2 was assessed by immunoblotting with their respective antibodies.

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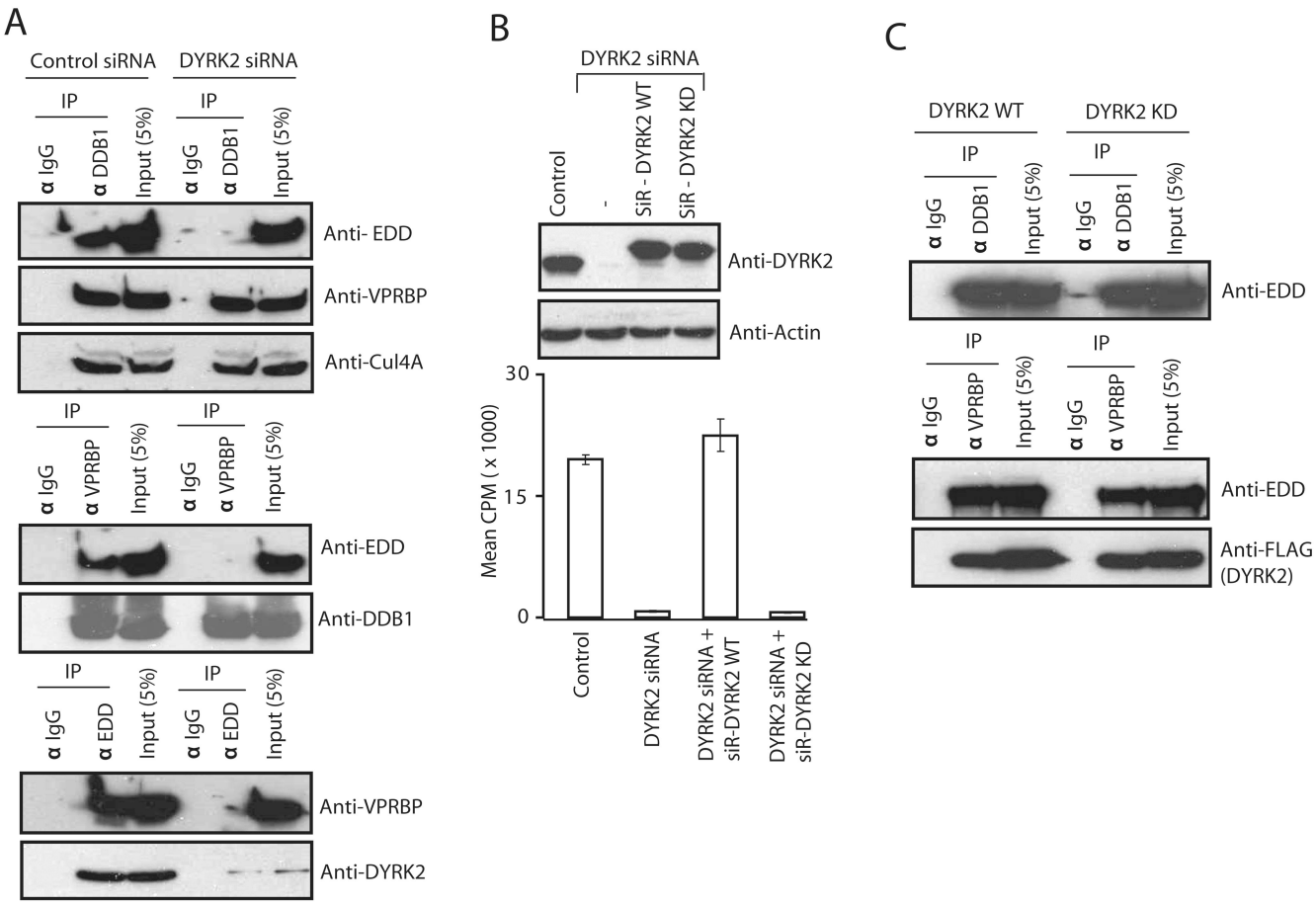

Figure 2. DYRK2 functions as an adaptor in the EDVP E3 ligase complex

(a) HeLa cells were transfected with control siRNA or DYRK2 specific siRNA and immunoprecipitations were performed using DDB1 (upper panel), VPRBP (middle panel) or EDD antibodies (lower panel). The presence of associated proteins in the immunoprecipitated complexes was assessed by immunoblotting with antibodies as indicated. **(b)** HeLa cells transfected with DYRK2 specific siRNA were retransfected with either siRNA resistant wild type DYRK2 (SiR-DYRK2 WT) or kinase dead DYRK2 (SiR-DYRK2 KD). The expression of endogenous DYRK2 and the transfected siRNA resistant DYRK2 was assessed by immunoblotting with anti-DYRK2 antibody. Actin was used as a loading control. The graph represents DYRK2 kinase activity (CPM; mean counts per minutes) measured after performing an in vitro kinase assay using DYRK2 immunoprecipitates prepared from indicated different cell lysates using woodtide peptide as a substrate (±s.d., n=3). **(c)** HeLa cells transfected with DYRK2 specific siRNA were retransfected with either siRNA resistant wild type (WT) DYRK2 or kinase dead (KD) DYRK2. Lysates prepared from these cells were used to immunoprecipitate DDB1 or VPRBP with their respective antibodies. The associated EDD in these immunoprecipitates was assessed by immunoblotting using anti-EDD antibody.

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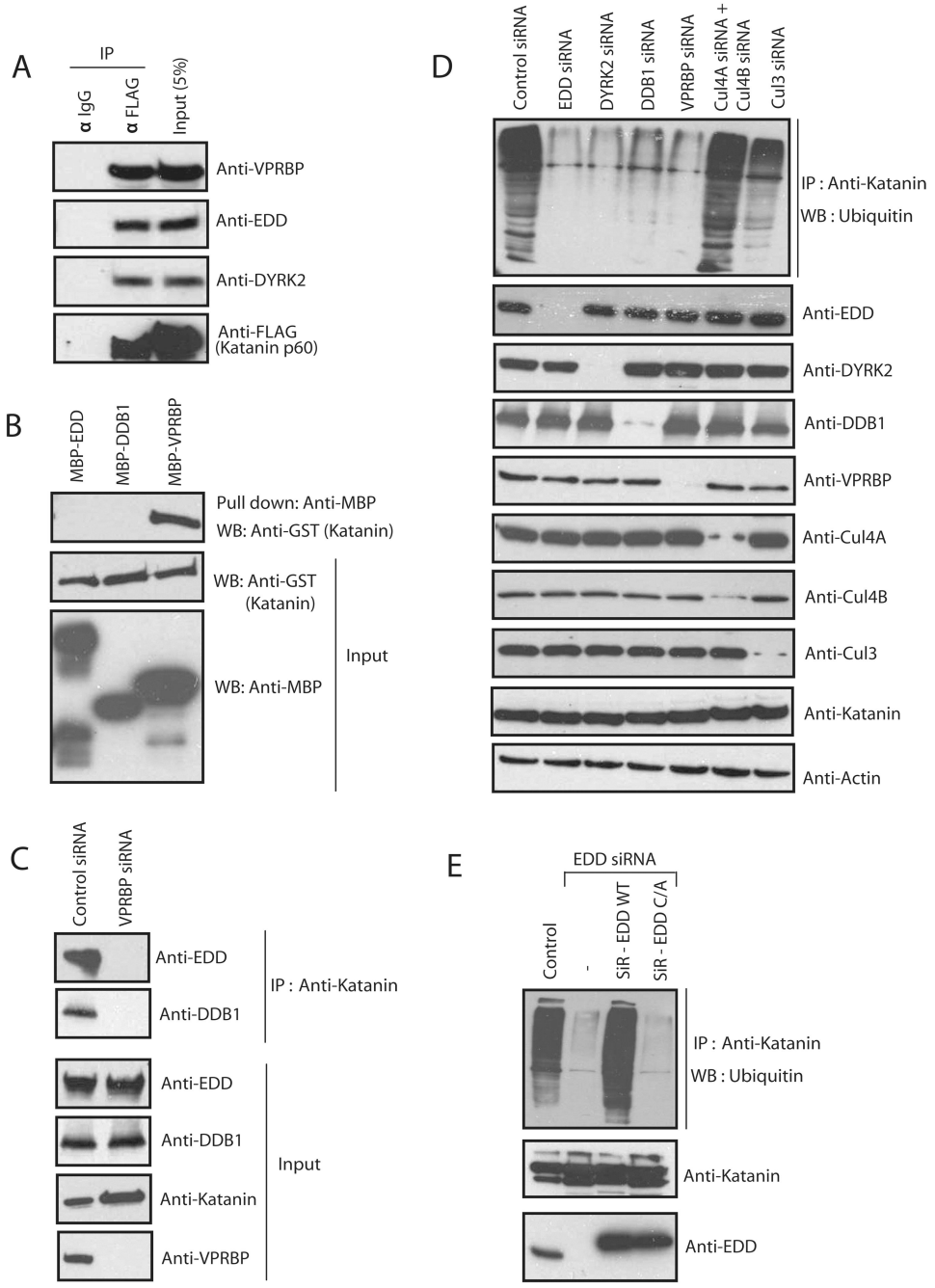

Figure 3. Katanin p60 is the ubiquitination substrate for EDVP E3 ligase complex

(a) Control (IgG) or anti-FLAG immunoprecipitates were prepared from 293T cells transfected with plasmid encoding a triple tagged Katanin. Western blotting was conducted using indicated antibodies to show a specific interaction between the DYRK2-EDVP complex and Katanin p60. **(b)** Bacterially expressed recombinant MBP-tagged EDD, DDB1 or VPRBP bound to amylose sepharose beads were incubated with recombinant GST-Katanin and the association of Katanin was detected by western blotting with anti-GST antibody. The expression of MBP-fusion proteins was detected by anti-MBP antibody. **(c)**

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HeLa cells were transfected with either control siRNA or VPRBP siRNA and the association of EDD and DDB1 with Katanin was assessed by immunoblotting with their respective antibodies after immunoprecipitation using anti-Katanin antibody. **(d)** HeLa cells were transfected with different siRNAs as indicated. Cell lysates prepared after 5 hour MG132 (10μM) treatment were subjected to immunoprecipitaton using anti-Katanin antibodies. The ubiquitinated Katanin was detected with anti-ubiquitin antibody. The protein expression and the specificity of different siRNAs were confirmed by immunoblotting of cell extracts using antibodies as indicated. **(e)** HeLa cells transfected with EDD specific siRNA were retransfected with either siRNA resistant wild type EDD (SiR-EDD WT) or catalytically inactive EDD (SiR-EDD C/A). Ubiquitination of Katanin was assessed by immunoblotting with anti-ubiquitin antibody after immunoprecipitating with anti-Katanin antibody. The expression of endogenous EDD and the transfected siRNA resistant EDD was assessed by immunoblotting with anti-EDD antibody.

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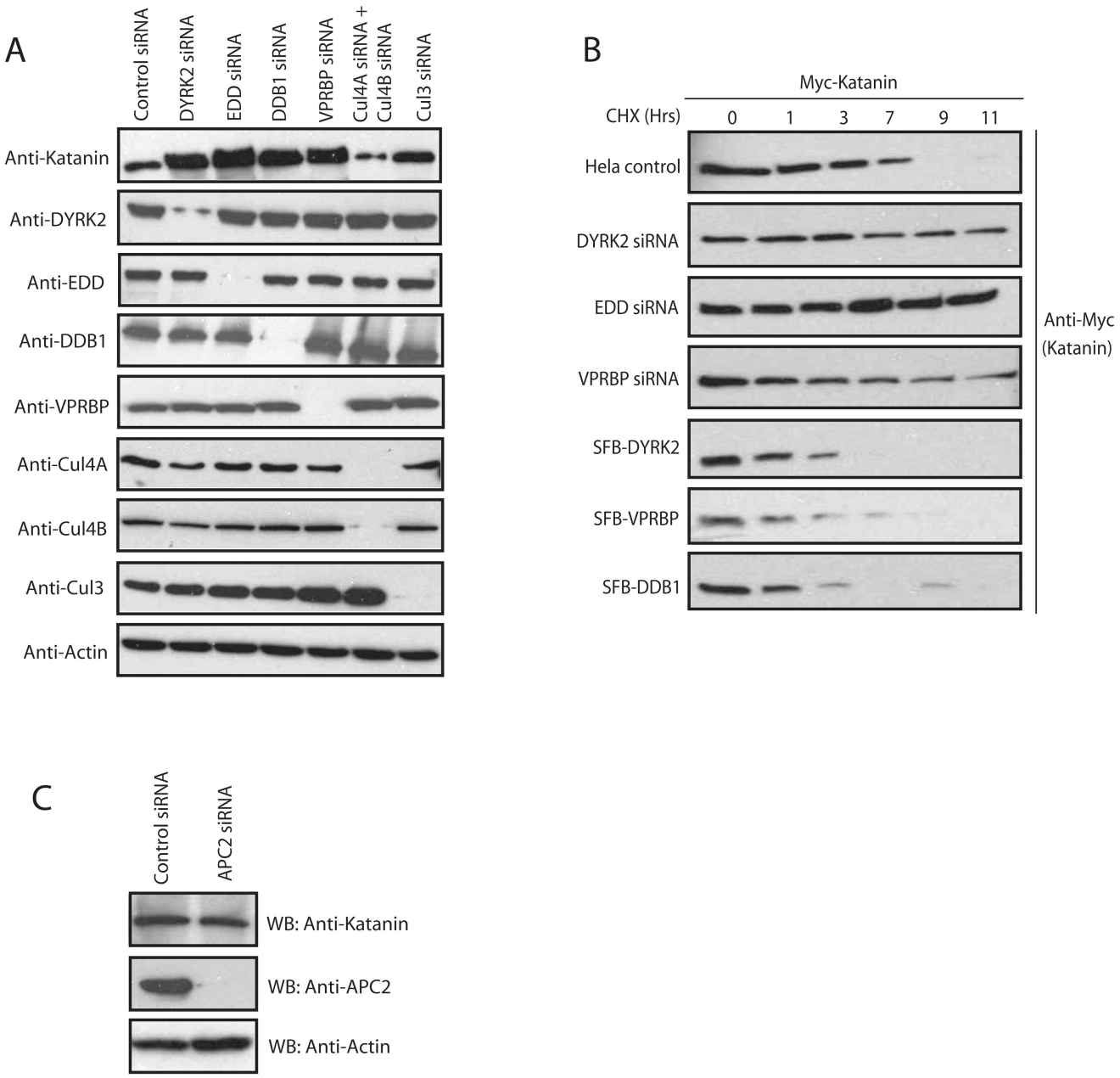

Figure 4. EDVP E3 ligase complex regulates Katanin p60 protein levels

(a) HeLa cells were transfected with control siRNA or siRNAs against DYRK2, EDD, DDB1, VPRBP, Cul4A/Cul4B and Cul3. The protein levels of Katanin were assessed by immunoblotting using anti-Katanin antibody and the efficiency of different siRNAs was shown by immunoblotting with the indicated antibodies. **(b)** HeLa cells transiently expressing Myc-tagged Katanin were either transfected with siRNAs against DYRK2, EDD and VPRBP or with plasmids encoding SFB-tagged DYRK2, VPRBP or DDB1. Twelve hours post-transfection, cells were treated with cyclohexamide and collected at the indicated times afterwards. The protein levels of Katanin were determined by anti-Myc immunoblotting. **(c)** Cells transfected with either control siRNA or anaphase promoting

complex 2 (APC2) siRNA were lysed and the expression of Katanin and APC2 was detected by western blotting with their respective antibodies. Actin is used as a loading control.

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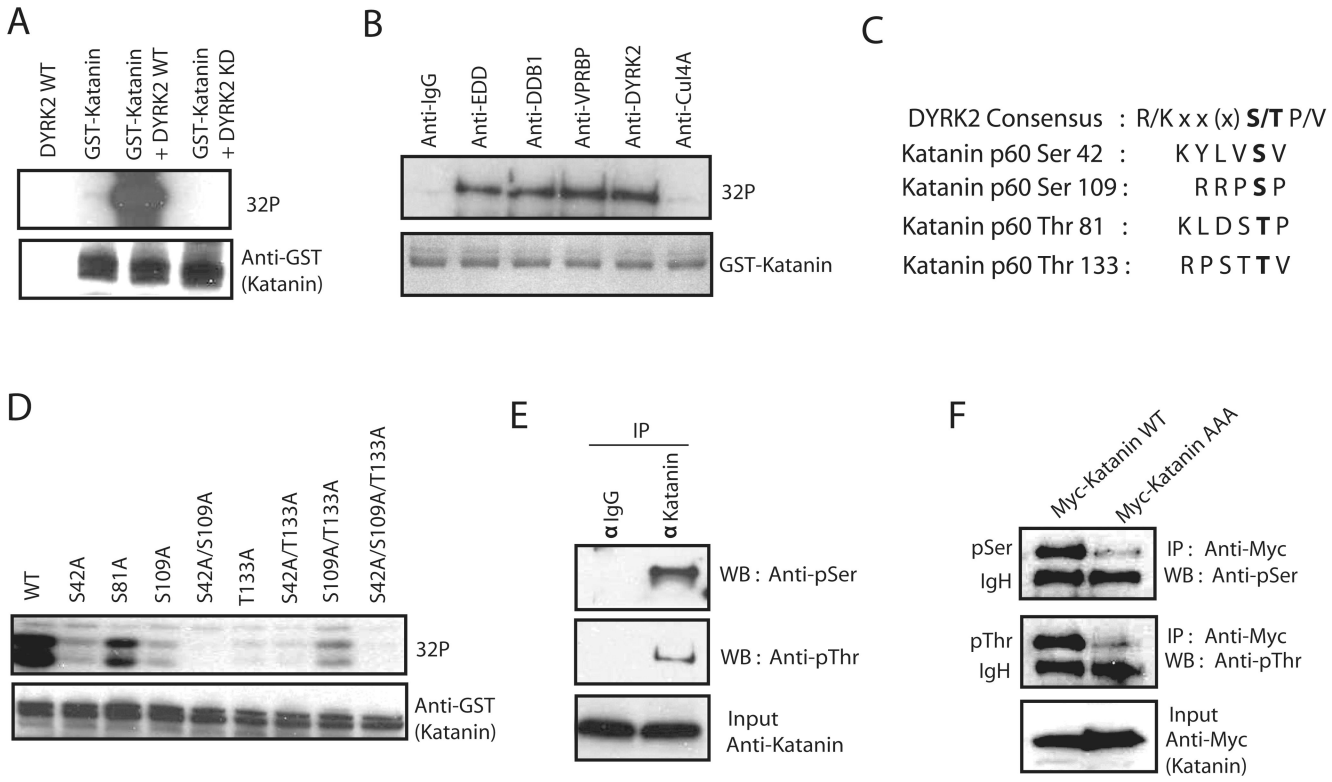

Figure 5. DYRK2 phosphorylates Katanin

(a) An *in vitro* kinase assay was performed with a bacterial expressed GST-Katanin and immunoprecipitated wild-type or Kinase inactive DYRK2. **(b)** An *in vitro* kinase assay was performed with a bacterial expressed GST-Katanin with immunoprecipitates prepared by using EDD, DDB1, VPRBP, DYRK2 and Cul4A antibodies. **(c)** The alignment of potential Katanin phosphorylation sites with DYRK2 consensus sequence is presented. Bold lettering indicates the phosphorylated residue. **(d)** *In vitro* DYRK2 kinase assays were conducted using different bacterially expressed GST-Katanin phosphorylation site mutants as indicated. **(e)** The *in vivo* phosphorylation of Katanin was detected by immunoblotting with anti-phospho-serine or anti-phospho-threonine specific antibodies following immunoprecipitation using control IgG or Katanin antibodies. **(f)** The *in vivo* phosphorylation of wild-type Katanin and the phospho Katanin mutant (AAA) was assessed by immunoblotting with phospho-serine or phospho threonine antibodies following anti-Myc immunoprecipitation of extracts prepared from 293T cells expressing Myc-tagged wild-type or mutant Katanin. IgH indicates IgG heavy chain.

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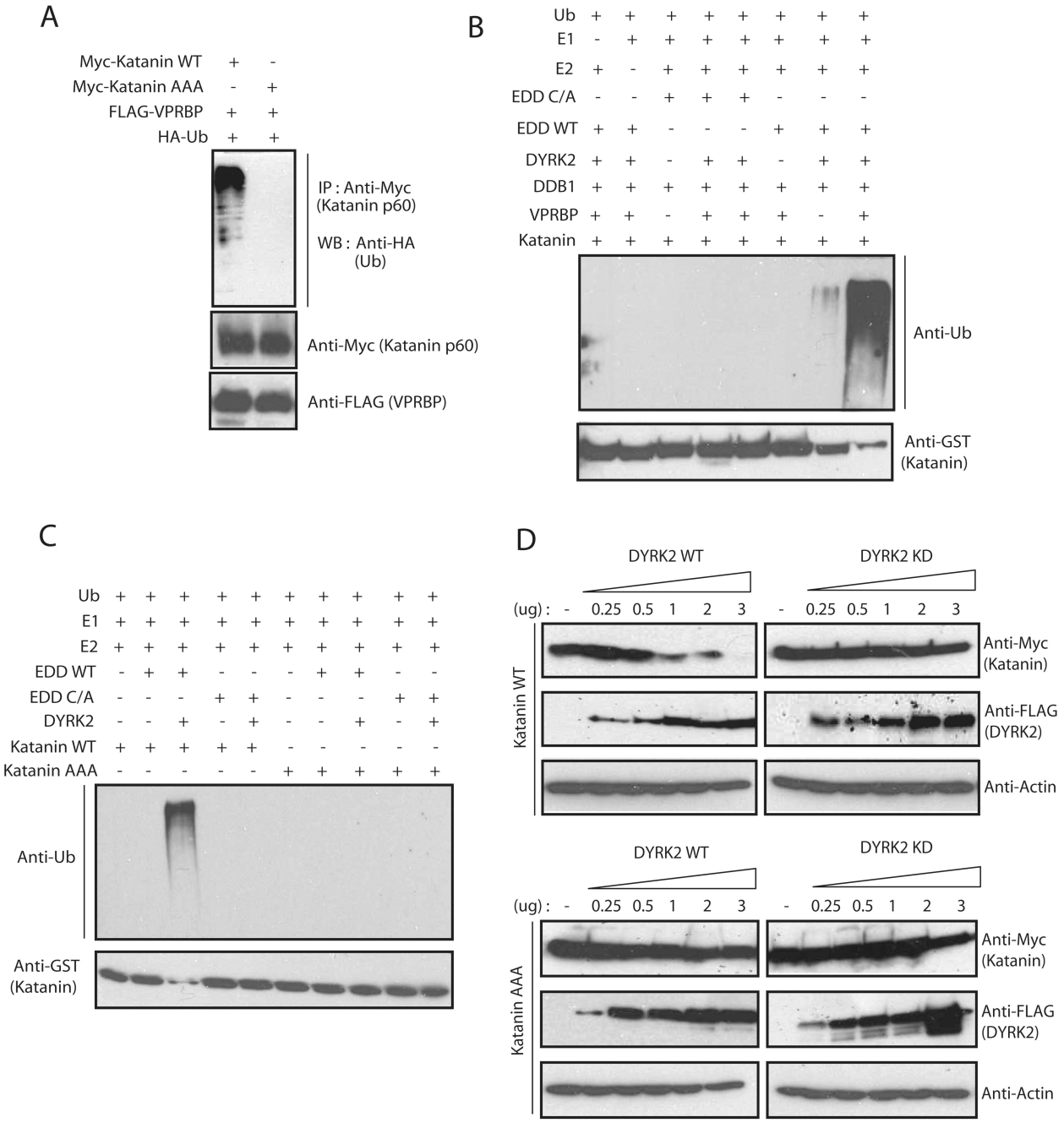

Figure 6. DYRK2 kinase activity is required for the regulation of Katanin degradation

(a) Myc-tagged wild-type or phosphomutant of Katanin was expressed in HeLa cells along with FLAG-VPRBP and HA-Ub. The levels of Katanin ubiquitination were evaluated by anti-HA immunoblotting following immunoprecipitation of Katanin from the cell extracts. **(b)** In vitro reconstitution experiments were performed using GST-Katanin as a substrate in the presence of recombinant ubiquitin, E1 (UBE1), E2 (UbcH5), MBP-tagged EDD, EDD C/A, DDB1, VPRBP and DYRK2 with various combinations as indicated. Ubiquitinated species of Katanin and GST-Katanin were detected by immunoblotting with anti-ubiquitin

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and anti-GST antibodies respectively. **(c)** In vitro reconstitution experiments were performed similar to figure 6B, using either wild type (WT) GST-Katanin or Katanin-AAA mutant as a substrate in the presence of various recombinant proteins as indicated. Ubiquitinated species of Katanin and GST-Katanin were detected by immunoblotting with anti-ubiquitin and anti-GST antibodies respectively. **(d)** The effect of DYRK2 kinase activity and Katanin phosphorylation on the regulation of Katanin protein levels was assessed by transient transfection experiments. 293T cells were transfected with the indicated expression vectors for DYRK2 and Katanin, and the protein levels were estimated by immunoblotting 24 hours post-transfection.

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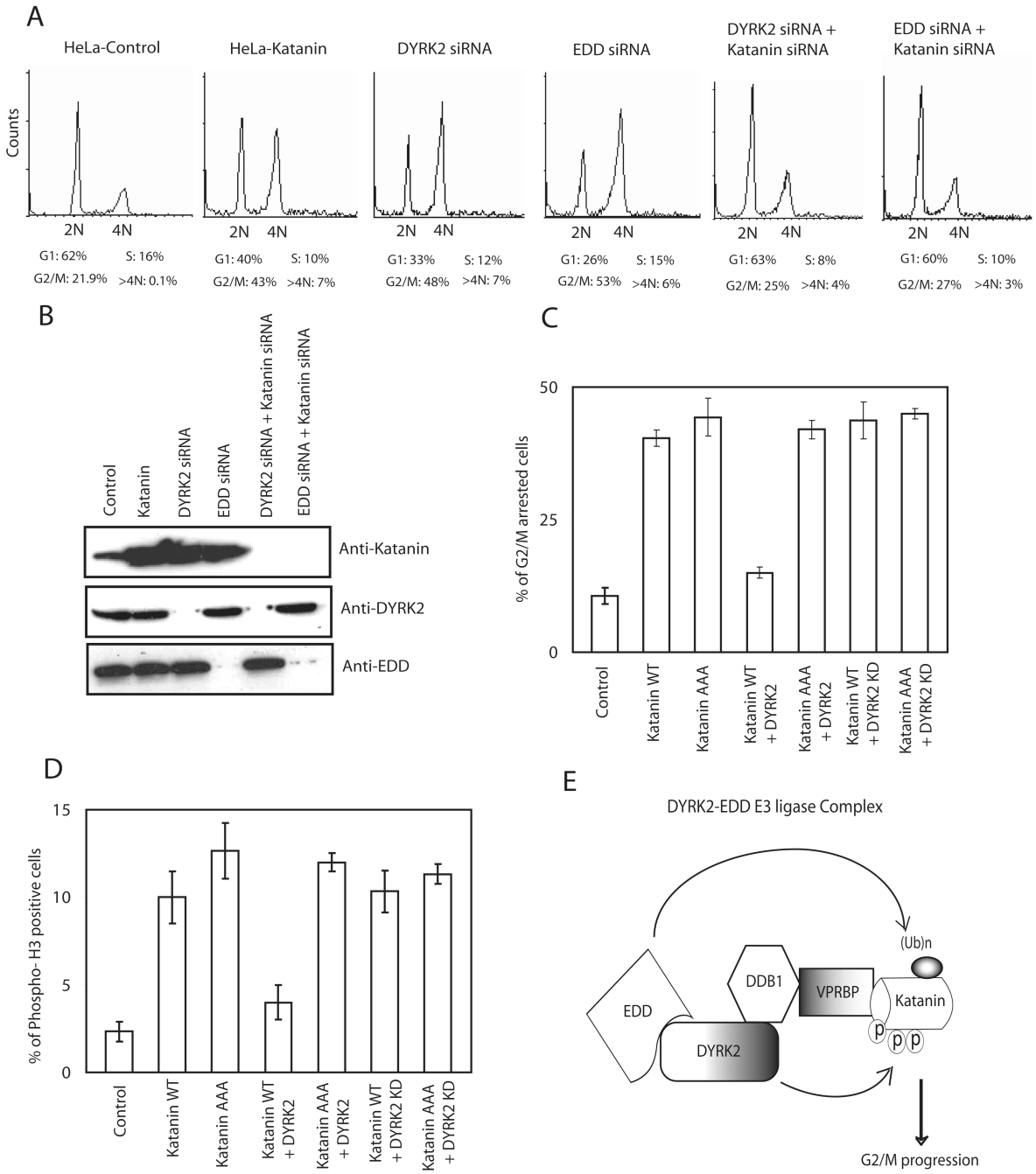

Figure 7. DYRK2 regulates mitotic progression via its adaptor and kinase function

(a) HeLa cells were transfected with plasmids encoding Katanin or with different siRNAs as indicated and the cell cycle profiles were determined by Propidium Iodide (PI) staining followed by Flow cytometric analysis. **(b)** The protein levels of Katanin, DYRK2 and EDD in HeLa cells transfected with plasmids encoding Katanin or different siRNA combinations were determined by Western blotting with the respective antibodies as indicated. **(c)** HeLa cells were transfected with plasmids encoding wild-type or phospho AAA mutant of Katanin along with plasmids encoding wild-type or kinase inactive DYRK2. The percentage of

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G2/M cells was determined by FACS analysis. Data were presented as mean ± SD from three different experiments. **(d)** The percentages of mitotic cells as measured by positive phospho-H3 staining were determined in HeLa cells transfected with indicated constructs. Data were presented as mean ± SD from three different experiments. **(e)** The model for a novel DYRK2-EDD E3 ligase complex demonstrates that DYRK2 functions both as an adaptor and a kinase and regulates G2/M cell cycle progression.