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Male germ cell-associated kinase is overexpressed in prostate cancer cells and causes mitotic defects via deregulation of APC/C-CDH1

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Abstract

Male germ cell-Associated Kinase (MAK), a direct transcriptional target of androgen receptor (AR), is a coactivator of AR. In this study, we determined the activating mechanism of MAK and identified a previously unknown AR-independent role of MAK in mitosis. We found that MAK kinase activity requires dual phosphorylation of the conserved TDY motif and that the phosphorylation is dynamic during cell cycle. MAK associates with CDH1 (FZR1, fizzy/cell division cycle 20 related 1) and phosphorylates CDH1 at sites phosphorylated by CDK. When MAK is overexpressed, the binding of CDH1 to Anaphase Promoting Complex/Cyclosome decreased, resulting in an attenuation of APC/C ubiquitin ligase activity and the consequential stabilization of the CDH1 targets such as Aurora kinase A and PLK1. As such, overexpression of MAK leads to mitotic defects such as centrosome amplification and lagging chromosomes. Our immunohistochemistry result showed that MAK is overexpressed in prostate tumor tissues, suggesting a role of MAK in prostate carcinogenesis. Taken with our previous results, our data implicate MAK in both AR activation and chromosomal instability, acting in both early and late prostate cancer (PCA) development.

Keywords

APC/C complex; CDH1/FZR1; MAK; Mitosis; Prostate Cancer

Introduction

For more than a decade in the United States, prostate cancer has been the most frequently diagnosed cancer in men and among the top three leading causes of cancer-related deaths. As a hormonally regulated malignancy, prostate cancer goes through different phases of hormone dependence. At early stage, growth and survival of the cancer cells rely heavily on androgen signaling and androgen receptor (AR). At a later stage, especially upon hormone deprivation therapy, a significant portion of prostate cancers transition into an androgen-independent or castration-resistant state, where the requirement for androgen is less (Chen *et al.*, 2004; Feldman & Feldman, 2001; Gregory *et al.*, 2001). Some castration-resistant cells derived from metastasis even lose androgen dependency altogether with no AR expression (Mendiratta *et al.*, 2009). Multiple mechanisms have been ascribed to account for the disease progression to castration-resistant and metastatic state. One mechanism that has received

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Conflict of Interest
The authors declare no conflict of interest.

less attention is chromosomal instability (CIN), although translocation, copy-number changes and gain/loss of oncogenes/tumor suppressor genes were frequently reported (Clark & Cooper, 2009; Shand & Gelmann, 2006). Mitotic defects associated with chromosomal aberrations are often observed in prostate cancer cell lines and patient tissues, and the degree of abnormalities positively correlates with tumor staging (Beheshti *et al.*, 2001; Pihan *et al.*, 2001; Tribukait, 1991).

Overexpression or mutations of proteins such as PIM-1, Aurora kinase A, PLK1 and Id1 are found in prostate cancer cells, causing defect in mitotic checkpoint, deregulation of anaphase-promoting complex/cyclosome (APC/C), or centrosome amplification which further lead to CIN and aneuploidy (Buschhorn *et al.*, 2005; Roh *et al.*, 2003; Wang *et al.*, 2008; Weichert *et al.*, 2004). The E3 ubiquitin ligase complex APC/C and CDH1 (or FZR1, fizzy/cell division cycle 20 related 1) plays a crucial role in maintaining proper levels of mitotic proteins and regulating the timely progression of cell cycle. In fact, deregulation of APC/C-CDH1 complex (APC/C^{CDH1}) is frequently seen in cancers with mitotic defects and CIN.

MAK (male germ cell-associated kinase) belongs to a protein kinase family characterized by a catalytic domain resembling a hybrid of the TXY motif found in mitogen-activated protein kinases (MAPK) and the TY motif in cyclin-dependent kinases (CDK) (Brown *et al.*, 1999; Fu *et al.*, 2005; Payne *et al.*, 1991; Xia *et al.*, 2002). Despite the hybrid signature, virtually nothing is known about the post-translational kinase-activating mechanism for MAK. We previously reported that MAK was transcriptionally activated by androgen (Xia *et al.*, 2002) and a high level of MAK transcript was found in several prostate cancer cell lines (Ma *et al.*, 2006; Robinson *et al.*, 1996). MAK, in turn, functions as a critical AR coactivator and is required for androgen-dependent cell proliferation (Ma *et al.*, 2006). These findings suggest that MAK may contribute to prostate carcinogenesis through AR signaling. However, our observation of MAK also being highly expressed in AR negative, castration-resistant cell lines (DU145 and PC3) (Xia *et al.*, 2002) suggests additional, AR-independent roles of MAK in prostate carcinogenesis.

Human MAK shares high degrees of homology to rat (90%) and mouse (89%) orthologues (Xia *et al.*, 2002), whose expression are exclusively enriched in testicular germ cells during meiosis (Koji *et al.*, 1992; Matsushime *et al.*, 1990). It is thus likely that human MAK is involved in meiosis and/or cell cycle control, potentially contributing to prostate cancer progression in an AR-independent manner.

In this study, we report that MAK is overexpressed in prostate cancer cell lines and clinical specimens. We demonstrate that dual-phosphorylation of the conserved TDY is required for MAK kinase activation, and that this phosphorylation displays a dynamic pattern during cell cycle. In addition, a novel function of MAK acting as a negative regulatory kinase of APC/C^{CDH1} is described. A new role of MAK in the context of prostate carcinogenesis will be discussed.

Results

MAK is overexpressed in prostate cancer cells

We previously reported that compared to PrEC normal prostate epithelial cell, MAK transcripts are elevated in several cancer cell lines (Xia *et al.*, 2002). In this study, we generated a highly specific antibody (Figure S1) capable of detecting endogenous MAK protein. Using this antibody, we found that similar to mRNA, as Xia *et al* previously reported (Xia *et al.*, 2002) and quantified by qRT-PCR in this study (Figure S2), high levels of MAK protein were detected in the castration-resistant cancer cell lines CWR22Rv1,

DU145 and PC3 (Figure 1A). We also found that it was localized in the nuclear fraction of the cell lysates (Figure 1B). Interestingly, MAK protein is barely detectable in androgen-sensitive LNCaP cell, even though the transcript level was shown to be increased by androgen (Figure 1A) (Xia *et al.*, 2002). Since MAK is an androgen-regulated gene, it was surprising that the AR-negative PC3 and DU145 cells overexpress MAK, suggesting an AR-independent mechanism regulating MAK expression during prostate carcinogenesis.

To study MAK expression in prostate cancer specimens, a human microarray containing prostate tissues from 15 normal, 25 hyperplasia and 26 malignant individuals was stained for MAK with our antibody (Figure 1C). The MAK expression level in each tissue core was quantified using 1) manual scoring by expert pathologists on a scale from 0 to 3, 3 indicating the highest intensity (Figure 1D upper) and 2) a computerized scanning analysis to quantify the percentage of cells with positive MAK staining (Figure 1D lower). The results were classified to 1–10%, 11–20%, 21–30% and >31% of MAK positivity. Both analyses revealed that MAK was significantly overexpressed in prostate tumors, with higher protein level within single cells and larger population of cells expressing MAK.

The subcellular localization of MAK is dynamic during cell cycle

Using the antibody, we further examined in details, the subcellular locations of MAK and found a dynamic pattern during the cell cycle. During interphase, MAK localizes uniformly in the nuclei, consistent with the results obtained from biochemical fractionation and tissue staining (Figure 1B, 1C, 2A). At metaphase and anaphase, MAK associates with the mitotic spindle and centrosomes (Figure 2B). At anaphase MAK also locates to the midbody and remained in this locale until telophase (Figure 2B bottom, 2C). This nuclear dynamic localization suggests a possible cell cycle-related role of MAK.

MAK is activated via phosphorylation of the TDY motif

Earlier studies on MRK (MAK-related kinase/ ICK; another member of the MAK kinase family) revealed that the dual-phosphorylation on the TDY motif, was carried out by its autokinase activity and by CCRK (human cell cycle related kinase) (Fu *et al.*, 2006; Fu *et al.*, 2005). This dual phosphorylation is required for MRK's full kinase activity. We therefore tested the possibility of MAK being activated in a similar manner. The conserved T157 and Y159 (of TDY) were substituted respectively with alanine and phenylalanine to yield the following mutants, ADY, TDF, ADF. Wild-type MAK strongly phosphorylated MBP *in vitro* (Figure 3B upper), and exhibited autophosphorylation on the TDY motif *in vivo* as detected by antibody that specifically recognizes the dual-phosphorylated TXY (Figure 3B lower). When the TDY motif is mutated however, the phosphorylation activity on MBP was diminished to similar level of MAK (KR), an inactive mutant carrying arginine substitution for the conserved lysine in the ATP-binding pocket (Figure 3B). We conclude that dual phosphorylation on the TDY motif is crucial for MAK activity, and that the autokinase activity is required for this phosphorylation.

This dual phosphorylation of MAK *in vivo* was enhanced by overexpression of CCRK (Figure 3C), which also physically interacted with MAK (Figure 3D). MKKs that are known to phosphorylate the TEY motif of MAPKs however, did not enhance MAK phosphorylation (data not shown). Taken together, MAK is specifically phosphorylated on the conserved TDY motif by CCRK and by autokinase activity; such phosphorylation status is indicative of its kinase activity.

Given its dynamic subcellular localization (Figure 2), we next determined whether the expression or activity of MAK is regulated along cell cycle. The endogenous MAK expression in DU145 and PC3 cells appeared to be generally constant at different stages

(data not shown), whereas the TDY-dual phosphorylation, an index of its kinase activity, oscillated during cell cycle (Figure 3E). HEK293 cells stably expressing MAK-V5H protein were synchronized at different cell cycle stages to examine the extent of MAK phosphorylation: it increased from S, peaked at G2 to early M phase, and decreased at late M phase (Figure 3E). The high level phosphorylation of MAK during G2/M suggests a role prior to the onset of anaphase, possibly during the metaphase-anaphase transition.

MAK binds to and phosphorylates CDH1

The transition between metaphase to anaphase is mediated by APC/C, whose E3 ubiquitin ligase is activated sequentially by binding to two activators: CDC20 and CDH1/FZR1. Activation of APC/C consequently triggers proteolysis of several mitotic proteins from anaphase to G1. A study in budding yeast reported that the MAK homolog Ime2 negatively regulates APC/C through Cdh1 during meiosis (Bolte *et al.*, 2002). To explore the role of MAK during cell cycle, we tested whether MAK is involved in the regulation of APC/C. As demonstrated by immunoprecipitation of ectopically expressed (Figure 4A) or endogenous (Figure 4B) MAK, CDH1 was found to associate to MAK, whereas no association between MAK and CDC20 was detected (data not shown). A reciprocal experiment of CDH1 immuoprecipitaion confirmed the MAK-CDH1 interaction (Figure S3A). The interaction domain was mapped to the WD40 domain of CDH1 (Figure S3B), a region involved in substrate binding and the subsequent ubiquitination mediated by APC/C^{CDH1} (Kraft *et al.*, 2005). Overexpression of CDH1 leads to CDH1-mediated substrate degradation, as shown by the reduced level of Aurora kinase A, a specific target of APC/C^{CDH1} (Figure 6C upper). However, the MAK level appeared to be unaffected (Figure S3C), suggesting that MAK is not a target of APC/C^{CDH1}, consistent with the data showing constant MAK level during cell cycle (Figure 3E).

We next determined whether MAK regulates CDH1 by phosphorylation. *In vitro* kinase assays showed that CDH1 is indeed phosphorylated by wild-type MAK, either immunoprecipitated from mammalian (Figure 5A) or purified from bacteria cells (Figure 5B). The MAK KR and ADF mutants however, exhibited very limited phosphorylation activity toward CDH1. Since the phosphorylation of MAK, an index of kinase activity, increased from G2 to early mitosis (Figure 3E), we tested whether MAK-mediated phosphorylation of CDH1 also displays a similar pattern during cell cycle. This turned out to be the case, based on phosphorylation of CDH1 by MAK IP from HEK293 cell (Figure 5B). To exclude the possibility that CDK is non-specifically bound in the MAK immunoprecipitated complex, contributing to the *in vitro* CDH1 phosphorylation, we blotted the IP samples for CDK1 and CDK2, and could not detect either (Figure S4). These results suggest a cell cycle-dependent phosphorylation of CDH1 by MAK.

Before entering and until the end of mitosis, the activity of APC/C^{CDH1} is mainly negatively regulated by CDK-dependent phosphorylation of CDH1 (Jaspersen *et al.*, 1999; Kramer *et al.*, 2000; Lukas *et al.*, 1999; Zachariae *et al.*, 1998). We developed two CDH1 mutants with alanine substitutions at the known CDK phosphorylation sites to determine whether MAK phosphorylates CDH1 on similar residues. Mutant CDH1(4A) lacks the four major phosphoacceptor sites of CDK (S40A, T121A, S151A, S163A) and mutant CDH1 (9A) lacks all nine acceptor sites (T32, S36, S40, S70, T121, S138, S146, S151, S163) (Jaspersen *et al.*, 1999; Kramer *et al.*, 2000; Zachariae *et al.*, 1998). Phosphorylation of CDH1(4A) by *E. Coli.*-purified MAK was significantly diminished, and only marginal phosphorylation of CDH1(9A) could be detected (Figure 5C). In addition to the bacterially expressed kinase, MAK isolated from mammalian cells also exhibited the same phosphorylation specificity toward CDK sites; only background level of phosphorylation of CDH1(9A) by the immunoprecipitated MAK could be detected (Figure 5B). Thus, MAK phosphorylates CDH1 during G2 to M phase on multiple sites overlapping with those by CDK.

Overexpression of MAK inhibits APC/C^{CDH1} activity

Upon CDK-mediated phosphorylation, CDH1 dissociates from CDC27, a subunit of APC/C, resulting in the inactivation of APC/C ubiquitin ligase and the consequent target stabilization (Kraft *et al.*, 2005). Phosphorylation of CDH1 by MAK on the CDK sites suggest a similar role of MAK as CDK. Indeed, when MAK was overexpressed, the association between CDH1 and CDC27 reduced significantly (Figure 6A, 6B), indicating that the CDH1-dependent activation of APC/C was inhibited. The APC/C E3 ligase activity was accordingly reduced, as reflected by the significantly decreased level of ubiquitinated Aurora A in MAK-overexpressing cell (Figure 6C). The ubiquitination of Aurora A in MAK(KR)-overexpressing cells was reduced to a lesser extent (Figure 6C). The inhibition of APC/C^{CDH1} thus results in increased level (Figure 6D) and stability (Figure 6E) of the target proteins Aurora kinase A and Polo-like kinase 1 (PLK1). Together, our results suggest that overexpression of MAK inhibits APC/C^{CDH1} activity *in vivo*, principally by a phosphorylation-dependent mechanism (Figure 8A).

Overexpression of MAK results in extra centrosomes and lagging chromosome

As described above, MAK is overexpressed in prostate cancer cells. This, together with the result that MAK affects APC/C activity and aberrantly stabilizes Aurora kinase A and PLK1, prompted us to further examine whether mitosis in prostate cancer cells is mis-regulated by MAK overexpression. With limited endogenous MAK expression (Figure 1A) and an intact p53/Rb pathway, LNCaP was a suitable cell line to study the role of MAK overexpression in mitosis. We generated a LNCaP cell line ectopically overexpressing MAK-V5H (LNCaP/MAK-V5H). Similar to what we observed in HEK293 and 293T cells, LNCaP/MAK-V5H cells showed stabilization of Aurora kinase A and PLK1 (Figure 7A), suggesting that MAK-mediated inhibition of APC/C^{CDH1} is not cell type specific. The mis-regulated APC/C^{CDH1} activity did not seem to affect cell cycle progression as no apparent change of overall cell proliferation rate and cell cycle distribution was observed in LNCaP/MAK-V5H cells, whether cultured in the presence or absence of androgen (data not shown).

Since it is well recognized that tight regulation of APC/C is critical for mitosis and genomic stability, the potential mitotic defects caused by MAK overexpression was examined. Compared to the cells expressing control vector (LNCaP/vec), LNCaP/MAK-V5H cell line showed a 2.3-fold increase of cells carrying extra centrosomes at metaphase (Figure 7B). Lagging chromosomes during anaphase occurred twice as frequently in LNCaP/MAK-V5H cell line compared to LNCaP/vec (Figure 7C), indicating an increased rate of chromosome mis-segregation and chromosomal instability. In light of these findings, we suggest that in addition to the activation of AR, MAK plays a role in chromosomal stability in prostate cancer cells (Figure 8B), affecting both early and late phase of carcinogenesis.

Discussion

In this study, we developed a highly specific MAK antibody, and confirmed that the expression levels of MAK are elevated in castration-resistant prostate cancer cell lines. In agreement with the cell line results, immunohistochemistry analysis shows that MAK is generally overexpressed in prostate tumor tissues. To our surprise however, MAK protein level is much lower in the androgen-sensitive LNCaP cell. Despite the low level, it is essential for LNCaP cell growth, as siRNA knockdown of MAK significantly reduced AR response and resulted in growth inhibition (Ma *et al.*, 2006). Immunostaining of endogenous MAK protein in DU145 and PC3 cells reveals a dynamic subcellular localization during cell cycle: It is localized to the mitotic spindle and centrosomes during metaphase and anaphase; and to the mitotic midbody from anaphase to telophase. Together, we speculate that while limited expression of MAK in LNCaP cell is sufficient to maintain survival and cell growth

through AR signaling, overexpression of MAK associated with castration-resistant cancer cells may contribute to malignancy via aberrant regulation of mitosis (Figure 8).

This is the first study on the post-translational regulation of MAK activity. Our results showed that, similar to human MRK and yeast Ime2, dual-phosphorylation of the conserved TDY motif is required for MAK to acquire full kinase activity. The dual-phosphorylation of MAK is mediated by autophosphorylation and partially by CCRK, which coincident with MAK, is enriched in testis (Wohlbold *et al.*, 2006). Interestingly, we found that this phosphorylation indicative of MAK activaty, oscillates during cell cycle. The phosphorylation of MAK increases between S and G2, reaches maximum at early mitosis, and drastically decreases at the end of mitosis.

We further provide evidence showing that MAK negatively regulates APC/C^{CDH1} through a phosphorylation scheme similar to CDK-dependent inactivation of CDH1. First, MAK physically interacts with CDH1, and colocalizes with CDH1 in the nucleus during G1 and at the centrosomes during mitosis (Zhou *et al.*, 2003). Second, bacterial and mammalian expressed MAK both show phosphorylation specificity toward CDH1 on a number of CDK-phosphorylation sites *in vitro*. Third, MAK mediates CDH1 phosphorylation with increasing levels from G2 to M phase, coincident with the APC/C^{CDH1} inactivation pattern. Forth, overexpression of MAK results in the following related events: a) the dissociation of CDH1 and APC/C via subunit CDC27, b) the attenuation of APC/C^{CDH1} activity on ubiquitination of Aurora kinase A, and c) stabilization of the substrates Aurora kinase A and PLK1. Our study therefore uncovers MAK as a new tissue-specific negative regulator of CDH1. Regarding the phosphorylation of CDH1, we showed that MAK was able to phosphorylate CDK sites, and this was not due to contaminating CDKs in the extracts. We however, cannot rule out the presence of other MAK-associated kinase (e.g., CCRK) may phosphorylate CDH1 at additional sites.

In mitosis, CDK is the major negative regulator of CDH1. Studies in yeast show that meiosis however, is driven by cooperation between Ime2 and CDK; they phosphorylate common substrates on similar, but also distinct residues (Holt *et al.*, 2007; Honigberg, 2004), which are acted upon by different phosphatases, allowing multiple layers of regulatory mechanisms (Holt *et al.*, 2007). The fact that CDH1(9A) with all CDK phosphorylation sites mutated still maintains a low level of phosphorylation by MAK suggests that MAK has a phosphorylation specificity distinct from CDK. Given the enriched MAK expression in testis germ cells and the molecular conservation between MAK and Ime2, we speculate that MAK may be normally involved in mammalian meiosis/spermatogenesis in cooperation with CDK. However, when it is aberrantly overexpressed in prostate epithelial cells, MAK has the capacity to perturb mitosis via CDH1.

Knockdown or inhibition of CDH1 is associated with centrosome amplification and chromosome mis-segregation, and is implicated in genomic instability and tumorigenesis (Engelbert *et al.*, 2008; Garcia-Higuera *et al.*, 2008; Ross & Cohen-Fix, 2003; Wasch & Engelbert, 2005). Cells dividing with extra centrosomes frequently end up with uneven segregation of chromosomes caused by errors on kinetochore-microtubule attachment and the subsequent lagging chromosomes (Ganem *et al.*, 2009). As predicted, we found that overexpression of MAK in LNCaP cell displayed stabilization of CDH1 substrates Aurora kinase A and PLK1, and defective mitosis including centrosome amplification and lagging chromosomes. Since overexpression of Aurora A is known to induce centrosome amplification (Zhou *et al.*, 1998), the extra centrosomes observed in MAK-overexpressing LNCaP cell is likely due to cellular accumulation of Aurora A.

Although the LNCaP cell line is derived from a lymph node metastasis, it is generally considered to be at relatively early stage of prostate cancer because of its androgen-dependent properties. LNCaP cell thus has been valuable for the study of prostate cancer progression. While the genome of LNCaP cell is hypotetraploid, the karyotype and chromosome numbers remain stable after repeated subculturing (Gibas *et al.*, 1984; Pan *et al.*, 1999). Additionally, the centrosome numbers is more stable in LNCaP cell compared to that in p53-deficient PC3 and DU145 cells (Ouyang *et al.*, 2001). For these reasons, LNCaP cell was a suitable cell line to study the effect of MAK overexpression on mitosis. It will be of interest to study whether long term MAK overexpression in LNCaP cells would ultimately induce CIN and a more aggressive tumorigenic phenotype.

Finally, we speculate that there are two mechanisms whereby MAK inactivates CDH1: the first is kinase-dependent inactivation, modeled after CDK, where phosphorylation dissociates CDH1 from APC/C; the second is through physical binding of CDH1 with MAK. The latter is reflected by the ability of MAK (KR) to inhibit CDH1-dependent ubiquitination, albeit to much less extent. There are precedents for the latter mechanism with examples provided by other CDH1 inhibitors such as Emi1, Mad2B and Id1; acting by preventing either substrate binding or substrate releasing (Pfleger *et al.*, 2001; Reimann *et al.*, 2001; Wang *et al.*, 2008). MAK physically interacts with CDH1 through the WD40 repeats, a domain known to be involved in substrate binding. It is therefore plausible that the physical interaction between MAK and CDH1 has inhibitory effect on CDH1 through substrate competition. These two mechanisms are not mutually exclusive and deserve further investigations.

In summary, this study shows that MAK is overexpressed in prostate tumors, and describes its previously unidentified role in negatively regulating APC/C^{CDH1}, leading to mitotic defects (Figure 8). We proposed that at early cancer stage, MAK serves as a coactivator of AR; at late stage, it may contribute to CIN via deregulating APC/C^{CDH1}. Given its male tissue specificity, MAK presents itself as a potential target.

Materials and methods

MAK antibody

The MAK rabbit polyclonal antibody was generated against fragment aa 290–400 in non-catalytic domain to avoid cross reactivity toward other family members. The GST-MAK(290–400) protein was purified from bacteria and submitted to Covance Inc. (Princeton, NJ) for animal immunization and serum collection. The crude sera were subjected to GST-immobilized column (Pierce Biotech) and MAK (290–400)-conjugated affinity column to purify specific-MAK antibodies.

Plasmids

The N-terminal Flag-tagged wild-type and kinase inactive MAK expression plasmids were obtained from the previous study (Ma *et al.*, 2006). Site-directed mutagenesis of MAK on the TDY motif was made by Quik-Change (Stratagene). Wild-type and mutated MAK genes were cloned into pEF6/V5-His TOPO TA expression vector (Invitrogen) to make C-terminal V5H-tagged expression plasmids. CDH1 and Aurora A were obtained from the IMAGE human cDNA library, CCRK DNA was a gift kindly provided by Dr. Robert P. Fisher (Wohlbold *et al.*, 2006). These genes were cloned into pcDNA3.1 expression vector with N-terminal HA or Flag tagged.

Cell culture and synchronization

Primary PrEC cells were purchased from Clonetics (Walkersville, MD) and cultured following the vendor's directions. Cell lines LNCaP (LNCaP-FGC), CWR22Rv1 (22Rv1), DU145, PC3, HEK293, HEK293T (293T) were purchased from ATCC, and were cultured under condition as recommended. Synchronization of the HEK293 cells at the G1/S boundary was induced by double-thymidine treatment: the culture was treated with 2mM thymidine for 16 hours, washed with PBS and released in complete medium for 9 hours, followed by second treatment of 2mM thymidinine for 15 hours.

Generation of MAK-overexpression stable cell lines

Stable HEK293/MAK-V5H cell line: the pEF6-MAK-V5H plasmid was transfected into HEK293 cells by Fugene 6 (Roche) and the stable cell line was obtained after 3 weeks of Zeocin selection. Stable LNCaP/MAK-V5H cell line: a Lentiviral vector pLenti4/TO/V5-DEST (Invitrogen) was modified to substitute the CMV promoter with EF1α for higher level of MAK transcription. Lentiviral particles containing MAK-V5H expression plasmid were generated according to standard procedure, and then used to infect LNCaP cells, followed by subsequent Zeocin selection. The mixed population of Zeocin-resistant cells was verified by Western blotting for protein expression.

Immunoprecipitation and immunoblotting

Cells were lysed with buffer: 50mM Tris-HCl pH7.5, 150mM NaCl, 0.5% Triton X-100, 10% glycerol, 1mM EDTA, plus protease inhibitors. Protein A/G pre-cleared lysates were incubated with antibodies, Flag-M2 (Sigma-Aldrich), V5 (Invitrogen), MAK, or CDC27 (Santa Cruz Biotech, Inc.), followed by conjugation of the immune complex to protein A/G sepharose beads. The immunoprecipitated proteins were analyzed by Western blotting with antibodies against MAK, V5, Flag-M2, β-actin (Sigma-Aldrich), HA (Covance), phospho-MAPK (Promega), Cyclin B1 (Santa Cruz Biotech, Inc.), phospho-histone H3 Serine 10 (Millipore), CDH1 (NeoMarkers), Aurora A (Millipore), PLK1 (Millipore) and CDC27. Nuclear and cytoplasmic fractionations were prepared using NE-PER Nuclear and Cytoplasmic extraction reagents (Pierce Biotech).

In vitro kinase assay and protein purification

The *in vitro* activity of MAK was detected on MAK immunoprecipitatnts or purified proteins. V5H-tagged wt and mutated (KR, ADY, TDF, ADF) MAK were transiently transfected into 293T cell, followed by immunoprecipitation using anti-V5. The captured immune complexes were tested for kinase activity by incubating with substrates (MBP [myelin basic protein], GST, or GST-CDH1) and 10μCi of [γ-^{32}P]ATP in reaction buffer (25mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 50mM NaCl, 1mM dithiothreitol, 100mM Na_3VO_4, 500mM NaF) at 30°C for 30 min. The reaction was terminated by SDS sample buffer at 80°C for 15 min. The same reaction condition was used to examine CDH1 phosphorylation by purified GST-MAK fusion proteins. After reaction, the proteins were visualized by SDS PAGE and coomassie blue staining. ^{32}P-ATP labeled proteins were detected and scanned by Bio-Rad Molecular Imager FX. Protein purification: pGEX plasmids containing GST, GST-CDH1 or GST-MAK were expressed in *E. coli*. The bacteria lysates were obtained using standard procedure, and then incubated with glutathione-sepharose beads. The glutathione beads bound proteins were washed and eluted in buffer containing excess free glutathione (50mM Tris-HCl pH8.0, 400mM NaCl, 1mM dithiothreito, 1% Triton X-100, 20mM glutathione).

Ubiquitination of Aurora A

Flag or Flag-Aurora A plasmid was co-transfected with HA-ubiquitin and empty or MAK-V5H plasmids into 293T cells. 24 hours after transfection, the cells were treated with 5μM of MG132 for 16 hours. The cells were lysed with buffer (as described above) containing 1ng/ml of NEM, followed by immunoprecipitation with anti-Flag M2. The immunoprecipitants were resolved by SDS PAGE, and the ubiquitinated proteins were detected by Western blotting by anti-HA antibody.

Immunofluorescence microscopy

All the cells subjected to immunofluorescence staining were seeded and cultured on coverslips for 2 days, fixed with 4% paraformaldehyde, permeabilized in 1% Triton X-100/PBS and 1% NP-40/PBS, blocked with 1% BSA/PBS, and incubated with primary and secondary antibodies using standard protocol. Primary antibodies and dilution used were as follows: rabbit anti-MAK 1:50, goat anti-RINT1 1:50 (Santa Cruz Biotech, Inc.), mouse anti-Aurora B 1:100 (abcam), mouse anti-α-Tubulin 1:100 (Sigma-Aldrich), rabbit anti-γ-Tubulin 1:100 (Sigma-Aldrich), human anti-centromere-FITC 1:100 (Antibodies Inc.). Secondary antibodies used were Alexa Fluor 488 donkey-anti-goat, 555 goat-anti-rabbit, 488 goat-anti-rabbit and 555 goat-anti-mouse (Invitrogen). DAPI (4',6-diamidino-2-phenylindole dihydrochloride) was used to label nuclei. Confocal images with a stack of 15 to 20 sections (0.215μm each) were taken by an Olympus FV1000 confocal microscope (Melville, NY), and images of the Z-sections were processed by maximal projection using ImageJ (http://rsb.info.nih.gov/ij/) to obtain the final presented images.

Immunohistochemistry

Immunohistochemistry was performed and analyzed by US Biomax, Inc. A prostate tissue microarray (PR805) consisting of 21 normal, 25 hyperplasia, and 32 malignant tumor tissues, was stained by the lab-generated MAK antibody. The tissue cores that were successfully stained were analyzed by two methods: (i) computerized scanning analysis using ScanScope and (ii) staining intensity scoring by a panel of US Biomax pathologists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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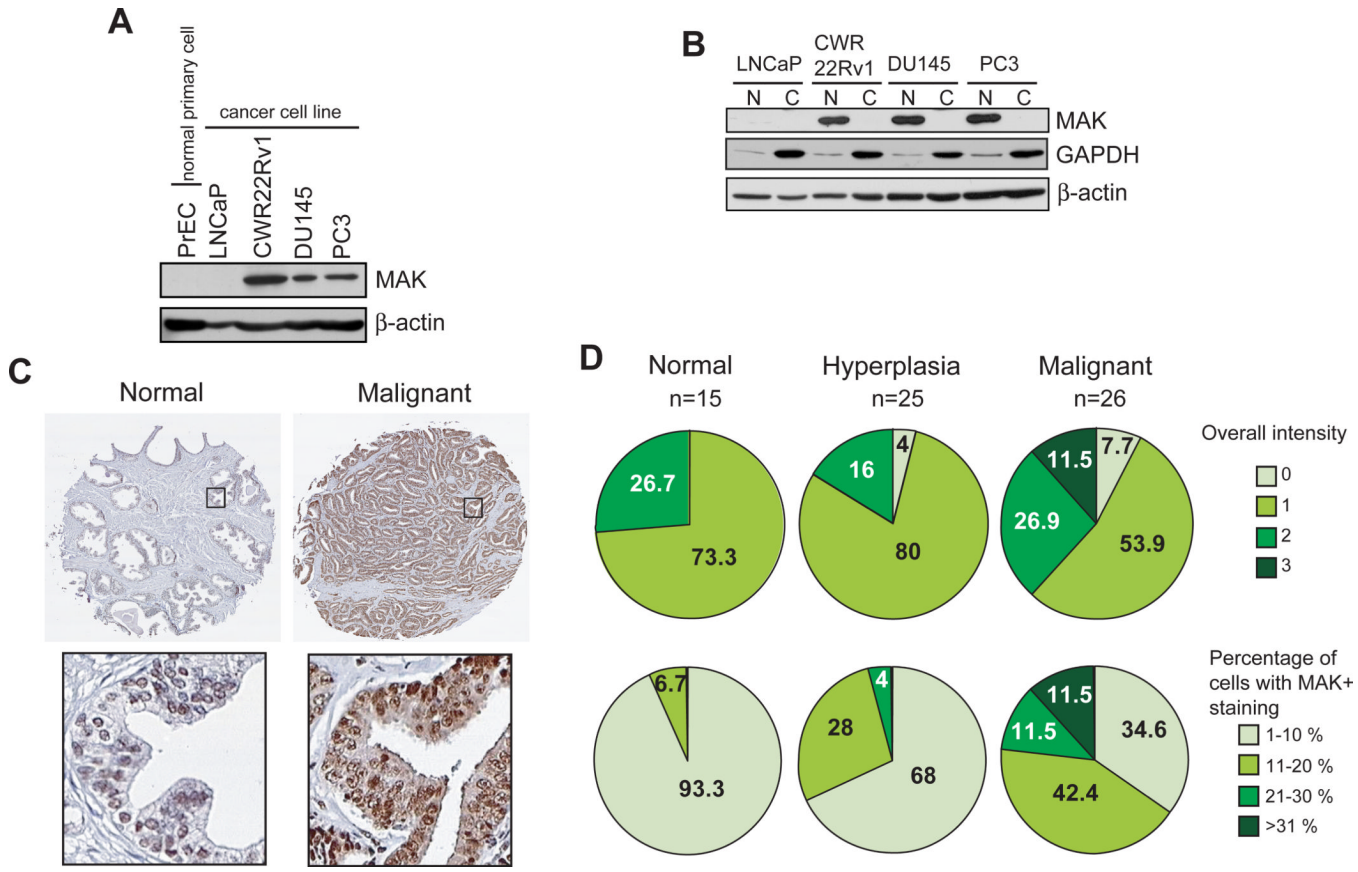

Figure 1.

MAK is overexpressed in prostate cancer cells. A) Western blotting analysis of MAK expression in normal prostate epithelial cell and cancer cell lines. B) Nulcear localization of MAK. Fractionation of cytoplasmic (C) and nuclear (N) cell lysates were subjected to Western blotting for analyzing MAK expression. C) Immunohistochemistry staining of MAK on prostate tissue microarray containing identically processed normal and malignant tissues. Representative malignant tissue showed abundant MAK staining in the nucleus and cytoplasm. D) 15 normal, 25 hyperplasia and 26 malignant prostate tissues on the microarray were analyzed for MAK staining. Upper, overall intensity of MAK was analyzed by pathologist and scored for 0 to 3, 3 indicating the highest intensity. Lower, percentage of the cells with positive MAK staining in each tissue core was analyzed by computerized scanning and quantification. The MAK positivity was classified into 1–10%, 11–20%, 21–30% and >31%. Distribution of the intensity scores and the positive percentages are shown by the pie graphs.

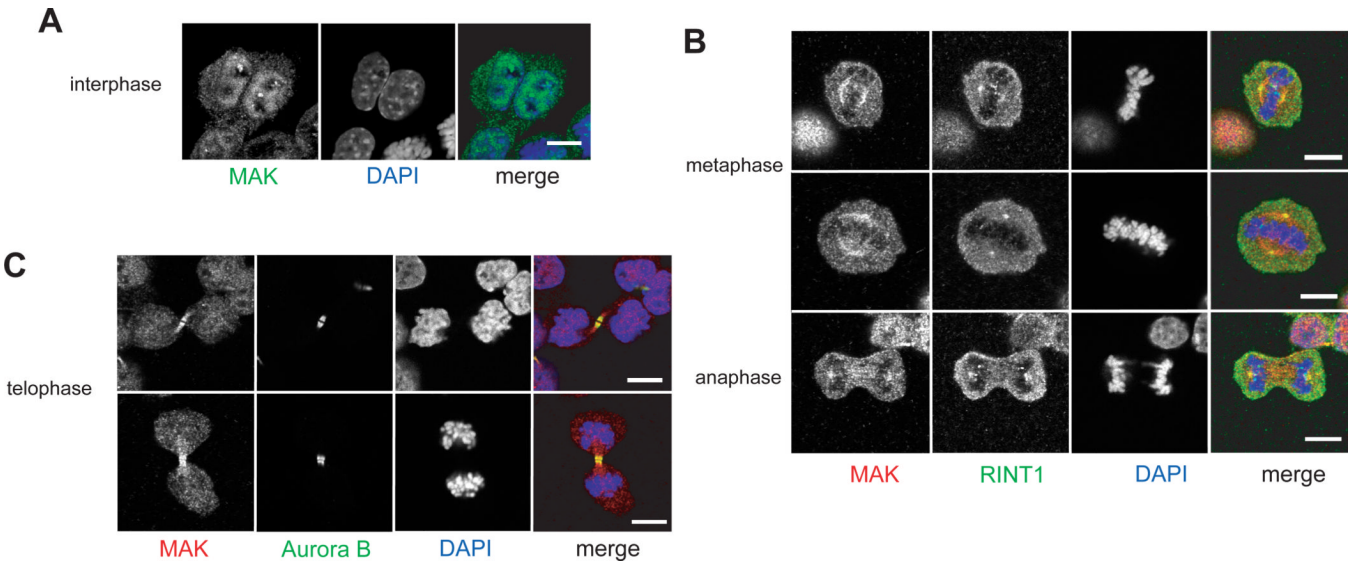

Figure 2.

Subcellular localization of the endogenous MAK protein in DU145 cells. A) Interphase cells showed nuclear localization of MAK immunostained with anti-MAK antibody (green). B) Immunofluorescent staining of MAK protein (red) and centrosomes using anti-RINT1 antibody (green). Upper and middle, metaphase cell; lower, anaphase cell. C) Telophase cells were immunostained for MAK (red) and Aurora B (anti-Aurora B antibody, green) labeling the midbody of dividing cells. Scale bar: 10μm.

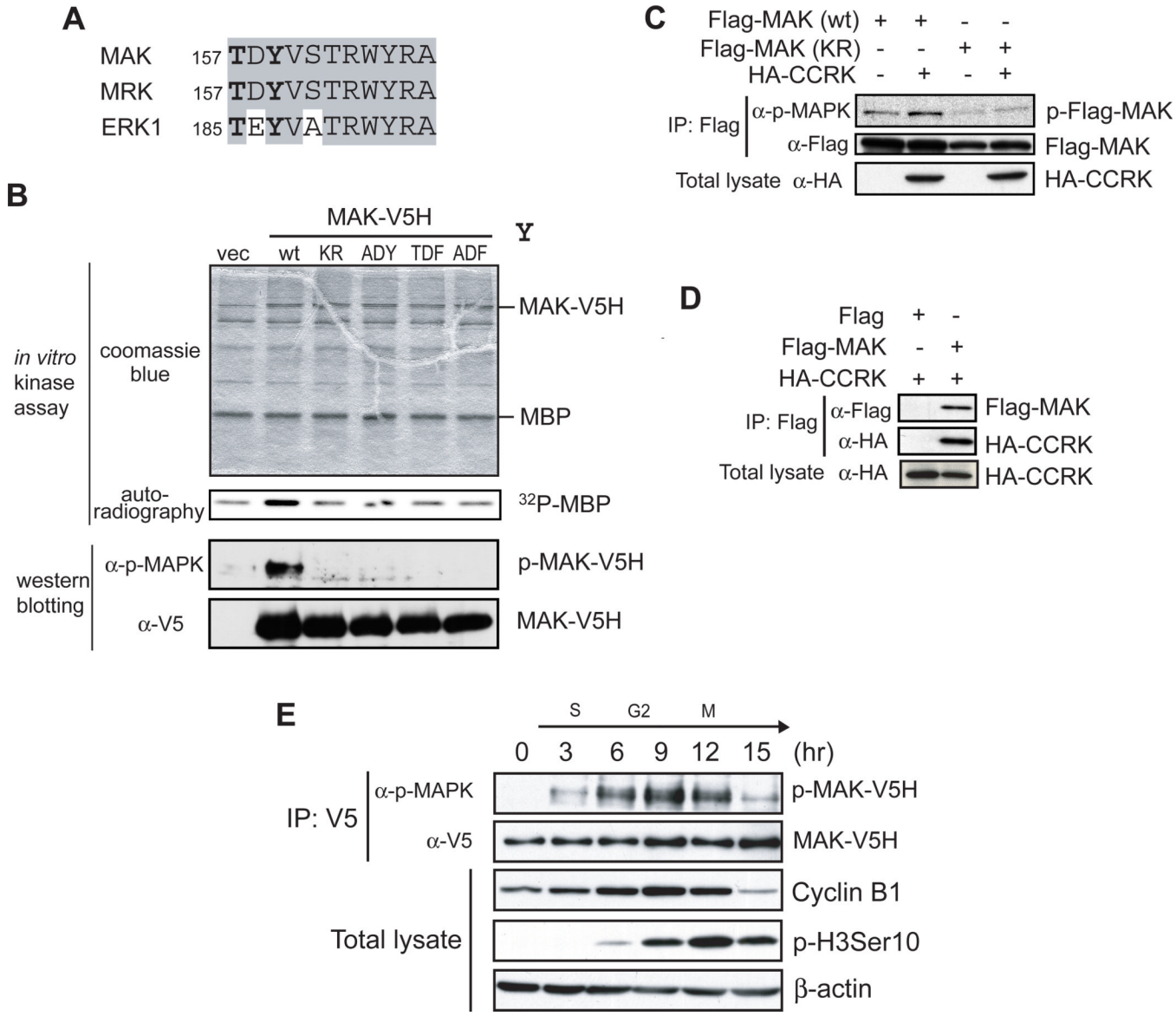

Figure 3.

Phosphorylation and activation of MAK. A) Sequence alignment of the conserved TXY motif in kinase domains of MAK, MRK and ERK. B) Phosphorylation of the TDY motif is required for MAK kinase activity. wt: wild-type; KR: kinase inactive mutant; ADY, TDF, ADF: TDY mutants. Empty vector or the variant V5H-tagged MAK constructs were expressed in 293T cells and immunoprecipitated with anti-V5 antibody, followed by *in vitro* kinase assay using MBP as the substrate. The immunoprecipitants were also subjected to Western blotting to examine phosphorylation of the TDY motif. C) CCRK enhances the dual phosphorylation of MAK. Wild-type or KR MAK were co-expressed with HA vector or HA-CCRK in 293T cells. Phosphorylation of the TDY motif was detected on MAK immunoprecipitants by anti-phospho-MAPK antibody. D) Co-immunoprecipitation of MAK and CCRK. HA-tagged CCRK was co-expressed with Flag vector or Flag-MAK in 293T cell, followed by immunoprecipitation of MAK using anti-Flag antibody. E) Dynamic MAK phosphorylation during the cell cycle. HEK293 cells stably expressing MAK-V5H were synchronized at G1/S transition by double-thymidine block and released in complete

medium at time 0. After release, cells were harvested at the indicated time points, and the cell lysates were analyzed by Western blotting. Phosphorylation and total level of MAK was detected by anti-phospho-MAPK and anti-V5 antibodies, respectively. Cyclin-B1 and phospho-histone H3Ser10 indicate the progression of cell cycle.

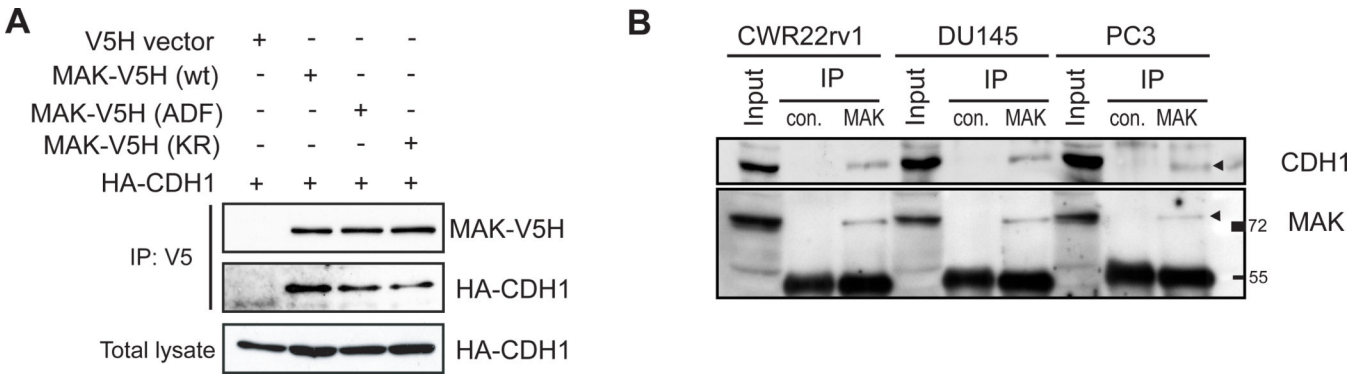

Figure 4.

MAK interacts with CDH1. A) Association of ectopically-expressed MAK and CDH1. 293T cells were co-transfected with HA-CDH1 and empty V5H or MAK-V5H plasmids, followed by immunoprecipitation of MAK with anti-V5. The co-precipitated CDH1 was detected with anti-HA antibody. B) Association of MAK and CDH1 protein at endogenous levels. Lysates of CWR22Rv1, DU145 and PC3 cells were used for MAK immunoprecipitation. Rabbit IgG was used as the negative control (con.) of immunoprecipitation. The co-precipitated CDH1 was detected by anti-CDH1 antibody.

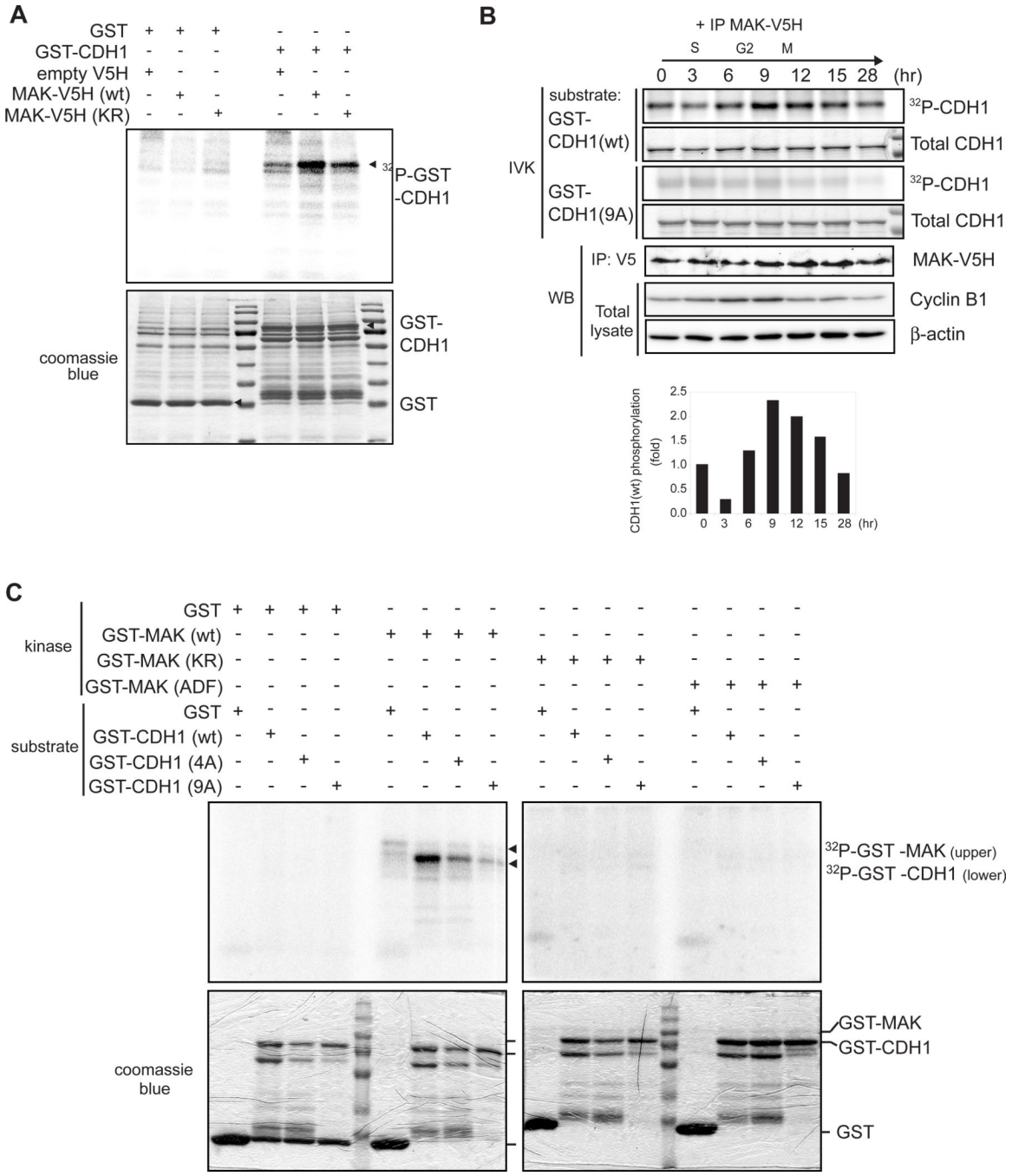

Figure 5.

In vitro phosphorylation of CDH1 by MAK. GST and GST-CDH1 fusion proteins used in this assay were expressed and purified from *E. Coli.*. A) Phosphorylation of GST-CDH1 by MAK immunoprecipitated from 293T cells using V5 antibody. ^{32}P-ATP was specifically incorporated to CDH1 but not GST. B) Cell cycle-dependent CDH1 phosphorylation by MAK. HEK293 cells expressing MAK-V5H were arrested at G1/S (time 0) for synchronization. Cells at indicated time points after releasing were harvested for MAK IP, followed by *in vitro* kinase assay (IVK) using GST-CDH1 (wt or 9A) as substrate. Western blot (WB) of immunoprecipitated MAK shows equal amount of MAK used for each IVK reaction. Cyclin B1 expression serve as a marker for different phases of cell cycle. Intensity

of the phosphorylated CDH1 (wt) was quantified by Quantity One. The value of each time point, after subtracting background signal, was divided by that of time 0, and the folds increase during cell cycle is shown in the bargraph. C) Phosphorylation of CDH1 by *E.Coli* expressed and purified GST-MAK. Purified wild-type, KR or ADF MAK was reacted with CDH1 protein wt, 4A or 9A to determine the phosphorylation specificity. Arrow heads indicate ^{32}P-labeled proteins, coomassie staining shows equal protein loaded. experimental repeats n= 2.

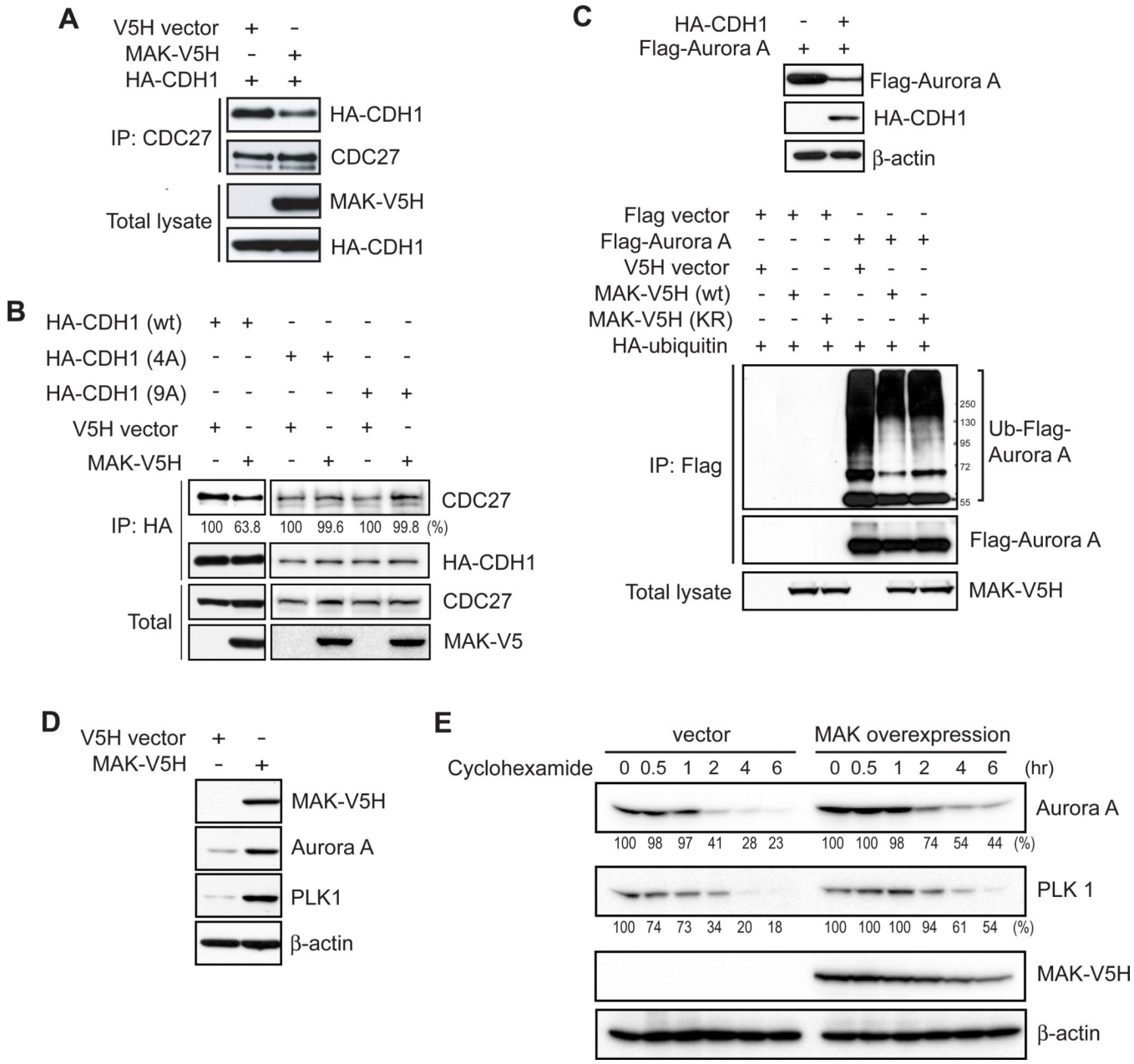

Figure 6.

Overexpression of MAK inhibits APC/C^{CDH1} activity. A, B) MAK-overexpression inhibits binding between CDH1 and CDC27. 293T cells were co-transfected with control vector or MAK-V5H with HA-CDH1 (wt, 4A or 9A). A) Endogenous CDC27 was immunoprecipitated and the co-immunoprecipitated CDH1 was detected by anti-HA. B) Reciprocal binding assay by IP CDH1 with anti-HA and detecting CDC27 in the immunoprecipitants. MAK-induced dissociation of CDH1 from CDC27 depends on the 9 phosphorylation sites. Numbers indicate percentage of the CDC27 band intensity. The CDH1-bound CDC27 in V5H control cells were set as 100%. C) Upper panel, Flag-Aurora A was co-transfected with empty HA or HA-CDH1 into 293T cells. Aurora A protein level was detected by Western blot. Lower panel, attenuated ubiquitination activity of APC/C^{CDH1} on Aurora A. Flag vector or Flag-Aurora A was co-expressed with V5H vector,

MAK-V5H wt or KR, as well as HA-Ubiquitin in 293T cells. The specific ubiquitination of Aurora A was detected in the Flag-immunoprecipitants by Western blotting with anti-HA. D) Higher protein level of Aurora A and PLK1 in MAK-overexpressing cell. The protein levels in 293T cells expressing V5H vector or MAK-V5H were analyzed by Western blot with specific antibodies. E) HEK293 cells stably expressing V5H vector or MAK-V5H were treated with 100nM nocodazole for G2/M-phased cell enrichment, followed by cyclohexamide treatment for the indicated time points. The protein levels were quantified by AlphaView SA (Cell Biosciences, Inc), and the quantified values were normalized against β-actin values. Compared to cyclohexamide-untreated sample (time 0), the relative levels of Aurora A and PLK1 at each time points were indicated by percentage. Same result was observed in at least 3 independent experiments. (n>3)

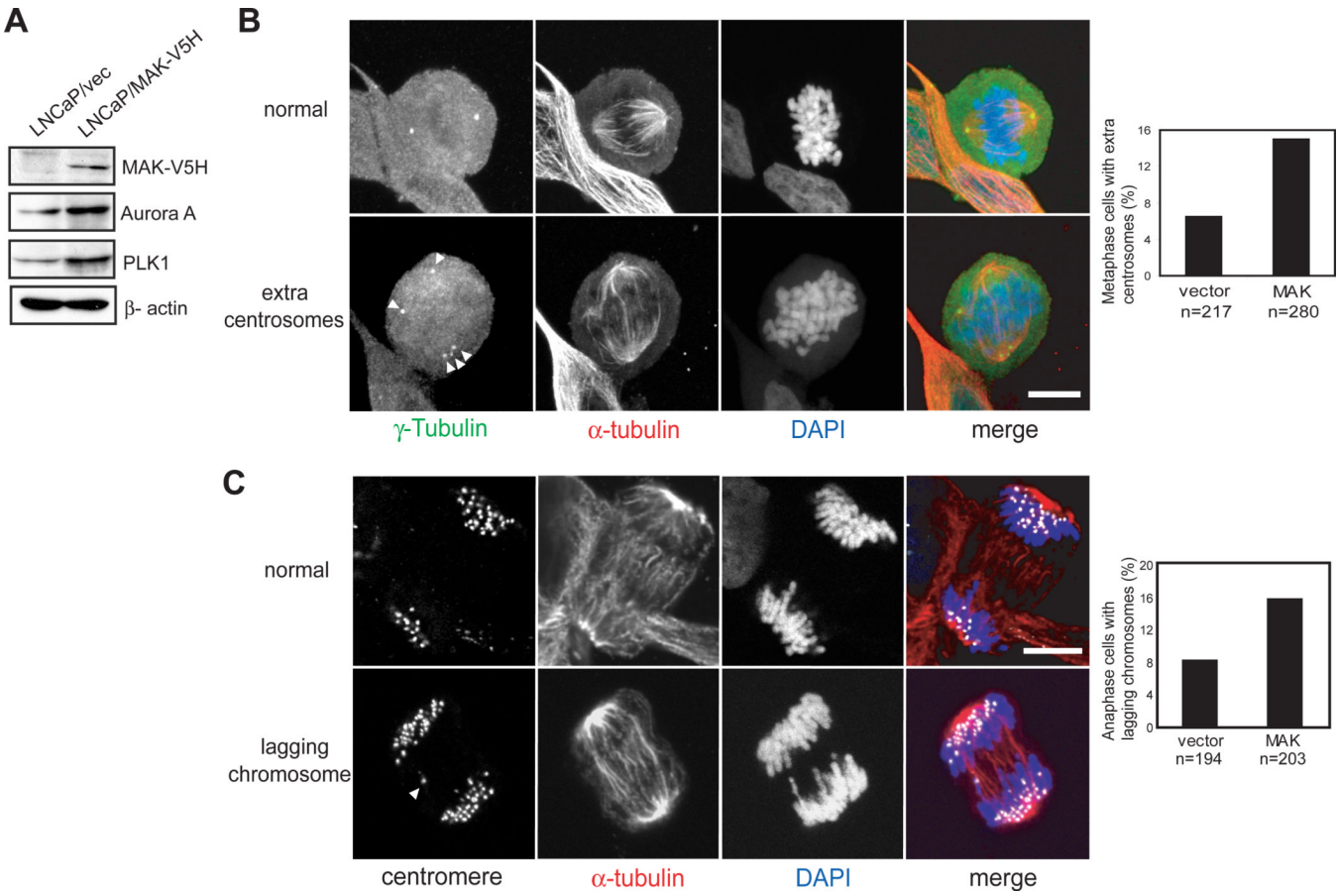

Figure 7.

Overexpression of MAK promotes centrosome amplification and lagging chromosome. A) Protein levels of Aurora kinase A, PLK1 and MAK in LNCaP cells stably expressing empty vector (LNCaP/vec) or MAK-V5H protein (LNCaP/MAK-V5H) were examined by Western blotting. B) Metaphase stage LNCaP/vec and LNCaP/MAK cells were stained for centrosomes (green), microtubules (red) and chromosomes (blue). MAK-expressing cells were more likely than LNCaP-vec cells to have extra (ie., >2) centrosomes (lower; arrowheads). Percentages of the cells containing extra centrosomes are shown in the bar graph. C) LNCaP/vec and LNCaP/MAK-V5H cells were stained for centromere (white), microtubules (red) and chromosomes (blue). Anaphase cells were analyzed for lagging chromosomes with distinct centromeres between the major two centromere-chromosome clusters at each pole (lower; arrowhead). The percentages of the cells with lagging chromosomes are shown in the bar graph. Scale bar: 10μm.

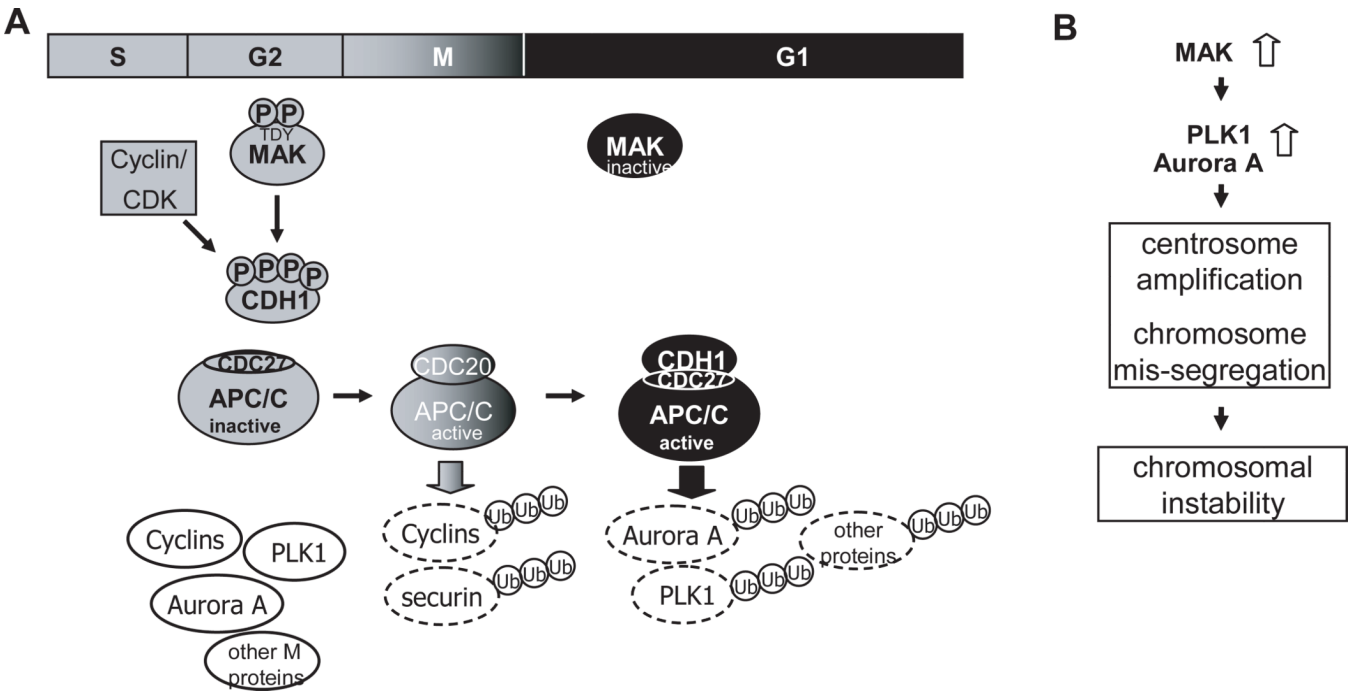

Figure 8.

The working model of MAK-mediated CDH1 regulation during cell cycle and its potential oncogenic role. A) During S to early M phase, MAK is activated by phosphorylation on the TDY motif. Active MAK phosphorylates CDH1 on CDK-phosphorylation sites, inhibiting association of CDH1 and CDC27 required for APC/C activation. At late mitosis, the dual-phosphorylation of MAK is decreased allowing APC/C activation by CDH1. Activated APC/C promotes disruption of cell cycle proteins for mitotic exit and stable G1 phase maintenance. B) Overexpression of MAK inhibits CDH1 and APC/C activation, and results in stabilization of Aurora kinase A and PLK1, which further induces extra centrosomes, chromosome mis-segregation and possible chromosomal instability in prostate cancer cells.