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Plk1 phosphorylation of Numb leads to impaired DNA damage response

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Abstract

Although Numb is well-recognized as a cell-fate determinant in stem/progenitor cells, accumulating evidence supports that Numb also has a critical role in adult tissues and cancers, in particular, in the context of regulation of tumor suppressor p53. Herein, we identified Numb as a novel substrate of Polo-like kinase 1 (Plk1). Of significance, we showed that Plk1-mediated phosphorylation of Numb leads to its enhanced proteasomal degradation and impaired Numb/p53 pathway, thus providing another mechanism how Plk1 antagonizes p53 during DNA damage response. In addition, the novel phosphorylation event identified by us further supports the notion that post-translational modifications of Numb uncouple Numb from p53 and lead to p53 destabilization. Finally, our data generated from both human cancer cell lines and mouse xenograft model showed that cancer cells carrying the unphosphorylated form of Numb by Plk1 are more sensitive to doxorubicin, a classical chemotherapeutic drug. Therefore, our work may provide future strategies for improving the efficacy of chemotherapy by targeting Numb phosphorylation by Plk1.

INTRODUCTION

Numb is a membrane-associated protein that was first identified to exert cell-fate determining functions in *Drosophila*.^{1,2} Numb exists in 4 isoforms in mammals, namely p65, p66, p70 and p71 based on their molecular weights. The different Numb isoforms result from the presence of two particular sequence inserts within the phosphotyrosine-binding domain and the central proline-rich region.³ Numb is well recognized as the Notch pathway inhibitor as it influences cell fate mainly through inhibition of the Notch pathway.⁴ Although the exact mechanism is still not clear, it was shown that Numb can promote the degradation of Notch 1 intracellular domain and Notch receptor, both of which are indispensable for Notch activation.⁵ Nevertheless, besides regulating developmental pathways like the Notch pathway, increasing evidence suggests that Numb has additional cellular functions. For

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CONFLICT OF INTEREST

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instance, Numb is an important p53 regulator by forming a tripartite complex with tumor suppressor p53 and its E3 ligase, Mdm2, thus inhibiting the degradation of p53 and promoting its tumor suppressive functions.⁶ Many other functions of Numb, such as muscle stem cell proliferation and regeneration,⁷ endocytosis and ubiquitination, have also been reported.⁸ Recently, it was shown that post-translational modifications, including phosphorylation and methylation, can affect Numb functions like asymmetric distribution as well as tumor suppressive roles based on its regulation on p53.^{9–11} p53, a well-studied transcription factor, is crucial for mediating cell cycle arrest and apoptosis in response to DNA damage.¹² Further, it has been documented that disruption of the p53 pathway has always been associated with therapeutic resistance and poor clinical outcomes.¹³ Of note, cancer cells without Numb are less sensitive to chemotherapeutic drugs than cells expressing a functional Numb.⁶ Thus, understanding how Numb is regulated will provide us novel opportunities for improving the efficacy of existing chemotherapy.

Polo-like kinase 1 (Plk1) is an essential serine/threonine kinase involved in many mitotic events, such as mitotic entry, bipolar spindle formation and sister chromatid segregation. Plk1 is overexpressed in many types of human tumor cells, and Plk1 inhibitors have been pre-clinically regarded as potential drugs for cancer treatment.¹⁴ Remarkably, Plk1 can directly interact with p53 and suppress its function.¹⁵ Mechanistically, previous studies of our laboratory have indicated that Plk1 can indirectly inhibit the p53 pathway by phosphorylating different p53-interacting proteins. Specifically, Plk1 promotes p53 degradation and the G2 DNA damage checkpoint recovery through inactivating p53.^{16,17} However, how Plk1 functions in the p53 pathway regulation still remain largely unexplored. Importantly, previous studies indicate a potential link between Plk1 and Numb. First of all, polo kinase was shown to directly phosphorylate Numb adaptor protein Pon, therefore regulating localization and function of Numb in flies.¹⁸ Consequently, this finding links one key cell cycle regulator to the asymmetric protein localization system. In addition, Numb can directly bind to Plk1 and ensure the proper function of Plk1 during mitosis.¹⁹ Nevertheless, whether and how these two proteins regulate each other remains largely unknown. As there is a direct interaction between Plk1 and Numb, and there is a reverse correlation between these two proteins in regulating p53 functions, we hypothesized that Plk1 may directly phosphorylate Numb and mediate its function on the p53 pathway.

Herein, we provide evidence to show that Numb is phosphorylated by Plk1 at serine 265 and 284 (amino acids sequence of isoform 4, p65) and that the phosphorylation event is conserved among different species and mammalian Numb isoforms. In addition, we demonstrated that Plk1-mediated phosphorylation of Numb results in enhanced Numb protein degradation and impaired Numb/p53 pathway, thus leading to diminished cellular response to DNA damage. Moreover, our cell line-based and mouse xenograft assays indicated that targeting Numb phosphorylation by Plk1 might be a promising strategy to increase the efficacy of chemotherapy.

RESULTS

Plk1 and Numb bind to and regulate each other

First of all, we focused on the interaction between Plk1 and Numb in mammalian cells. Consistent with the previous study in melanoma cells, we showed that both endogenous and over-expressed Plk1 can bind to Numb in HEK293T cells (Figures 1a and b). Remarkably, when Numb was depleted in HEK293T and HeLa cells, Plk1 protein level was significantly increased compared with wild-type (WT) cells (Figure 1c). This was likely induced by G2/M arrest as Plk1 is highly expressed in mitosis and obvious cell cycle changes were detected by using both immunoblotting against the mitotic marker Cyclin B1 and flow cytometry to follow cell cycle distribution (Figures 1c and d). On the other hand, experiments were designed to examine whether Plk1 status can affect Numb protein level as well. As shown in Figure 1e, overexpression of Plk1 using lentivirus significantly decreased Numb protein level and a similar result was observed using inducible Plk1 knock-in (KI) mouse embryonic fibroblasts (MEFs) (Figure 1f). Moreover, cells treated with a Plk1-specific kinase inhibitor BI2536 showed increased expression of Numb, with the level of p-Rictor (S1162) served as a control for Plk1 kinase activity (Figure 1g).²⁰ Overall, the data above showed that Plk1 and Numb bind to and regulate each other in mammalian cell lines.

Plk1 phosphorylates Numb at Serine 265 and 284 *in vitro*

Based on the previous report on the potential interaction between Plk1 and Numb as well as the data generated in Figure 1, we proposed that Numb is a direct substrate of Plk1. To test this hypothesis, we cloned different regions of Numb into a GST-vector and incubated purified glutathione *S*-transferase-tagged Numb fragments with purified Plk1 to conduct the *in vitro* kinase assay. As shown in Figure 2a, the fragment containing aa 199–300 of Numb (p65) was phosphorylated by Plk1. In order to map the individual sites for the phosphorylation, we further narrowed down the possible regions by testing all the constructs that express different parts of Numb and finally found that aa 241–300 was responsible for the phosphorylation event (Figure 2b). Next, we generated a series of point mutations to mutate all the serine/threonine within this region into alanine and repeated the kinase assays. To our surprise, compared with WT Numb, none of the mutants showed a convincing reduction of the ³²P signal (Figure 2c). Thus, we believed that there are multiple sites of Numb can be phosphorylated by Plk1 within this region. To figure out that, we generated different double mutations and found that the protein with serine 265 and Serine 284 to alanine mutations almost completely abolished the ³²p signal (Figure 2d). To confirm this, we cut the region into two parts and each part contains a predicted serine residue. Consistent with Figure 2d, single mutation within the shortened fragment showed significant reduction of the ³²P incorporation (Figure 2e). Importantly, we examined the protein sequences of Numb and found that these two mapped serine residues are conserved among different species and isoforms (Figure 2f). In sum, we demonstrated that Numb is phosphorylated by Plk1 *in vitro* at serine 265 and 284.

Plk1 phosphorylates Numb at Serine 265 *in vivo*

Next, we sought to validate the phosphorylation event *in vivo* by using the phospho-specific antibodies. We obtained the pS265-Numb antibody but failed to generate a functional

antibody that specifically recognizes phosphorylated S284 of Numb. Accordingly, we tested the efficacy of pS265-Numb antibody by conducting the cold-ATP based *in vitro* kinase assay. As indicated, the pS265-Numb signal was only detected when WT Numb, but not Numb-S265A, was incubated with Plk1, indicating that the pS265-Numb antibody is specific to recognize phosphorylated protein (Figure 3a). To investigate the role of Plk1 in regulating Numb phosphorylation level in cells, we performed the following experiments. First, HEK293T cells were arrested at different stages of the cell cycle, followed by immunoblotting against the pS265-Numb. As shown in Figure 3b, the pS265-Numb signal was hardly detectable in G1 phase but peaked during mitosis. More importantly, addition of Plk1 inhibitor BI2536 partially decreased the pS265-Numb level. A similar result was observed in Panc-1 cells, which have a relatively high level of endogenous Plk1 (Figure 3c). Moreover, when endogenous Plk1 was depleted with the lentivirus-based shRNA approach, the pS265-Numb level was also significantly decreased (Figure 3d). Consistent with the data above, overexpression of constitutively active form of Plk1 (T210D) increased the pS265-Numb level in HEK293T cells co-transfected with WT Numb but not Numb-S265A/S284A (2A) mutant (Figure 3e). In addition, we also detected obvious co-localization between Plk1 and pS265-Numb using immunofluorescence staining (Figure 3f). Thus, using a phospho-specific antibody, we were able to confirm the identified phosphorylation event *in vivo*.

Plk1 promotes Numb degradation in a phosphorylation-dependent manner

As we detected a marked decrease of Numb protein level upon overexpressing Plk1 in Figure 1, we predicted that Plk1 likely promotes Numb proteasomal degradation. To investigate that, we performed a series of typical protein degradation assays. Consistent with our previous data, overexpression of constitutively active Plk1 markedly decreased the protein levels of both endogenous and overexpressed Numb. However, when we co-treated the cells with proteasome inhibitor MG132, Numb protein levels were at least partially restored, indicating that the decreased level of Numb induced by Plk1 overexpression is proteasome-dependent (Figure 4a). To further validate this observation, we detected the poly-ubiquitination level of FLAG-tagged Numb upon modifying Plk1 status. As shown in Figure 4b, constitutively active Plk1 overexpression significantly increased the poly-ubiquitination level of Numb but the phenotype was rescued when we co-treated the cells with Plk1 inhibitor BI2536. Moreover, we also followed protein degradation in a time-dependent manner to support the data above. Compared with vehicle control, constitutively active Plk1 overexpression markedly facilitated the protein degradation of Numb (Figure 4c). Of note, when comparing the non-CHX treated groups (Figure 4c, lane 1 and lane 6), we were not able to observe a dramatic difference between endogenous Numb levels. This is probably due to the lower transfection efficiency and shorter treatment time compared to the experiments performed in Figure 1e. In addition, consistent with the data in Figure 4b, Plk1 kinase activity is required for Numb degradation as the kinase dead mutant of Plk1 (K82M) failed to induce obvious decrease of Numb protein level (Figure 4d). Next, we moved one step further to determine whether Plk1 promotes Numb decay in a phosphorylation-dependent manner. To achieve that, the unphosphorylatable (two serines were mutated to alanines, S265A/S284A (AA) mutant) and phosphomimic (two serines were mutated to aspartic acids, S265D/S284D (DD) mutant) mutants of Numb by Plk1 were generated to conduct the following experiments. First, we co-transfected the cells with FLAG-Numb

constructs (WT or AA) with Plk1-T210D. As indicated, Plk1-T210D overexpression reduced the level of WT Numb but not the unphosphorylatable form of Numb (Figure 4e). Consistent with that observation, the poly-ubiquitination level of Numb-AA mutant was much lower than that of WT Numb, whereas the Numb-DD mutant showed a significantly higher level of ubiquitination than that of WT Numb, indicating that phosphorylation of Numb by Plk1 at these two serine sites promotes the poly-ubiquitination of Numb and subsequent proteasome-mediated protein degradation (Figure 4f). Moreover, as shown in Figure 4g, we found that the AA form of Numb was much more stable than WT and DD form of Numb.

Plk1 negatively regulates the Numb/p53 pathway

Because Numb was demonstrated to be a crucial adaptor protein to ensure p53 stability and proper cellular response to DNA damage, and because Plk1 is involved in p53 inactivation and checkpoint recovery, we investigated whether Plk1 can affect the p53 and DNA damage response pathway via negatively regulating Numb. As expected, compared with WT MEFs, Plk1-overexpressing MEFs showed a reduced response to a traditional chemotherapeutic drug, doxorubicin, indicated by reduced levels of p53, p21 and -H2AX (Figure 5a). Consistent with this observation, we found that p53 transcriptional activity was suppressed after overexpression of constitutively active form of Plk1 (Figure 5b). More importantly, cells expressing unphosphorylated form of Numb by Plk1 showed a significantly higher level of p53 reporter activity than that of cells expressing WT Numb, whereas cells expressing phosphomimic form of Numb by Plk1 showed a diminished level of p53 transcriptional activity (Figure 5b). Because previous studies have indicated that post-translational modifications of Numb can uncouple Numb from p53 and affect the regulatory role of Numb on p53, we investigated whether Plk1-dependent phosphorylation of Numb will affect its association with p53. As shown in Figure 5c, the binding between AA mutant of Numb and p53 was much stronger than the binding between WT Numb and p53, whereas p53 was hardly detectable in the immunoprecipitate against DD form of Numb. To further assess whether the Numb/p53 pathway is affected by Plk1, we designed experiments to determine the stability of p53 upon modifying Numb phosphorylation status by Plk1 as the major role of Numb on p53 is to promote its stability. Accordingly, we confirmed the previous reported phenotype that Numb overexpression was able to stabilize p53 in the protein degradation assay (Figure 5d). More importantly, compared with cells expressing WT Numb, p53 degradation rate was reduced in cells expressing AA form of Numb, whereas cells expressing phosphomimic form of Numb by Plk1 showed a diminished level of p53 and a faster degradation rate (Figure 5d). Furthermore, compared with cells expressing WT Numb, cells expressing Numb-DD had a much higher poly-ubiquitination level of p53 (Figure 5e). Taken together, the data above suggest that Plk1 negatively regulates the Numb/p53 pathway.

Cells expressing unphosphorylated form of Numb by Plk1 are more sensitive to doxorubicin

Because the Numb/p53 pathway is crucial for cellular response to DNA damage, we sought to determine whether Plk1 phosphorylation of Numb affects the efficacy of doxorubicin. As shown in Figures 6a and b, cells expressing the AA mutant of Numb were more sensitive

to doxorubicin treatment than cells expressing WT Numb, indicated by both p53 levels and surviving cell numbers. On the other hand, cells expressing the DD mutant of Numb showed impaired response to doxorubicin. In addition, we were able to observe similar results in another p53 WT cell line, U2OS (Figures 6c and d). Remarkably, after doxorubicin treatment, cells expressing the AA form of Numb had more apoptotic and dead cells compared with cells expressing WT and the DD form of Numb, indicated by the Annexin V/PI staining (Figure 6e). Consistently, cells expressing Numb-AA almost completely lost their ability to form colonies after pre-treatment with doxorubicin (Figure 6f). Of note, without doxorubicin treatment, we were not able to observe dramatic differences in cell growth and death among different groups, suggesting that Plk1 phosphorylation of Numb at these two sites does not seem to significantly affect other cell surviving pathways.

Tumors carrying unphosphorylated form of Numb by Plk1 are more sensitive to chemotherapy

To better examine the role of Plk1 phosphorylation of Numb in the efficiency of chemotherapy, we conducted a xenograft study using U2OS cells stably expressing different forms of Numb. Consistent with the cell line-based studies, we found that tumors derived from cells expressing the AA form of Numb were much more sensitive to doxorubicin treatment as they had much smaller tumors compared to tumors derived from cells expressing WT and DD form of Numb (Figures 7a–c). In addition, compared with the WT plus doxorubicin group, we also observed significant higher percentage of tumor cells undergo apoptosis in AA plus doxorubicin group by performing IFC staining against cleaved-caspase 3 (Figure 7d). Of note, we did not observe significant differences among these groups without doxorubicin treatment. In addition, we also found that the signal of cell proliferation marker KI67 was much lower in AA+DOX group compared with all the other five groups (Figure 7f). Overall, the tumors we obtained were relatively small because cells stably expressing Numb have severe growth defect (data not shown).

DISCUSSION

Because the tumor suppressor p53 has crucial roles in the efficacy of existing chemotherapies, targeting pathways or specific proteins that disrupt p53 function provides an opportunity to overcome chemoresistance.^{12,21} Recently, the protein level and proapoptotic function of p53 were shown to be positively regulated by Numb, as Numb inactivates the E3 ubiquitin ligase of p53, Mdm2.⁶ Moreover, both methylation and phosphorylation of Numb were reported to inhibit its association to p53, thus decreasing p53 level and activity.^{9,11} Herein, we provided evidence to show that Plk1 acts as a novel kinase that directly phosphorylates Numb at two serine sites and that Plk1 phosphorylation of Numb inhibits the p53 pathway in the presence of DNA damage. Although the two serines we mapped were co-incident with two of the three reported PKC sites that also affect the binding affinity between Numb and p53, we were able to show that Plk1 was responsible for the phosphorylation events and mutations of these two serines to alanines were enough to have a strong phenotype.^{11,22} Interestingly, two studies conducted on liver cancer supported the similar working model to what we proposed above. Liu *et al.*²³ showed that HBV (Hepatitis B virus) infection can dissociate the Numb/p53 complex and decrease p53

activity. Of note, Plk1 is activated after HBV infection and promotes the HBV-induced liver carcinogenesis.²⁴ Therefore, it is likely that Plk1 phosphorylation of Numb is one of the mechanisms contributing to liver cancer progression after HBV infection. For experimental purpose, we used HEK293T and HeLa cells in the earlier parts of this study because they are easy to be transfected. However, when we started to check the p53 pathway, we used either U2OS or MCF-7 to study the molecular mechanism rather than focusing on one certain cancer type. It would be beneficial to check other cancer cell lines in the future using the similar strategy.

Moreover, we were able to demonstrate, for the first time, that Numb phosphorylation by Plk1 promotes its degradation. Thus, our findings reveal a novel phosphorylation-dependent mechanism that can affect both the level and function of Numb. It is very likely that the two phosphorylation sites of Numb by Plk1 serve as phosphodegrons for direct interaction with E3 ligase like F-box proteins.²⁵ Intrigingly, Mdm2 was also reported to be an E3 ubiquitin ligase of Numb and modulates Numb degradation.^{26–28} Based on what we observed, it is possible that phosphorylated form of Numb by Plk1 loses its binding affinity to p53, but somehow can interact with Mdm2 better and, subsequently, is degraded faster. Future study is needed to further investigate the exact mechanism how Numb functions in the p53/Mdm2 complex and whether post-translational modifications of Numb are involved in the interaction between Mdm2 and Numb.

In addition, our findings bridge the knowledge gap whether Plk1 can directly target Numb and affect its function. Besides multiple functions in regulating developmental pathway, ubiquitination and endocytosis, Numb was also shown to have critical roles in cell cycle progression in a Plk1-dependent manner, as previous studies suggested that adaptor partner of Numb (pon) is phosphorylated by Plk1 and that the phosphopon binding to Plk1 is crucial for Plk1 to release from its autoinhibitory state.^{18,29} In addition, the study by Travis *et al.* indicated that Numb is required for normal Plk1 function during mitosis and that Numb dysregulation leads to mitotic arrest, which was also confirmed by our data (Figures 1c and d).¹⁹ However, the exact role and regulatory mechanism of Numb during mitotic progression are largely unknown. By identifying Numb as a novel substrate of mitotic kinase Plk1, we are able to shed light on the future studies about regulation of Numb during mitosis.

On the other hand, our studies were able to provide another strong evidence that, in agreement with our laboratory's previous data, Plk1 negatively regulates p53 and the DNA damage response pathway.^{16,17} Based on both cell culture and mouse xenograft assays, we were able to demonstrate that modifying the phosphorylation status of Numb by Plk1 will significantly affect the cellular response to DNA damage reagent. Thus, targeting Plk1 phosphorylation may provide an effective strategy for cancer treatment, especially when Plk1 inhibitors are combined with existing chemotherapeutic drugs. Importantly, Plk1 inhibition has already been shown to exert strong synergistic effect with doxorubicin in renal carcinomas.³⁰ Therefore, future drug combination might be proposed in other cancers types with a high Plk1 expression level based on the molecular mechanism we identified.

MATERIALS AND METHODS

Chemicals

BI2536 was purchased from Symansis Ltd. (Timaru, New Zealand) and dissolved in dimethyl sulfoxide. Mimosine, Hydroxy urea, nocodazole, tamoxifen, doxorubicin, puromycin, G418, cycloheximide and MG132 were purchased from Sigma (St Louis, MO, USA).

Cell culture, transfection, shRNA and viral infection

HEK293T, HeLa, Panc-1 and U2OS cells were initially purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin in 5% CO₂ at 37 °C. MEFs were isolated from female mice with Rosa26-Cre/Plk1-KI genotype at a developmental stage of 13.5 days after pregnancy and cells were cultured in Dulbecco's modified Eagle's medium. For plasmid DNA transfection into cells, either MegaTran1.0 (Origene) or Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's recommended protocols. G418 was used to select single positive clones that stably express transfected FLAG-Numb constructs in U2OS cells. For shRNA experiments, human Numb shRNA lentiviral particles (sc-42146-v) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Numb-depleted cells were selected using puromycin as the protocol recommended. Lentivirus constructs were generated, and viral infections were performed according to previously described methods.³¹

Immunoblotting and immunoprecipitation

Cell lysates were prepared using TBSN buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet p-40, 5 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), 1.5 mM EDTA, 0.5 mM Na₃VO₄ and 20 mM p-nitrophenyl phosphate), supplemented with protease inhibitors (Sigma) and phosphatase inhibitor (Active Motif). For immunoprecipitation experiments, cell lysates were incubated with desired antibodies in TBSN buffer at 4 °C overnight, then protein A/G beads (Santa Cruz) were added to each sample, followed by four times wash with TBSN buffer containing 500 mM NaCl and four additional washes with TBSN buffer containing 150 mM NaCl. While the antibodies against Numb, Hes1, were purchased from Cell Signaling Technology (Danvers, MA, USA), the antibodies against GFP, Plk1, p53, p21 and ubiquitin were obtained from Santa Cruz. Sigma is the provider of antibodies against FLAG and β-actin.

Recombinant protein purification and *in vitro* kinase assays

Different parts of Numb open reading frame were cloned into pGEX-KG vector to generate glutathione *S*-transferase tagged constructs. The constructs were transformed into BL21 competent cells, which were induced with IPTG at 37 °C for 4 h after reaching OD₅₉₅ of 0.4–0.8. The bacterial pellet was resuspended in STE buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 8.0) and incubated with glutathione beads (Sigma) at 4 °C overnight, followed by 3 washes with STE buffer and subsequent elution. For *in vitro* kinase assays, purified recombinant Numb was incubated with purified Plk1 in the kinase reaction buffer

(50 mM Tris, pH 7.5, 10 mM MgCl₂, 2 mM EGTA, 0.5 mM Na₃VO₄, 25 mM DTT, 125 μM ATP) supplemented with 10 μCi of [γ -³²P]ATP at 30 °C for 30 min. After the reaction mixtures were boiled and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the gels were stained with Coomassie brilliant blue, dried and then subjected to autoradiography.

Immunofluorescence staining

Cells were grown on coverslips under the culture conditions described above, treated with PHEM buffer (60 mM piperazine-N,N'-bis (2-ethane-sulfonic acid), 25 mM HEPES, 10 mM EGTA and 2 mM MgCl₂; pH 6.9) plus 1% Triton X-100 for 5 min, fixed in PHEM buffer with 4% formaldehyde and blocked in phosphate-buffered saline with 5% bovine serum albumin and 0.1% Triton X-100 for 1 h. Primary and secondary antibody incubations were conducted for 1 h at room temperature. Immunofluorescence images were collected at room temperature with a Nikon C1+ microscope equipped with a $\times 60$, 1.4 numerical aperture oil immersion lenses.

Colony formation assay

After cells (with desired transfection) were seeded in six-well plates (1000 cells/well) for 15 days with medium refreshment every 2 days, cells were fixed with 10% formalin and stained with 0.05% crystal violet.

Annexin V/PI staining and flow cytometry analysis

Cells after desired transfections and drug treatments were stained with Annexin V-FITC Apoptosis Detection Kit (BIOVISION) according to the manufacturer's protocol and analyzed on a flow cytometer. For regular FACS, cells were trypsinized, fixed in 75% ethanol, stained with propidium iodide solution at a concentration of 50 μg/ml and subjected to flow cytometry analysis.

p53 luciferase reporter assay

The Signal p53 Pathway Reporter Assay Kit (Qiagen) was used to transfect desired constructs into cells to monitor p53 transcriptional activity. We purchased Dual-Luciferase Reporter Assay system (Promega) to measure the p53-inducible firefly luciferase activity and constitutively expressed *Renilla* luciferase activity and then monitored the relative luciferase unit as the read out for p53 transcriptional activity.

Mouse xenograft model

U2OS cells were transfected with different FLAG-Numb constructs (WT, AA or DD) and treated with 2 mg/ml G418 for 1 week to select transfection-positive cells. Then, 1 – 10⁶-selected cells were mixed with an equal volume of Matrigel (Corning) and inoculated into the right flank of a nude mouse (Envigo, 4 mice per group, 24 mice total). One week later, tumor volumes were measured twice a week using the following formula: $V = L \times W^2/2$ (V, mm³; L, mm; W, mm). Drug treatments were started one month after the tumor engraftment. Doxorubicin were dissolved in water and given to mice once a week using immunoprecipitation injection with the concentration of 2 mg/kg.

Histology and immunofluorescent chemistry staining

Xenograft tumors were fixed in 10% neutral buffered formalin, paraffin embedded and then immunofluorescent chemistry staining was performed using Elite Vectastain ABC kit, cleaved-caspase 3 antibody was purchased from Cell Signaling.

Statistical analyses

The level of significance indicated by *P*-values was calculated using standard two-sided Student's *t*-tests. *P*<0.05 was considered statistically significant.

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ABBREVIATIONS

Plk1	Polo-like kinase 1
GST	glutathione <i>S</i> -transferase
WT	wild type
KI	knock in
MEF	mouse embryonic fibroblast
AA	S265A/S284A
DD	S265D/S284D
PTB	phosphotyrosine binding
PRR	proline-rich region

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Figure 1.

Plk1 and Numb bind to and regulate each other. **(a)** HEK293T cells were treated with nocodazole (200 ng/ml) for 8 h and harvested for immunoprecipitation (IP), followed by immunoblotting (IB). **(b)** HEK293T cells were co-transfected with FLAG-Numb and GFP-Plk1 and harvested for IP, followed by IB using antibodies indicated. **(c, d)** After HEK293T and HeLa cells were infected with lentivirus that expresses shRNA to knock down (KD) Numb, cells were treated with puromycin to select infection-positive cells, the remaining cells were reseeded and harvested for IB **(c)** and FACS analysis **(d)**. **(e)** U2OS cells were infected with either control or Plk1-expressing lentivirus for 3 days and harvested for IB. **(f)** MEFs, derived from conditional Plk1-KI mice (Plk1 sequence was inserted in the Rosa26 locus and its expression is under the control of CreER), were treated with or without 1 μM tamoxifen for 2 days to induce Cre expression and harvested for IB. **(g)** HEK293T cells were treated with or without 20 nM BI2536 for 8 h and harvested.

Figure 2.

Plk1 phosphorylates Numb at serine 265 and 284 *in vitro*. (**a** and **b**) Purified GST-fusion recombinant proteins were incubated with purified Plk1 from insect cells at the presence of [γ - 32 P]ATP. The reaction mixtures were resolved by SDS-PAGE, stained with Coomassie brilliant blue (Coom.) (lower panels), followed by autoradiography (upper panels). (**c**, **d**) After site-directed mutagenesis Kit (Agilent) was used to generate specific mutants of GST-tagged Numb, the purified proteins were incubated with Plk1 as described above. (**e**) After GST-tagged Numb regions spanning aa 199–280 and 281–415 with or without specific serine to alanine mutations within the regions were generated, the purified proteins were subjected to Plk1 kinase assay. (**f**) Alignment of the two Plk1 phosphorylation sites in Numb homologs (human, mouse and rat) and different mammalian Numb isoforms (p72, p71, p66 and p65).

Figure 3.

Plk1 phosphorylates Numb at Serine 265 *in vivo*. **(a)** Recombinant Numb proteins (WT or S265A) were incubated with purified Plk1 in the presence of unlabeled ATP and immunoblotted with the pS265-Numb antibody. **(b)** HEK293T cells were treated with mimosine (0.3 mM, 20 h), hydroxyl urea (4 mM, 24 h), nocodazole (200 ng/ml, 12 h), nocodazole and BI2536 (20 nM 12 h), respectively, harvested in the presence of phosphatase inhibitor and subjected to IB. **(c)** Panc-1 cells were treated with mimosine, nocodazole or nocodazole plus BI2536 before harvested for IB. **(d)** Panc-1 cells were infected with control lentivirus or lentivirus to deplete Plk1 for 3 days and harvested for IB. **(e)** HEK293T cells were co-transfected with different plasmids indicated and harvested for IB. **(f)** Randomly growing HeLa cells were subjected to immunofluorescence (IF) staining with antibodies against Plk1 and pS265-Numb.

Figure 4.

Plk1 promotes Numb proteasomal degradation in a phosphorylation-dependent manner. **(a)** HEK293T and U2OS cells were transfected with indicated plasmids for 48 h, treated with or without 10 μ M MG132 for 6 h and harvested for IB. **(b)** HEK293T cells were co-transfected with indicated plasmids, treated with BI2536, incubated with MG132 for 6 h and harvested for IP, followed by IB. **(c)** U2OS cells were transfected with GFP-Plk1 (T210D) for 12 h, treated with 100 μ g/ml cycloheximide (CHX) for different time and harvested for IB. The relative band intensities (Numb/ β -actin) were calculated using Image Lab (Bio-Rad). **(d)** U2OS cells were transfected with indicated plasmids for 48 h and harvested for IB. **(e)** HEK293T cells were co-transfected with different plasmids, treated with MG132 and harvested for IB. **(f)** HEK293T cells were co-transfected with FLAG-Numb constructs (WT, AA, DD), treated with MG132 for 6 h and harvested for anti-FLAG IP, followed by IB. DD:

S265D, S284D. (g) HeLa cells were transfected with FLAG-Numb constructs (WT, AA or DD), treated with 100 µg/ml CHX for different time and harvested for IB.

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Figure 5.

Plk1 negatively regulates the Numb/p53 pathway. **(a)** MEFs derived from conditional Plk1-KI mice were treated with 1 μ M tamoxifen for 2 days, incubated with doxorubicin (DOX) for 24 h and harvested for IB. **(b)** U2OS cells were co-transfected with different FLAG-Numb constructs (WT, AA or DD), GFP-Plk1-T210D and a p53 luciferase reporter construct for 2 days, treated with 1 μ M doxorubicin for 24 h and harvested for IB (bottom panel) and luciferase assay (top panel). For p53 luciferase assay, p53 transcriptional activities were represented as relative luciferase units (firefly luciferase activities/*Renilla* luciferase activities), the results of each groups were repeated independently for at least three times. *, $P < 0.05$. **(c)** U2OS cells were transfected with different FLAG-Numb constructs (WT, AA or DD), treated with 1 μ M doxorubicin for 24 h and harvested for FLAG IP, followed by IB. **(d)** U2OS cells were transfected with vehicle or FLAG-Numb constructs (WT, AA or DD), and treated with 1 μ M doxorubicin for 24 h. Upon reseeding, cells were treated with CHX for different times as indicated and harvested for IB. **(e)** U2OS cells were transfected with different FLAG-Numb constructs (WT, AA or DD), treated with MG132 for 6 h and harvested for anti-p53 IP, followed by IB.

Figure 6.

Cells expressing unphosphorylated form of Numb by Plk1 are more sensitive to doxorubicin. (a, b) MCF-7 cells were transfected with different FLAG-Numb constructs (WT, AA or DD), treated with 1 μ M doxorubicin for 24 h and harvested for IB (a) and cell number counting using the Bio-Rad TC20 automatic cell number counter (b). *, $P < 0.05$, **, $P < 0.01$. (c, d) U2OS cells were transfected with different FLAG-Numb constructs (WT, AA or DD), treated with 1 μ M doxorubicin for 24 h and then harvested for IB (c) and cell number counting (d). *, $P < 0.05$, **, $P < 0.01$. (e) U2OS cells were transfected with different FLAG-Numb constructs (WT, AA or DD), treated with 1 μ M doxorubicin for 24 h and harvested

for Annexin V/PI staining using the BIOVISON apoptotic kit, followed by FACS analysis. After assays for each group were repeated for at least three times, the double-positive cells were quantified as late apoptotic cells (bottom panel). *, $P<0.05$. (f) U2OS cells were transfected with different FLAG-Numb constructs (WT, AA or DD) for 2 days, reseeded in six-well plates and treated with 1 μ M doxorubicin for 2 days. After cells were cultured in drug-free medium for additional 10 days, cells were fixed and stained with 0.5% crystal violet, followed by quantification at least three times of independent experiments (bottom panel). *, $P<0.05$, **, $P<0.01$.

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Figure 7.

Tumors carrying the unphosphorylated form of Numb by Plk1 are more sensitive to chemotherapy. **(a)** Images of the harvested tumors after 50 days of xenograft study. **(b)** Tumor growth curve of the study, with doxorubicin being given (IP, 2 mg/kg) once a week after 1 month of tumor inoculation. *, $P < 0.05$, **, $P < 0.01$. Error bars stand for s.d. At the same time, harvested tumors were blotted to detect the expression of transgenes. **(c)** Tumor weight measurement upon killing. **(d)** Representative images of IFC staining of the tumors using cleaved-caspase 3 antibody. **(e)** Quantification of cleaved-caspase 3-positive cells as percentage, *, $P < 0.05$. For quantification, at least 500 cells were scored on each field ($\times 20$ fields, more than three sections at different tumors/mice). **(f)** Representative images of IFC staining of the tumors using KI67 antibody, $\times 20$ magnification was used to capture the images.