PPM1H is a p27 phosphatase implicated in trastuzumab resistance

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ABSTRACT

The HER2 oncogene is overexpressed or amplified in 20% of breast cancers. HER2-positive cancer historically portends a poor prognosis, but the HER2-targeted therapy trastuzumab mitigates this otherwise ominous distinction. Nevertheless, some patients suffer disease recurrence despite trastuzumab, and metastatic disease remains largely incurable due to innate and acquired resistance. Thus, understanding trastuzumab resistance remains an unmet medical need. Through RNA interference screening, we discovered that knockdown of the serine/threonine phosphatase PPM1H confers trastuzumab resistance via reduction in protein levels of the tumor suppressor p27. PPM1H dephosphorylates p27 at threonine 187, thus removing a signal for proteasomal degradation. We further determined that patients whose tumors express low levels of PPM1H trend towards worse clinical outcome on trastuzumab. Identifying PPM1H as a novel p27 phosphatase reveals new insight into how cancer cells destabilize a well-recognized tumor suppressor. Furthermore, low PPM1H expression may identify a subset of HER2-positive tumors that are harder to treat.

SIGNIFICANCE: PPM1H is identified as a phosphatase impacting p27 stability. Low expression of PPM1H may be associated with poor outcome in breast cancer.

INTRODUCTION

Trastuzumab (Herceptin; Genentech) is an anti-human epidermal growth factor receptor 2 (HER2, ERBB2) therapeutic monoclonal antibody that provides significant clinical benefit for breast cancer patients whose tumors exhibit overexpression or amplification of the oncogene HER2 (1). Trastuzumab acts, at least in part, by blocking the interaction between overexpressed HER2 and its dimerization partner HER3, resulting in inhibition of oncopgenic phosphoinositide 3-kinase (PI3K) pathway signaling and subsequent upregulation of the cyclin-dependent kinase (CDK) inhibitor p27 (2–7). PI3K pathway inhibition also causes translocation of p27 from the cytoplasm to the nucleus, where it is able to inhibit CDK/cyclin complexes (8–10).

Given the molecular heterogeneity of cancer, not all patients with HER2-amplified tumors respond to HER2-targeted agents. In the metastatic setting, patients who derive initial benefit often exhibit evolution of the tumor with resultant progression on therapy. One major hypothesis on the mechanism of resistance to HER2-targeted therapy is that the PI3K pathway may be independently activated downstream of the HER2-HER3 receptor complex via loss of PTEN or acquisition of activating PIK3CA mutations (2, 11, 12). A recent preclinical study demonstrates that trastuzumab-resistant models harboring PTEN loss or PIK3CA activating mutations are sensitive to GDC-0941, a class 1A PI3K small molecule inhibitor (2). This finding illustrates how understanding the molecular nature of resistance can reveal potentially more effective diagnostic and therapeutic co-development strategies to better treat individual patients.

Although PTEN loss and PIK3CA activating mutations may play a role in trastuzumab resistance, we hypothesized that there could be additional resistance factors for several reasons. First, the PI3K signaling pathway is known to elicit a complex network of downstream events which may involve regulatory factors other than PTEN and PIK3CA (13). Second, there is ample evidence that the HER2-HER3 complex may activate signaling pathways other than PI3K, such as the MAPK pathway (14). Third, the downstream effect of trastuzumab primarily involves inhibition of the G1-S phase transition via stabilization of the cell-cycle inhibitor p27 (4–7), raising the possibility that downstream cell-cycle regulators may also impact response to trastuzumab.

RESULTS

siRNA Screen for Trastuzumab Resistance Genes

To determine if there are additional downstream trastuzumab resistance factors other than PTEN and PIK3CA, we conducted a functional screen to identify genes that, upon silencing, augment proliferation of trastuzumab-treated BT474 (HER2-amplified) breast cancer cells (Fig. 1A). Small interfering RNA (siRNA) knockdown of either p27 or PTEN increased proliferation in the presence of trastuzumab, consistent with the literature (11, 12, 15–17) (Fig. 1B). Notably, knockdown of p27 was more potent at abrogating trastuzumab response than was knockdown of PTEN. Both PTEN and p27 siRNAs were used as positive controls as we screened siRNA libraries of human kinases (795 genes) and phosphatases (159 genes). Each gene
PPM1H Loss Causes Trastuzumab Resistance

Of the 5 top hits, knockdown of PPM1H stood out as being the most potent at augmenting proliferation in the presence of trastuzumab (Fig. 2A), thus we focused on better understanding the role of PPM1H in cell proliferation and trastuzumab resistance. PPM1H is a member of the PP2C family of Ser/Thr phosphatases distinguished by the dependence on Mn" or Mg" for catalytic activity (18). Although PPM1H was recently implicated as an oncogene in colon cancer (19, 20), it was originally identified as a negative regulator of neurite outgrowth (21). In fact, many PP2C family members have been described as negative regulators of growth having substrates in the PI3K pathway, the JNK pathway, or in cell-cycle regulation (e.g., cyclin-dependent kinases) (18). Very little is known about the mechanism of PPM1H and how it might impact proliferation.

The same phenotype was observed with 3 of 4 independent siRNA oligonucleotides targeting PPM1H (Supplementary Fig. S1), suggesting that the proliferative effect is not likely due to off-target activity. Nevertheless, to further rule out any possibility of off-target effects, PPM1H shRNA was transfected into BT474 cells along with a control vector or a PPM1H expression vector carrying synonymous mutations within the shRNA targeted region, thus rendering the exogenous transcript resistant to knockdown. The PPM1H shRNA caused trastuzumab resistance, similar to the result with siRNA (Fig. 2B). However, when PPM1H shRNA was co-expressed with PPM1H carrying synonymous mutations, trastuzumab sensitivity was restored (Fig. 2B), providing further evidence that the observation with PPM1H is not likely due to off-target effects.

Many studies have suggested that 3-dimensional (3D) culture may more closely mimic the milieu of a tumor mass (22). To determine the impact of PPM1H knockdown in 3D culture, we created stable BT474M1 cell lines carrying doxycycline (dox)-inducible PPM1H shRNA. Treatment of the cell line with dox in 2D culture resulted in PPM1H knockdown at the mRNA and protein level and resulted in trastuzumab resistance, much like PPM1H siRNA. The same cell line was grown in 3D culture for 10 days in the presence or absence of dox, after which colony size was visualized and quantified. In the absence of dox, trastuzumab treatment resulted in significantly smaller colony size compared to untreated colonies;
proliferation, we examined the effect of PPM1H knockdown on the PI3K-Akt pathway, a key oncogenic signaling axis in HER2-amplified cells (23, 24). We speculated that the phosphatase activity of PPM1H might inactivate this pathway since PHLPP, a PPM1H-related family member, has previously been shown to dephosphorylate AKT (25). However, knockdown of PPM1H did not change the phosphorylation levels of AKT, HER2, or HER3. Furthermore, there was no evidence for modulation of the MAPK pathway as assessed by pERK (Supplementary Fig. S2).

We next examined the CDK inhibitor p27 and found significant modulation with PPM1H knockdown compared to both untreated and trastuzumab-treated control cells. p27 protein levels as assessed by Western blot and immunofluorescence dropped with PPM1H knockdown (Fig. 3A and B), and this was supported by quantitative image analysis of the immunofluorescence (Supplementary Fig. S2), consistent with the observed increase in cell proliferation. No change in p27 mRNA levels was observed with PPM1H knockdown, suggesting that loss of p27 is due to an alteration in protein stabilization.

p27 is known to undergo ubiquitin-mediated proteosomal degradation which is initiated by phosphorylation of p27 at T187 by CDK2 in complex with cyclin A or E (26, 27). T187 phospho-p27 is recognized by the F-box protein SKP2, which brings p27 to the COP9 signalosome (CSN) for ubiquitylation (28). Recently, SKP2 was shown to be phosphorylated and stabilized by AKT1 at S72 (29, 30). However, p27 also plays a role in regulating SKP2 via inhibition of CDK2, resulting in dephosphorylation and destabilization of SKP2 (31). Given this reciprocal regulation of p27 and SKP2, we hypothesized that PPM1H knockdown might be associated with increased SKP2 protein. This hypothesis was tested via Western blot and immunofluorescence, and the results clearly indicate that SKP2 protein increases while p27 decreases in the setting of PPM1H knockdown (Fig. 3A and C; Supplementary Fig. S2). No change in SKP2 mRNA was observed, consistent with the conclusion that PPM1H knockdown promotes SKP2 protein stability.

Because PPM1H belongs to a Ser/Thr phosphatase family, it is possible that PPM1H might directly dephosphorylate p27 or SKP2. To explore these hypotheses, we first examined the subcellular localization of PPM1H. Nuclear/cytoplasmic fractionation revealed that PPM1H is present in both the nucleus and the cytoplasm (Supplementary Fig. S3A). Enzymatically active recombinant FLAG-PPM1H was then screened for activity on in vitro synthesized phosphopeptides representing the major known phosphorylation sites on p27 (S10, T157, T187) and SKP2 (S72, S75, S64). Liberation of phosphate was observed with the p27 peptides, most notably with the T187 peptide (Fig. 4A). No evidence for modulation of the MAPK pathway as assessed by pERK (Supplementary Fig. S2).
was found to have decreased enzymatic activity in a synthetic phosphatase assay (Supplementary Fig. S3B). Wild-type PPM1H but not PPM1H-H153L dephosphorylated p27 at T187 (Fig. 4B). The prephosphatase (lower gel) and postphosphatase (upper gel) phospho-T187 bands were quantified by densitometry, which suggested that PPM1H diminished phosphorylation by about half. Although there appears to be more phospho-T187 in the PPM1H-H153L-treated lane (upper gel), the prephosphatase control reveals that this sample had more phospho-T187 before the addition of phosphatase, and the quantified ratio is 1.1, indicating little to no change with PPM1H-H153L treatment. Although the S10 site on p27 was not phosphorylated by CDK2/cyclin A, there was some endogenous phosphorylation observed. PPM1H exhibited no activity at the S10 site in this assay, suggesting specificity for T187.

Specificity of PPM1H was further explored via GST-tagged versions of PPM1H and the most closely related family member PPM1J. GST-PPM1H and GST-PPM1J exhibited similar enzymatic activity in a synthetic phosphatase assay (Supplementary Fig. S3C) but only PPM1H was effective at dephosphorylating p27 at T187 (Fig. 4C). Neither enzyme exhibited activity at S10. Together these data further support the hypothesis that PPM1H is a specific p27-T187 phosphatase.

Although PPM1H did not exhibit activity on SKP2 phosphopeptides, we nevertheless tested FLAG-PPM1H on full-length SKP2. SKP2 was phosphorylated in vitro by AKT in the presence of γ-32P-ATP and was then incubated with phosphatase. α-phosphatase was able to dephosphorylate SKP2 but PPM1H lacked activity (Supplementary Fig. S3D). These data further support the conclusion that unlike p27, SKP2 is not a substrate for PPM1H.

**Patients Whose Tumors Have Low PPM1H Expression Trend Toward Worse Clinical Outcome**

To explore whether expression of PPM1H might impact clinical outcome on trastuzumab, we developed an isotopic in situ hybridization probe to assay PPM1H mRNA in FFPE human breast cancer samples. PPM1H was examined in 87 HER2-positive tumor samples from patients who had been treated with trastuzumab. The sample set consisted of a mixture of first-line, second-line, and later-line patients from British Columbia, most of whom were treated with trastuzumab in combination with chemotherapy between 1998 and 2005 as previously described (33). Of 150 patient samples identified in the original study, 87 had sufficient tumor tissue with evidence of control β-actin signal and verified HER2 amplification by FISH. Areas enriched in invasive neoplastic cells were marked by a pathologist and a quantitative phosphor-imager analysis of PPM1H expression was performed in these regions. The samples were ranked based on PPM1H expression and the clinical outcome of the upper 50th percentile was compared to that of the lower 50th percentile. There was a trend toward poor outcome with low PPM1H expression (HR 1.6), although the data did not quite reach statistical significance ($P = 0.07,$
Role of PPM1H in p27 Regulation

Figure 3. PPM1H regulates expression of p27 and SKP2.

A. Western blot showing expression of p27, SKP2, PPM1H, and PTEN with and without siRNA knockdown of the same genes. B. p27 immunofluorescence with and without PPM1H knockdown and with and without trastuzumab treatment. C. SKP2 immunofluorescence with and without PPM1H knockdown and with and without trastuzumab treatment. Scale bar = 100 μm.
95% CI 0.96–2.6) (Fig. 5A). The data were also examined for differences in clinical outcome based on **PPM1H** expression in the estrogen receptor–negative (ER−) and estrogen receptor–positive (ER+) populations. The trend toward worse outcome with low **PPM1H** expression appeared largely restricted to the ER− population (Supplementary Fig. S4). These data suggest that low **PPM1H** expression could be a poor prognostic indicator in HER2-positive patients treated with trastuzumab. Further work in larger clinical datasets would be warranted to explore the significance of this trend.

To determine if the observation with **PPM1H** was independent from known PI3K pathway prognostic markers, we examined PTEN expression and **PIK3CA** mutation status in the same trastuzumab-treated samples. Low **PPM1H** expression was independent of **PIK3CA** mutation and PTEN expression status. Interestingly, in this sample set, neither decreased PTEN expression nor **PIK3CA** hotspot mutations were associated with poor outcome either alone or in combination (Supplementary Fig. S4).

In all cases in this sample set, **PPM1H** expression was observed to be low in normal breast epithelium and stroma, but seemed to be elevated in the epithelium of a proportion of the invasive breast cancers. In one case, **PPM1H** was observed to be elevated in premalignant glands (ductal...
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The basal-like subtype tends to lack expression of ER, progesterone receptor (PR), and HER2 and is associated with poor prognosis (34). The luminal subtype expresses ER and PR, lacks HER2 amplification, and is associated with a better prognosis (34). About 50% of HER2-positive cancers are ER⁺, and there are reports that ER status is reflected in expression profiles resulting in HER2-basal and HER2-luminal groups, with HER2-basal exhibiting a worse prognosis (17, 35). Interestingly, breast cancer cell lines classified as basal or HER2-basal exhibited lower levels of PPM1H than did the luminal counterparts (Fig. 5B). A similar observation was made in breast cancer tissues (Fig. 5C). To determine whether HER2 pathway signaling might regulate PPM1H transcription, HER2 and HER3 were knocked down in BT474M1 cells, and PPM1H expression was measured via qRT-PCR. There was no evidence for PPM1H transcriptional regulation by HER2 or HER3 (Supplementary Fig. S4).

Taken together, the data are consistent with a pattern of PPM1H expression tending to be increased in early stages of disease and decreased at later stages. PPM1H mRNA was also examined in a set of cell lines and cancer tissues representing the major breast cancer subtypes.

carcinoma in situ), but was low in adjacent invasive cancer (Supplementary Fig. S4E). This observation raises the possibility that PPM1H is upregulated earlier in the oncogenic process and in some cases is later downregulated. To further explore this possibility, we examined PPM1H expression in a database of 159 frozen breast tissue samples that had undergone gene expression profiling at Gene Logic (now Osimium Biosolutions). No clinical outcome data were available for this sample set. Although ductal carcinoma in situ was not included in this sample set, there was evidence for elevated PPM1H expression in some primary tumors compared to normal and benign fibrocystic breast tissue. However, metastatic tumor tissue exhibited decreased PPM1H expression compared to primary tumor (Supplementary Fig. S4F). Taken together, the data are consistent with a pattern of PPM1H expression tending to be increased in early stages of disease and decreased at later stages.

PPM1H mRNA was also examined in a set of cell lines and cancer tissues representing the major breast cancer subtypes.
that PPM1H expression is significantly decreased in glioblastoma and in renal cell carcinoma compared to normal brain and kidney, respectively (Supplementary Fig. S4). Interestingly, PPM1H is significantly increased in colon adenocarcinoma and prostate adenocarcinoma compared to normal colon and prostate, respectively (Supplementary Fig. S4). Thus, the role of PPM1H may depend on the cancer type or even subtype.

**DISCUSSION**

Diagnostic markers of poor outcome on therapy can be grouped into those that are prognostic (i.e., identify a particularly aggressive biology that is hard to treat) and those that are predictive of response to a specific therapy. Both types of diagnostic markers exhibit clinical utility and both can signify important disease biology. In the case of trastuzumab and lapatinib, prior barcode shRNA screens explored potential predictive markers of resistance by selecting for hits that impact long term cell growth in the setting of trastuzumab treatment, but not in the absence of treatment (11, 15). With this approach, the tumor suppressor PTEN was identified as the only hit. In the present study, we took a broader approach, seeking to identify any gene that increased proliferation in the setting of trastuzumab treatment, even if it also did so without treatment. We also used a different platform, choosing to use siRNA and directly measure proliferation rather than relying on long-term cell growth assays. With this different approach, we identified several novel hits, which we speculate may represent genes of broader prognostic significance.

The top novel trastuzumab resistance factor identified in the screen and after extensive validation was the PP2C family member PPM1H. The PP2C family consists of metal-dependent Ser/Thr phosphatases, of which there are 16 in humans (18). The family is conserved throughout evolution with 7 PP2C family members existing in yeast (PTC1-7) (36). One important difference from other Ser/Thr phosphatase family members is that the PP2C family acts as monomers as opposed to requiring several subunits to achieve specifically targeted enzyme activity. The better characterized family members in humans include PPM1A and PPM1B, which are negative regulators of growth and have been shown to dephosphorylate CDK2 as well as inhibit signaling through cellular stress pathways (18). The yeast PP2C family members (PTC2 and 3) are also described as negative regulators of growth by dephosphorylating and inactivating Cdc28, a primary regulator of cell-cycle progression (18). Other family members such as PTC1 are negative regulators of osmotic stress signaling pathways (36). Very little is known about PPM1H, although it was originally identified as a negative regulator of neurite outgrowth (21).

We explored whether PPM1H knockdown might impact total levels or phosphorylation of HER family members, immediate downstream PI3K or MAPK signaling components, or further downstream cell-cycle regulatory components. The only alteration that was consistently observed was a loss of nuclear p27, a key cell-cycle regulator. This observation raised the hypothesis that PPM1H could be a phosphatase for p27, which we confirmed in vitro. Based on the existing data, we propose a model in which PPM1H dephosphorylates the T187 site on p27, thus preventing its ubiquitylation and degradation (Fig. 6). Stabilized p27 would then be available to inhibit the cell cycle. Knockdown of PPM1H causes loss of nuclear p27 without evidence of cytoplasmic re-localization, consistent with the model that there is increased phosphorylation of p27 at T187 and subsequent proteasomal degradation of p27 in the nucleus. If PPM1H acted on the S10 and/or T157 phosphorylation sites on p27, one would have predicted to see evidence of cytoplasmic translocation and retention of p27.

This work is the first that we are aware of linking PPM1H to regulation of p27. This link raises interesting questions about what role PPM1H might play in regulating the cell cycle not only in HER2-positive breast cancer as described here, but also in other cancer types and in normal cell-cycle regulation.
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should be noted that although the observation that PPM1H can dephosphorylate p27 at T187 is intriguing, it is nevertheless possible that PPM1H might also act as a phosphatase for other proteins still to be determined. Furthermore, the antibody developed to detect PPM1H identifies 2 bands, both of which disappear with knockdown. This observation suggests that there are 2 different forms of PPM1H. How these forms differ molecularly and whether there are differences in substrate specificity or enzymatic activity is not known.

Consistent with the hypothesis that low PPM1H is associated with elevated proliferation, we found that there was a trend toward worse outcome in trastuzumab-treated patients whose tumors were HER2-amplified and had low PPM1H expression at the mRNA level. Based on the mechanism of PPM1H, this trend is consistent with many studies in the literature that have established low p27 expression and high SKP2 expression as poor prognostic indicators in breast cancer (26, 37–44). Furthermore, the cell-cycle inhibitory activity of p27 on cyclin E/CDK2 complexes can be abrogated by cyclin D/CDK4, which binds p27 and titrates it away from cyclin E/CDK2. Interestingly, inactivation of both cyclin D and CDK4 are capable of inhibiting HER2-mediated tumorigenesis in genetically engineered mouse models, highlighting the importance of this cell cycle pathway in HER2-mediated oncogenesis (45–47). Recently, it also was observed that amplification or overexpression of cyclin E is a mechanism of oncogenesis in genetically engineered mouse models, highlighting the importance of this cell cycle pathway in HER2-mediated oncogenesis (45–47). Recently, it also was observed that amplification or overexpression of cyclin E is a mechanism of HER2 signaling and trastuzumab resistance.

PPM1H expression was observed to be lower on average in the basal-like subtype of breast cancer, which is well documented to be associated with poor prognosis. The basal-like expression pattern has also been reported to be associated with decreased p27 and increased SKP2 protein levels (39, 42). It should be noted that although on average basal-like cancers have lower PPM1H expression, there are basal-like cancers that have PPM1H expression similar to luminal tumors. In fact, the trend toward low PPM1H being associated with poor outcome was observed in the ER− (i.e., basal-like) population in the sample set of HER2-positive tumors. We also observed that on average PPM1H expression was lower in metastatic compared to primary tumors, although clearly not all metastatic tumors had low PPM1H. Together the data in human tumor tissues are consistent with the hypothesis that low PPM1H may be associated with poor clinical outcome. However, further examination of PPM1H in larger clinical data sets would be necessary to make more definitive conclusions. Further, the role of PPM1H may differ depending on the cancer type.

The finding of PPM1H involvement in p27-mediated cell cycle regulation raises potential therapeutic opportunities. PPM1H itself is not likely a therapeutic target because inhibition causes elevated proliferation. However, it is possible that other proteins downstream or upstream of PPM1H could represent therapeutic targets. In that regard, small-molecule CDK inhibitors have been reported to work in synergy with trastuzumab (49). Further research to identify upstream factors that regulate PPM1H is thus of potential importance. The identification of PPM1H opens new avenues of research into a pathway that modulates p27 and raises potential new diagnostic and possibly therapeutic strategies for patients who progress on HER2-targeted therapies such as trastuzumab.

METHODS
siRNA screen

Libraries targeting human kinase (795) and phosphatase (159) genes were obtained from Dharmacon. Each of 4 independent siRNAs were transfected to duplicate 96-well plates followed by addition of lipofectamine 2000 (Invitrogen) diluted with OptiMEM (Gibco). Twenty minutes later, BT474 cells were plated (15,000 cells per well) resulting in a final siRNA concentration of 25 nM. Trastuzumab was added 24 hours after transfection, and cell proliferation was determined 72 hours later by measuring [3H]-thymidine incorporation (3). Data for each well were normalized to the plate average. Genes for which at least 2 siRNAs were 1.5 standard deviations above the mean were considered hits.

Cell lines

BT474M1 is a subclone of BT474 that was adapted for growth in vivo and was obtained from California Pacific Medical Center. All other cell lines were originally obtained from American Type Culture Collection and cultured as previously described (3). Transient BT474 shRNA transfection was done via electroporation (Nucleofactor, Lonza). Doxycycline-inducible PPM1H shRNA knockdown BT474M1 cells were produced via lentiviral transfection with a GFP-tagged PPM1H. Cultures were done using 3D methods as previously described (3). Phase contrast images were quantified using the Metamorph software package (Molecular Devices, a Danaher subsidiary). Briefly, a bottom hat filter was used to correct for nonuniform illumination to allow for a binary threshold to identify cell-specific regions. Sequential opening and closing was then performed to consolidate cells and remove small noncellular debris. Single-nucleotide polymorphism (SNP) genotypes are performed each time new cell line stocks are expanded for cryopreservation. Cell line identity is verified by high-throughput SNP genotyping using Illumina Golden Gate multiplexed assays. SNPs were selected based on minor allele frequency and presence on commercial genotyping platforms (Supplementary Methods). SNP profiles were compared to SNP calls from available internal and external data to determine or confirm ancestry. In addition, Short Tandem Repeat (STR) Profiling was performed for each line using the Promega Cell ID System in which 10 human loci were assessed (9 STR loci and Amelogenin for gender identification), including D21S11, TH01, TPOX, vWA, Amelogenin, CSF1PO, D16S539, D7S820, D13S317, and D5S818. The STR profile is determined once and compared to external STR profiles of cell lines to determine cell line ancestry.

Immunofluorescence

Cells grown on coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were stained with p27 antibody (BD Biosciences) or SKP2 antibody (Invitrogen) followed by Alexa Fluor 555-conjugated anti-mouse (Invitrogen) and then DAPI (Invitrogen). Images were acquired with theario SL-50 automated slide scanning platform (Genetix Ltd.) at 10× final magnification. Images were exported for analysis in the Metamorph software package (Molecular Devices, a Danaher subsidiary) as individual images. Standard morphological filters were used to remove staining artifacts, and the cells were counted with the Cell Scoring application module.

Preparation of recombinant PPM1H

Human embryonic kidney (HEK) 293T cells were transfected with GST-PPM1H or GST-PPM1H plasmid using manufacturer’s protocol for Fugene6 (Roche). Pelleted cells were re-suspended in ice-cold HKMT lysis buffer [20 mM Hepes pH 7.2, 20 mM KCl, 10 mM MgCl2,
0.5% Triton X100, 10% glycerol with PTPase inhibitors (Roche). Lysate was cleared by centrifugation and passed through a 1.5-ml GSH-Sepharose column for 3 hours at 4°C. The column was washed with HKMT containing 0.4 M NaCl for 10 column volumes, and eluted step-wise in 10 mM GSH, 20 mM Heps, 10 mM MgCl₂, 0.5% Triton X100, 20 mM NaCl, 20% glycerol, and 5 mM MnCl₂ with 0.6 ml elution fractions. Fractions containing full-length GST-PPM1H were pooled and dialyzed overnight in ice-cold HKM containing 50% Glycerol. Aliquots were made and stored at -80°C. Thirty-five plates (150 mm) of cells yielded 70 μg to 120 μg total protein with 70% full-length enzyme. A similar protocol was used for production of recombinant FLAG-PPM1H, except M2-anti-FLAG agarose (Sigma) was used as the resin and protein was eluted with sodium citrate at pH 3.0.

Biochemical and ISH assays

Antibodies used for Western blotting and immunoprecipitation were anti-FLAG (Sigma); anti-HA (Roche Applied Sciences); anti-p27 (Santa Cruz Biotechnology or BD Biosciences); anti-phospho-p27 Thr187 (Santa Cruz Biotechnology); anti-phospho-p27-Ser10 (Santa Cruz Biotechnology); anti-PTEN (Santa Cruz Biotechnology); anti-PPM1H (peptide rabbit polyclonal); anti-SKIP2 (Invitrogen); anti-α-tubulin (Sigma); and anti-β-actin (MP Biomedicals).

PPM1H activity was assessed in a phosphate release from synthetic phosphopeptides using the Innova Biosciences PicolorLock Gold assay. Briefly, 2 μg of peptide was treated with or without phosphatase in 0.1 ml 20 mM Heps, 20 mM MgCl₂, and 20 mM KCl. Absorbance of molybdate-complexed free phosphate was read at A635. λ-Phosphatase was used as a positive control. In another approach, an HA-tagged p27 construct was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). Cells were lysed in RIP buffer (Sigma) supplemented with a Complete Mini, EDTA-free protease inhibitor tablet (Roche). Three to five milligrams of cell lysate was incubated with anti-HA coupled agarose (Roche) overnight at 4°C, then pelleted and washed 3 times in RIP buffer. The immunoprecipitated material was equilibrated with 2 washes in kinase buffer (5 mM MOPS pH 7.2, 0.4 mM EDTA, 5 mM MgCl₂) then phosphorylated with CDK2/CyclinA kinase (Cell Signalling Technologies) for 2 hours at 30°C. Samples were then washed 3 times in HKM buffer (40 mM HEPES pH 7.2, 20 mM KCl, 20 mM MgCl₂) and then treated with an equal amount of full-length GST-tagged recombinant phosphatase overnight at 30°C. Phosphatase-treated material was then washed once with HKM buffer and then eluted by heating at 95°C for 5 minutes in SDS sample buffer (Invitrogen). Samples were resolved by SDS-PAGE and blotted onto nitrocellulose.

Isotopic in sit hybridization (ISH) was performed using a 591-bp probe starting at nucleotide 1597 of Genbank sequence NM_020700. The isotopically labeled slides were exposed to a phosphimager, which records the signal intensity per pixel. Separately, H&E-stained slides were marked for high tumor content areas. The population was ranked from lowest to highest for each sample. The population was ranked from lowest to highest for each sample. The population was ranked from lowest to highest for each sample.

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