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Screening of cell cycle fusion proteins to identify kinase signaling networks

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Abbreviations: PMA, Phorbol 12-myristate 13-acetate; S/B, Signal to background ratio; Z′, Z factor.

Kinase signaling networks are well-established mediators of cell cycle transitions. However, how kinases interact with the ubiquitin proteasome system (UPS) to elicit protein turnover is not fully understood. We sought a means of identifying kinase-substrate interactions to better understand signaling pathways controlling protein degradation. Our prior studies used a luciferase fusion protein to uncover kinase networks controlling protein turnover. In this study, we utilized a similar approach to identify pathways controlling the cell cycle protein $p27^{Kip1}$. We generated a $p27^{Kip1}$-luciferase fusion and expressed it in cells incubated with compounds from a library of pharmacologically active compounds. We then compared the relative effects of the compounds on $p27^{Kip1}$-luciferase fusion stabilization. This was combined with *in silico* kinome profiling to identify potential kinases inhibited by each compound. This approach effectively uncovered known kinases regulating $p27^{Kip1}$ turnover. Collectively, our studies suggest that this parallel screening approach is robust and can be applied to fully understand kinase-ubiquitin pathway interactions.

Introduction

Cell cycle transitions are finely controlled to ensure faithful transmission of genetic material. For instance, cyclin-dependent kinases are regulated to elicit unidirectional entry into S phase or mitosis. Cyclin-dependent kinase (CDK) activity is inhibited via a class of cyclin-dependent kinase inhibitors, or CKIs. CKIs bind to cyclin-dependent kinases, thereby inhibiting CDK-dependent phosphorylation of target proteins. In turn, CKI levels are controlled via ubiquitin-dependent degradation.[1-6]

$p27^{Kip1}$ is a key CKI at the G1/S checkpoint that binds and inhibits cyclin-CDK2 and –CDK4 complexes, causing cell cycle arrest.[7-9] $p27^{Kip1}$ protein levels are high during G1/G0, and decrease rapidly upon transition to S phase. Contact inhibition and mitogen deprivation in vitro induce increases in $p27^{Kip1}$ levels while mitogen stimulation causes $p27^{Kip1}$ levels to drop.[10-17] Overexpression of either a wild-type or a degradation-resistant version of $p27^{Kip1}$ inhibits cell cycle transit in vitro.[8,17-19] By contrast, antisense inhibition of $p27^{Kip1}$ prevents cell cycle arrest induced by mitogen depletion,[14] and full or partial deletion of $p27^{Kip1}$ in vivo increases cell proliferation and induces organ hypertrophy and tumorigenesis.[20-22]

Importantly, although $p27^{Kip1}$ levels have been shown to correlate with prognosis in multiple human cancers,[23-28] mutations in the $p27^{Kip1}$ gene are rare.[29-37] This observation led to the realization that $p27^{Kip1}$ protein levels are likely controlled by translation, protein sequestration, and post-translational modifications, including phosphorylation.[7,38] Phosphorylation of $p27^{Kip1}$ on T187 by CDK2 was shown to be necessary for recognition by Skp2 and subsequent degradation via the ubiquitin-proteasome system (UPS).[19,38-40] Similarly, multiple other phosphorylation sites on the $p27^{Kip1}$ protein have been implicated in modulating protein stability. For instance, phosphorylation at either S10 or T198 has actually been shown to increase the stability of $p27^{Kip1}$.[41,42] In all, 7 phosphorylation sites on the $p27^{Kip1}$ protein have been described. These include S10, Y74, Y88, Y89, T157, T187, and T198. Given the role of $p27^{Kip1}$ in controlling the cell cycle and tumorigenesis, uncovering the putative kinases mediating $p27^{Kip1}$ phosphorylation at these sites is critical.

To identify kinases controlling $p27^{Kip1}$ turnover, we adapted a protocol we utilized to identify kinases controlling degradation of the cell cycle regulator Wee1.[43] Like $p27^{Kip1}$, phospho-Wee1 is targeted for degradation via an SCF ubiquitin ligase. To identify possible kinases mediating Wee1 recognition by the ligase, we generated a Wee1-luciferase fusion protein, which we expressed in cells treated with putative small molecule kinase inhibitors. We identified one small molecule that selectively stabilized Wee1 relative to other luciferase fusion proteins. When we profiled this

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compound against 300 kinases, we found that it selectively inhibits CK1δ activity. We then showed that CK1δ phosphorylates Wee1 on N-terminal residues required for Wee1 turnover.[43]

Thus, our prior studies provided a successful workflow for identifying kinases that interact with cell cycle proteins and induce their subsequent degradation. However, these studies were dependent upon in vitro profiling of small molecules to determine their ability to inhibit or activate kinases. Here we demonstrate that *in silico* profiling of kinases inhibited by small molecules that also stabilize protein-luciferase fusions can identify signaling pathways controlling turnover of cell cycle proteins. Utilizing this approach we demonstrate that the PKC activator PMA stabilizes $p27^{Kip1}$-luciferase.

Results

Luciferase fusion proteins are stabilized by proteasomal inhibition

Several studies suggested that luciferase fusion proteins serve as accurate reporters of endogenous proteins.[44-48] In the case of cell cycle proteins, the N-terminus of cyclin B1 has been fused to luciferase and utilized in small molecule screens to identify small molecule inhibitors of the ubiquitin ligase APC/C, which controls cyclin B1 turnover.[44] Similarly, our prior studies have developed and characterized a luciferase fusion of the cell cycle kinase Wee1 to identify small molecule inhibitors that specifically affect endogenous Wee1 turnover.[45] In the case of Wee1, the small molecules inhibited kinases we described as novel regulators of Wee1 turnover.[45] Since kinases are known to control degradation of cell cycle proteins, we asked whether we could adapt our prior strategy to identify kinases controlling degradation of $p27^{Kip1}$. To do this, we created a luciferase fusion of $p27^{Kip1}$ for use in small molecule screens to identify kinases controlling endogenous $p27^{Kip1}$ turnover (**Fig. 1A**). We then determined whether this fusion protein is degraded via the UPS by measuring its steady-state level in the presence of the proteasome inhibitor MG132. As we had done previously for Wee1-luciferase, we transfected the construct encoding $p27^{Kip1}$-luciferase into HeLa cells and added either the proteasome inhibitor MG132 or 0.1% DMSO (vehicle control). As shown in **Figure 1B, C**, MG132 increased the steady-state level of $p27^{Kip1}$-luciferase. Further, we found that the screen was robust since it yielded an average Z' of 0.58 $+/-$ 0.13 and a S/B of 4.7 $+/-$ 0.4. Only one plate out of 4 did not pass Z' using $Z' > 0.5$ as a validation criteria (**Fig. 1B, C**).

Screening of LOPAC identifies compounds stabilizing luciferase fusion proteins

The observation that MG132 stabilized $p27^{Kip1}$-luciferase suggested that our screening approach could identify small molecules that inhibit degradation of this luciferase fusion protein. To identify such molecules, we expressed $p27^{Kip1}$-luciferase in HeLa cells and added small molecules from the library of pharmacologically active compounds (LOPAC). We chose this library since it contains a diverse set of compounds targeting different biological processes. As shown in **Figure 1B**, several small molecules stabilized $p27^{Kip1}$-luciferase to the same extent as MG132. When we rank-ordered these compounds based on stabilization relative to MG132, we found that phorbol 12-myristate 13-acetate (PMA) was one of the compounds that stabilized $p27^{Kip1}$-luciferase to the greatest extent (**File S1**).

PMA stabilizes multiple luciferase fusion proteins

Our screening and kinome prediction data suggest that the PKC activator PMA stabilizes $p27^{Kip1}$-luciferase. To validate our screening results further, we independently tested the effect of PMA on the steady-state levels of $p27^{Kip1}$-luciferase. We found that PMA does indeed stabilize $p27^{Kip1}$-luciferase (**Fig. 2A**), but, importantly, also stabilizes endogenous $p27^{Kip1}$ (**Fig. 2C**). Furthermore, PMA increases the levels of both $p21^{Cip1}$-luciferase and endogenous $p21^{Cip1}$ (**Figs. 2B and C**). Because Skp2 is one of the E3 ubiquitin ligases for both $p27^{Kip1}$ and $p21^{Cip1}$, we investigated whether PMA might modulate Skp2 activity. Interestingly, both 5 mM and 10 mM PMA were sufficient to decrease endogenous Skp2 to almost undetectable levels by Western analysis (**Fig. 2B**).

In silico kinase profiling of LOPAC libraries identifies putative signaling pathways controlling luciferase fusion turnover

To identify signaling pathways that may modulate the steady-state levels of $p27^{Kip1}$-luciferase, we measured the probability that compounds in the LOPAC library affect kinase activity. We used our previous predictive algorithm to identify the probability that any one compound either activates or inhibits a particular kinase.[49] As shown in **Figure 3** and **File S1**, several compounds in the LOPAC library likely bind and activate or inhibit kinases. Among these, PMA is a compound that is likely to bind PKCa, b, and d. Consistent with this prediction, several studies have shown that PMA potently activates PKCs.[41,50,51] Similarly, kenpaullone and indirubin-3′-oxime were predicted to bind GSK3β, on upstream regulator of many cell cycle proteins. Both kenpaullone and indirubin-3′-oxime have previously been implicated in interactions with GSK3b and CDKs.[52-56] These results suggest that our prediction algorithm can be supported by experimental results.

Discussion

Our studies provide a robust means of identifying kinase pathways controlling degradation of cell cycle proteins. We have demonstrated that luciferase fusions of $p27^{Kip1}$, $p21^{Cip1}$ and, previously, Wee1 can be used as reporters of endogenous protein turnover. Each of these fusion proteins was sensitive to MG132 proteasome inhibition, demonstrating that the UPS contributes to their turnover. Furthermore, PMA stabilized $p27^{Kip1}$-luciferase, which is consistent with prior studies linking PMA to both PKC activation and $p27^{Kip1}$ stabilization.[41,50,51] For instance, in 2012, De Vita et al. demonstrated that PMA activates PKCα to phosphorylate $p27^{Kip1}$ and thereby increase protein stability.[41] Consistent with these findings, an algorithm that predicts the

probability that a particular compound will bind a kinase also identified PMA as a likely regulator of PKC.

Our data, however, indicate that PMA may also inhibit degradation of other cell cycle proteins, as it also stabilized p21^{Cip1}-luciferase. Like p27^{Kip1}, p21^{Cip1} is phosphorylated to promote its proteasomal degradation (**Fig. 4**). The mechanism involves phosphorylation of the p21^{Cip1} protein on T145 by Pim-1.[57,58] However, PMA induces Pim-1 expression and, therefore, would be expected to promote p21^{Cip1} degradation, not stabilization as we observed.[59] This stabilization may instead be explained by alternative mechanisms of p21^{Cip1} regulation. Previous studies have suggested that PMA induces upregulation of Kruppel-like transcription factor 6 (KLF6) which, in turn, upregulates transcription of p21^{Cip1}.[60] Therefore, PMA-induced increases in p21^{Cip1} may indicate that transcriptional modulation plays a significant role in regulating p21^{Cip1}activity. However, given our finding that PMA induces significant Skp2 downregulation, it is likely that p21^{Cip1} protein stabilization is a secondary result of the decline in Skp2 activity. Skp2 is a common E3 ubiquitin ligase for p27^{Kip1} and p21^{Cip1}. Therefore, a decrease in Skp2 activity would not only stabilize p27^{Kip1}, but also p21^{Cip1}. Whether PMA causes Skp2 downregulation in a p27^{Kip1}-independent manner is not known, but evidence does exist to support the hypothesis that PMA stabilizes p27^{Kip1} independently of Skp2 inhibition.[41] Consistent with this possibility, we find that Skp2 inhibitors do not stabilize p27^{Kip1}-luciferase. This may suggest that the second ubiquitin ligase, KPC, known to modulate p27^{Kip1} levels may be operational in our system.[61]

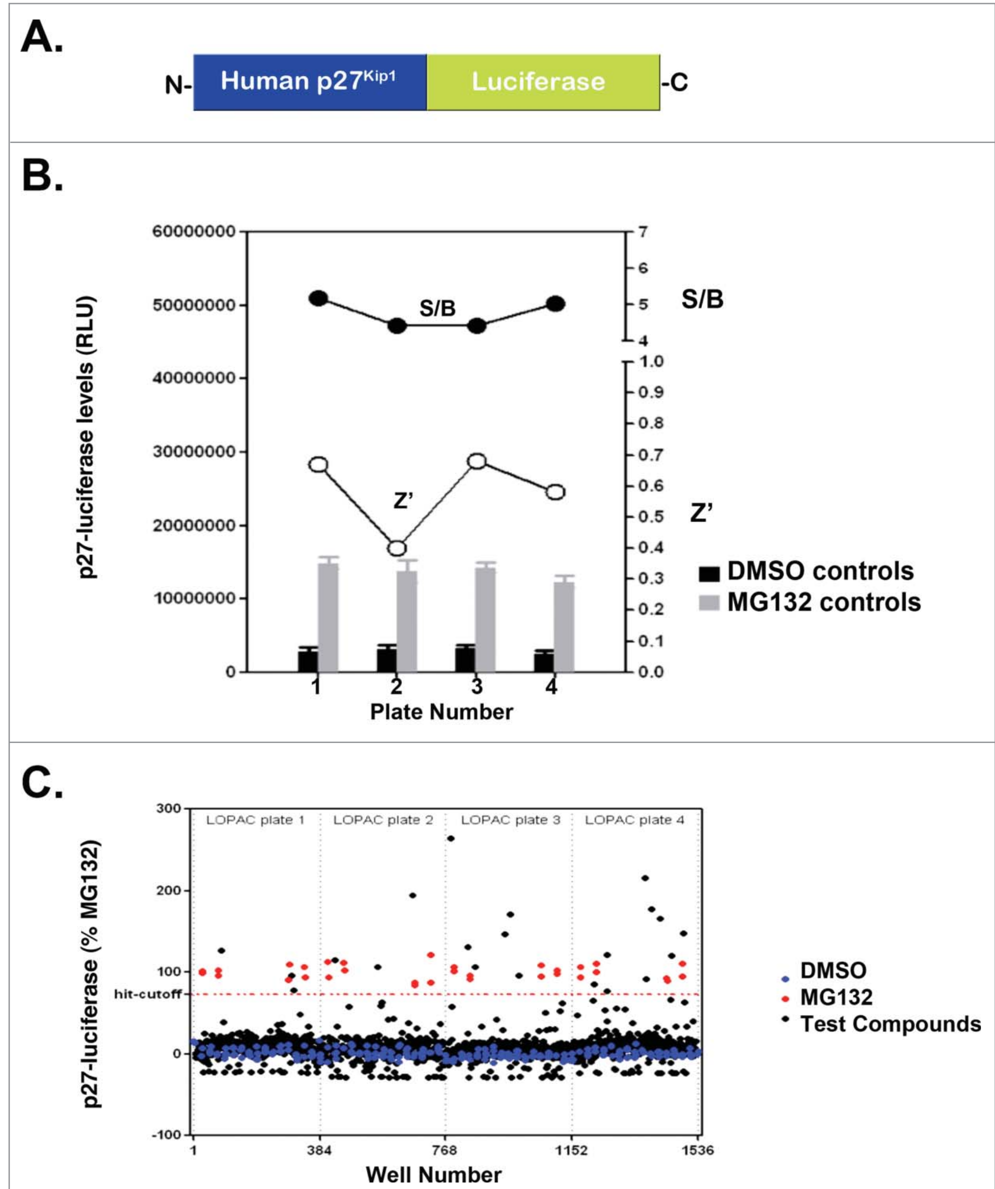

Figure 1. p27^{Kip1}-luciferase is stabilized by compounds from the Library of Pharmacologically Active Compounds and MG132. (**A**) Diagram of p27^{Kip1}-luciferase used in our studies. Note that luciferase is attached to the C-terminus of full-length human p27^{Kip1}. (**B**) HeLa cells were transfected with p27^{Kip1}-PGL4 plasmids and incubated with either MG132 or DMSO (vehicle control). After 24 hours, cells were lysed using BriteLite and the steady-state levels of luciferase measured. Results are from one representative experiment performed in quadruplicate. (**C**) p27^{Kip1}-luciferase is stabilized by compounds in the Library of Pharmacologically Active Compounds (LOPAC). HeLa cells were transfected with the p27^{Kip1}-luciferase plasmid and incubated with compounds from a library of pharmacologically active compounds (LOPAC). Cells were then lysed after 24 hours and the steady-state levels of luciferase measured. The hit-cutoff based on the average +3 SD calculation method was 73.16%. Based on this cutoff, 21 compounds were designated as hits, i.e., a hit rate of 1.64% (21/1280), which is in the expected range of 1–2% when screening a library of pharmacologically active compounds (otherwise a hit rate of $<$1% is expected). Further, there were no false positives among the DMSO wells and therefore the assay is not prone to false positives. The plate-to-plate variability of the raw luminescence values was kept below $<$15% (12% CV for DMSO control from one plate to another, 8% CV for the MG132 controls).

It is possible that PMA activates PKCs to phosphorylate and stabilize p27^{Kip1}. Increased p27^{Kip1} levels could then cause cell cycle arrest in G1, degradation of Skp2, and stabilization of p21^{Cip1}. Conversely, it is possible that PMA causes the degradation of Skp2, and the loss of Skp2 activity could increase p27^{Kip1}

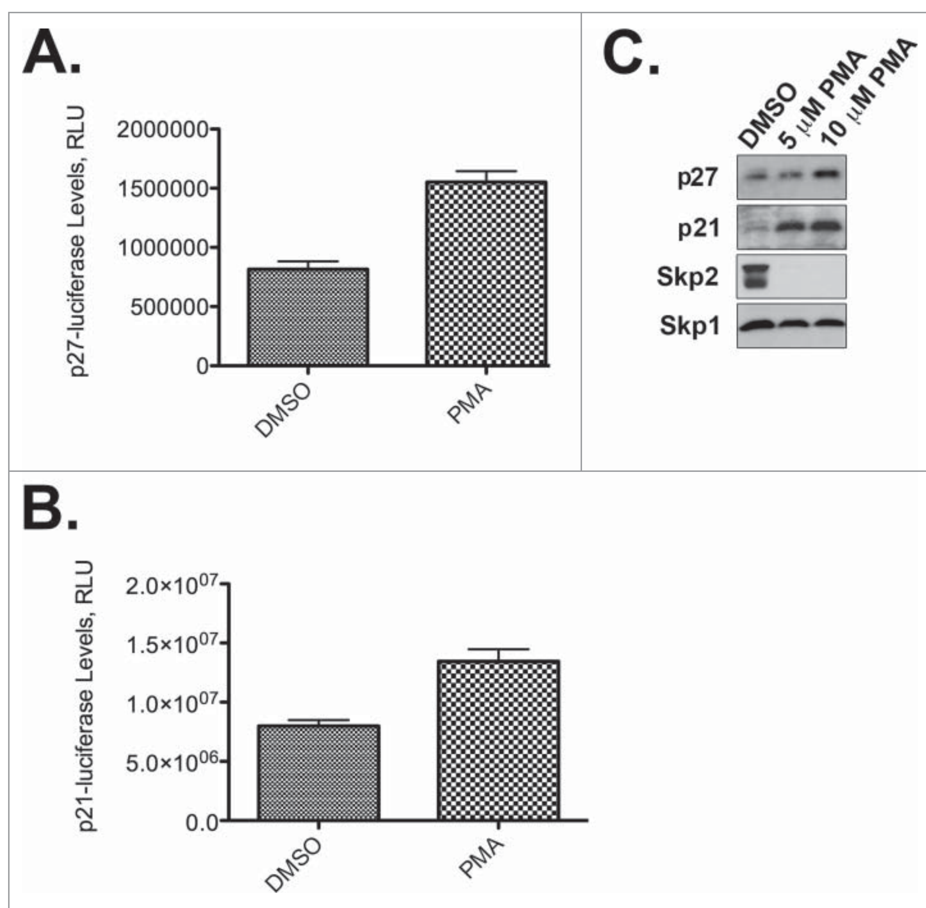

Figure 2. PMA stabilizes $p27^{Kip1}$ and $p21^{Cip1}$. (**A**) PMA stabilizes $p27^{Kip1}$-luciferase. Results are from one representative experiment performed in quadruplicate. (**B**) PMA stabilizes $p21^{Cip1}$-luciferase. Results are from one representative experiment performed in quadruplicate. (**C**) PMA stabilizes endogenous $p21^{Cip1}$ and $p27^{Kip1}$ and causes degradation of Skp2. HeLa cells were incubated with the indicated concentrations of PMA and processed for $p21^{Cip1}$, $p27^{Kip1}$, Skp2, or Skp1 immunoblotting. Skp1 is a loading control.

and $p21^{Cip1}$ stability. The question of which comes first is difficult to deduce from these cell-based screening approaches because of the interdependence of these proteins and the cell cycle. However, it is known that PMA activates PKCs and PKCα phosphorylates and stabilizes $p27^{Kip1}$.[41] An interaction between PMA and Skp2 has not been reported.

In addition to PMA, the small molecules kenpaullone and indirubin-3′-oxime stabilized $p21^{Cip1}$-luciferase and $p27^{Kip1}$-luciferase (**File S1**). Our *in silico* prediction suggested that kenpaullone and indirubin-3′-oxime are both potent inhibitors of CDKs and GSK3b. In addition, several studies suggested that kenpaullone and indirubin-3′-oxime are CDK/GSK3b inhibitors.[53,54,56,62,63] Further, CDKs have been implicated in $p27^{Kip1}$ turnover and GSK3b has been shown to control degradation of several cell cycle proteins.[52,55] These findings further support that our workflow can identify kinases implicated in cell cycle protein turnover.

One major consideration with our workflow is that compounds may be acting indirectly to stabilize cell cycle protein-luciferase fusions. For instance, one important possibility to rule out is that PMA, kenpaullone, and indirubin-3′-oxime may be acting not on the cell-cycle proteins, but the fused luciferase enzyme. However, these compounds do not affect luciferase as determined after searching the PubChem database. Another caveat with our screening approach is that compounds may arrest cells in a phase where the luciferase fusion proteins are most stable. This would confound the interpretation that the stabilization of the fusion proteins observed with small molecule treatment was due to interference with their degradation. Rather, some other phase-specific factor may be acting independently of the compound itself. However, in the case of PMA, convincing evidence exists that its mechanism of action includes activation of PKCα and phosphorylation of $p27^{Kip1}$ to decrease its degradation and cause cell cycle arrest.[41]

One means to identify novel kinases controlling phosphorylation of $p27^{Kip1}$ is to screen larger compound libraries. Our workflow can be adapted to include more compounds in order to identify multiple kinases inducing protein turnover. The compounds tested in the initial luciferase-fusion protein screen were limited to the 1280 compounds included in the LOPAC. With the screening of increasing numbers of compounds, one may expect to uncover additional interactions. However, by screening known pharmacologically active compounds, the probability of finding interactions is already many-fold greater than libraries that have not been built on known pharmacological activity. Collectively, our studies suggest that similar parallel screenings of cell-cycle fusion proteins may identify kinase cascades controlling protein turnover.

Materials and Methods

BriteLite assays

In a 50 mL conical, 87 uL *Trans*IT-LT1 Transfection Reagent (Mirus Bio, MIR2300) was mixed with 29 ug of luciferase conjugated-protein plasmid DNA and OptiMEM I Reduced-Serum Medium up to a final volume of 12 mL. This mixture incubated at room temperature for 20 minutes to allow complexes to form. In a second conical, 6×10^6 HeLa cells were resuspended in a final volume of 12 mL complete medium (10%

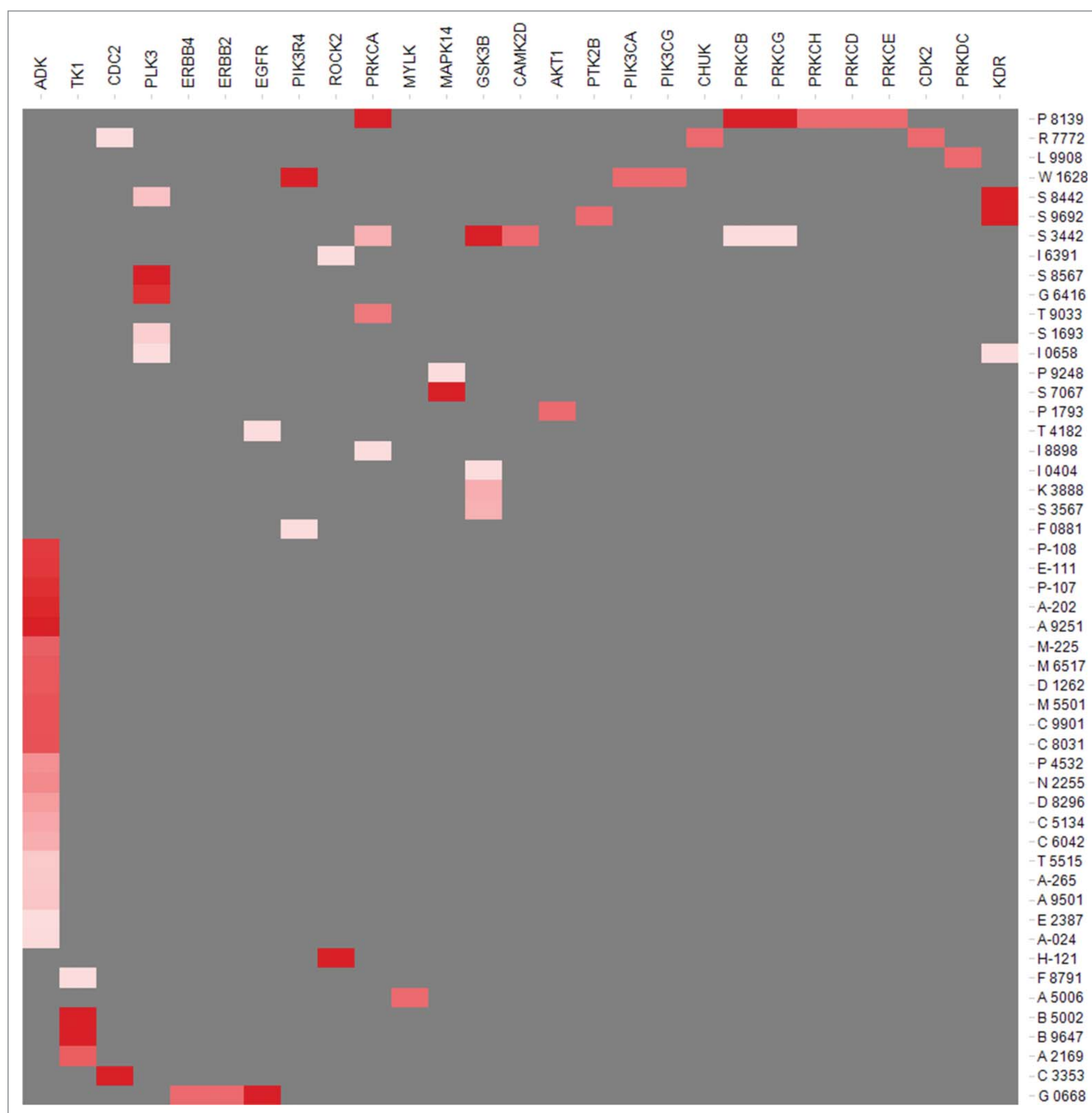

Figure 3. Estimated probability of a compound (right) being active (for 51 LOPAC compounds) against a set of kinases (top). The color intensity represents the probability value. Red is highest probability. P8139 refers to PMA.

FBS, 1% penicillin/streptomycin, DMEM). The two conicals were then combined into a flask and incubated 48 hrs, 37°C, 10% CO_2 in cell culture incubator. Transfected cells were then resuspended, passed through a cell filter, and counted. Complete medium was used to dilute the cells to 8.5 × 10^5 cells/mL and 50 μL of this suspension was added to each well of a 96-well plate. An additional 50 μL of 20 mM drug was added for a final concentration of 10 uM, and the cells were returned to the incubator overnight. Finally, 100 μL of reconstituted BriteLite plus (Perkin Elmer, #6066761) was added to each well and mixed well before reading on a Perkin Elmer EnVision 2104 Multilabel Reader.

In silico kinome profiling

For the purpose of computational kinase profiling, we used Laplacian-corrected naïve Bayesian classification models previously build in house.[49] The models were based on the Kinase Knowledge Base (KKB) data, including almost 500,000 tested kinase/compound pairs extracted from literature and patents. Data were subject to rigorous aggregation, standardization, and clustering procedures that resulted in over 180 distinct data sets covering all major groups of the human kinome. For each kinase in the data set, active and inactive compounds were described using extended connectivity fingerprints. Compounds considered inactive in the model training were either known inactives of a

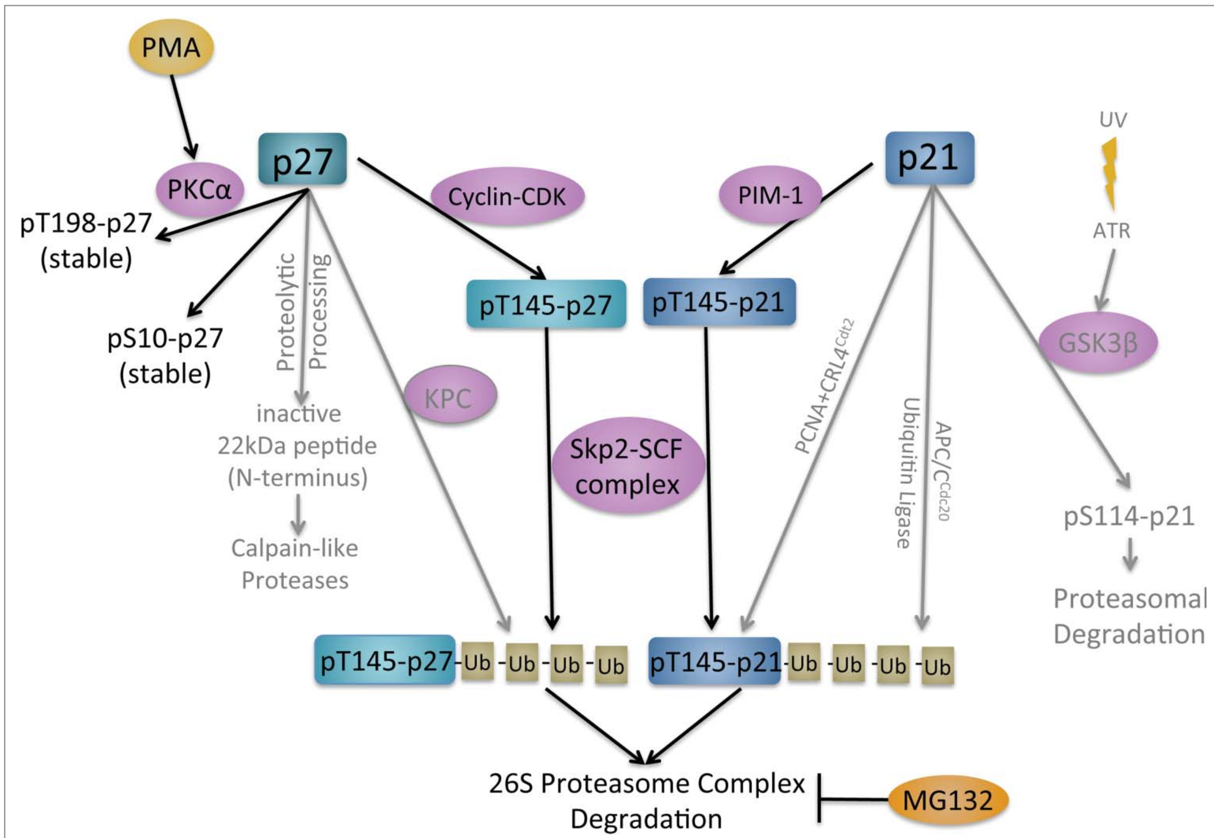

Figure 4. Model of kinase and proteolysis pathways controlling $p21^{Cip1}$ and $p27^{Kip1}$ degradation.

given kinase, or taken as the entire set of molecules not tested on that kinase. Rigorous cross-validation and characterization was performed and demonstrated highly predictive classification and quantitative models for the majority of kinase targets if a minimum required number of active compounds or structure-activity data points were available.

For the purposes of this study, we selected models based on 25 data points with 10 being active and applied them to the LOPAC library to predict compound kinase profiles. We identified 72 compounds with the estimated probability that the compound is in the active category (EstPGood) of 0.05 (data provided in Supporting Material), while 51 compounds had an EstPGood value above 0.10. These 51 compounds are shown in **Figure 3**.

PAM

In order to identify PAM screening results from PubChem,[64] we performed "Phorbol 12-myristate 13-acetate" search in the PubChem compound field. Three unique CIDs were identified with 2 corresponding to the 2 PAM stereoisomers (CID 27924 and CID 122634) while the third compound (CID 4792) has no stereochemistry annotated. All corresponding SIDs were identified and collected. That resulted in the total of 126 unique SIDs. The SIDs were matched to the previously annotated luciferase assays[65,66] and 20 screening data points were identified.

Similarly, kenpaullone was also identified via PubChem compound filed and the search resulted in one compound (CID 3820) and 75 SIDs. They were compared to the luciferase assay data and 69 data points were identified.

For Indirubin-3′-oxime the PubChem CID 5326739 was identified in the same way as described above. The CID corresponded to 101 SIDs and 71 data points in the luciferase assays.

Protein extract preparation, antibodies and western blot

Cells were homogenized and extracts were prepared using lysis buffer (50 mM Tris, 150 mM NaCl, 1 % Triton X-100, 1× Protease Inhibitor Cocktail, 1 μM Microcystin LR). Cells were lysed by the freeze-thaw method (liquid nitrogen/37°C water bath) and further sonicated. The soluble fraction was recovered by centrifugation at 14,000 RPM for 20 min at 4°C. Protein concentration was measured with the BCA Protein Assay kit (Pierce) and 30 μg of protein from each sample was resolved by SDS-PAGE. The resolved bands were transferred onto a nitrocellulose membrane by Western blotting and then probed with relevant antibodies. Primary Antibodies: anti-p21 (Abcam, ab7960), anti-p27 (Santa Cruz

Biotechnology, sc-776), anti-Skp1 p19 antibody (H-163, Santa Cruz Biotechnology, sc-7163); secondary antibody: anti-rabbit IgG-HRP antibody from GE Healthcare (Cat # NA9340V).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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