PHLDA1 is a crucial negative regulator and effector of Aurora A kinase in breast cancer

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Summary

Aurora A kinase is overexpressed in the majority of breast carcinomas. A chemical genetic approach was used to identify the malignant targets of Aurora A, which revealed pleckstrin-homology-like domain protein PHLDA1 as an Aurora A substrate. PHLDA1 downregulation is a powerful prognostic predictor for breast carcinoma, which was confirmed in our study. We further show that downregulation of PHLDA1 is associated with estrogen receptor (ER) expression in breast carcinoma. Aurora A directly phosphorylates PHLDA1 leading to its degradation. PHLDA1 also negatively regulates Aurora A, thereby triggering a feedback loop. We demonstrate the underlying mechanisms by which PHLDA1 upregulation strongly antagonizes Aurora-A-mediated oncogenic pathways, thereby revealing PHLDA1 degradation as a key mechanism by which Aurora A promotes breast malignancy. Thus, not surprisingly, PHLDA1 upregulation acts synergistically with Aurora A inhibition in promoting cell death. PHLDA1 overexpression might therefore be an alternative method to modulate Aurora A deregulation in breast carcinoma. Finally, this study led to the discovery of a mutation in the Aurora A active site that renders it amenable to the chemical genetic approach. Similar mutations are required for Aurora B, suggesting that this modified approach can be extended to other kinases that have hitherto not been amenable to this methodology.

Key words: Aurora kinase, Cancer, Chemical biology, PHLDA1, Analog-sensitive kinase

Introduction

The serine/threonine protein kinase 6 Aurora A is overexpressed in a high proportion of pre-invasive and invasive breast carcinomas (Tanaka et al., 1999; Miyoshi et al., 2001; Nadler et al., 2008). *AURKA* is one of the genes in the Oncotype Dx assay used for predicting the likelihood of breast cancer recurrence in early-stage, node-negative, estrogen-receptor-positive breast cancer (Cronin et al., 2007). It was the most important gene for predicting breast cancer outcome across multiple datasets in a 3D culture model (Martin et al., 2008). In a study of 638 breast cancer patients, high Aurora A expression was strongly associated, even after multivariate analysis, with node status and decreased survival (Miyoshi et al., 2001). Polymorphisms in the *AURKA* are also associated with an increased risk of breast cancer (Sun et al., 2004; Cox et al., 2006) and appear to work synergistically with prolonged estrogen exposure (Dai et al., 2004). In animal models, Aurora A overexpression induced tumor formation, and its inhibition significantly reduced tumor multiplicity and size (Wang et al., 2006). *AURKA* is also amplified in other types of cancers (Mountzios et al., 2008). Data such as these have resulted in an ongoing Phase II clinical trial of MLN8237, an orally available and potent inhibitor of Aurora A, in advanced solid tumors.

Despite the demonstrated potential of Aurora A as a cancer target, the underlying molecular mechanisms of Aurora-A-associated malignancy remain elusive. This information is critical for developing pharmacodynamic biomarkers for Aurora-A-targeted drugs in clinical trials, developing biomarkers predictive of breast cancer progression and selective targeting of critical malignant effectors of Aurora A independently, or in combination with Aurora A, in breast cancer.

In normal cells, Aurora A is expressed during the G2 and M phases of the cell cycle and localizes at the centrosome and mitotic spindle poles (Hirota et al., 2003). By contrast, in breast tumors, Aurora A is overexpressed in all phases of cell cycle, with a diffuse cytoplasmic distribution. Thus, aberrant phosphorylation of cytoplasmic proteins by mislocalized Aurora A is hypothesized to promote malignancy. More than a dozen Aurora A substrates are known (Mao et al., 2007; Katayama et al., 2001; Katayama et al., 2004; Katayama et al., 2007; Kunitoku et al., 2003; Ouchi et al., 2004; Toji et al., 2004; Cazales et al., 2005; Wu et al., 2005; LeRoy et al., 2007; Mori et al., 2007; Rong et al., 2007; Yu et al., 2005; Jang et al., 2008; Venoux et al., 2008), but few have been identified as potential targets in cancer. With the exception of BRCA1, none are known in breast cancer.

The goal of the present study was to identify cancer-related targets of Aurora A in breast cancer cells and use them to unravel the mechanisms by which it promotes breast malignancy. We used a chemical genetic approach that uses an analog-sensitive kinase and orthogonal ATP analog for global search of Aurora A substrates (Shah et al., 1997; Shah and Shokat, 2002; Shah and Shokat, 2003; Shah and Vincent, 2005; Kim and Shah, 2007; Sun et al., 2008a; Sun et al., 2008b; Chang et al., 2011). Analog-sensitive kinase is generated by the replacement of a conserved bulky residue (gatekeeper residue) in the kinase subdomain V with a glycine (analog-sensitive-1, as1). A complementary substituent on ATP is created by attaching bulky substituents at the N^6 position of ATP (e.g. N^6-benzyl ATP, N^6-phenethyl ATP, etc.). Because the ATP analog is not accepted by other wild-type kinases in the cells, this strategy allows for unbiased identification of direct substrates of any kinase in a global environment.

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An analog-sensitive mutant of Aurora A (Aurora-A-as1, L201G-Aurora-A) was generated; however, it poorly accepted the orthogonal ATP analogs. This led to the discovery of a mutation that renders Aurora A and Aurora B highly sensitive to orthogonal ATP analogs and PP1-derived inhibitors (Bishop et al., 1999). Using this modified strategy, several Aurora A substrates were identified, including known Aurora A substrates vimentin and p53. PHLDA1, a novel putative Aurora A substrate, was followed up in this study, which revealed a new mechanism by which Aurora A might promote breast malignancy.

Results

Cloning and characterization of analog-sensitive Aurora A kinase

An analog-sensitive mutation was created in the Aurora A active site by replacing the gatekeeper residue L201 (murine Aurora A numbering) with a glycine residue (AA-as1 kinase). To identify the most optimal orthogonal phospho-donor for the engineered kinase, several [γ-^{32}P]ATP analogs were synthesized and screened using wt Aurora A and engineered AA-as1 kinase. Aurora A kinase assay was conducted in the presence of 6-His tagged-TPX2(1–42), which is an Aurora A activator. Although AA-as1 kinase displayed high kinase activity, it poorly accepted any of the ATP analogs (data not shown). These results were surprising, because mutation of this single gatekeeper residue to G or A has been shown to confer analog-sensitivity in over 30 kinases (Shah et al., 1997; Shah and Shokat, 2002; Shah and Shokat, 2003; Shah and Vincent, 2005; Kim and Shah, 2007; Sun et al., 2008a; Sun et al., 2008b; Chang et al., 2011).

Engineering a novel mutation in Aurora A kinase: generation of a new analog-sensitive AA-as7 kinase

For the rational design of a mutant Aurora A that is sensitive to orthogonal ATP analogs, modeling studies were conducted using the published crystal structure of human Aurora A bound to ADP (at 2.5 Å resolution) (Nowakowski et al., 2002). The goal was to introduce subtle changes in the ATP binding pocket already possessing the gatekeeper mutation (L201G). The residues considered for mutagenesis had to be located near the N^6 position of the adenine ring, because the gatekeeper residue is in close contact with the N^6 position. Furthermore, ATP analogs possessing bulky groups are modified at N^6. These criteria suggested that an additional mutation at L185 (murine Aurora A numbering) to a smaller residue would allow the engineered kinase to use orthogonal ATP analogs. L185 is within 4 Å and gatekeeper L201 within 5 Å of the N^6 amino group (Fig. 1A, human Aurora A numbering: L194 and L210, respectively). Sequence alignment of Aurora A with other kinases including v-Src revealed that most kinases engineered previously to generate analog-sensitive alleles possess a Val residue at this position. Interestingly, Ipl1 kinase, a yeast homolog of aurora kinases, possesses a Thr residue at this position, which is isosteric to Val (Pinsky et al., 2006) (Fig. 1B). We postulated that the combined mutation of these two residues (L185V, L201G, murine numbering) would produce a mutant possessing a hydrophobic cavity that would enable it to accept orthogonal ATP analogs and inhibitors (Fig. 1C,D). In our very first study describing the chemical genetic approach using v-Src kinase, we mutated this residue (Val323) to an alanine (V323A), along with the gatekeeper mutation (I338A), to confer analog sensitivity (Shah et al., 1997). However, later, we found that V323A mutation was not required to generate analog and inhibitor-sensitive kinases (Shah and Shokat, 2002; Shah and Shokat, 2003; Shah and Vincent, 2005).

A double mutant of Aurora A (L185V, L201G) was generated and expressed in insect cells. Because previous studies have identified several mutations for generating analog-sensitive kinases (as-1, as-2, as-3, as-4, as-5, etc.), we named this new mutant AA-as7.

Catalytic efficiency of wild-type and AA-as7 kinases

Kinase assays were conducted in the presence of TPX2 and γ-^{32}P-labeled N^6-modified ATP analogs (Shah et al., 1997), which revealed N^6-phenethyl ATP as the most optimal orthogonal phosphodonor for AA-as7 kinase (Fig. 1D). The catalytic efficiency of the AA-as7 kinase with N^6-phenethyl ATP (A*TP) (K_{cat}/K_m=1.16×10^3 minute^{-1} M^{-1}) was comparable to the efficiency of the mutant with ATP (K_{cat}/K_m=1.84×10^2 minute^{-1} M^{-1}). More importantly, the catalytic efficiency of AA-as7 was similar to the efficiency of wild-type AA with ATP (K_{cat}/K_m=1.16×10^2 minute^{-1} M^{-1}) (Table 1).

Identification of the optimal orthogonal inhibitor for AA-as1 and AA-as7 kinases

A set of orthogonal 1-*tert*-butyl-3-phenylpyrazolo[3,4-*d*]pyrimidine-derived inhibitors (PP1-derived inhibitors) were synthesized and screened against the engineered kinases AA-as1 and AA-as7 to identify the most potent inhibitor as reported before (Bishop et al., 1999). AA-as1 kinase was poorly inhibited, similarly to the results obtained using ATP analogs, suggesting that the gatekeeper mutation alone is not enough to confer PP1-derived inhibitor sensitivity to Aurora A kinase (data not shown). However, AA-as7 kinase was strongly inhibited. 4-amino-1-*tert*-butyl-3-(1′-naphthylmethyl)pyrazolo[3,4-*d*]pyrimidine (1-NM-PP1) was identified as the most potent and selective inhibitor of the AA-as7 kinase (IC_{50}=1.7 nM).

Characterization of a new analog-sensitive mutant of Aurora A (AA-as7)

Previous studies have shown that Aurora A overexpression in NIH3T3 cells causes cellular transformation, which depends on its kinase activity (Zhou et al., 1998) Therefore, wild-type Aurora A and AA-as7 were overexpressed in NIH3T3 cells at similar levels to evaluate their relative transformation efficiency. NIH3T3 cells expressing wild-type or mutant Aurora A showed similar transformation potential in a soft agar assay (Fig. 1E), suggesting that the double mutations in AA-as7 kinase are functionally silent.

1-NM-PP1 inhibits colony formation in AA-as7 NIH3T3 cells

Our in vitro data showed that 1-NM-PP1 is highly potent and selective for AA-as7 kinase. To confirm this specificity in cells, AA-as7 NIH3T3 cells were treated with 0.5 μM 1-NM-PP1, which completely inhibited colony formation (Fig. 1E). Under identical conditions, cells expressing wild-type Aurora A demonstrated robust colony formation in soft agar assay (Fig. 1E). This result confirmed that 1-NM-PP1 is highly orthogonal and only inhibits AA-as7 kinase in cells.

Aurora B also requires double mutations for generating an analog-specific mutant

We next generated Aurora-B-as1 kinase (AB-as1) by mutating gatekeeper L154 to G. Similarly to the AA-as1 mutant, the AB-as1 mutant also poorly accepted ATP analogs and PP1-derived inhibitors (data not shown). Because Aurora B also possesses a Leu residue at 138 position (equivalent to L185 of Aurora A) (Fig. 1B), it was

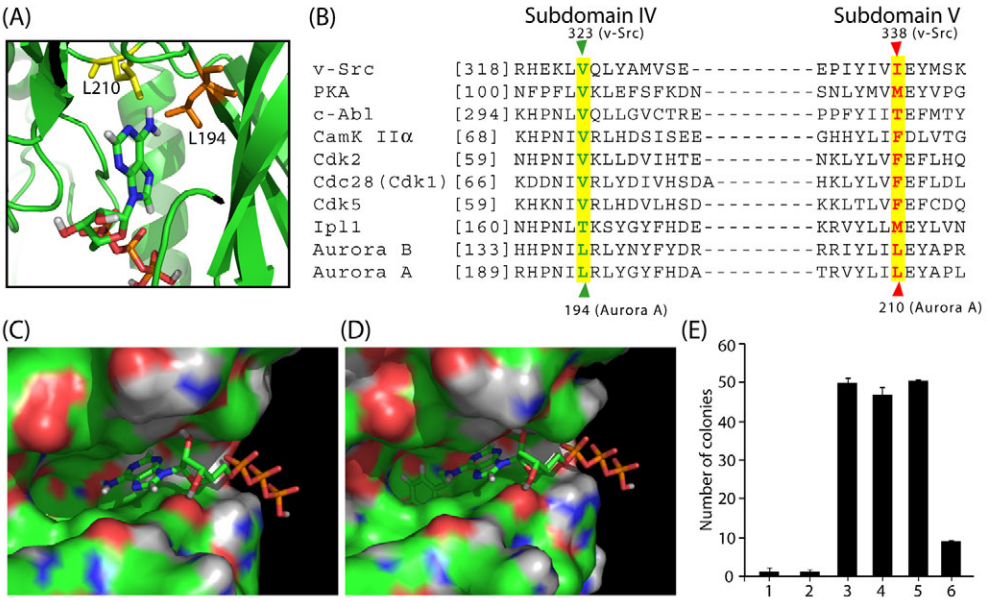

Fig. 1. A novel chemical genetic screen for Aurora A. (**A**) A close-up view of the ATP binding site in human Aurora A (1MQ4). Two residues within a 5 Å (0.5 nm) sphere of the N^6-amine of ATP (L194, L210, human numbering) are shown. ADP, L194 and L210 are shown in stick representation. The remainder of the protein is in ribbon format. L210 (yellow) is the gatekeeper residue. L194 (red) is the new residue identified in this study. Human residue L210 corresponds to L201 in murine Aurora A, and human L194 corresponds to mouse L185. (**B**) Sequence alignment of the ATP binding regions of several kinases. The residues shown in bold red correspond to the gatekeeper residue. The residue shown in bold green in Aurora A is within 4 Å of the N^6-amine of ATP in Aurora A. Most kinases have Val at this position. Ipl1 has a Thr at this position, which is isosteric with Val. All kinases shown here, with the exception of Ipl1, Aurora A and Aurora B have been previously engineered using a single gatekeeper mutation to generate analog-sensitive mutants. Ipl1 needs an extra mutation at T244A in subdomain VII (not shown in this figure) (Pinsky et al., 2006). Murine Aurora A and human Aurora B sequences are shown. (**C**) Docking of ATP was carried out using MacroModel. Amino acids L185 and L201 (murine Aurora A numbering) were mutated to Valine and Glycine residues, respectively. The resulting structure was then energy minimized to yield a unique favorable conformation visualized by pyMOL. (**D**) Docking of N^6-phenethyl ATP was achieved using MacroModel. (**E**) 1-NM-PP1 selectively inhibits colony formation in AA-as7 kinase-expressing NIH3T3 cells. Soft agar experiments were conducted as described in the Materials and Methods. Column 1, NIH3T3 and DMSO; column 2, NIH3T3 and 1-NM-PP1; column 3, AA-NIH3T3 and DMSO; column 4, AA-NIH3T3 and 1-NM-PP1; column 5, AA-as7-NIH3T3 and DMSO; column 6, AA-as7 and 1-NM-PP1.

mutated to Val and the double mutant (L138V, L154G) analyzed. AB-as7 (L138V, L154G-AB) kinase showed the highest catalytic efficiency with N^6-phenethyl ATP, which was comparable to that in the wild type with normal ATP (Table 2).

These results suggest that residue L185 is an important determinant of analog sensitivity in kinases. A small decrease in the side chain from Leu to Val is essential (in addition to the gatekeeper mutation) for generating a pocket capable of accepting orthogonal ATP analogs and inhibitors. Although a majority of protein kinases possess a Val at this position, there are a few that contain either Ile or Leu. Thus, the next generation mutant strategy (as7 kinases) should to be beneficial in conferring analog sensitivity to this class of kinases.

PHLDA1 is an Aurora A substrate

An in vitro kinase reaction was performed using unsynchronized MDA-MB-231 cell lysate, [γ-^{32}P]N^6-phenethyl ATP, AA-as7 and TPX2 to identify novel Aurora A substrates. Addition of N^6-phenethyl ATP to the cell lysate alone showed no signal, as expected (lane 1, Fig. 2A). N^6-phenethyl ATP is specific for the engineered kinase and is not accepted by wild-type kinases present in the cell lysate. By contrast, when AA-as7 and TPX2 were added to the cell lysate along with N^6-phenethyl ATP, it phosphorylated several proteins (Fig. 2A, lane 2). These proteins are the direct targets of Aurora A. To isolate these targets, cell lysates were fractionated using ion-exchange chromatography. Different fractions were dialyzed, concentrated and subjected to in vitro kinase assays (Sun

Table 1. Kinetic data of wild-type Aurora A and AA-as7 with ATP and N^6-phenethyl ATP

	AA-as7		Wild-type Aurora A
	N^6-phenethyl ATP	ATP	ATP
V_{max}	3.02×10^6	7.31×10^5	4.24×10^5
K_m (μM)	11.58	17.70	16.21
K_{cat} (minute^{-1})	1.34×10^4	3.25×10^3	1.88×10^3
E_{total} (μg)	225	225	225
K_{cat}/K_m	1.16×10^3	1.84×10^2	1.16×10^2

Table 2. Kinetic data of wild-type Aurora B and AB-as7 with ATP and N^6-phenethyl ATP.

	AB-as7		Wild-type Aurora B
	N^6-phenethyl ATP	ATP	ATP
V_{max}	7.44×10^4	3.30×10^4	5.12×10^4
K_m (μM)	11.4	30.4	23
K_{cat} (minute^{-1})	372	165	256
E_{total} (μg)	200	200	200
K_{cat}/K_m	32.63	5.42	11.13

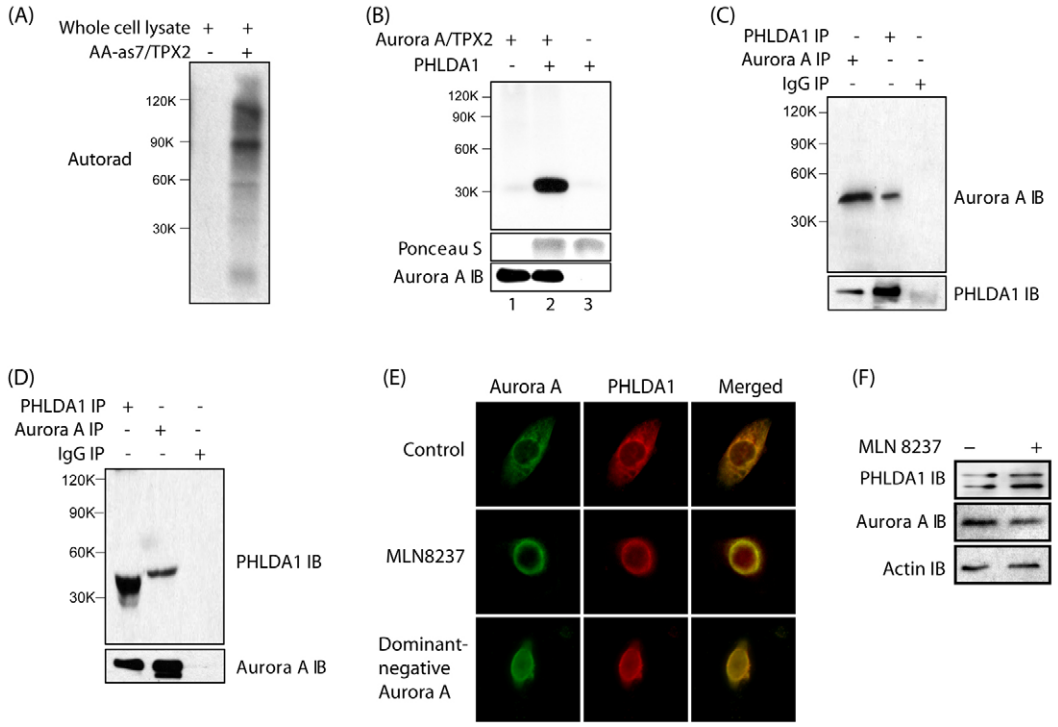

Fig. 2. Chemical genetic screen reveals PHLDA1 as a direct substrate of Aurora A. (**A**) Phosphorylation of direct Aurora A substrates using AA-as7, TPX2 and [γ-^{32}P]N^6-phenethyl ATP. Kinase reactions were conducted using unsynchronized MDA-MB-231 cell lysate in the presence of 100 μM ATP and N^6-phenethyl ATP in the absence (lane 1) or presence of 6-His–AA-as7 and TPX2 (lane 2) for 15 minutes at room temperature. (**B**) PHLDA1 is directly phosphorylated by Aurora A. 6-His–PHLDA1 was incubated with [γ-^{32}P]ATP in kinase buffer for 15 minutes either alone (lane 3), or with 6-His–Aurora-A and 6-His–TPX2 (lane 2) as described in the Materials and Methods. Lane 1 shows Aurora A and TPX2 with [γ-^{32}P]ATP, but without PHLDA1. (**C**) Aurora A and PHLDA1 associate in MDA-MB-231 cells. PHLDA1 was immunoprecipitated from MDA-MB-231 cells, and Aurora A binding analyzed (lane 2). Aurora A and IgG immunoprecipitates were used as positive and negative controls respectively (lanes 1 and 3). (**D**) Aurora A was immunoprecipitated from MDA-MB-231 cells, and PHLDA1 binding analyzed (lane 2). PHLDA1 and IgG immunoprecipitates were used as positive and negative controls respectively (lanes 1 and 3). (**E**) Aurora A regulates PHLDA1 cellular localization in MDA-MB-231 cells. Unsynchronized MDA-MB-231 cells were either treated with DMSO (top panel) or 1 μM MLN8237 for 12 hours (middle panel) or transfected with dominant-negative Aurora A for 30 hours, fixed and immunostained with Aurora A and PHLDA1 antibodies as described in the Materials and Methods. More than 100 cells were analyzed from multiple random frames. Representative data are shown. (**F**) Aurora A inhibition using MLN8237 (1 μM) for 4 hours increases unphosphorylated PHLDA1 (lower band). The two bands of PHLDA1 were separated using 10% SDS-PAGE gel.

et al., 2008b; Chang et al., 2011). The proteins were separated using 2D gel electrophoresis, isolated and visualized by autoradiography. Radiolabeled proteins were excised from the gel and, after trypsin digestion, the peptide cleavage products were identified by tandem mass spectrometry. This study identified several Aurora A substrates including PHLDA1, p53 and vimentin. In this study, we followed pleckstrin homology-like domain, family A, member 1 (PHLDA1/TDAG51) as a potential target of Aurora A. Expression of *PHLDA1* has been shown to be downregulated in the majority of breast cancer tumors and is a strong predictor of poor prognosis for breast cancer patients (Nagai et al., 2007).

PHLDA1 is directly phosphorylated by Aurora A

Because proteomics screening can often lead to false positives, we investigated Aurora-A-mediated phosphorylation of PHLDA1 using an in vitro kinase assay. PHLDA1 was generated as 6-His fusion protein and subjected to an in vitro kinase assay with Aurora A and TPX2. Aurora A directly phosphorylated PHLDA1 (Fig. 2B, lane 2).

PHLDA1 and Aurora A associate in MDA-MB-231 cells

Kinase substrate specificity in vivo is maintained by subcellular localization and protein–protein interactions. As a result, kinase assays using fractionated cell lysates might lead to artifacts. Therefore, the association between PHLDA1 and Aurora A was analyzed within cells. PHLDA1 immune complexes were isolated and Aurora A binding determined. Aurora A was co-immunoprecipitated with PHLDA1 (Fig. 2C). Similar results were obtained when the Aurora A immune complex was isolated and PHLDA1 binding analyzed (Fig. 2D). These findings show that Aurora A and PHLDA1 associate in MDA-MB-231 cells.

Subcellular localization of Aurora A and PHLDA1 was examined in unsynchronized MDA-MB-231 cells using immunofluorescence, which revealed a cytoplasmic localization for both (Fig. 2E, top panel). This result is consistent with previous findings showing diffuse cytoplasmic distribution of overexpressed Aurora A in cancer cells (Das et al., 2010). We next investigated whether Aurora A regulates subcellular localization of PHLDA1 using an Aurora-A-selective inhibitor, MLN8237 (Tomita and Nori, 2010). Inhibition of Aurora A caused perinuclear localization of both Aurora A and PHLDA1 (Fig. 2E, middle panel). To confirm this finding, dominant-negative Aurora A was transiently expressed, which also caused perinuclear localization of Aurora A and PHLDA1 (Fig. 2E, bottom panel). These findings show not only that Aurora A and PHLDA1

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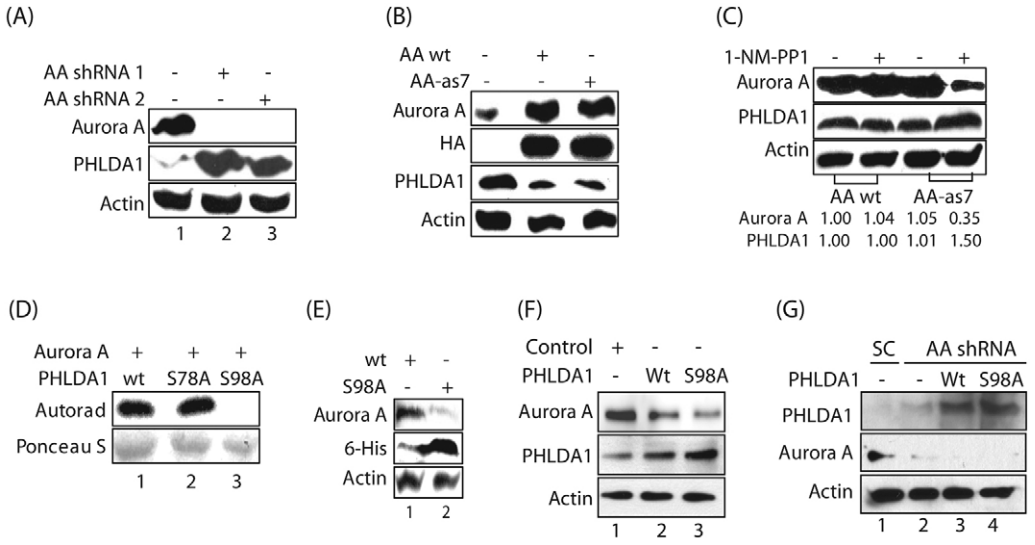

Fig. 3. Aurora A negatively regulates PHLDA1 protein levels. (**A**) Aurora A ablation upregulates PHLDA1 in MDA-MB-231 cells. MDA-MB-231 cells were transfected with scrambled shRNA (lane 1), Aurora-A-specific shRNA1 (lane 2) and Aurora A shRNA2 (lane 3) and Aurora A and PHLDA1 levels analyzed after 30 hours. Actin was used as loading control. (**B**) Aurora A overexpression decreases PHLDA1 levels. Wild-type HA-tagged Aurora A-MDA and mutant AA-as7-MDA cells were generated by infecting the corresponding retrovirus, followed by puromycin selection. Aurora A and PHLDA1 levels were analyzed in MDA-MB-231, and Aurora A-MDA cells, using β-actin as a control. (**C**) Inhibition of Aurora A kinase using 1-NM-PP1 activity upregulates PHLDA1 levels. Aurora A-MDA and AA-as7-MDA cells (AA and AA-as7) were treated with either DMSO or 250 nM 1-NM-PP1 for 12 hours and Aurora A and PHLDA1 levels analyzed. (**D**) Aurora A phosphorylates PHLDA1 at Ser 98. 6-His-tagged wild-type PHLDA1, (S78A)PHLDA1 and (S98A)PHLDA1 were phosphorylated using Aurora A, TPX2 and [γ-^{32}P]ATP for 15 minutes. (**E**) Aurora A promotes PHLDA1 degradation by phosphorylating S98. 6-His-tagged wild-type PHLDA1 or (S98A)PHLDA1 was transfected into Aurora A-MDA cells. After 24 hours, Aurora A and PHLDA1 levels were analyzed. (**F**) Aurora A downregulates PHLDA1 by phosphorylating S98. MDA cells were infected with either control, wild-type PHLDA1 or (S98A)PHLDA1 retroviruses. After 30 hours, Aurora A and PHLDA1 levels were analyzed. (**G**) Aurora A ablation prevents PHLDA1 degradation. Aurora A was ablated using Aurora A shRNA lentivirus in MDA-MB-231 cells (lane 2), stable PHLDA1-MDA cells (lane 3) or stable (S98A)PHLDA1-MDA cells (lane 4). After 30 hours, Aurora A and PHLDA1 levels were analyzed. SC, scrambled shRNA-treated MDA-MB-231 cells (lane 1).

colocalize, but also that Aurora A regulates the subcellular localization of PHLDA1.

Aurora A-mediated PHLDA1 phosphorylation was next confirmed in MDA-MB-231 cells using MLN8237. Aurora A inhibition using MLN8237 increased the level of unphosphorylated PHLDA1 (Fig. 2F, lane 2, bottom band), thereby showing that PHLDA1 is phosphorylated by Aurora A in these cells.

Aurora A negatively regulates PHLDA1 levels using its kinase activity

Aurora-A-mediated phosphorylation often promotes degradation (e.g. p53, NDEL1) or stabilization (e.g. AIP, HURP, ASAP1) of its substrates. Therefore, we investigated whether Aurora A affects the level of PHLDA1. Two different Aurora A shRNAs were generated and used to ablate Aurora A in MDA-MB-231 cells. Aurora A depletion increased the PHLDA1 level significantly in both cases (Fig. 3A, middle panel), suggesting that Aurora A degrades PHLDA1. To confirm this finding, control, Aurora-A-overexpressing MDA-MB-231 (Aurora A-MDA) cells and AA-as7-overexpressing stable MDA-MB-231 (AA-as7-MDA) cells were generated and PHLDA1 expression determined. Overexpression of Aurora A significantly decreased PHLDA1 levels (Fig. 3B). Together, these results show that Aurora A negatively regulates PHLDA1.

Aurora A can regulate its substrates using either its kinase activity or scaffolding function. Therefore, to dissect the mechanism further, MDA-MB-231 cells overexpressing Aurora A or AA-as7 were treated with 1-NM-PP1 for 12 hours and PHLDA1 expression was analyzed. Treatment with 1-NM-PP1 increased levels of PHLDA1 in AA-as7 cells, but not in wild-type Aurora A-MDA cells (Fig. 3C). Because 1-NM-PP1 selectively inhibits AA-as7 kinase activity, and not wild-type Aurora A, this finding suggests that Aurora-A-mediated downregulation of PHLDA1 is due to its kinase function, and not due to its scaffolding interactions. Interestingly, we also observed decreased Aurora A levels in 1-NM-PP1-treated cells, suggesting that Aurora A inhibition might also have a negative impact on its protein level.

Aurora A negatively regulates PHLDA1 levels by directly phosphorylating Ser98

Aurora A preferentially phosphorylates R/K/N-R-x-S/T-B, where B denotes any hydrophobic residue except Pro (Ferrari et al., 2005). This preference revealed Ser78 and Ser98 as putative Aurora A phosphorylation sites on PHLDA1. Ser78A and Ser98A PHLDA1 alleles were generated and their phosphorylation analyzed using Aurora A/TPX2. Aurora A predominantly phosphorylated PHLDA1 at Ser98 (Fig. 3D).

To elucidate the functional significance of this phosphorylation, both wild-type *6-His-PHLDA1* and *6-His-Ser98A-PHLDA1* were transfected in Aurora A-MDA cells and their levels analyzed after 24 hours using antibody against 6-His. Whereas (Ser98A)PHLDA1 showed robust expression in Aurora A-MDA cells, wild-type PHLDA1 was degraded significantly, presumably because of Aurora-A-mediated phosphorylation at Ser98 (Fig. 3E).

To confirm these results, MDA-MB-231 cells were either infected with control, wild-type PHLDA1 or (S98A)PHLDA1

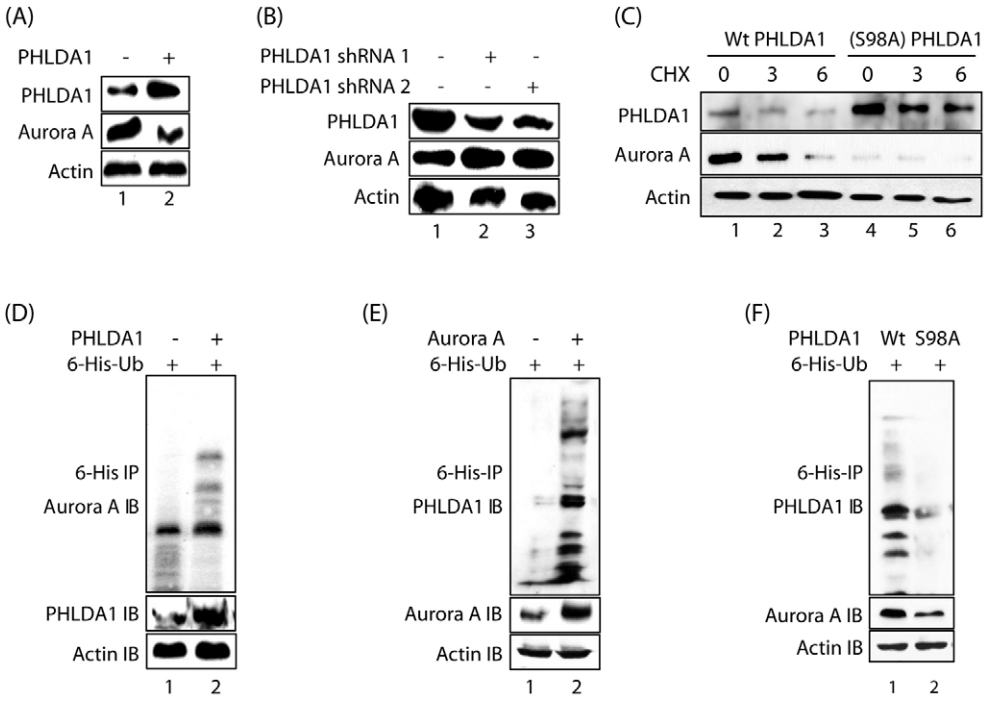

Fig. 4. PHLDA1 negatively regulates Aurora A protein levels. (**A**) PHLDA1 overexpression decreases Aurora A levels. PHLDA1-MDA cells were generated by infecting the corresponding retrovirus, followed by puromycin selection. Aurora A and PHLDA1 levels were analyzed in MDA-MB-231 and PHLDA1-MDA cells, using actin as control. (**B**) PHLDA1 ablation upregulates Aurora A in MDA cells. MDA-MB-231 cells were transfected with scrambled shRNA (lane 1), PHLDA1-specific shRNA1 (lane 2) and PHLDA1 shRNA2 (lane 3) and Aurora A and PHLDA1 levels analyzed after 30 hours. Actin was used as loading control. (**C**) Wild-type PHLDA1 degrades faster than (S98A)PHLDA1. Stable PHLDA1-MDA and (S98A)PHLDA1-MDA cells were treated with 10 μM cycloheximide (CHX) for 3 and 6 hours and Aurora A and PHLDA1 levels analyzed. (**D**) PHLDA1 overexpression increases Aurora A ubiquitylation. MDA-MB-231 cells were cotransfected with PHLDA1 along with 6-His–Ubiquitin. After 36 hours, MG132 was added (10 μM) for an additional 12 hours. Ubiquitinylated proteins were isolated using Ni-NTA beads. The proteins were separated and analyzed using antibodies against Aurora A and PHLDA1. (**E**) Aurora A overexpression increases PHLDA1 ubiquitylation. MDA-MB-231 cells were cotransfected with Aurora A and 6-His–Ubiquitin. Ubiquitylated proteins were isolated, separated and analyzed using antibodies against Aurora A and PHLDA1. (**F**) Aurora A degrades PHLDA1 by phosphorylating S98. Wild-type PHLDA1 (1) and (S98)PHLDA1 (2) were transfected along with 6-His–ubiquitin into Aurora A-MDA cells. Ubiquitylated proteins were isolated, separated and analyzed using antibodies against Aurora A and PHLDA1.

retroviruses, and Aurora A and PHLDA1 levels were analyzed after 30 hours. (S98A)PHLDA1 showed higher expression levels compared with wild-type PHLDA1, confirming that Aurora A downregulates PHLDA1 levels by phosphorylating it at Ser98 (Fig. 3F). These results were further validated in PHLDA1-overexpressing MDA-MB-231 (PHLDA1-MDA) cells and (S98A)PHLDA1-overexpressing MDA-MB-231 [(S98A)PHLDA1-MDA] cells, which showed similar expression levels of wild-type and (S98A)PHLDA1 (Fig. 3G). These results provide further evidence that the decrease in wild-type PHLDA1 levels in MDA-MB-231 cells depicted in Fig. 3F was due to Aurora-A-mediated phosphorylation. Because Aurora A is an oncogene and PHLDA1 promotes apoptosis, PHLDA1 downregulation might be one of the mechanisms by which Aurora A promotes breast tumorigenesis.

PHLDA1 negatively regulates Aurora A levels

A few Aurora A substrates are known to regulate Aurora A activity or expression via a feedback mechanism. Aurora A phosphorylates FAF1, which in turn degrades Aurora A (Jang et al., 2008). Similarly, protein phosphatase-1 inhibits Aurora A kinase activity upon phosphorylation by Aurora A (Katayama et al., 2001).

Because we observed lower levels of Aurora A in (Ser98A)PHLDA1-MDA cells compared with wild-type PHLDA1-MDA cells (Fig. 3E,F, top panels), it suggested that PHLDA1 might also negatively regulate Aurora A. Furthermore, lower Aurora A levels were observed when Aurora A was inhibited in AA-as7-MDA cells using 1-NM-PP1 (Fig. 3C), suggesting that the increase in PHLDA1 levels might in turn inhibit Aurora A by a negative-feedback loop.

PHLDA1 was stably expressed in MDA-MB-231 cells and Aurora A levels analyzed. PHLDA1 overexpression decreased Aurora A levels (Fig. 4A). Two different PHLDA1 shRNAs were generated and used to reduce PHLDA1 levels in cells, which increased the Aurora A level significantly (Fig. 4B). These findings show that PHLDA1 negatively regulates Aurora A levels, presumably by recruiting degradation machinery.

PHLDA1 promotes Aurora A degradation

To investigate whether PHLDA1 degrades Aurora A, its degradation profile was examined in PHLDA1- and (S98A)PHLDA1-MDA cells using cycloheximide. Because (S98A)PHLDA1 is resistant to Aurora-A-mediated phosphorylation and thus degradation, it showed higher expression levels, and lower degradation, than wild-type PHLDA1, as expected (Fig. 4C, top panel). Importantly, increased (S98A)PHLDA1 levels were associated with decreased Aurora A levels, thereby confirming that PHLDA1 increases Aurora A degradation (Fig. 4C, middle panel).

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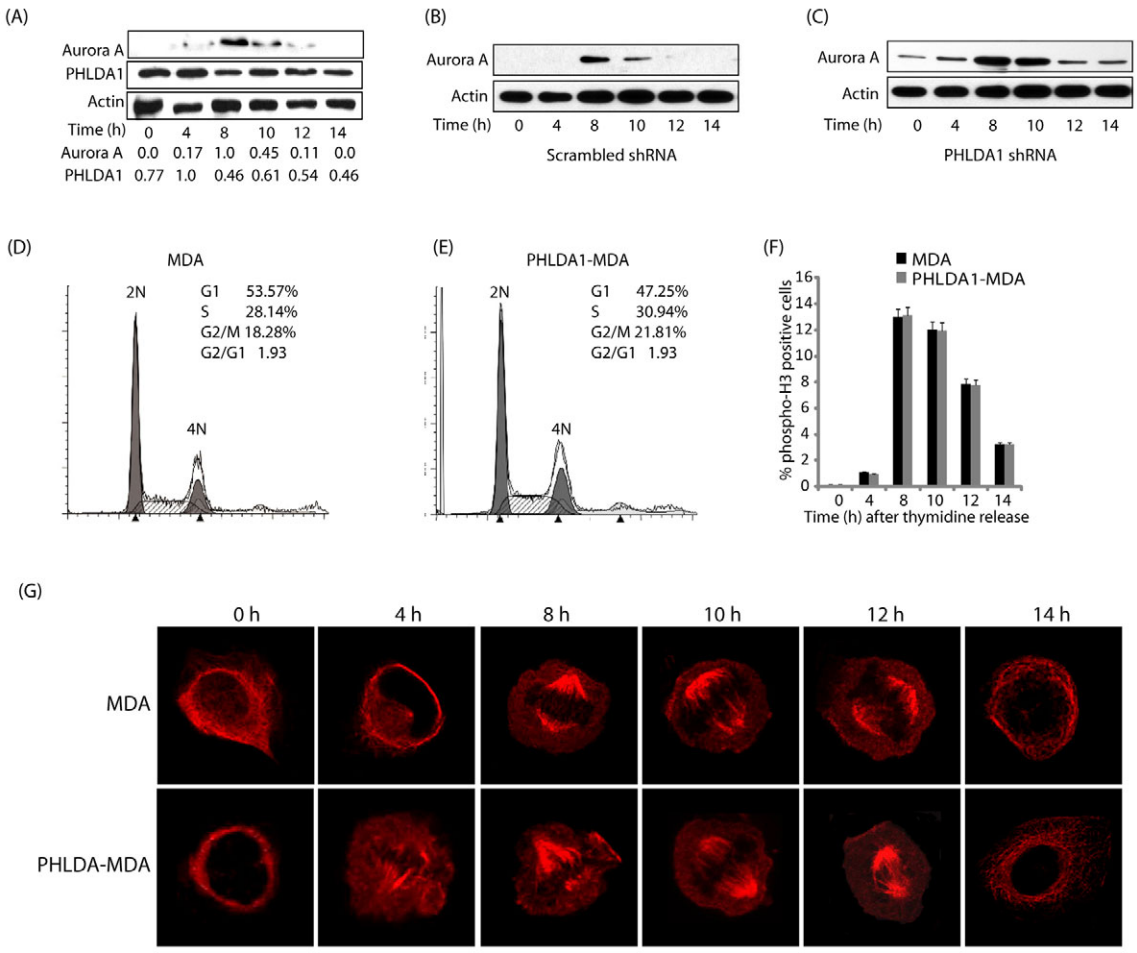

Fig. 5. PHLDA1 is not a mitotic target of Aurora A. (**A**) PHLDA1 expression is not cell cycle regulated. MDA-MB-231 cells arrested at G1–S using double thymidine block were released for varying periods and Aurora A and PHLDA1 levels analyzed. (**B**) MDA-MB-231 cells were infected with PHLDA1 shRNA lentivirus, followed by thymidine block. Aurora A and actin levels were analyzed following release for varying periods. (**C**) PHLDA1 ablation increases Aurora A levels in synchronized cells. PHLDA1 shRNA infected thymidine-blocked cells were released for varying periods and Aurora A and actin levels analyzed. (**D**) Unsynchronized MDA-MB-231 and (**E**) PHLDA1-MDA cells were analyzed by FACS analysis. (**F**) MDA-MB-231 and PHLDA1-MDA cells show a similar percentage of cells positive for phosphorylated histone H3. MDA and PHLDA1 cells plated on coverslips were blocked using thymidine and then released for varying periods. The cells were fixed and immunostained using phosphorylated histone H3. The percentage of cells showing positive staining for phosphorylated histone H3 were counted. More than 200 cells were counted from multiple random frames. (**G**) MDA-MB-231 and PHLDA1-MDA cells were synchronized with double thymidine block, released for different times, and co-stained with antibodies against α-tubulin. More than 100 cells were analyzed from multiple random frames. Representative data are shown.

To probe whether Aurora A degradation by PHLDA1 is mediated by ubiquitylation, 6-His–ubiquitin was transfected in control and PHLDA1-MDA cells, and Aurora A ubiquitylation was analyzed using 6-His antibody. PHLDA1 overexpression indeed increased ubiquitylated Aurora A (Fig. 4D, lane 2). In parallel, PHLDA1 degradation was analyzed in control and Aurora A-MDA cells; this also showed increased ubiquitylation of PHLDA1 upon Aurora A overexpression (Fig. 4E, lane 2). These results demonstrate that Aurora A and PHLDA1 negatively regulate each other by promoting ubiquitylation.

An examination of the ubiquitylation levels of wild-type PHLDA1 and (S98A)PHLDA1 in Aurora A-MDA cells revealed increased ubiquitylation of the wild-type allele, but not of the phosphorylation-resistant (S98A)PHLDA1 allele (Fig. 4F). These findings further confirm that Aurora A degrades PHLDA1 by direct phosphorylation at Ser98.

PHLDA1 is not a mitotic target of Aurora A

The role of PHLDA1 in mitosis has not been analyzed. Because Aurora A is predominantly expressed during mitosis in normal cells, we investigated whether PHLDA1 has a role in mitosis. We initially analyzed the protein levels of PHLDA1 in synchronized MDA-MB-231 cells. MDA-MB-231 cells arrested at G1–S using double thymidine block were released for varying periods, and PHLDA1 and Aurora A levels were analyzed. Interestingly, PHLDA1 was expressed almost uniformly throughout the cell cycle, whereas Aurora A expression markedly increased during mitosis (Fig. 5A), suggesting that PHLDA1 expression is not regulated by the cell cycle. Importantly, upon longer exposure, Aurora A expression was observed throughout the cell cycle, consistent with previous observations that in cancer cells and tissues Aurora A is expressed in all cell cycle phases (data not shown). PHLDA1 was ablated using PHLDA1-specific shRNA and Aurora A levels were analyzed

following thymidine release. Aurora A levels markedly increased upon PHLDA1 ablation, although it still peaked between 8 hours and 10 hours following thymidine release, similarly to levels in scrambled shRNA-treated cells (Fig. 5B,C).

To examine a potential role of PHLDA1 in mitosis, FACS analysis was conducted using unsynchronized MDA-MB-231 and stable PHLDA1-MDA cells. Both cell types showed a similar distribution of cells in different cell cycle phases and no aneuploidy, which further supported the notion that PHLDA1 overexpression does not affect the cell cycle (Fig. 5D,E).

Because FACS analysis might not differentiate subtle changes in the cell cycle, histone H3 (Ser10) phosphorylation (a mitotic marker) was analyzed in synchronized control and PHLDA1-MDA cells. Both cell types showed a similar percentage of phosphohistone-H3-positive cells at different times upon release from thymidine block, further supporting the idea that PHLDA1 is not a mitosis-regulated target of Aurora A (Fig. 5F). Importantly, PHLDA1 overexpression reduced Aurora A levels, but did not ablate it fully (Fig. 4A), suggesting that reduced Aurora A levels are sufficient to carry normal mitotic functions in MDA-MB-231 cells.

Because Aurora A has a vital role in mitotic spindle formation, we examined the consequences of PHLDA1 overexpression in mitotic spindle assembly. Control and PHLDA1-MDA cells arrested at G1–S were released for varying periods, and mitotic spindle was analyzed. PHLDA1 overexpression did not affect mitotic spindle assembly in the majority of the cells (~95%, Fig. 5G). PHLDA1-MDA cells are a pooled population, therefore variable PHLDA1 and Aurora A levels (due to the negative feedback loop) are expected in these cells. Thus the 5% of the cells with a defective mitotic spindle presumably have very low levels of Aurora A as a result of high levels of PHLDA1. These results suggest that PHLDA1 is not directly involved in mitosis; however, it might indirectly affect it by negatively regulating Aurora levels.

PHLDA1 is a negative regulator of Aurora-A-mediated breast oncogenesis

PHLDA1 reportedly acts both as an apoptotic and as an anti-apoptotic agent. In NIH3T3 cells expressing IGF1 receptors, PHLDA1 expression is essential for rescuing cells from serum starvation-induced apoptosis (Toyoshima et al., 2004). On the contrary, in many other cell lines, including T cells, neuronal, endothelial, melanoma, and cervical carcinoma, PHLDA1 reduces proliferation and induces cell death (Park et al., 1996; Gomes et al., 1999; Hossain et al., 2003). In Ras-transformed HME16C cells, reducing PHLDA1 protein level enhances cell growth under anchorage-independent, but not attached, conditions (Oberst et al., 2008). Furthermore, loss of PHLDA1 also favors apoptosis in these cells, although not significantly. The mechanism by which PHLDA1 could affect breast malignancy has not been analyzed.

To dissect the role of PHLDA1 in breast cancer cells, wild-type PHLDA1 and (S98A)PHLDA1 were overexpressed in MDA-MB-231 cells and cell proliferation was determined; both reduced cell

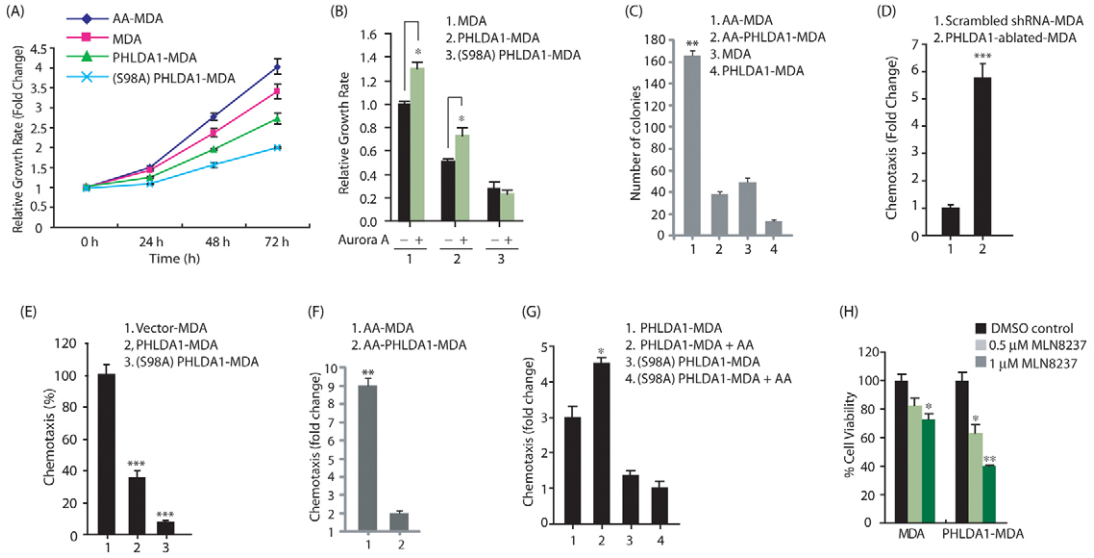

Fig. 6. PHLDA1 is a key oncogenic effector of Aurora A. (**A**) PHLDA1 inhibits cell proliferation in MDA-MB-231 cells. MDA-MB-231, Aurora A-MDA, PHLDA1-MDA and (S98A)PHLDA1-MDA cells were seeded in 96-well plates and cultured for 24, 48 and 72 hours. At the end of the incubation, MTT solution was added and absorbance measured. (**B**) Aurora A rescues growth inhibition induced by wild-type PHLDA1, but not (S98A)PHLDA1. MDA-MB-231, PHLDA1-MDA and (S98A)PHLDA1-MDA stable cells were seeded in 12-well plates for 12 hours, followed by Aurora A transfection. After 24 hours, growth rate was measured using MTT assay. The bar graph shows the mean ± s.e.m. **P*>0.05. (**C**) PHLDA1 inhibits anchorage-independent growth. Soft-agar colony formation assays were performed with MDA, Aurora A-MDA, PHLDA1-MDA, and PHLDA1-MDA and Aurora A-MDA cells. The bar graph show the mean ± s.e.m. ***P*>0.01. (**D**) PHLDA1 ablation increases chemotaxis. PHLDA1 was depleted in MDA cells, and chemotaxis measured using Boyden chambers. The bar graph show the mean ± s.e.m. ****P*>0.001. (**E**) PHLDA1 overexpression inhibits cell motility. Cell migration was measured in vector-expressing MDA-MB-231, PHLDA1-MDA and (S98A)PHLDA1-MDA cells. The bar graph show the mean ± s.e.m. (****P*>0.001). (**F**) PHLDA1 overexpression inhibits AA-mediated chemotaxis. The migrating abilities of MDA-MB-231 overexpressing both Aurora A and PHLDA1 (AA-PHLDA1-MDA) or only overexpressing PHLDA1 (PHLDA1-MDA) were determined. The bar graph show the mean ± s.e.m. ***P*>0.01. (**G**) Aurora A rescues motility inhibition induced by wild-type PHLDA1, but not (S98A)PHLDA1. MDA-MB-231, PHLDA1-MDA and (S98A)PHLDA1-MDA stable cells were seeded in 12-well plates for 12 hours, followed by Aurora A transfection. After 24 hours, cells were serum starved for 16 hours and cell motility was measured. The bar graph shows the mean ± s.e.m. **P*<0.05. (**H**) PHLDA1 overexpression and Aurora A inhibition synergistically promotes cell death. ~2000 cells were seeded per well overnight, followed by incubation with MLN8237 (0.5 μM and 1 μM) or vehicle (DMSO). After 48 hours, cells were analyzed using MTT assay. **P*<0.05 and ***P*<0.01, when compared with the control.

growth, but (S98A)PHLDA1 inhibited it more (Fig. 6A). These results differ from the previous findings in breast epithelial HME16C cells, which revealed no change in cell proliferation rate upon PHLDA1 downregulation under attached conditions (Oberst et al., 2008).

The reverse experiment was conducted by transiently transfecting Aurora A in control MDA-MB-231 cells or in MDA-MB-231 cells overexpressing wild-type PHLDA1 or (S98A)PHLDA1 and cell proliferation was measured (Fig. 6B). Although Aurora A expression increased cell proliferation in both control and PHLDA1-MDA cells, it did not affect the proliferation rate of (S98A)PHLDA1-MDA cells (Fig. 6B). This result was expected, because (S98A)PHLDA1 is resistant to Aurora-A-mediated degradation; instead, it efficiently degrades Aurora A (Fig. 4C).

The effect of PHLDA1 was further evaluated in control and Aurora A-MDA cells under anchorage-independent conditions, which revealed dramatic loss in colony-forming ability upon PHLDA1 overexpression in both cell types (Fig. 6C). Thus, we show that PHLDA1 upregulation inhibits cell proliferation under attached, as well as anchorage-independent, conditions in breast cancer cells. More importantly, the striking increase in the colony-forming ability of MDA-MB-231 cells upon Aurora A overexpression (Fig. 6C, compare columns 1 and 3) was almost completely lost upon PHLDA1 overexpression (compare columns 1 and 2), suggesting that downregulation of PHLDA1 is one of the key mechanisms by which Aurora A promotes tumorigenesis.

PHLDA1 is a negative regulator of chemotaxis

The role of PHLDA1 in cell motility has not been investigated. To examine a potential contribution of PHLDA1 in Aurora-A-mediated cell invasion, PHLDA1 was initially depleted from MDA cells and cell motility was measured. PHLDA1 ablation significantly increased cell motility, suggesting that it negatively regulates chemotaxis (Fig. 6D).

Next, cell motility was determined in control, PHLDA1 and (S98A)PHLDA1-MDA cells. Whereas expression of wild-type PHLDA1 decreased cell motility by more than 60%, the phosphorylation-resistant PHLDA1 mutant fully inhibited cell motility (Fig. 6E). These results further support the conclusion that PHLDA1 negatively regulates chemotaxis. Cell motility was also measured in serum-starved MDA-MB-231 cells overexpressing Aurora A and/or PHLDA1. Aurora A cells were highly motile; however, PHLDA1 overexpression dramatically reduced cell motility (Fig. 6F and supplementary material Fig. S1A). This result was further confirmed using a wound-healing assay, which also revealed PHLDA1 as a strong inhibitor of chemotaxis (supplementary material Fig. S1B).

Because PHLDA1 might inhibit chemotaxis independently of Aurora A, we examined whether Aurora A expression reverses PHLDA1-mediated inhibition of chemotaxis. Aurora A was transiently expressed in PHLDA1- and (S98A)PHLDA1-MDA cells and cell motility was determined. Aurora A expression increased motility in PHLDA1-MDA cells, but not in phosphorylation-resistant (S98A)PHLDA1-MDA cells (Fig. 6G). These results show that PHLDA1-mediated downregulation of Aurora A is one of the key mechanisms by which it inhibits chemotaxis.

PHLDA1 upregulation and Aurora A inhibition act synergistically to promote cell death

The negative role of PHLDA1 in Aurora-A-mediated oncogenic pathways suggested that PHLDA1 upregulation might work synergistically with Aurora A inhibition in promoting cell death. Aurora A was inhibited using MLN8237 in control and PHLDA1 MDA cells. Although ~20% loss in cell viability was observed in control cells, an ~60% loss was observed in PHLDA1 MDA cells (Fig. 6H). These findings suggest that PHLDA1 upregulation might be an alternative approach to modulate Aurora-A-mediated breast oncogenesis.

PHLDA1 protein expression correlates negatively with estrogen receptor expression in breast cancer tissue microarray

PHLDA1 expression was exclusively cytoplasmic in whole sections of normal and tumorous breast tissues. Of the 114 analyzable tumors in the tissue microarray (TMA), 90 (80%) were interpretable for PHLDA1 staining. The distribution of staining was as follows: no staining (n=59; 65.6%); mild staining (n=20; 22.2%); moderate staining (n=10; 11.1%) and strong staining (n=1; 1.1%) (Fig. 7). PHLDA1 expression was significantly associated only with estrogen receptor expression (P=0.012) (Table 3). We did not observe a significant correlation with any of the remaining clinicopathological markers, which included tumor size, type, grade, nodal status, and expression of HER2, PR and FOXA1.

Discussion

The chemical genetic approach for the identification of direct substrates of kinases is highly versatile and has been applied to over 40 kinases to date. For most kinases, a single mutation of gatekeeper residue to glycine or alanine renders them sensitive to orthogonal ATP analogs and PP1-derived inhibitors. Additional mutations are required for a few others (Shah and Shokat, 2003). In the case of Aurora kinases, a novel mutation was identified, which is required along with the gatekeeper mutation to render them amenable to the chemical genetic approach. This technique revealed PHLDA1 as a novel substrate of Aurora A in breast cancer cells.

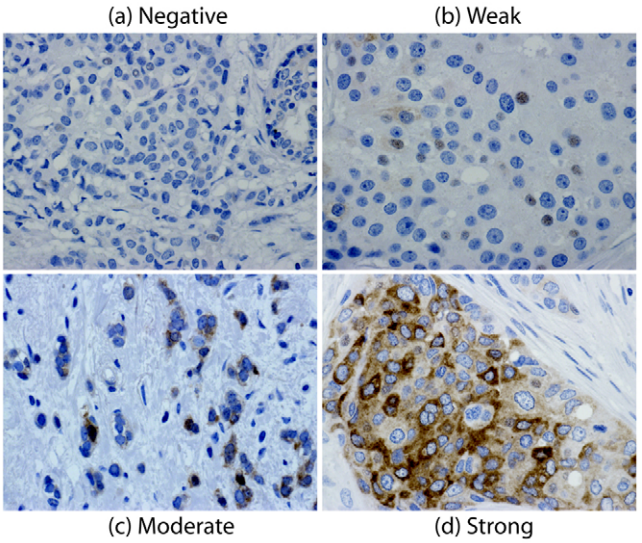

Fig. 7. Expression of PHLDA1 in a series of breast cancer samples. The majority of cases analyzed showed no expression of the protein (**a**). Although in some cases, nuclei showed brown staining (**b**), expression was predominantly cytoplasmic and of moderate intensity (**c**). In rare cases, strong cytoplasmic expression was noted (**d**).

Table 3. Description of patient population of tissue microarray

Variable	PHLDA1 negative	PHLDA1 positive	*P*-value
Age	55.4 ±11.8 years	56.7 ±13.8 years	NS[a]
Tumor size	2.5 ± 2.1 cm	2.4 ± 2.7 cm	NS[a]
ER			0.02[b]
Negative	4 (33.3%)	8 (66.7%)	
Positive	38 (70.4%)	16 (29.6%)	
PR			NS[b]
Negative	16 (57.1%)	12 (42.9%)	
Positive	26 (66.7%)	13 (33.3%)	
HER2			NS[b]
Negative	43 (65.2%)	23 (34.8%)	
Positive	1 (50.0%)	1 (50.0%)	
Nodal status			NS[b]
Negative	46 (71.9%)	18 (28.1%)	
Positive	12 (52.2%)	11 (47.8%)	
Grade			NS[b]
Grade I	17 (70.8%)	7 (29.2%)	
Grade II	26 (74.3%)	9 (25.7%)	
Grade III	15 (50.0%)	15 (50.0%)	
Type			NS[b]
IDC	46 (63.0%)	27 (37.0%)	
ILC	8 (100.0%)	0 (0.0%)	
Other types	5 (55.6%)	4 (44.4%)	

[a]Mann–Whitney test; [b]Chi squared test. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; NS, not significant.

PHLDA1 has been shown to be both pro-apoptotic and anti-apoptotic depending on the cell line and the experimental conditions. PHLDA1 was initially identified in T cell hybridoma, where it mediates apoptosis by inducing Fas expression, and was thus named T-cell death-associated gene 51 (TDAG51) (Oberg et al., 2004). However, other studies revealed no role of PHLDA1 in T cell apoptosis either in cells or in vivo (Rho et al., 2001). In IGFR NIH3T3 cells, PHLDA1 is a crucial mediator of the anti-apoptotic effect of IGF1 (Toyoshima et al., 2004). Similarly, PHLDA1 is highly expressed in pancreatic tumors, which are resistant to apoptosis and chemotherapeutic agents (Oberg et al., 2004).

PHLDA1 regulates apoptosis in vascular endothelial cells triggered by homocysteine (Hossain et al., 2003). In neuronal cells, PHLDA1 enhances cell death, but without Fas induction (Gomes et al., 1999). Downregulation of PHLDA1 expression is associated with the progression of malignant melanomas (Neef et al., 2002).

Our study confirmed previous reports of PHLDA1 being downregulated in primary breast tumors. In almost 66% of the cases in the current study, there were no detectable levels of the protein. This is similar to the 72% downregulation of PHLDA1 proteins previously reported (Nagai et al., 2007). The differences in association between PHLDA1 expression and estrogen receptors in the current study and that of Nagai and colleagues could be due, at least be in part, to different analytical methods (IHC versus ligand-binding assay). PHLDA1 expression does not correlate with patient age, tumor type, tumor size or nodal status (current study) (Nagai et al., 2007). In spite of this, downregulation of PHLDA1 protein was a strong predictor of poor prognosis for breast cancer patients, with the loss of protein being predictive of an adverse prognosis (Nagai et al., 2007). These results provide ample evidence that reduced PHLDA1 expression is important in breast cancer progression and could serve as a useful prognostic marker of disease outcome.

PHLDA1 and Aurora A have never been associated before. This is also the first study that shows that PHLDA1 protein level is regulated by a post-translational modification. We found that Aurora A negatively regulates PHLDA1 protein levels by directly phosphorylating Ser98 in breast cancer cells. PHLDA1 also negatively affects Aurora A protein levels, thereby engaging in a feedback loop. Phosphorylation-resistant (S98A)PHLDA1 strongly antagonizes Aurora-A-mediated oncogenic pathways, thereby revealing PHLDA1 degradation as a key mechanism by which Aurora A promotes breast malignancy. Thus, not surprisingly, PHLDA1 upregulation acts synergistically with Aurora A inhibition in promoting cell death.

PHLDA1 downregulation and Aurora A upregulation are strong predictors of poor prognosis for breast cancer patients. However, they have been not analyzed together. Thus, our finding that Aurora A and PHLDA1 are engaged in a feedback loop highlights the relevance of this study. We further show that PHLDA1 is a strong inhibitor of cell motility, proliferation and transformation in breast cancer cells, suggesting that PHLDA1 overexpression might be an alternative way to modulate Aurora A deregulation in breast cancer. Analysis of PHLDA1 and Aurora A levels could supplement standard staging information in primary biopsy samples. Results from these studies can facilitate the development of combination therapies using drugs targeted against both Aurora A and PHLDA1. Finally, a novel mutation was identified in this study, which is required (along with the gatekeeper mutation) to render Aurora kinases amenable to the chemical genetic approach. We expect that this new mutation will give researchers a versatile tool for creating allele-specific mutants of kinases that hitherto have not been amenable to chemical genetic methodology.

Materials and Methods

Materials

Antibodies against Aurora A (H-130), actin (C-2), α-tubulin (B-7), PHLDA1 (L-19) and phospho-histone H3 were purchased from Santa Cruz Biotech. MLN8237 was purchased from Chemie Tek.

Expression plasmids and constructs

AA-as1 (L201G) and AA-as7 (L185V, L201G) were generated using overlapping PCR and cloned using *Bam*HI and *Not*1 sites in VIP3 puro retroviral mammalian vector and baculoviral vector (Bac to Bac, Invitrogen). PHLDA1 was cloned in TAT-HA and VIP3 vectors at *Bam*HI and *Xho*I sites. TPX2 (a gift from Dirk Gorlich, Max Plank Institute for Biophysical Chemistry, Göttingen, Germany) was cloned into Fastbac vector at *Bam*HI and *Kpn*I sites. D274A Aurora A was a gift from Joan Ruderman (Crane et al., 2004).

Expression and purification of TPX2, wild-type Aurora A, Aurora A mutants and PHLDA1

For substrate labeling experiments, Aurora A (AA), AA-as1, AA-as7 and TPX2 were prepared from Sf9 insect cells using the Bac-to-Bac expression system (Invitrogen) according to the manufacturer's instructions. Protein concentration was determined using Bradford assay, and the protein purity was assessed using 6-His antibody. PHLDA1 was expressed in *E. coli* and purified as described previously (Sun, 2008a).

2D gel electrophoresis and MS spectrometry

To prepare a labeled sample for 2D gel electrophoresis, kinase reactions with whole cell lysate or fractionated lysate were carried out as published previously (Shah and Vincent, 2005; Sun, 2008b). Gel spots were manually excised and automatically processed for peptide mapping experiments using a Micromass MassPREP Station in conjunction with manufacturer-specified protocols.

Synthesis of [γ-^{32}P]N^6-phenethyl ATP and 1-NM-PP1

[γ-^{32}P]N^6-phenethyl ATP and 1-NM-PP1 were synthesized as described (Bishop et al., 1999; Shah et al., 1997; Shah and Shokat, 2002).

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Transfection and retroviral infection

Aurora A, PHLDA1 and TPX2 plasmids were transiently transfected into Phoenix cells. The retroviruses were harvested and used to infect MDA-MB-231 cells as reported previously (Shah and Shokat, 2002).

In vitro kinase assays

For in vitro labeling, Aurora A/TPX2 complex (on beads) was pre-incubated with 10 μM cold ATP for 10 minutes to activate the kinase. The beads were washed twice with kinase buffer, and then subjected to kinase assay with 2–5 μg of recombinant protein (such as PHLDA) and 1 μCi of [γ-^{32}P]ATP. Reactions were terminated by adding SDS sample buffer, separated by SDS-PAGE gel, transferred to PVDF membrane and exposed to Biomax MS film.

For kinase inhibition assays, various amounts of PP1-derived inhibitors were added to the kinase assay buffer containing 6-His-Aurora A/TPX2, 1 μCi of [γ-^{32}P]ATP and 3 μg of Aurora A substrate peptide (ALKRASLGAA) in a final volume of 30 μl for 30 minutes at room temperature (Sun et al., 2008a). Reaction mixtures (25 μl) were spotted onto a phosphocellulose disk, immersed in 10% acetic acid for 20 minutes, and washed with 0.5% H_3PO_4 (three times, 5 minutes each). The transfer of ^{32}P was measured by scintillation counting. IC_{50} values were determined by fitting the data to a sigmoidal dose response curve using GraphPad Prism 4.0 software. K_m and V_{max} values were derived from the assay described above using various concentrations of AA peptide substrate and ATP. K_m and V_{max} values were determined using GraphPad Prism 4.0 software.

Aurora A and PHLDA1 shRNA

Aurora A human short hairpin RNA (shRNA) sequences were designed as follows: (1) forward oligo, 5′-CCGGGCACCACTTGGAACAGTTTATCTCGAGATAAACTGTTCCAAGTGGTGCTTTTTG-3′ and reverse oligo, 5′-AATTCAAAAAGCACCACTTGGAACAGTTTATCTCGAGATAAACTGTTCCAAGTGGTGC-3′; (2) 5′-CCGGGCCAATGCTCAGAGAAGTACTCTCGAGAGTACTTCTCTGAGCATTGGCTTTTTG-3′ and reverse oligo, 5′-AATTCAAAAAGCCAATGCTCAGAGAAGTACTCTCGAGAGTACTTCTCTGAGCATTGGC-3′. For PHLDA1, the following sequences were designed: (1) forward oligo, 5′-CCGGGATGGTGCAGTACAAGAATCTCGAGATTCTTGTACTGCACCATCTTTTTG-3′ and reverse oligo, 5′-AATTCAAAAAGATGGTGCAGTACAAGAATCTCGAGATTCTTGTACTGCACCATC-3′. (2) forward oligo, 5′-CCGGTCCGCATCCACATCCACATCTCGAGATGTGGATGTGGATGCGGATTTTTG-3′ and reverse oligo, 5′-AATTCAAAAATCCGCATCCACATCCACATCTCGAGATGTGGATGTGGATGCGGA-3′. The sense and antisense strands were annealed at 95°C for 4 minutes to make at 20 μM concentration. This was followed by cooling to room temperature and subsequent cloning into pLKO.1 TRC vector (Moffat et al., 2006). pLKO.1 TRC vector was a gift from David Root (MIT, Boston, MA). Control shRNA (scrambled shRNA), PHLDA1 and Aurora A shRNA were transfected to MDA-MB-231 cells using Lipofectamine following the manufacturer's instructions. After 30 hours, transfected cells were harvested and analyzed for Aurora A and PHLDA1 expression. Alternatively, Aurora A shRNA and PHLDA1 lentiviruses were generated and used for infecting MDA-MB-231 cells.

Chemotaxis assay

Control, Aurora A-, PHLDA1-, and PHLDA1- and Aurora A-overexpressing MDA-MB-231 cells were serum starved in serum-free RPMI for 15 hours and isolated by limited trypsin digestion. Cell migration was determined as reported previously (Shah and Vincent, 2005). The assays were performed in triplicate, four independent times. To allow for comparison between multiple assays, the data were normalized, and expressed as a percentage of the number of cells present on the membrane.

Soft agar colony formation

Briefly, equal volumes of Noble agar (1%; DNA grade) and 2× RPMI 1640 (with 20% FBS) were mixed at 40°C to make 0.5% agar in six-well tissue culture plates (Corning) as a base agar. Cells (0.1 ml of 2.0×10^5/ml) were suspended in 3 ml of 2× RPMI 1640 (with 20% FBS) and 3 ml of 0.7% agar. 1.5 ml of this suspension was added to each well (as 0.35% top agar) with final concentration of 5000 cells per well. Top agar was covered with 500 μl of culture medium. Plates were incubated at 37°C for 3 to 4 weeks. Fresh medium was added every 3 days.

For 1-NM-PP1 experiments, fresh medium containing 1-NM-PP1 (100 nM) or DMSO were added to the cells every 3 days. Colony formation was observed by light phase-contrast microscope and visually after staining with 0.5 ml of 0.01% crystal violet in PBS for 45 minutes at room temperature. Experiments were repeated in quadruplicate, two independent times to ensure the reproducibility of the results.

Cell synchronization

Control or PHLDA1-overexpressing MDA cells were treated with 2.5 mM thymidine for 16 hours, released for 8 hours, and then treated with thymidine for an additional 16 hours. After two washes with phosphate-buffered saline (PBS), cells were cultured for different times as indicated in the experiment and harvested.

Immunoflorescence

MDA and PHLDA1-MDA cells were plated on poly-L-lysine-coated coverslips at a density of 50,000 cells per well in 24-well plates. Cells were arrested at G1–S using double thymidine block, followed by release for different time periods. Cells were immunostained using α-tubulin, phospho-histone H3 (S10), Aurora A or PHLDA1 antibodies, followed by FITC-labeled goat anti-rabbit or Texas-Red-labeled goat anti-mouse secondary antibodies. After washing with PBS, coverslips were mounted on microscope slides with Mowiol mounting medium. Images were taken either using a Fluoview laser scanning confocal microscope (Olympus, Melville, NY). The percentages of cells shown were counted in at least 100 cells from ten random frames in duplicate.

Peptide synthesis

Aurora A substrate peptide, ALRRASLGAA, was synthesized using solid-phase peptide synthesis using a standard Fmoc peptide synthesis protocol with WANG resin.

MTT assay

Cells were seeded in 96-well plates at 1500 cells in 100 μl per well and cultured for 24, 48 and 72 hours. At the end of incubation, MTT assay was conducted as published previously (Sun et al., 2009). Experiments were repeated three times in quadruplicate wells to ensure the reproducibility of results. MLN8237 was used at either 0.5 or 1 μM concentration.

Molecular modeling

Using PDB number 1MQ4, the docking of phenethyl ATP was carried out using MacroModel. Amino acids L194 and L210 (human Aurora A numbering) were mutated to valine and glycine residues, respectively. The resulting structure was then energy minimized to yield a unique favorable conformation visualized by pyMOL.

Immunohistochemical studies

To study the significance of PHLDA1 in human tissues, we analyzed a breast cancer tissue microarray (TMA) for protein expression using immunohistochemistry (IHC). The TMA was created at Indiana University, Department of Pathology. It consists of 1 mm tissue cores from 114 breast cancer patients treated at this institution. Data regarding age, tumor type IDC (invasive ductal carcinoma) compared with ILC (invasive lobular carcinoma) and others, grade (I vs II vs III), tumor size (<2 cm vs >2 cm), nodal status (No vs Yes), ER (Neg vs Pos), PR (Neg vs Pos) and HER2 (Neg vs Pos) status were available for this cohort. Expression of PHLDA1 was analyzed using mouse monoclonal antibody against human PHLDA1 (Santa Cruz Biotech, Santa Cruz, CA) by IHC. After de-waxing and hydration, 4 mm sections from formalin-fixed paraffin-embedded tissue were treated with target retrieval (Dako, pH 8.0), in a pressure cooker. Endogenous peroxidase activity was blocked by hydrogen peroxide for 10 minutes. The slides were then incubated with mouse monoclonal PHLDA1 antibody (1:50; Santa Cruz Biotech) for 1 hour at room temperature. The sections were incubated with donkey antigoat horseradish peroxidase polymer conjugate (Jackson Labs, West Grove, PA) according to the manufacturer's instructions. The stain was developed using diaminobenzidine (DAB) plus (Dako, Glostrup, Denmark) and hematoxylin QS (Vector Laboratories, Burlingame, CA) counterstain. To verify the specificity of staining, nonimmune goat serum and PBS-negative controls were used. Expression of PHLDA1 was evaluated for intensity of staining and scored as 0 (no expression), 1 (weak expression), 2 (moderate expression) and 3 (strong expression) by a single board certified pathologist (SB).

Statistical analysis

All statistical analyses were performed using SPSS v17.0. Expression of PHLDA1 protein was correlated with clinico-pathological variables as mentioned above using Chi-squared test, Fishers test or Student's *t*-test as appropriate. Bar graphs results are plotted as the average ± s.e.m. Significant results are displayed as follows: $*P>0.05$, $**P>0.01$, $***P>0.001$.

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