Review of: "Activation of the essential kinase PDK1 by phosphoinositide-driven trans-autophosphorylation"

Ricardo M. Biondi, Alejandro E. Leroux

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Levina et al. “Activation of the essential kinase PDK1 by phosphoinositide-driven trans-autophosphorylation”, published in Nature Communications (2022) 13: 1874.

The paper by Levina et al. (Activation of the essential kinase PDK1 by phosphoinositide-driven trans-autophosphorylation) investigates aspects of the regulation of the protein kinase PDK1. PDK1 is considered a master kinase that phosphorylates a number of substrates, participating in different signaling pathways. The basis for the current model are almost 20 years old and an increasing number of experimental findings on mammalian PDK1 cannot be fully explained with the current understandings. For example, the role of PDK1 dimers and PDK1 monomers, well described in cells in culture, remains controversial and the dimerization has not been measured *in vitro* with purified proteins. Also, Akt/PKB is now known to become phosphorylated and activated by PDK1 in a PIP3 independent manner, and the mechanism by which PDK1 phosphorylates Akt/PKB in the absence of PIP3 is not yet known. Therefore, it is welcome that the proposed mechanisms of PDK1 regulation are revisited and improved. However, the new hypotheses have to be put properly in the context of the previous published work using purified PDK1, cell culture experiments and studies in animals models, that describe the role of PDK1 and the different mechanisms by which PDK1 phosphorylates the different substrates (some substrates, like conventional PKCs are constitutively phosphorylated by PDK1, without growth factor/PI3-kinase stimulation).

The paper by Levina et al. has a first focus on the findings based on biochemical characterization of the *in vitro* autophosphorylation of PDK1 and complements the study with biophysical experiments and modeling. Based on their studies of PDK1 autophosphorylation, the authors aim to deduce new mechanisms of PDK1 regulation.

Unfortunately, we have criticisms on central aspects of the introduction to the topic, which misguides the reader on to the field of research. Also, the major biochemical results lack proper control experiments to sustain the conclusions and, biased by the weak conclusions from the biochemical part, the molecular models derived are most likely incorrect.

We will comment on the Levina et al paper and focus on three issues that we believe are important to discuss: 1- related to the Introduction, the aims of the work and the presentation of the current model of PDK1 regulation, 2- on the model of PDK1 dimer, 3- the studies of autophosphorylation and 4- on the claim that provides the title to the paper, that the PH domain has an autoinhibitory role and that this inhibition is released by PIP3.

Specific comments on the Introduction section of Levina et al. (Nature Comm., 2022).

1. There is an unfortunate flaw in the Levina et al. published paper, as it does not cite the previous work by Gao and Harris (Role of the PH domain in regulating in vitro autophosphorylation events required for reconstitution of PDK1

catalytic activity. Bioorg Chem, 2006. 34(4): p. 200-23), which overlaps in many aspects in the biochemical work of the paper and reaches similar conclusions, as depicted in the title.

2. A main theme of the paper is the phosphorylation of the activation loop of PDK1, at Ser241. The phosphorylation of Ser241 at the activation loop happens in PDK1 expressed in bacteria. There is no doubt that PDK1 can autophosphorylate at residue Ser241. In almost all studies (like in the study by Gao and Harris and by Levina et al.), purified PDK1 is fully or almost fully phosphorylated at the activation loop. Levina et al. cite one (1) paper to support the regulated phosphorylation of Ser241. In the same cited publication, the authors describe that Ser241 was constitutively phosphorylated in another cell line, HEK293, even after serum starvation. Over the years PDK1 was found constitutively phosphorylated at Ser241 in most other models where PDK1 does not become dephosphorylated upon serum starvation and PDK1 is phosphorylated at Ser241. In those cellular models, like HEK293 cells, fully Ser241-phosphorylated PDK1 does not phosphorylate Akt/PKB, S6K or SGK in the absence of growth factor/PI3-kinase stimulation and responds to growth factor/PI3-kinase signaling phosphorylating substrates Akt, S6K, SGK, with different timing after PI3-kinase stimulation. Therefore, the whole PI3-kinase regulatory downstream signaling is OFF when PDK1 is already phosphorylated at Ser241. Therefore, it is absolutely clear that PI3-kinase induced phosphorylation of Akt/PKB, S6K, SGK happens without the need of “activation” of PDK1 by Ser241 phosphorylation. In addition, it is very well established that classical/conventional PKCs are synthesized and constitutively phosphorylated by PDK1 in the absence of growth factor/PI3-kinase stimulation. The paper by Levina et al. misguides into this background knowledge that is very well established over the past 25 years.
3. In the Introduction, the authors argue that there is a need for the OFF-ON regulation of the intrinsic activity of PDK1: " These mechanisms, however, beg the question of why PDK1 is regulated by activation loop phosphorylation at all. Moreover, the presence of a constitutively active kinase in cells is likely to lead to spurious off target phosphorylation events, uncoupled from growth factor signaling, that are incompatible with the coordination of cellular events in a tightly regulated manner in both space and time. These contradictions prompted us to address the question of how PDK1 activity is regulated at a molecular level". The above statements do not reflect the current knowledge. First, the activation loop Ser241 phosphorylation is not a regulated phosphorylation in most cellular models studied to date. Second, the authors argue that PDK1 must have an OFF stage to only phosphorylate substrates in a PIP3-dependent manner. However, PDK1 DOES phosphorylate multiple substrates in the absence of PIP3 stimulation, like classical/conventional PKCs, which are phosphorylated by PDK1 constitutively. Also, in the last years it has become clear that Akt can also become phosphorylated by PDK1 in the absence of PIP3. In sharp contrast to the statements in the introduction of the paper by Levina et al., any model aimed to explain PDK1 mechanism of phosphorylation of substrates in a timely manner must take into consideration that indeed PDK1 phosphorylates some substrates constitutively, uncoupled from growth factor signaling!
4. To address the relevance of the main topic of research of the paper (Ser241 phosphorylation) we should keep in mind that even if PDK1 is already phosphorylated at Ser241 in cells in culture, PDK1 does not phosphorylate Akt/PKB, SGK, S6K in the absence of PI3-kinase stimulation. In other words, the constitutive phosphorylation of some substrates and the response to PIP3 phosphorylating Akt/PKB, SGK and S6K works fine even when PDK1 is constitutively phosphorylated at Ser241. PDK1 is indeed constitutively phosphorylated at Ser241 in HEK293 cells and the regulation

of insulin/growth factor signal downstream of PDK1 works well in HEK293 cells, as extensively described through the years by the Protein Phosphorylation Unit (Dundee) by Philip Cohen and Dario Alessi and hundreds of other researchers over the last 30 years.

5. The current model has an explanation for the phosphorylation of Akt/PKB, SGK and S6K, only upon PI3-kinase activation. The model is based on the discovery of two different mechanisms for the phosphorylation of substrates: 1- a mechanism used to phosphorylate Akt/PKB requiring colocalization with PIP3 (this phosphorylation happens immediately after PI3-kinase stimulation) and 2- the phosphorylation of protein kinase substrates requiring a docking interaction between PDK1 and its substrates, where the docking interaction itself would be the PIP3-dependent regulated step. This model also explains the different timing of phosphorylation of different substrates of PDK1 after growth factor stimulation of cells. The basic principles of the current model explain important aspects of the molecular mechanism of phosphorylation of PDK1 substrates from a wide range of organisms, from plants, worms, insects, yeasts and mammals. The basic principles of the model are conserved in yeasts, i.e. the PDK1 from Candida albicans (CaPkh2), although there are also fundamental differences in the regulation by lipids and by linker-PH domain regions.
6. Levina et al correctly cite the work by Morten Frödin and collaborators (2000) who described increased autophosphorylation of PDK1 by the interaction with the hydrophobic motif (HM) polypeptide derived from RSK2 (Frodin, M., C.J. Jensen, K. Merienne and S. Gammeltoft, “A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1”. Embo J, 2000. 19(12): p. 2924-34). However, Levina et al. fail to mention the allosteric activation of PDK1 upon the interaction of the C-terminal hydrophobic motif of substrates to the PIF-pocket of PDK1 that increases the specific activity of PDK1 (Biondi, R.M., P.C. Cheung, A. Casamayor, M. Deak, R.A. Currie and D.R. Alessi, “Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA”. Embo J, 2000. 19(5): p. 979-88). This mechanism of activation is central to the established mechanism of activation of PDK1 and many members of the AGC group of protein kinases (Frodin, M., T.L. Antal, B.A. Dummler, C.J. Jensen, M. Deak, S. Gammeltoft and R.M. Biondi, “A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation”. Embo J, 2002. 21(20): p. 5396-407; Hauge, C., T.L. Antal, D. Hirschberg, U. Doehn, K. Thorup, L. Idrissova, K. Hansen, O.N. Jensen, T.J. Jorgensen, R.M. Biondi and M. Frodin, “Mechanism for activation of the growth factor-activated AGC kinases by turn motif phosphorylation”. Embo J, 2007. 26(9): p. 2251-61; Yang, J., P. Cron, V. Thompson, V.M. Good, D. Hess, B.A. Hemmings and D. Barford, “Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation”. Mol Cell, 2002. 9(6): p. 1227-40). The allosteric system proposed to activate PDK1 by the binding of the HM of substrates to the PIF-pocket of PDK1 was confirmed by the development of small molecules that, binding to the PIF-pocket, increase the catalytic activity; the allosteric effect on the ATP-binding site was shown by hydrogen-deuterium exchange experiments and the crystal structure in complex with PS210. Allostery is bi-directional. The allosteric property between the ATP binding site and the PIF pocket was further confirmed by discovering and characterising compounds that bind to the ATP-binding site and affect, positively and negatively the binding of PIFtide at the PIF-pocket. The synergistic effect of HM peptide binding at the PIF-pocket and the ATP-binding site was confirmed by hydrogen deuterium exchange. It is quite conclusive: the binding of the C-terminus of substrates to the

PIF-pocket of PDK1 allosterically activates PDK1.

7. Numerous statements in the manuscript are wrong. For example in the discussion: “The PH domain of PDK1 is essential for its kinase activity”. This statement is overseeing 25 years of research by other scientists, including the not cited paper by Gao and Harris (Bioorg Chem, 2006. 34(4): p. 200-23). There are two mechanisms for the phosphorylation of substrates and the PH domain is required only for the phosphorylation of one of its substrates Akt/PKB. *In vitro*, the catalytic domain is equally active to phosphorylate physiological substrates like SGK or peptide substrates like T308tide.
8. The study of the autophosphorylation of a protein kinase can lead to knowledge on the protein structure. On the other hand, the autophosphorylation itself may not be a physiological phosphorylation. Classical/conventional PKCs were considered to autophosphorylate at the turn-motif site … until the discovery of the actual upstream protein kinase, mTORC2. We should keep in mind that PDK1 can autophosphorylate at its activation loop, and does so when expressed in bacteria, but this does not necessarily mean that physiologically the Ser241 phosphorylation is an autophosphorylation. We do not know if the autophosphorylation of PDK1 studied by Gao and Harris and now by Levina et al. reflects a physiological phosphorylation. Based on the results from both studies we should realize that the rate of autophosphorylation of full-length PDK1 is very slow. Gao and Harris studies show a very slow rate of phosphorylation at 30 °C (Bioorg Chem, 2006. 34(4): p. 200-23).

Comments on the Results of Levina et al. (Nature Comm. 2022)

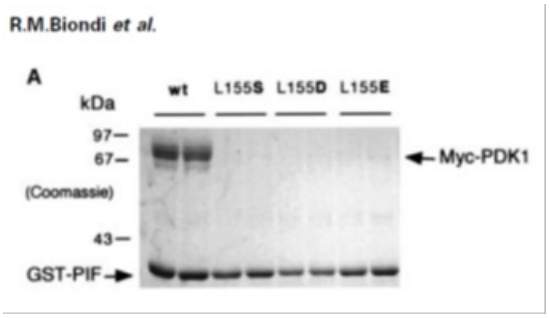
Centrally, to the Levina et al. paper, it deals with biochemical data on PDK1 autophosphorylation, replicating conclusions by Gao and Harris (2006). In addition, the paper deals with **A- a molecular model of the dimer (transient) that must form to enable the trans-autophosphorylation, B- the authors conclude that PDK1 is autoinhibited by the PH domain and C- that the autoinhibition is released by PIP3.**

A- Does the PDK1 dimer require a HM (from the linker) binding to a partner PDK1 molecule?

Levina et al. generate a model of the PDK1 dimers that must form, at least transiently, along the trans-autophosphorylation reaction.

Levina et al. conclude that a region on the linker of PDK1 interacts with the PIF-pocket of a neighbour molecule to form a dimer. As a first step into this part of the work, the authors fused the polypeptide PIFtide to PDK1 and investigated the formed PDK1-PDK1 dimer. It is unclear what new information you obtain from fusing PIF to PDK1 and studying the resulting “PDK1 dimers”. The fusion of GST to PIF (GST-PIF) interacts with high affinity with PDK1 just because the PIF-sequence has high affinity for PDK1. It happens with GST and is expected to happen with any protein fused to PIF, without any relevance to a “dimer” (see figure). The Figure is the Panel A from Fig.3 from Biondi et al. (Embo J, 2000. 19(5): p. 979-88). HEK293 cells were cotransfected to express PDK1 and GST-PIF. The lysates were incubated with glutathione-sepharose, the GST-PIF protein pulled-down by centrifugation, washed 4x (with low salt and high salt), run in SDP-PAGE and stained with Coomassie Blue. Result: strong formation of “GST-PIF / PDK1” (the duplicates two lanes on

the left), even visible by Coomassie staining. You should anticipate that anything you fuse to PIF will interact with high affinity with PDK1.



A key to the molecular model proposed by Levina et al. is the suggestion that a region within the linker between the catalytic domain and the PH domain can behave like a hydrophobic motif (HM) that can dock in trans to the PIF-pocket of a neighbour PDK1 molecule. The authors also cite a work by Morten Frödin where he observed an increase of autophosphorylation at Ser241 by addition of a hydrophobic motif (HM) polypeptide (similar to PIFtide (EMBO J., 2000, 19(12):2924-34)). This is recognized by Levina et al. “However, the binding of a HM peptide to the hydrophobic pocket has also been shown to promote PDK1 autophosphorylation”. There is a severe problem with the proposed model by Levina et al. because if their model was correct, there should be ***inhibition*** of autophosphorylation by the addition of a HM-polypeptide such as PIFtide. The authors did not perform the key experiment, but the result of the experiment is cited on the work by Morten Frödin: the HM-polypeptide ***enhances*** the autophosphorylation.

As a background information we must consider the well-established previous knowledge: that the PDK1 phosphorylations that are independent of the PIF-pocket may be enhanced by the addition of PIFtide while the phosphorylations that are dependent on the interaction with the PIF-pocket are inhibited by the addition of PIFtide. In summary, 1- the polypeptide PIFtide comprises the HM of a substrate, PRK2, and binds with high affinity to PDK1 (approximately Kd 50 nM). 2- PIFtide binds to a well characterized hydrophobic pocket termed PIF-pocket, on the small lobe of the kinase domain of PDK1. 3- *In vitro*, PIFtide enhances the PDK1 phosphorylation of a peptide substrate, T308ide, derived from the activation loop of PDK1. The enhanced phosphorylation is mediated by an allosteric mechanism between the PIF-pocket and the ATP-binding site, as shown by multiple approaches throughout the years. 4- Substrates of PDK1, like SGK, S6K and RSK use their C-terminal P-HM motifs to bind to the PIF-pocket of PDK1 and become phosphorylated at the activation loop. 5- In vitro, the addition of PIFtide competes for the docking of PDK1 to the HM of substrates like SGK, S6K and inhibits the ability of PDK1 to phosphorylate the substrates. This inhibition happens even if the binding of PIFtide enhances the catalytic activity of PDK1. 6- A long polypeptide consisting of PIFtide fused to T308tide (PDKtide) is an excellent *in vitro* substrate of PDK1 while addition PIFtide inhibits the phosphorylation of PDKtide (explained by the competition for the docking site). Together, the data from a large set of papers shows that PIFtide binds to the PIF-pocket and allosterically activates the kinase, increasing its ability to phosphorylate peptides or protein substrates that do not dock onto the PIF-pocket. On the other hand, PIFtide and small molecules binding to the PIF-pocket like PIFtide block the phosphorylation of substrates that require docking to the PIF-pocket, but do not block the phosphorylation of Akt/PKB, whose IGF1 induced phosphorylation does not require docking interaction with the PIF-pocket.

In the paper by Morten Frödin cited by Levina et al. (EMBO J., 2000, 19(12):2924-34), the authors found that the phosphorylated HM peptide (HM-P) of RSK (similar to PIFtide) enhanced the trans-autophosphorylation of PDK1 at Ser241. This is the standard finding when a substrate does not dock into the PIF-pocket. If the docking interaction between the presumed HM and the PIF-pocket occurred, then we should have expected the inhibition of the trans-autophosphorylation.

As stated above, the authors do not show the result of the key experiment, to test the effect of added PIFtide on the dimer interaction or on the autophosphorylation. But the result of the experiment on the autophosphorylation is cited and explicit in Levina et al. In the work by Morten Frödin: the HM-polypeptide enhances the autophosphorylation. This publicly known result cannot be easily reconciled with their derived molecular model.

B- The authors conclude, spelled out in the abstract and in Figure 6, that PDK1 is autoinhibited by the PH domain.

In contrast to the text in the abstract, the cartoon in the image of Figure 6A, etc. it is well established that the PH domain does not inhibit the activity of PDK1. If the PH domain were to inhibit PDK1 activity, we should have observed increased catalytic activity in a PDK1 construct lacking the PH domain. However, this contrasts the work of many of us: It is very well established that the full-length protein and the construct lacking the PH domain have similar specific activities towards the peptide substrate T308tide and towards physiological protein substrates, i.e. SGK and S6K. Gao and Harris (2006) also show that the full-length PDK1 and the catalytic domain (Delta PH) equally phosphorylate the peptide T308tide (Bioorg Chem, 2006. 34(4): p. 200-23). Of course, if there was an autoinhibition by the PH domain, it should inhibit all substrates, which is NOT the case. We know from the work of two decades by different people that this statement by Levina et al. is wrong. And since Levina et al. claim autoinhibition by the PH domain, they model the PH domain blocking the access to the active site of PDK1. Again, their derived molecular model depicting the PH domain blocking the active site must be wrong.

Neither the PH domain autoinhibits the kinase activity nor the PH domain is essential for kinase activity. Both statements in the paper by Levina et al. are not proven and should be read with caution: Those statements contrast with the results of many hundreds of experiments performed by different groups over two decades.

C- PDK1 Autoinhibition and release of inhibition by PIP3?

The slower autophosphorylation of full length PDK1 in relation to the rate of autophosphorylation of the catalytic domain (LKD) construct is taken as a proof that the PH domain hinders the activity of PDK1 (Figure 4a). And then the increased activity towards the substrate SUMO-Crosstide by the addition of PIP3-lipid vesicles is taken as a proof that the autoinhibited form can be released by PIP3.

One of the concerns about the Levina et al. claim of different rate of autophosphorylation of different constructs of PDK1 (Figure 4a), is the different mechanism of phosphorylation of constructs, cis- vs trans-. Gao and Harris (2006) described that the full-length construct autophosphorylates in cis while the catalytic domain construct autophosphorylates in trans. Autohosphorylation in cis is independent of the concentration while the rate of autophosphorylation in trans increases at higher concentrations. When the rate of phosphorylation of one construct is dependent on the concentration and the other

is not, the comparison is at least tricky, because the increased or decreased rate of phosphorylation will just depend on the assay conditions chosen.

Gao and Harris (2006) found that the addition of PIP3 vesicles increased the autophosphorylation and suggested that this was a release of an autoinhibition (similar to the current claim by Levina et al., although not cited by Levina et al). Notably, Gao and Harris showed that in the autophosphorylation of full-length PDK1 in the presence of PIP3 there is a trans-autophosphorylation. Therefore, the phosphorylation is dependent on the concentration of PDK1: this means that the higher concentration favours the encounter of PDK1 molecules and therefore the trans-autophosphorylation. The addition of PIP3 vesicles into the autophosphorylation assay (and not the addition of the isolated headgroup IP4 -a control performed by Gao and Harris-) produces the translocation of full-length PDK1 from the solution in 3-dimentions into the 2-dimentions of the surface of the vesicles, and produce an important increase in the local/efficient concentration of PDK1 at the surface of the vesicles. Being a trans-autophosphorylation, dependent on the concentration, the effect observed by Gao and Harris by the addition of PIP3 is the expected effect of the increase in the efficient concentration of PDK1, and does not require the existence of a release of autoinhibition. In short, we don't agree with the interpretation of release of autoinhibition by PIP3. When not fully phosphorylated at Ser241, or when artificially dephosphorylated (as in the work by Gao and Harris and now by Levina et al.) the increased local concentration of PDK1 by interaction with vesicles containing PIP3 will support the increased rate of autophosphorylation and an expected increase in activity… without any need of a model suggesting "autoinhibition" or release of autoinhibition or "activation" by PIP3.

D- Increased phosphorylation of the non-standard substrate SUMO-Crosstide in the presence of PIP3 vesicles.

In Figure 5, Levina et al. describe increased phosphorylation of SUMO-Crosstide in the presence of PIP3-vesicles. Although we don't know for certain which is the reason of the increased phosphorylation of this particular substrate (SUMO-Crosstide) in the presence of PIP3 we cannot support their strong conclusion that the increased phosphorylation measures "activation" of PDK1 because it has been well established throughout the years that the activity of PDK1 towards other substrates is not modified by PIP3-vesicles (i.e. the phosphorylation of SGK by PDK1 is not affected by PIP3; Kobayashi and Cohen, Biochem J. (1999) 339, 319–328) nor S6K phosphorylation is affected by PIP3 (Alessi et al. Current Biology (1997) 8, 69-81), to name only two examples).

What is the relevance of an increased activity towards SUMO-Crosstide if there is no change of specific activity using other physiological substrates? We should note that Levina et al. use SUMO-Crosstide as substrate in a **non-standard** *in vitro* assay for PDK1 activity. The sequence of Crosstide is derived from the Akt/PKB phosphorylation site on GSK3, and is a substrate traditionally employed to test the activity of Akt/PKB and other AGC kinases but not PDK1. PDK1 doesn't phosphorylate well the peptide substrate termed Crosstide (GRPRTSSFAEG). We know this because Crosstide is a traditional substrate of Akt/PKB and we and others have extensively measured Akt/PKB activation as a measure of PDK1 activity (Casamayor, A., P.D. Torrance, T. Kobayashi, J. Thorner and D.R. Alessi, Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. Curr Biol, 1999. 9(4): p. 186-97). Such assay of Akt/PKB in the presence of PDK1 is possible because PDK1 does not phosphorylate Crosstide. The non-standard substrate used by Levina et al. in the *in vitro* assay is Crosstide fused to SUMO-1. We must therefore consider the possibility that the phosphorylation of SUMO-Crosstide involves an additional interaction with PDK1 that is provided by SUMO-1. The

authors show that this substrate is not phosphorylated by the isolated catalytic domain of PDK1, suggesting to us that the additional interaction of SUMO-Crosstide with PDK1 that enables its phosphorylation is given by regions outside the catalytic domain of PDK1. Unfortunately, the authors don't state the specific activity of PDK1 towards this substrate, so we cannot compare with the data from previous studies using different substrates. The authors followed the phosphorylation of SUMO-Crosstide by SDS-PAGE and autoradiography, which suggest that the substrate is a weak substrate and, like the standard substrate T308tide, that the concentration of SUMO-Crosstide used was well below the Km of the substrate. In such cases, the changes in the concentration of the substrate can make important changes in the rate of phosphorylation. Why there is an increased activity towards SUMO-Crosstide in the presence of PIP3-vesicles but not an increase rate of phosphorylation in the presence of PIP3 when using other well characterized substrates employed previously by the scientific community? One possibility is an experimental artifact where SUMO-Crosstide also is enriched at the phospholipid vesicles and phosphorylation proceeds better due to increased efficient/local concentration in the proximity of PDK1 when PDK1 localizes at the lipid vesicles in the presence of PIP3. Interestingly, SUMO-1 has a positive charge surface patch that has been described by NMR to participate in binding to dsDNA ("SUMO-1 possesses DNA binding activity". BMC Res Notes, 2010. 3(1): p. 146); also, SUMO1 modification of PTEN (a process where SUMO-1 is covalently attached to PTEN) has been described to trigger membrane localization of PTEN-SUMO mediated by electrostatic interactions (where positive surface charges from SUMO-1 participate in the binding to membranes) ("SUMO1 modification of PTEN regulates tumorigenesis by controlling its association with the plasma membrane". Nat Commun, 2012. 3: p. 911); in this line, the fusion of SUMO-1 to Crosstide adds two Arginine residues, two additional positive charges that could further support the interaction of SUMO-Crosstide with the negative charges at the surface of the lipid vesicles. With the substrate SUMO-Crosstide being employed in the assay below its Km, and enriched at the surface of the phospholipid vesicles, the increased localization of PDK1 at the vesicles with the addition of PIP3 would be expected to increase the rate of phosphorylation of SUMO-Crosstide, but will just reflect the particular localization of the substrate and not a "release of autoinhibition" of PDK1. This is a possible explanation to the observed result by Levina et al. A proper characterization of the new substrate and many controls would be required to understand the effect of PIP3-vesicles on SUMO-Crosstide. The central point, however, is that the effect was not observed historically when using other peptide substrates and other physiologically relevant protein substrates.

In any case, the terminology "autoinhibition" mediated by the PH domain will certainly lead to confusion, because there is no traditional autoinhibition by the PH domain. Also, we would need to be very cautious about the terminology "activation" by PIP3 when the increased activity is observed apparently against only one substrate, an artificial substrate, SUMO-Crosstide. We are worried that the terminology employed by Levina et al. and the text in the title and abstract will lead to confusion because the PH domain does NOT autoinhibit PDK1 activity and PIP3 does not activate PDK1 by release of an autoinhibition.