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Regulation of ABCB1/PGP1-catalysed auxin transport by linker phosphorylation

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1st Editorial Decision 10 August 2011

Thank you for submitting our manuscript to the EMBO Journal. Three referees have now seen your study and their comments are provided below.

As you can see there is an interest in the study and the finding that PID regulates ABCD activity. However, significant work is also needed to substantiate the conclusions drawn. The referees raise many concerns with the analysis. I will not repeat all of them in this letter as they are clearly outlined below. However, some of the key points are that you need to resolve the discrepancies between the yeast and benthamiana auxin transport systems, further data is needed to support the biological significance of ABCB1 S634 phosphorylation using mutant analysis. Lastly, insight into the role of TWD1 in the described process and the significance of the TWD1/PID interaction has to be better sorted out. As you can see a lot of work is needed, but should you be able to address the concerns raised in full then we would be willing to look at a revised version. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. I recognise that a lot of work is needed and I can extend the revision time should you need it. Please contact me if you need more time.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors describe the identification of PID as an ABCB1/TWD1 interactor. PID was initially identified in pull downs with TWD1. The authors note that these pulldowns indicated the possibility of a direct or indirect interaction. The mascot score for PID is quite low, consistent with this being a minority protein. The TWD1 interaction was confirmed by in vitro pulldowns of a small amount of TWD1 with PID in fractions where both TWD1 and PID were overexpressed. These results are consistent with a weak interaction that might be expected of a kinase or where a third protein like ABCB1 might stabilize the interaction. The authors go on to show that the expression domains of PID and TWD1 are similar and overlap partially with ABCB1.
This portion of the manuscript is of potential concern, as the overlap of domains is apparently based on analysis of 35S-driven expression. In the case of ABCB1, the authors provide additional evidence of co-localization with PID under the control of native promoters, although the resolution and magnification do not really provide an adequate means to assess the co-localization. It is difficult to understand how visualization of these constructs indicates overlapping expression domains.

The authors also use BRET and co-localization to substantiate TWD1 and PID interactions. Although the co-localization is not particularly convincing, the BRET results are consistent with this interpretation. This result would be more convincing if an unrelated membrane associated protein was also expressed in the system as a Luc fusion and produced a negative result.

A negative effect on ABCB1-mediated transport is then shown in yeast with a kinase inactive form of PID used as a control. This result is convincing, although, if the logic of the pull downs from Figure 1 is followed, it would have to imply that PID is interfering with a TWD1-like activity in yeast to impact the activity.

The authors purport to show that it is an effect on ABCB1 interactions, by showing that expression of PID inhibits an apparent enhancement of ABCB1 activity by PIN1 and PIN2. These results introduce more questions than they resolve, as ABCB1 /PIN activity in the presence of PID appears to be reduced more than ABCB1 activity alone. This is difficult to reconcile with the established mode of action of PID regulating PIN protein localization and polarity in planta. Further, it is difficult to sort out what the effects are of multiple proteins in this system, as the relative protein levels are not provided.

The authors own previous work showed that activation of ABCB1 by TWD1 is highly dependent on levels of protein expression. These results with PIN1/2 and those with PIN1 in 3A might be best left out of the paper unless the PM localization and abundance of active protein levels of all of the proteins involved are better documented. The section also shouldn't have a heading describing the effects as "in planta" when only the last paragraph describes a plant system, and this is a heterologous protoplast system.

In these experiments , coexpression of ABCB1 with PID appears to have the opposite effect of a what is seen in yeast cells. Export mediated by ABCB1 is increased , while PIN1 mediated export is decreased. It would help to have verification that PIN1 and ABCB1 are localized to the PM in these experiments. Although some evidence of internalization of PID1 is seen in the +PID background, the PM co- localizations of PIN1 and ABCB1 are not clearly demonstrated.

Next, the authors show that loss of PID results in increased transport- again, apparently the opposite of what is observed in the benthiama assays. The explanation from the authors is that this is a case where the activity in yeast is the opposite of what is seen in planta and is similar to what is seen with TWD1 in yeast. However, this is a bit of a stretch as PID is a kinase and TWD1 is an FKBP chaperone that may have some PPIase activity. The more obvious interpretation is found in the conclusion that the authors reach- that PID regulates TWD1-ABCB1 interactions and these have complex impacts. It would be interesting to note whether any twd1 phenotypes are evident in 35S:PID or pid loss of function mutants.

A minor issue with these experiments is the question of the fate of the label in the 3H-IAA experiments. Work from Delbarre and others has shown that IAA is rapidly metabolized in tobacco cell cultures. It it is surprising that the NAA and IAA signals are almost identical if IAA metabolism is taking place.

The next set of experiments attempts to establish that quercetin reverses the effects of PID co-expression with ABCB1 in yeast. The result appears to be quite specific, although it would help to know the concentration used and to have information regarding the effect on another kinase. The authors show that, as expected, quercetin inhibits PID kinase activity. The authors also show that the presence of PID correlates with quercetin binding to microsomal membranes, that the linker domain appears to house a binding site, and that modification of this site correlates with altered effects of PID coexpression in yeast. These results are certainly consistent with quercetin binding, but do not conclusively demonstrate that quercetin binds directly to PID. The results and discussion should be modified to reflect this.

Finally, the authors show that NPA, quercetin, and chelerythin have similar effects on auxin influx in the region of the root in the vicinity of the elongation zone and that these are reflected in root gravitropism responses. It is interesting that treatment with quercetin and chelerytrin can partially rescue the agravitropism phenotype of pin2. It is an interesting suggestion that this is a result of activity against PID-ABCB1 interactions that is made more plausible by the observation that chelerythin, which appears to be far more specific than quercetin in inhibiting PKC activity, has such a clear effect.

Overall, this is a very interesting study that uses some unique biochemical tools to analyse PID-ABCB1 interactions. AS such, it is a refreshing change from studies that infer function just from the positioning of PINs in a membrane domain. Other than the minor controls that need to be supplied as noted, the manuscript needs some restructuring. First the authors should show what is demonstrated in PID-TWD1 interactions, then PID-ABCB1 interactions, then attempt to resolve the discrepancies and apply the results to what is observed in Arabidopsis. It is suggested that more attention be paid to evaluating what experiments are relevant and ordering them accordingly. Finally, as noted, the use of negative controls including other similar protein targets or kinases would strengthen the paper.

Minor: Some of the abbreviations in the manuscript are unnecessary- for instance NBP for NPA binding proteins. This is a term that is better to be avoided anyway and is not pertinent to this manuscript
Some editing is required for usage (example page 6 sentence starting "while AP2C1 is known to be involved.....". And "pepetide" in the same paragraph. Also "purified as described recently- references are from 2001 and 2002.

Referee #2 (Remarks to the Author):

In their ms the authors seek out to address the regulation of ABCB/MDR-dependent auxin transport in Arabidopsis and present some highly interesting findings.

Based on earlier findings made by the corresponding author's group, which identified the immunophilin-like protein TWD1 as regulator of ABCBs, the authors went on with a shotgun proteomics approach and identified protein interactors of TAP-tagged TWD by LC-MS/MS. Somewhat unexpectedly, PINOID, an AGC-type kinase known to affect transcytosis of PIN-type auxin carriers, turned out to interact with TWD in vivo and in vitro.

The authors then went on to analyze how PID might influence ABCB1-mediated auxin transport and determined auxin efflux in S cerevisiae and in N. benthamiana protoplasts, demonstrating an inhibitory effect in yeast, whilst in planta a substantial increase in auxin efflux could be obtained. This led the author to suggest that PID acts as a positive regulator of ABCB1 activity in auxin transport.
A potential role for PID kinase activity in mediating this response might be provided by pharmacological studies, since the authors demonstrate that staurosporin and quercetin seemingly antagonize PID-dependent inhibition of ABCB1 activity (I could not find information about the experimental set-up; neither in the result section nor in the figure legend, but I assume that this is the yeast-based assay). This effect could arise as a result of diminished kinase activity, since quercetin was found to bind to and to interfere with PID phosphorylation activity in vitro.
By using MS the authors also came up with ABCB1 S634 as phosphorylation site, potentially recognized by PID in planta, which is indicated by in vitro phosphorylation assays.
In a final set of experiments the authors determine effects of kinase inhibitors on PAT in root meristems and their ability to modulate root gravitropism. Whilst micro-electrode based measurements suggested an inhibitory effect of quercetin and chelerythrine on auxin influx in root meristems, analysis of root gravitropism in mutant combinations led to the suggestion that quercetin and chelerythrine effects depend on ABCB1 and ABCB19.

The finding that PID -apart from its function in PIN transcytosis- might modulate activity of ABCB-type auxin carriers comes somewhat unexpected and represents a highly interesting observation, suggestive of a scenario in which PID-type AGC kinases have evolved quite distinct functions. Having said that, the data presented is not sufficiently convincing to justify publication in EMBO J.

Some points:

PID was identified as protein interacting with TWD1. In all further experiments the authors address cross-talk between PID and ABCB (PINs) without assessing a potential effect of TWD co-expression. Whilst it is a valid argument that putative TWD1 orthologues could substitute for TWD1 in heterologous hosts, the impact of TWD1 still needs to be addressed in auxin transport assays. This seems of particular relevance, as the authors stress the important linker role of TWD1 in their model.

I am quite puzzled about the opposing activities of PID when comparing yeast and benthamiana auxin transport assays. I do not see how an altered membrane composition (as suggested in the discussion) could account for these different effects. It would be helpful to see the localization of the proteins in both hosts ("not shown", page 7). Again, it seems worthwhile to study consequences of TWD1 co-expression in these auxin transport assays.

the authors identify S634 as an ABCB1 phosphorylation site in planta. (page 9). In this context the authors compare predicted PID phosphorylation sites in PINs (TPRXS motifs) and the region flanking S634 in ABCB1, which lacks any similarity to the PIN sites. In neither case a conclusive (in vivo) proof for recognition/modification of these sites by PID has been provided. Thus, any statement about a direct involvement of PID in phosphorylating these motifs is still speculative. The authors should have addressed the biological significance of S634 by testing phosphomimetic alleles in their auxin transport assays. Such experiments would provide strong evidence for a mechanistic role of ABCB1 phosphorylation.

I do not fully agree with the interpretation of the root gravitropism - kinase inhibitor experiments (page 10-11). Based on another report (Santelia et al., 2008, JBC) the authors test the role of PIN and ABCB type auxin carrier proteins in mediating root gravitropism in the presence of kinase inhibitors. This section is a bit confusing. On page 10 (last paragraph, line 3) the authors suggest that chelerythrine effects on root gravitropism in pin2 are PIN1-dependent. On page 11 however (line 2) the authors point out that ".....Further analysis showed that the chelerythrine rescue was not PIN1 dependent (Fig. 7B), which is in contrast to what is found for flavonols....." In my opinion, the data presented is in agreement for a role of both PIN1 and ABCB1/19 activities.

Some minor issues:

The authors provide evidence for a role of kinase activity in the regulation of auxin carrier activity.

Zourelidou and others (2009; Development 136: 627) describe D6 PROTEIN KINASE as potential regulator of PIN activity. This reference should be cited.

Being not a native speaker myself, I would highly recommend using some of the spellchecking options offered by various programs.

I think this ms presents a lot of novel and interesting insights into control of PAT in higher plants. Some additional experiments would transform the current ms into a highly valuable contribution to the auxin field.

Referee #3 (Remarks to the Author):

In their manuscript, the authors examine the role of the AGC kinase PINOID (PID) in the regulation of auxin transport via the multidrug transporter proteins ABCB1 from Arabidopsis. The experiments are triggered by the observation that PID appears as an interactor of TWD1, a possible regulator of ABCB1, following immunoprecipitation and mass spec analysis. The authors present evidence that PID may control auxin transport via ABCB1 and identify a phosphorylation site in ABCB1 that may result from PID transphosphorylation.

Unfortunately, the authors have not made much use of the excellent genetic and biochemical tools that are available for Arabidopsis research to date but have chosen to go for a number of assays in heterologous systems, transient expression assays etc. that overall blur the significance of each individual finding. As it stands, the paper has insufficient quality to be published. The fact that TWD1 was used to identify PID is completely lost in the manuscript since most of the remaining part is concerned with the role of PID as a regulator of the interactor of TWD1, ABCB1. As such the paper is badly designed.

I have listed a range of comments below and my comments do not extend to the final parts of the results section or the discussion.

Comments to the experiments:

The filtering of mass spec data is not entirely clear to me. It should be stated clearly what was the negative control ("we substracted identified vector control proteins" - what are these vector control proteins? Myc-IP from an empty vector only control plant).

Since the key interactor, PID, was only recovered with a single peptide and its validity has to be seriously questioned. Since the authors have various GFP-tagged PID lines, they may use these in combination with TWD1-TAP for proper co-IP experiments.

For the BRET experiment, it would have been nice to have a positive control that allows to judge the strength of the observed BRET. Does the TWD1-rLUC construct complement the twd1 phenotype?

What is the role of TWD1 and its interaction with PID for the function of ABCD1?

In the introduction, the authors introduce PID, WAG1 and WAG2 as members of the AGC3 kinase family or subfamily and - at a latter stage in their introduction - refer to these kinases as WAG kinases but the definition of this family is not precisely given. If this includes PID kinases, then the name should be revised since the understanding of the PIDs is far more advanced than the understanding of the WAGs. A uniform nomenclature should be used for this protein family and its individual members throughout the manuscript.

There is no ref for the TAP-tag employed.

It remains unexplained why only a fragment of TWD1 was used for the pull-down (Fig. 1B). What does the TWD1 double band represent that is apparent after the pull-down?

Although overlaps are apparent between TWD1, ABCB and PID expression, the co-localization of the proteins at the subcellular level is not shown, e.g. at the membrane. Possible changes in ABCB

and TWD1 localization could be addressed with pid LOF and PID OX lines.

Figure 2 shows auxin transport data obtained in yeast following expression or coexpression of PID and ABCB1. Following coexpression of PID, auxin efflux is reduced but his may be attributed to a reduced expression of ABCB1. Using immunoblots, the authors should demonstrate that ABCB1 expression levels are constant.

Later in the ms, it is stated that a similar experiment did not have an effect on the abundance or localization of the overexpressed proteins, however, the quantitative nature of this experiment has to be seriously doubted. Although in the text the authors refer to similar experiments for the yeast auxin transport assay, these data are not shown (as far as I can judge).

The role of PID on ABCB1 is tested in yeast (FIg. 2) and plant cells (Fig. 3). Opposite effects are being observed and it is unclear which one is biologically significant.

I could not find a description of the auxin transport experiments shown in Fig. 3C and D.

In various experiments the authors use pid-/+, which I assume refers to a segregating line. For measurements, the authors should however by pheno- or genotyping identify pid homozygous mutants for their experiments. Working with a segregating line is not solid.

The experimental quantification in Fig. 5A (lower panel) refers to the experiments shown in the upper panels. However there is not "Chel" sample shown in the upper panels!?

The phosphorylation experiment and MS analysis does not proof that ABCB1 is phosphorylated by PID! Phosphorylation experiment with mutated ABCB1 and PID should be used to demosntrate that the site is a PID1 target, particularly since the site does not match the previously described consensus.

I am not a specialist in binding experiments but the experiments shown in Fig. 5B an C do not appear to be solid enough.

Comments to the text:

NBP is not properly introduced (first as binding protein) then later again as nucleotide binding protein.

At various places in the text I was not content with the phrasing and several sentences appeared unclear to me. Some examples:

"Mutant analysis and pharmacological treatment with kinase/phosphatase inhibitors, staurosporine/cantharidin, revealed..." should be "...with kinase and phosphatase inhibitors, staurosporine and cantharidin, respectively, revealed..."

"PIN efflux carriers show mainly polar locations in PAT tissues and are thought to be determinants of a reflux loop in the root apex characterized by strong developmental phenotypes (Refs)." Sentence unclear: Polar locations cannot be characterized by strong phenotypes.

"ABCB- and PIN-mediated auxin efflux can function independently...until the end of the paragraph" leaves a rather confusing picture of the ABCB and PIN interactions. What are "Interacting cells" in this context? Cells where PINs and ABCBs interact physically or neighbouring cells?

"However, several lines of evidence..." p. 3 "However" does not make sense.

"We chose second generation TAP tagging...because of its high degree of two-step affinity purification." ?

"While APC21 is known to be involved... p.6, middle." Sentence unclear.

1st Revision - authors' response 13 February 2012

In the following, we summarize here the points that were brought up in the reviews and how they are addressed in the revised manuscript:

Referee #1 (previous comments to the authors in italic):

1. The authors go on to show that the expression domains of PID and TWD1 are similar and overlap partially with ABCB1.
This portion of the manuscript is of potential concern, as the overlap of domains is apparently based on analysis of 35S-driven expression.

We agree entirely that 35S promoter driven locations need to be taken with care but would like to mention that they match quite nicely database expression profiles (Fig. 1B). However, the ratio to present these pictures was exactly to validate them as over-expressing constructs were used for transport studies, such as in Fig. 3 and 6.
Moreover, TWD1-PID and ABCB1-PID colocations on the plasma membrane were further substantiated by co-expression on tobacco protoplasts (Figs. 1D, 3B, 3B), which is now added to the Results (page 5, second last paragraph):
"TWD1-PID and ABCB1-PID colocations on the plasma membrane were further substantiated by co-expression in tobacco protoplasts (Fig. 1D, 3B, S3)."
2. In the case of ABCB1, the authors provide additional evidence of co-localization with PID under the control of native promoters, although the resolution and do not really provide an adequate means to assess the co-localization. It is difficult to understand how visualization of these constructs indicates overlapping expression domains.

We have substantially improved the quality of confocal images presented now in Fig. 1C, and close-ups demonstrate convincingly plasma membrane locations for all three proteins, as well as co-IL for ABCB1 and PID (Fig. 1F), which is now - as explained above - of relevance for functional PID-ABCB1 interactions in the absence of TWD1.

3. The authors also use BRET and co-localization to substantiate TWD1 and PID interactions. Although the co-localization is not particularly convincing, the BRET results are consistent with this interpretation. This result would be more convincing if an unrelated membrane associated protein was also expressed in the system as a Luc fusion and produced a negative result.

As suggested also by another reviewer, we have added a positive (TWD1-Rluc/ABCB1-YFP) and two negative controls (TWD1-Rluc/PIRK-YFP and TT12-Rluc/PID-GFP. PIRK is a plasma membrane (PM) protein kinase, while TT12 is a PM and vacuolar MATE transporter (Marinova et al. 2007). BRET ratios for PID/TWD1 were in the same range as found for established ABCB1/TWD1 interaction, supporting PID/TWD1 interaction in a plant system: (page 6, first paragraph):

"This interaction is specific since single expression of TWD1-Rluc, Rluc or YFP alone or TWD1-Rluc in combination with the non-related, plasma-membrane-bound protein kinase, PIRK (PIRK-YFP), only resulted in negligible BRET ratios. A similar result was found for PID-GFP co-expressed with TT12-Rluc, an unrelated plasma membrane control."

4. A negative effect on ABCB1-mediated transport is then shown in yeast with a kinase inactive form of PID used as a control. This result is convincing, although, if the logic of the pull downs from Figure 1 is followed, it would have to imply that PID is interfering with a TWD1-like activity in yeast to impact the activity.

As mentioned under the key point 1.), this important point holds based on our finding in yeast and plant systems indeed true, and is discussed in detail in the Discussion section Page 12, third paragraph:

"The obvious question that now arises is why PID has a negative impact on ABCB1 in yeast in the absence of TWD1. The most likely explanation is that yFKBP12 is able to functionally complement TWD1 in yeast as has been suggested for TWD1 modulation of ABCB1 (Bailly et al, 2008;

Bouchard et al, 2006). This is supported by the findings that ABCB1-mediated auxin efflux from yeast is strongly reduced in an *fkbp12* strain (Bouchard et al, 2006) in analogy to mammalian MDR3 that was shown to be dependent on yFKBP12 (Hemenway & Heitman, 1996). Finally, *yFKBP12* is able to widely complement *twd1* loss-of-function alleles (unpublished data)."

5. The authors purport to show that it is an effect on ABCB1 interactions, by showing that expression of PID inhibits an apparent enhancement of ABCB1 activity by PIN1 and PIN2. These results introduce more questions than they resolve, as ABCB1 /PIN activity in the presence of PID appears to be reduced more than ABCB1 activity alone. This is difficult to reconcile with the established mode of action of PID regulating PIN protein localization and polarity in planta. Further, it is difficult to sort out what the effects are of multiple proteins in this system, as the relative protein levels are not provided.

The authors own previous work showed that activation of ABCB1 by TWD1 is highly dependent on levels of protein expression. These results with PIN1/2 and those with PIN1 in 3A might be best left out of the paper unless the PM localization and abundance of active protein levels of all of the proteins involved are better documented.

As mentioned in our comments to the editor we agree fully to this point and have therefore removed all yeast and tobacco transport data from the ms. that concern PINs. We feel that like this the flow and key message of the ms. - as indicated by the title - has improved substantially.

The section also shouldn't have a heading describing the effects as "in planta" when only the last paragraph describes a plant system, and this is a heterologous protoplast system.

Thanks a lot for pointing this out. We have after PIN data removal, fused the heterologous transport data (yeast and tobacco) with Arabidopsis protoplast assays, and as tobacco triple ABCB1/PID/TWD1 transport data are in agreement with Arabidopsis data (both point to a negative impact of PID on auxin transport), we feel that "*in planta"* would now be appropriate. However, as we use only shoot-derived transport systems and things look in the root slightly different (Fig. 7). Therefore, we prefer to use the following title for this paragraph: "PID has a dual impact on ABCB1-mediated auxin efflux"

6. In these experiments, coexpression of ABCB1 with PID appears to have the opposite effect of a what is seen in yeast cells. Export mediated by ABCB1 is increased, while PIN1 mediated export is decreased. It would help to have verification that PIN1 and ABCB1 are localized to the PM in these experiments. Although some evidence of internalization of PID1 is seen in the +PID background, the PM co- localizations of PIN1 and ABCB1 are not clearly demonstrated.

We have carefully repeated imaging of ABCB1 co-expressed with PID, TWD1 and PID/TWD1 combinations and found no significant difference in ABCB1 localization (Figs. 3B, S3). Moreover, these pictures also clearly demonstrate PM locations for PID and TWD1 and co-IL using TWD1/PID, ABCB1/PID and ABCB1/PID/TWD1 combinations indicate co-locations, although some of these are limited to certain PM subdomains.

Finally, constant ABCB1 expression in tobacco co-expression experiments was further verified by Western detection of MYC-tagged ABCB1 in comparison to PM marker H^+-ATPase, AHA2 (Fig. S3B).

IL and Western controls after sucrose gradient centrifugation were also provided for yeast transport data (Fig. 2, Fig. S2) and mutational analyses of ABCB1 (Fig. 6).

7. Next, the authors show that loss of PID results in increased transport- again, apparently the opposite of what is observed in the benthiama assays. The explanation from the authors is that this is a case where the activity in yeast is the opposite of what is seen in planta and is similar to what is seen with TWD1 in yeast. However, this is a bit of a stretch as PID is a kinase and TWD1 is an FKBP chaperone that may have some PPIase activity. The more obvious interpretation is found in the conclusion that the authors reach- that PID regulates TWD1-ABCB1 interactions and these have complex impacts.

Discrepancies between yeast and plant expression/transport systems have now been addressed and clarified in detail (see under key points to the Editor).

Thanks for pointing out that PID might alter ABCB1-TWD1 interaction by ABCB1 phosphorylation. Although linker phosphorylation would require a long-distance mechanical

transition to the C-terminus that was shown to interact with TWD1, this is a valid option that is currently under investigation and was added to the Discussion section (page 15, last paragraph):

"Alternatively, ABCB1 linker phosphorylation might also alter ABCB1-TWD1 interaction, which is currently under investigation."

8. It would be interesting to note whether any twd1 phenotypes are evident in 35S:PID or pid loss of function mutants.

We have carefully looked at epidermal twisting in *pid* and *35S:PID* alleles but beside described root defects in the columella have not found any obvious "twisted syndromes", suggesting that PID regulation is eventually specific for ABCB1. It might be mentioned in this context that beside ABCB1, also ABCB19 and evt. also even ABCB4 have been shown to be dependent on TWD1 action (Wu et al. 2010).

9. A minor issue with these experiments is the question of the fate of the label in the 3H-IAA experiments. Work from Delbarre and others has shown that IAA is rapidly metabolized in tobacco cell cultures. It is surprising that the NAA and IAA signals are almost identical if IAA metabolism is taking place.

As described in detail in the ms. describing first this assay in Arabidopsis, we are performing these experiments essentially "on ice" (except for the 10 or 20 minutes for that we shift the samples to 25°C for initiating transport) in order to avoid auxin degradation. In that respect, we do not think that it is surprising that IAA and NAA rates are similar but take this comment as support of the quality of our transport data. However, when you have a careful look at the NAA data, you will see that PID effect on ABCB1 is even more pronounced for NAA than IAA, which is surprising as NAA is able to reflux into protoplasts again. Under this light, IAA degradation in tobacco protoplast might indeed have a minimal effect but should only shift the background baseline.

10. The next set of experiments attempts to establish that quercetin reverses the effects of PID co-expression with ABCB1 in yeast. The result appears to be quite specific, although it would help to know the concentration used and to have information regarding the effect on another kinase.

Yeast experiments were performed with inhibitor concentrations of 1 µM; this information is added now to the legends. Specificity of PID actin was demonstrated in yeast (Fig. 2) by employing unrelated GSK-3-like kinase BRASSINOSTEROID-INSENSITIVE2 (BIN2), a negative regulator of brassinosteroid signaling. Further, we have added a closely related AGC3 kinase, WAG1, in protoplast assays (Fig. 3). Both had no significant effect on ABCB1 activity (page 7, first paragraph):

"Moreover, closely related AGC3 kinase, WAG1 (39% protein sequence identity), sharing overlapping functionality with PID (Dhonukshe et al, 2010; Huang et al, 2010), had no significant effect on ABCB1."

11. The authors show that, as expected, quercetin inhibits PID kinase activity. The authors also show that the presence of PID correlates with quercetin binding to microsomal membranes, that the linker domain appears to house a binding site, and that modification of this site correlates with altered effects of PID co-expression in yeast. These results are certainly consistent with quercetin binding, but do not conclusively demonstrate that quercetin binds directly to PID. The results and discussion should be modified to reflect this.

We agree that binding studies using yeast and plant microsomes provide only indirect evidence that PID itself is a quercetin target and actually our work shows that ABCB1 phosporylation (probably by PID) or ABCB1 phospho-mimikry enhances quercetin binding to ABCB1 (Fig. 6D). In order to differenciate between these two options or to show that both options hold true, we performed quercetin binding assays using purified PID-GST (Fig. 5C) clearly demonstrating quercetin binding to PID.
Overall, binding to ABCB1 and PID is not that surprising as quercetin was shown to bind to ATP-binding pockets, such as the nucleotide binding folds of ABCBs, blocking ATP binding and hydrolysis (for a recent review, see Chen et al. 2010). This suggests that phosphorylation of the

linker, that is in direct connection to the N-terminal nucleotide binding fold (see Fig. 6), might also alter binding of ATP to these ATP pockets and is most probably not directly responsible for quercetin binding.
This information is briefly added to the Discussion section (page 13, second paragraph):

"Alternatively, phosphorylation of the linker, that is in direct connection to the N-terminal nucleotide-binding fold (see Fig. 6), might also alter ATP binding to these ATP pockets."

12. Overall, this is a very interesting study that uses some unique biochemical tools to analyse PID-ABCB1 interactions. AS such, it is a refreshing change from studies that infer function just from the positioning of PINs in a membrane domain.

Nice to hear, thank you very much!

13. Other than the minor controls that need to be supplied as noted, the manuscript needs some restructuring: First the authors should show what is demonstrated in PID-TWD1 interactions, then PID-ABCB1 interactions, then attempt to resolve the discrepancies and apply the results to what is observed in Arabidopsis. It is suggested that more attention be paid to evaluating what experiments are relevant and ordering them accordingly.

We have substantially re-organized the ms. and now have the feeling, that especially after removing PIN-related data, it reads much better. However, in some aspects we have also kept the original order as it represents the historical order of our approach.

14. Finally, as noted, the use of negative controls including other similar protein targets or kinases would strengthen the paper.

As mentioned above, positive and negative BRET controls and negative kinase controls have been added.

15. Minor: Some of the abbreviations in the manuscript are unnecessary- for instance NBP for NPA binding proteins. This is a term that is better to be avoided anyway and is not pertinent to this manuscript.

As suggested we have removed the abbreviation, NBP.

16. Some editing is required for usage (example page 6 sentence starting "while AP2C1 is known to be involved.....". And "pepetide" in the same paragraph. Also "purified as described recently- references are from 2001 and 2002.

The mentioned flaws have been corrected and the language has been edited by a native speaker.

Referee #2 (previous comments to the authors in italic):

1. A potential role for PID kinase activity in mediating this response might be provided by pharmacological studies, since the authors demonstrate that staurosporin and quercetin seemingly antagonize PID-dependent inhibition of ABCB1 activity (I could not find information about the experimental set-up; neither in the result section nor in the figure legend, but I assume that this is the yeast-based assay).

The information that these assays were performed in yeast was indeed missing, and is now added to the legends and results. Sorry!

The finding that PID -apart from its function in PIN transcytosis- might modulate activity of ABCB-type auxin carriers comes somewhat unexpected and represents a highly interesting observation, suggestive of a scenario in which PID-type AGC kinases have evolved quite distinct functions. Having said that, the data presented is not sufficiently convincing to justify publication in EMBO J.

Some points:

2. PID was identified as protein interacting with TWD1. In all further experiments the authors address cross-talk between PID and ABCB (PINs) without assessing a potential effect of TWD co-expression. Whilst it is a valid argument that putative TWD1 orthologues could substitute for TWD1 in heterologous hosts the impact of TWD1 still needs to be addressed in auxin transport assays. This seems of particular relevance, as the authors stress the important linker role of TWD1 in their model.

We fully agree to this point and have – as requested by you and pointed out by the editor – performed triple ABCB1/PID/TWD1 co-expression followed by auxin transport analyses using the tobacco *(N. benthamiana*) protoplast system. As explained already in detail above (see key points to Editor), triple co-expression revealed that it is essentially TWD1 itself that decides for positive or negative regulation of ABCB1 activity by PID (see Figs. 3, 6). Comparison between triple co-expression (Fig. 3A) and Arabidopsis transport data (Fig. 3C) reveals that PID *in planta* (or to be more accurate: in the shoot) in the presence of TWD1 functions as a negative regulator of export. Our tobacco data, together with ABCB1-S634 mutational analyses, indicate that in the absence of TWD1, PID phosphorylates S634 leading to enhanced ABCB1 export activity (page 7, second paragraph):

"In order to address the role of TWD1 in PID-mediated activation of ABCB1, we quantified auxin efflux from triple transfected ABCB1/PID/TWD1 protoplasts. Surprisingly, co-expression of ABCB1/PID/TWD1 entirely abolished ABCB1 auxin efflux. Co-expression of ABCB1/TWD1 revealed that at least for IAA, a portion of this inhibitory effect is caused by TWD1 itself. However, NAA efflux analyzed in parallel clearly demonstrated that PID, in the presence of TWD1, has a significant inhibitory effect on ABCB1 auxin efflux (Fig. 3A). The finding that TWD1 only affected IAA but not NAA export, as previously reported for *Arabidopsis* (Bouchard et al, 2006) was also found for yeast, where TWD1 as reported here has an inhibitory role on ABCB1 (Bailly et al, 2008; Bailly et al, 2012a; Bouchard et al, 2006), suggests that TWD1 besides its role as a regulator of activity also has an impact on ABCB1 specificity."
Confocal microscopy analyses revealed that GFP-tagged PID and TWD1 as well as YFP-tagged ABCB1 and WAG1 all reside on the plasma membrane (Fig. 3B, S3), where PID and TWD1 co-localize with ABCB1 (3B). Importantly, and in analogy to the yeast system, co-expression of PID and TWD1 did not alter expression of ABCB1 nor its location on the plasma membrane as monitored microscopically (Fig. 3B) and by Western detection of ABCB1-MYC when compared to the plasma membrane marker H^+-ATPase, AHA2 (Fig. S3A).
3. I am quite puzzled about the opposing activities of PID when comparing yeast and benthamiana auxin transport assays. I do not see how an altered membrane composition (as suggested in the discussion) could account for these different effects. It would be helpful to see the localization of the proteins in <u>both</u> hosts ("not shown", page 7). Again, it seems worthwhile to study consequences of TWD1 co-expression in these auxin transport assays.

Indeed, several studies have shown that the membrane composition alters transport activities of (ABC) transporters. That was also one of the reasons why the Murphy lab uses now the *S. pombe* auxin transport system because they could show that PIN1 and ABCB19, that are inactive in baker's yeast, are fully functional there.
However, comparison of yeast double expression and tobacco triple expression suggest that baker's yeast owns - as correctly pointed out by Reviewer 1- a functional TWD1 ortholog. Yeast does not contain a close, structurally related TWD1 orthologue, however, the most likely candidate for this, despite its weak amino acid sequence identity of 27%, is yeast FKBP12, which is supported by the following findings: First, our own work has shown that Arabidopsis ABCB1/PGP1 (like human MDR3; Hemenway and Heitman, 1996) is dependent on the presence of yFKBP12 (Bouchard et al. 2006). Second, yFKBP12 is able to functionally complement *twd1*. This information has been added to the Discussion of the ms. (page 12, second paragraph):

"The obvious question that now arises is why PID alone has a negative impact on ABCB1 in yeast in the absence of TWD1. The most likely explanation is that yFKBP12 is able to functionally complement TWD1 in yeast as has been suggested for TWD1 modulation of ABCB1 (Bailly et al, 2008; Bouchard et al, 2006). This is supported by the findings that ABCB1-mediated auxin efflux from yeast is strongly reduced in an *fkbp12* strain (Bouchard et al, 2006) in analogy to mammalian

MDR3 that was shown to be dependent on yFKBP12 (Hemenway & Heitman, 1996). Finally, *yFKBP12* is able to widely complement *twd1* loss-of-function alleles (unpublished data)."
Confocal microscopy studies using YFP–tagged ABCB1 versions in yeast (Figs. 2B, S2D and 4) and tobacco (Figs. 3B and 6G) have now been added to the ms., which are complemented by detailed Western controls (Figs. 2C, S2, S3, 4, 6G), indicating all that neither co-expression with mutated versions of PID, other kinase controls or TWD1 nor drug treatments altered significantly ABCB1 expression. This information was added to the Results section (page 6, second last paragraph):

"Moreover, PID or MPID co-expression did not substantially alter ABCB1-plasma- membrane expression or localization in yeast (Fig. 2B-C, Fig. S2A). ABCB1-YFP localizes primarily to raft-like structures at the boundaries of the plasma membrane as previously shown (Fig. 2B; (Bailly et al, 2008)) and to the plasma membrane as demonstrated by comparison of anti-GFP immune-positive fractions in comparison with the plasma membrane H^+-ATPase, PMA1 (Fig. S2)."

4. The authors identify S634 as an ABCB1 phosphorylation site in planta (page 9). In this context the authors compare predicted PID phosphorylation sites in PINs (TPRXS motifs) and the region flanking S634 in ABCB1, which lacks any similarity to the PIN sites. In neither case a conclusive (in vivo) proof for recognition/modification of these sites by PID has been provided. Thus, any statement about a direct involvement of PID in phosphorylating these motifs is still speculative. The authors should have addressed the biological significance of S634 by testing phosphomimetic alleles in their auxin transport assays. Such experiments would provide strong evidence for a mechanistic role of ABCB1 phosphorylation.

We here now have confirmed the mutational analyses in yeast by using binary, fully functional plant ABCB1-YFP constructs (Bailly et al, 2012) in an accepted plant system allowing for transport studies and location analyses at the same time. As described above, in analogy to the yeast data, S634E mutation strongly enhanced ABCB1 transport activity (Fig. 6F) mimicking ABCB1-PID co-expression, while S634A led to a loss of functionality, such as deletion of the linker in yeast. Again, mutation (or PID co-expression) did not significantly alter ABCB expression or location (Fig. 3B, 6G).

5. I do not fully agree with the interpretation of the root gravitropism - kinase inhibitor experiments (page 10-11). Based on another report (Santelia et al., 2008, JBC) the authors test the role of PIN and ABCB type auxin carrier proteins in mediating root gravitropism in the presence of kinase inhibitors. This section is a bit confusing. On page 10 (last paragraph, line 3) the authors suggest that chelerythrine effects on root gravitropism in pin2 are PIN1-dependent. On page 11 however (line 2) the authors point out that ".....Further analysis showed that the chelerythrine rescue was not PIN1 dependent (Fig. 7B), which is in contrast to what is found for flavonols....." In my opinion, the data presented is in agreement for a role of both PIN1 and ABCB1/19 activities.

Indeed, this paragraph was unclear. Our conclusions were based on the fact that agravitropism in *pin2* (28.6°) was partially rescued by chelerytrine (43.4°) and quercetin (44.8°) but in the *pin1 pin2* (12.2°) background only for chelerythrine (28.8°) and not for quecetin (15.7°), making the rescue by chelerythrine PIN1-independent. The following sentence was corrected (page 10, second last paragraph):
"Partial rescue by chelerythrine in *pin1 pin2* showed that the rescue by chelerythrine was, in contrast to what is found for flavonols, not PIN1-dependent (Fig. 7B)."
Some minor issues:

6. The authors provide evidence for a role of kinase activity in the regulation of auxin carrier activity. Zourelidou and others (2009; Development 136: 627) describe D6 PROTEIN KINASE as potential regulator of PIN activity. This reference should be cited.

Very good point, thank you. Although we have removed PIN transport data, we have added the following sentence to the end of the Discussion section (page 14, last paragraph):

"Moreover, also for PINs direct regulation by D6 protein kinases has been suggested (Zourelidou et al, 2009)."

7. Being not a native speaker myself, I would highly recommend using some of the spellchecking options offered by various programs.

As mentioned under the comments for Reviewer 1, the spelling and language of the ms. has ben corrected by a native speaker.

Referee #3 (previous comments to the authors in italic):

1. In their manuscript, the authors examine the role of the AGC kinase PINOID (PID) in the regulation of auxin transport via the multidrug transporter proteins ABCB1 from Arabidopsis. The experiments are triggered by the observation that PID appears as an interactor of TWD1, a possible regulator of ABCB1, following immunoprecipitation and mass spec analysis. The authors present evidence that PID may control auxin transport via ABCB1 and identify a phosphorylation site in ABCB1 that may result from PID transphosphorylation.
Unfortunately, the authors have not made much use of the excellent genetic and biochemical tools that are available for Arabidopsis research to date but have chosen to go for a number of assays in heterologous systems, transient expression assays etc. that overall blur the significance of each individual finding. As it stands, the paper has insufficient quality to be published. The fact that TWD1 was used to identify PID is completely lost in the manuscript since most of the remaining part is concerned with the role of PID as a regulator of the interactor of TWD1, ABCB1. As such the paper is badly designed.

We fully agree to the Reviewer's judgment that in its last version the ms. has been incomplete due to the lack of TWD1 in the transport experiments. As addressed already now several times in detail throughout this response (see key points addressed to the Editor and point 2. to Reviewer 2), this flaw has been addressed in detail by analyzing triple ABCB1/PID/TWD1 transport in tobacco. These data have been back-upped with kinase controls and expression studies arguing for a dual, counter-active role of PID in ABCB1 regulation (for details, see above).

Comments to the experiments:

2. The filtering of mass spec data is not entirely clear to me. It should be stated clearly what was the negative control ("we substracted identified vector control proteins" - what are these vector control proteins? Myc-IP from an empty vector only control plant).

Indeed, we have performed IP and MS/MS analyses for TAPa-TWD1 and vector control lines (TAPa tag alone) by anti-MYC Co-IP, and vector control MASCOT-identified proteins were subtracted from TAPa-TWD1 ones. This procedure reduced the amount of unspecific proteins binding to the MYC tag alone (or even to HIS/IgG domains of the TAPa-tag) enormously (see Table S1).
The procedure and the ratio behind it is now better explained in the Results section (page 5, first paragraph):

"To gain further specificity of TWD1 interacting proteins, we subtracted identified vector control (35S:TAPa) proteins from pulled-down TAPa-TWD1 proteins, allowing elimination of proteins binding to the TAPa-tag alone (Table S1)."

3. Since the key interactor, PID, was only recovered with a single peptide and its validity has to be seriously questioned. Since the authors have various GFP-tagged PID lines, they may use these in combination with TWD1-TAP for proper co-IP experiments.

Thanks for addressing this important point. Indeed, we have redone several reciprocal co-IP experiments using PID expressed under native (PID:PID-GFP) and strong constitutive (35S:PID-GFP) promoters but were unsuccessful in identifying TWD1 (or ABCB1) like in many other studies using membrane proteins but especially with kinases. However, we have not used lines that over-express both partners, simply because we wanted to avoid forcing PID and TWD1 into a complex that is caused by simple over-production.
In our eyes this does not question the validity of our findings but argues for a weak or transient

interaction as one might expect from an interaction with a kinase as has been pointed out by Reviewer 1. Moreover, it might be well the case that C-terminal GFP fusions has resulted in masked GFP epitopes or that GFP prevents TWD1 interaction. Finally, we would like to stress that these co-IP experiments on auxin export complexes covering at minimum three plasma membrane-bound components, ABCB, TWD1 and PID, are not trivial as we had to chose detergent conditions that balance between mild but efficient solubilization.
However, as shown in Fig. 1 we have used a whole range of imaging and *in vitro* to *in planta* techniques that strongly support a TWD1-PID interaction, such as co-IL, pull-downs and BRET. Inclusion of positive and negative BRET controls (Fig. 1D) argue for a weak but significant interaction as found for ABCB1-TWD1 (Bailly et al. 2008, 2012). Finally, expression of both TWD1 and PID is very low (Fig. 1B) and co-IL in Arabidopsis (Figs. 1C, F) and in tobacco protoplasts (Figs. 3B, S3) argue for the idea that PID-TWD1 (as well as PID-ABCB1) co-locations are limited to certain clusters on the plasma membrane further providing a further ratio for the low protein coverage during co-IP.
The following sentence was therefore added to the Discussion section (page11, second last paragraph):

"The low protein coverage after co-IP is in agreement with a weak or transient mode of protein interaction and kinase action and might explain also why we were unable to detect TWD1 in a reciprocal co-IP approach found in similar studies. Alternatively, the employed C-terminal GFP fusion might have resulted in a masked epitope or simply prevent protein interaction. Finally, expression of both TWD1 and PID is very low (Fig. 1B) and microscopical analyses in *Arabidopsis* roots (Figs. 1C, F) and tobacco protoplasts (Figs. 3B, S3) suggest that PID-TWD1 (as well as PID-ABCB1) co-locations are limited to certain clusters on the plasma membrane further leading to a low ratio for the protein coverage."
4. For the BRET experiment, it would have been nice to have a positive control that allows to judge the strength of the observed BRET.
As mentioned in the point above, we have added TWD1-Rluc/ABCB1-YFP as a positive control resulting in comparable BRET ratios, arguing for a weak but significant interaction as reported for TWD1/ABCB1 (Bouchard et al. 2006, Bailly et al. 2008, Bailly et al. 2012)
Negative control pairs, such as TWD1-Rluc/PIRK-YFP and TT12-Rluc/PID-GFP, employing unrelated PM-bound kinase, PIRK, and MATE transporter, TT12, did not result in significant BRET supporting specificity of TWD1-Rluc/PID-GFP interaction.
Further, we have provided confocal analysis of co-expressed TWD1-YFP/PID-GFP (Fig. 1D, inset), supporting co-localization on the PM of tobacco protoplasts used for the BRET assays (page 6, first paragraph):

"This interaction is specific since single expression of TWD1-Rluc, Rluc or YFP alone or TWD1-Rluc in combination with the non-related, plasma-membrane-bound protein kinase, PIRK (PIRK-YFP), only resulted in negligible BRET ratios. A similar result was found for PID-GFP co-expressed with TT12-Rluc, an unrelated plasma membrane control."

5. Does the TWD1-rLUC construct complement the twd1 phenotype?

Yes, both N- and C-terminal fusions of Rluc fully complement *twd1* in all respects as has been shown in detail in a ms. from our lab that is currently under resubmission in TPC (Bailly et al. 2012; uploaded for review). A photo demonstrating complementation has been provided as Suppl. Fig. 1E (page 5, last paragraph):

"TWD1-Rluc, like Rluc-TWD1 (Bailly et al, 2012a) was shown to be functional by complementation of *twd1-3* (Fig. S1E)."

6. What is the role of TWD1 and its interaction with PID for the function of ABCD1?

As explained throughout this reply and in the modified ms., our data support a model where TWD1 is responsible for a dual, counter-active regulation of ABCB1 activity by TWD1-PID interaction (see Fig. S8A) (page 13, second paragraph):

"Our data from mutational analyses (Fig. 6) are best in agreement with a model in which PID, in the absence of TWD1, does phosphorylate S634, resulting in ABCB1 activation (Fig. S8A). On the

other hand, negative ABCB1 regulation in the presence of TWD1 argues together with *in-planta* measurements of auxin transport (Fig. 3) for a second, PID-specific ABCB1 phosphorylation site that does not essentially need to be part of the linker. This aspect is currently under investigation."

7. In the introduction, the authors introduce PID, WAG1 and WAG2 as members of the AGC3 kinase family or subfamily and - at a latter stage in their introduction - refer to these kinases as WAG kinases but the definition of this family is not precisely given. If this includes PID kinases, then the name should be revised since the understanding of the PIDs is far more advanced than the understanding of the WAGs. A uniform nomenclature should be used for this protein family and its individual members throughout the manuscript.

This is indeed true and has been corrected throughout the text. We refer to members of clade 3 of AGC kinases now correctly as PID homologs. Moreover, we use now the term "AGC3 kinases" according to (Galvan-Ampudia & Offringa, 2007), which include PID, PID2/AGC3-4, WAG1 and WAG2 (page 4, second paragraph):

"PID belongs to the AGC family of serine/threonine kinases, and forms - together with AGC3-4/PID2, WAG1 and WAG2 - the clade AGC3 (Galvan-Ampudia & Offringa, 2007)."

However, we have kept PID and are not using PID1 because we feel that PID is a well-established name in the auxin and plant community.

8. There is no ref for the TAP-tag employed.

The TAPa tag was referenced in the Methods as Rubio et al. (2005):

"Rubio V, Shen Y, Saijo Y, Liu Y, Gusmaroli G, Dinesh-Kumar SP, Deng XW (2005) An alternative tandem affinity purification strategy applied to Arabidopsis protein complex isolation. *Plant J* **41:** 767-778"

9. It remains unexplained why only a fragment of TWD1 was used for the pull-down (Fig. 1B). What does the TWD1 double band represent that is apparent after the pull-down?

$TWD1^{1-337}$ covering the entire TWD1 protein (including all functional domains, such as the FKBD, the TPR and CaM-binding domain) but without in-plane membrane anchor (Scheidt et al. 2006) was used for the pull-down, as production of the entire TWD1 protein was not possible most probably due to the high hydrophobicity of the C-terminal membrane anchor. However, correct folding of this protein, at least of the FKB/PPIase domain has been shown by crystal structure analysis (Weiergräber et al. 2006).
Concerning the TWD1 double band, we have to admit that we do not yet understand the molecular reason behind it. However, such a double band is also found for Western detection of TWD1 protein produced *in planta* (Geisler et al. 2003). We believe that TWD1 might undergo post-translational modifications that might include as well also protein phosphorylation, which is currently under investigation.
The following sentence was added to the Results section (page 6, second paragraph):

"$TWD1^{1-337}$ purified as described (Kamphausen et al, 2002) contains all functional domains, such as the FKBD, the CaM-binding and TPR domain, except the C-terminal hydrophobic in-plane membrane anchor."

10.Although overlaps are apparent between TWD1, ABCB and PID expression, the co-localization of the proteins at the subcellular level is not shown, e.g. at the membrane.

The updated version of the ms. contains now better pictures that clearly indicate that PID expression overlaps with the ABCB1/TWD1 auxin export complex, shown by co-localization of PID:PID-VENUS and ABCB1:ABCB1-GFP (Fig. 1F).
Co-localization between ABCB1/PID (Figs. 3B S3) and TWD1/PID (Figs. 1D, 3B, S3) is further supported by imaging dual and triple co-expression in *N. benthamiana* protoplasts, indicating, first, that all three proteins co-localize on the PM and, second, that co-expression does not alter ABCB1 PM locations. The same holds true for ABCB1 expression levels that were verified by Western

analyses.

11. Possible changes in ABCB and TWD1 localization could be addressed with pid LOF and PID OX lines.

In the current version of the ms. we show that co-expression with PID or pharmacological inhibition of PID does not alter ABCB of TWD1 locations in yeast and tobacco. We are aware that these unicellular systems do not provide polarity. However, as ABCB1 and TWD1 have been shown to be expressed in an apolar fashion, we believe that altered expression caused by PID to be very unlikely *in planta.*
Although we agree that these data are of interest, we have to admit that these experiments are in our eyes slightly outside of the scope of this paper: we have aimed at investigating - on different biochemical levels - a novel mode of ABCB regulation by linker phosphorylation and identified PID as relevant kinase involved in this process.

12. Figure 2 shows auxin transport data obtained in yeast following expression or coexpression of PID and ABCB1. Following coexpression of PID, auxin efflux is reduced but his may be attributed to a reduced expression of ABCB1. Using immunoblots, the authors should demonstrate that ABCB1 expression levels are constant.

Western blots and confocal imaging have now been provided (see comment to point 6 of Reviewer 1).

13. Later in the ms, it is stated that a similar experiment did not have an effect on the abundance or localization of the overexpressed proteins, however, the quantitative nature of this experiment has to be seriously doubted. Although in the text the authors refer to similar experiments for the yeast auxin transport assay, these data are not shown (as far as I can judge).

As mentioned above, and described in detail throughout the comments to Reviewer 1 and 2, these expression and localization controls have now been provided for both yeast and tobacco expression systems (Figs. 2, S2, 3, S3, 6).

14. The role of PID on ABCB1 is tested in yeast (Fig. 2) and plant cells (Fig. 3). Opposite effects are being observed and it is unclear which one is biologically significant.

As addressed in the above comments in detail, opposite effects caused by PID co-expression between yeast and tobacco systems have been attributed to the presence/absence of TWD1 or functional orthologs in the test system, leading to a final model in which TWD1 decides for a dual, counter-active impact of PID on ABCB1 transport activity (see Fig. S8 and for details the comments above).

15. I could not find a description of the auxin transport experiments shown in Fig. 3C and D.

Fig. 3C represents a quantification of cellular efflux from Arabidopsis mesophyll protoplasts as described in the methods and in several publications by us and other groups (Geisler et al. 2005, Petrasek et al. 2006, Bouchard et al. 2006, etc.).
Fig. 3D represents a quantification of free IAA in roots and shoots of Arabidopsis seedling analyzed by GC-MS, which is now added to the figure legend (see Methods for details).

16. In various experiments the authors use pid-/+, which I assume refers to a segregating line. For measurements, the authors should, however by pheno- or genotyping identify pid homozygous mutants for their experiments. Working with a segregating line is not solid.

We are absolutely aware of the problematic of these experiments, concerning auxin transport (Fig. 3C), NPA binding studies (Fig. 5D) and analysis of free IAA (Fig. 3D) using *pid*+/- material. But we are afraid that for all methods we were forced to use segregating lines due to technical limitation, which might need some explanation:
For binding assays in Fig. 5D, we use usually 40 g of Arabdopsis seedlings as starting material for one replicate (n=1) that are therefore grown in liquid cultures, and therefore do not allow pheno/genotyping. However, these seedlings might be eventually grown in hydroponics or even on

plates for 9 dag, which would require genotyping of several thousand seedlings as phenotyping is not possible at this growth stage.
A similar calculation holds also true for the Arabidopsis protoplast assays (Fig. 3C): again, phenotyping is hindered by the fact that plants are grown under short day (8h) light conditions prevented bolting. As we are using for one replica of transport assays (n=1) the rosette leafs of ca. 50 plants, n = 4 would require a minimum of 800 genotyping PCR reactions, assuming a 1:4 segregation. Essentially the same number of PCR experiments would be required for the quantification of free IAA from Arabidopsis seedling (n=1 corresponds to 40-50 positive seedlings). Moreover, in respect to Arabidopsis seedlings also we have to admit that taking part of the cotyledons for the PCR reactions might have harbored also the risk of interfering with the auxin quantifications.
The following sentence has been added to the legends of Figs. 3 and 5 for explanation:

"Note that material was prepared from heterozygous *pid* (*pid*$^{+/-}$) plants because due to technical limitations a determination of homozygosity by shoot phenotyping or genotyping was not possible."

17. The experimental quantification in Fig. 5A (lower panel) refers to the experiments shown in the upper panels. However there is not "Chel" sample shown in the upper panels!?

We are sorry for this and have now added both Coomassie stain and autoradiography of the chelerythine treatment to Fig. 5A.

18. The phosphorylation experiment and MS analysis does not proof that ABCB1 is phosphorylated by PID! Phosphorylation experiment with mutated ABCB1 and PID should be used to demonstrate that the site is a PID1 target, particularly since the site does not match the previously described consensus.

In principle, we do agree with the Reviewer's judgment that the ultimate *in planta* proof that PID is phosphorylating S634 is missing. We have tried to address this by analyzing the phosphorylation status of ABCB:ABCB1-MYC/GFP by LC-MS/MS in PID gain-of function lines after anti-MYC/GFP IP. Unfortunately, we were unable to detect ABCB in these IPs. Therefore we have acknowledged this fact by choosing a very careful title and wording in the abstract ("a very likely target of PID phosphorylation").
Nevertheless, we believe that we provide a whole line of convincing correlations that PID phosphorylates ABCB1 in its regulatory linker, with S634 as a primary candidate site: First of all, we identified and verified PID as TWD1 interactor, itself an ABCB1 interacting protein. Second, we show that co-expression in heterologous non-plant and plant systems has a direct impact on ABCB1 and verify this action pharmacologically and by mutagenesis of PID and ABCB1. By co-expression in tobacco, we identify and verify S634 as relevant regulatory site in the ABCB1 linker and provide evidence that PID is able to phosphorylate this site *in vitro*.
However, we are now providing two more pieces of evidence: First, we repeated the *in vitro* phosphorylation by PID-GST using a mutated version of peptide 3 that contains beside S631 (that was identified as well as putative phosphorylation site) a S634A mutation. This peptide (peptide 5) is not phosphorylated at all by PID (Fig. 6A) but by PKC (Fig. S5) providing a definitive *in vitro* proof and indicating a higher specificity for PID compared to PKC.
Second, as suggested co-expression of ABCB1-S634A (and ABCB1-S634E) with PID in tobacco had no significant (or further additive) effect on ABCB1 transport, providing another strong argument that PID functionally phosphorylates this serine.

19. I am not a specialist in binding experiments but the experiments shown in Fig. 5B an C do not appear to be solid enough.

The underlying method of determining specific (here: NPA/quercetin/IAA) binding as difference between total drug binding (measured in the absence of a 1000-fold access of non radiolabelled drug) and unspecific drug binding (measured in the presence of a 1000-fold access of non radiolabelled drug) is a standard technique used by many labs, such as Michalke (Michalke et al. 1992), Muday (Cox and Muday, 1994) and Spalding (Noh et al., 2001) labs. We have adopted the assay essentially from the Noh et al. (2001) paper and modified it as described in Bailly et al. (2008).

This assay is used for yeast (Fig. 6D) and plant micrososomes (Fig. 5D) employing vacuum-filtration on nitrocellulose membranes. However, for column-bound PID-GST we corrected for unspecific (= background) binding to the column material or the GST alone by subtracting specific binding to column-bound GST from column-bound PID-GST.
This is now better explained in the figure legends and the Methods (page 18):

"Drug-binding assays using *Arabidopsis* or yeast microsomes or PID-GST were performed by vacuum filtration as described elsewhere (Bailly et al, 2008). [^{3}H](G)quercetin (10 Ci/mmol; 1.0 mCi/ml) was custom-synthesized by ARC Inc. (St. Louis, USA). In short, for determining specific drug binding, four replicates of each 20 µg of protein or 1 µg of column-bound PID-GST (or GST alone) were incubated with 10 nM radiolabelled drugs (30-60 Ci/mmol) in the presence and absence of the corresponding 10 µM non-radiolabelled drug. Reported values are the means of specific radiolabelled drug bound in the absence of cold drug (total) minus radiolabelled drug bound in the presence of cold drug (unspecific) from at least three independent experiments each with four replicates. Background drug binding to column material or GST alone (= background) was corrected by subtracting specific binding to column-bound GST from column-bound PID-GST."

Comments to the text:

20. NBP is not properly introduced (first as binding protein) then later again as nucleotide binding protein.

We removed the term NBP (nucleotide binding protein) from the text because we agreed with Reviewer 1, that it is a bit of a historical term that should be avoided. However, we kept the term NBD, which stands for "nucleotide binding fold", and might have led to this confusion.

21. At various places in the text I was not content with the phrasing and several sentences appeared unclear to me. Some examples:

"Mutant analysis and pharmacological treatment with kinase/phosphatase inhibitors, staurosporine/cantharidin, revealed..." should be "...with kinase and phosphatase inhibitors, staurosporine and cantharidin, respectively, revealed..."

"PIN efflux carriers show mainly polar locations in PAT tissues and are thought to be determinants of a reflux loop in the root apex characterized by strong developmental phenotypes (Refs)." Sentence unclear: Polar locations cannot be characterized by strong phenotypes.

"ABCB- and PIN-mediated auxin efflux can function independently...until the end of the paragraph" leaves a rather confusing picture of the ABCB and PIN interactions. What are "Interacting cells" in this context? Cells where PINs and ABCBs interact physically or neighbouring cells?

"However, several lines of evidence..." p. 3 "However" does not make sense.

"We chose second generation TAP tagging...because of its high degree of two-step affinity purification." ?

"While APC21 is known to be involved... p.6, middle." Sentence unclear.

The wordings above have been corrected, and the entire ms. has been corrected by a native speaker.

Literature

Bailly A, Sovero V, Vincenzetti V, Santelia D, Bartnik D, Koenig BW, Mancuso S, Martinoia E, Geisler M (2008) Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. *J Biol Chem* **283:** 21817-21826

Christensen SK, Dagenais N, Chory J, Weigel D (2000) Regulation of auxin response by the protein kinase PINOID. *Cell* **100:** 469-478

Galvan-Ampudia CS, Offringa R (2007) Plant evolution: AGC kinases tell the auxin tale. *Trends Plant Sci* **12:** 541-547

Kamphausen T, Fanghanel J, Neumann D, Schulz B, Rahfeld JU (2002) Characterization of Arabidopsis thaliana AtFKBP42 that is membrane-bound and interacts with Hsp90. *Plant J* **32:** 263-276

Sidler M, Hassa P, Hasan S, Ringli C, Dudler R (1998) Involvement of an ABC transporter in a developmental pathway regulating hypocotyl cell elongation in the light. *Plant Cell* **10:** 1623-1636

Zourelidou M, Muller I, Willige BC, Nill C, Jikumaru Y, Li H, Schwechheimer C (2009) The polarly localized D6 PROTEIN KINASE is required for efficient auxin transport in Arabidopsis thaliana. *Development* **136:** 627-636

2nd Editorial Decision 29 February 2012

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referees to review the revised version and referee #1 and 2 were able to do so. I have now heard back from the two referees. As you can see both appreciate the introduced changes and support publication here. Referee #1 has a few minor suggestions before final acceptance. I would like to ask you to respond to the remaining comments in a final revision. Once we receive the revision, we will proceed with its acceptance here.

Thank you for submitting your manuscript to the EMBO Journal.

Best wishes

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This second revision of the manuscript has answered most of this reviewers concerns expressed in the first review. The quality of the work presented is improved in the instances noted, important controls have been added, the confusing PIN data has been removed, and the characterization of the results is generally improved. The manuscript is certainly easier to read. This is appreciated. Some concerns remain that are easily addressed:
1. This reviewer is convinced by the results shown that TWD1 is indeed localized to the PM along with PID. However, the tobacco protoplast localization results do not address the overexpression issue as this is also an overexpression system. The authors should explicitly acknowledge that some of the intracellular localization data may reflect overexpression artefacts. In the response letter, the authors argue that expression data support the localizations observed. This may be true for comparisons of the expression domains, but not subcellular localization.
2. The complementation of some phenotypes by ScFKBP12 (yFKBP12 is not a very good designator) mentioned by the authors should be included as supplementary data. This is important as FKBP12 is not a PPIase/chaperone with TPR and CAM binding domains.

Referee #2 (Remarks to the Author):

This manuscript addresses a number of very important aspects of auxin transport regulation in plants. Most approaches so far, studied localization of auxin transport components, which then was

used for a variety of conclusions. Experimental work provided by Dr. Geisler and colleagues, follows a different, often times ignored but nonetheless equally important, more biochemical approach.
In their revised version the authors convincingly addressed most of my concerns. Of course, there are still several open questions, but this is exactly what good science is all about.

2nd Revision 29 March 2012

Referee #1 (previous comments to the authors in italic):

This reviewer is convinced by the results shown that TWD1 is indeed localized to the PM along with PID. However, the tobacco protoplast localization results do not address the overexpression issue as this is also an overexpression system. The authors should explicitly acknowledge that some of the intracellular localization data may reflect overexpression artefacts.

Indeed some of the intracellular localization might represent an artefact of the constitutive, strong over-expression, which has been now added to the text (Page 7, line 21): "TWD1 and PID, and to a lesser extend also ABCB1, revealed in some cases additionally to the plasma membrane signals some intracellular signals (Figs. 3, S3) that might represent artefacts caused by the constitutive, strong over-expression used in these assays."

In the response letter, the authors argue that expression data support the localizations observed. This may be true for comparisons of the expression domains, but not subcellular localization.

This is obviously correct, thanks for pointing this out. However, as this flaw was not included in the text, we did not include any changes here.

2. The complementation of some phenotypes by ScFKBP12 (yFKBP12 is not a very good designator) mentioned by the authors should be included as supplementary data. This is important as FKBP12 is not a PPIase/chaperone with TPR and CAM binding domains.

We have changed the designation of yFKBP12 into ScFKBP12 throughout the text. Concerning the complementation of ScFKBP12 of *twd1* we have left – as discussed with you - the presentation of the complementation phenotypes in the SD out but have according to the reviewer's comments added the following addition to the Discussion section (page 12, line 32):
"Finally, *ScFKBP12* is able to widely complement *twd1* loss-of-function alleles (unpublished data), which is slightly surprising as ScFKBP12 lacks functional TPR and calmodulin-binding domains as well as the C-terminal membrane anchor."

