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Chapter 5

MAB4/MEL scaffold proteins act in concert with AGC3 kinases to promote PIN plasma membrane abundance by microdomain formation

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Abstract

Plant growth and development is for a large part determined by polar cell-to-cell transport-generated differential distribution of the plant hormone auxin. The direction of auxin transport is determined by the asymmetric localization (polarity) of PIN-FORMED (PIN) auxin efflux carriers on the plasma membrane of cells. In *Arabidopsis*, PIN polarity is signalled through the phosphorylation of their hydrophilic loops by the AGC3 serine/threonine kinases PINOID (PID), WAG1 and WAG2. The MACCHI BOU 4 (MAB4) BTB-NPH3 scaffold protein and its homologs MAB4/ENP/NPY1-LIKE1,2,3 and 4 (MEL1,2,3,4) are also required for PIN-polarity establishment, but their mode of action is yet unknown. Here we show that MAB4 and PID interact with the PIN hydrophilic loop (PIN HL) and that this trimeric complex enhances PIN1 and PIN2 stability at the plasma membrane by reducing endocytosis and turnover of these PINs. We show that this complex formation contributes to the immobilization of PIN proteins in microdomains. Through this PIN stabilization, MAB4/MELs indirectly enhance PIN polarity, explaining the PIN polarity defects observed in *mab4 mel* higher order mutants.

Introduction

Polar auxin transport (PAT) is an essential process for the development and tropisms of plants, as it generates morphogenetic auxin gradients that position organ initiation and steer the direction of plant growth. The direction of this cell-to-cell auxin transport is determined by the polar subcellular plasma membrane distribution of “long” PIN-FORMED (PIN) auxin efflux carriers that are characterized by a long central hydrophilic loop (PIN HL) separating two transmembrane domain regions (Habets and Offringa, 2014). The polarity of long PIN proteins is regulated by the phosphorylation of three conserved serine residues in their central HL by the AGC protein serine/threonine kinases PINOID, WAG1 and WAG2 (Dhonukshe et al., 2010; Huang et al., 2010). The PID, WAG1 and WAG2 kinase-regulated PIN polarity is important for embryo, root and shoot development (Benjamins et al., 2001; Cheng et al., 2008; Dhonukshe et al., 2010; Huang et al., 2010) and for proper photo- and gravitropic growth (Dhonukshe et al., 2010; Ding et al., 2011; Preuten et al., 2013; Santner and Watson, 2006; Sukumar et al., 2009; Zhang et al., 2013). Overexpression of these kinases leads to main root meristem collapse in seedlings due to constitutive apical (shootward) localization of PIN proteins in the root meristem, thereby depriving the meristem of auxin (Benjamins et al., 2001; Dhonukshe et al., 2010; Friml et al., 2004). Single *pid* mutants develop pin-formed inflorescences, similar to the *pin1* mutant, and *pid wag1 wag2* triple loss-of-function mutant seedlings have no cotyledons due to a basal localization of PINs in the inflorescence meristem and in the early embryo (Benjamins et al., 2001; Bennett et al., 1995; Cheng et al., 2008; Dhonukshe et al., 2010).

A key question still is how PIN phosphorylation leads to a change in their polar distribution over the plasma membrane. A possible link was provided by the identification of the *ENHANCER OF PINOID/MACCHI-BOU4/NAKED PINS IN YUCCA1 (ENP/MAB4/NPY1)* gene, which codes for a Non-Phototropic-Hypocotyl 3 / Bric-a-brac/Tramtrack/Broad (NPH3/BTB)-domain protein. When the *mab4* loss-of-function mutation is introduced in the *pid* loss-of-function mutant background, seedlings fail to develop cotyledons and plants develop a stronger pin-formed inflorescence phenotype (Cheng et al., 2008; Furutani et al., 2007; Trembl et al., 2005). *MAB4* has four close homologs *MEL1,2,3 and 4* that act redundantly with *MAB4* in root gravitropism (Furutani et al., 2011; Li et al., 2010) and inflorescence development (Furutani et al., 2014). Subtle defects in PIN1 and PIN2 polarity have been detected in respectively the *mab4 mel1,2* triple and *mel1,2,3,4* quadruple mutants leading to pin-formed inflorescences and agravitropic roots (Furutani et al., 2011; Furutani et al., 2014; Li et al., 2010), however, the mechanism through which MAB4/MELs contribute to PIN polarity is not yet clear. MAB4/MEL proteins show a very striking co-localization with PIN1 and PIN2 in distinct polar domains: in the epidermis of the embryo at the sites of the cotyledon formation, in the shoot apical meristem at the sites of floral initiation, and in the root stele and epidermis (Furutani et al., 2011; Furutani et al., 2014). Recent data suggest that the PIN1 convergence points in the inflorescence meristem that mark the

positions for floral initiation are generated in a PID-dependent manner. The resulting enhanced auxin response leads to activation of *MAB4*, *MEL1* and *MEL2* transcription by the Auxin Response Factor (ARF) MONOPTEROS (MP) and to the subsequent switch of PIN polarity from the apical or basal to the inner site of convergence point L1 cells, resulting in auxin canalization from the epidermis towards the inner cell layers and subsequently to floral initiation via *LEAFY* (Furutani et al., 2014). This does not explain however, via which molecular mechanism *MAB4*, *MEL1* and *MEL2* achieve PIN1 reorientation.

The plant-specific NPH3 domain of *MAB4*/*MEL* proteins, which is named after the NON-PHOTOTROPIC HYPOCOTYL3 (NPH3) protein, is thought to act as a scaffolding domain. NPH3 acts on PIN2 and PIN3 plasma membrane localization and abundance in the process of shoot and root phototropism, possibly by inhibiting vacuolar degradation of PINs (Ding et al., 2011; Zhang et al., 2013). During the phototropic response, the scaffolding domain of NPH3 becomes phosphorylated by the blue light photoreceptor PHOTOTROPIN1 (PHOT1) (Pedmale and Liscum, 2007). BTB domains are known to create large interaction surfaces by homodimerization (Perez-Torrado et al., 2006), and can be found in proteins in all eukaryotes where they are combined with a wide variety of other domains such as zinc fingers, and NPH3, KELCH and MATH domains. The BTB domain in NPH3 and other NPH3/BTB domain proteins was shown to be necessary for association with the CUL3-Ring E3 Ubiquitin Ligase CRL3 (Roberts et al., 2011).

Here we show that PID interacts with the BTB domain of *MAB4*, and that PID and both the BTB and NPH3 domain of *MAB4* interact with the HL of PIN1 and PIN2. Our results suggest that through the scaffolding activity of *MAB4* PIN proteins are immobilized in microdomains at the apical polar domain of the plasma membrane. We propose that this microdomain formation is part of the mechanism through which the phosphorylation status of PIN proteins is translated into their asymmetric accumulation at the plasma membrane.

Results

***MAB4* and PIN1 colocalize independent of AGC3 kinases**

Previously it was shown that *MAB4*/*MEL* proteins colocalize with PIN1 and PIN2 at the plasma membrane and that they are required for maintenance of the polarity of these PINs (Furutani et al., 2011; Furutani et al., 2014). Since it is well established that AGC3 kinases instruct apical PIN plasma membrane polarity (Dhonukshe et al., 2010; Friml et al., 2004; Huang et al., 2010), we investigated how these AGC3 kinases and *MAB4*/*MELs* work together to establish PIN1 polarity by immunolocalizing PIN1 and *MAB4* in embryos of

wild-type *Arabidopsis* (Col-0) and the *pid wag1 wag2* triple AGC3 kinase mutant. In wild-type and *pid wag1 wag2* transition and heart stage embryos, MAB4 colocalized with PIN1 in the protodermal layer (Fig. 1A-D), but not in the vasculature where MAB4 is not expressed (Fig. 1C,D). PIN1 and MAB4 co-localized in the *pid wag1 wag2* mutant background (Fig. 1D,E) and at the shoot apical meristem of the *pid-3* loss-of-function mutant (Fig. S1A), which indicated that this co-localization is independent of the AGC3 kinases. Conversely, in *mab4 mel1 mel2* triple mutant shoot apical meristems, weakly expressed PID-YFP could still be observed at the plasma membrane (Fig. S1B), showing that PID plasma membrane localization is not dependent upon MAB4/MELs. Immunolocalization of MAB4 in wild-type and *pin1* mutant inflorescence meristems indicated that PIN1 enhances the plasma membrane localization of MAB4 (Fig. 1F). These colocalization results show that the AGC3 kinases are not necessary to establish MAB4 and PIN1 colocalization, but that they do determine their plasma membrane polarity.

MAB4/MELs are recruited to the plasma membrane by co-expressed PIN1 and PID

To further assess whether MAB4/MEL, PIN1 and PID colocalize and affect each other's subcellular localization, *Arabidopsis* protoplasts were transfected with 35S::PIN1-GFP (PIN1), 35S::PID-CFP (PID) and 35S::MAB4/MEL1/2/4-RFP (MAB4/MEL1/MEL2/MEL4) constructs. When transfected alone, MAB4, PIN1 and PID localized to the plasma membrane and PID and MAB4 were also found in the cytosol, whereas MEL1 showed a strong spotted localization with a varying plasma membrane signal and MEL2 and MEL4 mainly localized (to internal structures) in the cytosol (Fig. S2A-F). When MAB4 and PID were co-expressed they both localized on the plasma membrane and in the cytosol, but did not show perfect co-localization (Fig. 2A). Co-expression of PID with MEL1, MEL2 or MEL4 showed that these proteins did not change each other's subcellular localization compared to the single transfections (Fig. 2B-D, Fig. S2A-F). MAB4 MEL1/2/4 co-transfection with PIN1 resulted in the same pattern as with PID: MAB4/MEL1/2/4 and PIN1 co-localized to the plasma membrane, but not in a complete co-localization (Fig. 2E-H). However, PIN1 localization influenced that of MEL1/2/4 and vice versa: PIN1 appeared in the same plasma membrane spots as MEL1, while MEL2 and MEL4 were drawn to the plasma membrane. This result was similar to that of Fig. 1F, where MAB4 localization depended upon PIN1. Interestingly, when PID, PIN1 and MAB4/MEL were co-expressed, the three proteins showed perfect co-localization on the plasma membrane (Fig. 2I-L). Moreover, MEL1 did not show its spotty localization anymore, and the normally clear cytosolic localization of the MAB4/MEL proteins was significantly reduced. These results suggested that the MAB4/MEL proteins are actively recruited to the plasma membrane in the presence of PIN1 and PID. The plasma membrane localization of PID itself was independent of MAB4/MELs. PID plasma membrane localization has recently been reported to be mediated by electrostatic interactions between positively charged Arginine residues in the insertion domain of PID

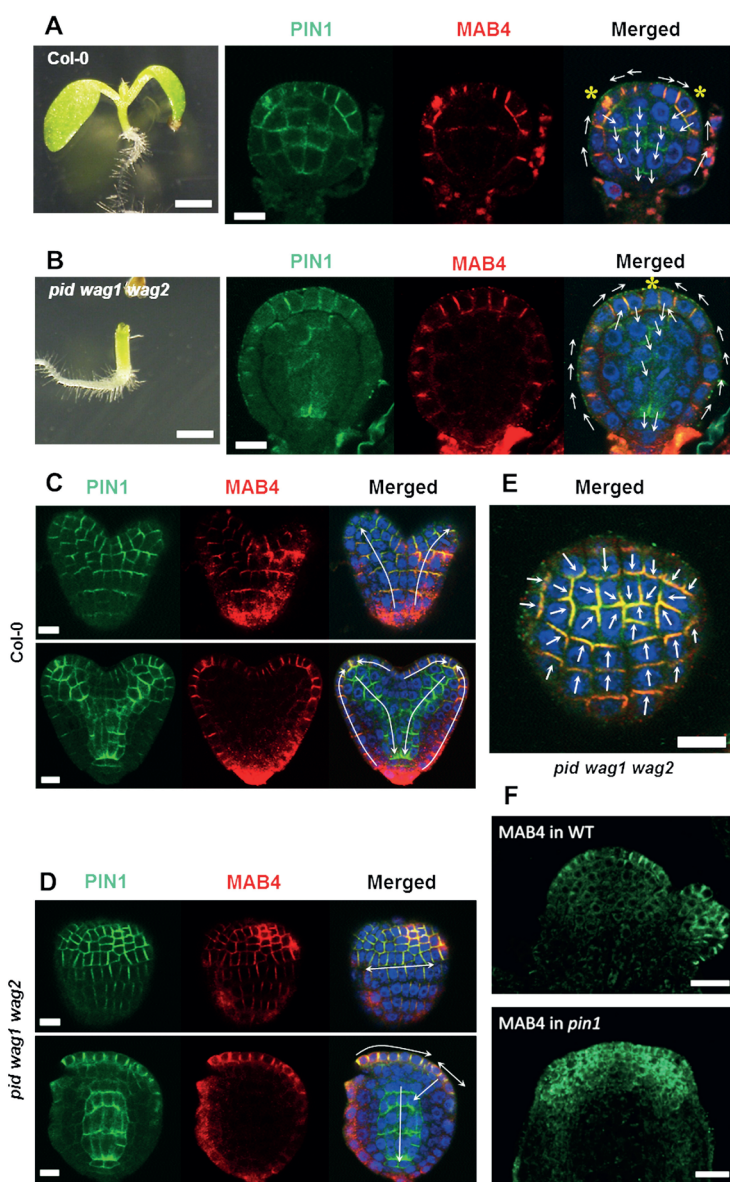
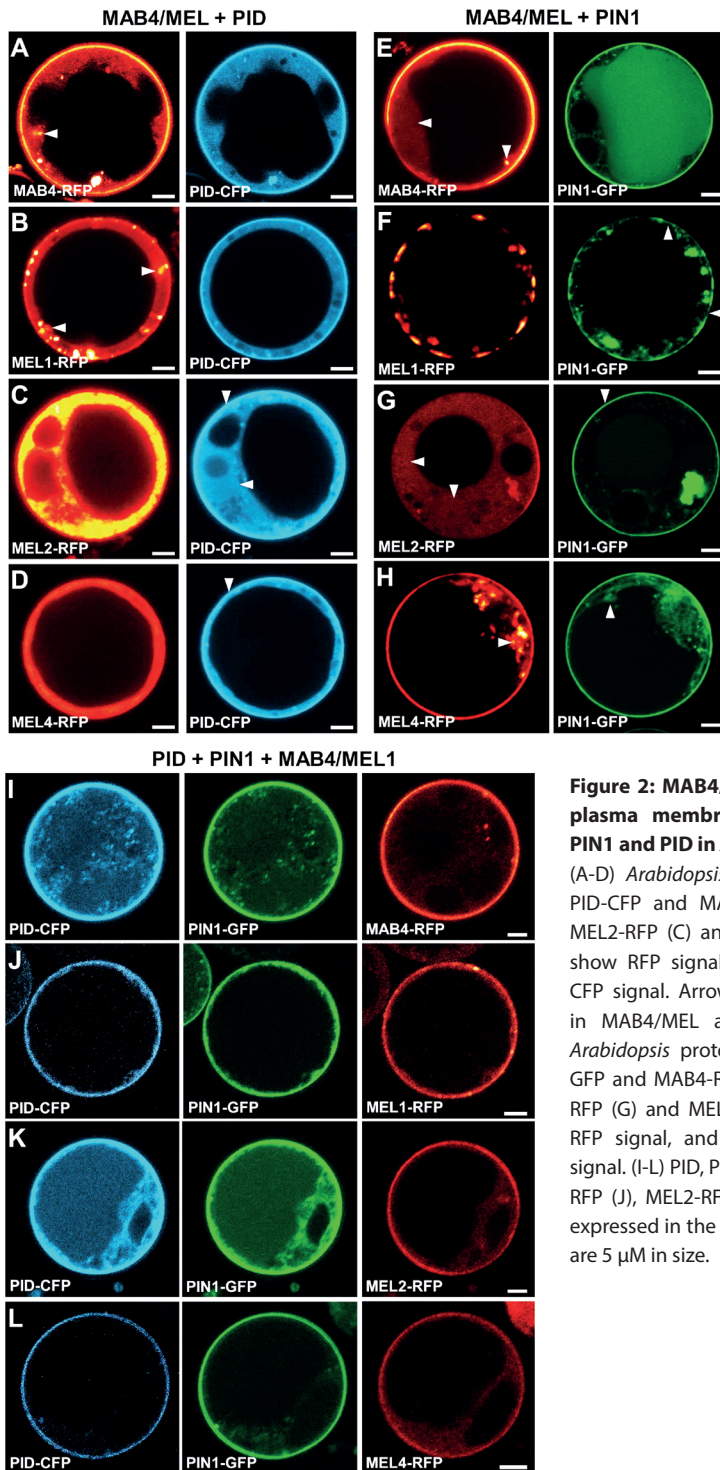


Figure. 1: MAB4 plasma membrane localization follows AGC3 kinase directed PIN1 polarity in the embryo protoderm.

(A,B) Left-most picture: seedling phenotypes of Col-0 and *pid wag1 wag2*. Anti-PIN1 and MAB4 immunolocalizations in (A) wild-type and (B) *pid wag1* and *wag2* transition stage embryos, (C) wild-type and (D) *pid wag1* and *wag2* heart stage embryos showing top (pictures above) and median (pictures below) confocal sections and (E) the embryo from (D) viewed from above. Blue colour shows a DAPI-staining and the white arrows indicate PIN1+MAB4 colocalization. (F) In the inflorescence meristem, MAB4 immunolocalization showed a polar plasma membrane signal in wild type, while this is lost in the *pin1* mutant. Scale bars of seedlings in (A) and (B) are 1mm, in embryo pictures of (A-E), scale bars are 10 μ m and in (F) scale bars are 20 μ m.



and negatively charged phosphoinositides residing in the plasma membrane (Simon et al., 2016). A mutant PID-YFP version with the insertion domain deleted co-localized to microtubule-like structures with co-expressed MAB4 and MEL1 (Fig. S2G-I), indicating that MAB4/MELs directly interact with PID and that they are also dependent upon PID for their plasma membrane localization. A kinase-dead PID mutant was also still able to colocalize with MAB4-RFP (Fig. S2J), showing that the MAB4-PID interaction is not dependent upon PID kinase activity. Together these results led us to hypothesize that PIN1, MAB4/MELs and PID proteins interact with each other, and that the interaction is stronger when all three proteins are present in the cell.

PID and MAB4 overexpression results in pin-formed inflorescences

The protoplast experiments suggested that PIN polarity could be changed by the combined expression of all three proteins in a cell. To test this *in planta* we combined overexpression of *PID* and *MAB4* by crossing *35S::MAB4-GFP-T7* and *35S::PID-GFP-MYC* transgenic lines. Adult flowering *35S::MAB4-GFP-T7* and *35S::PID-GFP-MYC* plants showed a more or less wild-type phenotype. However, to our surprise, when these mutant plants were crossed the resulting progeny developed pin-shaped inflorescences (Fig. 3). qRT-PCR analysis showed that this phenotype was not the result of silencing of either *MAB4* or *PID* (data not shown). A similar phenotype was observed in *pin1 PIN1::PIN1(S1,2,3E)-GFP* plants where the Serine (S) residues phosphorylated by PID in the TPRXS motifs were substituted for phosphomimic glutamic acid (E) residues (Huang et al., 2010). This substitution caused constitutive shootward localization of PIN1 proteins, resulting in an inability to create PIN1 convergence points where auxin reflux and organ outgrowth takes place. We hypothesized that a similar process was occurring in this double overexpression mutant, and that ectopically expressed PID and MAB4 cooperatively promoted apical PIN1 polarity.

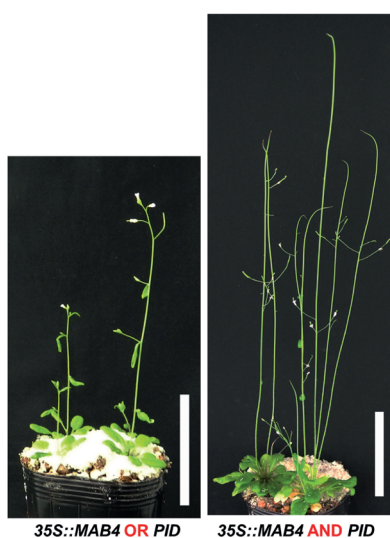


Figure 3: Overexpression of *PID* and *MAB4* enhance each other's action resulting in pin-formed inflorescences.

(A) *PID* and *MAB4* overexpression both lead to mild dwarfism phenotypes. (B) Combined, these overexpression constructs lead to pin-formed inflorescences. Scale bars are 5 cm.

PID interacts with the BTB domain of MAB4 and MEL1

The protoplast experiments suggested that the cooperative action of PID, MAB4/MEL and PIN might be mediated by their physical interaction. To investigate this, we performed Bi-molecular Fluorescence Complementation (BiFC) assays in *Arabidopsis* protoplasts using 35S::MAB4-VN, 35S::MEL1-VN and 35S::PID-VC constructs. PID interacted with both MAB4 and MEL1, mainly at the plasma membrane (Fig. 4A). The BiFC signal was strongest when the YFP parts were fused to the C-terminal end of the PID and MAB4 proteins, suggesting that the interaction is mediated by the N-terminal part of PID and MAB4. Next we used deletion mutants of the principal MAB4 domains to investigate the interaction site (Fig. 4B-I). Deleting the first 22 N-terminal amino acids

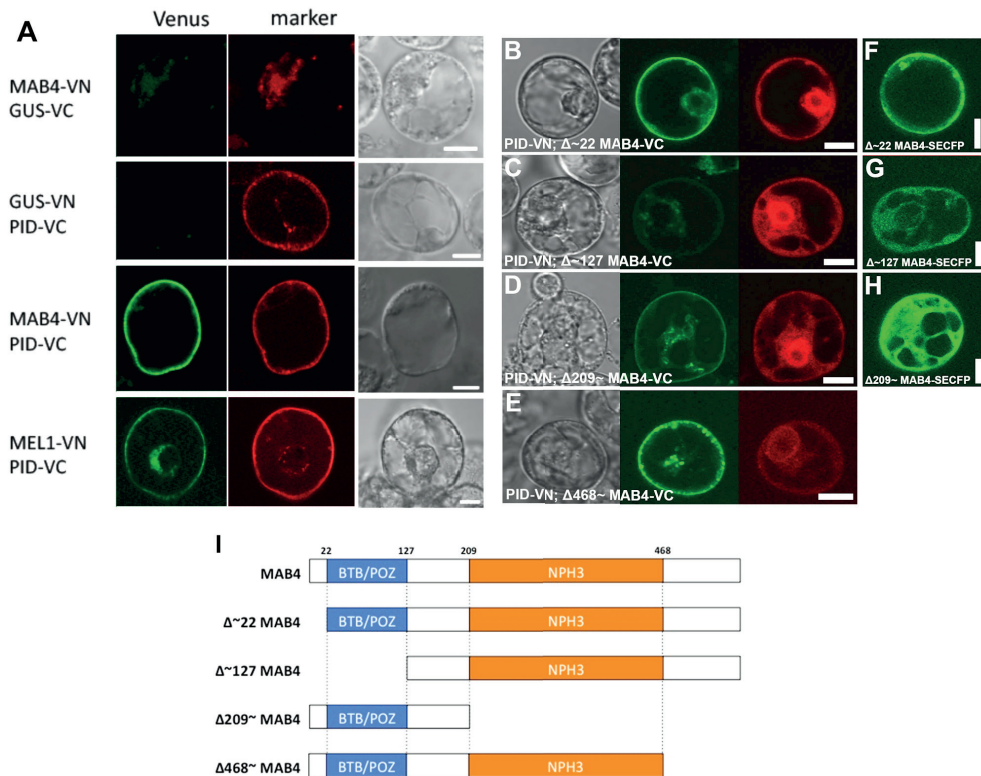


Figure 4: MAB4 and MEL interact through their BTB-POZ domain with PID at the plasma membrane in *Arabidopsis* protoplasts.

(A) BiFC experiment in *Arabidopsis* protoplasts between PID and MAB4/MEL1, using the GUS gene as a negative control. PID with C-terminally fused to the C-terminal YFP half interacted with MEL1/MAB4 C-terminally fused with the N-terminal YFP half. Green channel shows YFP BiFC signal, the red channel shows Propidium Iodide plasma membrane staining, with the accompanying bright field signal. (B-E) BiFC experiment between PID C-terminally fused to the N-terminal YFP half with various MAB4 domains C-terminally fused to the C-terminal YFP half, using DAPI as a counter stain. (F-H) Localization of MAB4 mutant versions used in B-D, fused to seCFP. (I) Diagram of the MAB4 mutants used in B-H. Scale bars are 5 μm.

of MAB4 or the last 103 C-terminal amino acids after the BTB domain did not alter the BiFC signal significantly (Fig. 4B,F), however deletion of the BTB domain disrupted the interaction (Fig. 4C,G). Deletion of the NPH3 domain or the c-terminal part changed MAB4 localization, but did not abolish the BiFC signal (Fig. 4D,E,H). These results show that PID interacts with the BTB-POZ domain of MAB4. Furthermore, MAB4 did not localize to the plasma membrane when either the BTB domain or the MAB4 domain were deleted suggesting that both domains confer plasma membrane localization to MAB4.

PID and MAB4 interact with the PIN2 HL

To investigate if the plasma membrane localization of MAB4 is dependent on an interaction with the PIN HL we performed *in vitro* pull-down assays using *E. coli* expressed GST- and HIS-tagged proteins. In this assay HIS-PIN2HL could be pulled down with GST-MAB4, and as expected from the BiFC experiments also HIS-PID could be pulled down with GST-MAB4 (Fig. 5A). However when both HIS-PID and HIS-PIN2HL were incubated with GST-MAB4 bound beads, only the PID signal remained (Fig. 5A), while at longer exposure a very faint HIS-PIN2HL band was visible (Fig. S3A). A repeat of this experiment confirmed that HIS-PIN2HL binding to –GST-MAB4 decreased in the presence of HIS-PID (Fig. 5B), suggesting that PID and PIN2HL compete for binding to MAB4, but that MAB4 preferentially binds to PID. A pull down of HIS-PID and HIS-MAB4 with GST-PIN2HL, however, did not show clear evidence for competition (Fig. 5C), suggesting that these proteins bind at non-overlapping positions in the PIN2 HL. In a similar experiment using GST-PIN1HL as bait, we could pull down PID, but experienced difficulties in detecting HIS-MAB4 (Fig. S3B). Interestingly, the pull down of PID seemed to be enhanced in the presence of HIS-MAB4. To confirm the interaction between PIN1 and MAB4 *in planta* we used a duo-link immunolocalization protocol, which showed that MAB4 and PIN1 indeed interact at the plasma membrane of epidermis cells in the inflorescence meristem (Fig. S4).

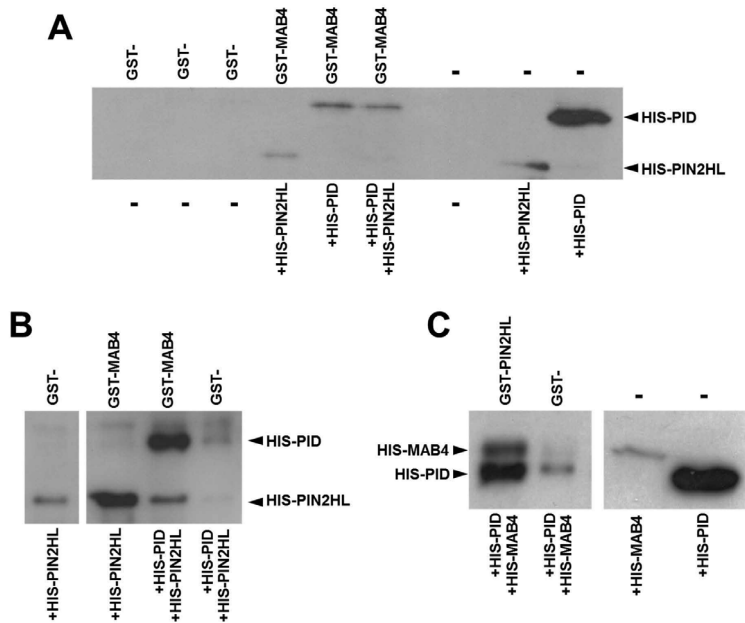


Figure 5: PID and PIN2HL competitively interact with MAB4.

(A) *In vitro* pull down of HIS-PIN2HL and/or HIS-PID with GST (negative control, left three lanes) or GST-MAB4 (middle three lanes). The input of HIS-tagged protein is loaded in the right three lanes. (B) *In vitro* pull down assay similar to (A) of HIS-PIN2HL or HIS-PIN2HL and HIS-PID with GST-MAB4 or (negative control) –GST only. (C) *In vitro* pull down of HIS-MAB4 and HIS-PID with GST-PIN2HL coated beads or GST-only beads (negative control).

MAB4 preferentially binds phosphorylated PIN2HL

Further *in vitro* pull down assays showed that binding was stronger between GST-MAB4 and the PIN2HL S1,2,3E phospho-mimic version, as compared to the wild-type or the HIS-PIN2HL S1,2,3A non-phosphorylatable version (Fig. 6A,B). A similar result was obtained when only the GST-BTB (AA 22-127) or the GST-NPH3 (AA 209-468) domains of MAB4 were tested for binding with HIS-PIN2HL, or the mutant versions thereof (Fig. 6C,D). This result suggested that indeed MAB4 binds more efficiently to the phosphorylated PIN2-HL, and that this binding can be mediated with equal efficiencies by either the BTB- or the NPH3 domain. Next we tested whether GST-WAG1 and GST-WAG2 could pull down the HIS-PIN2HL at a similar level as GST-PID, and whether binding was affected by the S1,2,3A or S1,2,3E mutations. Wild type PIN2HL was pulled down by all three GST-kinase fusions at similar efficiencies, however binding of both PIN2HL mutant versions was slightly reduced compared to the S1,2,3A and the bands were only just brighter than the control GST band (Fig. 6E), indicating that PID, WAG1 and WAG2 have a preference for binding to unphosphorylated PIN2HL. The data led to a model where at the plasma membrane unphosphorylated PIN2-HL effectively recruits PID, and that subsequent phosphorylation of the PIN2HL leads to recruitment of MAB4 to the complex (Fig. 10).

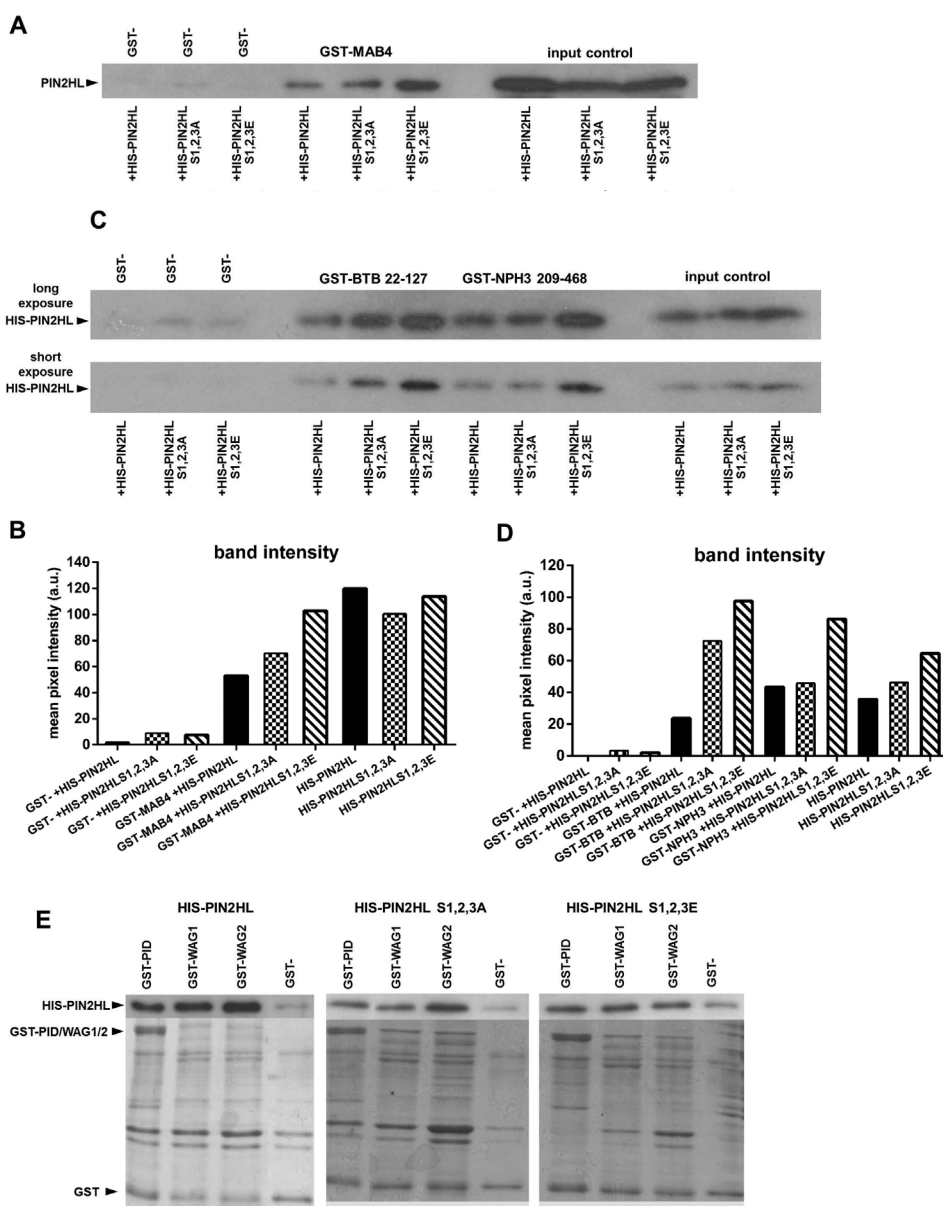


Figure 6: MAB4 interacts preferentially with the PIN2HL phosphorylation-mimic S1,2,3E mutant through both its BTB and NPH3 domains.

(A) *In vitro* pull-down assay of HIS-PIN2HL, HIS-PIN2HL S1,2,3A or HIS-PIN2HL S1,2,3E with GST- only or GST-MAB4 and the HIS-PIN2HL inputs on the right. (B) Quantification of the bands from the blot in (A). (C) *In vitro* pull-down assay of HIS-PIN2HL, HIS-PIN2HL S1,2,3A or HIS-PIN2HL S1,2,3E with GST- only, GST-BTB or GST-NPH3 and the HIS-PIN2HL inputs on the right. Upper lane shows longer exposure, while the lower lane shows the shorter exposure of the same blot. (D) Quantification of the bands from the blot in (C). (E) *In vitro* pull-down assay using either GST-PID, GST-WAG1, GST-WAG2, or a negative control, GST- only, as bait and HIS-PIN2HL, HIS-PIN2HL S1,2,3A or HIS-PIN2HL S1,2,3E as prey, with the corresponding coomassie stain of a similarly loaded SDS-PAGE gel shown below (The HIS-PIN2HLs are in too low a concentration to be seen on the coomassie staining).

MAB4/MELs reduce PIN vacuolar targeting in protoplasts and roots

Like all plasma membrane proteins, PINs are degraded in the vacuole and thus PIN vacuolar targeting is an important way for the plant cell to regulate the plasma membrane abundance and polarity of PINs (Abas et al., 2006; Baster et al., 2012; Kleine-Vehn et al., 2008; Laxmi et al., 2008; Wan et al., 2012). To investigate the effect of MAB4/MELs on vacuolar targeting of PIN1 we performed a vacuolar targeting assay in protoplasts. This assay is based on the observation that when a PIN1-GFP fusion is expressed in dark incubated protoplasts, the GFP signal is initially observed at the plasma membrane, but that over time the percentage of protoplasts showing vacuolar GFP signal gradually increases (Chapter 3). Dark incubation has been shown to induce PIN vacuolar targeting (Kleine-Vehn et al., 2008) and to stabilize GFP in the lytic vacuole (Tamura et al., 2003), thereby allowing to quantify the rate of vacuolar targeting by measuring the vacuolar GFP signal. Twenty hours after transfection the percentage of protoplasts showing vacuolar PIN1-GFP signal was significantly lower in MAB4, MEL1 or MEL2 co-transfected cells (Fig. 7A). In addition, we compared PIN2-GFP vacuolar targeting in wild-type and *mel1 mel2 mel3 mel4 (mel1234)* mutant roots by incubating seedlings in the dark. After 2 hours of incubation in the dark *mel1234* quadruple mutant root meristem epidermis and cortex cells showed significantly more vacuolar signal compared to wild-type cells (Fig. 7B,C). In the same cells, the PIN2-GFP plasma membrane abundance was significantly reduced in *mel1234* mutant roots compared to wild-type roots (Fig. 7D). These results indicated that MAB4/MELs promote plasma membrane abundance of PIN1 and PIN2 by decreasing vacuolar targeting.

PIN microdomain formation requires MAB4/MEL and PID-mediated phosphorylation

We observed *PIN2* promoter driven PIN2-GFP in lateral root cap cells and noticed that there are distinct spots visible on the plasma membrane that are very likely PIN2 microdomains described previously with super-resolution microscopy (Fig. 8A,B) (Kleine-Vehn et al., 2011; Titapiwatanakun et al., 2009). In the *mel1234* quadruple mutant PIN2-GFP was no longer clearly visible in the lateral root cap cells (Fig. 8C,D). We observed MEL1-GFP microdomains in *mel1 MEL1::MEL1-GFP* lateral root cap cells (Fig. 8E-J), which disappeared in *mel1234* mutant background (Fig. 8I,K). In addition, we used *PIN2::PIN2-YFP* and *PIN2::PIN2-YFP S1,2,3A* lines (Dhonukshe et al., 2010) to investigate the effect of PID-mediated phosphorylation on microdomain formation. PIN2-YFP spots were observed in the epidermis cells of the root meristem and interestingly were not seen in the PIN2-YFP S1,2,3A line, which also showed a marked decrease in PIN2 signal (Fig. 8L,M). This data showed that PIN2 microdomain formation is dependent on MAB/MEL function and PID phosphorylation. Microdomains have previously been reported to be necessary to generate the typical super polarity seen for PIN2 in wild-type root cells, by immobilizing PINs at the plasma membrane and restricting diffusion and endocytosis (Kleine-Vehn et al., 2011). This super polarity is clearly lost in *mel1234* mutant cells, again corroborating that MAB4/MELs are required for PIN microdomain formation.

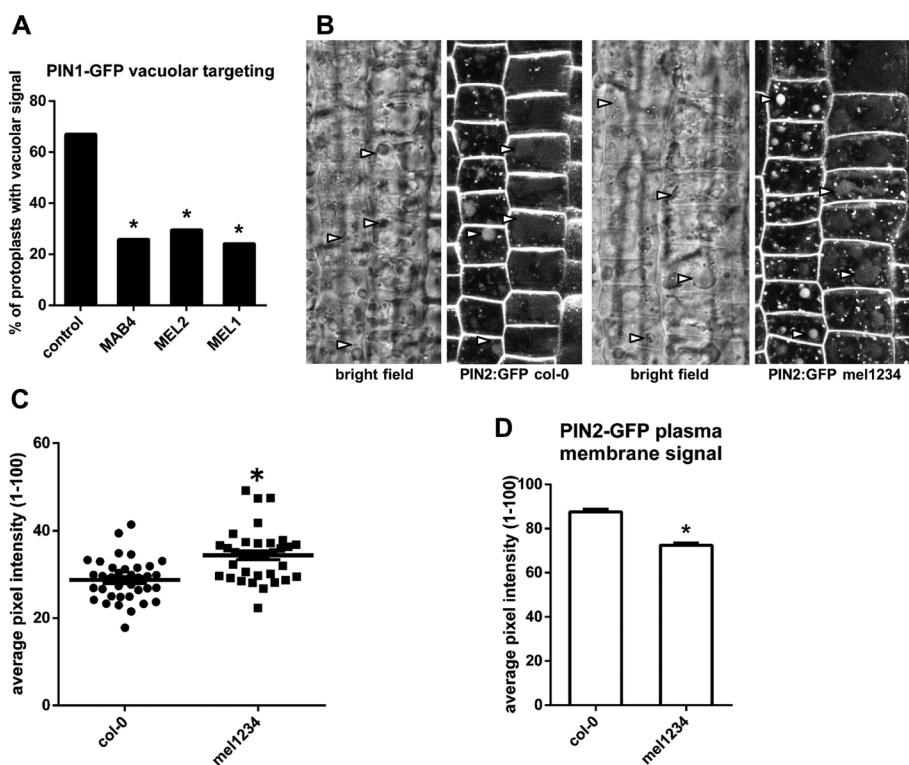


Figure 7: MAB4/MELs reduce PIN2 and PIN1 vacuolar targeting in roots and in protoplasts.

(A) PIN1-GFP vacuolar targeting assay in *Arabidopsis* protoplasts with co-expression of MAB4-RFP, MEL1-RFP and MEL2-RFP all under 35S promoter driven expression. After overnight incubation in darkness at least 40 protoplast/treatment were scored for presence of vacuolar signal). * $p < 0.05$ one-way ANOVA with post-hoc tukey. (B) Example of vacuolar targeting assay in the root meristem epidermis using (*mel1234*) PIN2-GFP after 2 hours of dark incubation (red arrows point towards vacuoles). (C) Quantification of vacuolar PIN2-GFP signal from experiment shown in (B). 10 seedlings/treatment, * $p < 0.05$ student's t-test. (D) Quantification of PIN2-GFP plasma membrane signal of experiment shown in (B), * $p < 0.05$ student's t-test.

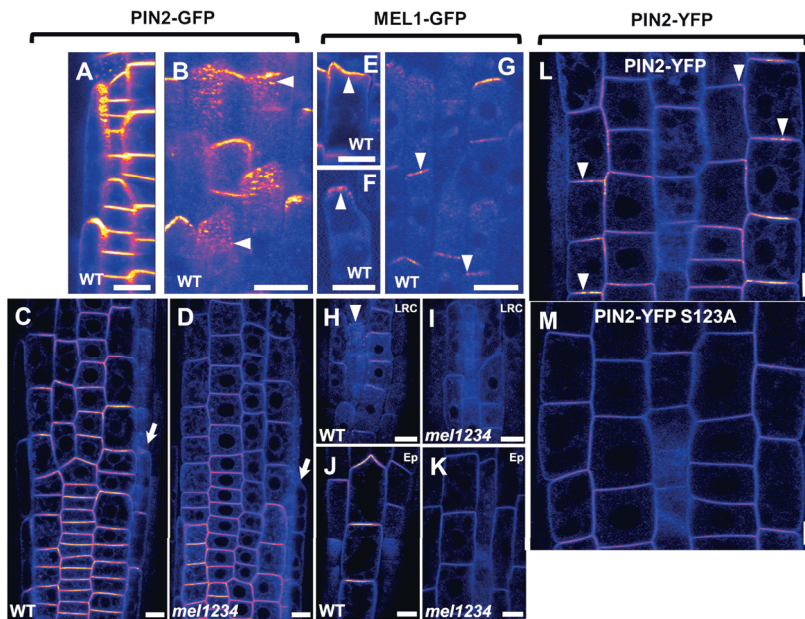


Figure 8: MEL and PID function are important for microdomain formation of PIN2 and MEL1.

(A-B) confocal microscopy images of PIN2-GFP in the lateral root cap cells (A) median section and (B) top of the cells (White arrowheads show PIN2-GFP microdomains). (C,D) PIN2-GFP in root epidermis cells in wild type and *mel1234* mutant background (White arrow shows lateral root cap cell). (E-G) MEL1-GFP in lateral root cap cells (white arrowheads point to microdomains) in the epidermis. (H-I) MEL1-GFP in the lateral root cap cells in wild type and *mel1234* background. (J,K) MEL1-GFP in the epidermis in wild type and *mel1234* background. (L) PIN2-YFP in the epidermis (White arrowheads show PIN2-GFP microdomains). (M) PIN2-YFP S1,2,3A in the epidermis. Scale bars are 10 μ m.

PID and MAB4 reduce mobility of PIN1 at the plasma membrane by inhibiting endocytosis

To further assess the effect of MAB4 and PID on PIN1 stability at the plasma membrane we performed Fluorescence Recovery After Photobleaching (FRAP) experiments on *Arabidopsis* protoplasts transfected with *35S::PIN1-GFP*. A region of the plasma membrane was bleached (Fig. 9A) and this bleached region was subsequently imaged for a duration of eight minutes. Afterwards the plasma membrane intensity of the bleached region was measured and compared to the pre-bleach intensity to yield a measure of recovery (Fig. 9B). During the eight minutes, PIN1-GFP plasma membrane intensity recovered to approximately 25% of pre-bleach intensity. Next we co-transfected *35S::MAB4-RFP* or *35S::PID-RFP* with *35S::PIN1-GFP* and again measured FRAP. The recovery when PID or MAB4 was present was significantly lower (~10%) compared to only PIN1-GFP transfection (Fig. 9B). When both MAB4, PID and PIN1 were co-transfected, the recovery was reduced to the same level. The observed FRAP rate in this triple co-expression may be the lowest

possible amount and that we did not observe any additional lowering of the FRAP rate in this experiment may be due to the fact that FRAP of PINs is already low compared to other transmembrane proteins (Kleine-Vehn et al., 2011). In the same experiment (but plotted separately for viewing convenience) we added Tyrphostin A23 (TyrA23) to the PIN1-GFP transfected cells. TyrA23 lowers the acidity of the cell, thereby decreasing clathrin mediated endocytosis of PIN proteins (Dejonghe et al., 2016; Dhonukshe et al., 2007; Konopka et al., 2008). With TyrA23 application we observed a similar decrease in FRAP as when MAB4 and/or PID were co-transfected (Fig. 9C). This TyrA23 mediated decrease in FRAP was not enhanced by the co-transfection of MAB4, suggesting that MAB4 acts on the same pathway as TyrA23. Addition of the inactive Tyrphostin A1 did not result in a significant reduction of FRAP. These results provide further evidence that PID and MAB4 decrease the mobility of PIN1 at the plasma membrane. When TyrA23 was used there was still a residual level of FRAP (+/-10%), which was most likely due to the diffusion of plasma membrane localized PIN1 in the bleached region. The fact that MAB4 and PID alone are sufficiently potent in this system to lower FRAP to this basic level, suggests that they act by inhibiting endocytosis. These results further strengthen the hypothesis that MAB4 and PID contribute to PIN1 stability at the plasma membrane.

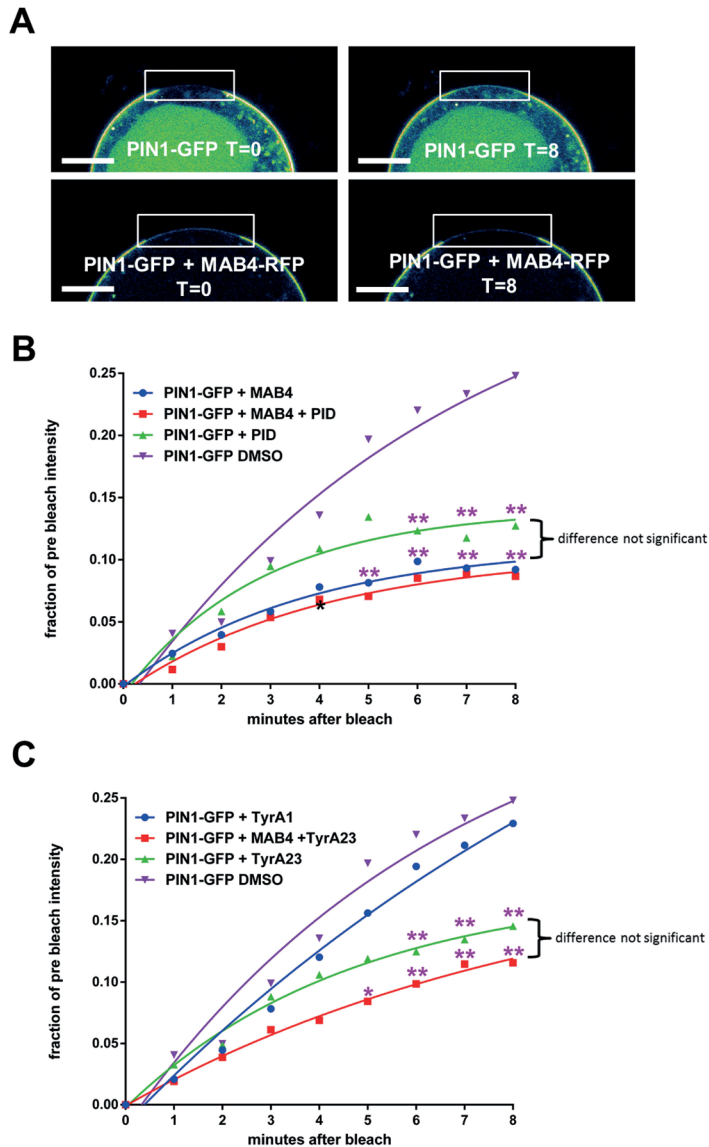


Figure 9: PID and MAB4 reduce PIN mobility at the plasma membrane, also by inhibition of endocytosis. (A-C) Fluorescence after Photobleaching (FRAP) experiment in *Arabidopsis* protoplasts expressing PIN1-GFP. (A) A small region of the protoplast plasma membrane was bleached and allowed to recover. Pictures show protoplast immediately post bleach and after 8 minutes recovery. scale bars are 10 μm . (B) FRAP experiments, every minute after bleaching a scan was made and the mean pixel intensity of PIN1-GFP relative to the pre-bleach intensity is plotted on the y-axis. CO-expressions of PIN1-GFP with MAB4 and PID, MAB4 or PID were performed and measured. (C) FRAP experiments similar to (B), but with chemical treatment of 30 μM TyrA23 and TyrA1 (negative control) combined with MAB4 co-expression. * $p < 0,05$, ** $p < 0,01$ significantly different to PIN1-GFP DMSO treatment, two-way ANOVA with post-hoc Tukey test.

Discussion

MAB4/MELs act as PIN scaffolding proteins

Previous studies have clearly shown a role for MAB4/MELs in the establishment and maintenance of PIN polarity (Furutani et al., 2011; Furutani et al., 2014; Li et al., 2010) and evidence was provided that MAB4/MELs act cooperatively with the AGC3 kinases (Cheng et al., 2008). However, the mode of action by which MAB4/MELs contribute to PIN polarity was still unclear. The data presented here suggest that MAB4/MELs promote PIN plasma membrane abundance by allowing micro domain formation by binding to PINs, which reduced vacuolar targeting and plasma membrane mobility of PINs. MAB4/MEL – PIN interaction is promoted by AGC3 kinase binding and possibly also by AGC3 kinase-mediated phosphorylation of the PINHL. We propose a model where PID-mediated phosphorylation not only results in apical PIN targeting, but also leads to recruitment of MAB4/MELs by enhancing their binding to the PINHL (Fig. 10). AGC3 kinases are peripheral membrane proteins. Their association with the plasma membrane through their interaction with phospholipids (Simon et al., 2016; Zegzouti et al., 2006) brings them in close proximity to the PIN proteins, which allows their interaction with and phosphorylation of PINs. This induces endocytosis of PIN and possibly also of the associated AGC3 kinases. Following their subsequent exocytosis to the correct polar domain, PINs are recruited by MAB4/MELs which bind the PINHL with both the BTB and NPH3 domain, and as such can function as a scaffolding protein between different PIN proteins at the plasma membrane to generate micro domains. We propose that by stimulating the formation of PIN microdomains, MAB/MELs reduce PIN diffusion, endocytosis and possibly also vacuolar targeting by shielding them from ubiquitination (Fig. 10). It has been shown that PINs are much less mobile in the plasma membrane than other plasma membrane proteins and that the formations of PIN microdomains may aid in immobilizing PINs, which is an important part of PIN polarity establishment (Kleine-Vehn *et al.*, 2011). Plasma membrane microdomains are structures where different fatty acids form a raft for dedicated protein complexes and very long fatty acid chain sphingolipids, found predominantly in plasma membrane microdomains, are necessary for proper PIN1 polarity (Markham et al., 2011). Furthermore it has been shown that clathrin mediated endocytosis can be actively regulated at plasma membrane microdomains (Li et al., 2012). Thus the MAB4/MELs enhance PIN plasma membrane polarity, leading to super-polarity (Kleine-Vehn et al., 2011), by protecting them against (the effects of) de-phosphorylation and ubiquitination and subsequent turnover and recycling through microdomain formation. Without MAB4/MELs, PIN2 super polarity is lost and lateral plasma membrane signals increase (Furutani et al., 2011).

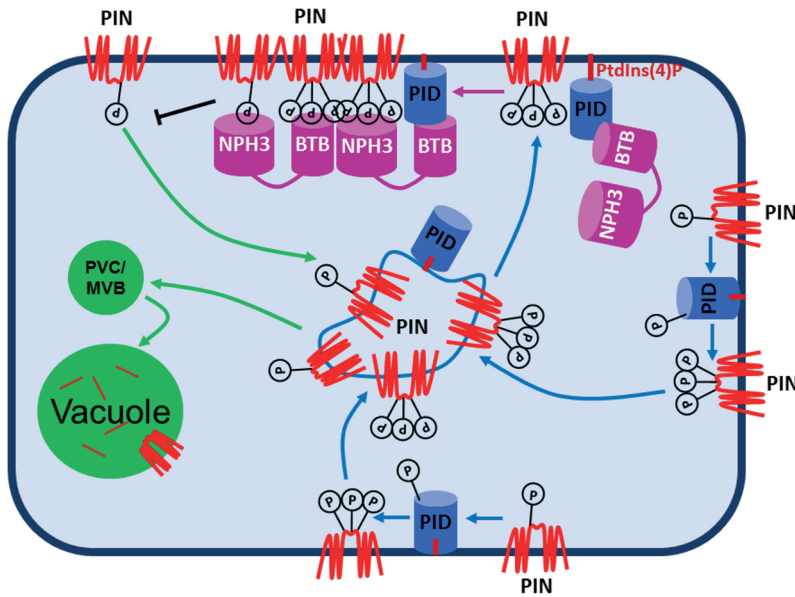


Figure 10: Model depicting MAB4 recruitment to PIN HLs, leading to stabilization of PINs at the plasma membrane.

PID (blue) is anchored to the plasma membrane through PtdIns(4)P (phosphoinositides). PID phosphorylation leads to directed targeting of PINs (red) to (apical) polar domains, where MAB4 (pink) preferentially binds to fully phosphorylated PINHL. Both MAB4 domains (NPH3 and BTB) can interact with the PINHL, while MAB4 proximity to PINs is further enhanced by the interaction between the MAB4 BTB domain and PID. MAB4 inducing microdomain formation of PINs, which protects them from endocytosis. This reduces the recycling of PINs and subsequent targeting to the pre-vacuolar compartment / multivesicular body (PVC/MVB) and vacuole.

Is there direct polarity control by MAB4/MELs?

An interesting question is whether MAB4/MELs only control PIN PM abundance, or that they are part of the mechanism by which PIN polarity is established. Our result whereby simultaneous overexpression of PID and MAB4 yielded PIN-formed inflorescences was very reminiscent of the *pin1 PIN1::PIN1 S1,2,3E* mutant phenotype (Huang et al., 2010), which has increased apical (shootward) PIN1 polarity. This result suggests that MAB4/MELs like AGC3 kinases are involved in PIN polarity establishment, and that in single overexpression lines of PID and MAB4 either one is rate limiting in promoting constitutive apical PIN1 polarity. The *mab4 mel1 mel2* mutant also has a pin-formed inflorescence and it has been shown that this is due to a defect in the canalization of auxin from the epidermis towards the vascular tissue (Furutani et al., 2014). Also it has been proposed that the strength of auxin transport is important in the formation of focal points strong enough to induce canalization in the meristem and subsequent floral meristem initiation (Kierzkowski et al., 2013). Our model indicates that the cooperative action of MAB4, MELs and AGC3 kinases strengthens PIN1 polarity oriented towards the convergence points of

auxin in the meristem epidermis. The question still remains how PIN1 polarity in these convergence-point cells switches from apical-basal to lateral. We hypothesize that the very high amount of auxin at convergence points creates a self-organising process fuelled by the positive feedforward loop between PINs and auxin (Benjamins and Scheres, 2008; Grieneisen et al., 2007; Kierzkowski et al., 2013). This auxin maximum can induce the activity of the ARF MONOPTEROS (MP), which upregulates MAB4/MEL expression (Furutani et al., 2014), thereby increasing stability of PINs to a degree that they are also stabilized in lateral domains and thus starting the canalization of auxin inward. MAB4/MELs show colocalization with basal PINs in the embryo, root and floral meristem, tissues where PIN polarity can be apical or basal, so their molecular function is not polarity control, but stabilization. Combined with differential binding to PINs, phosphorylated by AGC3 kinases, they can indirectly contribute to polarity by strengthening it.

Concluding remarks

Our data shows clearly that PINs, MAB4/MELs and AGC3 kinases can interact and we have indications that this is important for stimulating PIN plasma membrane abundance by PIN microdomain formation. It will be interesting to see how exactly microdomains can be formed and what serves as an organising structure. Advances in super-resolution microscopy now also enables live-cell imaging, opening up the possibility of following the process of micro-domain formation in a time-course experiment. It is unlikely that the MAB4/MELs are principle polarity determinants in the cell, but previous results (Furutani et al., 2011; Furutani et al., 2014) and our data show that they do contribute to PIN polarity. It will be intriguing to investigate which factors determine where in the cell PINs, AGC3 kinases and MAB4/MELs come together to cause asymmetric PIN distribution by immobilizing these auxin efflux carriers in micro domains.

Materials and methods

Plant material and growth conditions

All *Arabidopsis thaliana* lines are in Col-0 background except for *pid-3*, which is in Col-3. The *pid wag1 wag2*, *PIN2::PIN2-YFP (S1,2,3A)* (Dhonukshe et al., 2010), *MEL1-GFP*, *PIN2-GFP*, *mel1 mel2 mel3 mel4* lines (Furutani et al., 2011), *pid-3* and *pin1-201* (Furutani et al., 2004) were described previously. *35S::MAB4-GFP-T7* and *35S::PID-GFP-MYC* plants were constructed by crossing *35S::MAB4-GFP-T7* (Furutani et al., 2011) and *35S::PID-GFP-MYC* transformants. Seedlings were grown on vertical plates with ½ MS medium containing 0.8% agar and 1g/L MES, at 20°C and 16 hours photoperiod of 90 µmol/m²/s. Adult plants were grown on soil at 20°C, 70% relative humidity 16 hours photoperiod of 100 µmol/m²/s.

Molecular cloning

MAB4/MEL genes were amplified from cDNA provided by the Riken Institute in Japan, using primers with attB sites (Table S1). PCR products were cloned into pDONR207 by BP reaction and subsequently into pART7 (Gleave, 1992) modified with a Gateway cloning cassette between a Cauliflower Mosaic Virus 35S promoter and a *RFP* coding region with a Gateway LR reaction. The cloning procedure for *PID* was described previously (Dhonukshe et al., 2010) and the pDONR207:*PID* vector was recombined into pART7:35S:CFP. GST and HIS tagged *PID* and *MAB4/MEL* products were created by recombining the pDONR207 entry vectors into pGEX-GST (GE Healthcare) and pET16H-HIS (Novagen) vectors. BTB and NPH3 domains of *MAB4* were clones from cDNA using attB primers and Gateway recombination into pDONR207. *PID+PIDinsdom* and *PID-insdom* were created by introducing *SgsI* and *BspTI* restriction sites at the N- and C-terminal border respectively of the *PID* insertion domain by site-directed mutagenesis of the pDONR207:*PID* Gateway entry vector and subsequently deleting the insertion domain by restriction and ligation. Site directed mutagenesis was performed by using a long (45-50bp) 5' phosphorylated primer to introduce base changes by Phusion-PCR of the whole vector and subsequently degrading the template vector by *DpnI* digestion (protocol adapted from: Sawano and Miyawaki, 2000). The pBluescript-based 35S::*PIN1-GFP* vector used for protoplast transformation (Dhonukshe et al., 2007) and the *PIN1HL* and *PIN2HL* and S to A mutant constructs (Huang et al., 2010) were described before. pDONR:*PID* (Dhonukshe et al., 2010) and pDONR:*PID* D205A (Ding et al., 2011) were used to recombine into pART7:35S:CFP/YFP/RFP vectors. Vectors for the BiFC experiments were constructed using previously described vectors (Walter et al., 2004). *MAB4*, *MEL1* and *PID* were introduced into these vectors by gateway cloning. Primers used can be found in Table S1.

Pull-down and western blot

For the production of protein-containing *E. coli* lysate, 5ml of overnight culture was added to 50ml of LC medium supplemented with antibiotics and grown to an OD-600 of 0.4-0.8. The cultures were subsequently induced for 4 hours with 0.4 mM of IPTG. After induction the cultures were centrifuged at 4000 rpm for 20 min and the pellets were stored at -20°C. Pellets were resuspended in fresh extraction buffer (1x PBS, 2 mM EDTA, 2 mM EGTA, 2mM DTT, 150 mM NaCl pH 8, supplemented with 1 mM PMSF with protease inhibitor tablets from Pierce and 1 mg/ml lysozyme) and incubated for 1 hour at 4°C on a rocking table. Cells were sonicated (on ice) and cell debris was centrifuged at 14000rpm at 4°C for 30 minutes. After addition of 1% triton the supernatant (containing the expressed protein) was divided into 100µl aliquots. For pull-down, 100µl of GST-tagged lysate (in total 500 µl extraction buffer) was bound to glutathione agarose beads for 2 hours at RT. 100 µl of each HIS-tagged protein was loaded onto the beads and binding buffer (50 mM Tris pH 6,

200 mM NaCl, 0,1% Tween 20) was added to a total reaction volume of 400 µl. The samples were then incubated at RT for 2 hours on an Eppendorf rotator. After incubation, the resin was washed 3 times in 500 µl wash buffer (25 mM Tris-HCL pH 8, 10% glycerol, 300 mM NaCl, 20 mM imidazole, 0,05% Tween 20) with in-between centrifugation at 4000 rpm for 3 minutes. 40 µl of 1x SDS-PAGE loading buffer (40% glycerol, 240 mM Tris/HCl pH 6,8, 8% SDS, 0,04% bromophenol blue, 5% β-mercaptoethanol) was added to the precipitated resin and samples were boiled at 99°C for 10 minutes. The boiled resin was spun down and the supernatant was transferred to a 12,5% polyacrylamide gel for SDS page. Blotting was performed in a transblot semi-dry setup using top (60 mM Tris, 40 mM CAPS, pH 9,6 + 0,1% SDS) and bottom buffer (60 mM Tris, 40 mM CAPS, pH 9,6 + 15% MeOH) onto a PVDF membrane. Blocking was performed with 5% Elk-brand milk powder in TBS at 4°C overnight. The membrane was then probed for an hour at RT with an anti-HIS-HRP antibody (Roche, 1:1000 diluted) and chemiluminescence (LumiGlo, Cell Signalling) was detected using and X-Ray film (Fuji).

Protoplast isolation and transformation

Arabidopsis thaliana Col-0 cell suspension cultures were used for protoplast preparations. Culture maintenance, protoplast isolation and transfections were performed as previously described (Schirawski et al., 2000) with minor modifications. Four-to-six day old cultures were diluted 5-fold in Cell Medium (30 g /L sucrose, 3.2 g/L Gamborg's B5 basal medium with mineral organics, adjusted to pH 5.8 with KOH and sterilized by autoclaving), incubated overnight and used for protoplast isolation. Following transfection with 10µg plasmid DNA per 1*10⁶ protoplasts, the cells were incubated at 25 °C in the dark for 16-18 hours before observation or additional treatments.

Confocal microscopy and analysis

Protoplast analysis Fig. 2 and Fig. 9: 150-200 µl protoplasts in protoplast medium were pipetted into a Lab-Tek Chambered #1.5 German Coverslide System 8-chamber microscopy slide. Images were taken with a Zeiss inverted LSM5 an Axiolmager unit and Lasos RMC7821 Z1 Argon laser. Laser and filter settings for protoplast analysis can be found in **Table S2**. FRAP was performed by bleaching until the intensity reached <5% of original intensity using the ZEN-software built in bleach function (<http://www.zeiss.de/zen>).

Vacuolar targeting analysis Fig. 7: An upright Zeiss LSM5 confocal microscope with the same laser and detector units as described above was used. Four seedling roots were mounted without shoot parts on an object slide in standard mounting medium. Imaging was performed with a 488 nm excitation laser and a 505-530 nm band pass emission filter. Detector gain and pinhole settings were equal in all pictures and were chosen in such a

way that the vacuolar signal did not reach saturation. Quantification of pixel intensity was performed using ICY (<http://icy.bioimageanalysis.org/>) and statistically analyzed using graphpad.

BiFC analysis Fig.4: Images were captured with a standard confocal microscope with a 40x objective. For excitation of YFP an argon laser of 514 nm was used and for detection a standard band-pass filter combined with a CCD camera.

Immunolocalization and Duolink experiments

Antibodies to detect MAB4 and PIN1 were described before (Furutani et al., 2011; Gälweiler et al., 1998). Immunolocalizations were performed according to a previously published protocol (Sauer et al., 2006). Duolink experiments (Sigma-Aldrich) have been done according to the manufacturer's protocol. <https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Instructions/1/duolink-fluorescence-user-manual.pdf>

Images were captured using standard confocal microscopy.

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Supplemental data

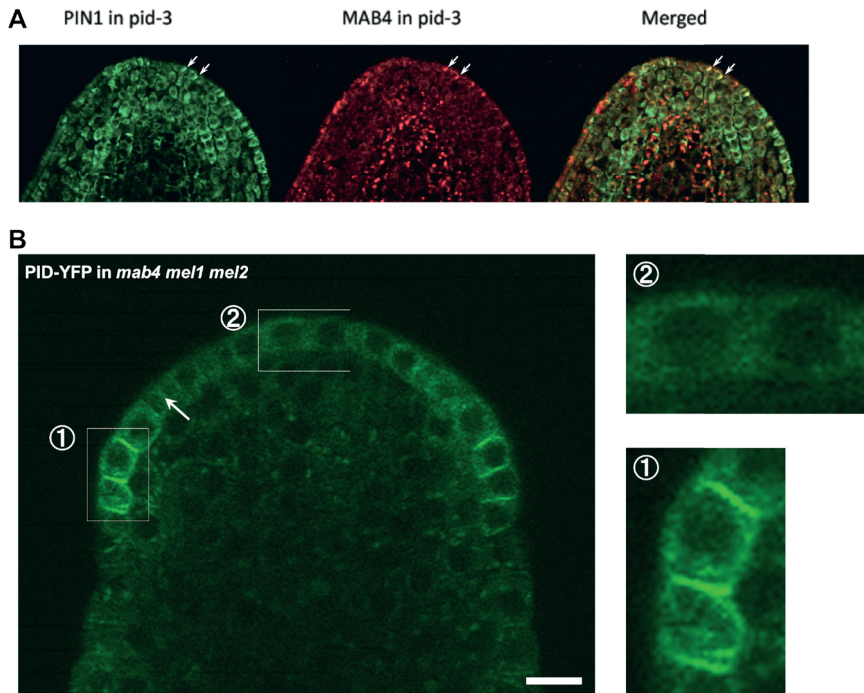


Figure S1: Interdependent PIN, PID and MAB4 plasma membrane localization in the inflorescence meristem.

(A) PIN1 (green) and MAB4 (red) immunolocalization in *pid-3*, arrows show colocalization. (B) PID-YFP in *mab4 mel1 mel2* background in the lateral part of the inflorescence meristem (1), PID-YFP at the top was absent (2).

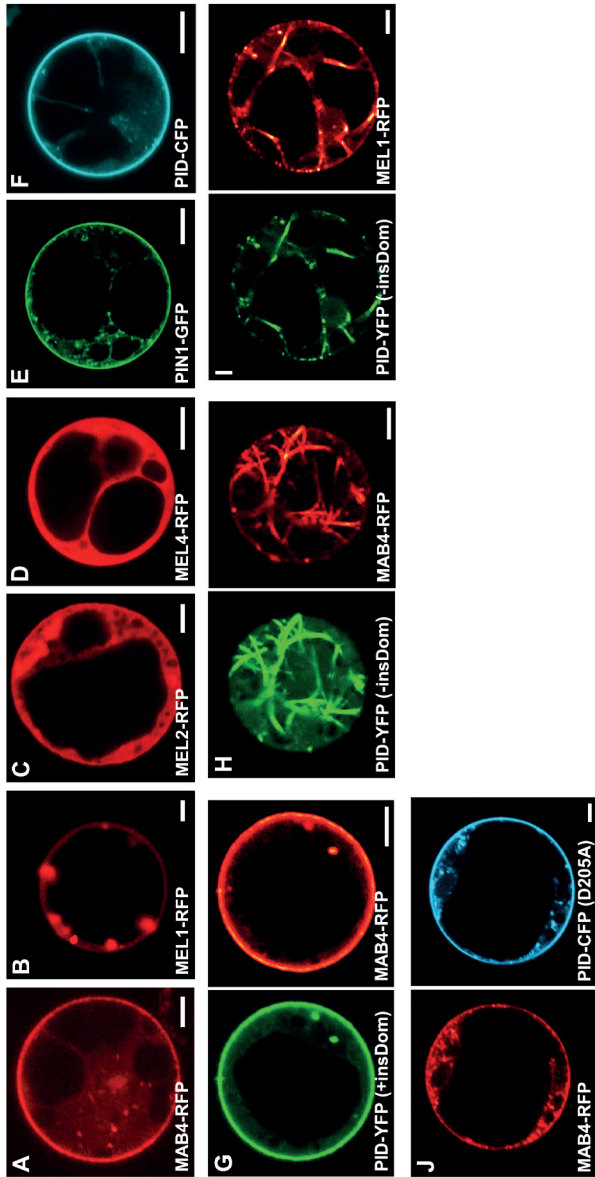


Figure S2: Transfection of single vectors and additional co-transfections of PID mutants with MAB4/MEL1 in *Arabidopsis* protoplasts.

Transfection of (A) 35S::MAB4-RFP, (B) 35S::MEL1-RFP, (C) 35S::MEL2-RFP, (D) 35S::MEL4-RFP, (E) 35S::PIN1-GFP and (F) 35S::PID-RFP revealed that MAB4, PIN1 and PID localized to the plasma membrane by themselves. (G-J) Co-transfections of (G) PID-YFP (with the insertion domain) and MAB4-RFP; (H) PID-YFP (without the insertion domain) and MAB4-RFP or (I) MEL1-RFP, MAB4-RFP and PID-CFP (D205A, kinase dead mutant). Size of scale bar is 5μm.

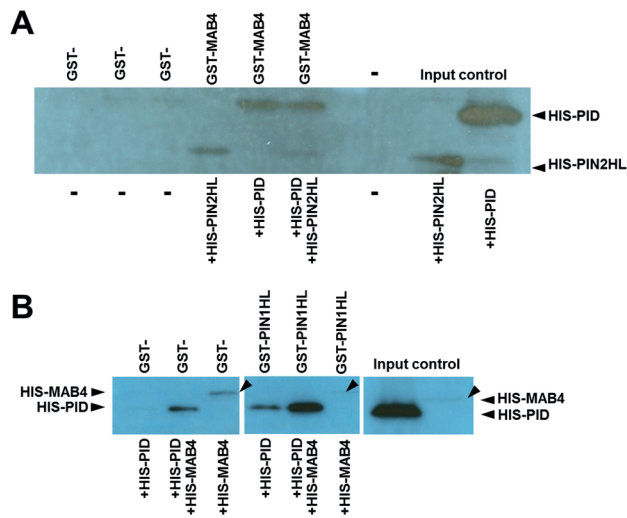


Figure S3: PID and PIN2HL competitively interact with MAB4. PID interacts with PIN1, while HIS-MAB4 is difficult to detect.

(A) Longer exposure of the same western blot as shown in Fig. 5A. (B) *In vitro* pull-down assay where GST-PIN1HL was bound to the agarose beads and probed with HIS-MAB4 and HIS-PID. HIS-MAB4 could almost not be detected when the pure lysate was loaded (most right lane).

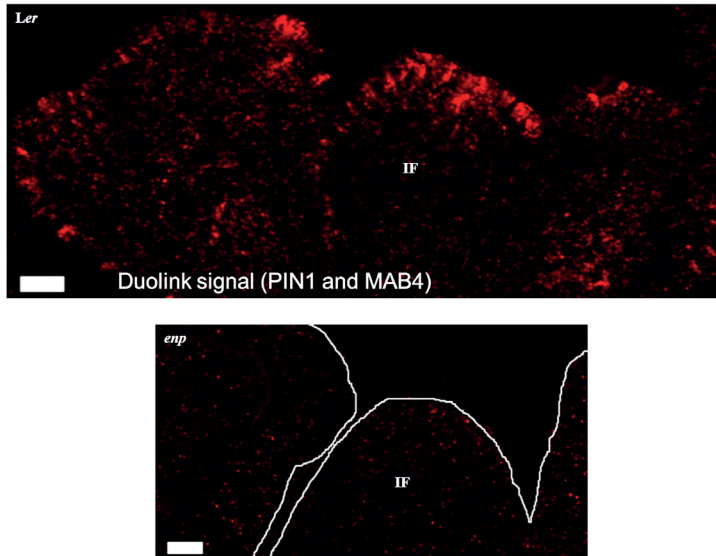


Figure S4: MAB4 and PIN1 interact *in situ* at the inflorescence meristem.

Duolink (Sigma-Aldrich) immunolocalization interaction experiment with anti MAB4 and PIN1 antibodies showing that PIN1 and MAB4 interacted at the apical meristem of *Ler* ecotype. The *enp* apical meristem (early stop in *MAB4*) was used as a negative control. Scale bars are 20 μm .

Table S1: Primers used in this study.

name	sequence	description
NPY1 S	GGGGACAAGTTGTACAAAAAGCAGGCTATGAAGTTCATGAAGCTAGG	MAB4 gateway attB forward primer
NPY1 AS	GGGGACCACTTTGTACAAAGAAAGCTGGGTACGATATCGAATGCTCGCGC	MAB4 gateway attB reverse primer
NPY3+4 S	GGGGACAAGTTGTACAAAAAGCAGGCTATGAAGTTTATGAAACTTGG	MEL2 and MEL4 gateway attB forward primer
NPY3 AS	GGGGACCACTTTGTACAAAGAAAGCTGGGTAAGACGATGACCTTCTCCGGC	MEL2 gateway attB reverse primer
NPY4 AS	GGGGACCACTTTGTACAAAGAAAGCTGGGTAAACTCTTCTCATGTGTC	MEL4 gateway attB reverse primer
NPY5 S	GGGGACAAGTTGTACAAAAAGCAGGCTATGAAGTTCATGAAACTTGG	MEL1 gateway attB forward primer
NPY5 AS	GGGGACCACTTTGTACAAAGAAAGCTGGGTAATGCTTCTTGTGTGAAG	MMEL1 gateway attB reverse primer
PID Deletion Ins Dom S	CTCTGACTTTGACCTCGGGCGGCCCTTAAGGCTGAACCGTTACTG	site directed mutagenesis primer for insdom deletion
PID Deletion InsDom AS	CAGTAACCGGTTACAGCCTTAAGGGCGCGCCCGAGGTCAAAGTCAGAG	site directed mutagenesis primer for insdom deletion
pDONR207 F	TCGCGTTAACGCTAGCATGGATCTC	pDONR207 colony PCR primer
pDONR207 R	GTACATCAGAGATTTTGAGACAC	pDONR207 colony PCR primer
NPY1 NPH3 domain Forward	GGGGACAAGTTGTACAAAAAGCAGGCTTTATGAAAGATTGGTGGTCAAG	MAB4 NPH3 domain attB forward primer
NPY1 NPH3 domain Reverse	GGGGACCACTTTGTACAAAGAAAGCTGGGTTTGATGCTTCATTTGTCACTTCC	MAB4 NPH3 domain attB reverse primer
NPY1 BTB domain Forward	GGGGACAAGTTGTACAAAAAGCAGGCTTCATGAAGCTAGGGTCTAAGCCCG	MAB4 BTB domain attB forward primer
NPY1 BTB domain Reverse	GGGGACCACTTTGTACAAAGAAAGCTGGGTTGCCTGAGTTGAGGAAAAC TTCGA	MAB4 BTB domain attB reverse primer

Table S2: Microscopy settings for protoplast analysis.

Fluorophores	Track 1			Track2			Track 3		
	Filter (BP)	Beam Splitter (HFT)	Laser (nm)	Filter (BP)	Beam Splitter (HFT)	Laser (nm)	Filter (BP)	Beam Splitter (HFT)	Laser (nm)
GFP	505-530	405/488	488						
RFP	560-615	405/488/543/633	543						
CFP	475-525	458/514	458						
YFP	530-600	405/514	514						
GFP RFP	505-530	405/488	488nm	560-615	405/488/543/633	543			
CFP RFP	475-525	458/514	458	560-615	405/488/543/633	543			
YFP RFP	530-600	405/514	514	560-615	405/488/543/633	543			
CFP GFP RFP	475-525	458/514	458	530-600	405/488	488	560-615	405/488/543/633	543