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SHORT COMMUNICATION

A new class of cancer-associated PTEN mutations defined by membrane translocation defects

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Phosphatase and tensin homolog (PTEN), which negatively regulates tumorigenic phosphatidylinositol (3,4,5)-trisphosphate (PIP3) signaling, is a commonly mutated tumor suppressor. The majority of cancer-associated PTEN mutations block its essential PIP3 phosphatase activity. However, there is a group of clinically identified PTEN mutations that maintain enzymatic activity, and it is unknown how these mutations contribute to tumor pathogenesis. Here, we show that these enzymatically competent PTEN mutants fail to translocate to the plasma membrane where PTEN converts PIP3 to PI(4,5)P2. Artificial membrane tethering of the PTEN mutants effectively restores tumor suppressor activity and represses excess PIP3 signaling in cells. Thus, our findings reveal a novel mechanism of tumorigenic PTEN deficiency.

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INTRODUCTION

More than 1500 different mutations in the phosphatase and tensin homolog (PTEN) gene have been identified in different cancers (http://cancer.sanger.ac.uk).[1–6] Approximately 40% of these are substitution missense mutations. These cancer-associated amino acid substitutions are distributed along the entire length of PTEN with the highest frequency in the lipid phosphatase catalytic residues, mutations of which abolish lipid phosphatase activity. However, there is a group of PTEN substitutions that maintain PTEN enzymatic activity,[7–9] and it is currently unknown how these mutations disrupt the tumor suppressor function of PTEN.

The membrane recruitment of PTEN is important for its lipid phosphatase function in phosphatidylinositol (3,4,5)-trisphosphate (PIP3) signaling.[10–15] Membrane association of PTEN is negatively regulated by phosphorylation of the inhibitory C-terminal tail domain. When phosphorylated at four serine/threonine residues, the C-terminal tail binds to the membrane-binding regulatory interface and masks the membrane-binding domain containing the CBR3 loop of the C2 domain.[10,11,16] When the phosphorylation sites are mutated to alanine (termed $PTEN_{A4}$), the C-terminal tail dissociates from the membrane-binding regulatory interface and promotes membrane recruitment of PTEN.[10,16] Therefore, we hypothesized that the cancer-associated mutations that retain enzymatic activity might affect the membrane recruitment of PTEN. To test our hypothesis and determine whether the enzymatically competent mutants represent a new class of tumorigenic PTEN deficiency, we took advantage of $PTEN_{A4}$. Although functionally important, PTEN membrane association is difficult to analyze because a large number of PTEN molecules are present in the cytosol.[10,11] The use of $PTEN_{A4}$ dramatically decreases the cytosolic pool of PTEN and allows us to visualize membrane localization.

RESULTS AND DISCUSSION

We focused on a group of PTEN missense mutants selected by two criteria. We first chose mutations that do not inhibit phosphatase activity.[7–9] Among these, we further narrowed the choice down to four mutations, S10N, G20E, L42R and F90S, which are located in or near the membrane-binding regulatory interface (Figure 1a and Supplementary Table S1).[10] We first biochemically confirmed intact PIP3 phosphatase activity of these mutants using purified PTEN as we described previously.[10] Wild-type (WT) and mutant forms of human PTEN fused with green fluorescent protein (GFP) were expressed and immunopurified from *Dictyostelium* cells using agarose bead-coupled anti-GFP antibodies. $PTEN_{S10N}$, $PTEN_{G20E}$, $PTEN_{L42R}$ and $PTEN_{F90S}$ showed enzymatic activities indistinguishable from that of WT PTEN (Figure 1b). Activity was completely abolished in the negative control $PTEN_{C124S}$, which carries a substitution in the catalytic residue C124. Fluorescence microscopy of *Dictyostelium* cells (Figures 1c and d) and the human cell line HEK293T (Figures 1f and g) expressing PTEN mutants fused to GFP showed that PTEN–GFP, $PTEN_{S10N}$–GFP, $PTEN_{G20E}$–GFP, $PTEN_{L42R}$–GFP and $PTEN_{F90S}$–GFP were mainly located in the cytosol and nucleus.

To determine whether the S10N, G20E, L42R and F90S mutations affect membrane association, we combined them with the A4 mutations. $PTEN_{A4}$–GFP was located at the plasma membrane in addition to the nucleus in *Dictyostelium* cells (Figures 1c and e) and HEK293T cells (Figures 1f and h), as previously reported.[10,11] In contrast, $PTEN_{S10N,A4}$–GFP, $PTEN_{G20E,A4}$–GFP, $PTEN_{L42R,A4}$–GFP and $PTEN_{F90S,A4}$–GFP were unable to localize at the plasma membrane in both cell types (Figures 1c and e for *Dictyostelium*, f and g for HEK293T). Their nuclear localization was increased, as expected from our previous study showing that dissociation of PTEN from the plasma membrane leads to accumulation in the nucleus.[10] The negative control

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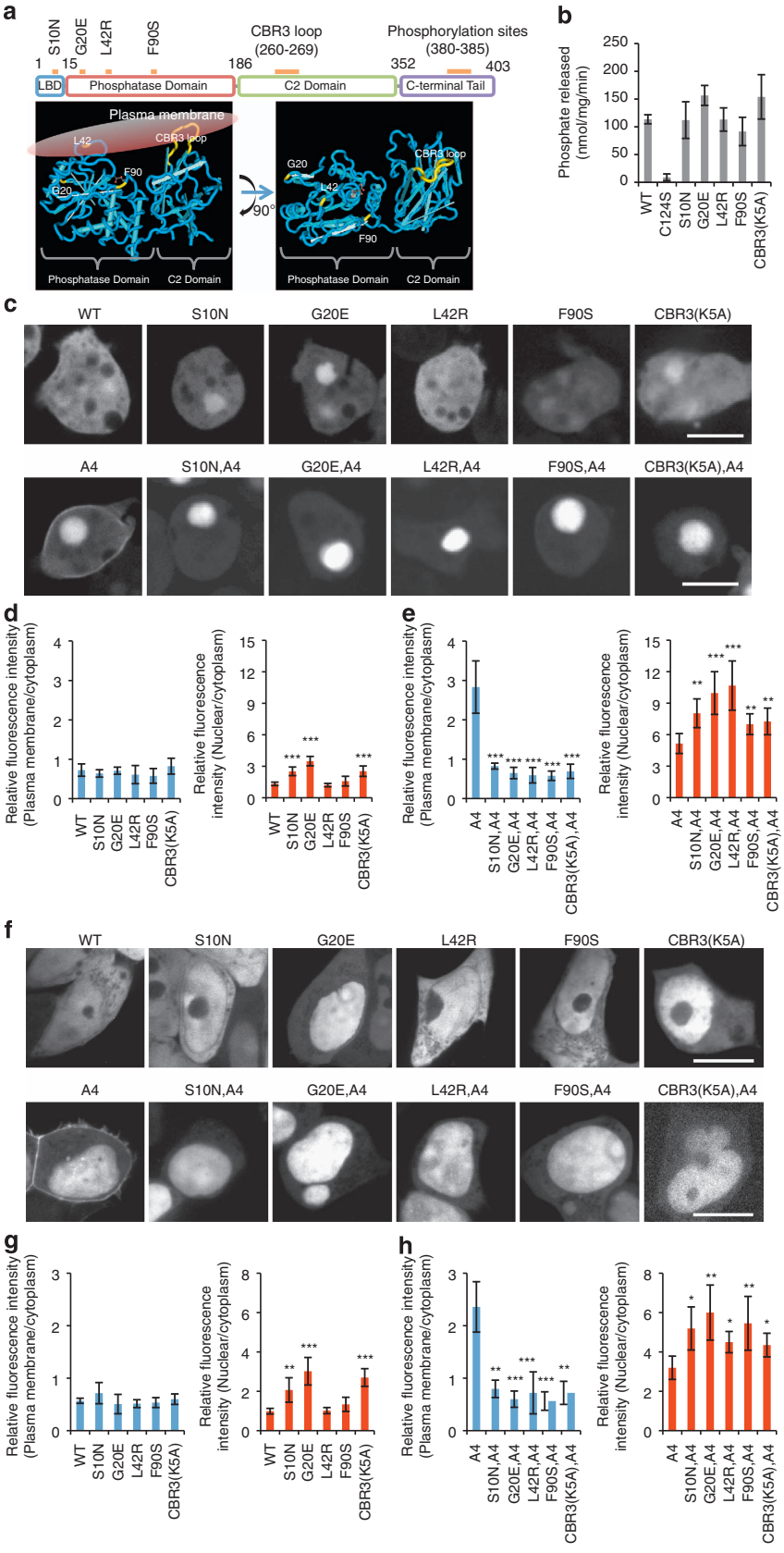

Figure 1. Cancer-associated PTEN mutations inhibit membrane association. (**a**) The domain structure of PTEN. S10N is located in the lipid-binding domain (LBD), whereas G20E, L42R and F90S are present in the phosphatase domain. The same mutations are indicated in the 3-D structure of PTEN with the phosphatase and C2 domains (R14-V351)[28] (http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid = 11638). (**b**) The S10N, G20E, L42R and F90S mutations do not decrease the PIP3 phosphatase activity of purified PTEN *in vitro*. (**c**–**h**) The indicated PTEN–GFP constructs were expressed in *Dictyostelium* cells (**c**–**e**) and HEK293T cells (**f**–**h**). Scale bar, 10 μm. Fluorescence intensity in the plasma membrane (blue bars) and the nucleus (red bars) was quantified relative to that in the cytosol ($n = 8$).

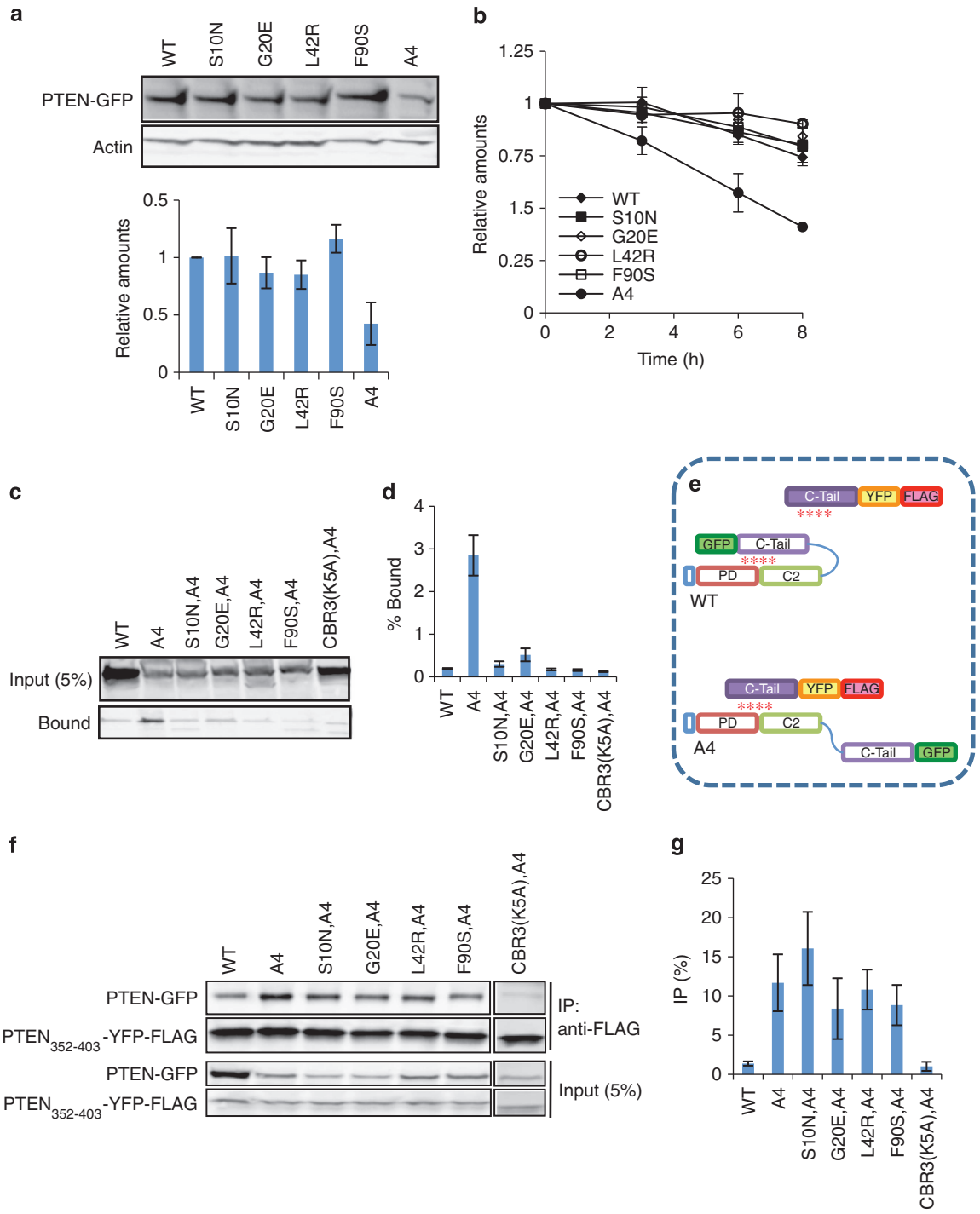

Figure 2. S10N, G20E, L42R and F90S mutations block interaction of PTEN with membrane lipids. (**a**) HEK293T cells were transfected with the indicated PTEN–GFP constructs. Whole-cell lysates were analyzed by immunoblotting with antibodies to PTEN and actin. The band intensity was quantified relative to WT PTEN–GFP. Values represent the mean ± s.d. ($n = 3$). (**b**) HEK293T cells were transfected with expression constructs containing GFP fused to the indicated forms of PTEN and were incubated with 100 μg/ml cycloheximide. Whole-cell lysates were analyzed by immunoblotting with anti-GFP antibodies at the indicated time points. Band intensity was quantified relative to a 0-h sample ($n = 3$). (**c** and **d**) Liposome-binding assay. Liposomes (PC:PS:PI(4,5)P2:PE-biotin = 70:20:5:5) were incubated with whole-cell lysates of *Dictyostelium* expressing the indicated PTEN–GFP proteins. Liposomes were collected using streptavidin-conjugated magnetic beads. The cell lysate (input) and bound fractions were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting using anti-GFP antibodies. Quantification of band intensity is shown in **d**. Values represent the mean ± s.d. ($n = 3$). (**e**) Association of the C-terminal tail of PTEN with full-length PTEN was examined by an 'in trans' interaction assay. Associations between the core and tail domains of the same PTEN molecule (close conformation) are maintained through phosphorylation of the tail (indicated by red asterisks). In contrast, the core region of $PTEN_{A4}$ dissociates from the tail (open conformation) and binds to an exogenously added tail domain. (**f**) Whole-cell lysates expressing the indicated PTEN–GFP proteins were incubated with $PTEN_{352–403}$–YFP–FLAG. $PTEN_{352–403}$–YFP–FLAG was immunoprecipitated with beads coupled to anti-FLAG antibodies. Bound fractions (IP) were analyzed with antibodies to GFP and FLAG. (**g**) Quantification of band intensity ($n = 3$).

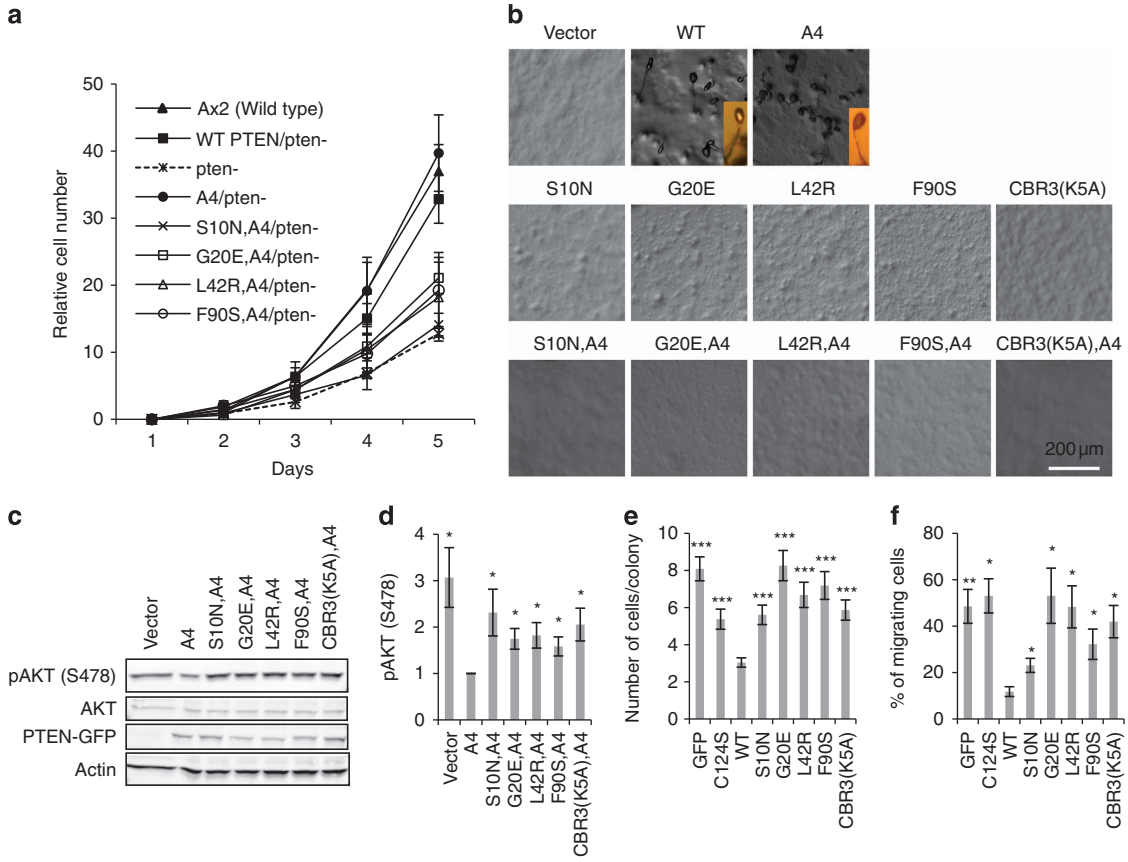

Figure 3. S10N, G20E, L42R and F90S mutations interfere with PTEN function in the regulation of PIP3 signaling. (**a**) The proliferation of PTEN-null *Dictyostelium* cells expressing different PTEN–GFP constructs is shown. The numbers of cells were normalized relative to those at day 1. Values represent the mean ± s.d. ($n = 3$). (**b**) PTEN-null *Dictyostelium* cells expressing different PTEN–GFP constructs were starved to induce differentiation into fruiting bodies. Pictures were taken at 36 h after the onset of starvation. PTEN-null cells expressing WT PTEN or $PTEN_{A4}$ formed fruiting bodies, whereas PTEN-null cells expressing vector alone did not aggregate and remained undifferentiated. PTEN and $PTEN_{A4}$ molecules carrying the cancer mutations did not differentiate into fruiting bodies. Inserts show side views of fruiting bodies. (**c**) HEK293T cell lysates containing the indicated PTEN–GFP constructs were analyzed by immunoblotting with antibodies to phospho-AKT, AKT, GFP (PTEN–GFP) and actin. (**d**) Quantification of band intensity ($n = 3$). (**e** and **f**) MCF-10A PIK3CA cells were infected with lentiviruses carrying the indicated PTEN constructs and analyzed for cell proliferation by counting the number of cells in individual colonies (**e**) and for cell migration using transwell plates (**f**). Values represent the mean ± s.d. ($n = 4$).

$PTEN_{CBR3(K5A)}$–GFP, which carries mutations in the CBR3 loop of the membrane binding C2 domain,[10] showed cytosolic localization (Figures 1c–h).

Because decreased stability of PTEN contributes to tumorigenesis,[17] we tested whether the S10N, G20E, L42R and F90S mutations affect the turnover of PTEN. Immunoblotting revealed similar levels of PTEN–GFP, $PTEN_{S10N}$-GFP, $PTEN_{G20E}$-GFP, $PTEN_{L42R}$-GFP and $PTEN_{F90S}$-GFP in HEK293T cells (Figures 2a). In contrast, $PTEN_{A4}$-GFP, which has decreased stability, showed lower steady-state levels (Figure 2a). Consistent with these results, PTEN–GFP, $PTEN_{S10N}$–GFP, $PTEN_{G20E}$–GFP, $PTEN_{L42R}$–GFP and $PTEN_{F90S}$–GFP had comparable half-lives in the presence of the protein synthesis inhibitor cycloheximide, whereas $PTEN_{A4}$–GFP showed a shorter half-life (Figure 2b). PTEN binds to another tumor suppressor, p53.[18,19] We tested whether the four mutations defective in membrane localization affected this protein–protein interaction using co-immunoprecipitation. The results showed similar binding for WT and mutant PTEN to p53 (Supplementary Figure S1).

To probe the mechanism by which the S10N, G20E, L42R and F90S mutations inhibited membrane localization of PTEN, we performed an *in vitro* liposome-binding assay. Liposomes containing PC (70%), PS (20%), PI(4,5)P2 (5%) and PE-biotin (5%) were incubated with lysates of *Dictyostelium* cells expressing different PTEN–GFP proteins (Figures 2c and d). Consistent with its cytoplasmic localization, WT PTEN–GFP bound only weakly to the liposomes. In contrast, $PTEN_{A4}$, which was enriched at the plasma membrane, showed approximately 10-fold higher association with the liposomes (Figures 2c and d). The $PTEN_{A4}$–liposome interactions were reduced to the WT level by the mutations S10N, G20E, L42R and F90S, thus $PTEN_{S10N,A4}$–GFP, $PTEN_{G20E,A4}$–GFP, $PTEN_{L42R,A4}$–GFP and $PTEN_{F90S,A4}$–GFP only weakly interacted with the liposomes. Therefore, the decreased localization of the PTEN mutants at the plasma membrane likely results from their inability to bind to membrane phospholipids.

The C-terminal tail of PTEN binds to the membrane-binding regulatory interface and closes the protein conformation, physically limiting access of the membrane-binding site to the membrane (Figure 2e).[10,16] Mutations in the C-terminal phosphorylation sites in $PTEN_{A4}$ release this inhibitory interaction (Figure 2e).[11] To determine whether the S10N, G20E, L42R and F90S mutations restore this interaction in $PTEN_{A4}$, we performed an 'in trans' interaction assay and examined the binding of PTEN–GFP to a separate tail domain fused to FLAG after immunoprecipitation with anti-FLAG antibodies. As previously reported,[10,11] although PTEN–GFP did not interact with $PTEN_{352–403}$–FLAG, $PTEN_{A4}$–GFP showed a strong interaction (Figures 2f and g). The S10N, G20E, L42R and F90S mutations did

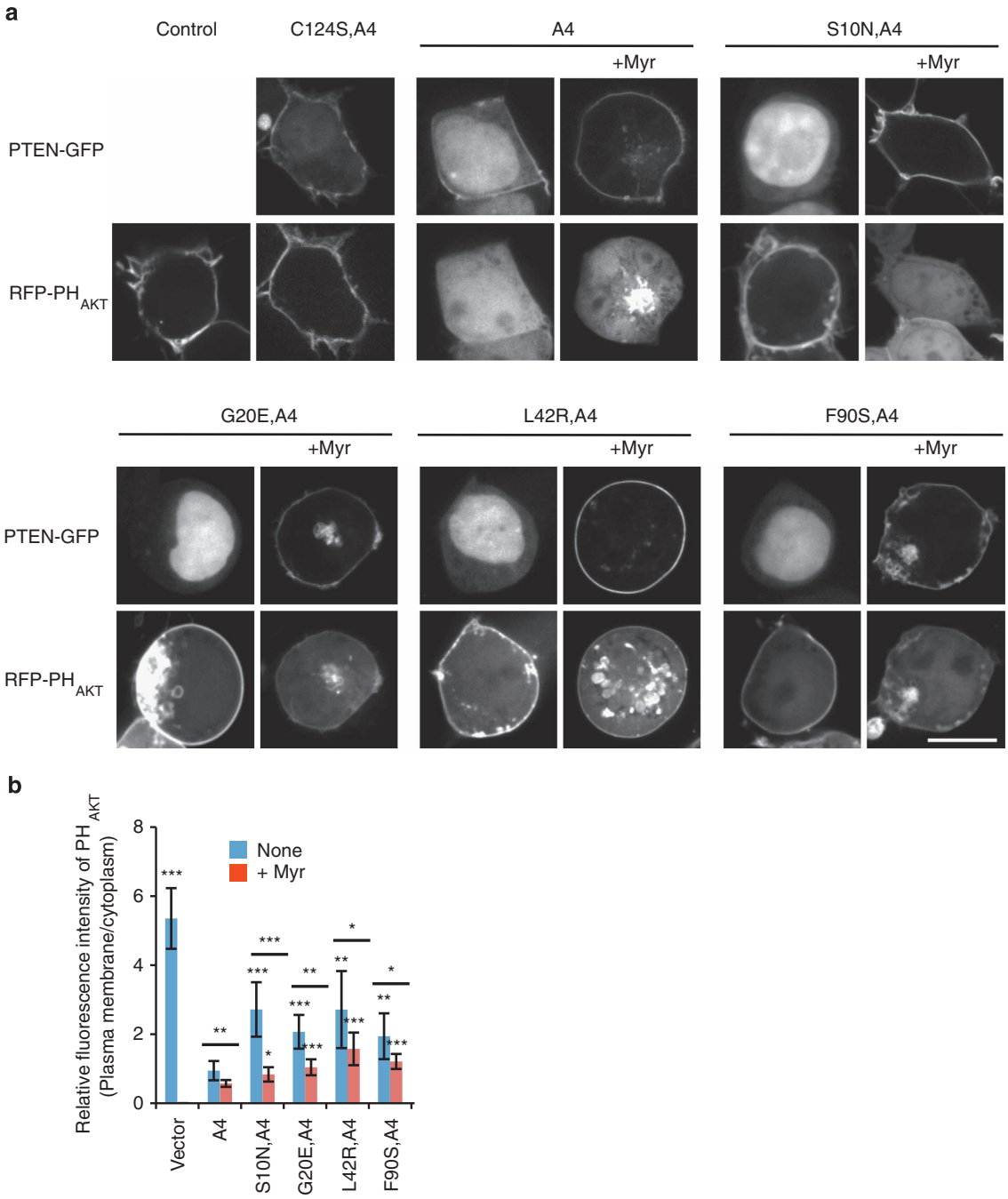

Figure 4. Artificial tethering to the plasma membrane neutralizes the effect of S10N, G20E, L42R and F90S on PIP3 signaling and PIP3-regulated cancer cell behavior. (**a**) HEK293T cells expressing a PIP3 biosensor (RFP–PH_{AKT}) together with different PTEN–GFP constructs were observed by fluorescence microscopy. Myristoylation motif derived from the plasma membrane protein PKBR1 was added to the N terminus of PTEN (+Myr). Scale bar, 10 μm. (**b**) Intensity of RFP at the plasma membrane was quantified relative to that in the cytosol. Values represent the mean ± s.d. ($n = 8$). RFP, red fluorescent protein.

not affect the interaction of $PTEN_{A4}$–GFP with $PTEN_{352–403}$–FLAG (Figures 2f and g). A negative control, $PTEN_{CBR3(K5A),A4}$–GFP that carries mutations in the tail-binding CBR3 loop,[10] did not interact with $PTEN_{352–403}$–FLAG. Therefore, S10N, G20E, L42R and F90S mutations do not stimulate interaction of the membrane-binding regulatory interface with the C-terminal tail.

To determine the function of $PTEN_{S10N}$, $PTEN_{G20E}$, $PTEN_{L42R}$ and $PTEN_{F90S}$, we first investigated cell proliferation and starvation-induced differentiation of *Dictyostelium* cells. PTEN function is evolutionarily conserved in *Dictyostelium* and human cells.[10,20,21] The loss of PTEN in *Dictyostelium* causes defects in cell proliferation and differentiation into stress-resistant fruiting bodies under starvation conditions.[20] The expression of WT PTEN and $PTEN_{A4}$ restored these phenotypes in *Dictyostelium* PTEN-null cells (Figures 3a and b). However, $PTEN_{S10N}$, $PTEN_{G20E}$, $PTEN_{L42R}$ and $PTEN_{F90S}$ were unable to rescue the defects in the presence or absence of the A4 mutation (Figures 3a and b). We then examined the role of PTEN in PIP3 signaling in HEK293T cells, in which PIP3 is overproduced, by measuring phosphorylation of AKT, a major downstream event of PIP3 signaling.[22] $PTEN_{A4}$ suppressed AKT phosphorylation by approximately 2.5-fold, whereas addition of the mutations (S10N, G20E, L42R or F90S) blocked the

suppressive activity of $PTEN_{A4}$ (Figures 3c and d). Furthermore, we examined the effect of these cancer mutations on PIP3-dependent cell proliferation and migration in the MCF-10A PIK3CA cell line, which carries a constitutively active form of PI3Kα and has increased PIP3 signaling.[23,24] When WT PTEN–GFP was expressed in MCF-10A PIK3CA cells, cell proliferation was significantly decreased approximately 2.5-fold compared with expression of the negative controls of GFP alone or the catalytic mutant $PTEN_{C124S}$ (Figure 3e). S10N, G20E, L42R and F90S mutations completely blocked the ability of PTEN to decrease proliferation rates. Similarly, the function of PTEN in cell migration was significantly impaired by these four cancer mutations (Figure 3f).

Finally, we tested whether artificial tethering of PTEN to the plasma membrane restored the function of $PTEN_{S10N}$, $PTEN_{G20E}$, $PTEN_{L42R}$ and $PTEN_{F90S}$. For this, we added a myristoylation motif derived from the plasma membrane protein PKBR1[25] to the N terminus of PTEN. The lipid anchor motif effectively recruited WT and mutant PTEN to the plasma membrane as revealed by fluorescence microscopy (Figure 4a). To assess the effect of membrane recruitment on the amount of PIP3 in the plasma membrane, we co-expressed a biosensor for PIP3, red fluorescent protein fused to the PIP3-binding PH domain from AKT1.[26] Bringing PTEN to the plasma membrane significantly improved the ability of $PTEN_{S10N,A4}$, $PTEN_{G20E,A4}$, $PTEN_{L42R,A4}$ and $PTEN_{F90S,A4}$ to remove PIP3 in the plasma membrane. These data further show that the primary defect of the PTEN cancer mutations can be traced to the defects in their membrane association.

In summary, we identified a new class of cancer-associated PTEN mutations that specifically inhibit the recruitment of PTEN to the plasma membrane as a result of impaired interactions with phospholipids. Our findings reveal the importance of membrane binding of PTEN in tumorigenic PIP3 signaling. As this class of mutations maintains normal PIP3 phosphatase activity and can be rescued by tethering to the plasma membrane, we envision that the pharmacologic promotion of membrane localization may be an effective strategy for anticancer therapy for this class of PTEN deficiency. According to the cBioportal and COSMIC databases, the four *PTEN* mutations analyzed in our study occur in ~1% (11/1210, COSMIC) of all human cancer sequencing studies. Thus, associations with progression and prognosis cannot currently be assessed given the relatively small number of patients in these studies and the lack of follow-up information for most sequencing studies outside of the Cancer Genome Atlas. However, the fact that these mutations recur, albeit at low frequencies, substantiates the functional relevance. In addition, as stated in our Supplementary Table S1, these mutations appear to occur in a variety of cancer types. The cBioportal database suggests that these mutations are more frequent in breast, lung, brain and uterine cancers. Although the overall frequency is small, 1% of breast cancers is equivalent to ~15 000 cases globally. This demonstrates the impact of our work; low-frequency mutations, which were previously of uncertain significance, now appear to be mutations of functional consequence. Thus, these mutations offer new insights into molecular mechanisms of cancer and possible new avenues for therapeutic intervention.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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