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Tetraspanin CD82 Regulates the Spatiotemporal Dynamics of PKC α in Acute Myeloid Leukemia

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Patients with acute myeloid leukemia (AML) have increased myeloid cells within their bone marrow that exhibit aberrant signaling. Therefore, therapeutic targets that modulate disrupted signaling cascades are of significant interest. In this study, we demonstrate that the tetraspanin membrane scaffold, CD82, regulates protein kinase c alpha (PKC α)-mediated signaling critical for AML progression. Utilizing a palmitoylation mutant form of CD82 with disrupted membrane organization, we find that the CD82 scaffold controls PKC α expression and activation. Combining single molecule and ensemble imaging measurements, we determine that CD82 stabilizes PKC α activation at the membrane and regulates the size of PKC α membrane clusters. Further evaluation of downstream effector signaling identified robust and sustained activation of ERK1/2 upon CD82 overexpression that results in enhanced AML colony formation. Together, these data propose a mechanism where CD82 membrane organization regulates sustained PKC α signaling that results in an aggressive leukemia phenotype. These observations suggest that the CD82 scaffold may be a potential therapeutic target for attenuating aberrant signal transduction in AML.

Acute myeloid leukemia (AML), the most common acute leukemia affecting adults, is characterized by increased immature myeloid blasts within the bone marrow, which interferes with normal hematopoiesis¹. While an increasing number of chemotherapy drugs are being made available, AML remains a highly fatal disease due to its significant relapse rate following standard treatment². Modeling studies have demonstrated that the expression and activation of signaling molecules can be used to predict AML patient remission attainment, relapse, and survival³. For example, increased expression of the protein kinase C (PKC) isoform PKC α correlates with poor survival in AML patients⁴. Therefore, therapeutic targeting of specific aberrant signaling in AML may be used to treat this aggressive disease.

The PKC family of enzymes are serine/threonine kinases that can be further classified into conventional, novel, and atypical PKCs⁵. The conventional PKC isoforms include PKC α , β 1, β 2 and γ , all of which require Ca²⁺ and diacylglycerol (DAG) to become activated. Upon activation, PKC is initially phosphorylated within the cytoplasm and translocates to the plasma membrane following full phosphorylation. This translocation process is controlled by DAG production but may be bypassed with the use of the PKC activator, phorbol 12-myristate 13-acetate (PMA)⁶. PKC activation initiates various signaling responses such as the activation of Rac1, RhoA, and the mitogen activated protein kinases (MAPK) signaling cascades⁶⁻⁹. As such, PKC activation controls many basic cellular processes including adhesion, migration, and proliferation, which all contribute to cancer progression.

In AML patients, PKC α gene expression is upregulated when compared to CD34⁺ normal donors¹⁰. Furthermore, treating AML cell lines with the PKC inhibitor, enzastaurin, blocks the phosphorylation of PKC α and its downstream target, ERK, and also prevents PKC α membrane recruitment¹⁰. Additional work suggests that increased levels of phospho-PKC are correlated with increased AML cell viability¹¹. However, the molecules and mechanisms that control PKC activation and downstream signaling remain poorly defined.

Tetraspanins serve as molecular scaffolds within the plasma membrane to generate highly organized membrane domains, termed tetraspanin enriched microdomains (TEMs)^{12,13}. TEMs consist of interactions between tetraspanins and with other membrane proteins including integrins and signaling receptors such as the epidermal growth factor receptor (EGFR) and c-kit¹⁴⁻¹⁶. The maintenance of TEMs promote cellular functions including

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cell adhesion, migration, and proliferation^{17–19}. The palmitoylation of tetraspanins regulate TEM organization through the control of protein–protein interactions^{14,20,21}, which can in turn mediate cellular signaling. For example, expression of the palmitoylation deficient form of CD151 weakens tetraspanin association with integrins, resulting in diminished AKT phosphorylation in response to laminin-5 engagement¹⁴. Moreover, inhibition of CD81 palmitoylation reduced signaling in B cells, as assessed by PLC γ 2 and VAV phosphorylation²². Therefore, tetraspanin palmitoylation can control various aspects of cellular signaling.

In addition to membrane proteins, tetraspanins interact with cytosolic proteins such as the serine/threonine binding protein 14-3-3²³ and G protein subunits²⁴. Moreover, previous work established that CD151 assists in the recruitment of Rac1 to the plasma membrane, in addition to associating with PKC α ^{23–25}. Interestingly, tetraspanins CD9, CD81 and CD82 were shown to associate with PKC α upon PMA activation²⁶, and coimmunoprecipitation studies with CD9 and CD151 detected PKC α associations. In the present study, we focus on identifying how this tetraspanin association modulates PKC signaling, with a specific emphasis on CD82.

Although it has been demonstrated that many tetraspanins can interact with PKC α , we have chosen to focus on CD82 due to previous work demonstrating that CD82 is upregulated in several human leukemias, including AML²⁷. Recently, CD82 upregulation was identified in chemotherapy-resistant CD34⁺/CD38[–] AML cells²⁸, which are often the cells responsible for disease relapse. The objective of this study is to determine how the CD82 scaffold and its membrane organization regulate PKC α -mediated signaling and influence AML progression. Using a combination of single molecule and ensemble imaging techniques, we find that CD82 modulates the spatial and temporal dynamics of PKC α signaling in AML cells. Our data demonstrate that the molecular organization of CD82 regulates PKC α stabilization and clustering at the plasma membrane, which controls downstream ERK signaling and AML colony formation. Together, our findings suggest that CD82 organization may be a suitable target for controlling AML progression through its regulation of PKC α signaling.

Results

The CD82 scaffold regulates PKC α expression and activation. To identify how CD82 membrane scaffolding affects PKC α signaling, we generated KG1a AML cell lines stably overexpressing wild type CD82 (CD82OE) or a palmitoylation mutant form of CD82 (Palm-CD82OE) tagged to the mCherry fluorescent protein. In the palmitoylation mutant, five membrane proximal cysteine residues are mutated to serines, preventing CD82 palmitoylation (Fig. 1A)²⁹. We also generated CD82 knockdown KG1a cells (CD82KD) cells, where stable expression of CD82-specific shRNAs reduces total CD82 expression by 50% and surface levels by 95% (Fig. 1B,C). To quantify total and surface CD82 expression, we used flow cytometry analysis of permeabilized (Fig. 1B) and non-permeabilized cells, respectively (Fig. 1C). We also measured the surface expression of other tetraspanins in these cell lines, finding similar levels of CD9 in all cell lines (Suppl. Fig. 1A), and decreased levels of CD151 (Suppl. Fig. 1B) in the CD82KD cells compared to controls. Interestingly, we find decreased levels of surface (Suppl. Fig. 1C) and total (Suppl. Fig. 1D) CD81 in CD82KD and CD82OE cells compared to control cells. Two additional myeloid leukemia cell lines (K562 and U937) were engineered to overexpress WT-CD82 or Palm-CD82 (Suppl. Fig. 2A–E,I–M). In K562 cells, CD81 and CD151 expression is similar across the cell lines, while CD9 expression is increased in the CD82OE cells (Suppl. Fig. 2F–H). U937 cells also display increased CD9 expression in the CD82OE cells and an increase in both CD9 and CD81 in the Palm-CD82OE cells, whereas CD151 expression remains unchanged (Suppl. Fig. 2N–P). In combination, these data suggest that changes in CD82 expression can regulate the tetraspanin profile expressed in leukemic cells.

To analyze how CD82 scaffolding regulates the expression and activation of PKC α , we first quantified the expression levels of total and activated PKC α using Western blot analysis. (Data presented utilize KG1a cells with additional cell line analysis quantified in supplemental data). Figures 1D–F demonstrate that the CD82OE cells have a twofold increase in total PKC α expression and a 1.3-fold increase in phosphorylated (active) PKC α expression compared to control cells. In contrast, we find that the Palm-CD82OE cells express approximately 50% less total PKC α and 60% less phospho-PKC α when compared to control cells. Similar changes in PKC α expression and activation were identified in the U937 cells overexpressing CD82 (Suppl. Fig. 2Q–R), whereas the K562 cells only display an increase PKC α activation. Upon CD82KD in KG1a cells, we are unable to detect the expression of PKC α or its active form by Western blot (Fig. 1D–F). RT-PCR analysis of the cell lines measures a transcriptional down regulation of PKC α in CD82KD cells and no change in PKC α transcript between the control and CD82OE cells (Fig. 1G). Together, these data suggest a critical role for CD82 expression and membrane organization in regulating PKC α expression and activation in AML.

Upon full activation, PKC α translocates to the plasma membrane from the cytoplasm, which is essential for PKC α signaling. Using immunofluorescence imaging, we find that under resting conditions PKC α is primarily localized within the cytoplasm, whereas, upon PKC α activation with PMA for 1 hr, PKC α translocates to the plasma membrane (Fig. 1H). We also observe by line scan analysis that the intensity plots for the CD82 and PKC α channels have a similar shape under PMA stimulated conditions, suggesting that PKC α activation stimulates PKC α to move to CD82 membrane regions. These data illustrate that despite the CD82 palmitoylation mutation, PKC α effectively translocates to the plasma membrane upon activation.

Following activation, PKC α can be dephosphorylated and degraded in order to down-regulate PKC α -mediated signaling^{30–33}. Therefore, we assessed whether CD82 scaffolding preserves PKC α protein levels upon activation, thereby providing a sustained signal. Upon PMA stimulation for 1 or 4 hrs, we find that total and phospho-PKC α expression is maintained at a higher proportion in the CD82OE cells when compared to Palm-CD82OE cells (Fig. 1I). Next, we investigated if the reduced PKC α expression upon activation is due to proteasomal degradation. Combining 4 hrs of PMA with the proteasomal inhibitor, MG132, we find that PKC α expression is rescued to basal levels in control and Palm-CD82OE cells (Fig. 1J). Collectively, these data suggest that CD82 scaffolding can stabilize PKC α levels upon activation.

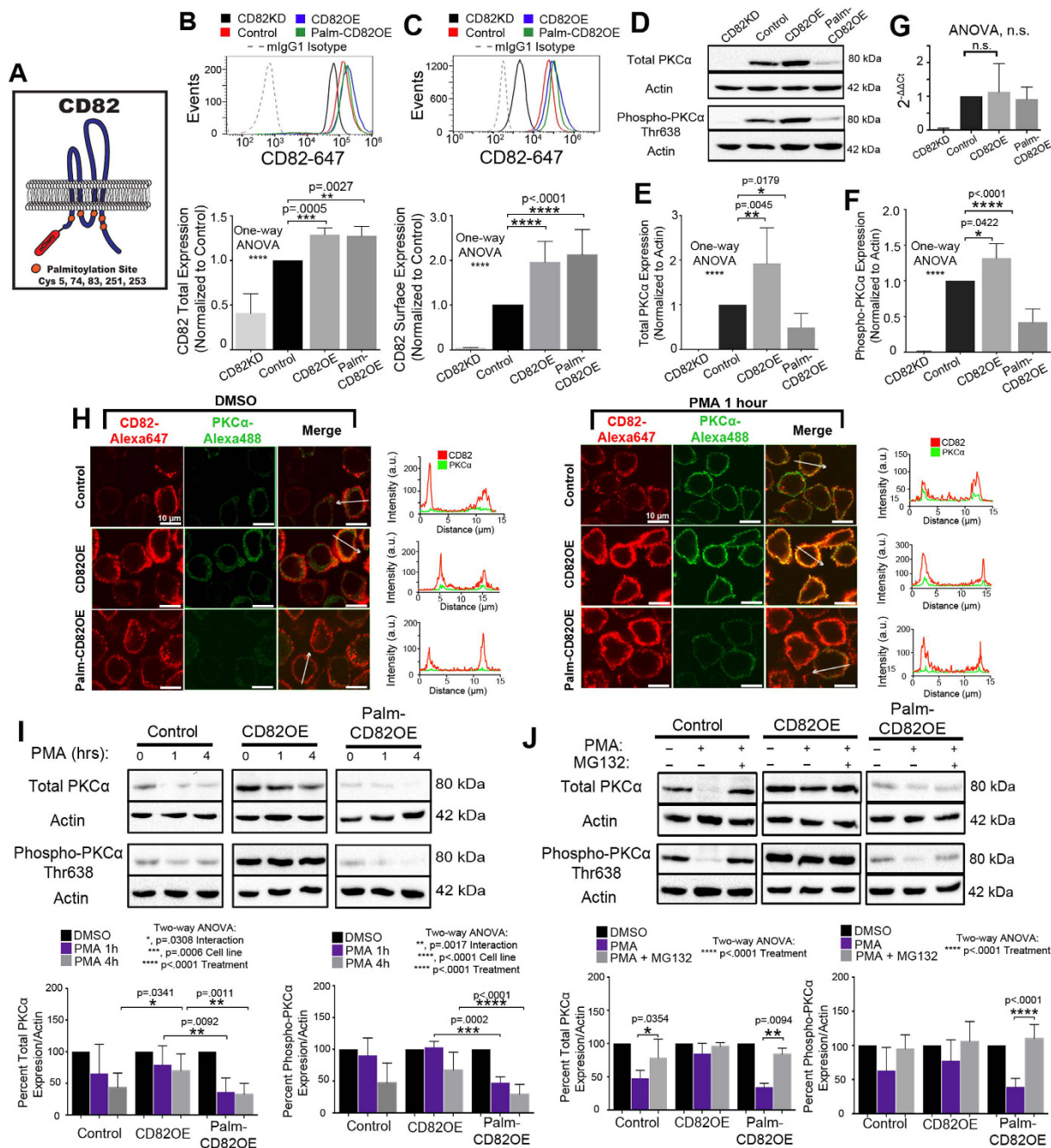


Figure 1. The CD82 scaffold regulates PKC α expression and activation. (A) Cartoon depicting mutated palmitoylation sites within CD82 and mCherry fusion. Flow cytometry analysis of (B) total and (C) surface CD82 expression using CD82KD, control, CD82OE, and Palm-CD82OE KG1a cells (Biologend, ASL-24). (n \geq 3 experiments; error bars indicate SD; mean fluorescence intensity normalized to control levels). (D) Western blot analysis for total PKC α (Cell Signaling #2056, polyclonal) and phospho-PKC α (Thr638) expression. Densitometric analysis of (E) total and (F) phosphorylated PKC α expression from Western blot analyses (n \geq 4 experiments; error bars indicate SD). (G) Real-time PCR analysis of KG1a cells. (H) Immunofluorescence imaging of CD82 (Biologend, ASL-24) and PKC α -488 (primary, abcam, Y124; secondary, Invitrogen, rabbit-488) under resting and 1 hr of PMA treatment with corresponding line scan plots for both channels. All channels were scaled equally across conditions. (I) Western blot analysis of total and phosphorylated PKC α expression following PMA stimulation (n \geq 4 experiments; error bars indicate SD). (J) Cells were treated with DMSO, PMA or PMA + MG132 (10 μ M) for 4 hrs and total and phospho-PKC α were quantified using Western blot analysis and densitometry. (n \geq 4 independent experiments; error bars indicate SD; post-hoc unpaired t-test).

The CD82 scaffold regulates short-term PKC α membrane association. One mechanism by which the CD82 scaffold could prolong PKC α activation is by stabilizing PKC α membrane recruitment. To visualize the

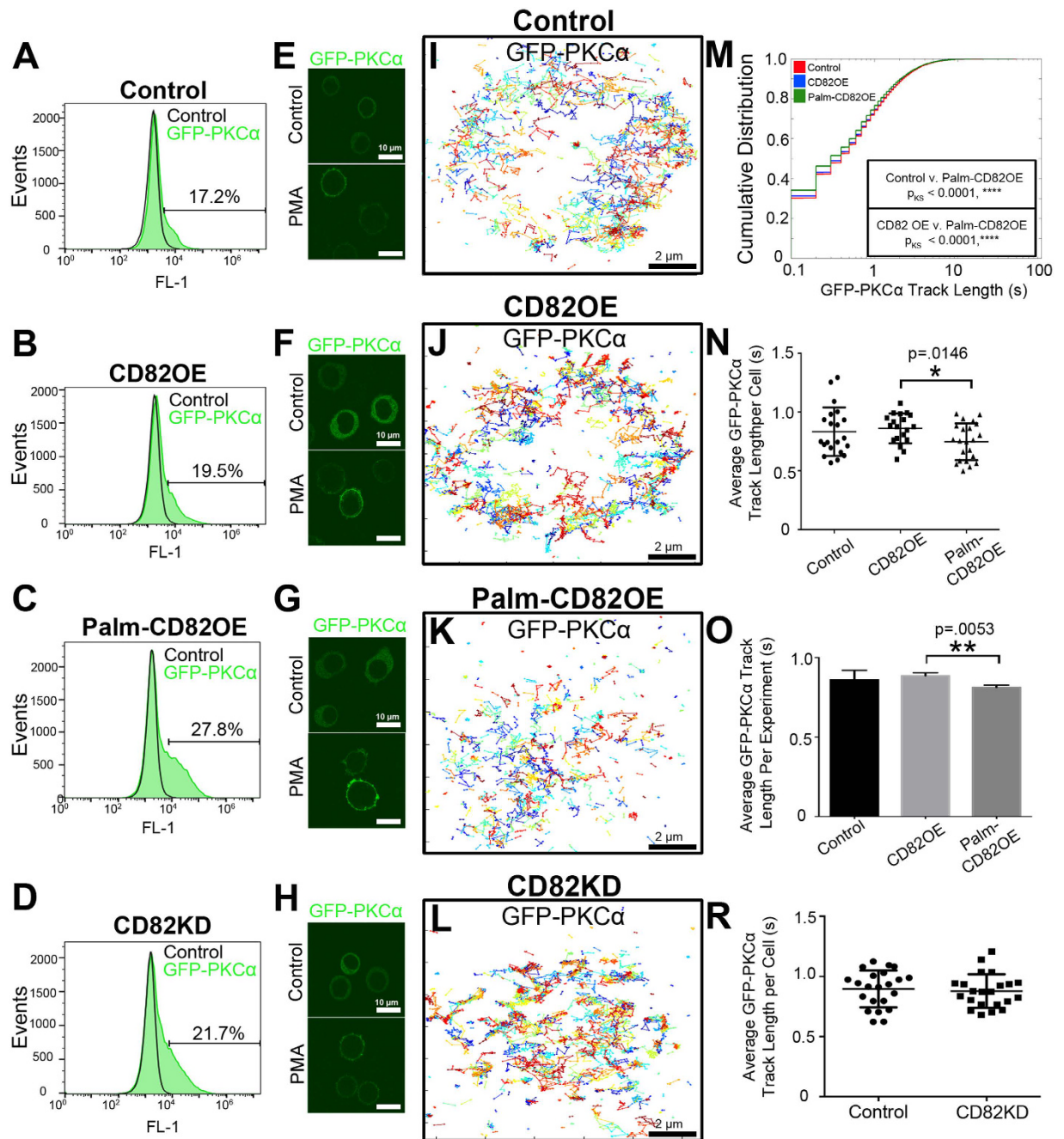


Figure 2. The CD82 scaffold regulates PKC α association with the membrane. (A–D) Flow cytometry analysis indicates the percentage of GFP-PKC α expression in transiently transfected cells. (E–H) Epifluorescence imaging of transfected cells showing GFP-PKC α localization +/- PMA. (I–L) PKC α trajectories from 600 frames of analyses are displayed. (M) Cumulative distribution plot of PKC α track length ($n \geq 31227$ tracks from $n \geq 19$ cells of each kind; the Kolmogorov-Smirnov test was used to compare cumulative distributions). (N) Average GFP-PKC α track length per cell and (O) per experiment (error bars indicate SD; $n \geq 19$ cells, $n = 3$ experiments; post-hoc unpaired t-test). (R) Average track length per cell was quantified in control and CD82KD cells (error bars indicate SD; $n = 22$ cells).

molecular recruitment of PKC α to the plasma membrane upon activation, we performed single particle tracking (SPT) analysis. Using transiently transfected GFP-PKC α cells (Fig. 2A–C) stimulated with PMA (Fig. 2E–G), we analyzed the membrane track length or “dwell time” of GFP-PKC α , which we define as the time between the membrane appearance and disappearance of GFP-PKC α . Figure 2I–K display representative GFP-PKC α trajectories, which were generated by filtering and connecting localizations with the parameters described in the Methods section. A cumulative distribution plot of the GFP-PKC α track lengths indicates that the Palm-CD82OE cells have an increased proportion of short-lived GFP-PKC α tracks compared to control or CD82OE cells, suggesting a

shortened PKC α dwell time (Fig. 2M). We also quantified PKC α dwell time based on the average track length per cell analyzed ($n \geq 19$ cells) (Fig. 2N) or per independently performed experiment ($n = 3$ experiments) (Fig. 2O), finding the same trend observed in our cumulative distribution plot. Interestingly, when analyzing GFP-PKC α dwell time in the CD82KD cells (Fig. 2D,H,L), we are unable to detect a change in track length (Fig. 2R), suggesting a potential compensatory scaffold function from other tetraspanins in the CD82KD cells, which may be inhibited by the palmitoylation mutant form of CD82. In combination, these data suggest that CD82 scaffolding has a modest effect on the initial membrane recruitment of PKC α .

PKC α is recruited and maintained at the CD82 scaffold upon stimulation. An extensive series of immunoprecipitation studies demonstrated that upon PMA stimulation, PKC α interacts with CD82²⁶, although little is known about the dynamics of this interaction. Our SPT analyses suggest that CD82 palmitoylation may regulate the membrane stabilization of PKC α on a short time scale. However, we are particularly interested in whether CD82 scaffolding can stabilize long-lived PKC α membrane interactions, which could potentiate prolonged signal transduction. Using Förster resonance energy transfer (FRET), we measured the recruitment and retention of PKC α relative to the CD82 scaffold over time. FRET was measured by quantifying fluorescence intensity changes in the donor fluorophore (GFP-PKC α) after the acceptor (mCherry-CD82) was photobleached. CD82OE and Palm-CD82OE cells transiently transfected with GFP-PKC α were imaged under resting conditions and upon PMA stimulation for 5 mins or 1 hr to assess both short and long-term PKC α recruitment, respectively. Under resting conditions, we detect minimal FRET between CD82 and PKC α in both the CD82OE and Palm-CD82OE cells, although the CD82OE cells have higher basal FRET than the Palm-CD82OE cells (Fig. 3A,B,G). Upon PMA stimulation, FRET is significantly increased in the CD82OE and Palm-CD82OE cells compared to resting cells (Fig. 3C,D,G), indicating that PKC α interacts with both the wild type and palmitoylation mutant form of CD82 upon activation. After of 1 hr of stimulation, we find that the increased FRET efficiency is maintained in the CD82OE cells, whereas the FRET is significantly reduced in the Palm-CD82OE cells over the same timeframe (Fig. 3E–G). These data suggest that disruption of the CD82 scaffold, in the case of the palmitoylation mutant, reduces the membrane association of PKC α with CD82. Together, these findings demonstrate that CD82 and PKC α have a prolonged membrane interaction that is hyperstabilized by overexpression of the CD82 scaffold.

PKC α clustering at the membrane is controlled by the CD82 scaffold. Tetraspanins can regulate the clustering of membrane proteins^{34–36}. Interestingly, PKC has also been shown to oligomerize³⁷ and aggregate upon activation³⁸. Therefore, we next wanted to determine how altered interactions between CD82 and PKC α described in our FRET studies could modulate PKC α clustering. Using the super-resolution imaging (SRI) technique, direct stochastic optical reconstruction microscopy (dSTORM), we resolved the molecular landscape of PKC α in control, CD82OE and Palm-CD82OE cells stimulated with PMA for 5 mins or 1 hr. The organization of signaling proteins into clusters may stabilize signaling by providing steric protection from negative regulators³⁹. Therefore, we applied the density-based spatial clustering of applications with noise (DBSCAN) algorithm⁴⁰ to our SRI data to quantify PKC α clustering (Fig. 4A). Under resting conditions, we detect a similar number of PKC α clusters between control and CD82OE cells, whereas the Palm-CD82OE cells display a significantly reduced number of PKC α clusters compared to control and CD82OE cells (Fig. 4B). Next, upon PMA stimulation for 5 min or 1 hr, we again measure no significant change in the number of PKC α clusters in either the control or CD82OE cells. However, in the Palm-CD82OE cells, PMA stimulation results in a significant increase in PKC α cluster number (Fig. 4B). In fact, upon PMA stimulation for 1 hr, the control, CD82OE and Palm-CD82OE cells all exhibit similar numbers of PKC α clusters (Fig. 4B). These data suggest that while Palm-CD82OE cells have reduced PKC α clusters under basal conditions, PMA treatment stimulates a similar number of PKC α clusters in all cells.

It has been previously suggested that the size of signaling molecule clusters is predicted to have a significant impact on signal transduction³⁹. Therefore, we next addressed how CD82 scaffolding affects PKC α cluster size. Further analysis of the DBSCAN data indicates that under resting conditions, PKC α cluster diameter is similar between control and CD82OE cells, but is reduced in the Palm-CD82OE cells (Fig. 4C). Upon PMA stimulation for 5 min, only the CD82OE cells exhibit an increase in PKC α cluster area. However, upon PMA stimulation for 1 hr, all the cells increase their PKC α cluster size with the CD82OE promoting even larger “superclusters”⁴¹.

Additionally, we assessed how CD82 scaffolding modulates PKC α molecular density, or the number of PKC α localizations found per cluster area, since this is another mechanism by which PKC α may be recruited into clusters upon activation. Our data demonstrate that upon PMA stimulation for 5 mins, control cells display increased PKC α molecular density compared to resting conditions (Fig. 4D). Meanwhile, the other cell lines exhibit similar PKC α molecular density upon resting or stimulated conditions. These data suggest that CD82 concentration affects the means by which PKC α is initially recruited to the membrane. More specifically, in the case of the control cells, a lower concentration of CD82 results in PKC α recruitment into densely packed clusters initially (5 m PMA) rather than larger-order superclusters as observed with CD82 overexpression. However, prolonged activation (1 hr PMA) continues to increase the PKC α molecules recruited to the membrane, ultimately leading to increased PKC α cluster sizes (Fig. 4B). These data demonstrate that CD82 concentration and scaffolding properties regulate unique aspects of PKC α membrane clustering.

We also assessed PKC α clustering in the CD82KD cells by transiently transfecting in GFP-PKC α and performing SRI analyses (Fig. 4E). Upon PMA stimulation for 1 hr, we measure an increase in the number of PKC α clusters, consistent with PKC α membrane translocation (Fig. 4F). However, in contrast to the other cell lines, PKC α cluster area remains unchanged in the CD82KD cells following PMA activation (Fig. 4G), suggesting that the CD82 scaffold is necessary to promote or stabilize the larger PKC α clusters measured following PMA stimulation. Combined, these data demonstrate that CD82 scaffolding significantly impacts PKC α cluster size.

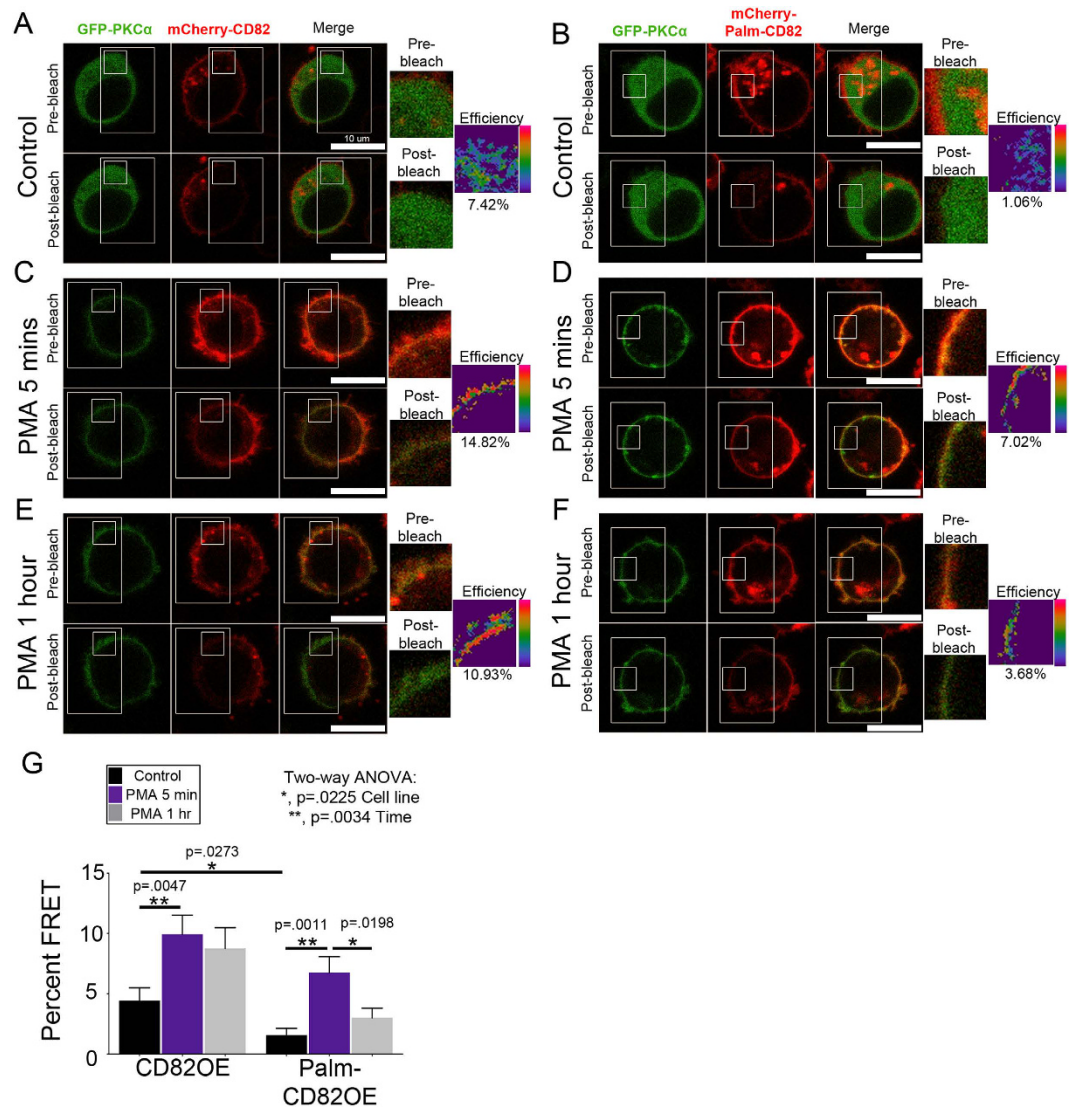


Figure 3. PKC α is stabilized by the CD82 scaffold. CD82OE or Palm-CD82OE KG1a cells were transfected with GFP-PKC α and imaged under (A,B) resting or upon PMA stimulation for (C,D) 5 mins or (E,F) 1 hr. (G) Percent FRET efficiencies were calculated in a region of interest per cell. ($n=4$ experiments, $n \geq 21$ cells per treatment, error bars indicate SEM, post-hoc unpaired t-test).

CD82 modulates ERK1/2 activity downstream of PKC α stimulation. The ability of PKC α to propagate a signal is dependent upon activation and sufficient membrane recruitment, which allows PKC α to phosphorylate a substrate and elicit a downstream response. Our findings suggest that CD82 stabilizes PKC α at the plasma membrane and promotes larger-scale clustering. We next examined how this stabilization and clustering affects PKC α -mediated signal propagation. One pathway that has been studied extensively with respect to PKC α is the MAPK pathway. Incidentally, it has been shown that MAPK can be constitutively active in leukemias and targeting this activation can help to promote AML blast susceptibility to apoptosis⁴². To determine how CD82 scaffolding affects PKC α -mediated signaling through MAPK, we stimulated cells with PMA and monitored p38 and ERK1/2 activation. Western blot analysis indicates that p38 expression and activation remain unchanged following PMA stimulation in all cell lines (Fig. 5A). Moreover, we find no change in total ERK1/2 expression between the cells (Fig. 5B,C) and detect only minimal phospho-ERK1/2 expression in unstimulated cells (Fig. 5D,E). However, upon PMA stimulation, phospho-ERK1/2 expression varies substantially between the cells. We find that there is increased phospho-ERK1/2 expression in the CD82OE cells compared to control and Palm-CD82OE cells upon 15 mins of PMA stimulation (Fig. 5D–F). Interestingly, the CD82OE cells maintain significantly higher phospho-ERK expression upon 1 hr of PMA stimulation compared to Palm-CD82OE cells (Fig. 5F). Together, these data demonstrate that CD82 scaffolding is critical for regulating the signaling kinetics of ERK1/2 downstream of PKC α activation.

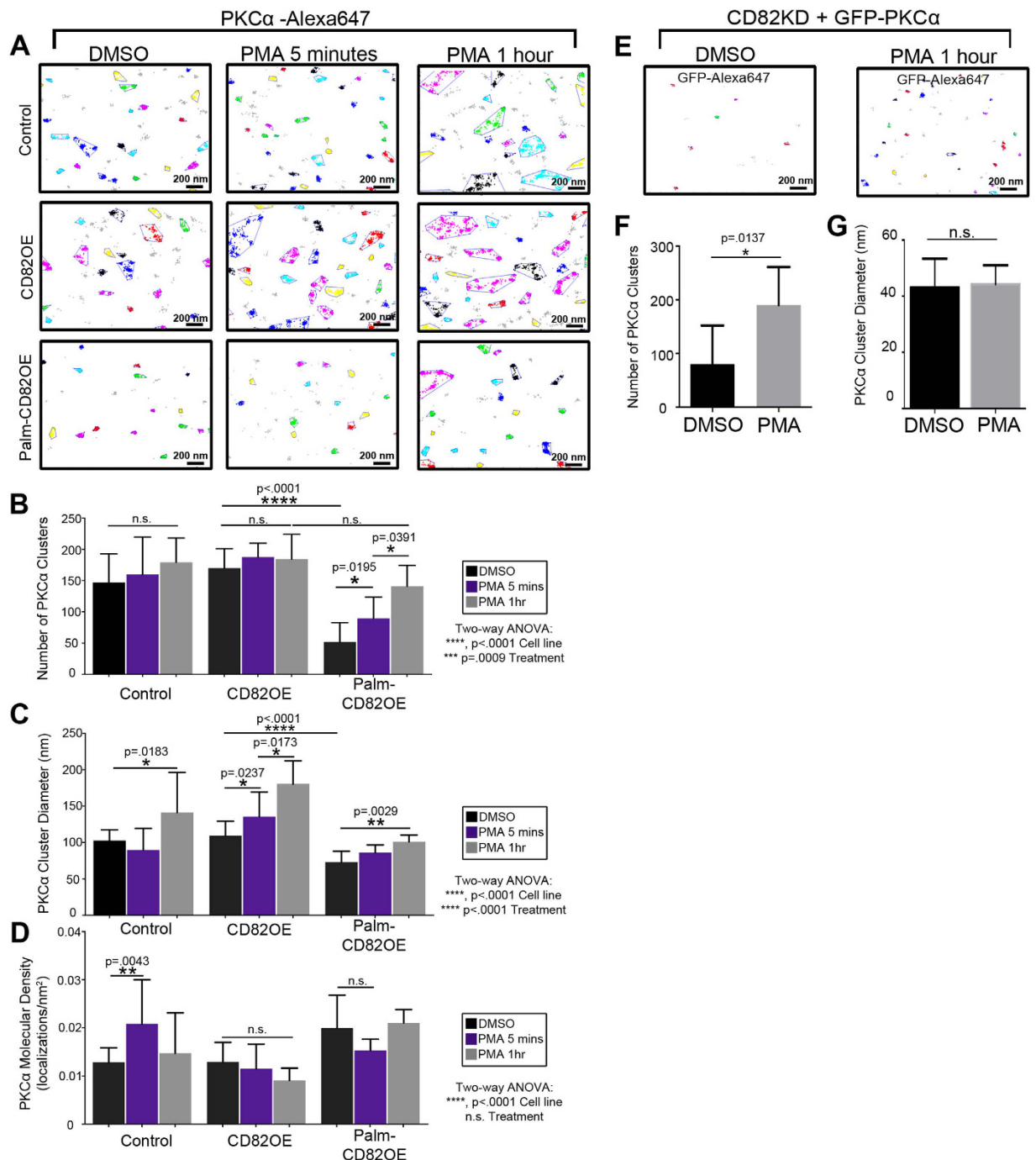


Figure 4. PKC α clustering at the membrane is controlled by the CD82 scaffold. Control, CD82OE and Palm-CD82OE KG1a cells were treated with DMSO, or PMA for 5 mins or 1 hr and imaged for PKC α (abcam, Y124; Invitrogen, rabbit-647) using dSTORM. (A) The DBSCAN algorithm was used to examine cluster organization within a subregion of the cells. Clustered localizations are indicated by color, whereas gray localizations did not meet the clustering parameters ($\epsilon = 50$ nm, $n = 30$ localizations). The DBSCAN algorithm was used to determine the (B) number of PKC α clusters, (C) PKC α equivalent cluster diameter, and (D) PKC α molecular density ($n \geq 4$ cells of each condition, error bars indicate SD, post-hoc unpaired t-test). CD82KD cells were transfected with GFP-PKC α and imaged using dSTORM. (E) PKC α clustering was quantified using the DBSCAN clustering algorithm in cells treated with DMSO or PMA ($\epsilon = 50$ nm, $n = 10$ localizations). (F) The number of clusters ($n = 7$ cells, error bars indicate SD) and (G) the cluster diameters were quantified ($n \geq 151$ clusters, error bars indicate SEM, unpaired t-test).

CD82 regulates AML colony formation in a PKC α -dependent manner. Finally, we wanted to determine how PKC α activation and ERK signaling affect the leukemia colony forming potential of AML cells. We

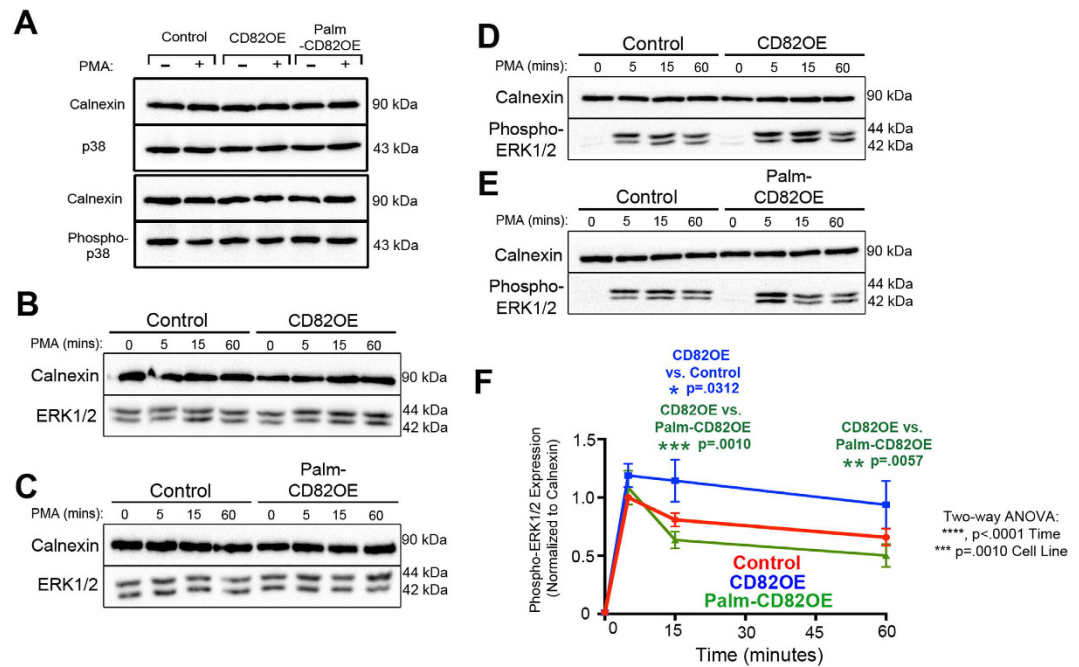


Figure 5. CD82 modulates ERK1/2 activity downstream of PKC α stimulation. (A) Control, CD82OE and Palm-CD82OE cells were treated with DMSO or PMA for 1 hr and analyzed by Western blot analysis for total (D13E1) and phospho-p38 (Thr180/Tyr182). Representative Western blot showing control and (B) CD82OE cells or (C) Palm-CD82OE cells treated with PMA for 0, 5, 15, or 60 mins and analyzed for total ERK1/2 (137F5) expression. Representative Western blot depicting (D) control and CD82OE or (E) Palm-CD82OE cells treated with PMA for 0, 5, 15, or 60 mins and analyzed for phospho-ERK1/2 (Thr202/Thr204) expression. (F) Graphical depiction of phospho-ERK expression over time quantified by Western blot analysis. (n \geq 4 experiments, error bars depict SEM; post-hoc unpaired t-test).

treated cells with DMSO, PMA, or PMA in combination with the ERK1/2 inhibitor, FR180204. Cells were then plated in MethoCult H4334 media for 14 days, after which, the leukemia colony-forming units (CFU-L) were counted via microscopy. Following PMA treatment, we find that all cells increase CFU-L formation with the CD82OE cells displaying more than four times as many colonies compared to control and Palm-CD82OE cells (Fig. 6A,B). Interestingly, the combined treatment of PMA and ERK1/2 inhibitor significantly reduced colony growth in all cell lines. These data indicate that the CD82- and PMA-mediated increases in CFU-L formation are dependent upon ERK1/2 signaling. Collectively from these data, we suggest the current model (Fig. 6C) where the CD82 scaffold recruits and stabilizes PKC α in membrane clusters, which can sustain ERK1/2 signaling for the development of an aggressive leukemia phenotype.

Discussion

In this study, we provide new insights into how tetraspanins can serve as membrane scaffolds that control signal transduction in AML. As PKC α is a critical signaling hub for controlling AML cell proliferation and survival³, we focused on identifying the properties of tetraspanins that contribute to aberrant PKC α signaling. Numerous studies defined an interaction between PKC α and tetraspanins, but the mechanisms regulating this association and the downstream signaling consequences remain poorly understood. Our study describes a role for CD82 membrane organization in regulating PKC α expression, membrane stabilization and signaling.

Increased phospho-PKC α expression has been correlated with poor survival rates in AML patients⁴ and increased AML cell viability¹¹. Consistent with previous findings⁴³, data from our study demonstrate that the overexpression of CD82 increases total and phospho-PKC α expression (Fig. 1D–F). Upon mutation of the palmitoylation sites within CD82, we detect decreased total and phospho-PKC α expression (Fig. 1D–F) when compared to control or CD82OE cells, suggesting that the scaffolding properties of CD82 modulate PKC α expression. Previous studies⁴³ observed a similar decrease in PKC α expression upon knock down of CD82, further supporting the importance of the CD82 scaffold for maintaining PKC α expression. Similarly, we detect a reduction in PKC α expression upon CD82KD in our study, which we link to reduced PKC α transcript levels. At this point, the mechanism behind this reduction in PKC α mRNA in the CD82KD cells remains undefined. In contrast, we detect similar PKC α transcript levels between the control, CD82OE and Palm-CD82OE cells (Fig. 1G). As palmitoylation has been demonstrated to regulate TEM organization^{14,44}, we speculate that TEM disruption by CD82 mutation renders PKC α more susceptible for degradation.

Interestingly, another difference identified between the CD82OE and Palm-CD82OE cells is the expression of CD81, which has been shown to interact with PKC α ²⁶ and is significantly reduced in the CD82OE cells (Suppl. Fig. 1C,D). We speculate that compensatory roles of CD81 and CD82 within TEMs may result in the decreased

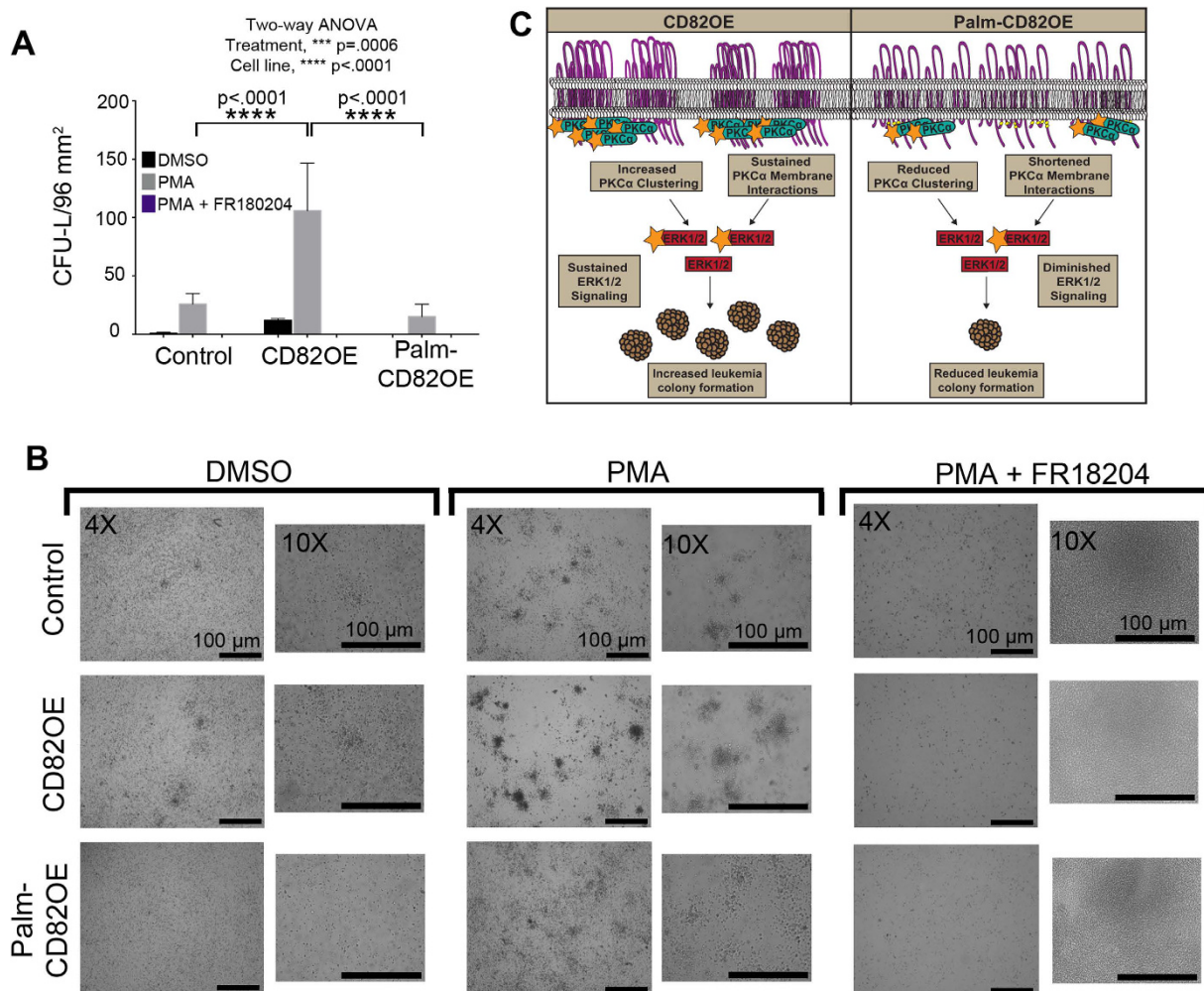


Figure 6. CD82 regulates AML colony formation in a PKC α -dependent manner. (A,B) Control, CD82OE and Palm-CD82OE cells grown in clonogenic assays in the presence of PMA alone (10 ng/ml), or PMA + FR180204 (100 μ M), or equal volumes of DMSO and assessed after 14 days by microscopy for the number of leukemia colony-forming units per 96 mm² ($n \geq 4$ experiments, error bars indicate SD). (C) Proposed model whereby the scaffolding function of CD82 regulates the membrane clustering and stabilization of PKC α , which controls ERK1/2 signaling and AML colony forming potential.

CD81 expression in the CD82OE cells. Perhaps to maintain the appropriate tetraspanin concentration, scaffold structure, or functional TEM signaling, CD81 is down regulated in response to the overexpression of CD82. However, in the Palm-CD82OE cells, which have disrupted TEMs³⁶, CD81 expression may be maintained in an effort to conserve the functionality of these domains. Additionally, since CD81 is a PKC interacting protein, its expression may be maintained in the Palm-CD82OE cells in an attempt to preserve PKC α membrane recruitment and signaling under conditions where PKC α expression is diminished.

A number of previous studies have proposed that tetraspanins serve as protein recruitment platforms. For example, the presence of CD82 was shown to enhance the PKC α phosphorylation of c-Cbl following HB-EGF activation, which led the authors to suggest that CD82 could in fact serve to recruit PKC α ⁴⁵. Additionally, a described role for CD151 was to recruit PKC α into proximity with the $\alpha 6 \beta 4$ integrin, which significantly impacted tumor initiation and progression⁴⁶. Our SPT data suggest a decrease in PKC α membrane dwell time in the Palm-CD82OE cells (Fig. 2M–O), indicating that disruption of the CD82 scaffold organization may shorten PKC α membrane interactions. Our PKC α tracking experiments used GFP, which has a relatively short fluorescence lifetime. As such, we detect sub-second PKC α track lengths, which may contribute to the modest change seen in PKC α track length in the Palm-CD82OE cells. More pronounced track length differences might be observed with the use of a more stable tracking probe. Additionally, the differential CD81 expression observed in the cell lines may mask a more substantial change in PKC α membrane stabilization as detected with SPT. Despite experimental limitations, our data suggest that CD82 organization alters the interaction of PKC α at the membrane.

Biochemical characterization of tetraspanins suggests that PKC α and PI4K may have distinct tetraspanin recruitment sites, indicating the potential for differential recruitment of signaling enzymes to specific

tetraspanins²⁶. One possible explanation for how decreased PKC α dwell time could occur is through diminished interactions with the CD82 signaling platform. Our FRET analyses (Fig. 3) indicate that PKC α interacts with both wild type and Palm-CD82, demonstrating that CD82 palmitoylation is not essential for the interaction to occur. However, following 1 hr of PMA stimulation, the FRET efficiency between PKC α and CD82 is sustained, while it is significantly diminished in the Palm-CD82OE cells. These data suggest that CD82 scaffolding contributes to the long-lived protein interactions between PKC α and CD82 at the membrane. Interestingly, PKC α can also be palmitoylated, which was shown to facilitate its membrane recruitment⁴⁷. Therefore, future studies may be directed at understanding how PKC α palmitoylation contributes to the robust membrane interaction between tetraspanins and PKC α .

While tetraspanins have been described to regulate membrane protein clustering^{34–36}, this study explores how tetraspanins modulate cytosolic protein clustering. Previous work has demonstrated that the number and size of Ras clusters contributes to its downstream signaling response^{48,49}. Moreover, increased expression of galectin-1, a Ras membrane scaffold, can enhance Ras-mediated signaling⁵⁰. The current study has uncovered a role for CD82 in regulating PKC α oligomerization, a concept that was hypothesized to have physiological signaling consequences³⁷. By taking advantage of the newly developed SRI techniques, we provide visual and quantitative evidence at the nanometer scale for how tetraspanins regulate the spatial arrangement of cytosolic proteins. First, our data suggest that CD82 scaffolding can modulate PKC α cluster number. In the Palm-CD82OE cells, we detect reduced PKC α clusters until PMA stimulation for 1 hr, which results in a similar number of clusters in all cells (Fig. 4B). These data suggest that a limited number of PKC α membrane recruitment sites exist and that PKC α cluster number is dependent upon both the scaffolding of CD82 as well as the concentration of PKC α .

Additionally, our data implicate CD82 expression and scaffolding as regulators of PKC α cluster size. For example, CD82OE cells support PKC α cluster diameters that are approximately 30% and 80% larger than those in control and Palm-CD82OE cells, respectively (Fig. 4C). It is important to take into account the ratio of surface CD82 to PKC α in our cell lines for interpretation of these data. By setting the control cells at a 1/1 ratio of CD82/PKC α , the CD82OE cells have a ratio of 2/2, while the Palm-CD82OE cells have a ratio of 2/0.5. We suggest that the additional CD82 expression in CD82OE cells creates a larger platform to enhance the size of PKC α clusters formed at the membrane. Despite the fact that the Palm-CD82OE cells have the same concentration of CD82, the scaffolding capacity is disrupted and the PKC α concentration is reduced, resulting in smaller PKC α clusters observed. Therefore, it appears that the concentration of CD82 and its scaffold organization work in concert to provide a membrane platform to regulate the recruitment, stability, and cluster size of PKC α .

Aberrant activation of the ERK pathway is implicated in AML progression⁵¹. Previous studies have shown that inhibiting MAPK signaling in AML can lead to increased apoptosis and reduced proliferation^{52–55}. Additional studies have shown that the treatment of lymphoid cells with CD81 and CD9 antibodies modulate proliferation through alterations in the ERK1/2/MAPK pathway^{12,56,57}. Our data indicate that increased expression of CD82 results in a robust and sustained activation of ERK1/2 upon PMA stimulation that is maintained out to 1 hr (Fig. 5F). However, in the Palm-CD82OE cells, the ERK1/2 activation is abrogated to approximately 50% of the CD82OE response at 1 hr following PMA stimulation. We postulate that the sustained levels of activated PKC α in the CD82OE cells (Fig. 11) serve to stimulate and maintain the activation of ERK1/2. Conversely, we suggest that the reduced levels of PKC α seen in the Palm-CD82OE cells upon activation (Fig. 11) leads to a quick turnover in ERK1/2 signaling. It has been hypothesized that membrane clustering of signaling molecules can regulate signal transduction, with smaller, short-lived “nanoclusters” responsible for rapid signaling and larger “microclusters” promoting sustained signal transduction³⁹. Our findings are consistent with this notion, demonstrating that increased PKC α “microcluster” formation seen in the CD82OE cells (Fig. 4C) correlates with sustained ERK1/2 signaling. Our findings suggest that CD82 scaffolding primarily affects the long-lived phase of ERK signaling, which further implicates that the CD82-mediated effects on the spatial and temporal dynamics of PKC α can significantly impact the prolonged downstream ERK1/2 effector signaling.

ERK activity has been linked to cell proliferation and leukemia chemoresistance^{58,59}. Additionally, CD82 expression was shown to be increased in the chemotherapy-resistant CD34⁺/CD38[−] cells in AML²⁸. Our leukemia colony-forming unit assays indicate that CD82OE cells form significantly more AML colonies when compared to control or Palm-CD82OE cells, suggesting that CD82OE cells have a colony forming advantage independent of PKC α stimulation. Interestingly, following PMA treatment, CD82OE cells generate an even greater increase in leukemia CFU formation, indicating that PKC α activation and downstream signaling regulate the aggressiveness of AML. Moreover, we measure a significant reduction in CFU-L formation upon treatment with the ERK1/2 inhibitor FR180205, suggesting that both CD82- and PMA-mediated colony formation are dependent on ERK1/2 signaling. Together, these data suggest that targeting the CD82 scaffold may provide an alternative route towards regulating PKC α and its downstream signaling response in AML. Tetraspanins are already being used in clinical trials for the treatment of chronic lymphocytic leukemia⁶⁰. Therefore, the ability to specifically disrupt the CD82 membrane organization, where aberrant signaling can be initiated and sustained, may represent a novel approach to the treatment of AML.

Methods

Cell Culture. The KG1a, K562 and U937 cell lines (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10%FBS, 2 mM l-glutamine, 100 u/ml penicillin, and 100 μ g/ml streptomycin. Cells were incubated at 37 °C, 95% humidity, and 5%CO₂. For stimulation experiments, cells were treated with 10 ng/ml of PMA alone (Sigma), or combined with FR180204 (Tocris) at 100 μ M or equivalent volumes of DMSO.

Plasmids/Cell Line Generation. The mCherry-CD82 and mCherry-Palm-CD82 plasmids were constructed as previously described³⁶. Cells were nucleofected with the aforementioned plasmids or the mCherry-C1 plasmid (Invitrogen) and then sorted for mCherry expressing cells using fluorescence activated cell sorting at the

Flow Cytometry Facility, UNMHSC to generate a stable pool and kept under selection using 500 µg/ml of G418. Stable CD82 knockdown was established using KG1a cells transfected with the CD82 shRNA plasmid (Santa Cruz Biotechnology, sc-35734-SH); cells were put under puromycin selection for 4 weeks and sorted for negative CD82 surface expression. The GFP-PKC α plasmid, cloned in the pEGFP-N3 vector, was generously provided by Dr. Yousuf Hannun from Stony Brook University, Stony Brook, NY. Cells were transiently nucleofected with GFP-PKC α according to the manufacturer's protocol (Amaxa, Lonza Group).

Western Blotting. Western blots were performed as previously described³⁶. Antibodies used for Western blotting were purchased from Cell Signaling Technology as follows: calnexin (C5C9), PKC α (#2056, polyclonal), phospho-PKC α (Thr638), ERK1/ERK2 (137F5), phospho-ERK1/ERK2 (Thr202/Thr204), p38 (D13E1), phospho-p38 (Thr180/Tyr182), or β -Actin (Sigma, AC-74); all antibodies were used at a 1:1000 dilution. Horseradish peroxidase conjugate enzymes were stimulated with SuperSignal West Pico Chemiluminescent Substrate or Femto Maximum Sensitivity Substrate (Life Technologies). Blots were imaged using the ChemiDoc XRS Imager (Bio-Rad) and analyzed using ImageJ (National Institutes of Health) densitometry software.

Flow Cytometry. For surface expression, cells were labeled with antibody or the corresponding isotype control in 1%BSA/PBS for 30 mins on ice. For total expression, cells were fixed with 4% paraformaldehyde and blocked with 1%BSA/PBS/0.2%Tween for 1 hr before labeling. Cells were washed 3 times and analyzed using an Acuri C6 flow cytometer; histograms were generated using FlowJo software. Mean fluorescence values were normalized to the "control" cell line level. Antibodies used were CD82-647 (Biolegend, ASL-24), CD81-FITC (Biolegend, 5A6), CD151-PE (BD Biosciences, 14A2.H1), and CD9-647 (Bio-Rad, MM2/57).

Real-time PCR. The TRIzol Reagent protocol was used to isolate total RNA; cDNA was synthesized using qScript cDNA SuperMix protocol. Fast SYBR Green Master Mix was used for PCR reaction. The following primers were used for amplification: PKC α forward: 5' ATC CGC AGTGGG ATG AGT CCT TTA CAT 3', PKC α reverse: 5' TTG GAA GGT TGT TTC CTG TCT TCA GAG 3', GAPDH forward: 5'-GTCGGTGTCAACGGATTT-3', human GAPDH reverse: 5'-ACTCCACGACGTACTGAGC-3'. The PCR plate was read using the 7500 Fast Real-Time PCR System (Applied Biosystems). The Ct value from the sample was normalized to the expression of GAPDH. Expression values were averaged from three independent experiments and expression level changes were calculated using the $2^{-\Delta\Delta CT}$ method.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde and then blocked/permeabilized with 1%BSA/PBS/0.2%Tween. Cells were then incubated with primary antibodies (CD82-Alexa647, 1:125, Biolegend ASL-24; PKC α , 1:200, abcam, Y124). Cells were then labeled with a rabbit-Alexa488 secondary antibody (1:200, Invitrogen). Cells were imaged by laser scanning confocal microscopy with a Zeiss Axiovert 100 M inverted microscope (LSM 510) system (Carl Zeiss, Jena, Germany) using an excitation wavelength of 488 or 633 nm and a 63X/1.2 numerical aperture oil immersion objective. Image analysis was performed using the Zeiss LSM 510 software.

Super-Resolution Microscopy. Cells were plated on chamber slide wells that were treated with fibronectin (25 µg/ml, Millipore). Cells were fixed with 4% paraformaldehyde and blocked/permeabilized (1%BSA/PBS/0.2%Tween). Cells were labeled with an anti-PKC α antibody (1:200, abcam, Y124), washed, and incubated with a goat anti-rabbit AlexaFluor647 secondary antibody (1:200; Invitrogen). Cells transfected with GFP-PKC α were labeled with an anti-GFP Alexa647 antibody (Biolegend, FM264G). Cells were washed and post-label fixed with 4% paraformaldehyde. Cells were washed and imaged in dSTORM imaging buffer consisting of 50 mM Tris, 10 mM NaCl, 10% w/v glucose, 168.8 u/ml glucose oxidase (Sigma #G2133), 1404.0 U/ml catalase (Sigma #C9332), and 50 mM MEA, pH8.5. Red reference beads were used to stabilize the sample during imaging; drift corrections were performed using MCL NanoDrive stage controller (Mad City Labs, Nano-CLP100). The sample was imaged for 10,000 frames using a custom TIRF microscope system as described previously⁶¹ that uses an inverted microscope (IX71, Olympus America Inc.). A 637 nm laser (HL63133DG, Thorlabs) is coupled along with a 405 nm laser (Crystal laser), into two mode fibers and focused onto the objective lens with a 1.45 NA (UAPON 150XTIRE, Olympus America, Inc.) for data acquisition. For imaging, emission light was filtered using bandpass filter (FF01-692/40-25, Semrock) and data was collected on an electron-multiplying charge-coupled device (EMCCD) Camera (iXon 897; Andor Technologies, South Windsor, CT). Pixel size was 106.7 nm. Images were acquired at ~20 ms (50 frames/second) for a 256 × 256 pixel region. All of the instrumentation is controlled by custom-written software in Matlab (MathWorks Inc.). For one color imaging, the 637 nm and 405 nm lasers were used concurrently. The 561 nm laser was used for bead stabilization.

Data collected was then analyzed using a method previously described, where the pixel values are converted to photon counts and a 2D localization algorithm is used to determine the x and y positions of emitters, total photon counts, and the background photon counts⁶². The localized emitters were then put through a series of thresholds of various fitting parameters. The fitting parameters used are maximum background photons = 80 and minimum photons per frame per emitter = 500.

The SuperCluster Matlab software (<http://stmc.health.unm.edu/tools-and-data/index.html>) was used for SRI cluster analysis using the DBSCAN module; a 6 µm × 6 µm region of a cell was analyzed with DBSCAN which outputs the number of clusters and their corresponding areas. Clusters in Fig. 4A–D were determined as having at least 30 localizations within a 50 nm search radius, while clusters in Fig. 4E–G required 10 localizations. Areas were calculated using a convex hull around all points identified as a cluster. Equivalent diameter of a cluster was calculated as $\text{area} = \pi \cdot R^2$. Molecular density is calculated as the number of localizations in a cluster divided by the cluster area.

Förster resonance energy transfer (FRET). Stable KG1a cells were transfected with GFP-PKC α and plated on 25 $\mu\text{g}/\mu\text{l}$ of fibronectin overnight. Cells were imaged using the Leica SP8 System using a 63X water objective equipped with an objective heater which maintained samples as 34 °C throughout imaging. The excitation light source was a white-light laser system set at 488 nm (GFP) and 561 nm (mCherry). Fluorescence from the 488 nm channel was collected using a HyD1 detector and fluorescence from the 561 nm channel was collected using the HyD SMD2 in standard mode. Photobleaching was performed at 100% 561 nm laser power for 5 frames. GFP and mCherry levels in cells outside of the field of bleaching demonstrate that inherent photobleaching did not play a significant role in reducing GFP or mCherry fluorescence over the course of imaging (Supplemental Fig. 4). FRET efficiencies were calculated using the formula: $\text{Efficiency} = (\text{Donor}_{\text{post-bleach}} - \text{Donor}_{\text{pre-bleach}}) / \text{Donor}_{\text{post-bleach}}$ where D is the fluorescence intensity in a plasma membrane region of interest of fixed shape and size (3×7 ellipse). Analysis was performed using the Leica Application Suite AF Lite software.

Single Particle Tracking (SPT). SPT was performed using the TIRF microscope optical setup as described in the “Super-resolution Microscopy” section. A 488 nm laser (Cyan Scientific; Spectra-Physics) was used for GFP excitation. The sample emission light was detected using an EMCCD camera (iXon 897; Andor Technologies). 500 frames per cell were acquired at 20 frames/sec. An objective heater maintained samples as 34 °C throughout imaging.

SPT data processing was performed as described previously⁶³. The algorithm first finds box centers from raw data, and then fits these centers to determine the location of single particles. The localizations are then filtered and trajectories are built by connecting localizations. The minimum number of photons to threshold a box was 1.5 photons. Once boxes were determined, the box region size used to determine the localization of single molecules was 7 pixels. In order to filter localizations, the minimum number of photons to consider a localization was 20 photons, while the minimum distance between localized fits was 3 pixels. The maximum number of pixels to search for connections was 8 pixels in x or y. The maximum number of frame gaps to search for connections was 5 frames. The minimum track length to consider valid before gap closing assignments was 2 frames.

Leukemia Colony-Forming Unit Assay. 100,000 KG1a cells were treated with PMA (10 ng/ml) alone, PMA + FR180204 (100 μM) or equivalent volumes of DMSO. Cells were plated in MethoCult H4434 Classic Medium and allowed to grow for 14 days and then leukemia colony forming units (>30 cells) were counted.

Statistics. Statistical analyses were performed using GraphPad Prism 6 software. For multiple comparisons, one or two-way ANOVA was performed, followed by a Bonferroni multiple comparison analysis. Post-hoc unpaired t-tests were performed as referenced, using Welch’s correction if variances were unequal. Alpha = .05 in all analyses. The Kolmogorov-Smirnov test was used to compare cumulative distributions. (*<0.05, **<0.01, ***<0.001, ****<0.0001).

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Author Contributions

C.M.T. and J.M.G. wrote the manuscript. C.M.T. performed experiments, analysis and prepared figures. C.M.T. and J.M.G. conceived experiments. K.A.L. assisted with data analysis. All authors reviewed the manuscript.

Additional Information

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