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CD82 membrane scaffolding regulates hematopoietic cell functions

Christina M. Termini
University of New Mexico Health Sciences Center

Christina Marie Termini
Candidate

Biomedical Sciences
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Jennifer Gillette, Chairperson

Angela Wandinger-Ness

Matthew Campen

Keith Lidke

Tione Buranda

CD82 MEMBRANE SCAFFOLDING REGULATES HEMATOPOIETIC CELL FUNCTIONS

BY

CHRISTINA MARIE TERMINI

B.S., Biology, University of Maryland, 2011
B.A., Music, University of Maryland, 2011
M.M., Music Performance, University of New Mexico, 2015

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Ph.D., Biomedical Sciences, 2017

ABSTRACT

Through their ability to self-renew and differentiate, hematopoietic stem/progenitor cells (HSPCs) maintain the adult blood and immune systems. The microenvironment, or niche, in which HSPCs reside, serves as a critical regulator of HSPC functions. As previous work has identified the tetraspanin CD82 as a mediator of HSPC-niche interactions, we aimed to determine the mechanism by which this occurs. Our data demonstrate that CD82 expression and scaffolding regulate HSPC interactions with niche components by organizing the α4 integrin subunit into tightly packed nanoclusters. The HSPC niche can also protect acute myeloid leukemia (AML) cells from therapeutics. Therefore, we next examined how CD82 regulates AML cell interactions with the niche. Our data show that the organization of CD82 mediates N-cadherin clustering in a glycosylation-dependent manner for the control of AML-niche interactions. As

AML blasts can exhibit uncontrolled signaling, we also examined how CD82 promotes Protein Kinase C α (PKCα) signal transduction in AML. Our data demonstrate that CD82 scaffolding promotes sustained PKCα signaling for the control of AML growth. From these studies, we suspect that targeting the molecular organization of CD82 may provide a means by which AML cells can be released from the bone marrow, while attenuate uncontrolled signaling in AML. Collectively, these data shed light on the mechanisms by which CD82 and the domains within CD82 contribute to cellular adhesion and signaling. We believe that these data offer CD82 and palmitoylation as molecular targets for enhancing HSPC transplantations and improving the efficacy of AML therapeutics.

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Chapter 1 – Introduction

1.1 Hematopoietic stem cells

1.1.1 History of hematopoietic stem cells

The use of the atomic bomb during World War II and the subsequent radiation exposure experienced by civilians inspired research aimed towards understanding methods by which bone marrow failure occurred and could be restored (Henig and Zuckerman, 2014). Researchers began their work by using murine models of radiation and came across the discovery of hematopoietic stem cells (HSCs) and their therapeutic potential in transplantation, which is now a standard of care for several diseases.

The first inkling of the therapeutic nature of HSCs was observed during experiments monitoring the survival rate of mice with various tissues protected. Jacobson and colleagues demonstrated that by lead-shielding the spleen, a hematopoietic organ, they could achieve complete hematopoietic recovery in mice that were irradiated (Jacobson et al., 1951). A series of follow-up reports from this group further postulate upon the mechanism by which protection of the spleen might enhance mouse survival. It was hypothesized that the cells of the protected tissue, in this case, the spleen, produced the components responsible for enhanced mouse survival (Jacobson, 1952). In order to further investigate this idea, Lorenz and colleagues transplanted bone marrow from non-irradiated mice into irradiated mice to determine if the hematopoietic components of the bone marrow were sufficient to promote animal survival (Lorenz et al., 1952; Lorenz et al., 1951). Indeed, the authors found that mortality following injections of bone marrow protected animals from death and enhanced the production of erythrocytes, reticulocytes, and leukocytes compared to animals that did not receive a bone marrow transplantation. Further studies attempted to track the fate of transplanted bone marrow cells throughout the animal, finding that donor derived circulating erythrocytes as well as injected bone marrow cells are capable of repopulating the marrow of irradiated animals (Nowell et al., 1956; Smith et al., 1957). These studies demonstrate that bone marrow components

can protect animals from death following irradiation, though it was unclear if the cellular or humoral components (or both) are responsible for such actions.

A closer examination of the cellular components of the bone marrow demonstrated that injection of bone marrow components into irradiated mice promotes the formation of colonies of erythrocytes, myelocytes and metamyelocytes within the spleen (Till and Mc, 1961). Additional repopulation studies by Wu and colleagues suggested that hematopoietic and immune cells are derived from a common stem cell (Wu et al., 1968), which is considered the accepted principle of hematopoietic stem cell lineage today.

1.1.2 Identification of hematopoietic stem cells

Hematopoietic stem cells are defined as cells capable of self-renewal and differentiation into blood and immune cells. HSCs can differentiate into a variety of cell types, which are depicted in Figure 1.1. The general hierarchy of differentiation begins with long-term HSCs (LT-HSCs), which are capable of self-renewal or differentiation, which then become short-term HSCs (ST-HSCs) with reduced self-renewal capacity. ST-HSCs can undergo self-renewal or differentiation into multipotent progenitor cells (MPPs), which ultimately become lineage restricted progenitor cells, and subsequently mature effector cells (Ivanova et al., 2002). These distinct populations of cells express unique combinations of surface markers that allow them to be isolated.

1.1.3 Long-term hematopoietic stem cells

Long-term HSCs are defined as HSCs that when transplanted into lethally irradiated recipients can repopulate the recipient's hematopoietic system for life. The first identifying marker of LT-HSCs that was explored was CD34 (Baum et al., 1992). CD34 is a transmembrane glycoprotein, which is expressed on the cell surface and is a ligand for L-selectin. CD34 has been demonstrated to regulate cellular proliferation, differentiation, adhesion, and morphogenesis (Nielsen and McNagny, 2008). Early work demonstrated that human cells within the CD34(+) fraction were responsible for the establishment of long-term myeloid and lymphoid cultures (Baum et al., 1992). The researchers went on to evaluate additional markers for HSCs, demonstrating that CD34 combined with Thy-1(+)

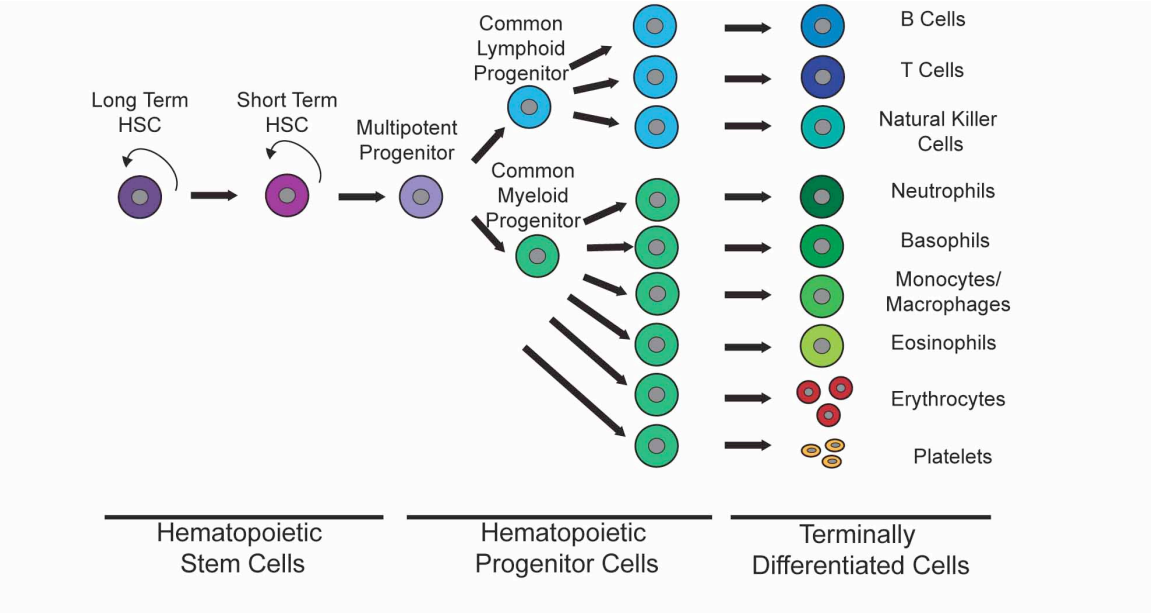

Figure 1.1: Diagram of hematopoiesis. Hematopoietic stem cell differentiation first occurs when long-term HSCs differentiate into short-term HSCs, which ultimately become multipotent progenitor cells. Multipotent progenitor cells can then become lymphoid or myeloid progenitors. Once these cells become myeloid or lymphoid progenitors, they are restricted in terms of the kinds of cells they can ultimately become. Upon further differentiation, progenitor cells have the potential to become a variety of terminally differentiated cells.

(CD90(+)) and lineage negative (Lin (-)) cells further purify the HSC population. Lineage negative cells do not express surface markers that define them as lineage committed or differentiated cells.

Additional work has demonstrated that the CD38 glycoprotein also serves as a marker for HSCs. CD38 is an ectoenzyme, serving as an adenosine diphosphate (ADP) ribosyl cyclase (Deaglio et al., 2008). Huang and Terstappen demonstrated that CD34(+) and CD38(-) human fetal bone marrow cells were enriched for the ability to differentiate into lymphoid and myeloid cells. Furthermore, they hypothesized that the CD34(+)/CD38(-) fraction of cells could recapitulate all phases of hematopoiesis (Huang and Terstappen, 1994). Further analysis of CD34(+)/CD38(-) cells demonstrated that these cells represented only 0.02% of the mononuclear cells from the bone marrow and cord blood and exist mostly in the G0/G1 cell cycle state (Hao et al., 1996). Additional work by Miller and colleagues show that CD34(+)/Lin(-)/CD38(-) cells grown under differing culture conditions can differentiate into natural killer cells, B-lineage cells, dendritic cells, as well as myeloid cells (Miller et al., 1999).

Conversely, the CD45 glycoprotein serves as a distinguishing marker for committed myeloid progenitors, B cells and naïve T cells (Altin and Sloan, 1997; Civin and Gore, 1993; Fritsch et al., 1993). As such, it was recently shown that the fraction of cells that express low levels of the CD45 isoform, CD45RA, further purifies LT-HSCs (Majeti et al., 2007). Subsequent analysis of these markers demonstrates that the Lin(-)/CD34(+)/CD38(-)/CD90(+)/CD45RA(-) has improved long-term engraftment potential compared to the CD90(-) population (Majeti et al., 2007). As such, the current standard set of markers used to identify LT-HSCs in humans is Lin(-)/CD34(+)/CD38(-)/CD90(+)/CD45RA(-).

1.1.4 Hematopoietic progenitor cells

As was previously mentioned, Majeti et al. showed that CD90 expression serves as a marker for LT-HSCs. Although the CD90(-) population has some repopulation capacity, it is severely diminished compared to CD90(+) cells (Majeti et al., 2007). As such, the Lin(-)/CD34(+)/CD38(-)/CD90(-)/CD45RA(-) population of cells is considered to contain hematopoietic multipotent progenitor

cells. The authors also find that when individual cells were plated in methylcellulose media, the Lin(-)/CD34(+)/CD38(-)/CD90(+)/CD45RA(-) cells repopulate the lymphoid and myeloid cells of the bone marrow more efficiently compared to the Lin(-)/CD34(+)/CD38(-)/CD90(-)/CD45RA(-) fraction. Furthermore, the authors demonstrate that Lin(-)/CD34(+)/CD38(-)/CD90(+)/CD45RA(-) LT-HSCs become Lin(-)/CD34(+)/CD38(-)/CD90(-)/CD45RA(-) expressing multipotent progenitor cells and ultimately Lin(-)/CD34(+)/CD38(-)/CD90(-)/CD45RA(+) expressing multipotent progenitor cells.

Downstream of multipotent progenitor cells are the lineage committed progenitor cells, which include common lymphoid and myeloid progenitors and further downstream, megakaryocyte/erythrocyte and granulocyte/macrophage progenitor cells (Akashi et al., 2000). Common lymphoid progenitors will first become lineage restricted progenitors, which include pro-dendritic cells, pro-B-cells, pro-T-cells or pro-natural killer cells. Ultimately, these restricted progenitors will become dendritic cells, B-cells, T-cells or natural killer cells, respectively (Galy et al., 1995). Common myeloid progenitors (CMPs) follow a different track; these cells can first become megakaryocyte/erythrocyte progenitors or granulocyte/macrophage progenitors (Seita and Weissman, 2010). Megakaryocyte/erythrocyte progenitors can then become megakaryocyte progenitors or erythrocyte progenitors, while CMPs can differentiate into granulocyte/macrophage progenitors. CMPs can also differentiate into pro-dendritic cells and ultimately dendritic cells.

The process of hematopoiesis is a complicated hierarchy that requires several levels of regulation. In the next section, we will address some of the signaling cascades that help to maintain proper numbers of HSCs and differentiated cells.

1.1.5 Regulation of hematopoietic stem cells

HSCs are regulated by a variety of intrinsic and extrinsic factors that contribute to their continued self-renewal and differentiation. It is estimated that HSCs only replicate once every 40 weeks *in vivo* (Catlin et al., 2011). Upon division, cells must decide to remain HSCs and undergo self-renewal,

differentiate, or undergo apoptosis. The pathways that will be discussed with regards to maintenance of HSCs are the Wnt/β-catenin pathway and Notch signaling cascades.

The Wnt signaling pathway is generally accepted as a regulator of HSC self-renewal and differentiation, though conflicting studies exist that challenge this notion. In cells that are not undergoing Wnt signaling, the cytoplasmic protein, β-catenin, is usually degraded (Reya and Clevers, 2005). However, upon Wnt signal initiation through binding to the receptor complex of Frizzled and Lrp5/6, β-catenin is stabilized. This stabilization allows β-catenin to ultimately translocate to the nucleus and interact with the T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factors, which promotes gene transcription. It has been described that HSCs and their surrounding microenvironmental cells can produce Wnt proteins (Austin et al., 1997; Hackney et al., 2002; Reya et al., 2000; Van Den Berg et al., 1998). Furthermore, studies from the laboratory of Irving Weissman have demonstrated using several experimental approaches that Wnt signaling promotes HSC maintenance (Rattis et al., 2004; Reya et al., 2003; Staal and Luis, 2010; Willert et al., 2003). In contrast, follow up studies using modified experimental setups challenged the findings from the Weissman laboratory (Baba et al., 2005; Baba et al., 2006; Kirstetter et al., 2006; Scheller et al., 2006). However, more recent *in vitro* studies have recapitulated some of the Weissman findings, showing that Lin(-)/Sca(+)/c-Kit(-) cells engineered to express Wnt3a exhibit a decrease in the proportion of myeloid or lymphoid committed cells compared to total cells, suggesting that Wnt signaling indeed plays a role in regulating HSC differentiation (Malhotra et al., 2008).

The Notch signaling pathway has also been implicated in regulating HSC self-renewal and differentiation. Notch protein exists as a transmembrane receptor, which is cleaved upon its engagement with a transmembrane ligand as presented by an adjacent cell. This cleavage product, the Notch intracellular domain, can then translocate to the nucleus to promote the transcription of target genes, many of which contribute to HSC maintenance (Kopan, 2012). Experiments from Varnum-Finney and colleagues determined that constitutive

expression of the Notch1 intracellular domain in murine hematopoietic progenitor cells shifted the cell population to hematopoietic stem cells (Varnum-Finney et al., 2000). Furthermore, the experimenters determined that Notch signaling promotes HSC self-renewal and differentiation into granulocytes, macrophages, erythroid, and megakaryocyte lineages, while Notch signaling reduces differentiation into B-cells. An additional study from the David Scadden laboratory also expressed constitutively activated Notch1 in murine progenitor cells (Stier et al., 2002). They found that these cells have an increased stem cell population, as quantified by the ability for progenitor cells to repopulate lethally irradiated mice.

It is important to note that the environment in which HSCs reside can also greatly influence their self-renewal and differentiation properties. This can be achieved through initiation of the aforementioned signaling cascades through paracrine signaling or adhesive signaling. These topics will be discussed later in the “Stem cell niche” section of Chapter 1.

1.1.6 Clinical usage of hematopoietic stem cells

Due to the multipotent capacity of hematopoietic stem cells, they can be transplanted to help treat a variety of diseases that affect the blood and immune system. Early studies demonstrated that bone marrow transplants can protect mice that were lethally irradiated from death (Spangrude et al., 1988).

The type of HSC transplant performed is defined by the relationship of donor to recipient (Appelbaum, 2003). For example, syngeneic transplants involve the transplantation of HSCs from one identical twin to another. The most common forms of HSC transplants are autologous and allogeneic transplants. Autologous transplants isolate cells directly from a patient to be transplanted back into the same patient under a treatment regimen. Numerous cancer treatment regimens require radiation therapy and chemotherapy, which can greatly diminish the efficiency of the patient’s immune system. In order to combat this, patients will often times undergo an autologous transplantation during their treatment regimen. Allogeneic transplantation requires cells to be isolated from a donor and transplanted into a different recipient. This type of transplantation is

used primarily for the treatment of leukemias and blood diseases, as the recipient will benefit from having their hematopoietic system replaced with a new one.

1.1.7 Hematopoietic stem cell isolation from patients

In order to improve the success of HSC transplants, it is essential that high numbers of pure HSCs are isolated. HSCs reside in specialized microenvironments within the bone marrow, vasculature, and spleen amongst other locations within the body (Taichman et al., 2001). In order to reduce the invasiveness of patient HSC isolations, it has become routine to mobilize HSCs, or to release them from their niche to the peripheral blood prior to isolation (Appelbaum, 2003). It was demonstrated that the mobilized cells exhibit properties unique from the residual niche HSCs, including low expression levels of vascular cell adhesion molecule-1 (VCAM-1), c-Kit and integrins as well as an increased proportion of cells within the G0 cell cycle phase (Bonig et al., 2009a; Graf et al., 2001; Scott et al., 1997; Yamaguchi et al., 1998).

One of the most commonly used agents to mobilize HSCs is granulocyte-colony stimulating factor (G-CSF). The receptor for G-CSF is expressed on hematopoietic progenitor cells, terminally differentiated cells, and surrounding endothelial cells (Bocchietto et al., 1993). It is hypothesized that G-CSF promotes the cleavage of stromal cell-derived factor-1 (SDF-1, also known as CXCL12), which is the ligand for the C-X-C chemokine receptor 4 (CXCR4), thereby reducing CXCR4-mediated HSC retention (Liu et al., 2000; Petit et al., 2002). Furthermore, treatment of human CD34(+)/CD38(-)/(lo) cells with anti-CXCR4 antibodies decreases the ability for G-CSF treatment to mobilize HSCs (Petit et al., 2002). Additionally, in patients that do not mobilize HSCs effectively with G-CSF alone, the CXCR4 antagonist AMD3100 (also known as Plerixafor or Mozobil) is used in combination with G-CSF (Bonig et al., 2009a; Bonig et al., 2009b; Burroughs et al., 2005; Devine et al., 2004; Devine et al., 2008; Flomenberg et al., 2005; Larochelle et al., 2006).

Additional work has identified several other molecules and mechanisms by which HSCs can be mobilized from their niches. However, the clinical relevance of these pathways remains to be explored. For example granulocyte-macrophage

colony-stimulating factor (GM-CSF) has also been demonstrated to promote HSC mobilization in combination or sequence with G-CSF treatment (Lane et al., 1999; Sohn et al., 2002). Work from Molineux and colleagues demonstrated that mice treated with G-CSF in combination with stem cell factor (SCF), the ligand for the c-Kit receptor, increased the frequency of blood-borne colony-forming cells, which is an indicator of an increase in HSC release (Molineux et al., 1991). Although this study has yet to be followed up with more sophisticated technologies, it indicates that synergism between G-CSF and SCF may exist to enhance HSC mobilization. Interleukins IL-2 and IL-8 have also been implicated in mediating HSC mobilization in concert with G-CSF (Burns et al., 2000; Watanabe et al., 1999). Furthermore, work has also demonstrated that treatment with the chemotherapeutic agents paclitaxel and cyclophosphamide can enhance HSC mobilization (Burtness et al., 1999; Fernandez et al., 2008; Verma et al., 1999). Future clinical analyses of the aforementioned pathways in mediating HSC mobilization will be required to improve mobilization efficiency.

Beyond the mobilization and isolation of HSCs, umbilical cord blood has also been evaluated as a source for HSCs for transplantation (Appelbaum, 2003). Though the success rate is diminished compared to bone marrow transplants (estimated at 31% versus 43%, respectively), cord blood has a lower T cell content, reducing the risk of graft-versus-host disease (GVHD) occurrence (Rocha et al., 2001). The major setbacks regarding umbilical cord blood transplantations are low cell number, delayed engraftment, and reduced ability to reconstitute the immune system (Ballen et al., 2013). As such, it will be valuable to continue to evaluate means by which we can enhance the efficacy of umbilical cord blood transplantations.

1.1.8 Regulation of hematopoietic stem cell transplant success

Once the donor HSCs are mobilized and collected, they are then infused into the recipient. The sign of a successful transplantation is the repopulation of the adult hematopoietic system. There are a number of complications that can prevent the success of HSC transplantation. GVHD occurs when immune cells from the transplant cause injury to the recipient (Appelbaum, 2003). This

primarily occurs when the donor and recipient are ineffectively matched for their human leukocyte antigen, which causes the donor cells to view the recipient's cells as foreign. In cases with two or more mismatched gene loci, poor survival is expected (Anasetti et al., 1989). In cases of autologous transplantations, it is possible that the isolated HSCs may contain tumor cells, which when re-infused may result in disease development. There is evidence that purification of the HSC population or treatment of the isolated cells with chemotherapeutics can improve patient outcome, but this has yet to become standard in the clinical setting (Appelbaum, 2003; Gribben et al., 1991).

Finally, the success of the transplantation is greatly impacted by the ability for HSCs to effectively home to the recipient's bone marrow. As the bone marrow is the primary site of hematopoiesis, when HSCs reach this microenvironment, they can undergo self-renewal and differentiation, two processes critical to repopulating the recipients hematopoietic system (Calvi and Link, 2015). There are numerous molecules know to regulate this process of bone marrow homing, including integrins (α4, α5) as well as CXCR4 and it's ligand, SDF-1 (Kollet et al., 2001 ; Lanzkron et al., 1999; Scott et al., 2003). The role of these molecules and signaling events in regulating HSC interactions with the bone marrow niche will be discussed further in the "Stem cell niche" section of this introduction.

1.2 Acute myeloid leukemia

1.2.1 Acute myeloid leukemia disease properties

Acute myeloid leukemia (AML) is a blood cancer that results from a defective hematopoietic system, which generates an increase in myeloid progenitor cells (Lowenberg et al., 1999). The diagnosis of AML is primarily performed through morphological identification of leukemic myeloblasts within patient peripheral blood and bone marrow samples. Generally speaking, a blast count of 20% or more results in a diagnosis of AML. Upon diagnosis, flow cytometry is used to further characterize the disease based on the expression patterns of myeloid markers, usually CD33 and CD13 (Estey and Dohner, 2006).

Genetic mapping of patient samples has demonstrated that chromosomal abnormalities are associated with AML. For example, AML can result from

chromosomal translocations, which often lead to the production of oncogenic fusion proteins (Tenen, 2003). One of the most common pathways affected by the production of fusion proteins in AML is the AML1-CBFβ heterodimeric transcription factor. Core binding factors (CBFs) include one alpha and one β subunit (Hart and Foroni, 2002). There are three different potential α subunits, Runx1-3 (also known as AML1, CBFA2 or PEBP2αB), and one common β subunit, CBFβ (Lund and van Lohuizen, 2002). Under normal conditions, the AML1 transcription factor interacts with CBFβ, which allows transcription of genes that regulate hematopoiesis (Lowenberg et al., 1999; Okuda et al., 1996). However, in AML, the generation of AML1 or CBFβ fusion proteins renders the transcription factor nonfunctional. For example, translocation of t(8;21) results in the generation of the eight-twenty-one (ETO)-AML1 fusion protein, while inv(16) leads to the production of the CBFβ-MYH11 chimera (Downing et al., 2000). It is estimated that the incidence of these fusion proteins in AML is between 6-7% (Estey and Dohner, 2006). Another commonly generated fusion protein is the promyelocytic leukemia (PML)- retinoic acid receptor alpha (RARα) fusion resulting from t(15:17), which has been estimated to occur in 4-7% of AML patients (Estey and Dohner, 2006; Papaemmanuil et al., 2016). The expression of the PML-RARα fusion protein has been shown to deregulate the differentiation of myeloid progenitor cells (Grignani et al., 1993).

The mixed-lineage leukemia (MLL) gene can also be subject to fusions with several partner genes to generate leukemia. Under normal conditions, MLL controls expression of homeobox (*HOX)* genes, which contribute to the maintenance of hematopoietic stem and progenitor cells (Alharbi et al., 2013). However, in AML, MLL can become fused to the AF9 protein, which prevents complete erythroid and myeloid maturation (Abdul-Nabi et al., 2010). Researchers have taken advantage of this fusion protein system and integrated the MLL-AF9 fusion protein into mouse models to study AML in mice (Corral et al., 1996).

1.2.2 Identifying markers of acute myeloid leukemia

Because AML is a heterogeneous disease, there is no single immunophenotype associated with diagnosis. Rather, the blast count serves as the best indication of disease. However, recent advances have characterized the surface markers associated with the cancer stem cell population in leukemia. Cancer stem cells are defined as a rare subset of cancer cells, which have stem cell properties, making them particularly difficult to target. More specifically, cancer stem cells are described as tumorigenic cells with the ability to self-renew and to become any cell of the tumor population (Guo et al., 2006; Jordan et al., 2006 ; Yu et al., 2012). AML has been described to contain a population of cancer stem cells, which are referred to as leukemia stem cells (LSCs) (Bonnet and Dick, 1997; Lapidot et al., 1994). Early work using patient sample xenografts into mouse models demonstrated that LSCs are found exclusively within the CD34(+)/CD38(-) AML blast population (Bonnet and Dick, 1997; Jordan, 2002; Lapidot et al., 1994). Further characterization has identified numerous other surface markers that can be used to isolate LSCs, including CD33, CD123 and CD13 (Horton and Huntly, 2012; Taussig et al., 2005). However, from patient to patient, there is a large degree of heterogeneity; as such, personalized targeting of LSCs may prove to be more fruitful for AML therapeutics.

1.2.3 Aberrant signaling in acute myeloid leukemia

As is the case in most cancers, signaling in AML is significantly altered compared to normal cellular signaling. For example, mutations in the FMS-like tyrosine kinase 3 (FLT3) and the c-Kit receptor tyrosine kinase (RTK) have been well documented in AML. As such, targeting RTKs is of significant therapeutic interest. Under normal conditions, FLT3 is expressed on healthy c-Kit(+)/CD34(+) progenitor cells, while FLT3 expression is frequently increased on AML blasts (Drexler, 1996; Rosnet et al., 1996). Upon ligand engagement, FLT3 signal transduction activates various downstream targets including phosphoinositide 3-kinase (PI3K), Ras, signal transducer and activator of transcription 5 (STAT5), phospholipase C-γ (PLC-γ) and Src (Gilliland and Griffin, 2002). The two most common FLT3 mutations associated with AML are an internal tandem duplication

(ITD) in exons 14 and 15 or a missense point mutation in exon 20, both of which produce a constitutively active form of FLT3 (Nakao et al., 1996; Stirewalt and Radich, 2003). FLT3 mutations have been found in approximately 15-35% of AML cases, demonstrating potential for FLT3 targeting as an AML therapeutic (Nakao et al., 1996; Stirewalt and Radich, 2003). As such, several FLT3 inhibitors are undergoing phase 1 and 2 clinical trials to determine the appropriateness of their use as single agents of in combinational therapy with chemotherapeutics (Grunwald and Levis, 2013).

The c-Kit receptor tyrosine kinase is expressed on HSCs and contributes to the maintenance of their stemness (Thoren et al., 2008). c-Kit (also known as CD117) is a receptor for stem cell factor (SCF) and c-Kit positive blasts are found in approximately 80% of AML cases (Ikeda et al., 1991). Furthermore, mutations in c-Kit are found in approximately 17% of AML cases, but in patients with CBF AML, the incidence is 52% (Boissel et al., 2006; Corbacioglu et al., 2006; Goemans et al., 2005; Paschka et al., 2006). The most well characterized mutations of c-Kit in AML are ITD of exon 11, insertion/deletion of exon 8, or a single amino acid substitution of a valine or tyrosine for aspartate at codon 816, referred to as D^{816} (Longley et al., 2001; Park et al., 2011). The activation of c-Kit can induce various signaling cascades including PI3K, Src family kinases, mitogen-activated protein kinase (MAPK) and phospholipase C and D (Lennartsson and Ronnstrand, 2012). Though tyrosine kinase inhibitors (TKIs) are already in use clinically to treat AML, current work is focusing on the development and efficacy of c-Kit specific TKIs, such as dasatinib and midostaurin (Dohner et al., 2015).

Intracellular kinases as well as other kinds of intracellular molecules have also been shown to exhibit aberrant signaling in AML. For example, the Ras family of guanosine triphosphate (GTP)-binding proteins has increased activation in several leukemias, including AML. In particular, N- and K-RAS have been shown to have increased activation in 20-40% of AML cases (Reuter et al., 2000). More specifically, mutations within Ras itself, or upstream regulators, such as c-Kit or FLT3, can render Ras constitutively activated (Bos et al., 1987; Dosil

et al., 1993; Farr et al., 1988; Senn et al., 1988). Ras activation requires adequate tethering of Ras to the plasma membrane, which is mediated through the post-translational modification of farnesylation. Though palmitoylation has also been demonstrated to contribute to this process, it seems that farnesylation is most critical for mediating membrane recruitment and subsequent signaling (Heimbrook and Oliff, 1998). Therefore, the use of farnesyltransferase inhibitors to attenuate Ras signaling has been demonstrated in cellular and animal models, but had disappointing results when examined in human clinical trials (Reuter et al., 2000). As such, direct inhibition of the MAPK and Akt pathways is currently under investigation in AML patients with Ras mutations (Johnson et al., 2014).

1.2.4 Acute myeloid leukemia treatment

Conventionally, AML treatment is administered to first achieve remission (induction therapy) and then to further ablate the disease (post-induction therapy) (Appelbaum, 2003). In order to induce remission, patients undergo conventional chemotherapy with the use of daunorubicin, cytarabine or a combination (Coombs et al., 2016). Upon the achievement of remission, younger patients will undergo high dose cytarabine, followed by a myeloablative allogenic or autologous bone marrow transplantation. Because older patients cannot tolerate the toxicity of high dose chemotherapy, this treatment recommendation is not given. Instead, older patients are recommended to undergo further chemotherapy but not at as high of a concentration or a non-myeloablative transplant (Lowenberg et al., 1999). There are significant ongoing efforts for the use of therapies targeting tyrosine kinases, farnesyltransferases, methyltransferases as well as the proteasome for the use in older patients (Kuendgen and Germing, 2009). Unfortunately, the overall survival rate for adults remains low at only about 10%, primarily due to persistent or relapsed AML (Appelbaum et al., 2001; Tallman et al., 2005). Furthermore, significant development of post-remission and relapse therapies, particularly for older patients, will be necessary to successfully eradicate this disease.

1.3 Stem cell niche

1.3.1 History of the stem cell niche

The concept of the stem cell microenvironment or “niche” was coined first by Schofield in 1978 whereby he hypothesized that the specialized surrounding environment of stem cells contributed to their reconstitution capacity (Schofield, 1978). Furthermore, the niche contributes to the maintenance of stem cells as well as their differentiation when appropriate. Though this dissertation will take particular focus on the HSC niche, it is important to note that the concept of the stem cell niche has been heavily characterized with respect to hematopoietic, skin/hair follicle, intestine, neural and gonadal stem cells (Morrison and Spradling, 2008). Work has also identified the role of the niche in regulating germline stem cells in invertebrates including *Drosophila melanogaster* and *Caenorhabditis elegans*. In particular, it was determined that the surrounding terminal filament, cap, and inner sheath cells make up the ovarian niche in *Drosophila*. Researchers determined that cap cells are essential for the regulation of the gonadal niche structure, meanwhile, cap cells and terminal filament cells participate in direct cellular contact with germline stem cells (GSCs), critical for the maintenance of GSC differentiation (Xie and Spradling, 2000). Another study from around the same time characterized the GSC niche interactions in the testis, determining that associations exist between cyst cells and GSCs, which may contribute to GSC division and differentiation (Kiger et al., 2000). Meanwhile, distal tip cells have been demonstrated to regulate GSC division in *Caenorhabditis elegans* (Kimble and White, 1981). These fundamental studies of how niche interactions regulate stem cell fate provided the framework for future researchers to characterize the hematopoietic stem cell niche.

1.3.2 Hematopoietic stem cell niche

The bone marrow and vasculature represent the primary hematopoietic stem cell niches, while the spleen is also known to be a site of extramedullary hematopoiesis. Additionally, the fetal liver is a critical niche for HSC expansion during development before HSCs migrate to and reside within the bone marrow (Samokhvalov et al., 2007). The bone marrow niche is comprised of several cellular components including osteoblasts, osteoclasts, stromal cells, mesenchymal stem cells (MSCs) and adipocytes, as diagrammed in Figure 1.2.

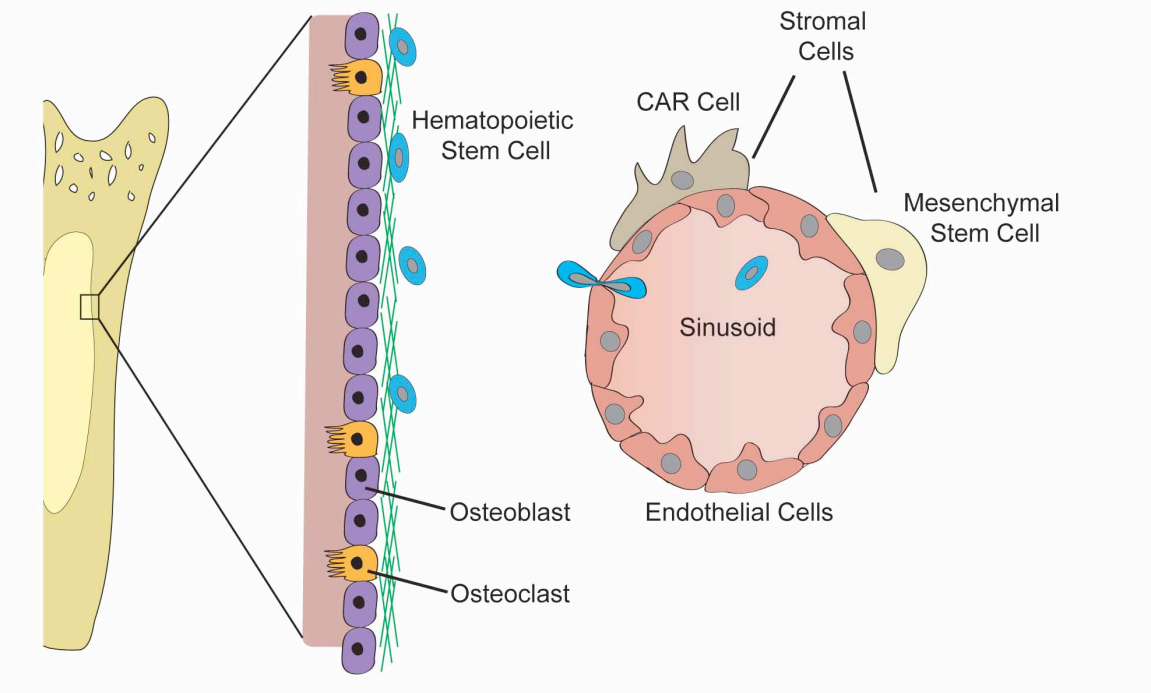

Figure 1.2: Components of the hematopoietic stem cell niche. The endosteal and vascular stem cell niches are depicted in the cartoon above. The spongy bone is magnified on the left to depict osteoblasts and osteoclasts, which are the main cellular components of the bone marrow niche. These cells can also deposit extracellular matrix components, to which HSCs may adhere. On the right is a sinusoid depicting the main cellular components of the vascular niche. HSCs may enter the vasculature by extravasating through endothelial cells. Stromal cells, such as CAR cells and mesenchymal stem cells are on the outside of the endothelial layer.

Early work shows that osteoblasts produce G-CSF, and when CD34(+) cells were cultured with osteoblasts, there was an increase in the production of hematopoietic cells (Taichman and Emerson, 1994). Defining studies from the laboratory of David Scadden demonstrate that an increase in the number of osteoblasts significantly increased the hematopoietic cell population, as defined as Lin(-)Sca-1(+)c-Kit(+) cells, within the bone marrow (Calvi et al., 2003). Furthermore, the authors also determined that these cells have increased engraftment capacity compared to normal control counterparts. The authors propose that increased γ-secretase activity contributes to enhanced Notch signaling, thereby increasing HSC numbers. A concurrently published article demonstrates that the presence of a particular type of osteoblast termed the spindle-shaped N-cadherin(+)CD45(-) (SNO) cell is critical for maintenance of HSC number (Zhang et al., 2003). The authors conclude that SNO cells enhance bone morphogenic protein expression, which contributes to the maintenance of niche size. It is important to note that the role of SNO osteoblasts remains highly controversial, as reports have disputed claims of their importance in regulating HSCs (Kiel et al., 2007).

There are several signaling pathways enacted by osteoblasts that are implicated in regulating HSCs within the niche. For example, it has been shown that HSCs that express the Tie2 receptor tyrosine kinase, which are determined to be a quiescent population, are found in contact with the endosteal bone surface (Arai et al., 2004). Furthermore, previous work has demonstrated that signaling along the myeloproliferative leukemia (MPL)/thrombopoietin (THPO) axis is implicated in regulating HSC quiescence (Yoshihara et al., 2007). More specifically, the authors found that long-term MPL expressing HSCs adhered to THPO expressing osteoblasts, indicating a potential regulatory role. The involvement of Notch signaling from osteoblasts in regulating HSCs is somewhat controversial. Initial reports in mice demonstrate that osteoblasts within the bone marrow niche express the Notch ligand, Jag1 (Calvi et al., 2003). The authors then examined the levels of the Notch intracellular domain (NCID) in murine HSCs, finding an increase in NCID in transgenic mice with increased osteoblast

and HSC numbers. From these data, the authors suggest that osteoblast-mediated Notch signaling may play a role in regulating HSC numbers. However, a follow-up study in 2005 concluded that Notch signaling is dispensable for the regulation of HSC self-renewal and differentiation (Mancini et al., 2005). This study also utilized transgenic mice, but these mice had an Mx-Cre-inducible system whereby Jagged1 could be deleted. The authors show that HSC self-renewal and differentiation are not affected by the absence of Jagged1 or Notch1, providing conflicting results to the initial studies. Future work should focus on uncovering the role of osteoblastic-mediated Notch signaling in regulating HSCs, as it remains unclear within the field.

As the bone marrow microenvironment is highly vascularized, it is not surprising that the endothelial cells that line the bone marrow comprise their own vascular niche for the regulation of HSCs. These lining endothelial cells are essential for allowing HSCs to enter and exit the bloodstream. Anatomical studies have shown that bone marrow sinusoids are unique from regular veins; they consist of a single layer of endothelial cells and lack any other supporting cells (Kopp et al., 2005; Tavassoli, 1981). As was previously mentioned, HSCs move to the fetal liver to expand prior to entering the bone marrow microenvironment. It was found that mice lacking SDF-1 expression had defective bone marrow colonization. Meanwhile, enforced expression of SDF-1 in vascular endothelial cells could rescue this defect, demonstrating that signaling from the vascular niche can regulate HSCs (Ara et al., 2003). Furthermore, early work from the laboratory of Sean Morrison determined that LT-HSCs can interact directly with endothelial cells within the femur in mice, providing visual and quantitative evidence that HSCs may be regulated by interactions with these cells (Kiel et al., 2007).

Beyond their role in regulating HSC trafficking, these endothelial cells can also regulate HSC signaling. Recently, it was discovered that bone marrow endothelial cells promote the maintenance of the LT-HSC compartment through Notch-dependent signaling. The authors found that when LT-HSCs were incubated with endothelial cells with neutralized Notch ligands, there was a

significant decrease in proliferation and number of LT-HSCs compared to incubation with control endothelial cells (Butler et al., 2010). A recent report has shown that endothelial-selectin (E-selectin) is critical for regulating HSC proliferation, whereby knocking out E-selectin in mice or treating animals with an E-selectin agonist enhanced HSC self-renewal and slowed HSC cycling (Winkler et al., 2012). The authors mention that the ligand by which E-selectin propagates it's action remains unknown, but they suggest that glycoproteins are likely involved in regulating E-selectin signaling for the control of HSCs. Another recent report has utilized a variety of SCF knockout mice to examine how the origin of SCF regulates HSC signaling (Ding et al., 2012). The authors find that SCF secreted from endothelial and perivascular cells is critical for maintaining the repopulation capacity of HSCs, while SCF secreted from osteoblasts and nestin(+) stromal cells is dispensable. The authors do note that other signaling components from different niche components likely contribute to HSC maintenance, although their study focuses on SCF signaling.

Although the osteoblastic and vascular niches represent the most well researched regulatory niches for HSCs, HSCs may also interact with other types of stromal cells. The bone marrow stroma includes all cells found between the outer bone marrow blood vessels and the marrow surface which are not of the hematopoietic lineage (Krebsbach et al., 1999). Mesenchymal stem cells (MSCs) have been demonstrated to be in direct contact with HSCs within the niche. By using nestin as a marker for MSCs, researchers found that these MSCs highly express numerous genes that regulate HSCs, including genes that encode for SDF-1, SCF, angiopoietin-1, IL-7, VCAM-1 and osteopontin. Furthermore, the researchers determined that the presence of these nestin(+) MSCs within the bone marrow is critical for maintaining HSC number within the bone marrow and ultimately the presence of MSCs significantly impacts the ability for HSCs to home to the bone marrow in transplantation assays (Mendez-Ferrer et al., 2010). Additionally, perivascular stromal cells known as C-X-X motif ligand 12 (CXCL12)-abundant reticular cells (CAR cells) are known to regulate HSCs through the SDF-1-CXCR4 signaling axis (Sugiyama et al., 2006). Additionally,

an examination of the HSC repopulation capacity using HSCs from sites of differing fatty content demonstrate that adipocytes are negative regulators of HSCs (Naveiras et al., 2009). Further analysis into the mechanism by which this occurs will shed light on how adipocytes regulate HSC fitness, which will be critical towards tailoring HSC transplant therapies towards patients with obesity.

Beyond cellular components, HSCs may also interact with extracellular matrix (ECM) components, which can control HSC signaling. For example, integrins on HSCs may interact with ECM components deposited by osteoblasts including fibronectin, collagen I, III and IV as well as laminin (Nilsson et al., 1998). Hyaluronic acid (HA), which is produced by stromal and hematopoietic cells, is also found within the bone marrow. HA is the ligand for CD44, which is expressed on HSCs and can regulate HSC homing (Avigdor et al., 2004; Wight et al., 1986). Furthermore, osteopontin has also been demonstrated to exist within the endosteal region of the bone marrow niche, which can contribute to HSC proliferation (Nilsson et al., 2005). We will discuss the molecules on HSCs that regulate niche interactions, with a particular emphasis on integrins and cadherins, later in this introduction.

1.3.3 Leukemia stem cell niche

The bone marrow niche provides a supportive microenvironment to promote HSC maintenance. Unfortunately, cancerous cells can also take advantage of this specialized microenvironment in order to evade treatment efforts, which is a major contributor to AML patient relapse. In particular, AML LSCs can take residence within the bone marrow niche, meanwhile remodeling the microenvironment to meet the needs of LSCs. It has been shown that when human AML LSCs are injected into NSG mice, they home to and engraft within the endosteal region of the bone marrow niche. Furthermore, this microenvironment renders LSCs resistant to cytarabine treatment (Ishikawa et al., 2007). As was mentioned in the hematopoietic stem cell section of this introduction, the CXCR4 chemokine receptor is critical for maintaining HSCs within the niche, whereby inhibition of CXCR4 signaling can mobilize HSCs into the bloodstream. In line with this finding, it has been determined that CXCR4

signaling can also be targeted in leukemias to disrupt LSC-niche interactions and sensitize AML cells to therapeutics. For example, treatment with AMD3100, novel CXCR4 blocking peptides, or CXCR4 blocking antibodies can mobilize leukemia cells from the niche and improve their chemosensitivity (Kuhne et al., 2013; Nervi et al., 2009; Zeng et al., 2006). Interestingly, it has also been demonstrated that cytarabine treatment of a variety of leukemia cell lines can actually increase the expression of CXCR4, indicating that perhaps CXCR4 should be targeted in conjunction with chemotherapeutic regimens (Sison et al., 2013). Further studies have determined treatment with the SDF-1 blocking peptide AMD3465 renders AML cells susceptible to death from treatment with cytarabine as well as FLT3 inhibitors (Zeng et al., 2009). As FLT3 inhibitors have not proven to be successful in the clinical setting, future studies should aim to re-evaluate FLT3 inhibition in the context of CXCR4 inhibition to determine if these molecules should be targeted in combination with one another.

Beyond the endosteal region of the niche, the vascular niche appears to also interact with leukemia cells. Initial reports using *in vivo* confocal imaging of the mouse skull demonstrate that the pre-B acute lymphoblastic leukemia cell line Nalm6 interacts with the vasculature upon injection into NSG mice. The authors determine that inhibition of the SDF-1/CXCR4 signaling axis diminishes this recruitment, indicating that perhaps there is a conserved role for CXCR4 signaling in regulating leukemia interactions with both the endosteal and vascular niche (Sipkins et al., 2005). Interestingly, the presence of leukemia cells within the bone marrow microenvironment can dislodge healthy CD34(+) cells from their niches, causing them to enter unconventional niche sites (Colmone et al., 2008). Therefore, leukemia cells can significantly alter the normal landscape of the niche, which ultimately disrupts “normal” interactions between HSCs and their microenvironment.

1.4 Molecules regulating niche interactions

1.4.1 Introduction to integrins

One of the key molecular regulators of niche interactions is the integrin family of adhesion receptors. Integrins are heterodimeric transmembrane

proteins consisting of one α and one β subunit, which have been shown in numerous systems to regulate cellular adhesion and migration (Campbell and Humphries, 2011). In vertebrates, there are 18 α subunits and eight β subunits, which can generate 24 known integrin heterodimers (depicted in Figure 1.3), which have binding specificity for ECM or membrane bound ligands based on the combination of subunits (Takada et al., 2007). It is thought that integrins exist in two conformations; a closed (bent) inactive conformation or an open, fully extended active conformation. Structurally, the α integrin subunit ectodomain contains a β-propeller, a thigh, and two calf domains (Barczyk et al., 2010)**;** additionally, nine of the known integrin α subunits contain a α-I domain within the β-propeller domain (Larson, 1989). Within the β-propeller region, there is a Ca^{2+} binding site; Ca^{2+} binding to this site has been demonstrated to affect integrin ligand interactions (Campbell and Humphries, 2011 ; Humphries et al., 2003; Oxvig and Springer, 1998). There is also a Mg^{2+} binding site within the metal-ion-dependent adhesion site (MIDAS) of the α subunit, which has been demonstrated to contribute to integrin ligand binding and adhesion (Humphries et al., 2003; Lee et al., 1995). There are domains within the α subunit that contribute to the ability for integrins to tether between the open and closed conformations. Within the α subunit, the linker domain between the β-propeller and calf as well as the “knee” or “genu” region between the thigh and calf domain contribute to integrin flexibility (Humphries et al., 2003; Xiong et al., 2001). The integrin β subunit ectodomain is made up of seven domains, which are a β-I-domain, a hybrid domain, plexin-semaphorin-integrin (PSI) domain, four cysteine rich epidermal growth factor-like repeats and a tail. The β-I-domain contains a Mg^{2+} MIDAS, while also containing an inhibitory Ca^{2+} binding site next to the MIDAS, termed the adjacent to MIDAS (ADMIDAS). This ADMIDAS can also bind Mn^{2+}, which promotes the change from the closed to open (active) integrin conformation (Barczyk et al., 2010; Humphries et al., 2003; Lee et al., 1995).

Integrin activation can be characterized as occurring in an ‘inside-out’ or ‘outside-in’ manner. In the case of inside-out activation, signaling from the inside

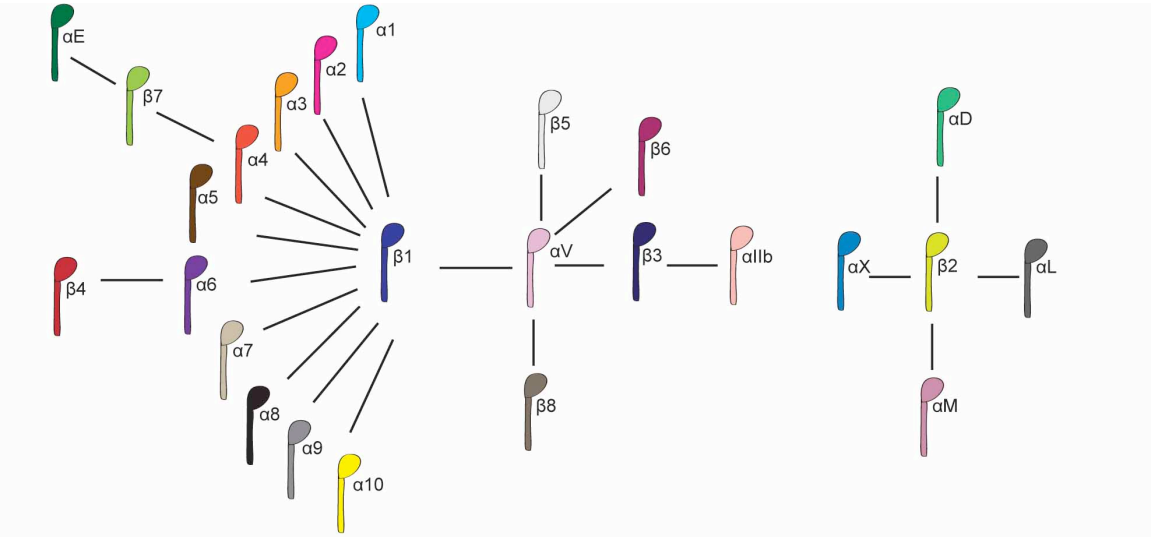

Figure 1.3: Integrin heterodimer combinations Depicted are the 24 known human integrin subunits. The heterodimeric combinations of alpha and beta subunits are shown. Some integrin subunits can form dimers with more than one other subunit (ex: α4 can dimerize with β1 or β7) Schematic adapted from (Takada et al., 2007).

of the cell promote the conformational shift of the integrin into the open or active conformation. This phenomena can be regulated by cytosolic proteins, such as focal adhesion kinase (FAK), integrin linked kinase (ILK), or talin, amongst others, interacting directly with the integrin cytoplasmic tails (Honda et al., 2009). In the case of outside-in activation, interactions between the integrin ectodomain and ligand can promote integrin activation (Emsley et al., 2000; Zhang et al., 2008). There is substantial evidence from mutational studies that the transmembrane domains of integrins form disulfide bonds with one another when in the closed conformation (Lu and Springer, 1997; Luo et al., 2004). Meanwhile, it appears that non-disulfide bonded integrin subunits can bind ligands, which has led the acceptance of a model whereby integrin activation requires the physical separation of the alpha and beta transmembrane domains. Interestingly, the need for separation for integrin activation can be bypassed by the use of Mn^{2+}, which activate integrins in an 'outside-in' manner (Kim et al., 2003).

1.4.2 Integrin ligand interactions

The integrin ligand binding site (or pocket) has been determined to exist between the α subunit β-propeller and β subunit I-domain (Xiong et al., 2001). Meanwhile, the substrate binding specificity is conferred by the combination of alpha and beta subunits, which interact with specific amino acid sequences within integrin ligands. Integrin ligands include ECM components, cellular receptors (VCAM-1, intracellular adhesion molecule-1 (ICAM-1)) and microorganisms, pathogens and venoms (Arnaout et al., 2002; Gould et al., 1990; Humphries et al., 2006; Isberg and Tran Van Nhieu, 1994; Nemerow and Cheresh, 2002; Plow et al., 2000; Rieu et al., 1994). Although there is significant diversity within the kinds of integrin ligands that exist, the amino acid binding motifs remain somewhat conserved. For example, most integrins bind to ligands with aspartic acid containing sequences, such as the RGD (α5β1, α8β1, αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, αIIbβ3), LDV (α4β1), KQAGDV (αIIbβ3), RLD/KRLDGS (αVβ3, αMβ2), YYGDLR/FYFDLR (α2β1), and R...D (α1β1) (Plow et al., 2000; Ruoslahti, 1996). Furthermore, a significant amount of research has determined that the RGD amino acid motif is found within several integrin

ligands, including fibronectin, vitronectin, fibrinogen, and collagen, which explains why there are numerous integrins which recognize this particular sequence. Additionally, much work has focused on identifying the ligand for the α4β1 integrin, determining that it is localized within the CS-1 region of fibronectin to the amino acid sequence LDV (Dominguez-Jimenez et al., 1996; Komoriya et al., 1991; Mould et al., 1991; Wayner et al., 1989).

When integrins move from their closed to open conformation, they also move from a low to high ligand binding affinity state. With the use of integrin membrane proximal cytoplasmic domain deletion mutants, it was determined that the integrin subunit cytoplasmic domains are critical regulators of integrin affinity (Crowe et al., 1994; O'Toole et al., 1994). Beyond the membrane proximal domains, it has also been determined that the integrin C-terminal domains contribute to their affinity regulation (Hughes and Pfaff, 1998). More specifically, the NPxY motif within the β integrin tail is critical for regulation of integrin affinity, as several integrin activating proteins, including talin and kindlin, bind to the NPxY motif (O'Toole et al., 1995; Tadokoro et al., 2003). Talin is an intracellular protein that can bind to integrin cytoplasmic tails as well as vinculin and actin filaments, which allows it to connect integrins to the actin cytoskeleton (Ziegler et al., 2008). It is currently accepted that talin binding to the membrane proximal region of the β integrin subunit alters the spacing of the α and β subunits, ultimately initiating integrin activation (Wegener and Campbell, 2008). The contribution of integrin affinity to regulating integrin-mediated adhesion has been explored. For example, it was shown that integrin α4β1 avidity contributes to the adhesion of T cells to VCAM-1 and fibronectin (Feigelson et al., 2001). Furthermore, it was shown that increased integrin affinity could also increase Chinese hamster ovarian (CHO) cellular adhesion to the αVβ3 ligand, penton base (Pampori et al., 1999).

It is thought that integrins become activated, bind to their ligand and then form lateral interactions amongst each other to assume a ‘high avidity’ organization within the membrane (Cluzel et al., 2005). Ultimately, this clustering is hypothesized to lead to the formation of highly stable focal adhesions, which

link integrins to the actin cytoskeleton. The currently accepted sequence of events first involves integrins to bind their ligand and then clustering into nascent adhesions (Choi et al., 2008). Nascent adhesions can then mature into focal complexes, which become focal adhesions and ultimately fibrillar adhesions (Geiger et al., 2001). Previous work has demonstrated that outside-in activation using Mn^{2+} can induce the formation of integrin clusters, demonstrating that integrin activation precedes integrin rearrangement within the membrane (Cluzel et al., 2005). Conversely, reports have demonstrated that integrin clustering precedes leukocyte function-associated-antigen (LFA-1) integrin activation as well as ligand binding (van Kooyk and Figdor, 2000; van Kooyk et al., 1994). Additionally, work has demonstrated that integrin clustering is regulated by the membrane composition. For example, it has been shown that membrane clustering through the formation of membrane rafts can induce LFA-1 avidity changes (Krauss and Altevogt, 1999). Furthermore, with the use of tetraspanin CD151 knockout mice, it has been shown that CD151 contributes to α3β1 clustering in glomerular epithelial cells (Sachs et al., 2012).

1.4.3 Integrin mediated signaling

Though integrins lack catalytic activity, they can sequester intracellular molecules to mediate signaling. Integrins are bidirectional signaling molecules whereby intracellular signaling can promote integrin ligand binding (inside-out) and ligand binding can also initiate intracellular signaling (outside-in) (Das et al., 2014; Legate et al., 2009).

1.4.3.1 Outside-in signaling

Upon integrin activation, clustering, and ligand engagement, outside-in signaling can be initiated, which helps integrins to maintain adhesions with extracellular ligands. Recently the integrin “adhesome” was characterized with respect to integrin binding interactions as well as signaling interactions (Zaidel-Bar et al., 2007). The authors identify more than 156 components within this network with more than 500 interactions amongst components, illustrating the complexity of the integrin adhesive signaling network. One of the most well characterized downstream consequences of integrin outside-in signaling is the

control of actin dynamics. For example, in T cells, LFA-1 outside-in signaling can mediate the formation of an “actin cloud” in T cells in the cell center, which ultimately contributes to T cell activation (Suzuki et al., 2007). Furthermore, outside-in signaling induced by the engagement of integrin αIIbβ3 engagement in platelets initiates the formation of actin containing filopodia (Hartwig et al., 1996; Varga-Szabo et al., 2008). Additionally, another actin-dependent cellular process, cell spreading, is also mediated by the activation of integrin α2β1 in platelets (Inoue et al., 2003). Collectively, these studies demonstrate that outside-in integrin activation can regulate actin-mediated cellular processes, which ultimately can contribute to cellular adhesion and migration.

1.4.3.2 Inside-out signaling

Integrin inside-out signaling occurs when stimuli (internal or external) initiate integrin activation. Several external stimuli can initiate integrin inside-out signaling, such as T cell receptor activation (Burbach et al., 2007), selectin engagement (Green et al., 2004), purinergic receptor stimulation (Jung and Moroi, 2001) as well as chemokine receptor activation (Laudanna et al., 2002). In addition to external stimuli, integrin inside-out signaling can also be mediated through the activation of cellular signaling pathways, including protein kinase C (PKC), PI3K, and G proteins including Ras and Rho (Kinashi, 2005; Kolanus and Seed, 1997; Shen et al., 2012).

One of the cellular consequences associated with integrin inside-out signaling is the sequestration of intracellular molecules, such as talin and kindlin (Ye et al., 2011). The recruitment and activity of these molecules can be regulated by particular characteristics within the integrin cytoplasmic tail. Interactions between intracellular molecules and integrins are mediated by the NPxY motif within the cytoplasmic tail of β integrins. More specifically, proteins that contain a phosphotyrosine-binding (PTB) domain, such as talin and the kindlins can recognize the NPxY motif (Calderwood et al., 2003). Meanwhile, the phosphorylation of the tyrosine residue within this motif can regulate the particular proteins that can bind to this motif (Legate and Fassler, 2009 ; Legate et al., 2009 ; Oxley et al., 2008). Beyond the NPxY motif, amino acid residues

within integrin tails can also be phosphorylated by intracellular proteins. For example, the β1 integrin can be phosphorylated at threonine 788/789 by protein kinase C isoforms (Stawowy et al., 2005), meanwhile PKC can also promote the phosphorylation of the serines within the β4 integrin subunit (Li et al., 2013a; Rabinovitz et al., 2004). Furthermore, the β3 integrin subunit can be phosphorylated at Thr799 by AKT and PDK1 (Kirk et al., 2000) and also by extracellular signal-regulated kinase 2 (ERK2) in platelets (Lerea et al., 2007).

1.4.4 Integrins and hematopoietic stem cells and leukemia

As the niche is composed of numerous types of extracellular matrix components, integrins have been described to be important regulators of hematopoietic stem cell interactions with the niche. For example, early reports demonstrate that bone marrow cells expressing the α4 integrin subunit had increased stemness, as assessed by the ability to form colonies in a colony forming unit (CFU) assay (Williams et al., 1991). Furthermore, the authors used polyclonal antibodies to block the β1 integrin subunit, finding that injection of antibody treated cells significant inhibited the ability for cells to colonize the spleen or bone marrow. In the same year, a separate report also demonstrated that the α4β1 integrin (also known as VLA-4) is a critical regulator of hematopoiesis. Through the use of novel VLA-4 antibodies, the authors demonstrate that blocking the α4β1 integrin diminishes the lymphoid potential of long term bone marrow murine isolates, while slowing down the production of myeloid cells (Miyake et al., 1991). Furthermore, with the use of $CD34^{hi}$ human progenitor cells, it was determined that the VLA-4, VLA-5 and β2 integrins are critical for regulating HSPC adhesion to stromal cells (Teixido et al., 1992). With the use of isolated ECM components, they determined that VLA-4 and VLA-5 are specifically regulating HSPC adhesion to VCAM-1 and fibronectin. Later studies demonstrated that anti-VLA-4 antibodies inhibited the ability for murine bone marrow cells to home to the bone marrow compared to PBS or rat IgG treated cells (Vermeulen et al., 1998). This study also examined the ability for HSPCs to be maintained within the niche by treating animals with VLA-4 antibodies and examining the efflux of HSPCs into the blood. They find that anti VLA-4 antibody

treatment significantly increases the presence of HSPCs within the blood compared to controls. Finally, the authors isolated the mobilized cells from VLA-4 or control treated animals and injected them into a lethally irradiated recipient, finding that the VLA-4 treated mobilized cells had increased repopulation capacity, indicating the presence of long-term hematopoietic stem cells. Other reports have demonstrated that blocking VLA-4 can inhibit HSPC homing (Kollet et al., 2001), and may possibly act in concert with the α6 integrin subunit (Qian et al., 2006). Moreover, numerous reports have demonstrated that antibodies targeted to VLA-4 can be used to mobilize HSPCs into the blood (Papayannopoulou and Nakamoto, 1993; Ramirez et al., 2009; Zohren et al., 2008). Therefore, the α4β1 integrin is an attractive target with the potential to be used to improve bone marrow homing, as well as HSPC yield during the isolation process.

Integrins are also expressed on AML cells, which can contribute to AML chemosensitivity and serve as a prognostic marker in certain cases. An early study characterizing the expression of integrins on primary human AML blasts showed that integrins VLA-4, VLA-4, LFA-1, and LFA-3 were expressed on CD34(+) AML patient samples (De Waele et al., 1999). Additionally, it was shown that AML adhesion to fibronectin occurs through VLA-4 and VLA-5, while laminin binding occurs through VLA-6 (Bendall et al., 1993). Furthermore, the authors used β1 and β2 integrin blocking antibodies and inhibited AML adhesion to bone marrow fibroblasts, demonstrating a potential role for integrins in regulating AML niche adhesion. Further studies demonstrate that the α4β1 integrin can regulate HSC and AML pseudoemperipolesis, which refers to cellular migration below stromal cells (Burger et al., 2003). Collectively, these studies show that integrins present on AML cells can regulate AML interactions with niche components. Clinical reports have also evaluated the suitability for VLA-4 as a predictor of AML outcome. Contrary to several reports in adults (Matsunaga et al., 2003), researchers demonstrate that high VLA-4 expression is associated with improved patient outcome in child AML (Walter et al., 2010a).

As the bone marrow may protect AML cells from therapies (Ishikawa et al., 2007), much work has also focused on how integrins can regulate niche interactions for chemosensitivity. Using U937 myeloid cell lines, authors demonstrate that cells that have adhered to fibronectin have increased survival upon daunorubicin treatment (De Toni et al., 2006). The authors go on to demonstrate that crosstalk between the Wnt/β-catenin pathway and integrins occurs to promote cellular survival, indicating that integrins, with other signaling molecules, contribute to AML. Additional work focused specifically on VLA-4 mediated niche interactions shows that U937 cells adoptively transferred into NOD scid gamma (NSG) mice can be dislodged from the bone marrow with the administration of VLA-4 antibodies (Matsunaga et al., 2003). Furthermore, the authors demonstrate that adhesion to fibronectin can protect patient samples from cell death by administration of cytarabine and daunorubicin. Another report utilizes a peptide that prevents β1 engagement with fibronectin to disrupt myeloid adhesion. Using cell line models of leukemia, the authors show that peptide treatment enhances cell death upon treatment with cytarabine, similarly to treatment with VLA-4 or VLA-5 monoclonal antibodies. Further examination into how this translates into *in vivo* systems show that in a mouse model of minimal residual disease, treatment with this blocking peptide and cytarabine enhances animal survival compared to cytarabine treatment alone. Collectively, these studies demonstrate that integrins are critical regulators of AML niche interactions as well as survival signaling. Therefore, integrins should continue to be considered as potential targets for improving AML patient outcome (Matsunaga et al., 2008).

1.4.5 Cadherins

Another critical regulator of cell-cell adhesion is the cadherin family of membrane proteins. Cadherins are adhesion molecules that have structural characteristics that identify them as classical, atypical, desmosomal, atypical, and proto-cadherins (Nollet et al., 2000). Cadherins contain extracellular cadherin (EC) domains, which are amino acid repeats found on the cadherin extracellular region, as depicted in Figure 1.4. Cadherins propagate their

adhesive function by binding to other cadherins on the surface of cells; this involves both homophilic and heterophilic cadherin interactions (Niessen and Gumbiner, 2002; Shapiro and Weis, 2009). As classical cadherins have been determined to regulate hematopoietic stem/progenitor and acute myeloid leukemia cell interactions, we will focus on these molecules throughout this section.

Several classical cadherins are named for the tissue from which they were identified. For example, N-cadherin was originally identified in chicken nerves and has since been named neural cadherin (Hatta et al., 1988; Matsunaga et al., 1988), while E-cadherin was characterized early on in human epithelia (Mansouri et al., 1988; Shimoyama et al., 1989). Meanwhile, P-cadherin was characterized in mouse placenta (Nose et al., 1987) and R-cadherin in retina (Inuzuka et al., 1991). The ectodomain of classical cadherins, contain five extracellular (EC) domains, which allow cadherins to participate in homophilic interactions (Koch et al., 1999; Yap et al., 1997a). It is believed that the HAV amino acid domain within EC1 at the amino-terminus of classical cadherins mediate their adhesive potential in concert with hydrophobic pockets and tryptophan residues (Cavallaro and Christofori, 2004). Interestingly, this motif is suggested to regulate interactions between cadherins on the same cell, as well as interactions with cadherins on adjacent cells (Bunse et al., 2013; Harrison et al., 2011). It is important to note that both classical and atypical cadherins contain five EC domains (Niessen et al., 2011). As such, one of the more definitive characteristics of classical cadherins is the ability to interact with β-catenin and p120-catenin at the cytoplasmic tail (Harris and Tepass, 2010; Nollet et al., 2000). Classical cadherins also contain four calcium binding sites in their ectodomain, which have been demonstrated to contribute to cadherin-mediated adhesion (Angst et al., 2001).

Several models of the mechanism by which the ectodomain of cadherins physically interact with each other on the same cells (*cis* interactions) and adjacent cells (*trans* interactions) have been explored. The models range from

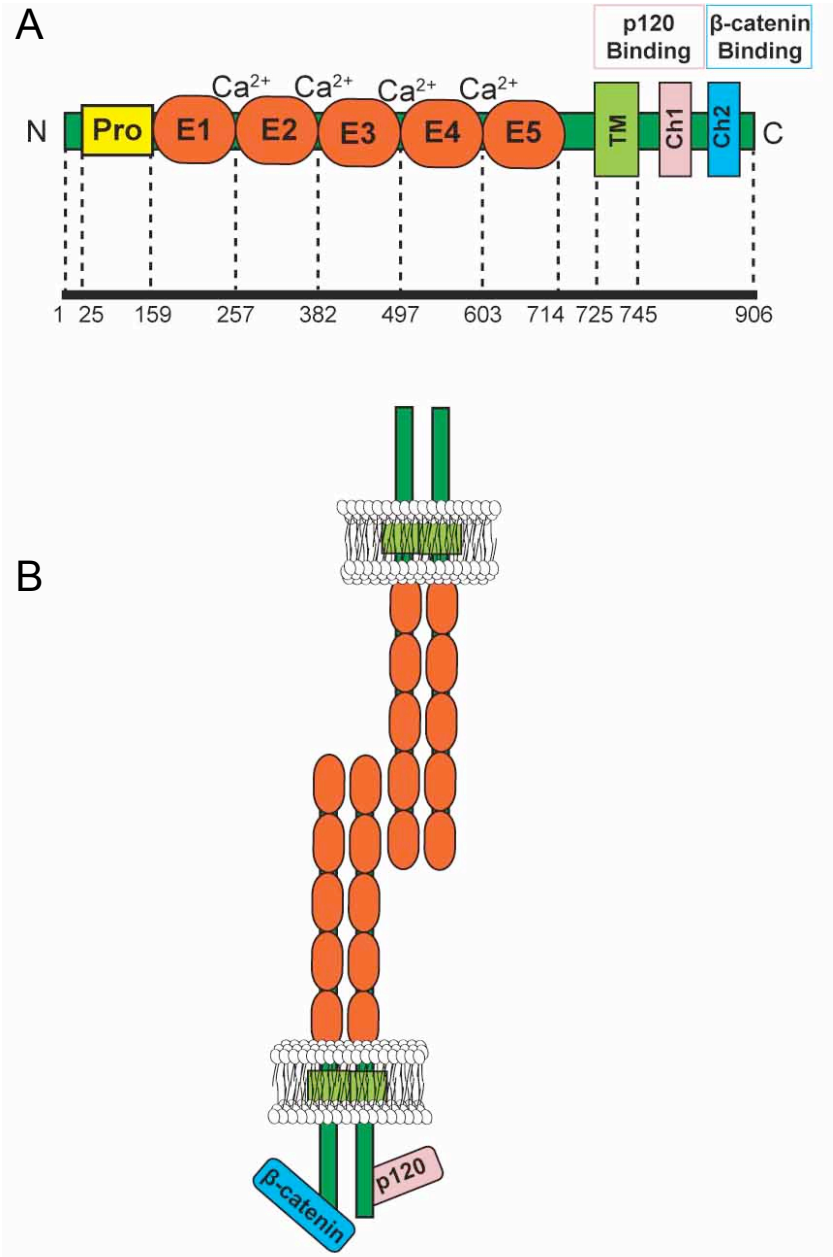

Figure 1.4: N-cadherin structure and proposed *cis* and *trans* binding models (A) cartoon depicting the full-length structure of N-cadherin. At the N-terminus is the pro-cadherin domain (pro), which is cleaved as N-cadherin moves from the Golgi apparatus to the plasma membrane. N-cadherin consists of five extracellular cadherin domains (E1-E5), which make up four Ca^{2+} binding sites. There is a small transmembrane region, followed by a cytoplasmic Ch1 and Ch2 domain, which control p120 and β-catenin binding to N-cadherin, respectively. (B) A model depicting N-cadherin interactions within the same cell (*cis*) and a potential model for how the EC1 and EC2 domains mediate N-cadherin interactions between cells (*trans*). Model adapted from (Langer et al., 2012).

cadherins existing in highly ordered straight conformations to cadherins assuming a bent conformation to promote EC1 domain interactions with neighboring cadherins (Koch et al., 1999; Niessen et al., 2011). The jury is still out regarding which models correctly predict cadherin *cis* and *trans* molecular interactions. Beyond self-interactions, cadherins can also associate with intracellular signaling molecules, such as β-catenin. This interaction helps to link cadherins to α-catenin, vinculin and ultimately to the actin cytoskeleton, which is critical for sustained cellular adhesion. (Cavallaro and Christofori, 2004 ; Yap et al., 1997a).

The most well explored cadherin in regulating HSCs is N-cadherin, although the role of N-cadherin in regulating HSPC-niche interactions remains extremely controversial (Li and Zon, 2010). Reports have found N-cadherin expressed on osteoblasts to be critical for regulating niche interactions (Arai et al., 2004; Calvi et al., 2003; Zhang et al., 2003), meanwhile others have found no such evidence for this phenomena (Kiel et al., 2009; Kiel et al., 2007). It remains to be determined whether N-cadherin is expressed on hematopoietic stem cells and if N-cadherin is a critical regulator of HSCs and their niche interactions.

Cadherins have a much better accepted role in regulating acute myeloid leukemia. In particular, N-cadherin expression has been identified as being enriched on human AML stem cells (as identified as CD34(+)/CD38(-)/CD123(+) cells) (Zhi et al., 2010). The authors characterize the proportion of N-cadherin positive AML stem cells following chemotherapy treatment, finding that this population increases under treatment conditions. These data indicate that N-cadherin could mark a chemotherapy resistant population. A follow-up study utilizing the CD34(+)/CD38(-) population of cells to represent human AML stem cells demonstrated that N-cadherin(+) LSCs have an improved ability to induce leukemia in xenograft models (Qiu et al., 2014). These studies demonstrate that N-cadherin should be considered as a potential therapeutic target for AML. However, the mechanisms underlying N-cadherin mediated chemoresistance remains to be elucidated.

1.4.6 Molecular clustering

The clustering of adhesion and signaling molecules has been demonstrated to be a critical regulator of molecular functions and in particular, cellular signaling. Early reports have identified a role for growth factor receptor dimerization/clustering in mediating downstream signaling. For example, the dimerization of the fibroblast growth factor receptor is tightly controlled by extracellular receptor regions to prevent spontaneous dimerization, which ultimately controls unwanted downstream signaling (Kiselyov et al., 2006). Additionally, the epidermal growth factor receptor requires dimerization to promote *trans*-autophosphorylation and subsequent activation of downstream signaling cascades (Schlessinger, 2000). Additional work has demonstrated that cytoplasmic proteins, such as Ras, can form short-lived nanoclusters or long-lived microclusters, which have differential signaling capacities (Cebecauer et al., 2010). As such, understanding how molecular clustering is controlled is critical for developing therapies that can be used to attenuate aberrant signaling.

Beyond the control of cellular signaling, the formation of clusters of adhesion receptors, such as integrins, can promote and strengthen cellular adhesion. In order for integrins to cluster, they must first bind ligand and assume the activated conformation and associate with talin at their cytoplasmic tails (Cluzel et al., 2005). The means by which this ultimately results in the formation of oligomers of α and β integrin subunits is not clearly defined. However, it has been hypothesized that the local lipid environment may contribute to integrin oligomerization and thus, the formation of integrin clusters (Kaiser et al., 2011). Additional evidence supports a model whereby the force generated by the cell also contributes to integrin clustering (Wehrle-Haller, 2012). Several reports have determined that the spatial arrangement of integrins is a critical component of early adhesion, adhesion strengthening, and integrin mechanotransduction (Koo et al., 2002; Roca-Cusachs et al., 2009; Selhuber-Unkel et al., 2008).

In addition to integrins, the lateral organization of cadherins can also contribute to cadherin-mediated adhesion. N-cadherin can exist as monomers and dimers on the same cell. Cadherin dimers have been determined to have an

increased probability of ligand binding compared to monomers (Zhang et al., 2009). Furthermore, through interactions between adjacent cells, N-cadherin can oligomerize into larger scale platforms, which have been demonstrated to contribute to the generation of strong cadherin-mediated adhesions (Yap et al., 1997b). The underlying mechanisms that regulate cadherin clustering are poorly defined. As such, understanding the means by which N-cadherin clustering is regulated can provide us with means aberrant N-cadherin-mediated adhesion may be attenuated.

1.5 Tetraspanins

Section adapted from (Termini and Gillette, 2017)

Tetraspanins Function as Regulators of Cellular Signaling. *Frontiers in Cell and Developmental Biology*. 2017 Apr 06 doi: 10.3389/fcell.2017.00034

1.5.1 Introduction to tetraspanins

Tetraspanins are membrane-spanning proteins with a conserved structure that function primarily as membrane protein organizers. Phylogenetic analysis identified 33 tetraspanins in humans, 37 in *Drosophila melanogaster* (Charrin et al., 2014), and 20 in *Caenorhabditis elegans* (Huang et al., 2005), while only 17 were identified in *Arabidopsis thaliana* (Boavida et al., 2013). Tetraspanins have also been identified in the ameoba, *Dictyostelium discoideum*, which exists as both a unicellular and multicellular organism (Albers et al., 2016). While some tetraspanins are expressed ubiquitously in humans, others are cell or tissue specific (de Winde et al., 2015; Maecker et al., 1997), providing a means to regulate the signal transduction associated with a breadth of cellular processes.

Members of the tetraspanin family of proteins have four transmembrane domains, which contribute to the creation of a small (EC1) and large (EC2) extracellular loop (Figure 1.5). The large extracellular loop contains a conserved Cys-Cys-Gly amino acid motif (CCG-motif), as well as two other conserved cysteine residues. EC2 of CD81 was resolved using crystallography (Kitadokoro et al., 2001), where the authors demonstrated that the four conserved cysteine

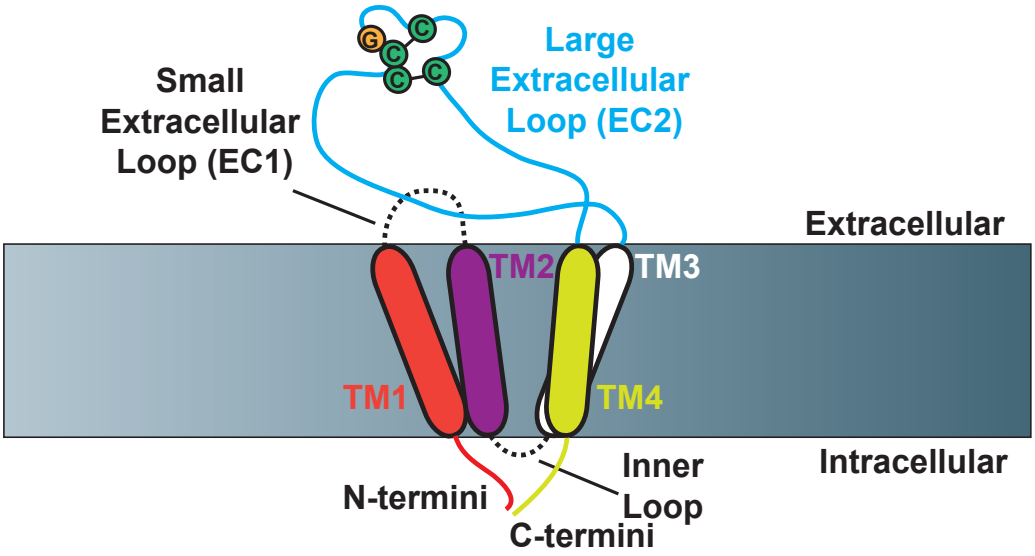

Figure 1.5: Schematic of tetraspanin molecular structure (Based on (Zimmerman et al., 2016). Cartoon depicting the structural characteristics of tetraspanins. Tetraspanins have four transmembrane domains (TM1-TM4), which create one small (EC1) and one large (EC2) extracellular loop as well as a short inner loop. The N- and C-termini of tetraspanins are localized to the intracellular side of the membrane. The Cys-Cys-Gly amino acid motif is depicted in addition to the two characteristic disulfide bonds that are formed in EC2.

resides within EC2 promote the formation of disulfide bridges, as had been suggested by previous reports (Levy et al., 1991; Maecker et al., 1997; Tomlinson et al., 1993). Moreover, molecular modeling studies using the CD81 EC2 structure as a template predicted the topography of several other tetraspanins including CD37, CD53, CD82 and CD151 (Seigneuret, 2006; Seigneuret et al., 2001). These studies demonstrated that the EC2 domain of tetraspanins consist of one conserved and one variable domain, with the conserved domain consisting of a three-helix bundle while the variable domain is unique to particular tetraspanins. A recent report resolved a crystal structure of full-length CD81, finding that the four transmembrane domains create a cholesterol-binding pocket (Zimmerman et al., 2016). Furthermore, the authors performed molecular dynamics simulations that suggest CD81 can adopt an open or closed conformation depending on whether or not cholesterol is bound.

In addition to the defining features of tetraspanins, many members of the tetraspanin family also contain post-translational modifications. For example, tetraspanins may be palmitoylated at membrane proximal cysteine residues, which was demonstrated to regulate protein-protein interactions (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002; Yang et al., 2004). Meanwhile, tetraspanins can also be N-linked glycosylated at asparagine residues, which is less clearly understood (Marjon et al., 2015; Ono et al., 1999 ; Stuck et al., 2012). Tetraspanins may also be ubiquitinated at cytoplasmic sites, which contributes to their down-regulation (Lineberry et al., 2008; Wang et al., 2012b). An example structure of tetraspanin CD82 is depicted in Figure 1.6, with the post-translational modifications highlighted. How these tetraspanin post-translational modifications impact signal transduction will be addressed in more detail later in this review.

Through their function as molecular scaffolds, tetraspanins contribute to organismal development, reproduction, and immunity (Garcia-Frigola et al., 2001; Han et al., 2012; Jarikji et al., 2009; Kaji et al., 2002; Kaji et al., 2000; Le Naour et al., 2000; Levy and Shoham, 2005; Miyado et al., 2000; van Spriel, 2011). Consistent with their expression being primarily found in multicellular organisms, it is not surprising that many processes to which tetraspanins contribute center

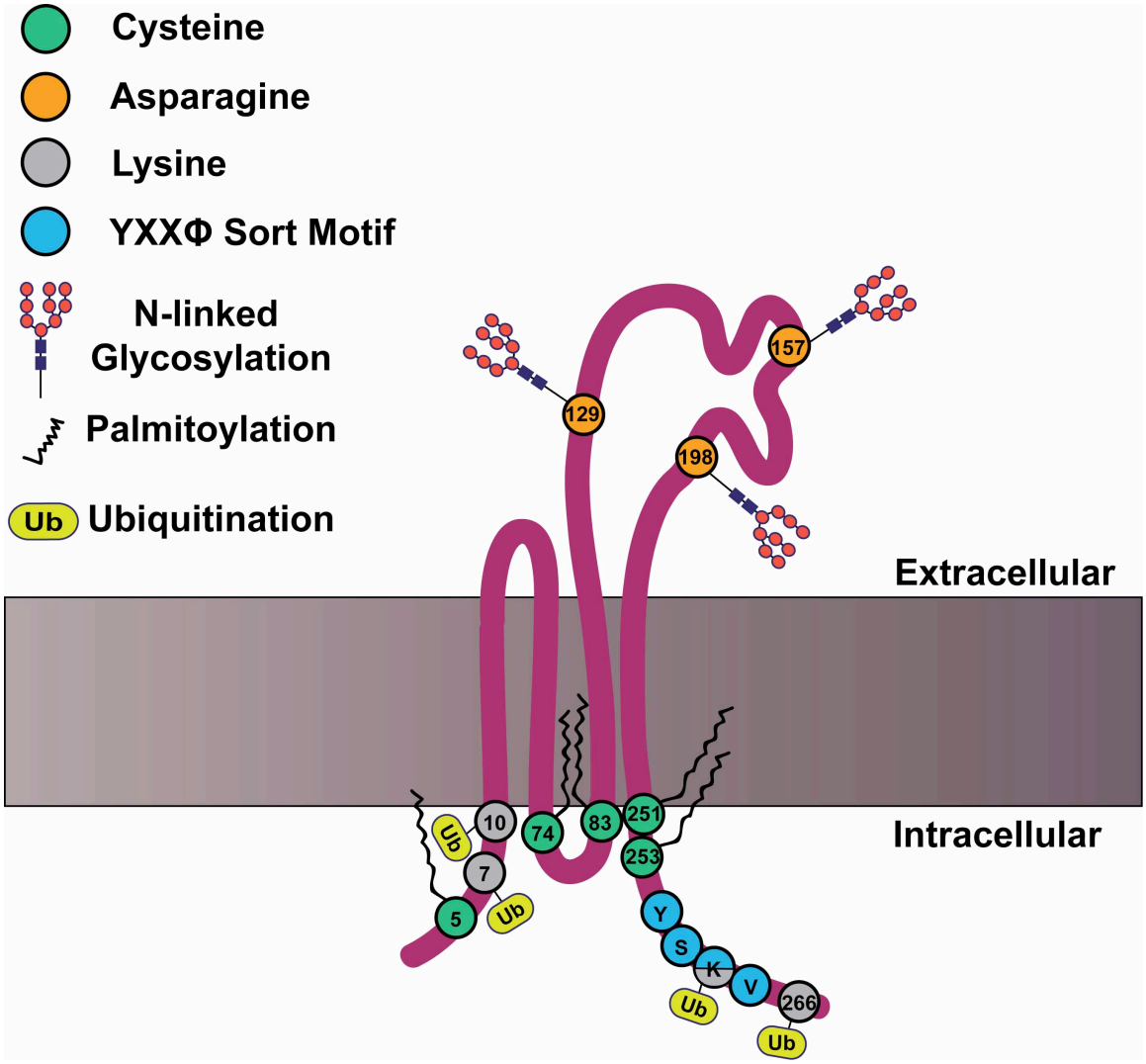

Figure 1.6: CD82 structure and motifs. Cartoon depicting CD82 topology within the plasma membrane and important motifs. CD82 contains five membrane proximal cysteine residues (shown in green) at residues 5, 74, 83, 251 and 253, which can be palmitoylated. There are three asparagine residues in EC2 (shown in orange) that are predicted to be N-linked glycosylated at residues 129, 157, 198. There are four cytoplasmic lysine residues 7, 10, 263, and 266 (shown in grey), which are predicted to be ubiquitinated. The C-terminal tyrosine based sort motif (YXXø) is depicted in blue at amino acids 261-264; for CD82 this motif is Tyr-Ser-Lys-Val.

around cell-cell- interactions. Additionally, numerous tetraspanins are also associated with the development and progression of disease, in particular, with respect to cancer and cancer cell-niche interactions (Hemler, 2013; Zoller, 2009). Although tetraspanins do not have known adhesive ligands or catalytic activity, they contribute to cellular physiology by organizing molecules within the plasma membrane into microdomains.

The proposed function of tetraspanins is to organize the plasma membrane by facilitating the formation of what are termed tetraspanin enriched microdomains (TEMs). TEMs consist of homophilic and heterophilic interactions amongst tetraspanins, interactions between tetraspanins and other membrane proteins, as well as interactions between tetraspanins and proteins at the membrane/cytoplasm interface (Charrin et al., 2014; Charrin et al., 2009b ; Hemler, 2005; Stipp, 2010). Moreover, these protein associations can occur through direct binding between tetraspanins and other proteins or through tetraspanin interactions with a common binding partner.

Interactions between tetraspanin and signaling molecules have been detected for various types of proteins, including adhesion and signaling receptors, and cytosolic signaling molecules, which are depicted in Figure 1.7. The downstream cellular consequences of these interactions vary, ranging from regulation of cellular adhesion, migration, contractility and morphology. As recent comprehensive reviews focused on tetraspanin regulation of immune signaling are available (Halova and Draber, 2016 ; Levy and Shoham, 2005), we will discuss other major classes of signaling molecules regulated by tetraspanins, as well as the cellular consequences of such regulations.

1.5.2 Tetraspanins as regulators of cellular adhesion

Through their service as molecular scaffolds, tetraspanins can interact with integrins to promote cellular adhesion. Several reports have demonstrated direct or indirect interactions between tetraspanins and integrins using biochemical approaches. For example, tetraspanins CD81, CD82, CD63 and CD53 were shown to associate with integrin α4β1 with the use of extensive

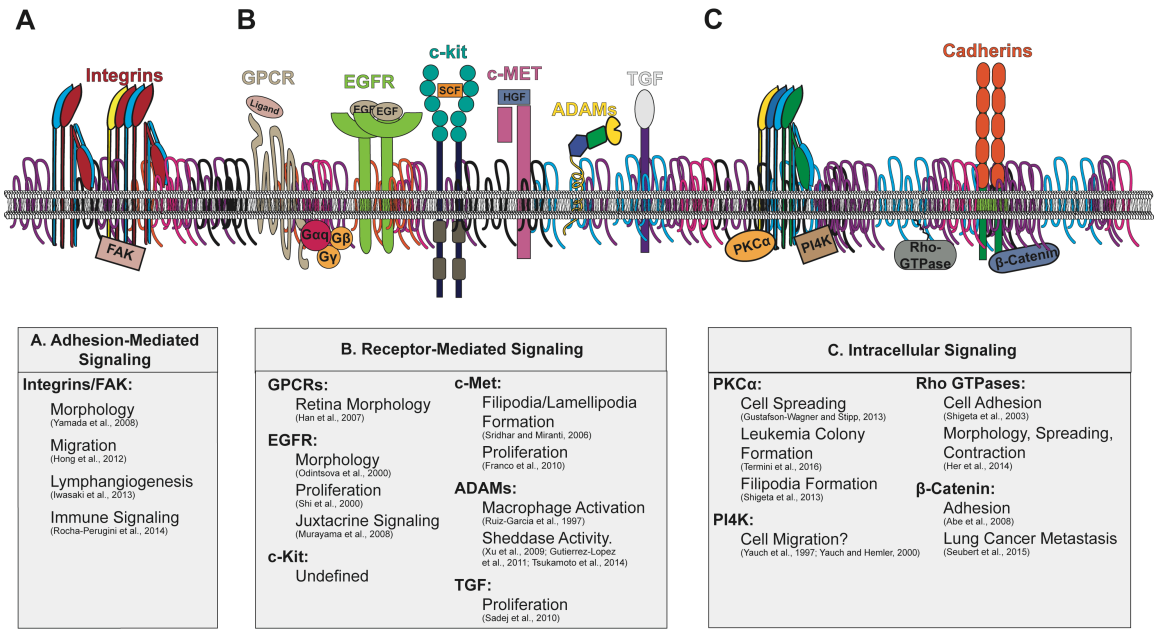

Figure 1.7: Tetraspanin enriched microdomains with signaling molecules. Illustration of the plasma membrane depicting tetraspanin interactions with membrane and cytosolic signaling molecules. The downstream signaling consequences attributed to tetraspanin regulation are indicated beneath. Key signaling molecules modulated by tetraspanins include: (A) Adhesion-Mediated Signaling (Integrins/FAK), (B) Receptor-Mediated Signaling (GPCRs, EGFR, c-Kit, c-Met, ADAMs, TGF), and (C) Intracellular signaling (PKC, PI4K, Rho-GTPases, and β-catenin).

biochemical approaches (Mannion et al., 1996). Additional work has demonstrated that tetraspanins CD9and CD151 can interact with laminin-binding integrins, such as α3β1 and α6β1 (Gustafson-Wagner and Stipp, 2013; Stipp, 2010; Zoller, 2009). Beyond the interaction between tetraspanins and integrins, tetraspanins have also been shown to regulate integrin-dependent adhesion. For example, the expression of CD82 was shown to control αVβ3-mediated adhesion (Ruseva et al., 2009) as well as α6 (He et al., 2005) and β1-dependent (Jee et al., 2007) adhesion, while tetraspanin CD37 can control β2-mediated adhesion (Wee et al., 2015).

Tetraspanins can regulate several aspects of integrins, including integrin expression, internalization, organization, and integrin-dependent signaling, all of which can contribute to cellular adhesion. For example, loss of CD82 expression led to increased αIIβ3 expression in mice (Uchtmann et al., 2015), while decreased expression of CD9 can reduce β1 integrin expression in ovarian cancer cells (Furuya et al., 2005). One mechanism by which integrin expression can be controlled is through alterations in integrin internalization and recycling. The internalization of α3β1 has been shown to be reduced in cells with decreased CD151 expression (Winterwood et al., 2006), suggesting that tetraspanins control integrin trafficking. More specifically, the YXXφ motif at the C-terminus of tetraspanins was demonstrated to mediate integrin trafficking (Liu et al., 2007). Tetraspanins can also regulate the organization of integrins, which is critical to generate stable adhesions. For example, tetraspanin CD37 can regulate the organization of α4β1 (van Spriel et al., 2012). Moreover, CD81 was shown to promote cellular adhesion to VCAM-1 by increasing the avidity of α4β1 under shear flow (Feigelson et al., 2003).

1.5.3 Tetraspanins function as regulators of cellular signaling

1.5.3.1 Tetraspanins and adhesion-mediated signaling

One of the most prominent classes of adhesion receptors which tetraspanins are known to regulate is the integrin family of proteins. Integrins are heterodimeric proteins consisting of one α and one β subunit, and this combination of subunits dictates their ligand specificity (Humphries et al., 2006).

Numerous studies identified direct and indirect interactions between integrins and tetraspanins (Berditchevski, 2001; Berditchevski et al., 1996; Mannion et al., 1996; Rubinstein et al., 1994; Slupsky et al., 1989; Stipp and Hemler, 2000; Yanez-Mo et al., 1998; Yanez-Mo et al., 2001). Though integrins lack intrinsic catalytic activity, they propagate signals through a variety of cytoplasmic signaling molecules, many of which are components of focal adhesions (Schwartz, 2001). Through a combination of imaging and biochemical studies, researchers showed that tetraspanins colocalize with the focal adhesion proteins vinculin and talin as well as myrstoylated alanine-rich C-kinase substrate, (MARCKS), which is involved in PKC-mediated signaling (Berditchevski and Odintsova, 1999). Moreover, signaling downstream of integrins is also mediated by the focal adhesion kinase, which is further regulated by tetraspanins as indicated below.

1.5.3.1.1 Focal adhesion kinase

Focal adhesion kinase (FAK) is a cytosolic protein which can interact directly with the integrin cytoplasmic tail, thereby allowing integrins to link to the actin cytoskeleton and promote downstream signaling (Schlaepfer et al., 1999). Immunoprecipitation studies demonstrated that tetraspanins CD9, CD63, CD81, CD82, and CD151 interact with the phosphorylated form of FAK (Berditchevski and Odintsova, 1999). Additionally, cells plated on anti-tetraspanin monoclonal antibodies demonstrated reduced FAK phosphorylation, further suggesting that tetraspanin scaffolding can contribute to FAK activation.

As suggested, a number of tetraspanins have been implicated in FAK regulation. It was shown that the siRNA knockdown of CD151 resulted in diminished phosphorylation of FAK, p130Cas, paxillin and Src (Yamada et al., 2008). In fact, treatment with a CD151 monoclonal antibody, which reduced CD151 interactions with α3β1, also led to a reduction in FAK phosphorylation. In an attempt to rescue this phenotype, control or CD151 knockdown cells were treated with a β1 integrin activating antibody and these data demonstrated that FAK phosphorylation could not be rescued under enforced integrin activation. As such, this study provides evidence that tetraspanins may regulate integrin-

mediated signaling through a mechanism independent of initial integrin activation. The authors quantified FAK autophosphorylation (Tyr397), which is a FAK modification stimulated by integrin clustering (Schlaepfer et al., 1999). As tetraspanins have been previously demonstrated to regulate integrin clustering (Termini et al., 2014; van Spriel et al., 2012), perhaps the loss of CD151 diminishes integrin clustering, thereby reducing FAK phosphorylation. Additionally, the presence of CD151 increased FAK and Src phosphorylation in response to plating on extracellular matrix components, which modulated GTPase activation and downstream cell migration (Hong et al., 2012). The authors demonstrated that there is a greater increase in FAK and Src activation in response to plating on laminin than fibronectin, which is consistent with previous findings that CD151 is closely associated with laminin binding integrins (Berditchevski et al., 2002; Stipp, 2010).

Another tetraspanin identified to regulate FAK activity is CD9. In the case of lymphatic dermal endothelial cells, CD9 knockdown diminished FAK phosphorylation in response to VEGF-1 administration, demonstrating that tetraspanin regulation of FAK signaling can occur through multiple activating stimuli (Iwasaki et al., 2013). The authors further demonstrated that this CD9-mediated reduction in post-adhesion signaling impaired lymphangiogenesis. Consistent with previous studies of CD151 (Yamada et al., 2008), Rocha-Perugini et al. demonstrated that silencing of CD151 or CD9 reduced the expression of phospho-FAK and phospho-ERK in response to T-cell engagement (Rocha-Perugini et al., 2014). A decrease in the accumulation of activated β1 integrins and phospho-FAK was also detected at the immune synapse in CD9 and CD151 knockdown cells, suggesting that CD9 and CD151 promote the recruitment to and retention of integrins at the immune synapse, which results in diminished integrin downstream signaling. Therefore, the influence that tetraspanins have on integrin localization provides a critical means to regulate integrin-mediated signaling.

Though not technically considered a tetraspanin, the L6 tetraspan protein, TM4SF5, has sequence characteristics and structural properties similar to

tetraspanins (Wright et al., 2000). It was shown that the intracellular loop of tetraspan TM4SF5 is critical for promoting an interaction between TM4SF5 and FAK (Jung et al., 2012). The authors performed *in vitro* pull-down assays using the N- or C-terminal cytoplasmic regions of TM4SF5 or the TM4SF5 intracellular loop to assess FAK binding. It was found that only the intracellular loop interacted with FAK, although the precise sites of association remain unknown. Future studies focused on identifying the particular amino acid residues within tetraspans that promote this association may offer potential targets to attenuate FAK signaling, which can be deregulated in numerous types of cancer (Sulzmaier et al., 2014).

1.5.3.2 Tetraspanins and receptor-mediated signaling

1.5.3.2.1 G-protein coupled receptors

G-protein coupled receptors (GPCRs) are seven membrane-spanning proteins that transmit signals with the help of intracellular G proteins (Kobilka, 2007). Upon ligand binding, GPCRs can be coupled to Gα, Gβ, and Gγ subunits to activate numerous cellular responses including calcium and potassium channel regulation, as well as phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K) signaling (Tuteja, 2009). With the use of model systems such as *Drosophila*, it was determined that tetraspanins can regulate GPCR-mediated signaling. For example, the *Drosophila*-specific tetraspanin, Sunglasses or Sun, is required for the light-induced down-regulation of rhodopsin, a light-sensitive GPCR (Xu et al., 2004). Interestingly, Sun was concentrated in the retina and removal of Sun resulted in retinal degeneration. Moreover, the authors determined that in flies with reduced Sun expression, extended exposure to light resulted in the diminished ability to down regulate rhodopsin. In line with these findings, Sun is most closely related to human tetraspanin, CD63, which is enriched within the lysosome (Metzelaar et al., 1991). Therefore, it is likely that Sun assists with GPCR signal attenuation by directing its endosomal trafficking in a similar manner to CD63. Additionally, an interaction between Sun and the Gq subunit of rhodopsin was identified, which was further proposed to help Sun promote the endocytosis of rhodopsin (Han et al., 2007).

The regulation of GPCRs by human tetraspanins has also been explored. It was shown that the GPCR, GPR56, associates with tetraspanins CD9 and CD81 (Little et al., 2004; Xu and Hynes, 2007), two tetraspanins which have also been demonstrated to interact with one another (Stipp et al., 2001). Through the use of mass spectrometry, it was also determined that the G protein subunits, $G\alpha_{11}$ $G\alpha_q$ and Gβ associate with CD81 and further immunoprecipitation studies demonstrated that this association is not detected with tetraspanins CD63 or CD151 (Little et al., 2004). The authors postulate that perhaps the regulatory role of tetraspanins with respect to GPCRs may be to enhance ligand binding and downstream signaling, though this has yet to be directly tested. Important future studies will involve the analysis of downstream signaling through tetraspanin-mediated changes in GPCRs, including the potential regulation of GPCR-ligand affinity.

1.5.3.2.2 Epidermal growth factor receptor

In addition to GPCRs (Han et al., 2007; Metzelaar et al., 1991; Xu et al., 2004) and integrins (He et al., 2005; Termini et al., 2014; Winterwood et al., 2006), tetraspanins have also been demonstrated to regulate the trafficking and signaling downstream of the epidermal growth factor receptor (EGFR). EGFR is a transmembrane receptor that can be activated by numerous ligands including epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). Ligand binding induces EGFR dimerization, which enhances EGFR catalytic activity (Jura et al., 2009; Valley et al., 2015). Moreover, EGFR endocytosis can serve as both a positive and negative regulatory signaling mechanism (Tomas et al., 2014). The contribution of tetraspanins in mediating EGFR trafficking has been extensively studied (Berditchevski and Odintsova, 2007; Odintsova et al., 2000; Odintsova et al., 2003).

Through a series of immunoprecipitation studies, it was shown that tetraspanin CD82 associates with EGFR and the overexpression of CD82 controls the phosphorylation kinetics of EGFR, Grb2, and Shc (Odintsova et al., 2000). It was determined that this regulation mediates the morphological response of HB2 cells to EGF stimulation. Interestingly, in cells expressing

CD82, there was a more rapid down-regulation of EGFR upon EGF stimulation compared to cells that do not express CD82, indicating that CD82 contributes to EGFR down-regulation through modified internalization kinetics. This led the authors to suggest that the presence of CD82 modulates the signaling potency of the receptor even before it is activated. Furthermore, the authors speculate that the combination of reduced CD82 and increased EGFR expression may lead to uncontrolled signaling. Therefore, CD82, and likely other tetraspanins, may provide a means to attenuate signaling through modulations in EGFR trafficking. A follow-up study found that CD82 negatively regulates ligand-induced dimerization of EGFR, but does not affect the dimerization of ErbB2 or ErbB3 (Odintsova et al., 2003). Although the authors did not examine the downstream effects of altered dimerization, they suggest that the differential compartmentalization of EGFR by CD82 might alter cellular signaling.

Further studies examined the role of the vesicular associated membrane protein (VAMP), TI-VAMP, and CD82 in regulating the surface dynamics of EGFR. In this study, knockdown of CD82 led to increased EGFR endocytosis upon EGF stimulation through increased AP-2 recruitment (Danglot et al., 2010). Furthermore, CD82 knockdown also altered the EGFR diffusion patterns on the plasma membrane and reduced ERK phosphorylation upon EGF stimulation, providing evidence that tetraspanins can regulate the spatial dynamics of proteins for controlling downstream signaling. This report provides a unique mechanism by which CD82, through cooperation with TI-VAMP and AP-2, can regulate EGFR signaling and surface dynamics. Moreover, the authors propose that these regulatory mechanisms may be in part controlled by CD82-mediated alterations in actin dynamics or the membrane lipid composition.

EGFR regulation by CD82 was also shown to mediate ganglioside production. More specifically, the overexpression of CD82 in combination with inhibition of ganglioside production resulted in increased EGFR phosphorylation in response to EGF stimulation (Li et al., 2013b). The authors speculate that significant interplay occurs between glycosphingolipid enriched microdomains and TEMs, which cooperatively regulate cellular signaling. The overexpression of

CD82 might promote EGFR clustering, which may stimulate dimerization and thereby enhance downstream EGFR signaling. Alternatively, the reduction in ganglioside production might improve EGFR phosphorylation by reorganizing the receptors into clusters within TEMs, since gangliosides have been demonstrated to contribute to TEM organization (Odintsova et al., 2006).

Beyond the prominent role of CD82 in regulating EGFR, additional studies also identified CD9 as a mediator of EGFR signaling. With the use of an autocrine system of MDCK cells co-expressing CD9 and TGF-α, TGF-α stimulation promoted EGFR activation (Shi et al., 2000). The authors also utilized a paracrine system whereby CHO cells expressing TGF-α alone or TGF-α and CD9 together were plated with 32D cells expressing EGFR. This experiment demonstrated that co-expression of TGF-α and CD9 increases EGFR activation, although the precise mechanism by which CD9 modulates EGFR signaling remains unclear. Regardless, this study provides unique insight into how CD9 may regulate cellular signaling initiated through contact between adjacent cells. Interestingly, another report investigated the effect of CD9 expression on EGFR signaling, finding that increased expression of CD9 resulted in decreased phosphorylation of EGFR, Shc, and total Grb2 expression (Murayama et al., 2008). Though these studies demonstrate opposing effects of CD9 on EGFR, they also indicate that TNF-α plays a role in mediating EGFR activation through CD9. These studies open the possibility that other tetraspanins such as CD82 may also work in concert with TNF-α, similar to CD9 and TNF-α in mediating EGFR activation. Therefore, future analyses would benefit from examining the interplay of TNF-α with other tetraspanins in regulating EGFR signaling.

1.5.3.2.3 c-Kit

The stem cell factor receptor or c-Kit (CD117) is a receptor tyrosine kinase that binds to its ligand, stem cell factor (SCF), which is also known as steel factor (SLF) or kit ligand (Lennartsson and Ronnstrand, 2012). c-Kit signaling can activate several signaling cascades, including PI3K, Src family kinases, and MAPK to name a few. Moreover, c-Kit mediated signaling can control numerous cellular processes including migration, survival and the differentiation of

hematopoietic progenitor cells. With the use of immunoprecipitation studies, it was determined that c-Kit associates with tetraspanins CD9, CD63 and CD81 and this interaction was enhanced upon treatment with SCF (Anzai et al., 2002). Although the authors found increased phosphorylation of c-Kit within the immunoprecipitated fraction, they determined that this does not enhance kinase activity in response to SCF treatment. Rather, the kinetics of SCF binding to c-Kit were altered when c-Kit associated with CD63. The authors suggest that this might be because free c-Kit is internalized upon SCF binding, implying that perhaps the CD63/c-Kit complex is more stable on the cellular surface. While this study alludes to a role for tetraspanins in regulating c-Kit phosphorylation, further analysis is necessary to determine the downstream consequences of tetraspanin mediated c-Kit activation. Additionally, the possibility that tetraspanins, such as CD63, might stabilize c-Kit and modulate signaling through alterations in protein trafficking could have significant impact on specific leukemias where c-Kit expression and activation are known to be dysregulated (Boissel et al., 2006; Corbacioglu et al., 2006; Goemans et al., 2005; Ikeda et al., 1991; Paschka et al., 2006).

1.5.3.2.4 c-Met

c-Met is a receptor tyrosine kinase that can activate numerous pathways to promote cellular survival, motility, and proliferation (Birchmeier et al., 2003). Hepatocyte growth factor (HGF) binding to c-Met causes c-Met dimerization, which helps to initiate various cellular signaling cascades including AKT, ERK/MAPK, and JNK (Organ and Tsao, 2011). Furthermore, the overexpression of CD82 diminished the phosphorylation of c-Met in response to integrin ligand engagement, resulting in reduced Src phosphorylation (Sridhar and Miranti, 2006). In the case of invasive tumor situations, the authors' data suggest that the loss of CD82 leads to enhanced activation of c-Met through integrin activation. Although the regulatory mechanism remains unknown, this study provides a clear indication that tetraspanins can modulate c-Met mediated signaling downstream of integrin engagement.

It was also shown through immunoprecipitation studies that CD82 and c-Met interact (Takahashi et al., 2007). Moreover, the authors demonstrated that upon the ectopic expression of CD82, activation of c-Met with HGF led to increased formation of lamellipodia and filipodia through modulations in GTPase activities. Additionally, the ectopic expression of CD82 also prevented c-Met association with Grb2 and PI3K, implicating that CD82 has an inhibitory role with respect to these binding events. As such, perhaps the Grb2 and PI3K binding sites within c-Met become inaccessible in the presence of the c-Met/CD82 interaction.

The regulatory role of CD82 with respect to c-Met-mediated signaling has also been extended to controlling ERK1/2 and AKT signaling in hepatocellular carcinoma cells (Li et al., 2013b). An alternative report focused on CD151 with respect to Met signaling, showing that knockdown of CD151 led to diminished HGF-induced proliferation (Franco et al., 2010). The researchers determined that CD151 knockdown decreased tyrosine phosphorylation of the β4 integrin subunit, which decreased MAPK signaling through ERK in response to HGF. Therefore, this study suggests that the c-Met-CD151-β4 complex is critical for MAPK signaling. While the molecular link between tetraspanins and ERK or AKT downstream of c-Met remains an open question, this work implicates integrins as a possible connection.

1.5.3.2.5 Transforming growth factor signaling

Transforming growth factor α (TGF-α) is synthesized as a transmembrane protein, which can become cleaved by metalloproteinases to release soluble TGF-α (Pandiella and Massague, 1991). This cleavage is stimulated by endotoxins (Breshears et al., 2012; Liu et al., 2013b) and reactive oxygen species (Boots et al., 2009) and is mediated primarily by ADAM17 (Peschon et al., 1998), but also by ADAM10 (Hinkle et al., 2003) and MeprinA (Bergin et al., 2008; Minder et al., 2012; Singh and Coffey, 2014). Moreover, TGF-α can interact with and activate EGFR on neighboring cells (Moral et al., 2001; Schlessinger and Ullrich, 1992; Thorne and Plowman, 1994). An association between CD9 and transmembrane TGF-α was identified and found to be

dependent on the TGF-α ectodomain (Shi et al., 2000). The experimenters illustrated that the cleavage of TGF-α was inhibited by CD9, implicating a role for the association between CD9 and TGF-α as a means of protecting TGF-α from proteolytic cleavage. The authors suggested that the inhibition of TGF-α cleavage feeds into enhanced TGF-α induced EGFR activation, which can increase cellular proliferation. This study provides evidence that tetraspanins, such as CD9, can promote cellular signaling by stabilizing transmembrane proteins, thereby providing a potent activation stimulus to mediate juxtacrine signaling. Protein kinase C (PKC) and MAPK signaling can also regulate TGF-α cleavage (Baselga et al., 1996; Fan and Derynck, 1999). As tetraspanins can regulate PKC and MAPK signaling (Termini et al., 2016; Zhang et al., 2001), a closer examination into the interplay between these molecules in mediating TGF-α signaling may provide a more comprehensive view of the complex regulatory networks at play within TEMs.

A follow up study demonstrated that CD9 expression enhances TGF-α expression at the cell surface using MDCK cells (Imhof et al., 2008). Here, CD9 was shown to promote the trafficking of TGF-α from the Golgi to the cell surface by stabilizing the glycosylated and prodomain-removed forms of TGF-α. Furthermore, the authors demonstrated that the expression of TGF-α and CD9 alters actin organization and focal adhesion formation, supporting the notion that the combination of CD9 and TGF-α expression produces dramatically different signaling responses than the expression of TGF-α alone. Therefore, the tetraspanin expression profile should be considered when characterizing TGF-α signaling, particularly in many cancers where TGF-α expression is thought to support cancer progression (Kenny and Bissell, 2007).

Additionally, the contribution of tetraspanins to the regulation of the TGF isoform TGF-β1 has been assessed. Researchers used CD151 knockdown MDA-MB-231 cells and determined that in the presence of TGF-β1, CD151 knockdown cells had a significantly decreased proliferative rate compared to control cells (Sadej et al., 2010). More specifically, in the CD151 knockdown cells, TGF-β1 stimulation led to reduced p38 phosphorylation, resulting in

decreased metastasis. Mechanistically, the authors propose that CD151 modulations of the plasma membrane may alter the distribution of TGF-β1 receptors and downstream signaling. Future studies may focus on determining how CD151 modulates the molecular organization of the TGF receptor, as this may provide a mechanism to regulate downstream signaling.

1.5.3.2.6 A disintegrin and metalloproteases

The A Disintegrin and Metalloprotease (ADAM) family of transmembrane and secreted proteins contribute to the regulation of cellular adhesion, migration, proliferation and signaling (Seals and Courtneidge, 2003). As the name states, ADAMs contain a disintegrin and a metalloprotease domain. While the metalloprotease domain can cleave extracellular matrix components and mediate ectodomain shedding of cytokines, growth factors, the disintegrin domain can interact with integrins. Recent comprehensive reviews provide insight on the role that tetraspanins play in regulating membrane proteases, with a particular emphasis on their role in regulating ADAM10 and ADAM17 (Matthews et al., 2016 ; Yanez-Mo et al., 2011). Initial reports demonstrated that ADAM10 is associated with CD9, CD81 and CD82, indicating that ADAM10 likely exists within TEMs. Interestingly, treatment with anti-tetraspanin antibodies stimulated the release of TNF-α and EGF in an ADAM10-mediated manner. Furthermore, through mass spectrometry studies and extensive immunoprecipitation studies, Tspan12 was found to associate with ADAM10, which contributed to the ability of ADAM10 to process amyloid precursor protein for shedding (Xu et al., 2009b). Using several mutated TSPAN12 constructs, this association was determined to be regulated by EC1, the C-terminal tail and TSPAN12 palmitoylation. More recent co-immunoprecipitation studies revealed that the subgroup of TspanC8 tetraspanins (Tspan5, 10, 14, 15, 17 and 33) interact with ADAM10 (Dornier et al., 2012). Additionally, ADAM17 was also found to associate with tetraspanin CD9 in leukocytes and endothelial cells, which diminishes ADAM17-mediated TNF-α and ICAM-1 shedding. Interestingly, CD9 can regulate the catalytic activity of ADAM17 with regards to shedding of LR11 in monocytes, promonocytes and B-lymphoblastoid cell lines (Tsukamoto et al., 2014). As ADAMs are implicated in

regulating various cancer cell types (Mochizuki and Okada, 2007), the role of tetraspanins in regulating ADAMs in malignant cells will provide significant insight and perhaps a means to attenuate aberrant ADAM activity.

ADAMs are produced as immature, inactive, preforms in the endoplasmic reticulum (ER). During trafficking from the ER to the plasma membrane, the enzyme's prodomain is removed and ADAMs are then rendered catalytically active (Seals and Courtneidge, 2003). Interestingly, it was determined that TspanC8 contributes to ADAM10 maturation and ultimately the stabilization of ADAM10 at the cell surface (Prox et al., 2012). Furthermore, Tspan33 knockdown in erythrocytes resulted in diminished ADAM10 surface expression. Meanwhile, ADAM10 surface expression remained unchanged in platelets, demonstrating that tetraspanin regulation of ADAM10 is likely cell-type specific (Haining et al., 2012). Additionally, the role of Tspan33 in regulating ADAM10 for the control of macrophage activation was recently explored (Ruiz-Garcia et al., 2016). Researchers utilized Tspan33 overexpressing Raw 264.7 cells and demonstrated that increased Tspan33 expression results in increased ADAM10 processing, consistent with the earlier aforementioned studies.

1.5.3.3 Tetraspanins and intracellular signaling

Although tetraspanins are known to primarily affect the properties of other membrane proteins, they have also been shown to regulate cytoplasmic signaling molecules. Signaling proteins are often recruited to the cytoplasmic interface of the plasma membrane where they initiate signaling and TEMs can serve as a potential membrane recruitment site. Therefore, in the following section, we will review how tetraspanins control the localization, kinetics, and signaling properties of cytosolic proteins.

1.5.3.3.1 Protein kinase C

The Protein Kinase C (PKC) family of enzymes are regulators of numerous cellular processes, many of which can be deregulated under cancerous conditions (Griner and Kazanietz, 2007). There are 10 identified PKC isozymes, which can be classified into three different types. Classical PKC isozymes, which include PKCα, PKCβI, PKCβII and PKCγ, are calcium

dependent enzymes. Meanwhile, the calcium independent novel PKC isozymes are PKCδ, ε, η and θ and the atypical PKC isozymes are PKCζ and PKCι. The structure of PKC isozymes can be broken into several components. Firstly, all PKC isozymes consist of one catalytic and one regulatory region (Mackay and Twelves, 2007). Secondly, PKCs are built from four conserved domains, C1-C4 (Figure 1.8) (Coussens et al., 1986; Parker et al., 1986). Conventional PKC isozymes consist of C1-C4, while novel PKC isozymes contain a modified C2 domain and atypical isozymes lack a C2 domain completely, and instead contain a modified C1 domain (Newton, 2010). These structural differences have significant consequences on the means by which these enzymes are activated and signal. For example, the C2 domain of conventional PKC isozymes has a Ca^{2+} binding site, while the C1 domain has a binding site for diacylglycerol (DAG)/phorbol esters. Meanwhile, the modified C2 domain within novel PKC isozymes lacks Ca^{2+} binding ability (Newton, 1995). Due to their involvement in leukemia and HSC regulation, we will focus on classical PKCs.

Classical PKCs (cPKCs) contain a flexible hinge region between the C2 and C3 domains, allowing autoinhibition during times of inactivation (Newton, 2010). In order to ensure appropriate signal regulation, cPKCs contain a pseudosubstrate region at the N-terminus, which interacts with the substrate binding pocket within the kinase domain to promote this autoinhibition (Rosse et al., 2010). Upon activation, the pseudosubstrate can be cleaved by proteolysis (Orr et al., 1992). As PKCs can be activated by various stimuli, it is important to note that Ca^{2+} binding to the C1 domain promotes PKC interactions with DAG or phorbol esters, while Ca^{2+} binding to the C2 domain promotes the interaction of PKC with anionic phospholipids at the membrane (Dempsey et al., 2000). During times of activation, cPKCs can also interact with scaffold proteins at the membrane to induce their activation. For example, the receptors for the activated C kinase (RACKs) proteins, can interact with PKCs to relieve their autoinhibition (Ron and Mochly-Rosen, 1995). Beyond RACK proteins, PKCs can also interact other types of scaffolding proteins, such as tetraspanins (Zhang et al., 2001),

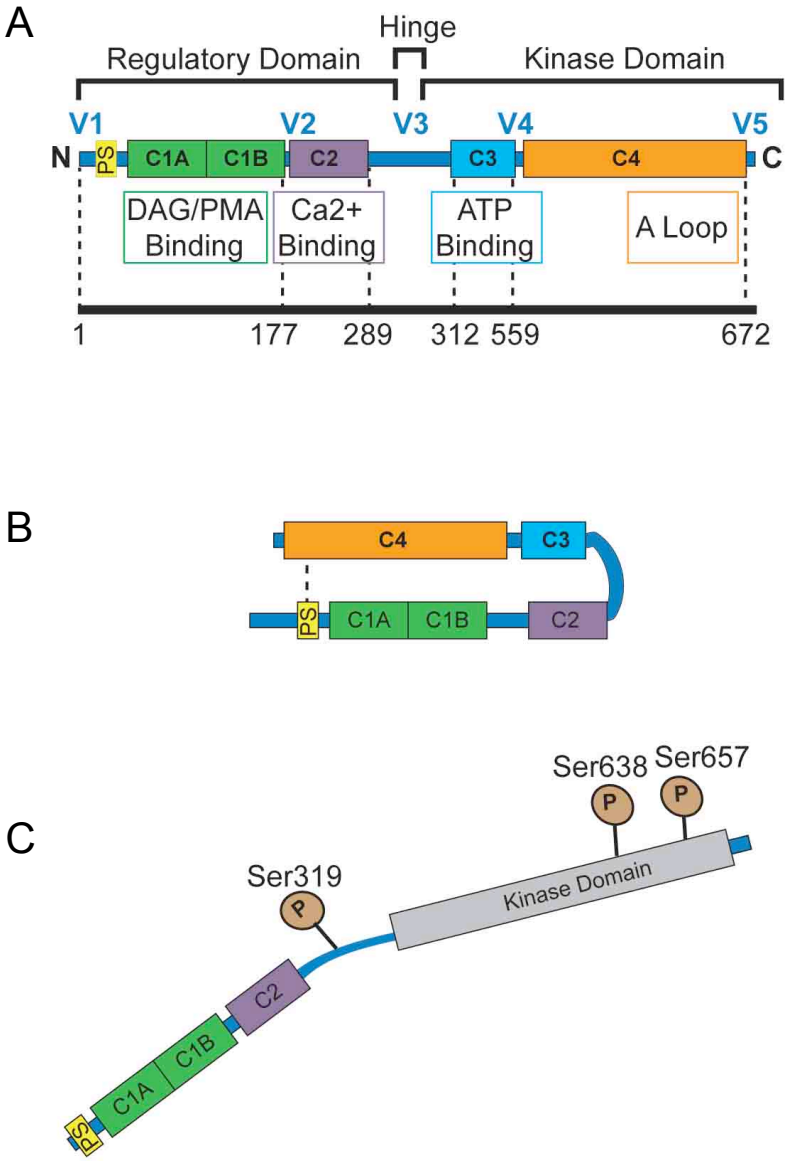

Figure 1.8: PKCα structural domains and protein conformations. (A) PKCα is a 672 amino acid protein with structural features depicted above. PKCα consists of a regulatory domain, made up of the PS pseudosubstrate), V1, C1A, C1B, V2, and C2 domains, while the kinase domain consists of the C3, V4, C4 and C5 domains. The V3 domain is the hinge region, which allows PKC to autoinhibit itself. The C1 domains are responsible for DAG/PMA binding, while the C2 domain promotes Ca^{2+} binding. Meanwhile, the C3 domain confers ATP binding and the C4 domain contains the activation loop, which helps PKCα autoinhibit itself. (B) In an inactive state, the hinge region provides PKCα with flexibility, which allows an interaction between C4 and the pseudosubstrate. (C) Upon PKCα activation, the kinase domain becomes free. PKCα becomes phosphorylated on Ser319 within the hinge region, as well as Ser638 and Ser657 within the C4 region.

caveolin (Oka et al., 1997), annexins (Hoque et al., 2014) and A-kinase anchoring proteins (Greenwald et al., 2014).

Once activated and recruited to the plasma membrane, PKC can now elicit a downstream response**.** Although PKCs have been discovered for decades, there is still very little known about their downstream targets (Steinberg, 2008). PKC enzymes have been shown to phosphorylate myrstoylated alanine-rich c-kinase receptor substrate (MARCKS) (Herget et al., 1995), which is an actin-filament binding protein (Hartwig et al., 1992). PKCs can also phosphorylate the myosin light chain II (Liu et al., 2013a), PKD2 (Navarro and Cantrell, 2014; Waldron et al., 2001), and Ras GEFs (Jun et al., 2013). It will be valuable for future work to continue to examine PKC substrates, as many remain undiscovered. Beyond the direct substrates, PKC can induce several well-known signaling pathways. For example, PKCs can serve as an anti- or pro-apoptotic signaling regulator depending on the cell type (Lucas and Sanchez-Margalet, 1995) and a role for PKC in mediating apoptosis through caspases has been established (Gutcher et al., 2003; Nowak, 2002). PKCs can also promote cellular growth and proliferation signaling through MAPK signaling, in particular through ERK1/2 activation (Clerk and Sugden, 2001). Meanwhile, PKCs also play a role in mediating differentiation, which has been extensively studied in myeloid lineages (Clemens et al., 1992).

PKC can also mediate differentiation signaling in hematopoietic progenitor cells. For example, when granulocyte-macrophage colony-forming cells were transfected with a constitutively activated form of PKCα, there was an increase in the production of macrophages when cells were treated with macrophage colony stimulating factor, G-CSF, or IL-3, indicating that PKC activation can regulate progenitor differentiation (Pierce et al., 1998). Another group used real time polymerase chain reaction (rtPCR) to quantify PKC isozyme transcript levels in CD34(+) cells and progenitor cells, finding fluctuations in isozyme expression upon differentiation (Oshevski et al., 1999). Beyond differentiation, PKC has also been shown to regulate HSC homing. A previous study demonstrates that treatment of CD34(+) human HSPCs with chelerythide chloride, which inhibits

PKCs, reduces HSPC homing to the bone marrow and spleen (Kollet et al., 2001). An additional report shows that treatment with the PKC inhibitor, GF109203X, reduces cellular migration and appears to do so through the activation of FAK (Wang et al., 2000).

There is evidence that PKCs can also be deregulated in several cancers (Griner and Kazanietz, 2007), in particular in leukemias (Redig and Platanias, 2008). For example, PKCs can be deregulated in chronic lymphocytic leukemia (CLL), giving CLL cells a survival advantage (Alkan et al., 2005; Barragan et al., 2002). Additionally, the expression of PKCα (Guzman et al., 2007) and PKCβ (Ruvolo et al., 2011) is increased in AML cells compared to healthy CD34(+) counterparts. Meanwhile, it was found that patients with expression of active BCL-2 coupled with active PKCα exhibited decreased overall survival, demonstrating the clinical potential of PKC in leukemias (Kurinna et al., 2006). However, the precise mechanisms by which PKC regulates leukemia cell and patient survival remain unclear. Future work focused on providing mechanistic insight into the action of PKC in regulating these processes will have significant clinical value.

The interaction between tetraspanins and PKC was originally demonstrated in K562 cells using an elaborate series of immunoprecipitation experiments (Zhang et al., 2001). The experimenters used phorbol 12-myristate 13-acetate (PMA), which mimics diacylglycerol (DAG) to activate PKC (Castagna et al., 1982). Under PMA stimulated conditions tetraspanins CD9, CD53, CD81, and CD82 individually interact with PKCα and not with PI3K. Additionally, they determined that CD81 and CD151 associate with PKCβII. Moreover, in a PKCα pull-down, β1, α3, and α6 integrins were detected in complex with PKC. Therefore, it was suggested that tetraspanins serve to link PKC to integrins. In order to assess the tetraspanin domains that control PKC associations, chimeric mapping was performed by replacing portions of CD9 with portions of the non-PKC associating tetraspanin, A15/Talla1. These findings demonstrated that PKC association with tetraspanins occurs outside of the short inner loop, the large outer loop, and transmembrane 3 or 4.

A recent report also demonstrated that tetraspanin CD151 regulates skin squamous cell carcinoma through STAT3 and PKCα signaling (Li et al., 2013a). Utilizing wild type or CD151 ablated A431 epidermoid carcinoma cells, it was shown that the loss of CD151 reduces STAT3 activation in response to 12-O-Tetradecanoylphorbol-13-acetate (TPA) stimulation, which is another known activator of PKCα. The authors found that PKCα only associates with α6β4 upon TPA stimulation when CD151 is present. Together, these data suggest that perhaps the role for CD151 is to recruit PKCα into close proximity with the α6β4 integrin, which ultimately aids in the phosphorylation of α6β4. As such, these data build upon previous implications that tetraspanins link PKC to integrins (Zhang et al., 2001), but also provide evidence that this scaffolding is important for epidermal proliferation and STAT3 activation.

Another interesting report investigated how CD9, CD81 and CD151 expression affects PKCα association with TEMs (Gustafson-Wagner and Stipp, 2013). It was demonstrated that CD9/CD81 knockdown diminishes the ability for the α3 integrin to associate with PKCα, which delays cell spreading on laminin and directed migration. In contrast, CD151 knockdown enhanced the association of PKCα with the α3 integrin, while promoting cell migration on collagen-I. The authors propose that CD9/81 may serve as linkers of PKC to the α3 integrin subunit, or there might be an indirectly associating molecule at play. Furthermore, the authors propose that perhaps upon CD151 depletion, there is increased association between PKC and α3 due to the loss of CD151, which makes CD9/81 available to fully associate with α3, thereby promoting PKC-integrin association. This study provides substantial evidence that the roles of tetraspanins CD9, CD81 and CD151 are unique in their regulation of PKCα-integrin interactions.

Further examination into the regulatory role of tetraspanins with respect to PKC-mediated signaling has uncovered many unique cellular responses. For example, treatment of A431 cells with Calphostin C to inhibit PKCα reduced filipodia extensions as well as E-cadherin puncta formation, demonstrating the involvement of actin in tetraspanin-regulated PKC signaling (Shigeta et al.,

2003). The authors suggest that CD151 directly or indirectly associates with PKCα, which they propose may activate Cdc42 to promote filipodia formation.

A more recent report from our laboratory demonstrated that CD82 regulates PKCα signaling in acute myeloid leukemia (AML) (Termini et al., 2016). Using quantitative FRET imaging and KG1a AML cell lines that overexpress wild type CD82 or a palmitoylation deficient form of CD82 (Delandre et al., 2009), we found that upon PMA stimulation, PKCα was recruited to the plasma membrane where it associates with CD82. However, upon extended PMA stimulation, this PKCα/CD82 association is reduced in cells overexpressing the palmitoylation deficient form of CD82, demonstrating that the palmitoylation of CD82 regulates the stability of the PKCα interaction. We went on to use super-resolution imaging to examine how the scaffolding properties of CD82 regulate the macromolecular clustering of PKCα and found that upon disruption of the CD82 scaffold, there is a significant reduction in the size of PKCα clusters. Moreover, using CD82 knock-down cells, we found that while PKCα is still recruited to the membrane upon PMA stimulation, large-scale PKCα clusters are not detected. This change in PKCα clustering was then linked to alterations in downstream ERK1/2 signaling that influenced the aggressive phenotype of AML (Termini et al., 2016). Interestingly, the kinetics of PKCα oligomerization were recently quantified and modeled using HEK cells where they found that the intramolecular clustering of PKCα contributes to downstream phosphorylation (Bonny et al., 2016). Collectively, these studies illustrate that the modulation of signaling molecule clusters may serve as an important regulatory mechanism for stabilizing and/or attenuating signal transduction pathways. Moreover, our work implicates tetraspanins as critical mediators of cluster size and stability. Future super resolution imaging studies focused on identifying how the clustering of tetraspanins can modulate downstream signaling through PKC and other molecules such as Rac or Cdc42 would be valuable to help clarify how tetraspanins and PKCα mediate cytoskeleton-dependent cellular responses such as adhesion and migration.

An interesting link was also discovered between PKC and EGFR-mediated signaling that is enhanced by CD82. c-Cbl is an ubiquitin ligase recruited to EGFR where it assists with receptor down-regulation (Joazeiro et al., 1999). The authors found that PKC mediates c-Cbl phosphorylation upon EGF stimulation in CD82 expressing H2B cells (Odintsova et al., 2013). The phosphorylation of c-Cbl serves as a negative regulator of enzyme function (Ryan et al., 2006), which may be responsible for inhibiting EGFR downregulation. Therefore, without CD82 present, EGFR can be quickly downregulated as PKC is not present to regulate c-Cbl. Collectively, these studies provide substantial evidence that implicates tetraspanins as signaling scaffolds that promote the close proximity of PKC with integrins, EGFR and cytoplasmic proteins like c-Cbl.

1.5.3.3.2 Phosphatidylinositol 4-kinase

Phosphatidylinositol 4-kinase (PI4K) catalyzes the conversion of phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate (PI4P), which is an important intermediate for lipid-mediated signaling (Clayton et al., 2013). A series of biochemical experiments demonstrated that PI4K exists within α3 integrin and CD63 containing TEMs (Berditchevski et al., 1997). The authors suggest that perhaps TEMs are responsible for linking the α3β1 integrin to PI4K. A follow up study from the same group explored this further, demonstrating that immunoprecipitation of α3 or CD151 yields similar levels of PI4K activity based upon PI4P production (Yauch et al., 1997). Additionally, using cells with diminished α3 expression, CD151 was pulled down, demonstrating that there is still PI4K associated with the complex. Conversely, immunodepletion of CD151 resulted in significantly diminished lipid kinase activity associated with α3, while CD63 and/or CD81 deletion did not have as significant of an effect. Collectively, these data implicate CD151 as a critical linker between PI4K and α3β1, which the authors suggest may support cell migration.

A subsequent follow up study demonstrated that PI4K associates with tetraspanins A15/TALLA1, CD63, CD151, CD9 and CD81, however it does not appear to associate with NAG-2, CD53, CD37 or CD82 (Yauch and Hemler,

2000). Moreover, PI3K and PI4P5K activity were not detected in CD63, CD81 and CD151 complexes, indicating that perhaps the association is specific to PI4K. Studies with CD9/CD82 chimeras were unsuccessful at determining the site of association with PI4K. Therefore, a closer examination into the structural domains within tetraspanins that contribute to their association with PI4K could provide insight into the mechanism by which tetraspanins may regulate the catalytic activity of PI4K and downstream responses.

1.5.3.3.3 GTPases

Rho GTPases mediate signal transduction by switching between a GTP-bound (active) and GDP-bound (inactive) state (Bishop and Hall, 2000). There are numerous effector proteins downstream of GTPases including PI3K, PI-4-P5K, MEKK1, and DAG kinase. The Rho family GTPases Rac1, RhoA, and Cdc42 as well as the Ras family of GTPases translocate to the plasma membrane upon activation (Collins, 2003), where their regulation by tetraspanins continues to be defined.

For example, CD151 was demonstrated to regulate Cdc42 for the control of cellular adhesion. Using A431 cells, CD151 antibody treatment or CD151 overexpression was found to increase Cdc42 activation, which the authors suggest controls actin reorganization, promoting filopodia-based adhesions (Shigeta et al., 2003). Another study assessed how the coexpression of CD9 and TGF-α regulates GTPase signaling, finding increased and decreased levels of activated Rac1 and RhoA respectively, with Cdc42 levels remaining unchanged upon coexpression of CD9 and TGF-α (Imhof et al., 2008). This shift in signaling was determined to be due to enhanced EGFR signaling, which ultimately contributed to enhanced stress fiber formation. Additionally, the overexpression of CD82 was shown to decrease the proportion of GTP-bound Rac1, while RhoA and Cdc42 levels remained unchanged (Liu et al., 2012).

Previous work also demonstrated that CD151 promotes the association between CD151-β1 complexes and Ras, Rac1 or Cdc42. Immunofluorescence imaging showed that CD151 regulates the translocation of Rac1 and Ras to the membrane and promoted colocalization with β1 integrins (Hong et al., 2012).

Interestingly, through the use of a CD151 chimera with disrupted α3β1 integrin association, the authors showed that this mutant is unable to recruit Rac1 to the membrane. Therefore, integrins also have the capacity to link GTPases to tetraspanins in a manner similar to what was previously proposed for PKC and tetraspanins (Li et al., 2013a; Zhang et al., 2001). An association between Rac1 and the C-terminal, cytoplasmic region of CD81 has also been suggested based on the use of an eight amino acid C-terminal tail peptide (Tejera et al., 2013). Future experiments that mutate or delete the CD81 C-terminal tail will be important to demonstrate that such a mutation eliminates Rac1 association, further validating the interaction. Furthermore, upon EGF stimulation, it was shown that knockdown of CD81 increases Rac activation. A more recent study identified a correlation between CD9 expression and GTP bound Rac1 expression in acute lymphoblastic leukemia patient samples (Arnaud et al., 2015). Moreover, this group also determined that the C-terminal tail of CD9 is important for regulating Rac1 activation. Interestingly, the C-terminal region of CD9 has two known palmitoylation sites (Charrin et al., 2002), and Rac can also be palmitoylated (Tsai and Philips, 2012). Therefore, it is possible that these post-translational modifications may help to anchor tetraspanins and GTPases into similar membrane compartments.

Tetraspanin regulation of RhoA signaling, which can promote changes in cytoskeletal organization, has also been characterized (Sit and Manser, 2011). Using human aortic smooth muscle cells, CD9 knockdown decreased the expression of GTP-bound RhoA, leading to defects in cellular morphology, spreading and contraction (Herr et al., 2014). The authors suggest that integrins are involved in CD9-mediated alterations in RhoA activation by possibly stabilizing integrin-ECM interactions, augmenting RhoA activation. Interestingly, a recent report demonstrates that the loss of CD151 in breast cancer cells resulted in increased RhoA activation as quantified using FRET biosensors (Novitskaya et al., 2014). These data are contrary to Hong et al. (Hong et al., 2012), who showed no change in Rho activation upon CD151 depletion. However, the change in FRET efficiency detected was less than 5%, which

would likely be below the detection of the small GTPase protein pull-down assays used by Hong et al. Moreover, a separate report demonstrated that the knockdown of CD151 in human dermal microvascular endothelial cells resulted in an increase in RhoA-GTP and decreased Rac1-GTP (Zhang et al., 2011). Future studies focused on the mechanism by which tetraspanins can modulate GTPase activation will be important for determining how certain tetraspanins may be targeted to control specific GTPase activities in specialized cell types.

1.5.3.3.4 β-Catenin

β-catenin is a component of the Wnt signaling pathway that binds to the cytosolic portion of cadherins to initiate cellular signaling (Valenta et al., 2012). Through this complex formation, β-catenin promotes the internalization and recycling of E-cadherin, thereby destabilizing the complex and ultimately reducing cell-cell adhesion. Researchers determined that ectopic CD82 expression in h1299 cells relocalizes β-catenin to E-cadherin at the cell membrane, which stabilizes complex formation (Abe et al., 2008). Furthermore, they showed that ectopic CD82 expression increased cancer cell aggregation. To assess the downstream consequences of altered β-catenin localization, the authors stimulated cells with EGF or HGF, demonstrating that ectopic expression of CD82 diminished β-catenin phosphorylation. While β-catenin phosphorylation is known to destabilize the E-cadherin complex, the mechanism for tetraspanin involvement remains to be clearly defined. Based on our previous work with N-cadherin (Marjon et al., 2015), we speculate that the CD82 scaffold might contribute to cadherin clustering, which may stabilize β-catenin membrane interactions, thereby protecting β-catenin from phosphorylation and down-regulation.

More recently, CD63 was shown to stabilize β-catenin signaling. In this study, shRNA knockdown of CD63 decreased β-catenin protein expression levels, which was suggested to occur through diminished levels of inactive GSK3β, leading to increased levels of phosphorylated β-catenin (Seubert et al., 2015). Furthermore, decreased levels of the β-catenin targets, MMP-2 and PAI-1, were detected, demonstrating CD63-mediated changes in downstream β-catenin

signaling. The authors went on to find that the reduced expression of CD63 diminishes the metastatic potential of lung cancer cells, while the overexpression promoted tumor aggressiveness. However, modulations in signaling induced by CD63 overexpression were not explored. A previous study provided evidence that disrupting the interaction between the α3β1 integrin and CD151 enhanced β-catenin phosphorylation (Chattopadhyay et al., 2003). Therefore, it is plausible that the combination of integrins and tetraspanins serves to stabilize β-catenin within TEMs.

1.5.3.4 Tetraspanin post-translational modifications and signaling

1.5.3.4.1 Palmitoylation

S-palmitoylation is the addition of a 16-carbon fatty acid chain, palmitate, to cysteine residues of either cytoplasmic or membrane proteins (Blaskovic et al., 2013). Palmitoylation of cytoplasmic proteins promotes membrane anchoring, while palmitoylation of membrane proteins facilitates trafficking and membrane organization. Palmitoylation has been confirmed for tetraspanins CD9, CD151 (Yang et al., 2002), CD81 (Delandre et al., 2009), and CD82 (Mazurov et al., 2007), however other tetraspanins also contain conserved cysteine residues that are predicted to be palmitoylated. The defined role for palmitoylation is to modulate TEM formation (Yang et al., 2004). Therefore, we took a closer examination of how tetraspanin palmitoylation contributes to the signaling that occurs downstream of TEM associated proteins.

For example, the expression of the palmitoylation deficient form of CD151 weakened its association with integrins (Berditchevski et al., 2002), resulting in diminished phosphorylation of AKT in response laminin-5 engagement. These data indicate that palmitoylation-mediated disruption of TEMs can reduce downstream signaling responses. Additionally, a palmitoylation deficient form of Tetraspanin12 was shown to have diminished association with ADAM10, resulting in decreased ADAM10 activity as assessed by amyloid precursor protein (APP) shedding (Xu et al., 2009b). Recent work from our lab has shown that overexpression of a palmitoylation-deficient form of CD82 diminishes PKC

membrane stabilization, reducing ERK1/2 activation and downstream leukemia colony formation (Termini et al., 2016). Collectively, these studies demonstrate that tetraspanin palmitoylation contributes significantly to the regulation of downstream cellular signaling. Intracellular signaling molecules such as Ras (Eisenberg et al., 2013), Rac (Tsai and Philips, 2012), and PKC (Ford et al., 1998) can themselves be palmitoylated to assist with their membrane anchorage. As tetraspanin palmitoylation is thought to regulate lateral protein associations within TEMs, perhaps tetraspanin palmitoylation functions in concert with the palmitoylation of cytoplasmic proteins to produce stable membrane interactions critical for sustained signaling.

1.5.3.4.2 Glycosylation

Although the large extracellular loop of many tetraspanins has been demonstrated to have one or more potential N-linked glycosylation sites, little is known about the functional consequences of this post-translational modification. The N-glycosylation pattern of CD82 was recently identified using proteomics and glycomics, determining that there are three putative N-glycosylation sites (Wang et al., 2012a). Previously, these sites were suggested to regulate apoptosis, however the researchers did not examine the signaling that led to these apoptotic changes (Ono et al., 1999). Interestingly, the photoreceptor-specific tetraspanin retinal degeneration slow (RDS) can also be glycosylated (Conley et al., 2012; Kedzierski et al., 1999). More recently, the function of RDS glycosylation was re-examined by expressing a glycosylation deficient version of RDS in mice, which identified differential functional outcomes in cones versus rod photoreceptor cells (Stuck et al., 2015). Moreover, the authors determined that glycosylation regulates the formation of RDS complexes with another tetraspanin ROM-1, demonstrating that glycosylation can modulate tetraspanin complex formation. A recent report from our laboratory examined the role of CD82 glycosylation with respect to acute myeloid leukemia homing (Marjon et al., 2015). In this study, we demonstrated that mutation of the three glycosylation sites within CD82 to inhibit glycosylation resulted in increased AML cell homing to the bone marrow, which we linked to increased molecular packing of N-

cadherin via super resolution imaging. Although we have yet to examine signaling deficits in cells with disrupted CD82 glycosylation, it is possible that these changes in the molecular organization of N-cadherin may modulate the activation or stability of p120 catenin or β-catenin signaling downstream of N-cadherin.

1.5.3.4.3 Ubiquitination

Protein ubiquitination is important for regulating cellular signaling by selectively targeting proteins for degradation. Both CD81 and CD151 were shown to interact with gene related to anergy in lymphocytes (GRAIL), which promotes tetraspanin ubiquitination, ultimately downregulating surface tetraspanin expression (Lineberry et al., 2008). Interestingly, it was determined that these tetraspanins can only be ubiquitinated at their N-terminus. Through mutational studies, it was shown that mutation of K8 and K11 diminished the ubiquitination of CD81, while mutation of K8, K11 and K17 ablated the ubiquitination of CD151. More recently it was demonstrated that TSPAN6 interacts with the adaptor mitochondrial antiviral signaling (MAVs) in 293T cells to inhibit RIG-I-like receptor (RLR) mediated signaling (Wang et al., 2012b). The authors went on to show that induction of RLR signaling promoted the ubiquitination of TSPAN6 at K11, K16, and K43, which are sites found within the TM1 of TSPAN6. Additionally, the authors determined that TSPAN6 ubiquitination serves to inhibit the formation of the signalosome, effectively down-regulating RLR signaling. As ubiquitination can target proteins for degradation, we suspect that tetraspanin ubiquitination will be a regulatory mechanism to allow for specific and efficient attenuation of tetraspanin-mediated signaling.

1.5.4 Concluding remarks

Tetraspanins and their formation into TEMs enable the compartmentalization of membrane receptors within the plasma membrane. In this review, we focus on how tetraspanins also serve to connect these membrane-associated molecules with intracellular signaling complexes. It is now clear that tetraspanins regulate diverse cell signaling pathways that impact a breadth of biological processes. However, though numerous signaling molecules

have been demonstrated to associate with tetraspanins, the mechanisms by which tetraspanins precisely modulate signal transduction remains relatively undefined. Future studies focused on how domains and motifs within tetraspanins promote or perhaps attenuate cellular signaling will help us understand the specific mechanisms used by this family of proteins to control signaling. Many laboratories are now using sophisticated imaging techniques to provide novel insight into the spatiotemporal interactions mediated by tetraspanins and TEMs. These studies will help to define how the scaffolding properties of tetraspanins contribute to the formation, stabilization and dynamics of signal transduction complexes at the plasma membrane. Moreover, these studies may provide the needed insight to establish tetraspanins and TEMs as potential therapeutic targets for the modulation of aberrant signal transduction that mediates processes such as inflammation, wound healing, and various types of cancer.

1.6 Tetraspanins and leukemia

One of the earliest clues of the involvement of tetraspanins in regulating leukemia comes from a study first examining the expression profile of tetraspanins in healthy HSCs (Burchert et al., 1999). The authors utilized flow cytometry to determine that CD82 is expressed in peripheral blood leukocytes from healthy donors, with differential expression patterns found depending on the class of leukocyte examined. The authors also examined blood from leukemic patients, findings increased CD82 surface expression in blood samples from patients with chronic myeloid leukemia, chronic lymphoid leukemia and AML. These data demonstrate that CD82 expression may serve as a marker of the aggressiveness of blood cancers, a notion that researchers have more recently examined in mechanistic detail.

Previous studies demonstrate that the CD34(+)/CD38(-) AML cell fraction highly express CD82, which contributes to cell migration to mesenchymal stem cells. The authors also show that treatment with CD82 shRNA can significantly reduce AML colony growth in the CD34(+)/CD38(-) fraction, indicating that CD82 is critical for AML propagation in these cells (Nishioka et al., 2013). Two follow up

reports from the same laboratory demonstrate that CD82 expression can also regulate STAT5 signaling in AML cells, which promotes cellular survival (Nishioka et al., 2015a; Nishioka et al., 2014). Furthermore, the Yokoyama laboratory has also shown that the utilization of a CD82 monoclonal antibody can enhance AML cell death under chemotherapeutic conditions (Nishioka et al., 2015c). In line with these findings, there are also tetraspanin antibodies under clinical evaluation for the treatment of chronic lymphocytic leukemia (Beckwith et al., 2015).

It has been shown that tetraspanin CD9 (Leung et al., 2011) as well as CD82 (Larochelle et al., 2012) can regulate the adhesion and homing of CD34(+) HSPCs. As tetraspanins can promote healthy interactions between HSCs and their microenvironment, several other groups have also examined how leukemic cells can utilize tetraspanins to integrate into the niche and propagate disease pathologies. For example, a recent report from the Reya laboratory demonstrated using Tspan3 knockout mice that Tspan3 is essential for the migration of AML cells into the bone marrow niche and ultimately AML disease progression (Kwon et al., 2015). Collectively, these studies have provided significant insight regarding the role of tetraspanins in mediating disease progression and survival. Future work that examines the mechanism underlying tetraspanin mediated leukemia progression will likely lead to more specific treatment options for patients with aberrant tetraspanin expression.

1.7 Summary and discussion

Although the work described throughout the introduction has provided significant insight regarding the mechanisms by which HSCs and AML are regulated, there are still several unanswered questions within the field. For example, the involvement of integrins in mediating HSC adhesion and homing has been established, the precise molecular means by which integrins assist with this task remain unclear. Based on previous evidence demonstrating the importance of the α4 integrin subunit in regulating HSC adhesion and homing, as well as reports that tetraspanins can regulate the molecular avidity of integrins, we hypothesize that CD82 regulates the molecular organization of the α4 integrin

subunit to promote HSPC adhesion. Furthermore, previous studies indicate that the palmitoylation of tetraspanins can regulate protein-protein interactions. Therefore, we also hypothesize that CD82 palmitoylation controls the organization of the α4 integrin subunit. In Chapter 2, we utilize cell lines to model HSPCs and determine how CD82 expression and palmitoylation control HSPC adhesion to ECM components. Our findings demonstrate that the overexpression of CD82 results in increased adhesion to fibronectin, and that this increased in mediated through the α4β1 integrin. Furthermore, overexpression of a palmitoylation mutant form of CD82 does not result in an increase in HSPC adhesion to fibronectin. Additionally, using quantitative imaging techniques, we determine that CD82 palmitoylation is a critical regulator of the molecular density of the α4 integrin subunit.

As cadherins are critical regulators of AML-niche interactions, we also examined the role of CD82 in mediating AML homing and adhesion. In Chapter 3, we use cell line models of AML as well as AML blast patient samples to examine how CD82 expression and post-translational modifications regulate AML bone marrow homing. Our data demonstrate that knockdown of CD82 expression leads to a reduction in bone marrow homing compared to control cells. Furthermore, in patient samples with increased CD82 expression, we find that there is an increase in bone marrow homing, demonstrating a role for CD82 expression in mediating this process. Further examination shows that this adhesion occurs in an N-cadherin dependent manner, whereby CD82 palmitoylation regulates the number of N-cadherin clusters, meanwhile, CD82 N-linked glycosylation controls the packing of N-cadherin clusters. These data provide a role for the molecular organization of N-cadherin in mediating AML niche-interactions, which can be regulated by CD82.

We have also studied the role of tetraspanin CD82 in regulating signaling in AML with a particular focus on PKCα signaling. In Chapter 4, we use AML cell lines to demonstrate that the scaffolding of CD82 is critical for sustained PKCα signaling. Using quantitative imaging techniques, we find that CD82 palmitoylation regulates PKCα membrane interactions as well as interactions with

CD82. Additionally, we find significant defects in PKCα clustering upon palmitoylation mutation, demonstrating that disruption of the scaffold diminishes PKCα organization at the membrane. Furthermore, increased CD82 expression leads to sustained ERK1/2 signaling, which ultimately feeds into propagating a more aggressive AML phenotype in clonogenic assays.

Collectively, these chapters establish tetraspanin CD82 as a critical regulator of HSPC adhesion, AML homing/adhesion and AML signaling. Furthermore, through its role in controlling integrins, cadherins and signaling proteins, CD82 may be a valuable target for therapeutics addressing a variety of different cancers. Additionally, our work provides significant insight into the role of tetraspanin palmitoylation, which may prove to be a suitable therapeutic target on CD82 and potentially several other palmitoylated tetraspanins.

Chapter 2: The membrane scaffold CD82 regulates cell adhesion by altering α4 integrin stability and molecular density.

Christina M. Termini[a], Maura L. Cotter[a], Kristopher D. Marjon[a], Tione Buranda[a], Keith A. Lidke[b], and Jennifer M. Gillette[a*]

[a]Department of Pathology, University of New Mexico Health Sciences Center, and [b]Department of Physics and Astronomy, University of New Mexico, Albuquerque, NM 87131

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2.1 Abstract

Hematopoietic stem and progenitor cell (HSPC) interactions with the bone marrow microenvironment are important for maintaining HSPC self-renewal and differentiation. In recent work, we identified the tetraspanin protein, CD82, as a regulator of HPSC adhesion and homing to the bone marrow, although the mechanism by which CD82 mediated adhesion remained unclear. In the current study, we determine that CD82 expression alters cell-matrix adhesion as well as integrin surface expression. By combining the super-resolution microscopy imaging technique, direct stochastic optical reconstruction microscopy (dSTORM), with protein clustering algorithms, we identify a critical role for CD82 in regulating the membrane organization of α4 integrin subunits. Our data demonstrate that CD82 overexpression increases the molecular density of α4 within membrane clusters, thereby increasing cellular adhesion. Furthermore, we find that the tight packing of α4 into membrane clusters is dependent upon CD82 palmitoylation and the presence of α4 integrin ligands. In combination, these results provide unique quantifiable evidence of CD82's contribution to the spatial arrangement of integrins within the plasma membrane and suggest the regulation of integrin density by tetraspanins as a critical component of cell adhesion.

2.2 Introduction

Cells receive signals or cues from their surrounding environment and respond in ways to optimize survival, maintain quiescence, promote proliferation and differentiation. Stem cells, in particular, rely on physical interactions with their surrounding microenvironment or "niche" for the regulation and maintenance of proper stem cell function. In the case of hematopoietic stem/progenitor cells (HSPCs), which reside primarily in the bone marrow, direct contact with the surrounding microenvironment is essential for regulating HSPC proliferation, multipotentiation, and self-renewal (Renstrom et al., 2010; ter Huurne et al., 2010; Zhang and Li, 2008). The bone marrow niche is a complex microenvironment consisting of a number of different cellular and extracellular matrix (ECM) components including fibronectin, collagen I, III and IV, as well as laminin (Klein, 1995). In addition to the bone marrow, HSPCs can traffic into and

out of the peripheral blood, which is utilized clinically for stem cell isolations and transplantation. Furthermore, under stress conditions and/or injury, HSPCs can migrate to other tissues such as the spleen, the liver, and even the heart to aid in tissue repair and remodeling (Losordo et al., 2011; Oostendorp et al., 2000; Taniguchi et al., 1996). However, the molecular mechanisms orchestrating the interactions between HSPCs and various niche components are not well understood.

The tetraspanins are a family of multi-spanning membrane scaffold proteins that regulate intercellular interactions. CD82 (also known as Kai1) is a member of the tetraspanin family of proteins, which are evolutionarily conserved proteins present in most eukaryotes that function in many aspects of cell physiology as mediators of cell adhesion, membrane trafficking and cell signaling (Charrin et al., 2009a). One of the most distinct features of tetraspanins is their ability to associate in *cis* with other tetraspanins, integrins, members of the immunoglobulin superfamily of cell adhesion molecules and signaling receptors, thereby forming tetraspanin-enriched microdomains (TEMs) (Bassani and Cingolani, 2012; Charrin et al., 2009a; Hemler, 2008a). Formation of TEMs enables tetraspanins to serve as molecular facilitators or organizers for a number of transmembrane proteins. Tetraspanins also recruit and maintain intracellular signaling molecules in close proximity with membrane proteins, thus regulating downstream biochemical pathways (Choi et al., 2009; Hemler, 2005; Li et al., 2010; Wang et al., 2007a).

In its role as a protein scaffold, CD82 can form TEMs hypothesized to be critical for the organization and function of several membrane proteins including integrins (Han et al., 2012; He et al., 2005). Integrins are heterodimeric cell adhesion receptors consisting of one α and one β subunit and are expressed by all multicellular organisms. Components of the ECM as well as specific cell surface receptors serve as integrin ligands (Barczyk et al., 2010; Harburger and Calderwood, 2009; Johnson et al., 2009). Integrins are capable of transmitting signals across the plasma membrane, which can promote cell migration, survival, differentiation and motility. Specifically, the α4 integrin, which is highly enriched in

HSPCs, regulates HSPC migration, homing, proliferation, and differentiation (Arroyo et al., 1999; Coulombel et al., 1997; Papayannopoulou and Nakamoto, 1993). Furthermore, previous studies in mice show that defects occur in HSPC homing and short-term engraftment upon conditional α4 knockout (Scott et al., 2003), and anti-α4β1antibodies mobilize HSPCs into the bloodstream (Papayannopoulou and Nakamoto, 1993). How CD82 can regulate integrin-mediated cellular and molecular functions including migration, adhesion, and signaling remains unclear. Furthermore, fundamental questions concerning the formation and regulation of TEMs and their potential modulation of integrin organization also still exist.

Previous work from our lab identified CD82 as a regulator of HSPC homing and osteoblast adhesion (Larochelle et al., 2012). Using monoclonal antibodies specific to CD82, we demonstrated an inhibition of HSPC homing to the bone marrow and were able to reduce HSPC adhesion to osteoblasts. In the current study, we set out to identify the mechanism by which CD82 regulates HSPC niche interactions. We find that CD82 expression alters integrin expression by contributing to integrin stabilization on the plasma membrane through modulation of integrin internalization and recycling. Furthermore, we apply direct stochastic optical reconstruction microscopy (dSTORM) analysis to evaluate how CD82 and modifications in the palmitoylation sites of CD82 regulate the nanoscale clustering of integrins. Our data suggest that CD82 modulates the molecular packing of α4 molecules within clusters, thereby regulating the local molecular density of α4. As such, CD82 functionally regulates niche adhesion by modifying the organization of integrins into tightly packed clusters, which serves to strengthen cell adhesion to the ECM.

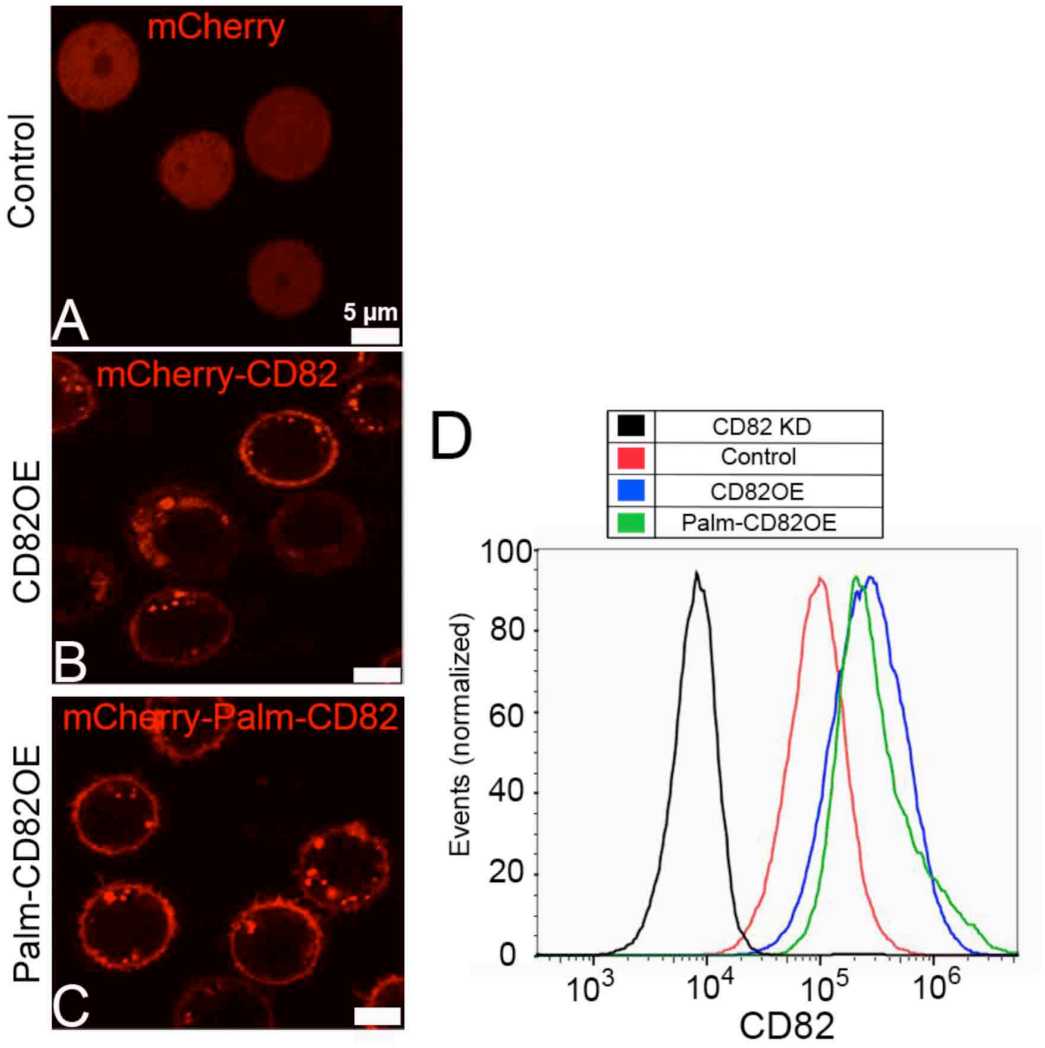
2.3 Results

2.3.1 CD82 expression regulates cell-matrix adhesion

To begin analyzing the molecular mechanism by which CD82 regulates HSPC interactions with niche components, we generated a CD82 overexpressing cell line (CD82OE) using the human acute myelogenous leukemia progenitor-like cells, KG1a. We created a fusion protein where CD82 was tagged with the

mCherry fluorescent protein at the amino-terminus. Stably transfected cells were selected and sorted. Figure 2.1B illustrates the plasma membrane and endosomal localization of mCherry-CD82, which is consistent with the localization of endogenously expressed CD82 (Larochelle et al., 2012). A stably expressing mCherry control cell line (control) was also generated (Fig.2.1A). Flow cytometry analysis indicates a two-fold increase in CD82 surface expression between overexpressing and control cells (Fig.2.1D). Since our previous data suggested that CD82-specific antibodies alter *in vitro* adhesion and *in vivo* homing, we evaluated the CD82OE cells for changes in ECM adhesion. Using a fluorescence-based adhesion assay to quantify changes in cell-matrix adhesion to various substrates, we identified an increase in cell adhesion with the CD82OE cells (Fig.2.1E). More specifically, we found that CD82OE cells display an increase in cell adhesion to laminin and an even greater increase in adhesion to fibronectin when compared to control cells. Similarly, we found that the reduction of CD82 expression could also affect cell-matrix adhesion. CD82 knockdown cells (CD82KD) were generated in the KG1a cell line using siRNA and shRNA. The CD82KD cells were found to express less than 10% of wild type CD82 expression, as determined by Western blot and flow cytometry analysis (Fig.2.1F,G). When the CD82KD cells were assessed for cell adhesion, we detected a significant decrease in cell-matrix adhesion to fibronectin (Fig.2.1H) that was rescued when the CD82KD cells were transiently transfected with mCherry-CD82 (Suppl. Fig.S.2.1A-D). In combination these data suggest a role for CD82 expression in the regulation of cell-matrix adhesion.

The function of CD82 as a molecular organizer can be regulated by the ability of CD82 to cluster and form TEMs. Based primarily on biochemical studies, palmitoylation of the intracellular cysteines of tetraspanins has been suggested to play an important role in the maintenance of tetraspanin-tetraspanin interactions and to facilitate the oligomerization and dynamic reorganization of proteins into TEMs (Berditchevski et al., 2002; Charrin et al., 2002; Kovalenko et al., 2004; Stipp, 2010; Yang et al., 2002). To assess whether the five, membrane proximal cysteine residues known to be palmitoylated in CD82 are critical for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Control
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Palm-CD82OE
Events (normalized)
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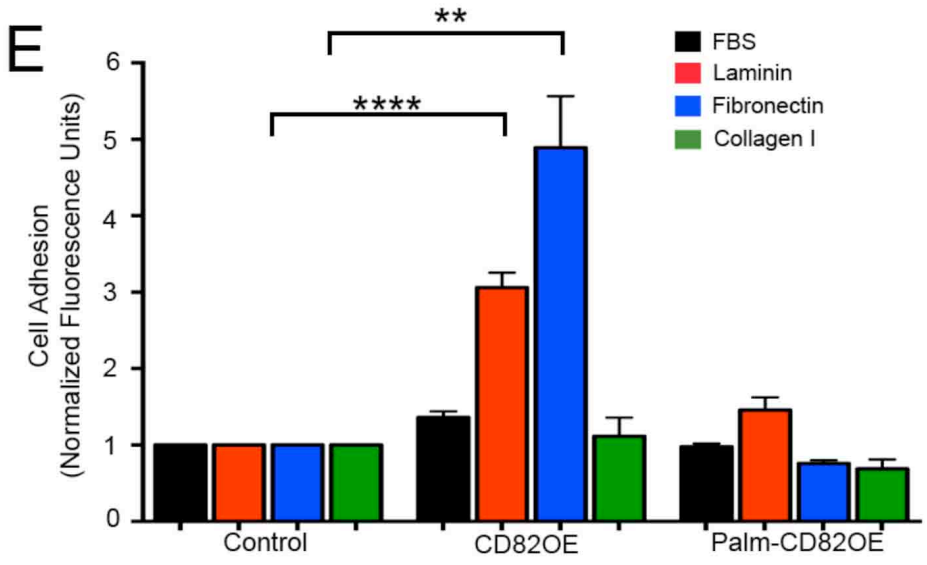
10^6
CD82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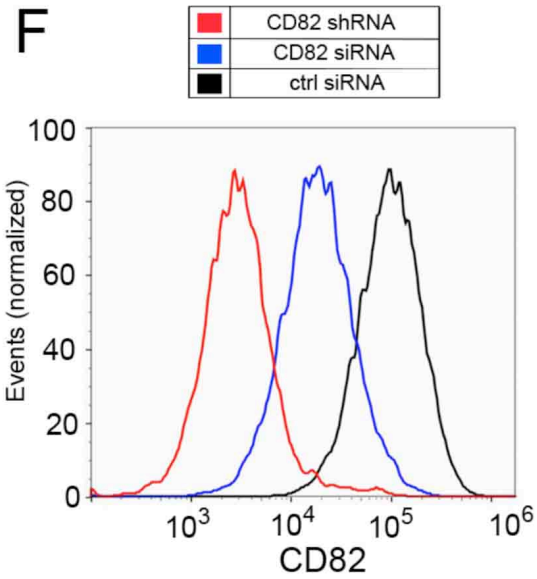
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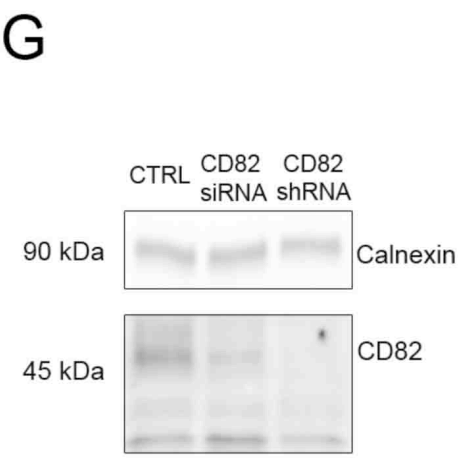
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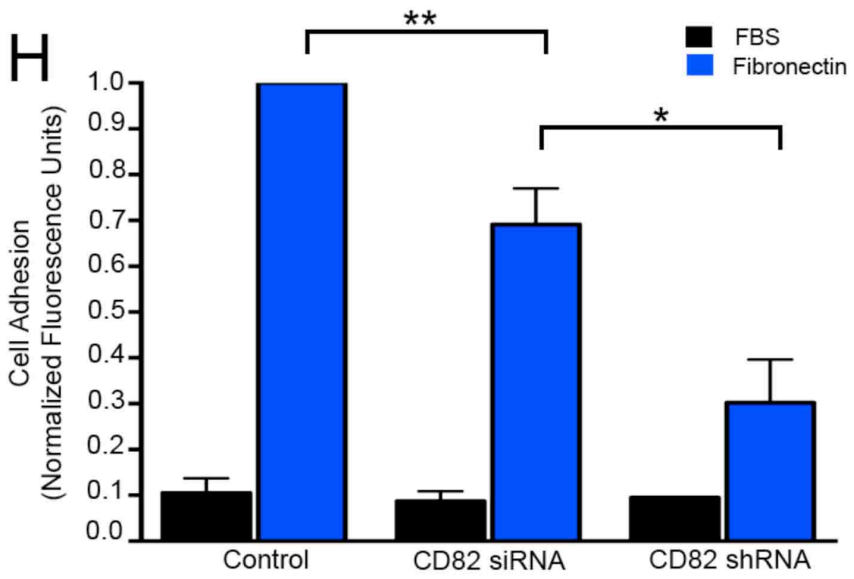
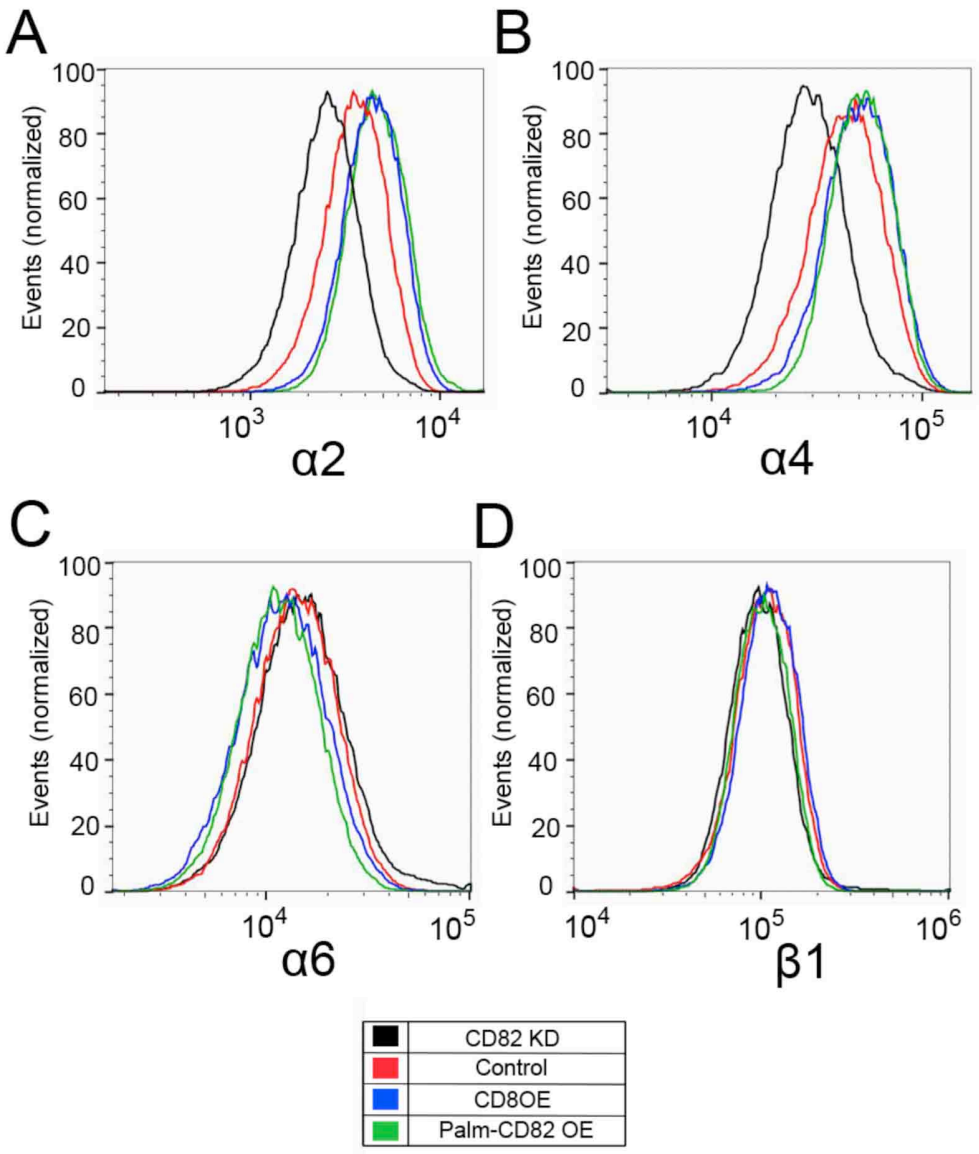


Figure 2.1: CD82 expression mediates HSPC adhesion to fibronectin. Epifluorescent images depicting stable KG1a cell lines generated with (A) mCherry, (B) mCherry-CD82, and (C) mCherry-Palm-CD82 constructs. (D) The surface expression of CD82 was analyzed for each cell line using flow cytometry. (E) Cellular adhesion of each cell line was measured using a fluorescence-based adhesion assay. Cells were plated on FBS as a control or the indicated ECM proteins. To knock-down CD82, KG1a cells were transfected with control siRNA, CD82 siRNA, and CD82 shRNA. The reduction of CD82 surface and total expression was measured by flow cytometry (F) and Western blot analysis (G). The adhesive abilities of these KD cells were then measured with the fluorescence adhesion assay (H). Error bars indicate SD; $n \geq 3$ (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

HSPC adhesion, we generated the mCherry-Palm-CD82 KG1a cells (Palm-CD82OE). The Palm-CD82 construct was generated by mutating the five cysteine residues at 5, 74, 83, 251 and 253 to serine, thereby preventing their palmitoylation (Mazurov et al., 2007). Characterization of the Palm-CD82OE cells indicates that the localization and expression of mCherry-Palm-CD82 is consistent with that of mCherry-CD82 based on epifluorescence imaging (Fig.2.1C) and flow cytometry analysis (Fig.2.1D). To assess whether the palmitoylation sites alter the ability of CD82 to regulate cell-matrix adhesion, we performed matrix adhesion assays with the Palm-CD82OE cells and found a significant decrease in adhesion when compared to the CD82OE cells. These data indicate that the palmitoylation of CD82 is essential for its ability to regulate cell-matrix adhesion.

2.3.2 CD82 expression modifies the profile of surface integrin expression

Cell adhesion to ECM proteins such as laminin and fibronectin occurs through specific integrin heterodimers. Tetraspanins form complexes with integrins, which can regulate ligand binding and integrin signaling properties (Johnson et al., 2009; Kotha et al., 2008; Nishiuchi et al., 2005; Sridhar and Miranti, 2006). Furthermore, recent studies suggest that tetraspanins can also regulate integrin trafficking and complex assembly (He et al., 2005; Liu et al., 2007; Xu et al., 2009a). Therefore, we set out to determine whether CD82 expression levels affect the surface expression of specific integrins that are critical for HSPC adhesion. Flow cytometry analysis suggests that while the levels of CD82 expression have minimal effect on α3, α5, β1, or β7 surface levels (Fig.2.2D, Suppl. Fig.S.2.2), CD82 overexpression results in an increase in α2 and α4 expression (Fig.2.2A,B). This observed increase in α2 and α4 is consistent with the detected increase in adhesion to laminin and fibronectin, respectively (Fig.2.1E). In contrast, we detect a significant reduction in α6 expression in the CD82OE and the Palm-CD82OE cells. This decrease in α6 likely results in the availability of β1 to bind to α4, which could explain the lack of β1 expression increase in the CD82OE cells (Fig.2.2C). Our cytometry data also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Events (normalized)
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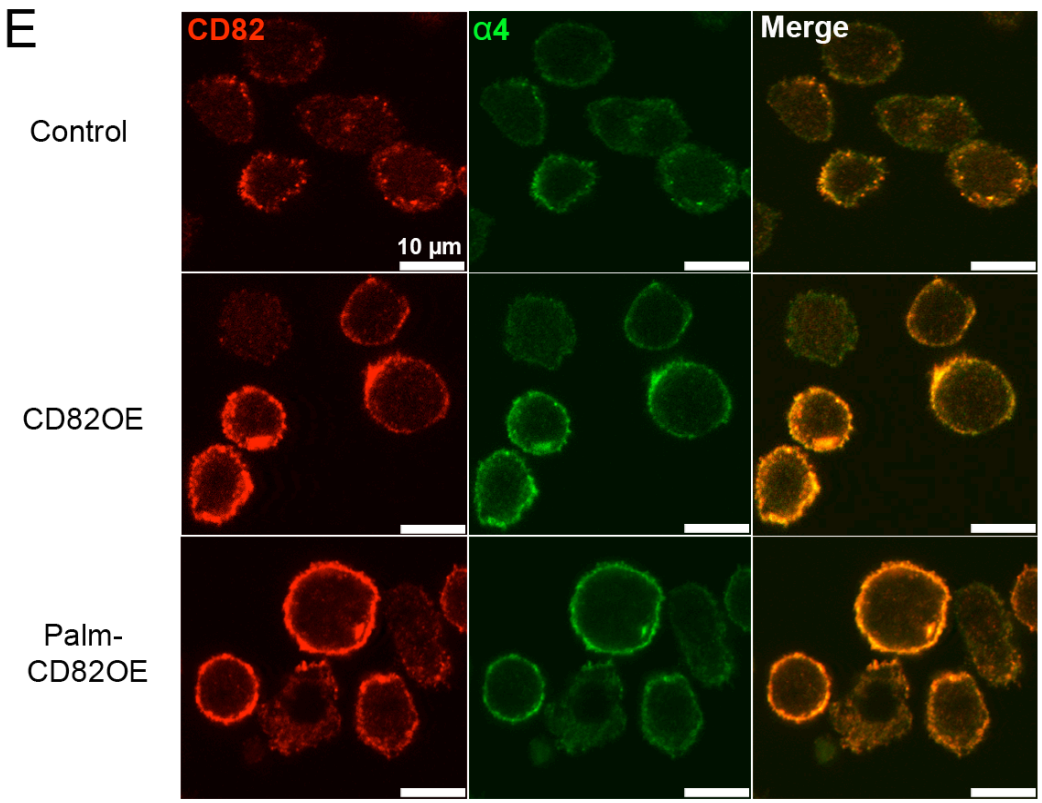
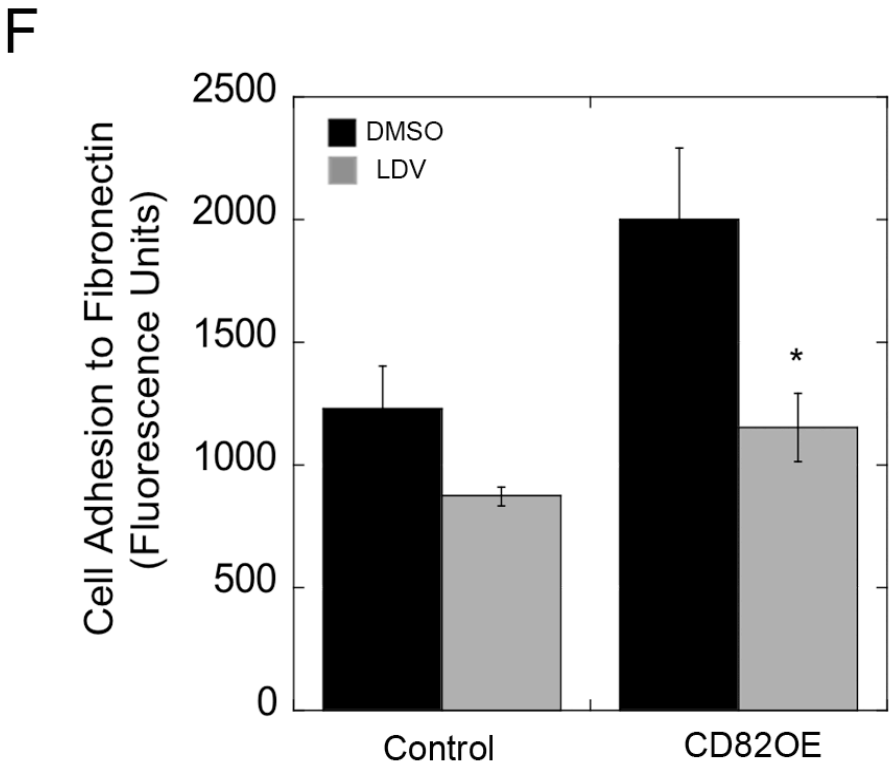
α4
α6
β1
CD82 KD
Control
CD8OE
Palm-CD82 OE


E
CD82
α4
Merge
Control
10 μm
CD82OE
Palm-
CD82OE


F
Cell Adhesion to Fibronectin
(Fluorescence Units)
2500
2000
1500
1000
500
0
DMSO
LDV
*
Control
CD82OE

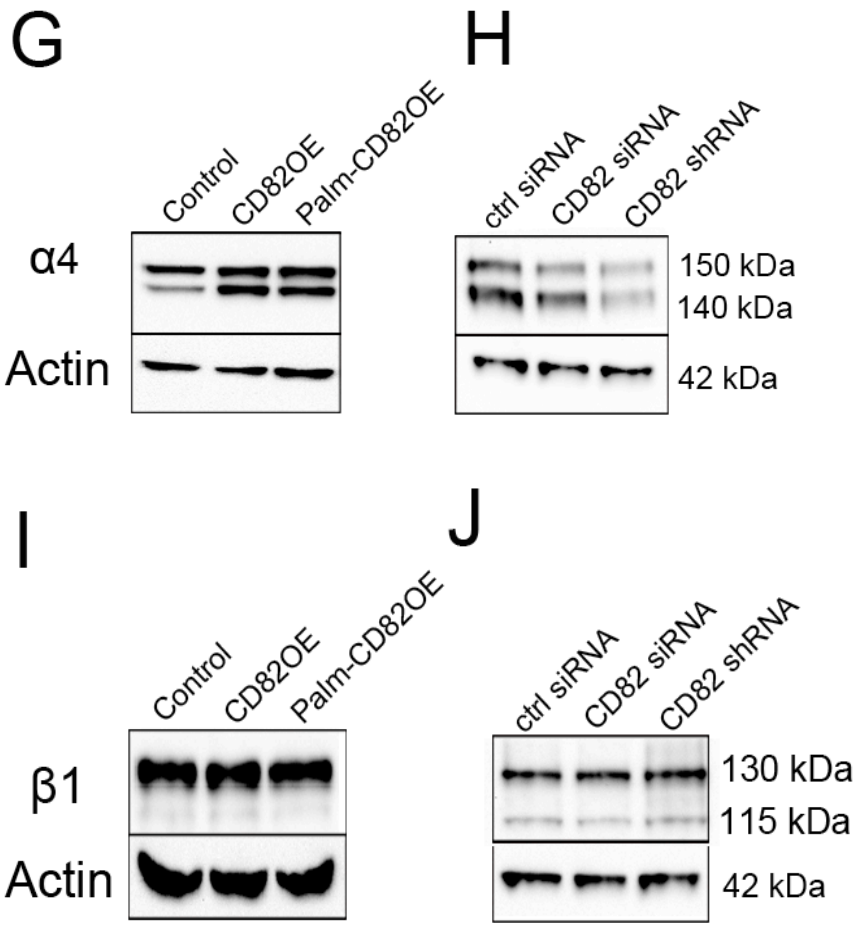


Figure 2.2: CD82 expression modulates integrin expression. Protein surface expression was assessed by flow cytometry for the (A) α2, (B) α4, (C) α6, and (D) β1 integrin subunits. (E) Confocal microscopy was used to assess colocalization of α4 and CD82 in each cell line. Pearson's correlation was determined using ImageJ analysis (R = 0.99 for each image). (F) Control and CD82OE cells were treated with the α4β1-specific monovalent blocking peptide, LDV, and adhesion to fibronectin was quantified using the fluorescence adhesion assay (* $p < 0.05$). Western blot analysis of total α4 protein expression in (G) control, CD82OE and Palm-CD82OE cells or upon CD82 knock-down in KG1a cells transfected with (H) control siRNA, CD82 siRNA and CD82 shRNA vectors. Western blot analysis of total β1 protein expression in (I) control, CD82OE and Palm-CD82OE cells or upon CD82 knock-down in KG1a cells transfected with (J) control siRNA, CD82 siRNA and CD82 shRNA vectors.

indicate that CD82KD results in a decrease of α2 and α4 surface expression (Fig.2.2A,B). In combination, these data suggest that modifications in CD82 expression levels can serve to regulate the surface expression of specific integrins.

2.3.3 CD82-mediated adhesion to fibronectin is modulated by the α4β1 integrin

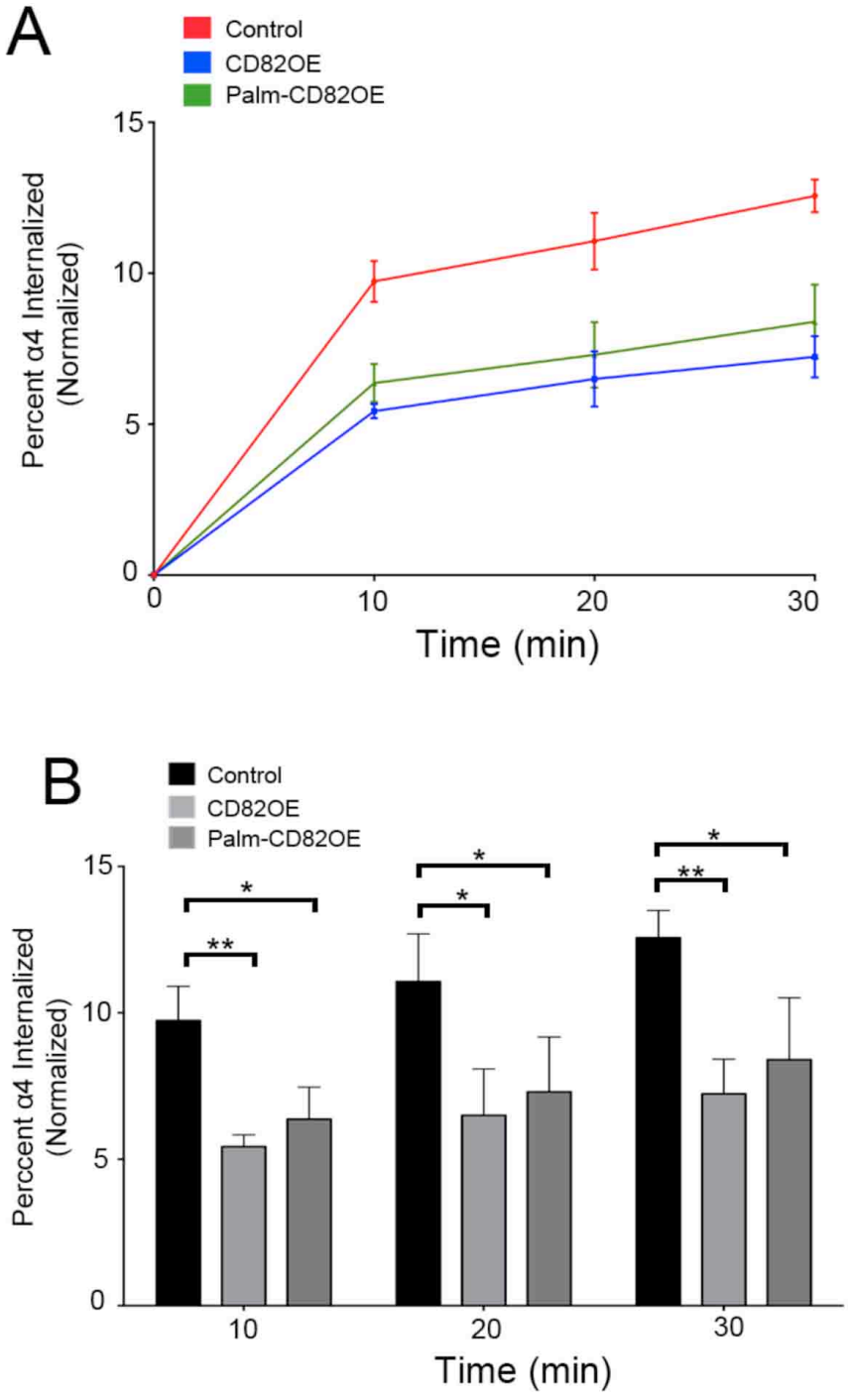
On the surface of HSPCs, the predominant integrins involved in fibronectin binding are αVβ3, α5β1, α4β7, and α4β1 (Coulombel et al., 1997; Mazo et al., 2011). Of these fibronectin-binding integrins, α4β1 is well-established as a critical regulator of HSPC/niche cell contact, mobilization and homing. As CD82 was described previously to interact with α4β1 (Mannion et al., 1996), we first analyzed the localization of CD82 with respect to the α4 integrin. Confocal images in Figure 2.2E suggest a similar membrane localization of CD82 with α4 further indicating a potential interaction. To determine more specifically whether the CD82-mediated increase in fibronectin adhesion occurs through the regulation of α4β1, we added a specific blocking peptide to the adhesion assay. Using a saturating concentration (1 μM) of the α4β1-specific monovalent ligand, LDV, which binds to $\alpha 4\beta 1$ and subsequently blocks its function (Jackson et al., 1997), we observe an inhibition of the CD82-mediated increase in adhesion to fibronectin (Fig.2.2F). These data suggest that the CD82-mediated adhesion to fibronectin involves the $\alpha 4\beta 1$ integrin. In addition to fibronectin, we also evaluated adhesion to the $\alpha 4\beta 1$-specific ligand, vascular cell adhesion molecule-1 (VCAM-1) (Suppl. Fig.S.2.3). Consistent with our fibronectin data, CD82 expression also regulates adhesion to VCAM-1 further supporting the involvement of the α4β1 integrin.

Next, we evaluated whether CD82 alters the global expression levels of either α4 or β1, which could affect cell adhesion to fibronectin. Western blot analysis indicates that CD82 overexpression increases the expression of mature and immature forms of $\alpha 4$ (Fig.2.2G). Based on densitometry analysis, the increase in mature α4 expression is approximately 20%, which correlates with the increase in α4 surface expression observed by flow cytometry.

Overexpression of the Palm-CD82 mutant also results in an increase in α4 expression (Fig.2.2G), whereas knock-down of CD82 using si- or sh-RNAs leads to decreased expression of α4 (Fig.2.2H) with no perturbations of β1 expression (Fig.2.2J). Similarly, we were unable to detect differences in β1 expression in the CD82OE or Palm-CD82OE cells by Western blot analysis (Fig.2.2I). In addition, we were unable to detect a direct interaction between CD82 and α4 via immunoprecipitation analysis, consistent with previous reports (Serru et al., 1999 and Suppl. Fig.S.2.4A). Finally, evaluation of α4 mRNA levels by real-time PCR indicates that the α4 expression decrease in CD82KD cells does not result from changes in mRNA expression (Supp. Fig.S.2.4B). These data suggest that CD82 alters the integrin expression profile of the cells and specifically affects α4 expression, which may alter cell-fibronectin adhesion.

2.3.4 CD82 expression alters the endocytosis and recycling of the α4 integrin

Recently a number of tetraspanins, including CD82, were shown to regulate integrin turnover during *Drosophila* oocyte development (Han et al., 2012). As such, one mechanism by which CD82 could alter the surface expression of α4 is through changes in internalization. To monitor the internalization rate of the α4 integrin, we performed a fluorescence-quenching internalization assay using flow cytometry. Following surface labeling of α4 at 4°C with a specific Alexa-488 conjugated-antibody, we quenched the surface fluorescence of α4 with an anti-Alexa-488 antibody and quantified the remaining fluorescence as internalized α4 integrin. Figure 2.3A,B illustrates the percent of total surface α4 internalized over time and demonstrates that the CD82OE cells have reduced internalization when compared to control cells. The internalization of α4 in the Palm-CD82OE cells is similar to the internalization of the CD82OE cells suggesting that the palmitoylation of CD82 has no affect on the internalization of α4. Therefore, one mechanism by which CD82 can modify the surface expression of integrins is by altering their endocytosis.


A
Control
CD82OE
Palm-CD82OE
Percent α4 Internalized (Normalized)
15
10
5
0
0
10
20
30
Time (min)
B
Control
CD82OE
Palm-CD82OE
Perccent α4 Internalized (Normalized)
15
10
5
0
*
**
*
*
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**
10
20
30
Time (min)

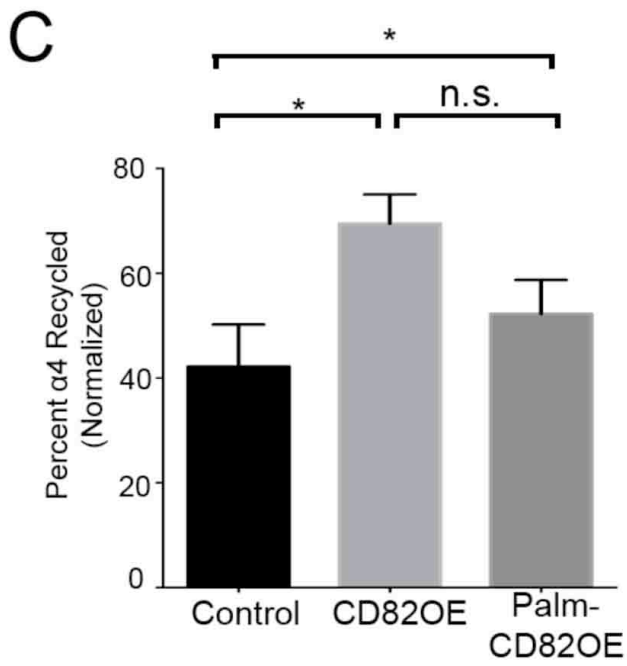


Figure 2.3: CD82 expression regulates α4 stability on the cell surface. (A, B) Control, CD82OE and Palm-CD82OE cells were assessed for α4 internalization using a fluorescence based internalization assay. Cells were labeled using an Alexa Fluor 488 integrin α4 antibody, allowed to internalize for 10, 20, and 30 minutes, and the surface fluorescence was quenched using an anti-Alexa Fluor 488 antibody (Invitrogen). The remaining fluorescence indicates internalized protein, which was compared to 100% α4 surface labeling. (C) α4 recycling after 30 minutes was quantified from three independent experiments using a modified version of the internalization assay. Cells were allowed to internalize protein for 30 minutes. Cells were then quenched and allowed to recycle protein back to the cell surface for 30 minutes. Surface fluorescence was quenched again and the difference between the first and second quench represents the amount of protein recycled back to the plasma membrane. Error bars indicate SD; n = 3 (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

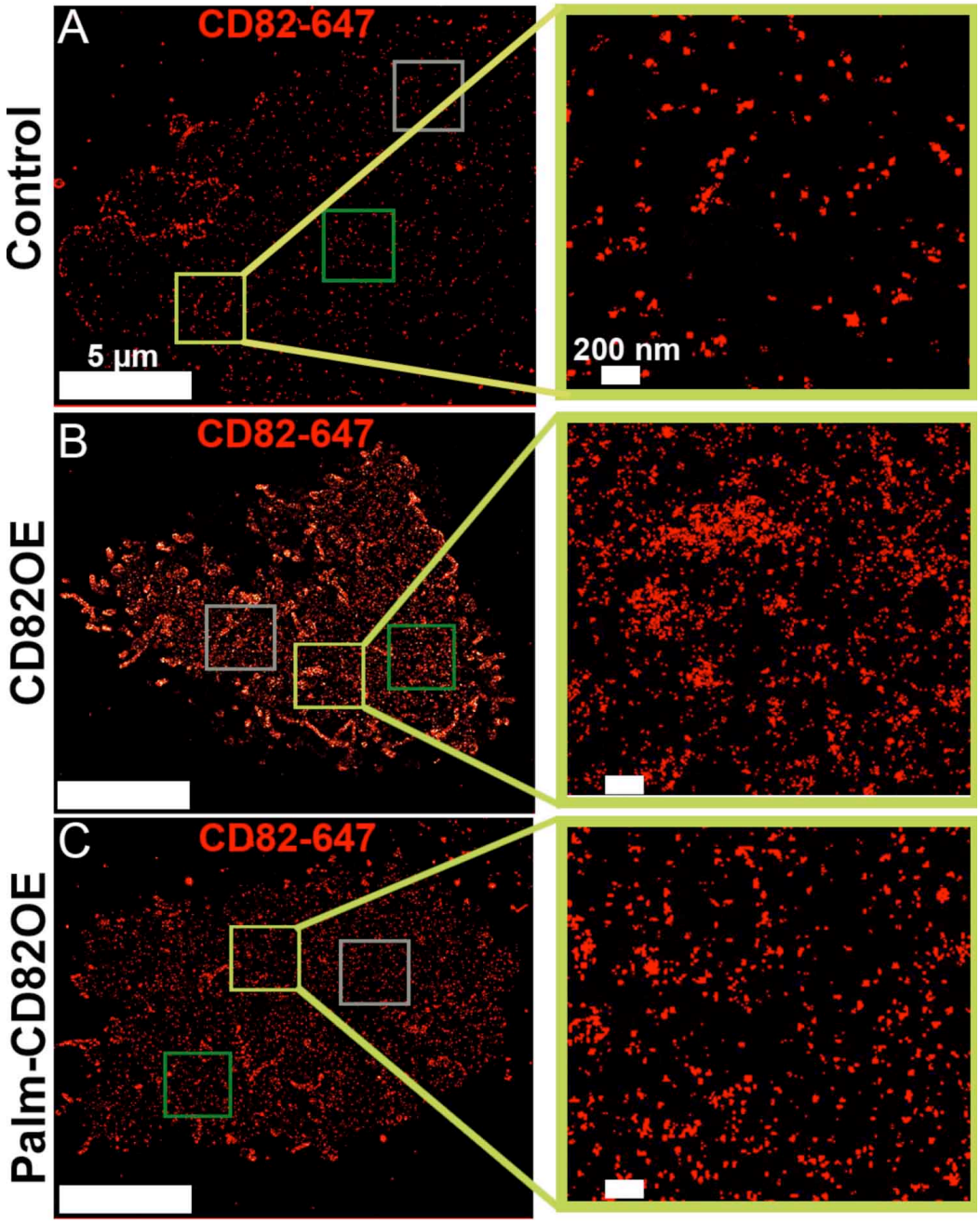
Once internalized, integrins can either be degraded through trafficking to the lysosome or they can be recycled to the surface via the recycling endosome. To evaluate whether the reduced α4 internalization observed in the CD82OE cells (Fig.2.3A) could be due to changes in integrin recycling, we quantified $\alpha 4$ recovery to the surface. Following the internalization and quench described for the endocytosis assay, we placed the cells back at 37°C for 30 minutes to evaluate the rate of α4 recycling. Figure 2.3C illustrates that in both the CD82OE and Palm-CD82OE cells, we detect an increase in α4 recycling to the plasma membrane. These data suggest that the decreased rate of α4 internalization observed with CD82 overexpression is likely mediated by an increase in α4 recycling. Together these data support a role for CD82 in regulating integrin expression through modulation of endocytosis and the recycling endosome pathway.

2.3.5 CD82 expression does not affect the α4β1 affinity state

Our data suggest a role for CD82 in the regulation of α4 integrin expression and its trafficking. However, in addition to differences in the expression of integrins, changes in cell adhesion can also be modulated by changes in integrin affinity. As such, we wanted to determine whether CD82 expression could change the α4β1 affinity state. To quantify potential differences in $\alpha 4\beta 1$ affinity, we measured the binding affinity of LDV-FITC to cells using a flow cytometer (Chigaev et al., 2007). Binding isotherms or Langmuir plots were generated by incubating increasing concentrations of LDV-FITC with cells, and the measured fluorescence was fit to a suitable non-linear regression function to calculate the K_d values. These data suggest that CD82 overexpression does not statistically alter the affinity state of the α4β1 integrin (Suppl. Fig.S.2.5A). Next, we used real-time flow cytometry to analyze the dissociation kinetics or “off rate” of LDV-FITC upon addition of a saturating, competitive concentration of unlabeled LDV (1 μM) (Suppl. Fig.S2.5B). The dissociation rate constant, K_{off}, was determined from the nonlinear fit and indicates that CD82 overexpression does not affect the off rate of LDV. Taken together, CD82 overexpression does not appear to alter the affinity state of the α4β1 integrin.

2.3.6 Palmitoylation of CD82 regulates its surface clustering

The overall strength of cellular adhesiveness is regulated by a combination of the affinity of individual integrins and their local density or surface geometry. As tetraspanins are known to organize proteins into clusters or "webs", which could potentially alter the organization and density of surface integrins, we set out to evaluate the membrane distribution of CD82 and its effects on α4. Using the super-resolution imaging technique, dSTORM (Heilemann et al., 2008), we reconstructed images of the single molecule distribution of CD82 on the surface of each of the cell lines (Fig.2.4A-C). From the magnified images, we were able to observe clusters of CD82 on the surface of the control cells (Fig.2.4A), the CD82OE cells (Fig.2.4B) and the Palm-CD82OE cells (Fig.2.4C). To quantify the sizes of the identified CD82 clusters, we used the pair auto-correlation function (Fig.2.4D-F). Previously described for dSTORM, the pair auto-correlation analysis establishes the probability of finding a molecule at a given distance from another molecule and does not depend on the number of times an average molecule is counted (Sengupta et al., 2011; Veatch et al., 2012). Applying this analytical method, we quantified the average, radial, protein cluster sizes of CD82 within the cell membrane. CD82 clusters measured on the control cells were significantly smaller (92 nm) than the average clusters in the CD82OE cells (140 nm) (Fig.2.4G). Interestingly, even though the CD82 surface expression is the same between the CD82OE cells and the Palm-CD82OE cells (Fig.2.1D), the measured cluster size of CD82 on the surface of the Palm-CD82OE cells is significantly smaller (97 nm). These data illustrate that the palmitoylation sites within CD82 are critical for the molecular organization of CD82 into clusters. While the role of palmitoylation in regulating tetraspanin clustering has been implicated from biochemical experiments (Berditchevski et al., 2002; Charrin et al., 2002; Stipp, 2010; Yang et al., 2002; Yang et al., 2004), our data provide quantifiable imaging evidence that illustrates the importance of these sites for CD82 organization.


A
CD82-647
Control
5 μm
200 nm
B
CD82-647
CD82OE
C
CD82-647
Palm-CD82OE

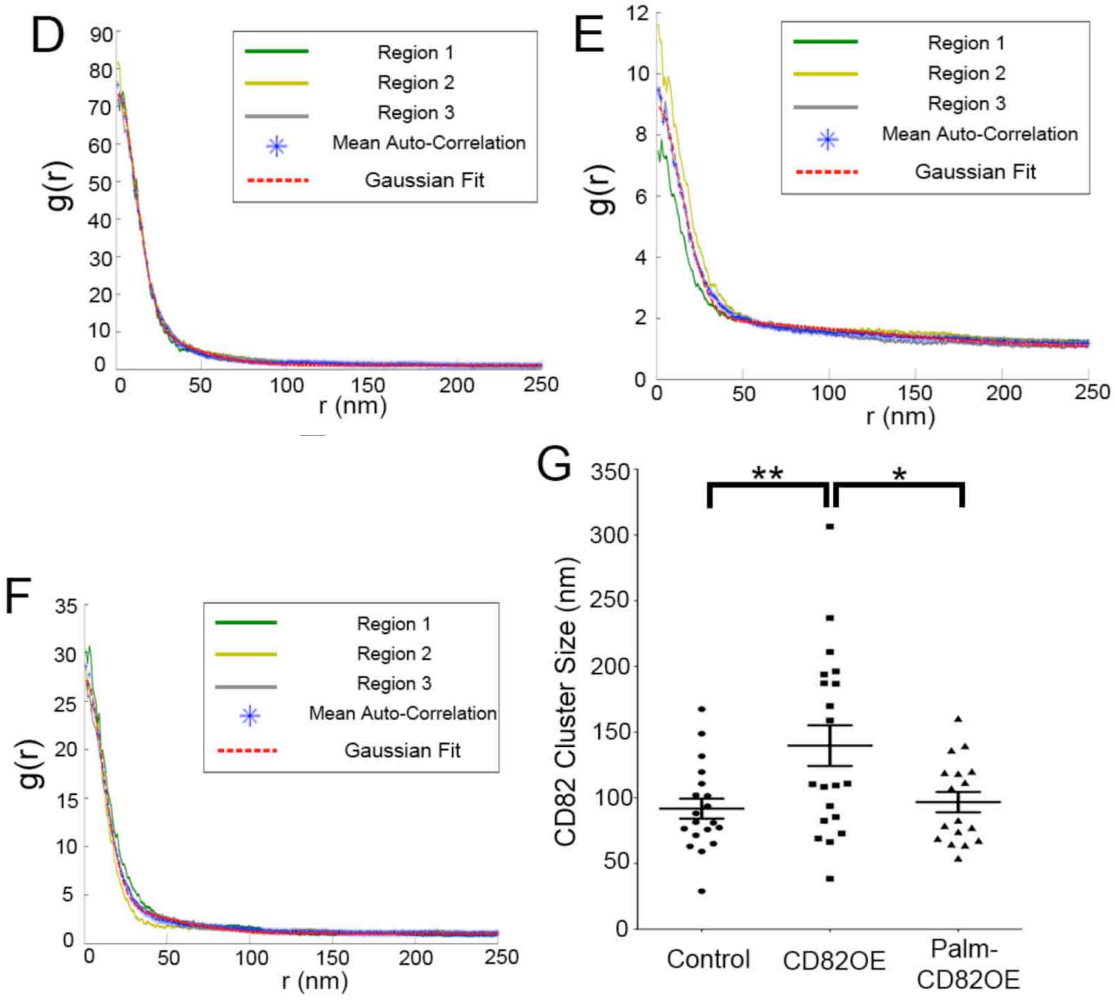


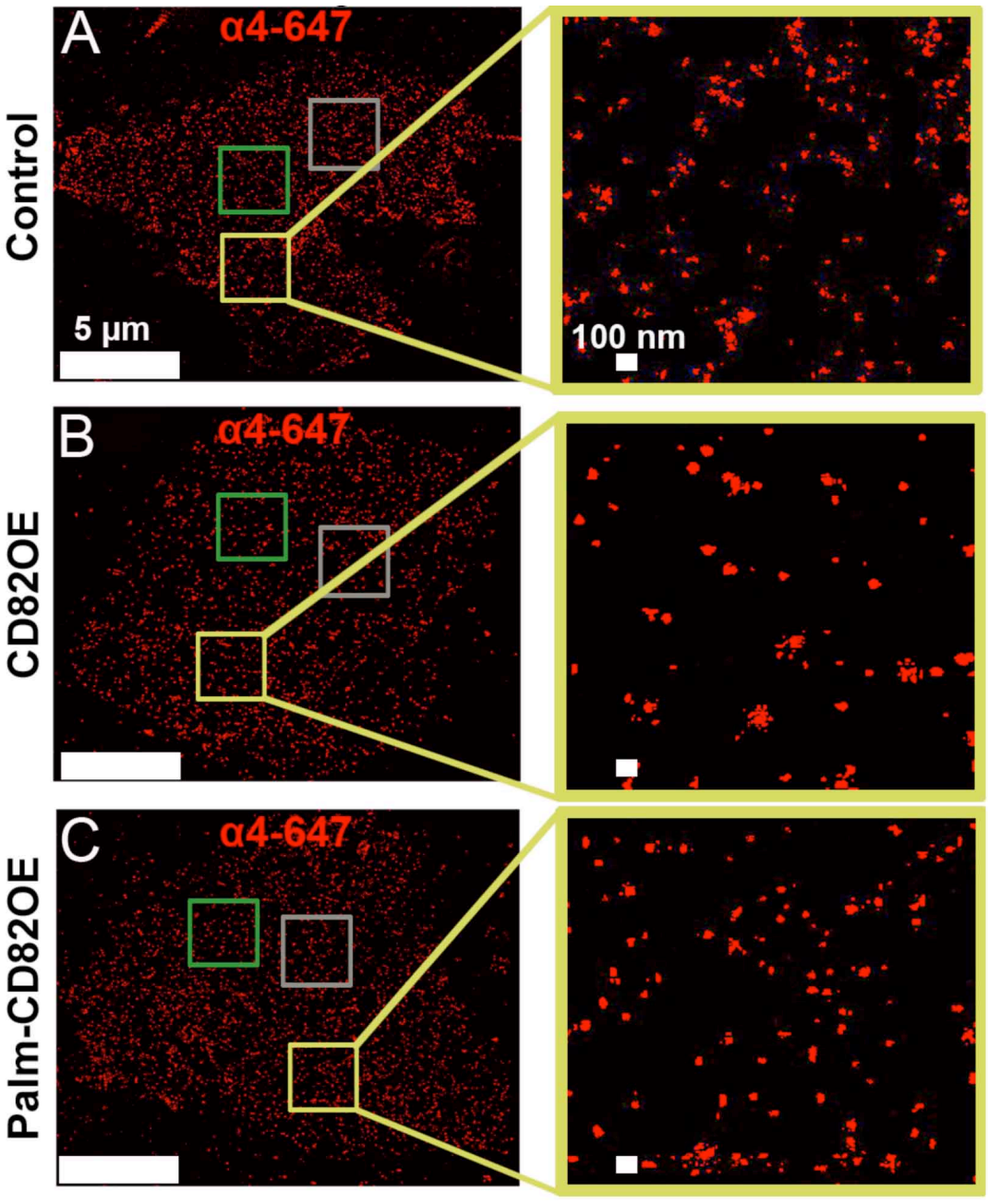
Figure 2.4: CD82 palmitoylation contributes to CD82 oligomerization. Reconstructed dSTORM images of representative (A) control, (B) CD82OE, and (C) Palm-CD82OE cells plated on fibronectin and labeled with an Alexa Fluor 647 anti–human CD82 antibody. CD82 cluster size was assessed using the pair autocorrelation function (Veatch et al., 2012) for control (D), CD82OE (E), and Palm-CD82OE (F); this function determines the probability, g(r), of localizing a molecule a given radius, r, away from another localized molecule. Radially averaged autocorrelation functions were calculated from three 3 × 3 μm regions of each cell as described in Materials and Methods, Super resolution imaging. The mean autocorrelation function from these three regions is shown in blue. (G) Average CD82 cluster size, σDom, extracted from the fitting equation for each cell and plotted for each population of cells. Error bars, SEM; n = 19 cells for control, 20 cells for CD82OE, and 17 cells for Palm-CD82OE (**p < 0.01, *p < 0.05).

2.3.7 The α4 integrin is organized into small-scale clusters

Once we established the distribution of CD82 on each of our cell lines, we determined whether the expression and/or organization of CD82 had any effect on the clustering of the α4 integrin. Again, we used dSTORM imaging to assess potential changes in the α4 surface distribution between the control, CD82OE and the Palm-CD82OE cells. From the dSTORM images (Fig.2.5A-C), we detect small-scale clusters of α4 in each cell line. Using the pair auto-correlation function described above, we fit the α4 localization data (Fig.2.5D-F) and extracted cluster sizes that were significantly smaller than those calculated for CD82 (Fig.2.5G). The average cluster size for each of the cell lines is approximately 35 nm. These data suggest that the α4 integrin is organized into small-scale membrane clusters on the order of 35 nm, which is not affected by CD82 expression.

2.3.8 Palmitoylation of CD82 regulates molecular density of α4 clusters

The contribution of integrin clusters to cellular adhesion is heavily dependent upon their larger scale molecular organization and protein density, which can alter the strength of the adhesive complex. While the pair auto-correlation function is effective at quantifying the average uniform size of α4 clusters, the function output is representative of a singular α4 cluster. In order to assess the potential for CD82 to regulate the large-scale organization of α4, we analyzed the dSTORM images with the density-based spatial clustering of applications with noise (DBSCAN) data clustering algorithm (Ester et al., 1996a). DBSCAN quantifies cluster size in terms of cluster area, providing valuable information about the two-dimensionality of protein clusters (Kim et al., 2013). This density-based clustering algorithm identifies clusters by evaluating the number of localizations that are within a density-reachable area and outputs the cluster sizes (in μm^2) found within a region of a cell. As such, the identified clusters are no longer dependent upon a radial cluster size. Evaluating sections


A
α4-647
Control
5 μm
100 nm
B
α4-647
CD82OE
C
α4-647
Palm-CD82OE

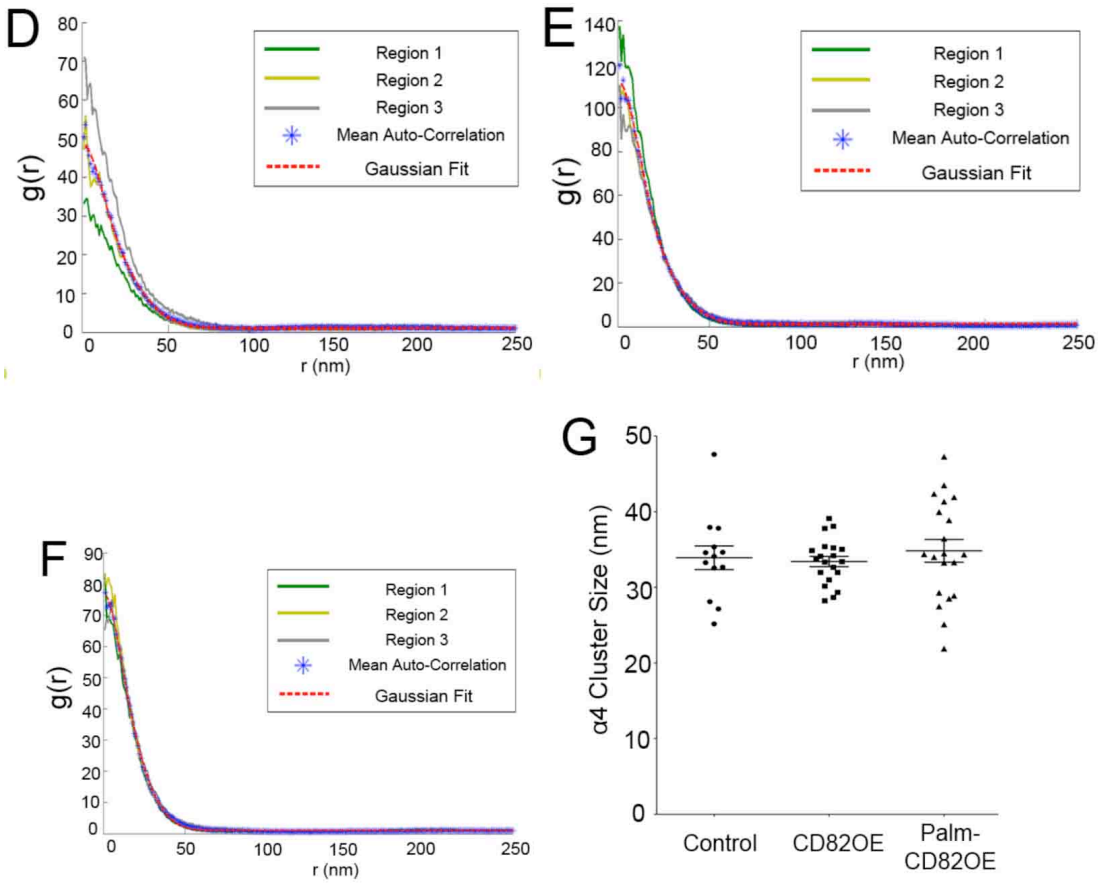
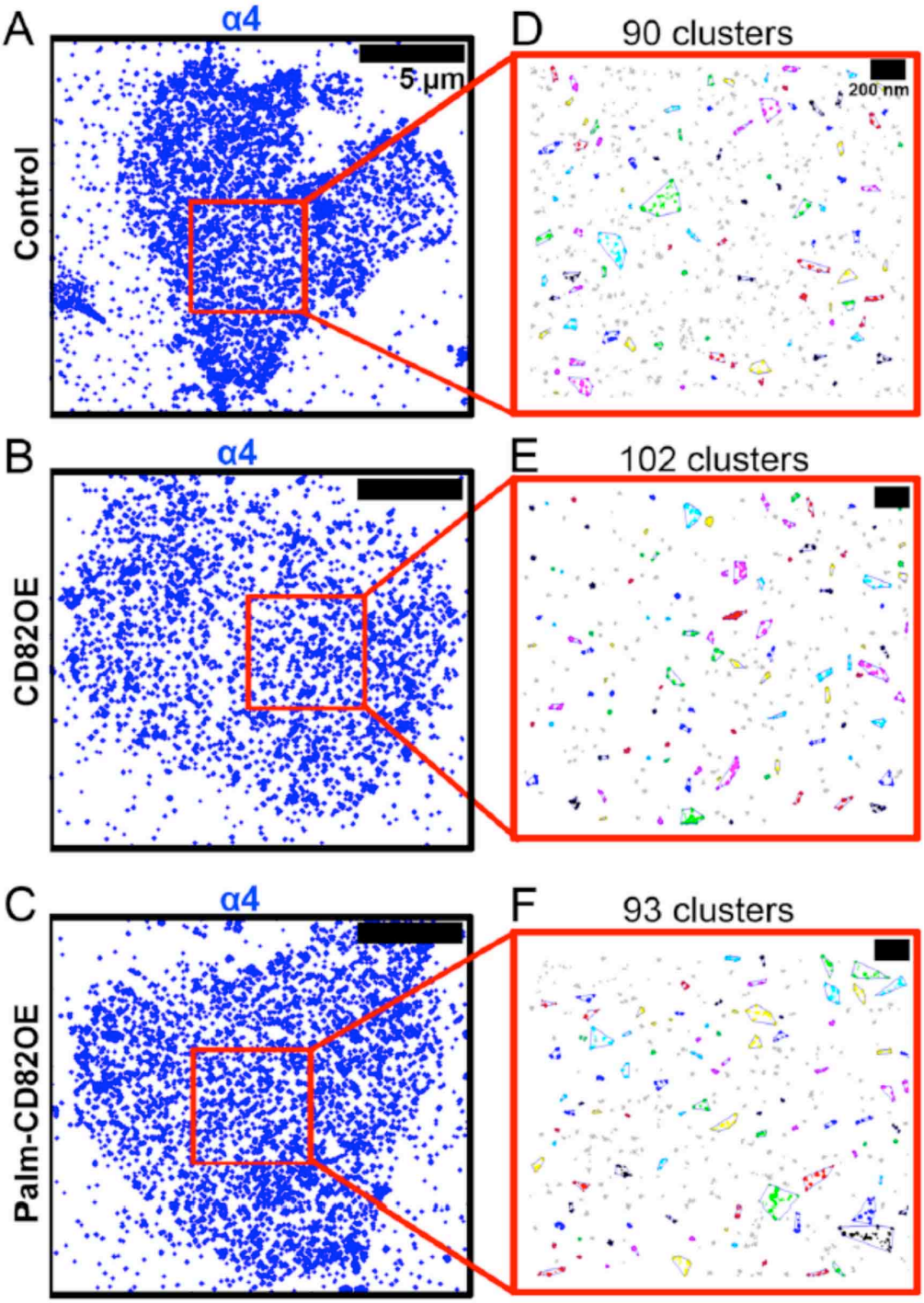


Figure 2.5: The α4 subunit is organized into small-scale clusters. Reconstructed dSTORM images of representative (A) control, (B) CD82OE, and (C) Palm-CD82OE cells plated on fibronectin and labeled fluorescently for the α4 integrin subunit using a monoclonal α4 integrin primary antibody and goat anti-mouse Alexa Fluor 647 secondary antibody. The α4 cluster size was assessed using the pair autocorrelation function (Veatch et al., 2012) for control (D), CD82OE (E), and Palm-CD82OE cells; this function determines the probability, g(r), of localizing a molecule a given radius, r, away from another localized molecule. Radially averaged autocorrelation functions were calculated from three 3 × 3 μm regions of each cell as described in Materials and Methods, Superresolution imaging . The mean autocorrelation function from these three regions is shown in blue. (G) Average α4 cluster size, σDom, extracted from the fitting equation for each cell and plotted for each population of cells. Error bars, SEM; n = 13 cells for control, 20 cells for CD82OE, and 20 cells for Palm-CD82OE.

of the reconstructed dSTORM images from cells with approximately the same number of α4 localizations, (Fig.2.6A-C) we quantified the number of larger-scale α4 clusters or “DB clusters”. Using DBSCAN, we observed an increased number of total DB clusters of α4 in the CD82OE and Palm-CD82OE cells (Fig.2.6G), which we attribute to the aforementioned increase in α4 surface expression in these cell lines (Fig.2.2A).

Integrins must organize into adhesive clusters that can resist the strong forces present at sites of adhesion, while maintaining ligand engagement (Balaban et al., 2001; Roca-Cusachs et al., 2009; Selhuber-Unkel et al., 2008). Therefore, we used the DBSCAN to quantify the organization of α4 localizations into clusters as a mechanism of increased HSPC adhesion. More specifically, we set out to determine the percent of α4 localizations determined to be clustered, as well as the number of α4 localizations found within clusters. In both the CD82OE and the Palm-CD82OE cells, we calculated an increase in the percent of localizations that are considered clustered (Fig.2.6H) by the DBSCAN. Furthermore, we found an increase in the average number of α4 localizations found within DB clusters for CD82OE as well as Palm-CD82OE cells (Fig.2.6I). These increases are likely due to our previous finding that the CD82OE and Palm-CD82OE cells exhibit an increase in α4 surface expression as compared to the control cells (Fig.2.2A). Moreover, since both the CD82OE and Palm-CD82OE cells show an increase in the percent of α4 clustered as well as number of α4 localizations within a cluster, these results are unlikely to account for the change in adhesion between the CD82OE and Palm-CD82OE cells.

Adhesion complex stability can be strengthened by the tight packing of multiple integrins into clusters (Geiger et al., 2001; Kiessling et al., 2006; Mammen et al., 1998; Selhuber-Unkel et al., 2008). Upon further evaluation of the DBSCAN clusters, we observed a striking difference in the size of α4 clusters as well as the spatial organization of α4 molecules within these clusters. When we magnify the reconstructed images to analyze the size and shape of the DB clusters (Fig.2.7A-C), we find that the CD82OE cells have smaller, more tightly


A
α4
5 μm
Control
D
90 clusters
200 nm
B
α4
CD82OE
E
102 clusters
C
α4
Palm-CD82OE
F
93 clusters

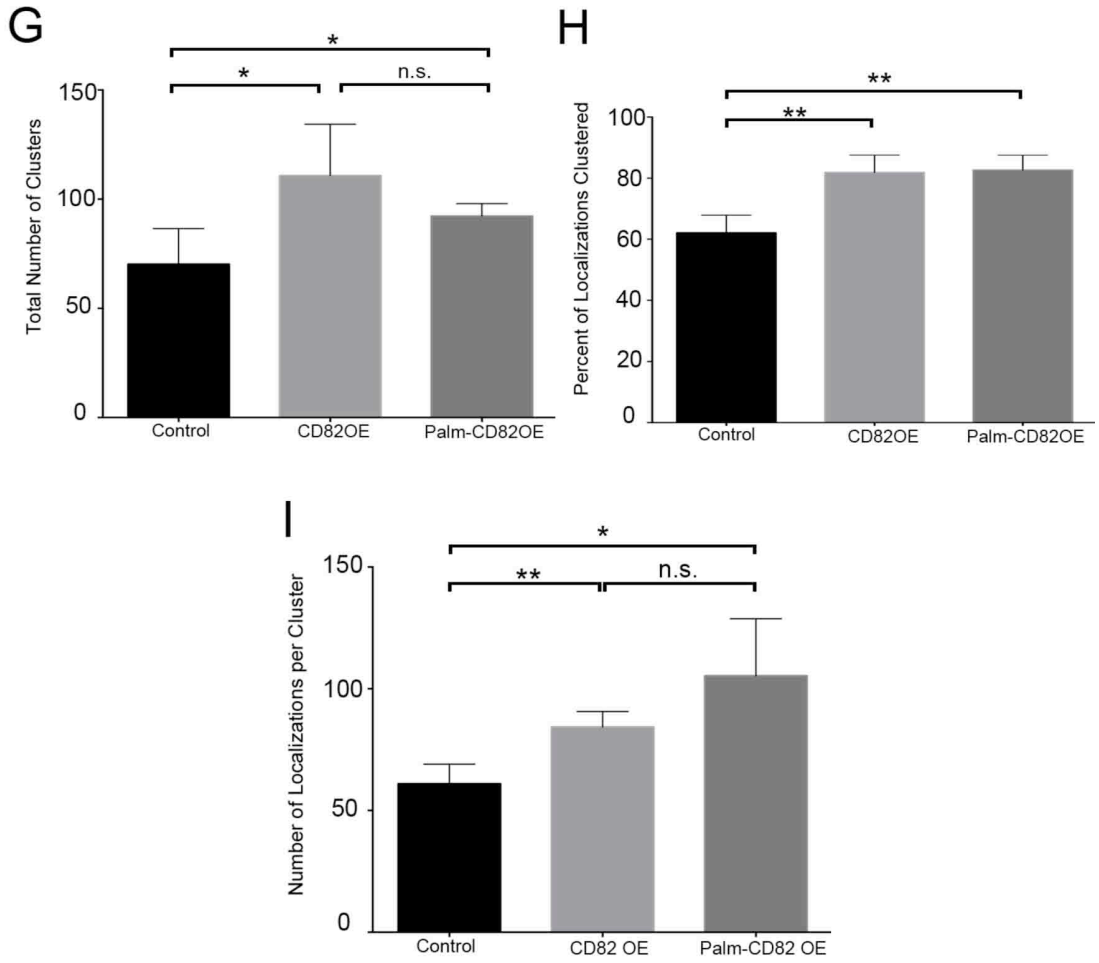
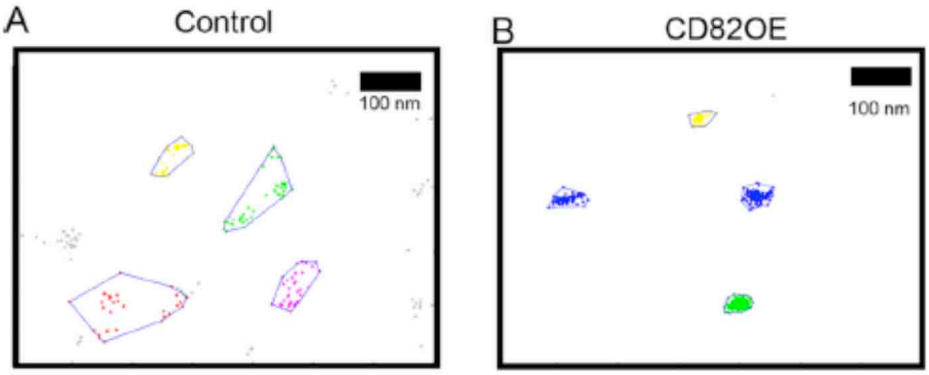


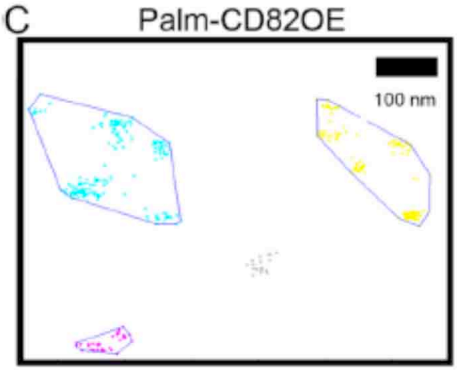
Figure 2.6: CD82 expression regulates α4 molecular organization. Reconstructed dSTORM images of representative (A) control, (B) CD82OE, and (C) Palm-CD82OE cells plated on fibronectin and labeled fluorescently for α4. (D–F) The DBSCAN algorithm was used to examine cluster organization within a subregion of the cells. The DBSCAN parameters used were ε = 1 pixel and n = 30 localizations. Colored localizations denote localizations organized into a cluster, and gray localizations indicate molecules not organized, as they did not meet the DBSCAN parameters. (G) Quantification of the total clusters determined by DBSCAN. (H) Quantification of the percentage of α4 localizations determined to be organized into clusters. (I) Average number of α4 localizations per cluster as determined by DBSCAN. Error bars, SD; n = 4 cells (*p < 0.05, **p < 0.01).

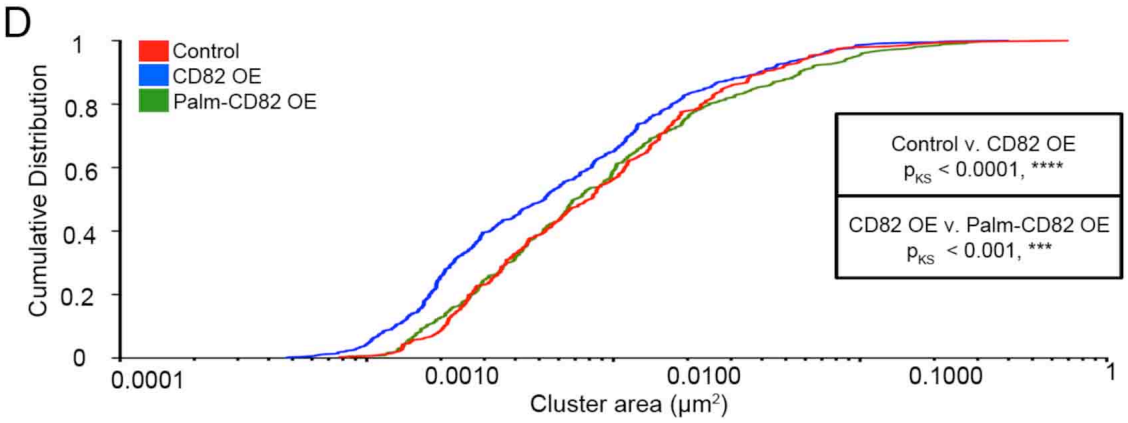
packed clusters of α4 when compared to control or Palm-CD82OE cells. (Additional larger fields of view are illustrated in Suppl.Fig.S.2.6A-C.) In order to assess differences in the distribution of clusters found using DBSCAN, we generated cumulative distribution plots of DB cluster sizes (Fig.2.7D)(Suppl. Fig.S.2.7A-B). The cumulative distribution plot illustrates the overall cluster sizes from all of the cells, which enables us to assess the percentage of clusters within a given size. Using the Kolmogorov-Smirnov test to assess if two data sets differ significantly, we find that the distribution of clusters found in the CD82OE cells differs from the distribution found in the control cells, as well as the Palm-CD82OE cells. These data suggest that the size of the α4 DB clusters present on the CD82OE cells are smaller than those measured on the control and Palm-CD82OE cells.

We next examined the size distribution of clusters found by DBSCAN by binning the data by α4 cluster area (μm^2). This allows us to extract the relative percentages of various sized DB clusters detected and quantify differences in the types of DB cluster sizes identified as well as their relative abundance. We found that in the CD82OE cells, there is an increase in the percent of DB clusters that fall within the smaller 0-0.0025 μm^2 bin (Fig.2.7F). In contrast, an increase in the larger DB clusters (> 0.005 μm^2) are detected upon Palm-CD82OE. In Figure 2.7E, we provide a visual reference for the length dimensions that would result in each of the square cluster areas. Taken together, these data indicate that there is a difference in the relative abundance of small and large α4 clusters between the overexpressing cell lines. Furthermore, these data suggest a functional difference between the ability of the CD82OE and Palm-CD82OE cells to contribute to α4 cluster size. More specifically, the palmitoylation mutant form of CD82 is less effective at tightly packing the α4 molecules into a cluster, and as such, the α4 clusters in the Palm-CD82OE cells contain an increased proportion of clusters > 0.005 μm^2.

In addition to identifying CD82-mediated changes in α4 cluster size, we also detected a difference in the spatial organization of α4 localizations within the







E Representative molecular geometry, dimensions and area

Cluster molecular geometry	Length Dimensions	Area
	50 nm	0.0025 μm²
	71 nm	0.005 μm²
	100 nm	0.01 μm²

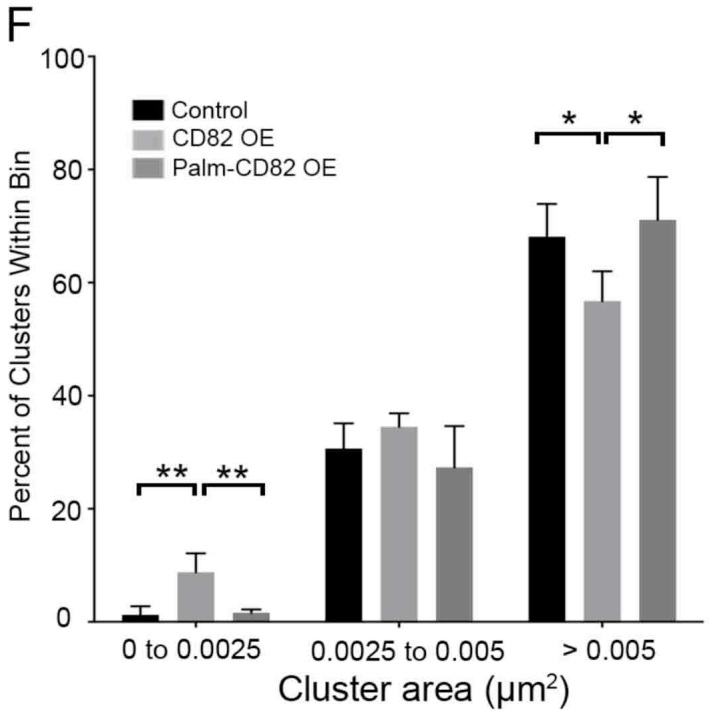


Figure 2.7: CD82 palmitoylation regulates the α4 density within clusters. Enlarged DBSCAN regions of (A) control, (B) CD82OE, and (C) Palm-CD82OE cells showing representative α4 clusters. (D) Cumulative distribution plot of the clusters compiled from n = 4 cells of each cell line plated on fibronectin; >250 clusters. Statistics determined using the Kolmogorov–Smirnov test. (E) Representative cluster geometry as depicted by black squares. Corresponding dimensions that give rise to cluster areas (0.0025, 0.005, and 0.01 μm2) drawn to scale of images in A–C. (F) Percentage of total clusters that fall within the cluster area bins determined for cells plated on fibronectin. (G) Average number of α4 molecular localizations/0.01 μm2 determined for cells plated on N-cadherin, fibronectin, and VCAM-1 using DBSCAN. Error bars, SD; n = 4 cells (*p < 0.05, **p < 0.01). the cells are plated on an 4 ligand (Fig.7G)(Suppl.Fig.8A-C,G). When the cells are plated on N-cadherin, we no longer detect a change in integrin density (Fig.7G)(Suppl.Fig.8D-F,H). In combination, these data suggest a critical role of tetraspanins in promoting the organization of integrins into adhesion complexes, which allows for proper cell-ECM interactions. More specifically, our data suggest that CD82 mediates the tight packing of 4 into clusters upon ligand engagement, which increases the molecular density of 4 and enhances cell-matrix adhesion. Furthermore, our data indicate that CD82 palmitoylation is required for the effective formation of tightly packed integrin clusters.

clusters. To quantify these differences, we calculated the number of α4 localizations per unit cluster area of the cell (0.01 μm^2) (Fig.2.7G). From these data, we found an increase in the average number of α4 localizations per 0.01 μm^2 in the CD82OE cells indicating an increase in the number of α4 molecules packed into a smaller area of the membrane. When we compare these results to the α4 packing in the Palm-CD82OE cells, we find that Palm-CD82OE does not promote the compact lateral packing of α4 molecules within clusters. Next, we assessed whether the presence of α4 ligand has an effect on the CD82-mediated changes in α4 density. To quantify this potential difference, we completed the dSTORM imaging and analysis on cells that were plated on VCAM-1 (α4 ligand) or N-cadherin (non-ligand). Interestingly, our data indicate that the increase in α4 density measured in the CD82OE cells occurs only when occurs only when the cells are plated on an α4 ligand (Figure 2.7G and Supplemental Figure S.2.8, A–C and G). When the cells are plated on N-cadherin, we no longer detect a change in integrin density (Figure 2.7G and Supplemental Figure S.2.8, D–F and H). In combination, these data suggest a critical role of tetraspanins in promoting the organization of integrins into adhesion complexes, which allows for proper cell–ECM interactions. More specifically, our data suggest that CD82 mediates the tight packing of α4 into clusters upon ligand engagement, which increases the molecular density of α4 and enhances cell–matrix adhesion. Furthermore, our data indicate that CD82 palmitoylation is required for the effective formation of tightly packed integrin clusters.

2.4 Discussion

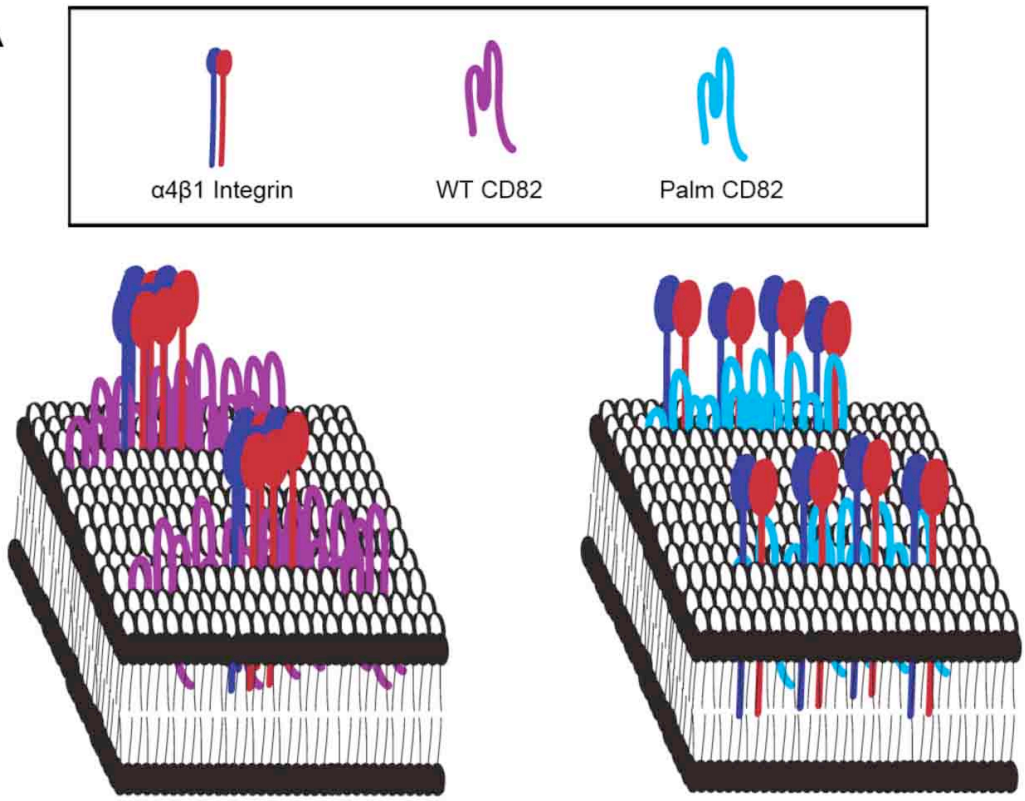
Tetraspanin-tetraspanin and tetraspanin-integrin interactions modify cell-cell and cell-matrix adhesion; although, the molecular mechanisms that mediate these processes remain unclear. Our study provides strong evidence that the tetraspanin, CD82, can regulate the membrane organization of integrins resulting in the formation of tightly packed integrin “nanoclusters”, which increases matrix adhesion. Moreover, if we inhibit lateral CD82 clustering by overexpressing a mutant form of CD82, which cannot be palmitoylated, we diminish the organization and molecular packing of α4 integrins and ultimately block cell-

matrix adhesion. These results have led us to propose a model whereby CD82 TEMs serve to regulate the molecular density of integrins by recruiting integrins into and/or stabilizing them within plasma membrane clusters in a ligand dependent manner (Fig.2.8).

Trafficking of HSPCs into and out of the bone marrow is essential throughout life to maintain homeostasis of the hematopoietic system and participate in innate immune responses. It is also critical in the clinical setting where HSPCs can be isolated from normal donors and transplanted back into patients to replenish a compromised hematopoietic system. Previous work from our group identified the enrichment of CD82 at HSPC contact sites with osteoblasts, which led us to evaluate its potential role in HSPC/bone marrow interactions (Gillette and Lippincott-Schwartz, 2009). In a follow up study, we found that treatment of human CD34(+) cells with CD82 monoclonal antibodies inhibited CD34(+) cell adhesion and homing to the bone marrow, although the mechanism for this CD82-mediated effect on adhesion and homing remained unknown (Larochelle et al., 2012). In this study, we evaluate how CD82 expression regulates cell adhesion, with a particular focus on modifications in integrin interactions. First, we demonstrate a role for CD82 in cell adhesion, finding that CD82OE increases cell adhesion to fibronectin, whereas CD82KD results in decreased adhesion. Interestingly, it is important to note that this CD82-mediated increase in fibronectin adhesion requires the palmitoylation of CD82, since an increase in cell adhesion was not observed with the Palm-CD82OE mutant.

Previously, the expression of CD82 was shown to modify adhesion through the αVβ3 (Ruseva et al., 2009) as well as the α6 (He et al., 2005) and β1 integrins (Jee et al., 2007). As such, we went on to evaluate CD82-mediated differences in integrin surface expression and identified changes in α4 expression. Signaling through the α4β1 integrin is known to regulate HSPC adhesion and homing to the bone marrow (Hartz et al., 2011). For example,

A



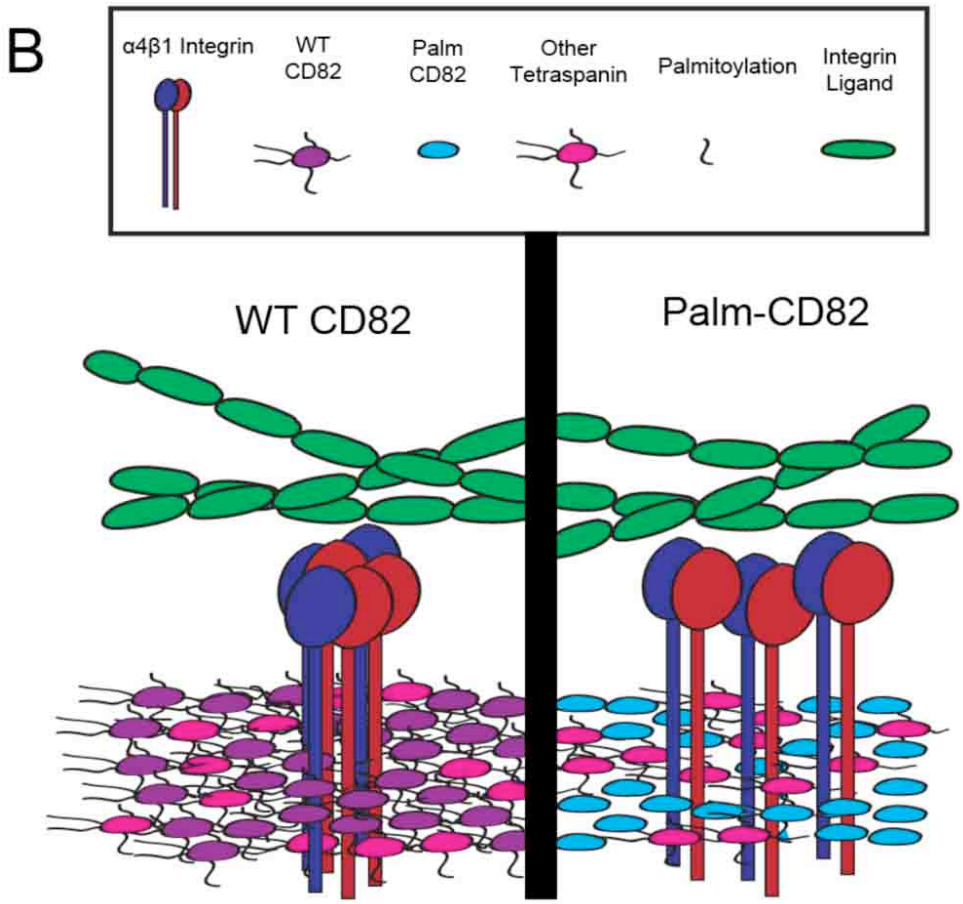


Figure 2.8: Proposed model for CD82 regulation of molecular clustering and protein density. On the basis of super-resolution microscopy data, we propose a model in which CD82 expression and clustering modulate α4 protein density. (A) CD82OE facilitates the membrane clustering of CD82, which leads to larger CD82 clusters and more tightly packed α4 clusters. The detected increase in α4 density upon CD82OE depends on α4 ligand engagement. In contrast, Palm-CD82OE is unable to promote effective TEMs, which results in smaller CD82 clusters and reduced molecular density of α4. (B) The spatial arrangement of molecules within TEMs is essential for organizing adequate adhesion and signaling platforms, which are weakened by palmitoylation site mutation. The association of CD82 with other tetraspanins, a process strengthened by tetraspanin palmitoylation, could indirectly affect the molecular density of α4 clusters by disrupting the organization within the TEM required to establish effective packing of α4 molecules. The increased α4 molecular density results in α4 molecules within close proximity of one another, which contributes to the overall strength and activity of the adhesion complex.

treatment of mice with α4-blocking antibodies results in HSPC mobilization into the blood (Craddock et al., 1997; Papayannopoulou et al., 1995). In addition, HSPC homing to the bone marrow is perturbed by α4-blocking antibodies indicating that α4 plays a role in regulating HSPC/bone marrow niche interactions (Papayannopoulou et al., 1995). Our results demonstrate that the CD82-mediated increase in fibronectin adhesion occurs primarily through the α4β1 integrin. Furthermore, we identified an increase in the surface expression of α4 upon CD82OE or Palm-CD82OE and a decrease in surface expression with CD82KD. Recent studies have shown that tetraspanins can modulate integrin surface distribution and function through the regulation of integrin internalization (Liu et al., 2007; Winterwood et al., 2006) and trafficking through the endosomal pathway (Caswell et al., 2009). Previous studies have shown that the rate of α4β1 internalization was significantly reduced in CD151-silenced cells (Winterwood et al., 2006). In addition, the YXXϕ motif in CD151 was identified as a structural element that determines the trafficking of its associated integrins (Liu et al., 2007). In both the CD82OE and Palm-CD82OE cells, we detect a decreased rate of α4 internalization as well as an increased rate of α4 recycling when compared to control cells. These data suggest that CD82OE can increase the surface expression of integrins, independent of palmitoylation status, by enhancing their plasma membrane recycling rate. In combination, these data implicate a mechanism for our measured expression increase of surface α4; however, it is clear that α4 expression alone cannot account for the observed change in cell adhesion, since both CD82OE and Palm-CD82OE cells express approximately the same amount of surface α4 yet illustrate dramatically different adhesion abilities.

TEMS have been proposed to enhance cell adhesion by clustering functionally related molecules or tightly packing specific receptors into the plasma membrane (Yanez-Mo et al., 2009). Palmitoylation can play a key role in the stable association of tetraspanins with each other (TEMs) and adhesion-related proteins. In fact, several reports have shown that mutation of the intracellular membrane proximal cysteines reduces interactions between

tetraspanins (Berditchevski et al., 2002; Charrin et al., 2002; Delandre et al., 2009; Stipp, 2010; Yang et al., 2002; Yang et al., 2004; Zhou et al., 2004). In this study, we utilize the dSTORM super-resolution imaging technique to visualize and quantitatively demonstrate palmitoylation-mediated alterations in tetraspanin organization. Our data indicate that mutation of the CD82 palmitoylation sites reduces the size of CD82 clusters within the plasma membrane and leads to changes in the membrane organization of the α4 integrin. While previous work has established that CD82 does not directly interact with α4β1 (Serru et al., 1999), it is clear that α4β1 and CD82 exist within the same membrane complex (Mannion et al., 1996). Therefore, the interaction of CD82 with other tetraspanins, which is stabilized by palmitoylation, likely contributes to the indirect linking of α4β1 into TEMs.

Previous work has shown that tetraspanin association facilitates the recruitment of cell adhesion molecules such as VCAM and ICAM into adhesive "nanoclusters" (Barreiro et al., 2008). Similarly, CD81 was proposed to generate rapid adhesion strength to VCAM-1 through the augmentation α4β1 avidity (Feigelson et al., 2003). More recently, CD37 was also shown to regulate the mobility and clustering of α4β1 in B cells (van Spriel et al., 2012). Our clustering data indicate that CD82OE results in α4 clusters, which are smaller in area (μm^2) when compared to clusters found in control or Palm-CD82OE cells. However, despite the difference in average α4 cluster area, the number of α4 molecular localizations within each cluster remains the same. The molecular density of a protein cluster can be modified by altering the number of localizations found within a cluster area. Therefore, fitting the same number of localizations into a smaller area results in the increased molecular density of α4 in the CD82OE cells. The number and strength of bonds between integrins and ECM components can contribute to the overall strength of the adhesion complex (Maheshwari et al., 2000). Our data suggest that it is the tight packing of α4 molecules, as promoted by CD82 upon α4 ligand engagement, which enhances the overall adhesive contribution of α4 clusters. The increase in laminin adhesion by CD82OE cells may also suggest that CD82 alters the clustering and

potentially the density of laminin binding integrins α3 and α6. Previous studies assessing the importance of integrin spacing for adhesive contribution postulate that proper positioning is necessary to maintain integrin linkages with one another, as well as adequate integrin binding to ECM components (Arnold et al., 2004; Selhuber-Unkel et al., 2008). Moreover, Arnold et. al show that improper integrin binding site separation results in limited cell attachment due to restricted integrin clustering. In our study we find that CD82OE can facilitate the organization of α4 integrins into densely packed structures implicating the importance of α4 molecular density for cell adhesion. Furthermore, we speculate that the compromised α4 receptor clustering observed in the Palm-CD82OE cells reduces adhesion by limiting the recruitment or stability of structural and/or signaling elements.

Among the tetraspanins, CD82 is largely studied in cancer where its expression is inversely correlated with metastasis formation (Miranti, 2009; Tsai and Weissman, 2011; Zoller, 2009). The ability of CD82 to regulate metastasis is likely related to its ability to modulate integrin function, which we demonstrate in this study involves molecular density regulation. Taken together, CD82 can modify not only the assembly of membrane protein structures, but also the molecular concentration of integrins within these structures. As such, we propose that the molecular crowding of α4, which is regulated by CD82 and its palmitoylation state, modulates the overall adhesive strength of cells to the ECM. Finally, our detailed insight into how CD82 contributes to the coordinated molecular regulation and organization of α4 implicates CD82 as an attractive potential therapeutic target to improve HSPC mobilization and engraftment capabilities.

2.5 Materials and methods

2.5.1 Cell culture

KG1a human hematopoietic myeloid progenitor cells (ATCC CCL-246.1, Manassas, VA) were cultured in RPMI 1640 Medium (Mediatech, Manassas, VA.), supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 100 Units/mL penicillin and

100 µg/mL streptomycin (PenStrep; Invitrogen, Carlsbad, CA). Human microvascular endothelial cells (hMVECs) were purchased and cultured as indicated by Cell Applications. Cells were incubated at 37°C, 5% humidity, and 5% CO_2.

2.5.2 Overexpression and knockdown vector constructs

To generate the mCherry-CD82 plasmid, CD82 was subcloned from the YFP-CD82 construct (Addgene) into the mCherry-C1 Vector (Invitrogen) using the XhoI and SacII restriction sites. The YFP-Palm-CD82 (CD82 palmitoylation mutant) construct was a generous gift from D. Derse (NIH) (Mazurov et al., 2007). To crease the mCherry version of the construct, the PALM--CD82 insert was PCR amplified with the following primers (Forward: 5'-CTCGAGCGATGGGCTCAGCC-3' and Reverse: 5'-CCGCGGAAGCTTTCAGTACTTGGG-3') and inserted into the mCherry-C1 with the XhoI and SacII restriction enzymes. The CD82 shRNA plasmid (Santa Cruz Biotechnology, Santa Cruz, CA) consisted of a pool of three to five plasmids encoding 19-25 nucleotides (plus hairpin). CD82-targeted siRNAs consisting of pools of three 20-25 nucleotide siRNA sequences and the scrambled control siRNA were also purchased from Santa Cruz Biotechnology.

2.5.3 Nucleofection

KG1a cells were transfected according to the manufacturer's instructions using the Lonza Nucleofection Kit (Lonza, Walkersville, MD). Stable cell lines expressing mCherry, mCherry-CD82, and mCherry-Palm--CD82 constructs were selected for with 500 µg/mL Geneticin® (G418; Invitrogen, Carlsbad, CA). Stably expressing cells were isolated via fluorescence-activated cell sorting (FACS; UNM Facilities).

2.5.4 Flow cytometry

Cells were labeled in PAB buffer (PBS + 1% BSA + 0.02% sodium azide) for 30 minutes on ice with either Alexa Fluor 647 CD82 (clone ASL-24; BioLegend), Alexa Flour 488 integrin α4 (clone 7.2R; R&D), FITC integrin α6 (clone GoH3; BioLegend), Alexa Flour 488 integrin α3 (clone ASC-1; BioLegend), APC integrin α5 (clone NKI-SAM-1; BioLegend), PE integrin α2 (clone HAS3; R&D), FITC

integrin β7 (clone FIB27; BioLegend), or Alexa Flour 647 integrin β1 (clone TS2/16; BioLegend). Separate tubes of cells were labeled with either Alexa Flour 488 mouse IgG1, κ, isotype control (clone 11711; R&D), FITC rat IgG2a, κ, isotype control (clone RTK2758; BioLegend), Alexa Flour 647 mouse IgG1, κ, isotype control (clone MOPC-21; BioLegend), PE mouse IgG2a, κ, isotype control (clone MOPC-173; BioLegend), APC mouse IgG2b, κ, isotype control (clone MPC-11; BioLegend). Cells were washed 3 times with PAB buffer and analyzed using Accuri C6 flow cytometer. Histograms were created using FlowJo software; fluorescence values were normalized to the mode.

2.5.5 Western blot and immunoprecipitation

Cells were lysed in RIPA buffer. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce). 25 µg of protein was subjected to 8% or 10% SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane. Membranes were blocked with 5% dry milk in PBS with 0.22% Tween-20 for 1 hour at room temperature. Membranes were then incubated with either β-Actin (clone AC-74; Sigma Aldrich), Calnexin (clone C5C9, Cell Signaling), integrin α4 (clone EPR1355Y; Novus), integrin β1 (Cell Signaling), CD82 (clone ab66400; AbCam) or integrin α6 (clone ab97760; AbCam) diluted in 5% milk/PBST overnight at 4°C. The membranes were washed three times for 10 minutes in PBS/0.22% Tween-20. Membranes were then incubated with peroxidase-conjugated AffiniPure goat anti-rabbit IgG or peroxidase-conjugated AffiniPure goat anti-mouse IgG secondary antibody diluted in 5% dry milk in PBS/0.22% Tween-20 for 1 hour at room temperature. The membrane was washed three times for 10 minutes in PBS/0.22% Tween-20. HRP conjugate enzymes were stimulated with SuperSignal® West Pico Chemiluminescent Substrate (Pierce). Blots were imaged using the ChemiDoc XRS Imager (Bio-Rad) and analyzed using ImageJ densitometry software. For immunoprecipitation experiments, BRIJ O10 cell lysates were incubated with CD82 antibody overnight at 4°C. Protein A/G Beads (Santa Cruz Biotechnology) were washed and added to the lysates for 30 min at room temperature. The supernatants were removed

and the beads were washed 3x before the beads and supernatants were analyzed for CD82 and α4 by Western blot as described above.

2.5.6 Adhesion assay

96-well microplates were coated with either fibronectin (10 µg/mL in phosphate-buffered saline (PBS); Millipore, Billerica, MA), collagen I (10 µg/mL in PBS; Sigma-Aldrich, St. Louis, MO), laminin (10 µg/mL in PBS; BD Biosciences, Franklin Lakes, NJ), or 10% FBS as a control. For the VCAM-1 adhesion assay, 10 µg/ml of recombinant VCAM-1 (R&D) was used to coat wells. Cells were labeled for 20 min with 2 µM calcein AM fluorescent dye (Invitrogen, Carlsbad, CA) in Hank's buffered salt solution (HBSS). After washing twice with HBSS, the cells were plated at 100,000 cells/well and incubated at 37°C for 2 hrs. The microplate was washed to remove non-adherent cells and the remaining adherent cells were measured using a fluorescence plate reader with excitation wavelength of 488 nm emission detected at 512 nm. Fluorescence data were then normalized to the mean fluorescence obtained for control cells. To measure $\alpha 4\beta 1$ specific adhesion, cells were treated with either dimethyl sulfoxide (DMSO) or blocked with the monovalent peptide LDV (1 µM), which was a generous gift from Drs. Larry Sklar and Tione Buranda (UNM).

2.5.7 Immunofluorescence

Cells were fixed in 4% PFA then blocked and permeabilized with PBS + 1.0 % BSA + 0.1% tween 20. Alexa Fluor 647-conjugated anti-human CD82 (Clone ASL-24, Biolegend) and Alexa Fluor 488-conjugated anti-human integrin α4 (clone 7.2 R; R&D) were added to the sample. Immunofluorescence of VCAM-1 was completed with the mouse anti-human VCAM-1 primary antibody (Abd Serotec) and the Alexa Fluor-488 goat anti mouse secondary antibody (Life Technologies). Cells were labeled for 30 minutes. Cells were washed 3 times with PBS + 1% BSA and then imaged in an 8 well chamber slide. Cells were imaged by laser scanning confocal microscopy with a Zeiss Axiovert 100M inverted microscope (LSM 510) system using excitation wavelengths of 488 or 633 nm and a 63X 1.2 N.A. oil immersion objective. Image analysis was performed using the Zeiss LSM 510 software of Image J (NIH, Bethesda, MD).

2.5.8 Internalization assay

Cells were labeled for 1 hour on ice using an Alexa Fluor 488 integrin α4 antibody (clone 7.2R, R&D). Cells were washed three times using cold medium and resuspended in RPMI medium. An aliquot of cells was used to determine median fluorescence using the Accuri C6 flow cytometer; this is considered 100% surface labeling. The remaining cells were put into the incubator (37° C, 5% CO_2) for 10, 20 and 30 minutes. At the respective time point, 150,000 cells were moved to individual tubes. Cells were treated with 1 µg of anti-Alexa Fluor 488 antibody, (Clone A-11094, Invitrogen) which quenches surface fluorescence; cells were quenched on ice for 1 hour, with > 90% quenching efficiency. After quenching, cells were then fixed for 20 minutes with 4% PFA. Median fluorescence in the FL-1 channel was read using Accuri C6 flow cytometer. Percent internalized was calculated by dividing the median fluorescent intensity quenched value (normalized to background quench) by the median total α4 surface label intensity.

2.5.9 Recycling assay

Cells were labeled for 30 minutes on ice using an Alexa Fluor 488 integrin α4 antibody (clone 7.2R, R&D). Cells were washed three times using cold medium and resuspended in RPMI medium. Before allowing internalization, two aliquots of cells were removed. The first is to determine 100% α4 surface labeling. The second aliquot was quenched, and fixed; this aliquot represents the quenched background fluorescence. The remaining cells were put back into the incubator (37° C, 5% CO_2) and allowed to internalize for 30 minutes. Cells were then treated with 1 µg of anti-Alexa Fluor 488 antibody, (Clone A-11094, Invitrogen) to quench surface fluorescence; cells were quenched on ice for 1 hour. Cells were then moved back to the incubator (37° C, 5% CO_2) and allowed to recycle for 30 minutes. After 30 minutes, the samples were moved back on ice and quenched again for 1 hour. Cells were then fixed with 4% PFA, and median fluorescence was determined using Accuri C6 flow cytometer. The difference between the internalized value and the recycled value gives the amount of α4 recycled back to the membrane. To calculate recycled α4, the fluorescent intensity values were

normalized. Since fluorescent recycling changes are relatively small, quenched background was subtracted from the internalized and recycled median fluorescent values. The percent of α4 recycled was calculated by taking the difference between the normalized internalized and normalized recycled fluorescent median values, and dividing this number by the normalized internalized value. This gives the percent of α4 that was labeled, allowed to internalize for 30 minutes, and quenched upon recycling back to the plasma membrane.

2.5.10 Super resolution imaging

25 µg/ml of human plasma fibronectin (Millipore) diluted in PBS was used to coat the wells of an 8 well chamber slide for 20 minutes. 15 µg/ml of recombinant N-cadherin (R&D) diluted in PBS and wells were coated for 30 minutes. 10 µg/ml of recombinant VCAM-1 (R&D) was also used to coat wells for 1 hour. Cells were then plated on the coated wells and incubated overnight at 37°C. The following day cells were fixed with 4% PFA for 20 minutes, washed once with 1% BSA/PBS and then blocked with 1% BSA/PBS for 1 hour. For CD82 staining, cells were labeled with Alexa Fluor 647 anti-human CD82 antibody (1:125) (clone ASL-24; BioLegend) diluted in 1% BSA/PBS. The wells were then washed three times with 1% BSA/PBS and fixed again with 4% PFA. For α4 staining, cells were first labeled with monoclonal α4 integrin primary antibody (1:200) (clone Bu49; ThermoScientific) diluted in 1% BSA/PBS for one hour. The well was then washed three times with 1% BSA/PBS and subsequently labeled with goat-anti-mouse Alexa Fluor 647 secondary antibody (1:200) (Invitrogen) diluted in 1% BSA PBS for one hour. The wells were then washed three times with 1% BSA/PBS and fixed again with 4% PFA.

Labeled cells were imaged in a reducing buffer including 50 mM β-mercaptoethylamine as a reducing agent. Reference beads were used as a reference point to stabilize the sample during imaging; drift corrections were performed using MCL NanoDrive stage controller. The sample was imaged for 10,000 frames using the microscope set up previously described (Huang et al.,

2011; van den Dries et al., 2013). After obtaining molecule localization estimates and uncertainties, super resolution images were reconstructed using MATLAB analyses (Huang et al., 2011).

The pair autocorrelation function (Veatch et al., 2012) was used to analyze CD82 and α4 cluster size. Radially averaged autocorrelation functions were calculated from three 3 x 3 micron sized areas in each cell. Autocorrelation functions from the same cell were averaged and fit to the functional form $g_{meas}(r)=B*\exp\{-r^2/4\sigma_{PSF}^2\}/(4\pi\sigma_{PSF}^2\rho) + g(r>0)*g_{psf}$. In order to decouple cluster sizes from broadening due to finite localization precision, domains are evaluated as 2D Gaussian shapes, giving $g(r>0)= A*\exp\{-r^2/4\sigma_{Dom}^2\}/(4\pi\sigma_{Dom}^2)$ and therefore the fitting function $g_{meas}(r)=B*\exp\{-r^2/4\sigma_{PSF}^2\}/(4\pi\sigma_{PSF}^2\rho)+ A*B*\exp\{-r^2/4(\sigma_{Dom}^2+\sigma_{PSF}^2)\}/(4\pi(\sigma_{Dom}^2+\sigma_{PSF}^2)\rho)+1$. Here A is the number of molecules per domain, B is the number of repeat observations per molecule, σ_{PSF} is the fluorophore localization precision, σ_{Dom} is the cluster size, and ρ is the observed localization density. The value for ρ was calculated directly from the selected regions, while A,B, σ_{PSF}, and σ_{Dom} were simultaneously estimated by performing a non-linear least-squares fit of the average autocorrelation to $g_{meas}(r)$. The magnitude of $g(r)$ is a function of both the density and number of repeat observations of each molecule and can therefore differ with expression level, labeling efficiency and imaging conditions, whereas the cluster size is extracted from the shape of the curve and is independent of these effects. The average cluster size for a population of cells was assessed statistically using Student's unpaired t-test.

The DBSCAN cluster algorithm was used to assess larger scale α4 clustering. A 56 x 56 pixel box (5.975 x 5.975 μm box) was examined for clustering. Epsilon value of 1 pixel (106.7 nm) and n value of 30 localizations were used to examine α4 cluster area. In order to validate our parameters, we also tested the modified parameters epsilon = 0.5, n = 30 and epsilon = .5, n = 20 and saw the same trends of the cumulative distribution plots as assessed using the Kolmorgov-Smirnof test (Suppl. Fig. S.2.7A-B)

2.5.11 Statistics

All experiments were performed at least three times independently. Results are expressed as mean ± SD or SEM. Student's *t* test was used for mean comparisons. The Kolmogorov-Smirnov test was used for comparison of cumulative distributions. Statistical analyses were performed using Prism 5 (Graphpad software). Significant differences are indicated using asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

2.6 Acknowledgements

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Chapter 3: Tetraspanin CD82 regulates bone marrow homing of acute myeloid leukemia by modulating the molecular organization of N-cadherin

Kristopher D. Marjon[1*], Christina M. Termini[1*], Karin L. Karlen[1], Chelsea Saito-Reis[1], Cesar E. Soria[1], Keith A. Lidke[2], Jennifer M. Gillette[1#]

[1]Department of Pathology, University of New Mexico, 1 University of New Mexico, Albuquerque, NM 87131. [2]Department of Physics and Astronomy, 1 University of New Mexico, Albuquerque, NM 87131. [*]Equal Contribution.

[#]Corresponding Author. Jennifer M. Gillette, 1 University of New Mexico, MSC 08-4640, Albuquerque, NM 87131. (505) 272-0835. JGillette@salud.unm.edu

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3.1 Abstract

Communication between acute myeloid leukemia (AML) and the bone marrow microenvironment is known to control disease progression. Therefore, regulation of AML cell trafficking and adhesion to the bone marrow is of significant interest. In this study, we demonstrate that differential expression of the membrane scaffold CD82 modulates the bone marrow homing of AML cells. By combining mutational analysis and super-resolution imaging, we identify membrane protein clustering by CD82 as a regulator of AML cell adhesion and bone marrow homing. Cluster analysis of super-resolution data indicates that N-linked glycosylation and palmitoylation of CD82 are both critical modifications that control the microdomain organization of CD82 as well as the nanoscale clustering of associated adhesion protein, N-cadherin. We demonstrate that the inhibition of CD82 glycosylation increases the molecular packing of N-cadherin and promotes the bone marrow homing of AML cells. In contrast, we find that the inhibition of CD82 palmitoylation disrupts the formation and organization of N-cadherin clusters and significantly diminishes bone marrow trafficking of AML. Taken together, these data establish a mechanism where the membrane organization of CD82, through specific posttranslational modifications, regulates N-cadherin clustering and membrane density, which impacts the in vivo trafficking of AML cells. As such, these observations provide an alternative model for targeting AML where modulation of protein organization within the membrane may be an effective treatment therapy to disrupt the bone marrow homing potential of AML cells.

3.2 Introduction

AML, the most common acute leukemia affecting adults, is characterized by an increase of immature myeloid blasts in the bone marrow that results from a loss of normal differentiation and proliferation of hematopoietic stem/progenitor cells (HSPCs) (Machida et al., 1999). Multiple subtypes of AML exist with a range of aggressiveness and treatment sensitivity (Guzman and Allan, 2014). One sign of disease aggressiveness is the ability of AML cells to home to the bone marrow and displace HSPCs (Konopleva et al., 2002). Homing requires multiple steps

including the ability to respond to a chemotactic gradient, extravasation, and adhesion to specialized niches within the bone marrow. In fact, adhesion-mediated interactions between AML cells and the bone marrow play an important role in disease progression and chemoresistance (Bradstock and Gottlieb, 1995; Gibson, 2002; Jin et al., 2006; Zhang et al., 2013). Therefore, identifying the molecules and mechanisms that mediate AML-bone marrow adhesion and homing are fundamental to the development of future therapeutic treatments.

Recently, an AML protein profile was identified for a subpopulation of leukemic blasts, the leukemia stem cells (LSCs). This mass spectrometry study found an enrichment of specific adhesion-related proteins including CD44, integrin α6, CD47 and CD82 on LSCs (Bonardi et al., 2013). An alternative AML screen also identified the upregulation of CD82 in LSCs where it was suggested to modulate AML adhesion to the bone marrow (Nishioka et al., 2013). Following its initial cloning (Gil et al., 1992; Imai et al., 1992; Lebel-Binay et al., 1994), the tetraspanin CD82 (or Kai1) was described as a metastasis suppressor in solid tumors (Dong et al., 1995). Tetraspanins are evolutionarily conserved membrane proteins present in most eukaryotes that function as mediators of cell adhesion, trafficking, and cell signaling (Boucheix and Rubinstein, 2001). Through their ability to associate in *cis* with other tetraspanins, cell adhesion molecules, and signaling receptors, tetraspanins form tetraspanin-enriched microdomains (TEMs) (Bassani and Cingolani, 2012; Hemler, 2008b). Formation of TEMs enables tetraspanins to serve as molecular organizers for membrane proteins (Hemler, 2008b). Our recent work identified a role for CD82 in the homing of human HSPCs, which we linked to the membrane organization of CD82 and associated adhesion and signaling molecules (Larochelle et al., 2012). Currently, basic questions concerning the formation and regulation of TEMs and their modulation of adhesion receptors, which specifically impact bone marrow homing, still remain.

N-cadherin is a classical cadherin that interacts homophilically with cadherins on neighboring cells to form adherence junctions, which mechanically link cells and relay signaling information from the extracellular environment

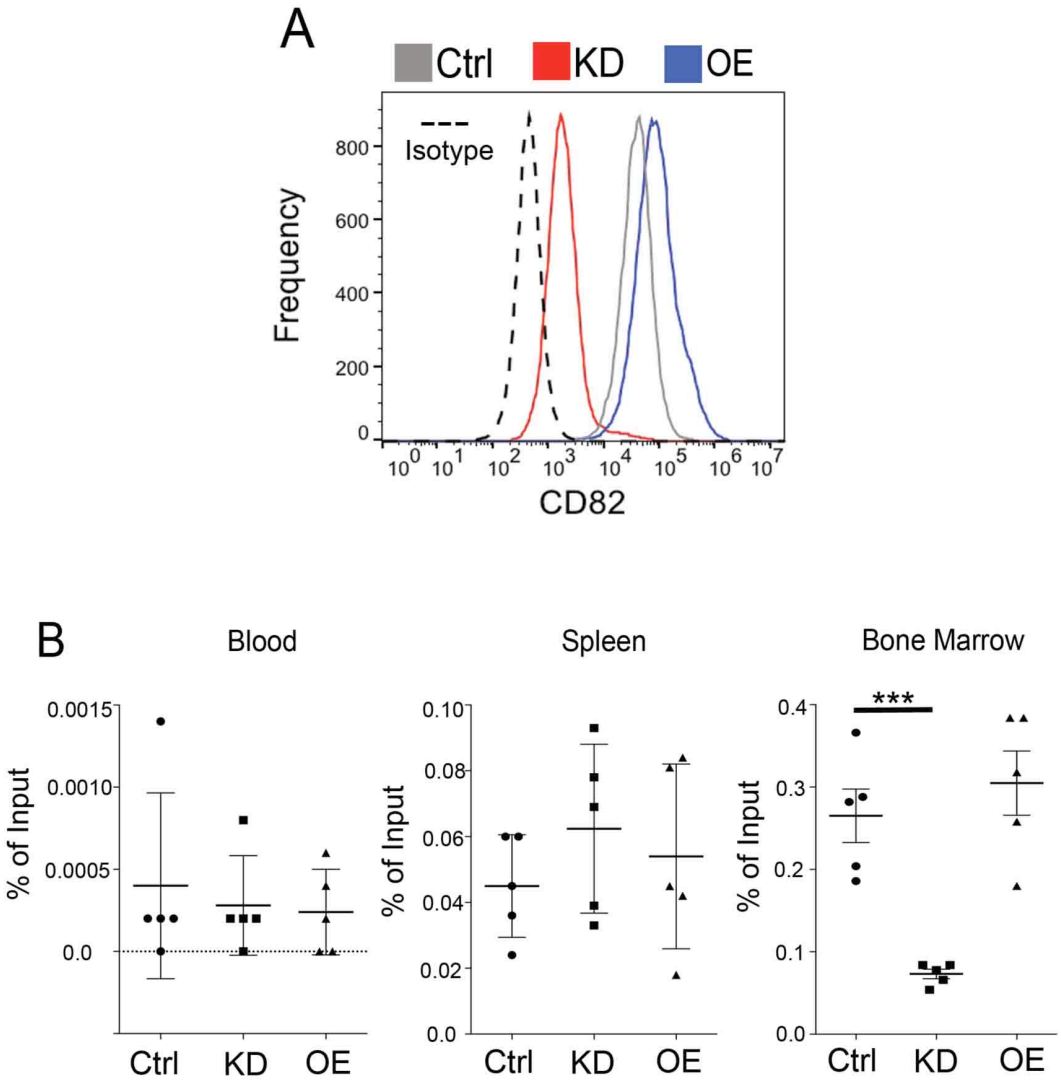
(Kemler, 1993; Takeichi, 1995). While the function of N-cadherin remains controversial for HSPCs (Bromberg et al., 2012; Calvi et al., 2003; Greenbaum et al., 2012), its role in the regulation of specific leukemias is more evident. In AML, the LSC compartment that expresses N-cadherin is relatively resistant to chemotherapy treatments and highly enriched following chemotherapy (Zhi et al., 2010). Subsequent studies suggest that N-cadherin expression facilitates LSCs to initiate and induce AML development (Qiu et al., 2014). In combination, these data indicate that N-cadherin participates in the protection of LSCs and the relapse of AML; therefore, the regulation of N-cadherin function in AML is of significant interest.

The dynamic regulation of cadherin-mediated adhesiveness is thought to involve modulation of cadherin density arrangement on the cell surface (Hong et al., 2013). Moreover, clustering of cell surface cadherins is known to modify cadherin-mediated adhesion and signal transduction, but the mechanism of cadherin clustering is poorly understood (Nelson, 2008). Combining super-resolution imaging, CD82 mutational analysis, and *in vivo* functional studies, we utilize a multiscale approach that identifies CD82 as a regulator of AML cell adhesion and bone marrow homing. Our work establishes a mechanism where the membrane organization of CD82, which is dependent upon specific post-translational modifications, regulates N-cadherin clustering and membrane density. We demonstrate that the spatial regulation of N-cadherin by CD82 leads to functional *in vivo* consequences for AML cell behavior.

3.3 Results/Discussion

3.3.1 CD82 expression increases AML cell homing to the bone marrow and modulates N-cadherin mediated adhesion.

To gain mechanistic insight into how CD82 affects bone marrow homing, we used the previously described control, CD82 overexpression (CD82OE), and CD82 knock down (CD82KD) human KG1a cells (Fig.3.1A) to monitor changes in AML cell homing using NSG mice. Sixteen hours following injection, we detected no difference in AML cell localization to the spleen or blood (Fig.3.1B). However, when we analyzed the bone marrow, we identified a marked reduction in bone


A
Ctrl
KD
OE
Isotype
Frequency
800
600
400
200
0
10^0 10^1 10^2 10^3 10^4 10^5 10^6 10^7
CD82
B
Blood
Spleen
Bone Marrow
% of Input
0.0015
0.0010
0.0005
0.0
0.10
0.08
0.06
0.04
0.02
0.0
0.4
0.3
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Ctrl
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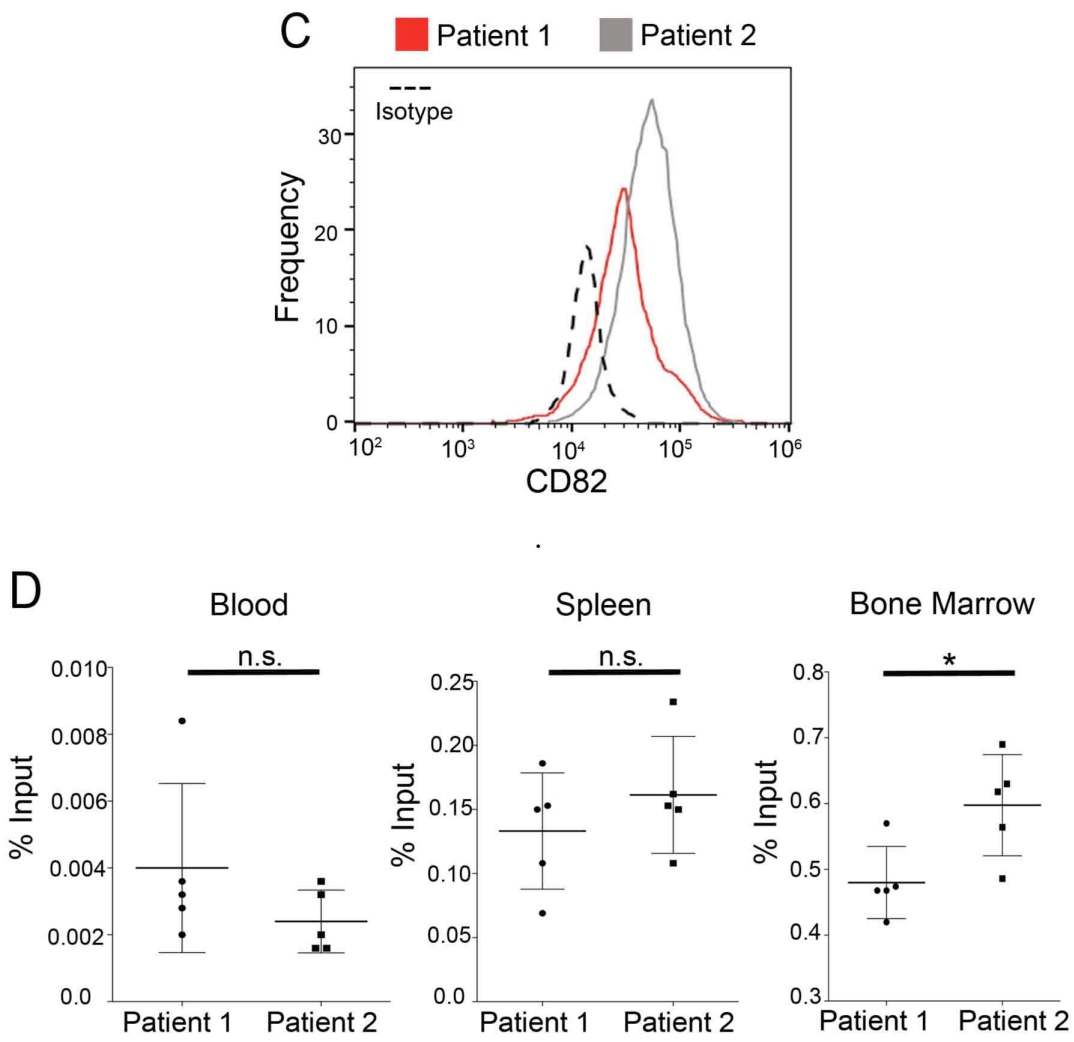
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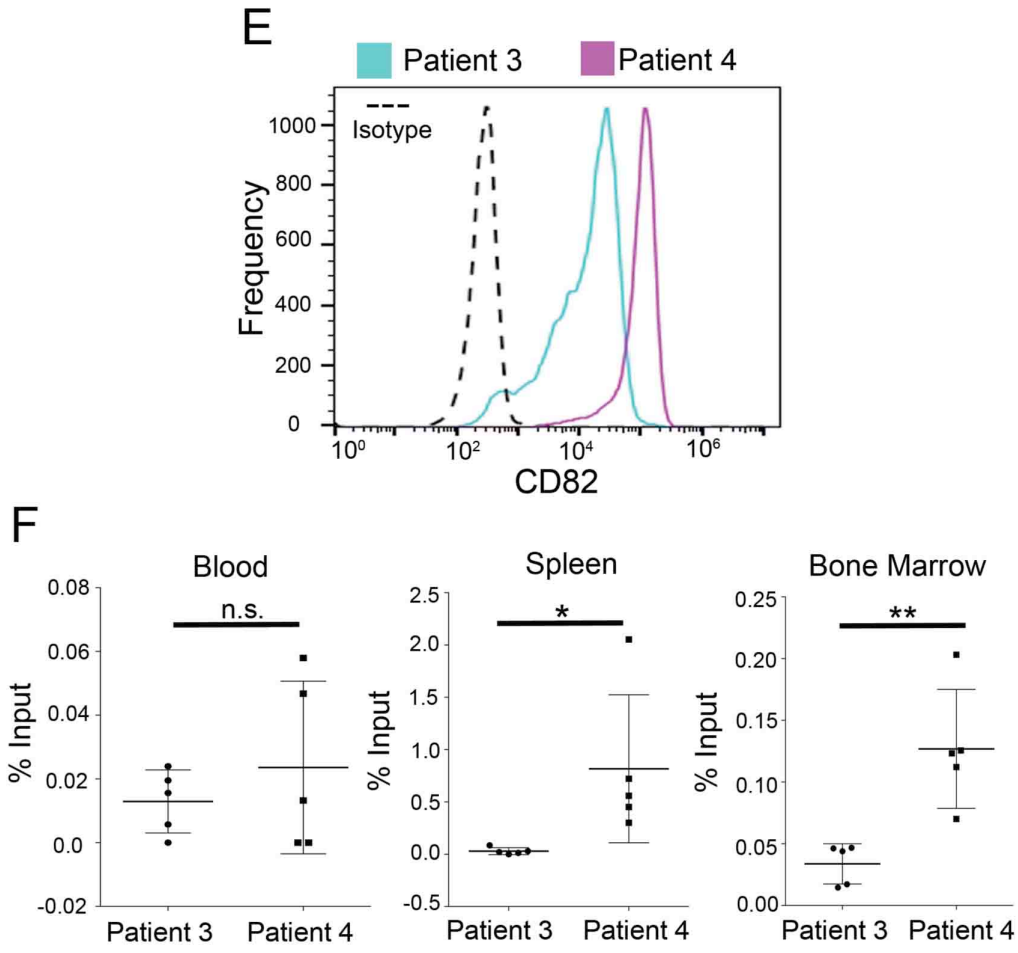
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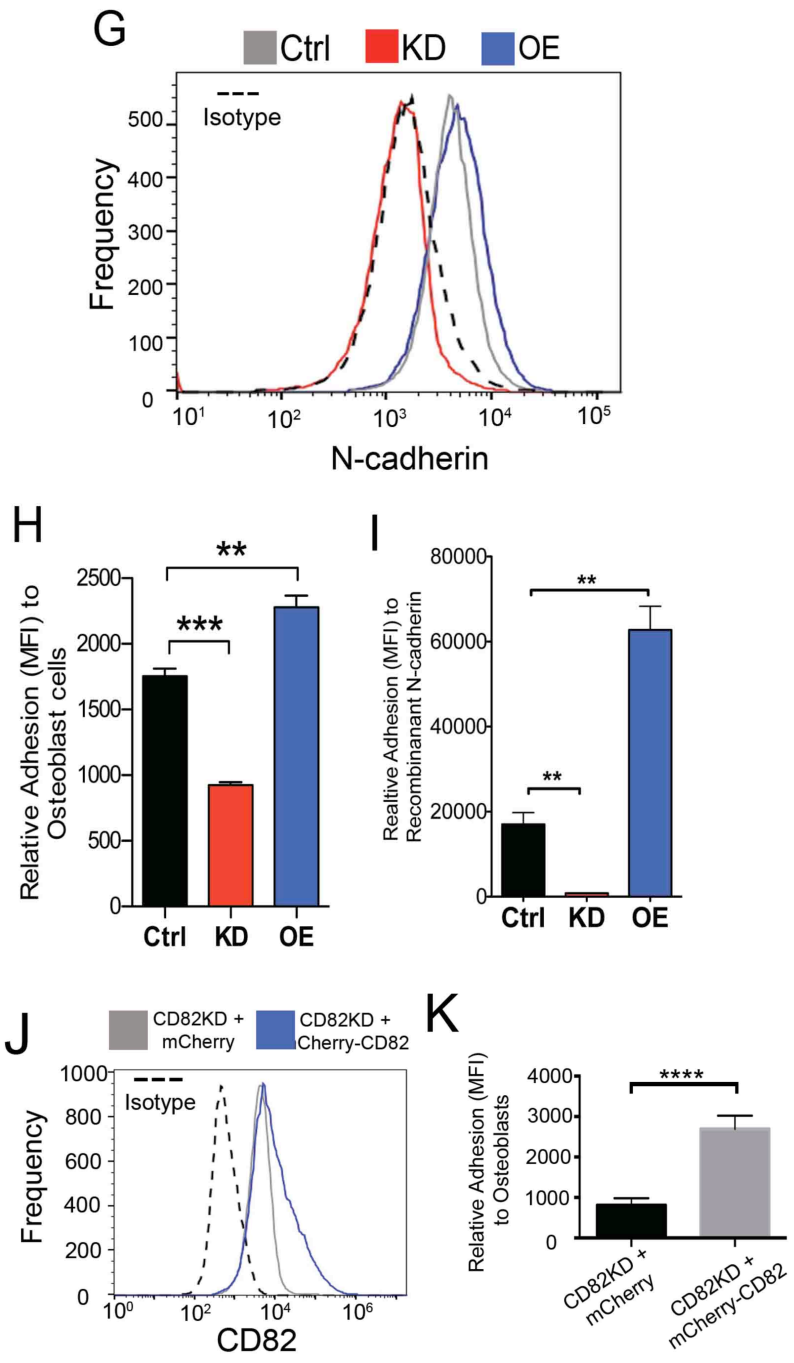


Figure 3.1: CD82 expression regulates homing to the bone marrow and adhesion to niche components. (A) Flow cytometry analysis of CD82 surface expression using previously described CD82OE, CD82KD and control KG1a cell lines27 (ATCC, Manassas, VA, USA; CCL-246.1). Cells were characterized using Alexa Fluor 647 anti-human CD82 (clone ASL-24, BioLegend, San Diego, CA, USA). Data were acquired using an Accuri flow cytometer C6 (BD Bioscience, San Jose, CA, USA) and analyzed with FlowJo X software (Tree Star, Inc., Ashland, OR, USA). (B) Bone marrow homing of CD82OE, KD or Ctrl

KG1a cells. Cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer's protocol. After labeling, 1×10^6 cells were injected intravenous into female NOD. Cg-PrkdcscidIl2rgtm1wjl/SzJ (NSG) mice 8–12 weeks of age. NSG mice were housed and bred at the Animal Research Facility under specific pathogen-free conditions at the University of New Mexico Health Sciences Center (Albuquerque, NM, USA). All procedures were approved by the University of New Mexico Institutional Animal Care and Use Committee and carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Sixteen hours after injection, the blood, spleen and bone marrow were harvested. A single cell suspension was generated and red blood cells were lysed with ACKs buffer (15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA). Cells were treated with Fc block, and then stained for human-CD45 and analyzed by flow cytometry for CFSE and huCD45 (Clone HI30, BioLegend) double positive cells. Percent input was calculated on the basis of the number of double positive events multiplied by total tissue cell number divided by the number of cells injected all multiplied by 100 (n=5 mice). (C and E) Flow cytometry analysis of CD82 on the surface of primary AML cells. (D and F) Tissue harvest from 8- to 10-week-old male and female NSG mice 16 h after intravenous injection of CFSE-labeled primary AML cells (1×10^6 cells) using the protocol described above (n=5 mice/patient sample). AML patient samples were deidentified and obtained from the UNM Health Science center (HSC) cell bank. Flow cytometry analysis of (G) N-cadherin (Clone 8C11, BioLegend) surface expression on Ctrl, CD82KD or CD82OE KG1a cells. Fluorescence-based cell adhesion assay using Ctrl, CD82KD and CD82OE cells. Cells were labeled with 2 μM calcein (Invitrogen, Carlsbad, CA, USA) and allowed to adhere to (H) SaOS-2 osteoblastic cells (ATCC) or (I) purified N-cadherin (R&D Systems, Minneapolis, MN, USA) for 1 h. Non-adherent cells were removed by washing and remaining fluorescent cells were measured by using synergyH1 plate reader (Biotek, Winooski, VT, USA) and analyzed with the Gen5 2.00.18 plate reader software (n=3 replicates). (J) Flow cytometry analysis for CD82 following the nucleofection of mCherry or the mCherry-CD82 vectors into the CD82KD cells. (K) Osteoblastic cell adhesion analysis (as previously described) for CD82KD cells upon CD82 reintroduction. For all graphs, mean is displayed with error bars denoting s.d., all variances were determined to be similar; no randomization or blinding methods were used; statistics were performed using two-sided unpaired t-test. (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

marrow homing of the CD82KD cells along with a modest increase in the bone marrow homing of CD82OE cells when compared to control cells. Therefore, CD82 expression can modify the *in vivo* trafficking of AML cells. To further evaluate this finding, we compared the homing capacity of primary human AML cells with differential CD82 expression (Fig.3.1C,E). Consistent with the cell line data, we find that AML cells with higher CD82 expression display improved bone marrow homing when compared to AML cells with lower expression of CD82 (Fig.3.1D,F). The combined cell line and primary AML cell data suggest that CD82 expression modulates AML cell homing to the bone marrow microenvironment, which is an indicator of aggressive AML.

Bone marrow homing of AML cells requires a series of complex steps involving a combination of cell migration and adhesion signaling. The chemokine receptor, CXCR4, with its ligand, stromal derived factor-1 (SDF-1), is the major receptor signaling pathway used for bone marrow homing by HSPCs (Aiuti et al., 1997) and various types of leukemic cells (Zaitseva et al., 2014). While functional interactions between tetraspanins and CXCR4 signaling were shown previously (Yoshida et al., 2008), we did not detect any CXCR4 expression differences between the control, CD82OE, and CD82KD cells (Suppl.Fig.S.3.1A,B). Additional analysis of cell migration toward SDF-1 illustrates no difference in the migratory behavior of these cells in a transwell assay (data not shown). Therefore, these data suggest that the observed changes in bone marrow homing are not likely due to CD82-mediated effects on the CXCR4 homing signal.

Next, we turned to evaluate whether CD82 expression may affect AML cell adhesion within the bone marrow by screening the cell lines for expression changes in the cadherin family of cell-cell adhesion molecules (Kemler, 1993; Takeichi, 1995). While we were unable to detect differences in the expression of E-cadherin and P-cadherin (Suppl.Fig.S.3.1C,D), the surface expression of N-cadherin was significantly reduced in the CD82KD cells (Fig.3.1G). Recently, N-cadherin enrichment was identified on the surface of LSCs, which was proposed to enable the cell adhesion of AML cells to the bone marrow (Qiu et al., 2014; Zhi

et al., 2010). Therefore, we used a fluorescence-based adhesion assay to measure changes in cell adhesion to osteoblasts and purified N-cadherin. Consistent with the homing experiments, we find that CD82KD results in a decrease in cell adhesion to osteoblastic cells as well as purified N-cadherin, whereas CD82OE cells display an increase in cell adhesion (Fig.3.1H,I). Furthermore, the reintroduction of CD82 back into the CD82KD cells recovered the reduced adhesion phenotype (Fig.3.1J,K). Together, these data implicate a specific role for N-cadherin in CD82-mediated AML cell adhesion.

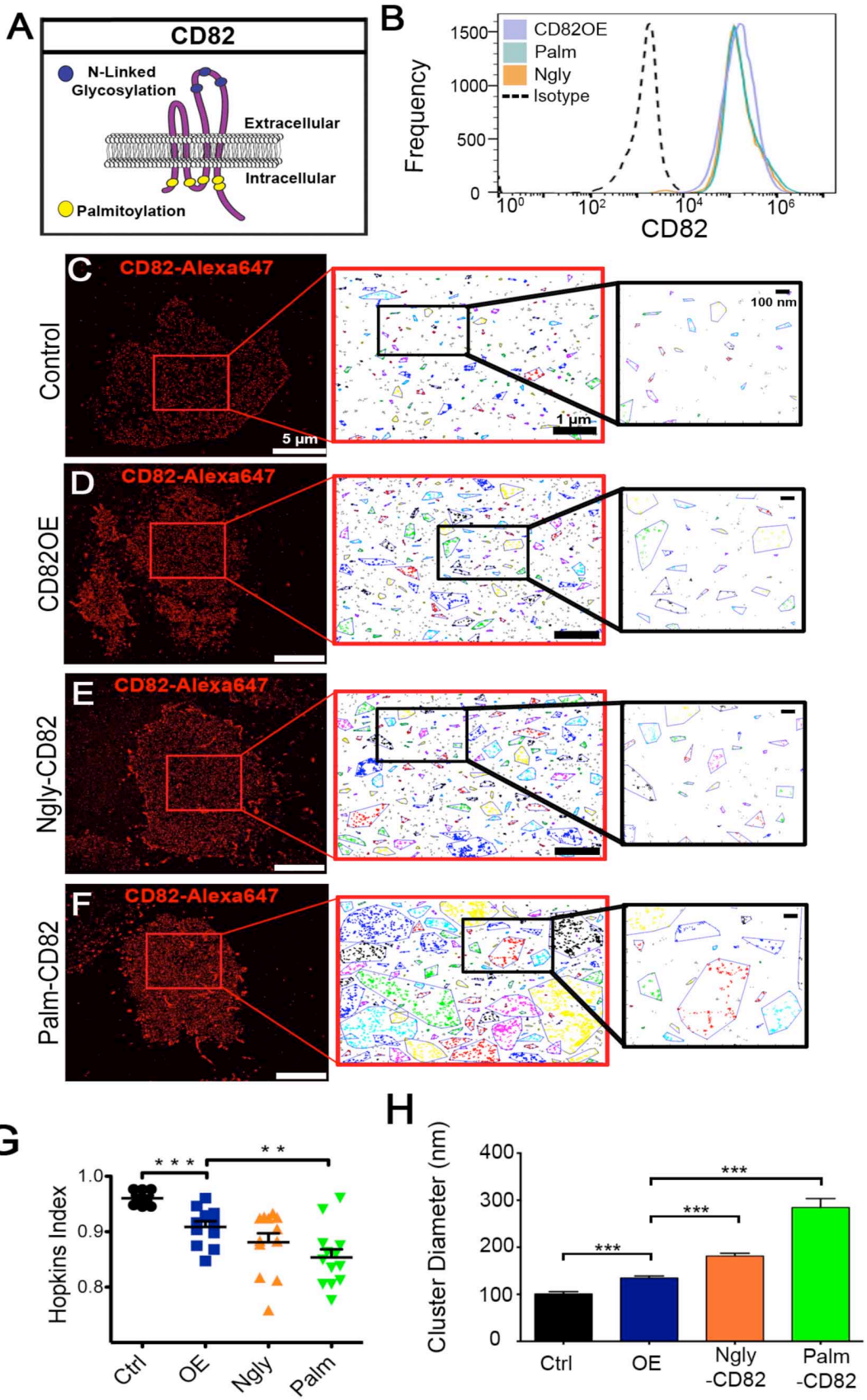
3.3.2 CD82 membrane clustering is altered by glycosylation and palmitoylation status.

A distinct feature of tetraspanins is their ability to associate with other tetraspanins, cell adhesion molecules and signaling receptors, thereby serving as molecular facilitators for membrane proteins (Bassani and Cingolani, 2012; Hemler, 2008b). Therefore, the mechanism by which CD82 regulates AML cell adhesion and homing is likely to be dependent upon its ability to form higher order protein complexes in the cell membrane. Moreover, the regulation of TEM formation and stability is of significant interest. Previously, our group and others showed that the palmitoylation of the membrane proximal cysteines of CD82 promotes the oligomerization and dynamic reorganization of proteins into microdomains (Berditchevski et al., 2002; Termini et al., 2014; Yang et al., 2004; Zhou et al., 2004). Furthermore, cell surface glycosylation, which can alter protein-protein interactions, also regulates the membrane organization of proteins. The glycosylation of membrane bound proteins is perturbed in many cancers and can be regulated by oncogenic factors (Dwivedi et al., 1988; Seales et al., 2003; Swindall et al., 2013). Recently the membrane glycosylation of CD82 was shown to play a role in cell adhesion and motility in specific cancers (Wang et al., 2012a; White et al., 1998). To evaluate how palmitoylation and glycosylation of CD82 affect its membrane organization and the aggressive potential of AML, two constructs were generated where: 1) the membrane proximal cysteines were mutated to serine, preventing palmitoylation (Palm-CD82) (Mazurov et al., 2007; Termini et al., 2014), and 2) the three N-linked

glycosylation sites were mutated to glutamine, inhibiting glycosylation (Ngly-CD82) (Fig.3.2A). These constructs were stably transfected into KG1a cells and Figure 3.2B indicates that the Ngly-CD82 and Palm-CD82 cells express similar CD82 surface levels as the CD82OE cells. Interestingly, both mutants contain intracellular CD82, which may further suggest changes in CD82 protein trafficking that are regulated by these post-translational modifications.

Next, we assessed how these CD82 mutations affect the membrane organization of the CD82 scaffold. To measure differences in microdomain organization between control, CD82OE, Ngly-CD82 and Palm-CD82 cells, we used the super-resolution imaging technique, direct stochastic optical reconstruction microscopy (dSTORM) (Heilemann et al., 2008). Super-resolution imaging allows us to quantify changes in CD82 membrane organization at the level of individual molecules on the nanometer scale (Fig.3.2C-F). Initially, the reconstructed dSTORM images were analyzed using the Hopkins index, which determines the extent to which CD82 is present in a random distribution on the cell surface (Mattila et al., 2013; Zhang et al., 2006). Consistent with our visual observations, we find that each of the CD82 expressing AML cells has a Hopkins index that is significantly higher than what would be expected for a random distribution of molecules (0.5), demonstrating that CD82 is not randomly distributed, but organized into membrane clusters (Fig.3.2G).

The CD82 dSTORM images were also analyzed using the density-based spatial clustering of applications with noise clustering algorithm (DBSCAN) (Fig.3.2C-F,zoom) as previously described (Ester et al., 1996b). From these measurements, we determined that CD82OE cells have an increased CD82 cluster diameter and area with respect to control cells, which is likely due to the increased expression of CD82 (Fig.3.2H,I). Interestingly, the CD82 cluster size quantified for both the Ngly-CD82 and Palm-CD82 cells indicates an even further increase in CD82 cluster diameter and area when compared to CD82OE cells (Fig.3.2H,I). Measurements of the Palm-CD82 cells detect the most significant increase in CD82 cluster size and decrease in CD82 cluster organization, which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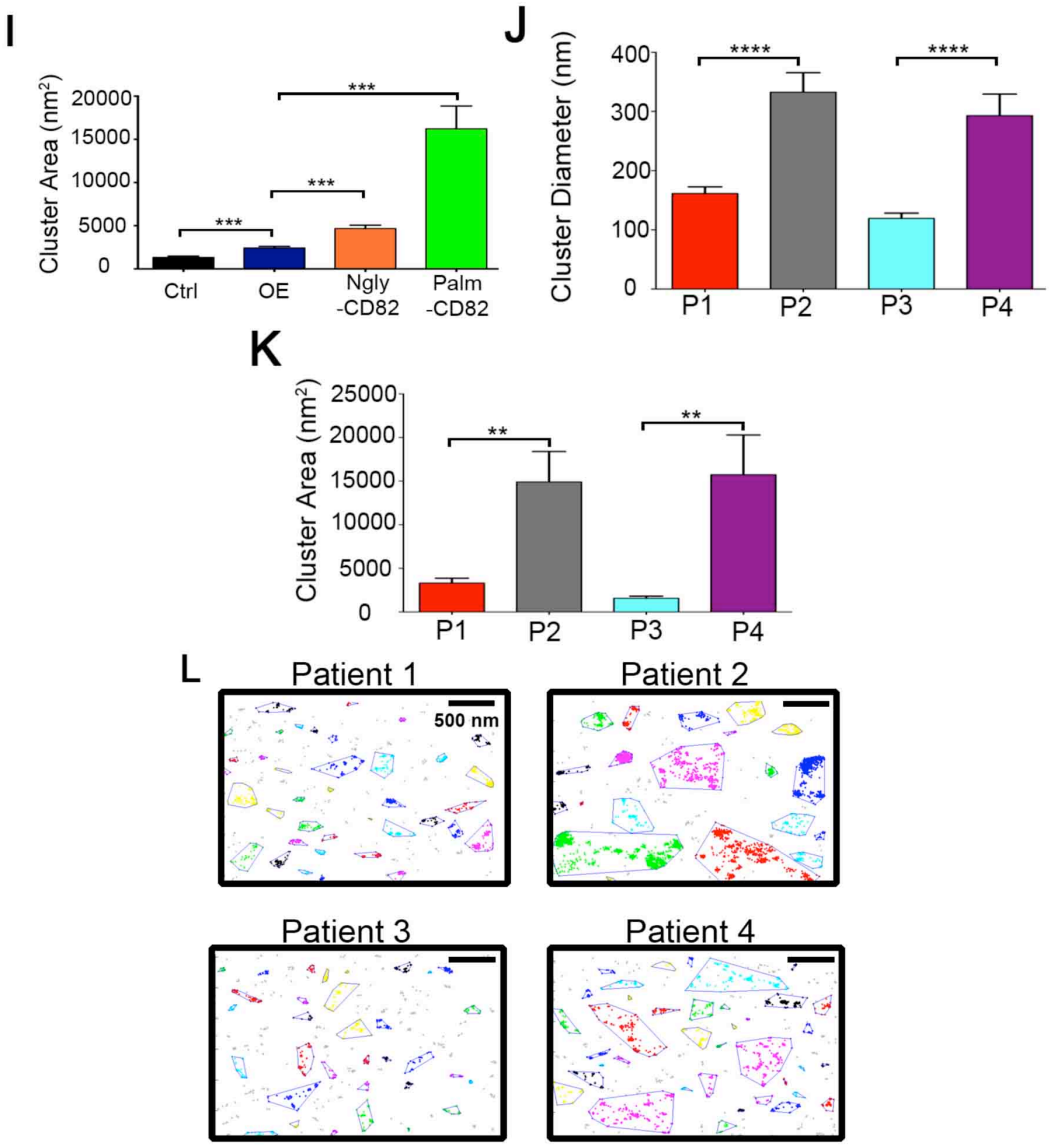


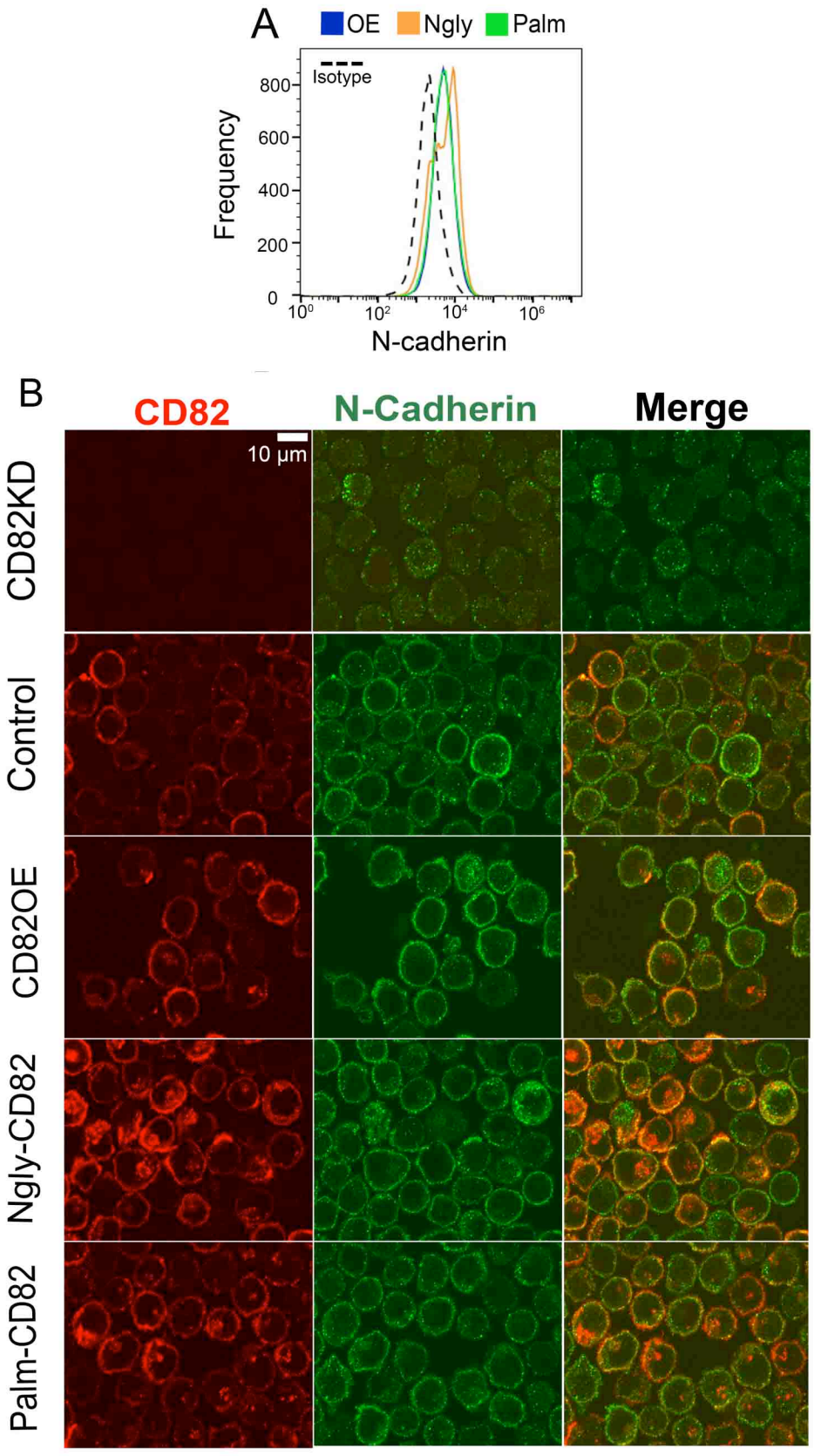
Figure 3.2. Palmitoylation of CD82 is critical for CD82 membrane organization. (A) Cartoon of CD82 highlighting N-linked glycosylation and palmitoylation sites. Using the mCherry-CD82 plasmid (Termini et al., 2014), three N-linked glycosylation sites on CD82, Asparagine 129, 157 and 198 were mutated individually to glutamine using a QuickChange II site-directed mutagenesis kit (Agilent) according to the manufacture's instructions (Ngly-CD82). All mutations were confirmed by DNA sequence analysis (ACGT Inc.). CD82 palmitoylation mutant was generated as previously described (Palm-CD82) (Termini et al., 2014). (B) Flow cytometry analysis of CD82 surface expression on CD82OE, Ngly-CD82, and Palm-CD82 cells. (C-F) Reconstructed dSTORM images of CD82 distribution on each cell line (n ≥ 3 cells per cell line). The previously described labeling, imaging, and fitting protocols were followed (Huang et al., 2011; Termini et al., 2014). (G) Hopkins analysis of CD82 cellular membrane organization using reconstructed dSTORM images was performed

using SuperCluster Matlab software from the UNM Spatiotemporal Modeling Center. The reconstructed dSTORM images were also analyzed with the DBSCAN algorithm to generate DBSCAN images (C-F zooms), which represent clustered CD82 localizations in color and non-clustered CD82 localizations in gray. A 6 x 6 μm box was examined for clustering using an epsilon value of 100 nm and an n value of 10 localizations. Quantification of (H) CD82 cluster diameter and (I) CD82 cluster area based on DBSCAN analysis ($n \geq 3$ cells per cell line). (J-L) dSTORM imaging and DBSCAN analysis for CD82 cluster area and diameter was performed on four primary AML samples. (** $p < 0.01$, *** $p < 0.001$; one-way ANOVA, post-hoc t-test with Welch's correction for groups with unequal standard deviations).

is consistent with previous work demonstrating the importance of the palmitoylation sites in the lateral packing of CD82 (Termini et al., 2014; Zhou et al., 2004). Previous work from our lab identified smaller CD82 cluster sizes in the Palm-CD82 cells using pair-auto correlation function analysis, which is an averaged radial cluster measurement. In contrast, the DBSCAN algorithm enables the quantification of larger scale clusters of varying shapes and sizes, which is what we find for CD82. As for the N-glycosylation mutation, the effects on CD82 cluster size are more modest, however we do detect an increase in CD82 cluster diameter and area. We also imaged and analyzed the CD82 cluster area and diameter in primary AML cells. Consistent with the cell line data, Fig.3.2J-L further illustrate the differentiation clustering of CD82 in primary patient samples. In combination, these data illustrate that while the CD82OE, Ngly-CD82 and Palm-CD82 cells all have similar CD82 surface expression, the Ngly- and Palm- mutations change the CD82 membrane distribution into larger ordered CD82 clusters. Therefore, these specific post-translational modifications regulate the membrane organization of CD82, which may in turn modulate protein-protein interactions important for bone marrow homing and adhesion.

3.3.3 N-cadherin clustering is regulated by CD82 membrane organization.

Next, we set out to determine whether the described changes in CD82 membrane organization affect the expression and distribution of N-cadherin. First, we confirmed that N-cadherin surface expression is consistent between the CD82OE, Ngly-CD82, and Palm-CD82 cell lines (Fig.3.3A). Next, we performed confocal immunofluorescence imaging to analyze N-cadherin distribution in the cells. Figure 3.3B illustrates that both CD82 and N-cadherin are localized to the plasma membrane in each of the cells except for the CD82KD cells, which have reduced expression levels of CD82 and a punctate distribution of N-cadherin. In addition to the change in N-cadherin distribution upon CD82KD, a reduction in N-cadherin expression is observed, which is consistent with the flow cytometry data (Fig.3.1F). Moreover, double staining of primary AML cells suggests a similar surface expression profile for CD82 and N-cadherin (Fig.3.3C). To further assess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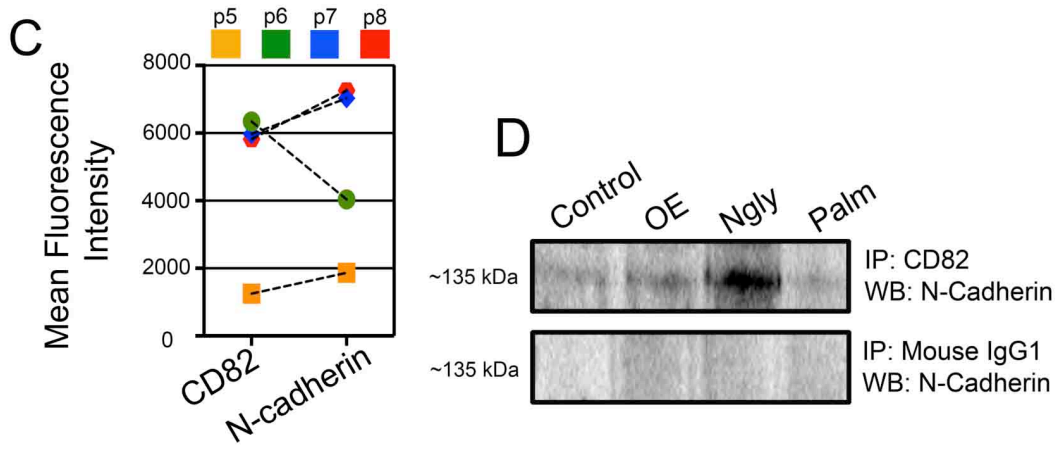
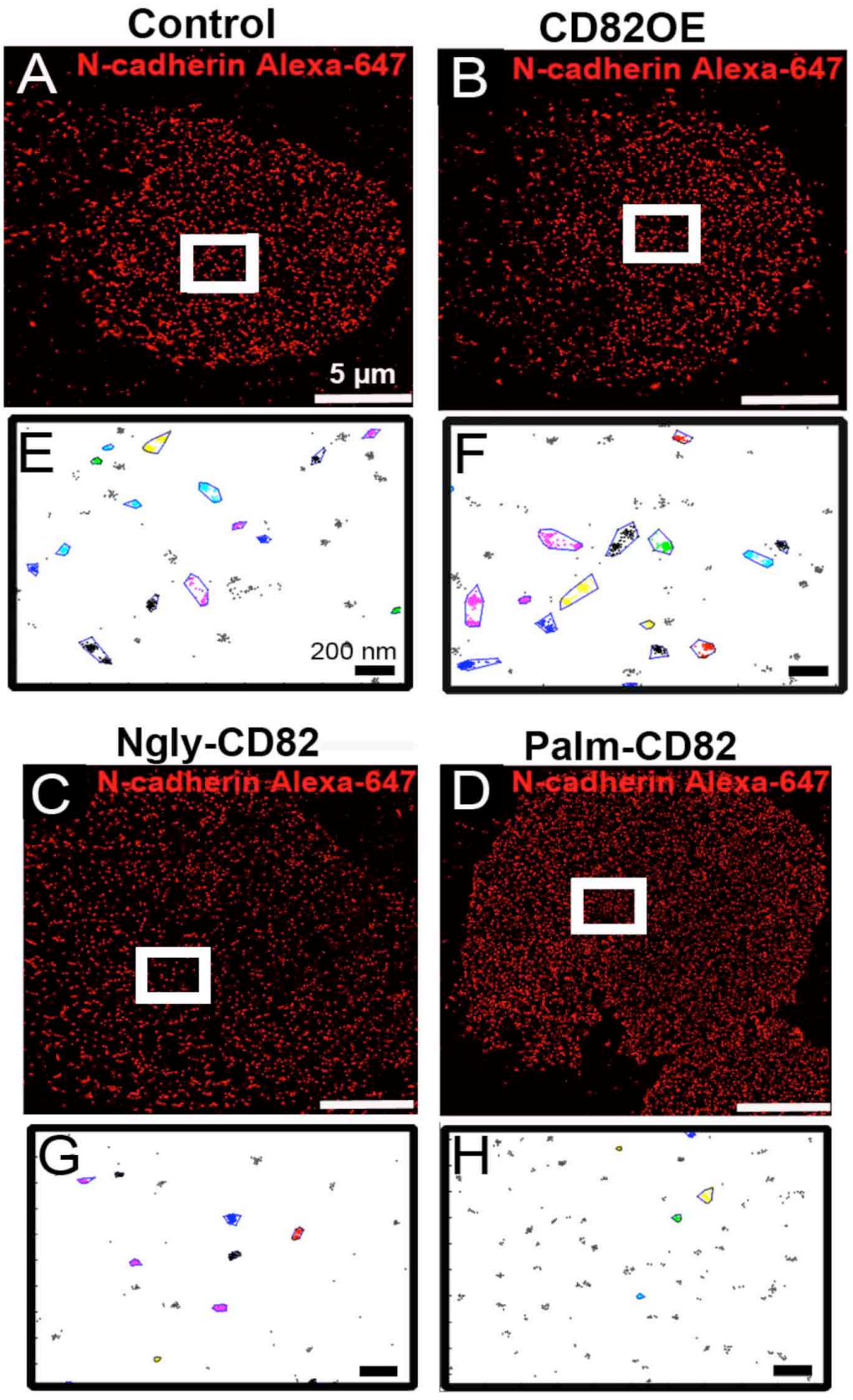


Figure 3.3: CD82 interacts with N-cadherin on the plasma membrane. (A) Flow cytometry analysis of N-cadherin surface expression on CD82OE, Ngly-CD82, and Palm-CD82 cells (Clone 8C11, BioLegend). (B) Confocal immunofluorescence imaging of CD82 and N-cadherin. Cells were fixed in 4% PFA then blocked and permeabilized with PBS + 1.0 % BSA + 0.1% tween 20. Alexa Fluor 647-conjugated anti-human CD82 (Clone ASL-24, BioLegend) and anti-human N-cadherin (clone 32/N-cadherin, BD Bioscience) antibodies were diluted 1:500 in permeabilization buffer and added to the sample overnight at 4C. Cells were washed and then Alexa Fluor 488-goat-anti-mouse secondary antibodies were added to the cells for 1hr at room temperature. Following PBS washes, cells were imaged by laser scanning confocal microscopy with a Zeiss Axiovert 100M inverted microscope (LSM 510) system using excitation wavelengths of 488 or 633 nm and a 63X 1.2 N.A. oil immersion objective. Image analysis was performed using the Zeiss LSM 510 software and Image J (NIH, Bethesda, MD). Double surface expression analysis by flow cytometry for (C) CD82 and (D) N-cadherin on primary AML cells. (E) Co-immunoprecipitation of CD82 and N-cadherin. Co-immunoprecipitations were performed using BRIJ O10 cell lysates incubated with CD82 antibody (Clone B-L2, Abcam) or control IgG antibody (Santa Cruz Biotechnology) and then immunoprecipitated using protein A/G Beads (Santa Cruz Biotechnology). Western blots were performed as previously described (24) using the N-cadherin antibody (32/N-Cadherin, BD Biosciences).

potential protein-protein interactions between CD82 and N-cadherin, we completed co-immunoprecipitation experiments using Brij lysates. The ability of CD82 to pull down N-cadherin in this mild detergent (Fig.3.3D) suggests that CD82 and N-cadherin are present in a protein complex.

Surface clustering of N-cadherin can trigger signaling events, which promote cell adhesion (Hong et al., 2013). Furthermore, the regulatory mechanism of cadherin clustering is a critical aspect of cadherin adhesion since the adhesive capacity of individual cadherins is negligible (Nelson, 2008). Therefore, the lateral association between cadherin receptors is a prerequisite for the formation of adhesive dimers (Chitaev and Troyanovsky, 1998). To quantify how changes in CD82 membrane organization affect the nanoscale organization of N-cadherin, we again used dSTORM (Fig.3.4A-D). Analysis of the N-cadherin dSTORM images with the DBSCAN algorithm (Fig.3.4E-H) suggests that N-cadherin cluster size and diameter is significantly decreased in Ngly-CD82 and Palm-CD82 cells when compared to control and CD82OE cells (Fig.3.4I,J). More importantly, Palm-CD82 cells display a marked decrease in the number of N-cadherin clusters when compared to CD82OE or control cells (Fig.3.4K). Additional analysis of Palm-CD82 cells also identified that the majority of the N-cadherin molecules are distributed diffusely throughout the membrane and not localized to organized clusters (Fig.3.4L). Thus, the palmitoylation of CD82 and its lateral assembly significantly affects the formation of N-cadherin adhesive protein complexes. Interestingly, we also find that the Ngly-CD82 cells demonstrate a significant increase in the density or molecular confinement of N-cadherin molecules into a cluster (Fig.3.4M), which is predicted to modulate N-cadherin function. We find that N-glycosylation of CD82 maintains N-cadherin clusters at approximately 80 nm. However, when the N-linked glycosylation sites on CD82 are mutated, the average size of N-cadherin clusters shrinks to approximately 65 nm, which leads to an increase in the molecular confinement of N-cadherin in each cluster. Together, these data suggest that while palmitoylation of CD82 regulates N-cadherin assembly into clusters, N-glycosylation of CD82 affects the nanoscale packing of N-cadherin. Therefore, in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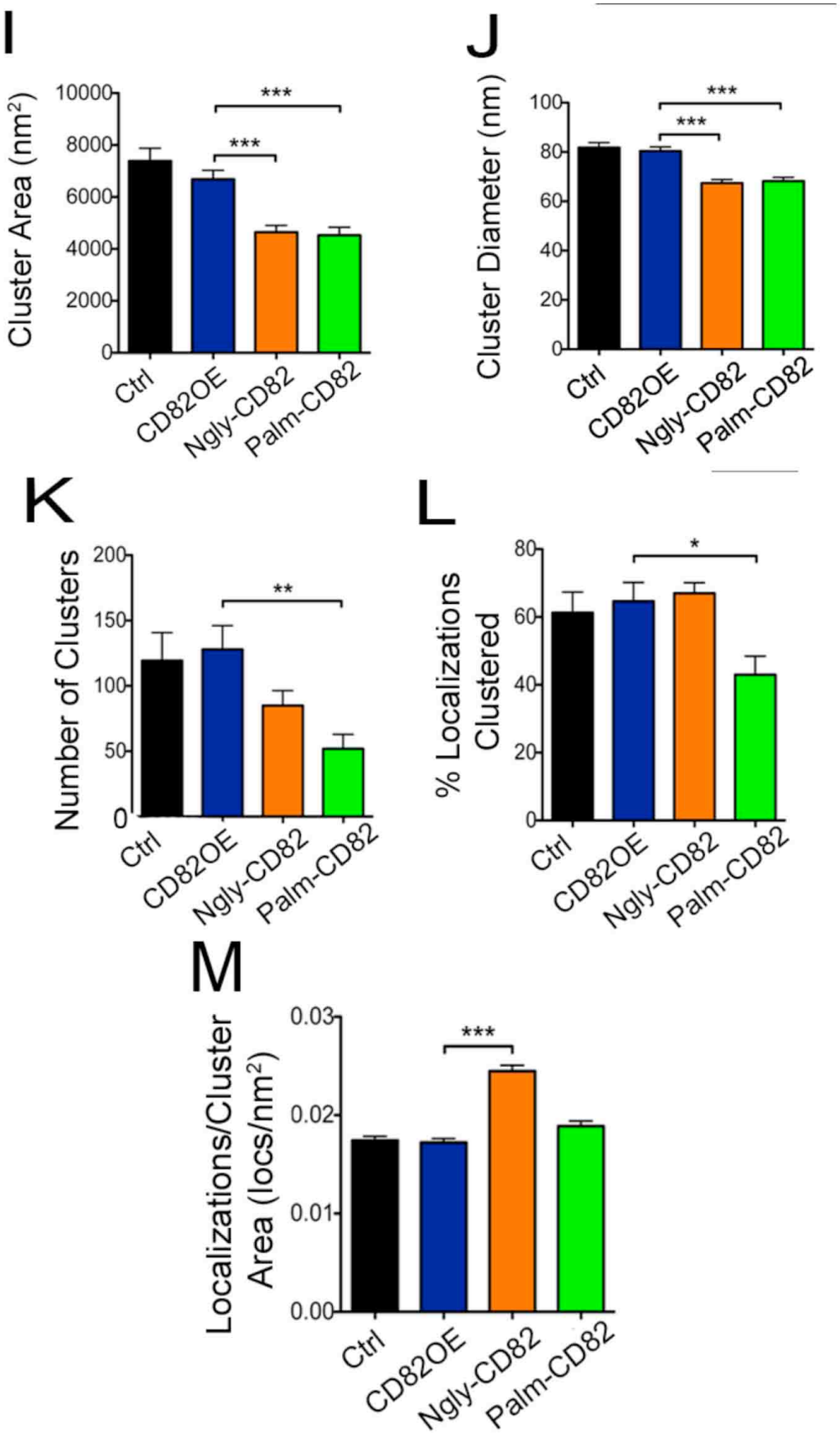
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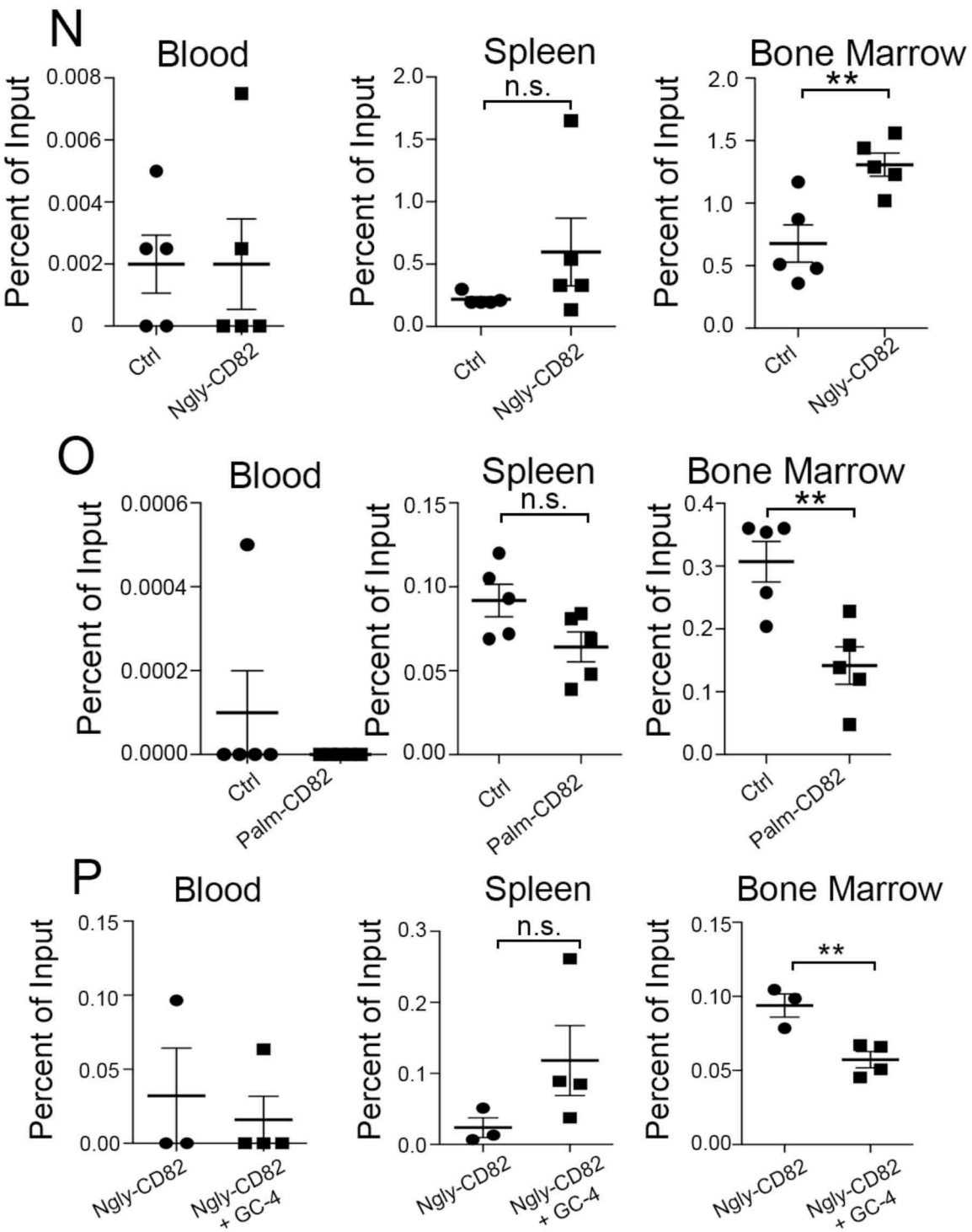
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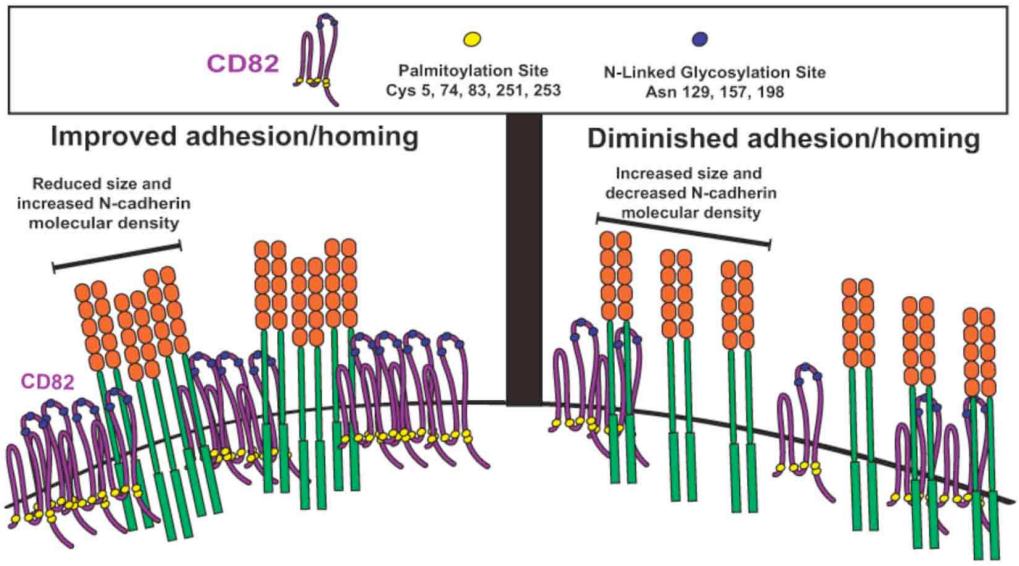

Figure 3.4. CD82 regulates N-cadherin cell membrane organization and AML homing. (A-D) Reconstructed dSTORM images of N-cadherin distribution on each cell line. (E-H) DBSCAN images of N-cadherin clustering generated from DBSCAN analysis from the highlighted white boxes from the reconstructed dSTORM images (n ≥ 6 cells per cell line). Clustered N-cadherin localizations are displayed in color and non-clustered N-cadherin localizations are in gray. An epsilon value of 50 nm and an n value of 30 localizations were used to examine N-cadherin clustering. Quantification of (I) N-cadherin cluster diameter, (J) N-cadherin cluster area, (K) number of N-cadherin clusters, (L) percent N-cadherin localizations clustered, and (M) density of N-cadherin in a cluster based on DBSCAN analysis (n ≥ 6 cells per cell line). Bone marrow homing analysis of CFSE labeled control, (N) Ngly-CD82 and (O) Palm-CD82 cells injected i.v. into NSG mice and analyzed as described in figure 1. (P) Ngly-CD82 cells were pretreated with 40 g of N-cadherin blocking antibody (GC-4: Sigma) or IgG control (Santa Cruz Biotechnology) for 30 min at 37C prior to i.v. injection into NSG mice. Sixteen hours following injection, homing analysis was completed as previously described. (Q) Working model of how CD82 post-translational modifications regulate N-cadherin protein organization and confinement, thereby contributing to functional differences in adhesion and homing. (** $p < 0.01$, *** $p < 0.001$; one-way ANOVA, poc-hoc t-test with Welch's correction for groups with unequal standard deviations).

addition to N-cadherin expression, the regulation of N-cadherin membrane organization by CD82 may also be an important regulatory mechanism for controlling N-cadherin function and subsequent behavior of AML.

3.3.4 Molecular scale organization of CD82 alters the bone marrow homing capacity of AML cells.

The lateral assembly of cadherins in the membrane can stimulate signaling events and promote cell adhesion (Hong et al., 2013). Therefore, we assessed whether the CD82-mediated changes in N-cadherin clustering affect the homing of AML cells into the bone marrow. We injected the Ngly-CD82, Palm-CD82 and control cells into NSG mice to measure potential differences in bone marrow homing. Interestingly, we detect a significant increase in the ability of the Ngly-CD82 cells to home to the bone marrow when compared to control cells, while the Palm-CD82 cells display a substantial decrease in bone marrow homing (Fig.3.4N,O). Analysis of the blood and spleen for Ngly-CD82 and Palm-CD82 cell localization identified no differences. To assess the role of N-cadherin in the enhanced homing of the Ngly-CD82 cells, we pretreated the cells with the N-cadherin blocking antibody (GC-4) prior to injection. Fig.3.4P shows a disruption in Ngly-CD82 cells homing when N-cadherin is inhibited. Together these data demonstrate that CD82 and its post-translational modifications regulate N-cadherin cluster size, organization, and density, which modulate AML bone marrow homing.

While protein expression plays a critical role in AML (Xu et al., 2014), our study suggests that protein organization can be equally important. We define a pathway by which CD82 regulates bone marrow homing of AML cells through the membrane clustering of N-cadherin (Fig.3.4Q). Establishment of AML within the bone marrow has extremely poor patient outcomes and we speculate that N-cadherin clustering may serve as a valuable marker to predict the aggressive behavior of AML. In addition, these findings provide an alternative model for targeting AML where modulation of protein organization within the membrane may be an effective treatment to dislodge AML cells from the protective environment of the bone marrow. Although N-cadherin is a focus of this study,

we propose that N-cadherin will most likely model other adhesive proteins expressed on the cell surface such as selectins and integrins. In fact, CD82 regulation of specific integrin organization has been previously described in a variety of cellular systems (Malik et al., 2009; Miranti, 2009; Termini et al., 2014).

In summary, these observations strengthen the significance of tetraspanin-mediated membrane organization within a complex multi-step process such as bone marrow homing. Moreover, we reason that CD82 serves as to regulate cellular behavior by modulating the topological distribution of protein networks on the cell membrane. It is plausible that this regulation ultimately leads to more robust signaling and adhesive potential that can be harnessed in disease states such as AML where cancer stem cells have a greater fitness advantage over normal HSPCs. Together, these data suggest that membrane clustering of proteins can regulate the aggressive potential of AML cells and may serve as a novel therapeutic target for future disease treatments.

3.4 Acknowledgements

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Chapter 4: Tetraspanin CD82 regulates the spatiotemporal dynamics of PKCα in acute myeloid leukemia

Christina M. Termini[1], Keith A. Lidke[2] & Jennifer M. Gillette[1]

[1]Department of Pathology, University of New Mexico Health Sciences Center, University of New Mexico, MSC 08-4640, Albuquerque, NM 87131, USA. [2]Department of Physics and Astronomy, University of New Mexico, MSC 07-4220, Albuquerque, NM 87131, USA. Correspondence and requests for materials should be addressed to J.M.G. (email: jgillette@salud.unm.edu)

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4.1 Abstract

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 (PKCα)-mediated signaling critical for AML progression. Utilizing a palmitoylation mutant form of CD82 with disrupted membrane organization, we find that the CD82 scaffold controls PKCα expression and activation. Combining single molecule and ensemble imaging measurements, we determine that CD82 stabilizes PKCα activation at the membrane and regulates the size of PKCα 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 PKCα 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.

4.2 Introduction

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 hematopoiesis (Colmone et al., 2008). 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 treatment (Walter et al., 2010b). Modeling studies have demonstrated that the expression and activation of signaling molecules can be used to predict AML patient remission attainment, relapse, and survival (Kornblau et al., 2006). For example, increased expression of the protein kinase C (PKC) isoform PKCα correlates with poor survival in AML patients (Kurinna et al., 2006). Therefore, therapeutic targeting of specific aberrant signaling in AML can 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 PKCs (Newton, 1995). The conventional PKC isoforms include PKCα, β1, β2 and γ, all of which require Ca^{2+} 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) (Nakashima, 2002). PKC activation initiates various signaling responses such as the activation of Rac1, RhoA, and the mitogen-activated protein kinases (MAPK) signaling cascades (Chang et al., 1998; Kolch et al., 1993; Nakashima, 2002; Schonwasser et al., 1998). As such, PKC activation controls many basic cellular processes including adhesion, migration, and proliferation, which all contribute to cancer progression.

In AML patients, PKCα gene expression is upregulated when compared to CD34(+) normal donors (Ruvolo et al., 2011). Furthermore, treating AML cell lines with the PKC inhibitor, enzastaurin, blocks the phosphorylation of PKCα and its downstream target, ERK, and also prevents PKCα membrane recruitment (Ruvolo et al., 2011). Additional work suggests that increased levels of phospho-PKC are correlated with increased AML cell viability (Zabkiewicz et al., 2014). 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) (Charrin et al., 2009b; Hemler, 2005). 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-kit (Anzai et al., 2002; Berditchevski et al., 2002; Odintsova et al., 2000). The maintenance of TEMs promote cellular functions including cell adhesion, migration, and proliferation (Lammerding et al., 2003; Shi et al., 2000; Yanez-Mo et al., 1998). The palmitoylation of tetraspanins regulate TEM organization through the control of protein-protein interactions (Berditchevski et

al., 2002; Charrin et al., 2002; Yang et al., 2004), 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 engagement (Berditchevski et al., 2002). Moreover, inhibition of CD81 palmitoylation reduced signaling in B cells, as assessed by PLCγ2 and VAV phosphorylation (Cherukuri et al., 2004). 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 14-3-3 (Clark et al., 2004) and G protein subunits (Little et al., 2004). Moreover, previous work established that CD151 assists in the recruitment of Rac1 to the plasma membrane, in addition to associating with PKCα(Clark et al., 2004; Hong et al., 2012; Little et al., 2004). Interestingly, tetraspanins CD9, CD81 and CD82 were shown to associate with PKCα upon PMA activation (Zhang et al., 2001), while CD9 and CD151 were also shown to coimmunoprecipitate with PKCα (Gustafson-Wagner and Stipp, 2013). In the present study, we focus on identifying how this tetraspanin association modulates PKC signaling, with a specific emphasis on CD82.

CD82 is upregulated in several human leukemias, including AML (Burchert et al., 1999) and recent work identified CD82 upregulation in chemotherapy-resistant CD34(+)/CD38(-) AML cells (Nishioka et al., 2015b), often responsible for disease relapse. The objective of this study is to determine how the CD82 scaffold and its membrane organization regulate PKCα-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 PKCα signaling in AML cells. Our data demonstrate that the molecular organization of CD82 regulates PKCα 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

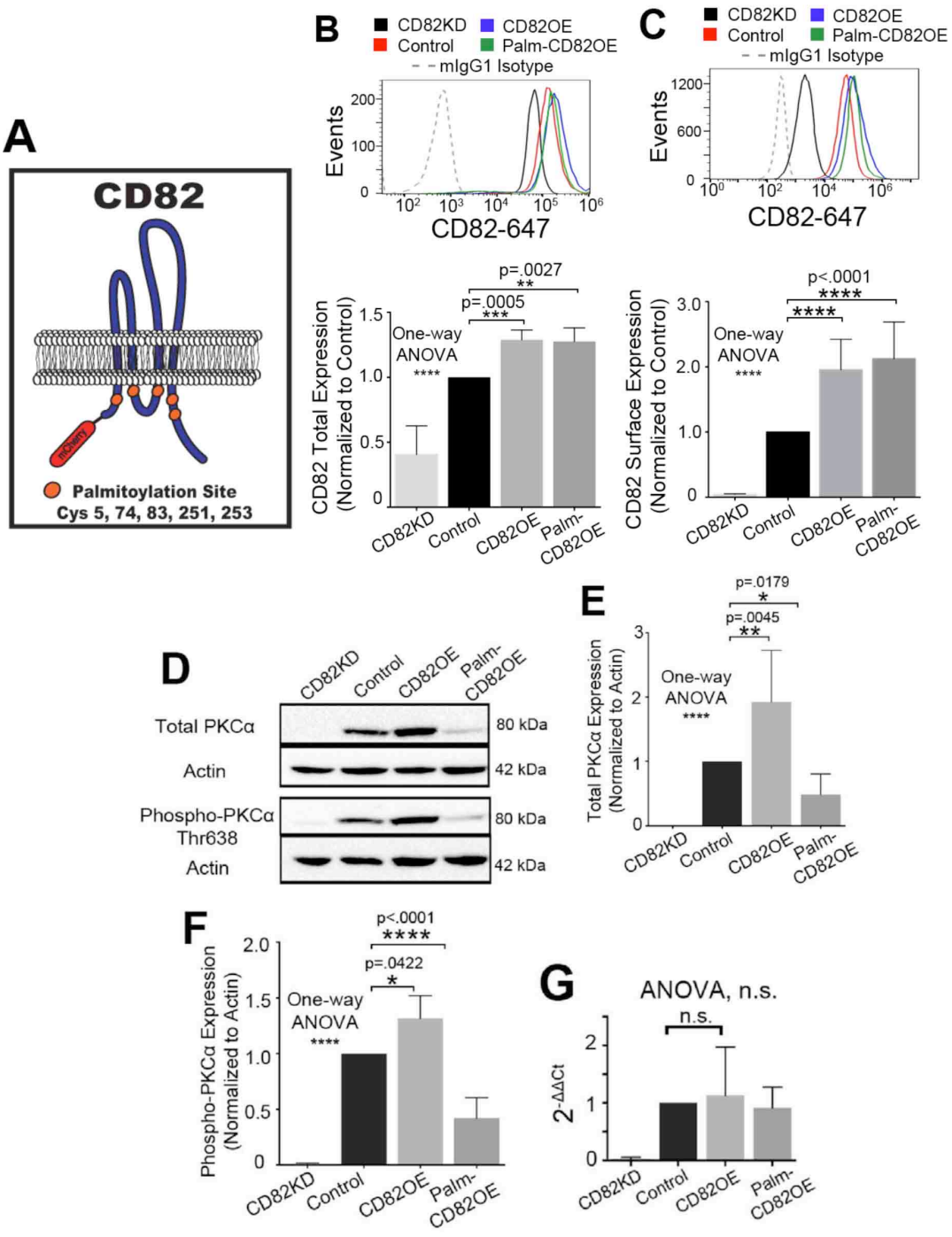
a suitable target for controlling AML progression through its regulation of PKCα signaling.

4.3 Results

4.3.1 The CD82 scaffold regulates PKCα expression and activation.

To identify how CD82 membrane scaffolding affects PKCα signaling, we generated KG1a AML cell lines stably overexpressing wild type CD82 (CD82OE) or a palmitoylation mutant (Palm-CD82OE) form of CD82 tagged to the mCherry fluorescent protein. In the palmitoylation mutant, five membrane proximal cysteine residues are mutated to serines, preventing CD82 palmitoylation (Fig.4.1A)(Mazurov et al., 2007). We also generated CD82 knockdown KG1a cells (CD82KD) cells, where stable expression of a CD82-specific shRNA reduces total CD82 expression by 50% and surface levels by 95%**.** To quantify differential CD82 total and surface expression, we used flow cytometry analysis of permeabilized (Fig.4.1B) and non-permeabilized cells, respectively (Fig.4.1C). We also measured the expression of other tetraspanins in these cell lines, finding similar levels of CD9 in all cell lines (Suppl.Fig.S.4.1A), and decreased levels of CD151 (Suppl.Fig.S.4.1B) in the CD82KD cells compared to controls. Interestingly, we find decreased levels of surface (Suppl.Fig.S.4.1C) and total (Suppl.Fig.S.4.1D) CD81 in CD82KD and CD82OE cells compared to control cells. We also checked the tetraspanin profile of two additional myeloid leukemia cell lines (K562 and U937) overexpressing WT-CD82 or Palm-CD82 (Suppl.Fig.S.4.2A-E,I-M). While K562 cells display increased CD9 expression in the CD82OE cells, the CD81 and CD151 expression levels remain unchanged (Suppl.Fig.S.4.2F-H). U937 cells display increased CD9 expression in CD82OE and Palm-CD82OE cells, whereas Palm-CD82OE cells also exhibit a slight increase in CD81 expression; CD151 remains unchanged (Suppl.FigS.4.2N-P). Therefore, CD82 overexpression regulates the tetraspanin expression in leukemic cells.

To analyze how CD82 scaffolding regulates the expression and activation of PKCα, we first quantified the expression levels of total and activated PKCα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CD82
mCherry
Palmitoylation Site
Cys 5, 74, 83, 251, 253
B
CD82KD
CD82OE
Control
Palm-CD82OE
mIgG1 Isotype
Events
CD82-647
C
CD82KD
CD82OE
Control
Palm-CD82OE
mIgG1 Isotype
Events
CD82-647
CD82 Total Expression (Normalized to Control)
One-way ANOVA
****
p=.0027
**
p=.0005
***
CD82KD
Control
CD82OE
Palm-CD82OE
CD82 Surface Expression (Normalized to Control)
One-way ANOVA
****
p<.0001
****
****
D
CD82KD
Control
CD82OE
Palm-CD82OE
Total PKCα
Actin
Phospho-PKCα Thr638
Actin
80 kDa
42 kDa
80 kDa
42 kDa
E
Total PKCα Expression (Normalized to Actin)
One-way ANOVA
****
p=.0179
*
p=.0045
**
F
Phospho-PKCα Expression (Normalized to Actin)
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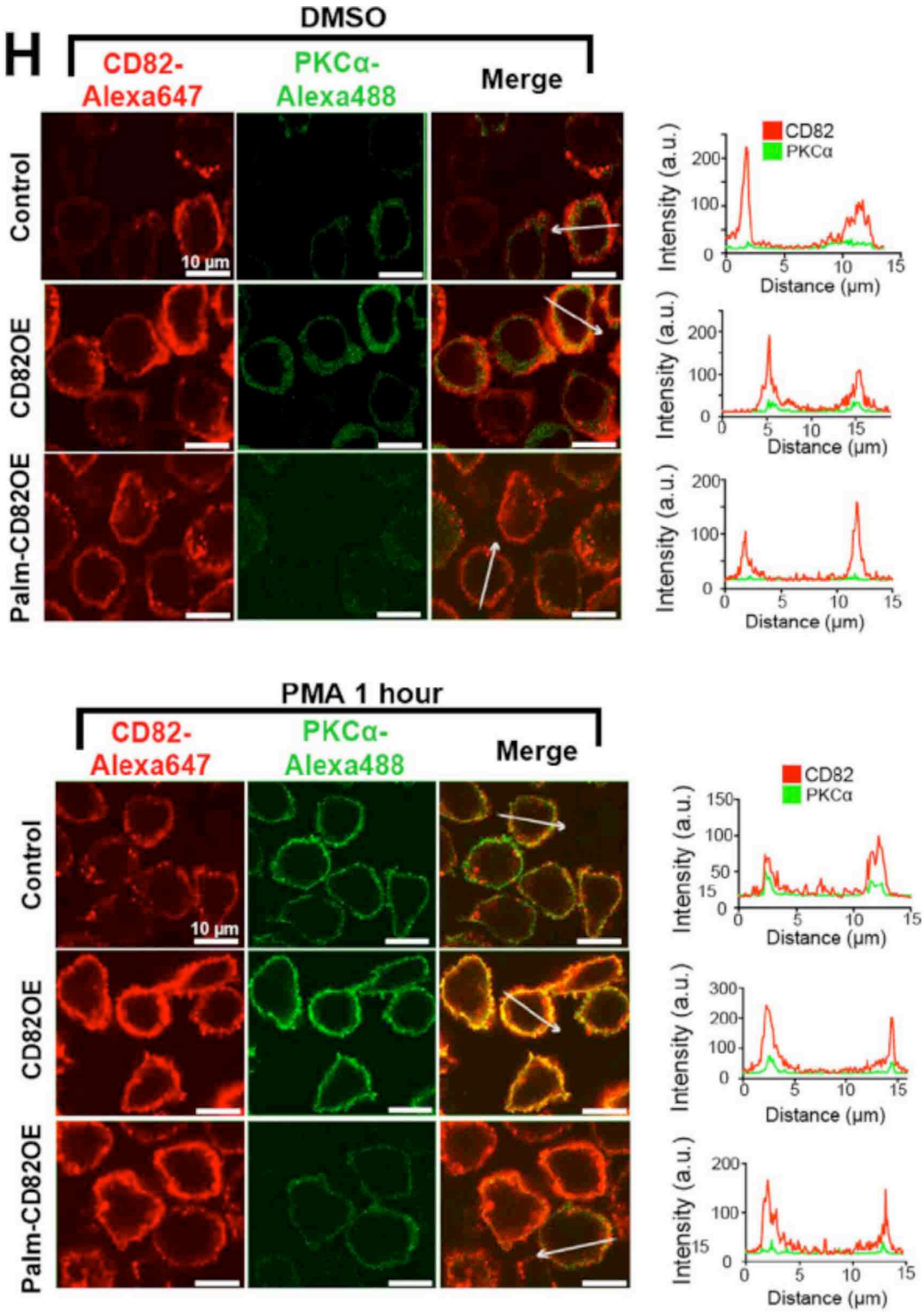
p<.0001
****
p=.0422
*
G
ANOVA, n.s.
n.s.
2-ΔΔCt


H
DMSO
CD82-Alexa647
PKCα-Alexa488
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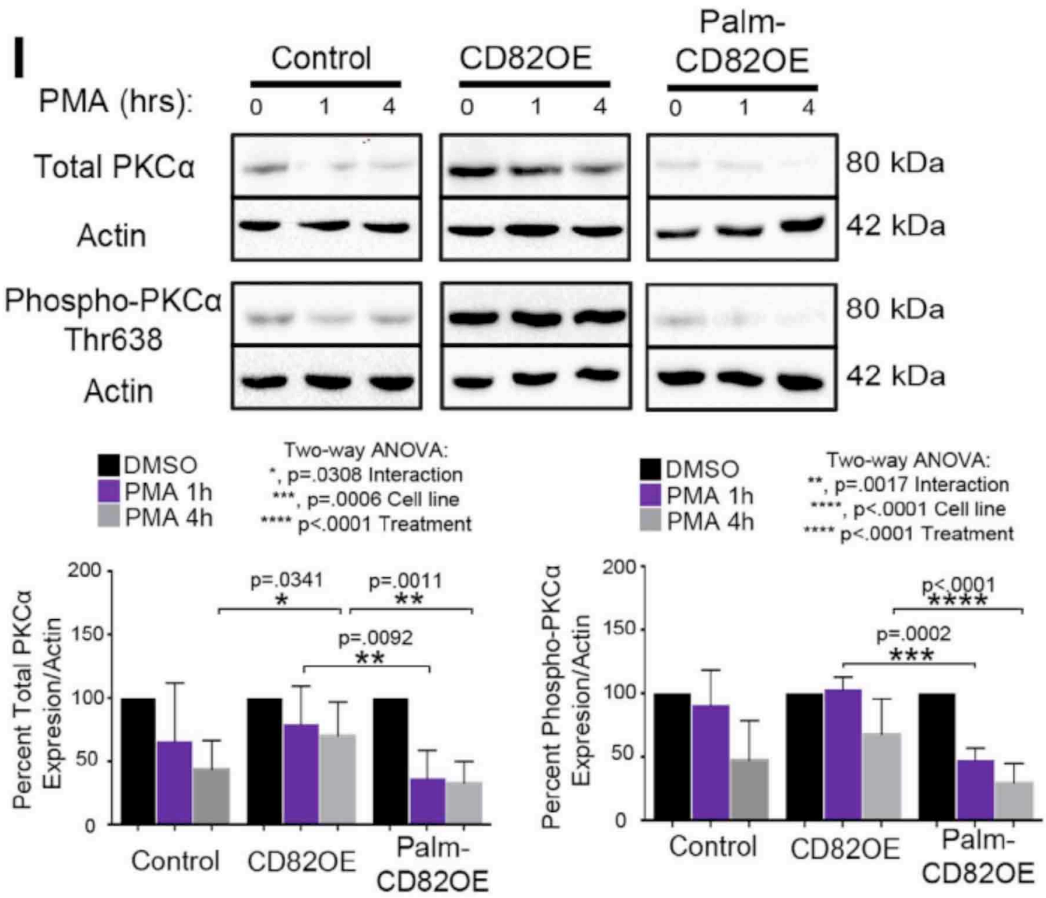
Control
CD82OE
Palm-CD82OE
10 μm
CD82
PKCα
Intensity (a.u.)
Distance (μm)
PMA 1 hour
CD82-Alexa647
PKCα-Alexa488
Merge
Control
CD82OE
Palm-CD82OE
10 μm
CD82
PKCα
Intensity (a.u.)
Distance (μm)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Palm-CD82OE
PMA (hrs):
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Total PKCα
Actin
Phospho-PKCα Thr638
Actin
80 kDa
42 kDa
80 kDa
42 kDa
DMSO
PMA 1h
PMA 4h
Two-way ANOVA:
*, p=.0308 Interaction
***, p=.0006 Cell line
**** p<.0001 Treatment
Percent Total PKCα Expresion/Actin
p=.0341
*
p=.0011
**
p=.0092
**
Control
CD82OE
Palm-CD82OE
DMSO
PMA 1h
PMA 4h
Two-way ANOVA:
**, p=.0017 Interaction
****, p<.0001 Cell line
**** p<.0001 Treatment
Percent Phospho-PKCα Expresion/Actin
p<.0001
****
p=.0002
***
Control
CD82OE
Palm-CD82OE

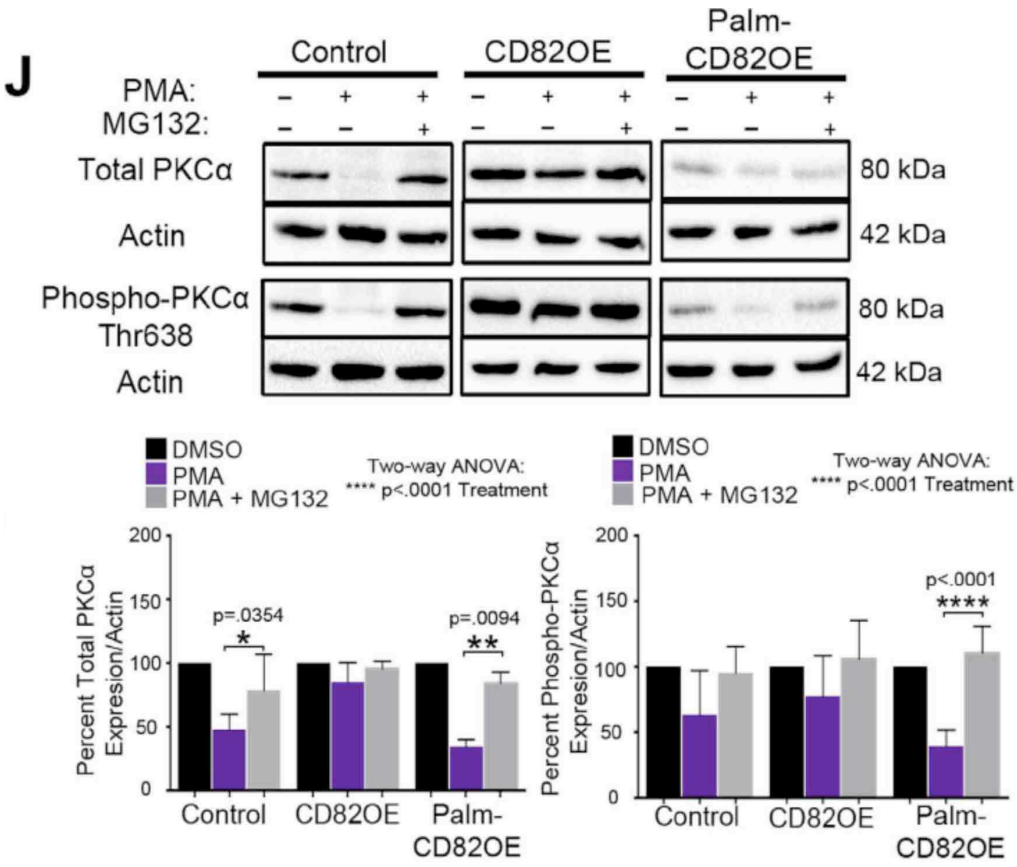


Figure 4.1 The CD82 scaffold regulates PKCα expression and activation. (A) Cartoon depicting mutated palmitoylation sites within CD82 and mCherry fusion. Flow cytometry analysis of (B) total and (C) surface CD82 expression using CD82KD, control, CD82OE, and Palm-CD82OE KG1a cells (Biolegend, ASL-24). (n≥3 experiments; error bars indicate SD; mean fluorescence intensity normalized to control levels). (D) Western blot analysis for total and phospho-PKCα expression. Densitometric analysis of (E) total and (F) phosphorylated PKCα expression from Western blot analyses (n≥4 experiments; error bars indicate SD). (G) Real-time PCR analysis of KG1a cells. (H) Immunofluorescence imaging of CD82 (Biolegend, ASL-24) and PKCα-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 PKCα expression following PMA stimulation (n≥4 experiments; error bars indicate SD). (J) Cells were treated with DMSO, PMA or PMA+MG132 for 4 hrs and total and phospho-PKCα were quantified using Western blot analysis and densitometry. (n≥4 independent experiments; error bars indicate SD; post-hoc unpaired t-test).

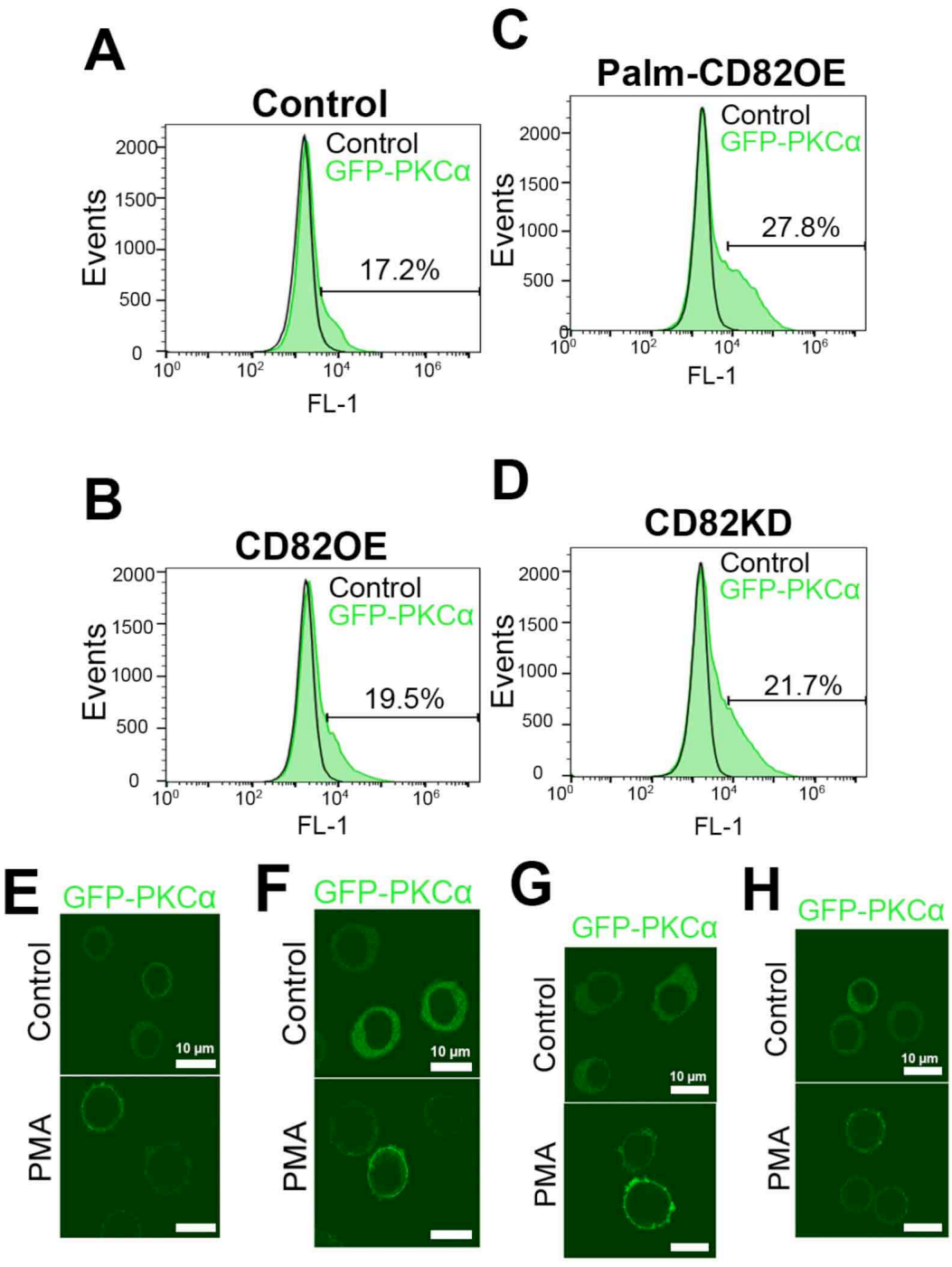
using Western blot analysis. (All data presented in this manuscript except supplementary utilize KG1a cells with additional cell line analysis quantified in supplemental data). Figures 4.1D-F demonstrate that the CD82OE cells have a twofold increase in total PKCα expression and a 1.3-fold increase in phosphorylated (active) PKCα expression compared to control cells. In contrast, we find that the Palm-CD82OE cells express approximately 50% less total PKCα and 60% less phospho-PKCα when compared to control cells. Similar changes in PKCα expression and activation were identified using stable U937 and K562 cells overexpressing wild type CD82 or Palm-CD82 (Suppl.Fig.S.4.2Q-R). Upon CD82KD in KG1a cells, we are unable to detect the expression of PKCα or its active form by Western blot (Fig.4.1D-F). RT-PCR analysis of the cell lines measures a transcriptional down regulation of PKCα in CD82KD cells and no change in PKCα transcript between the control and CD82 overexpressing cells (Fig.4.1G). Together, these data suggest a critical role for CD82 expression and membrane organization in regulating PKCα expression and activation in AML.

Upon full activation, PKCα translocates to the plasma membrane from the cytoplasm, which is essential for PKCα signaling. Using immunofluorescence imaging, we find that under resting conditions PKCα is primarily localized within the cytoplasm, whereas, upon PKCα activation with PMA for 1 hr, PKCα translocates to the plasma membrane (Fig.4.1H). We also observe by line scan analysis that the intensity plots for the CD82 and PKCα channels have a similar shape under PMA stimulated conditions, suggesting that PKCα activation stimulates PKCα to move to CD82 membrane regions. These data illustrate that despite the CD82 palmitoylation mutation, PKCα effectively translocates to the plasma membrane upon activation. Following activation, PKCα can be dephosphorylated and degraded in order to down-regulate PKCα-mediated signaling (Hansra et al., 1996; Lee et al., 1996; Melnikov and Sagi-Eisenberg, 2009; Wang et al., 2016). Therefore, we assessed whether CD82 scaffolding preserves PKCα protein levels upon activation, thereby providing a sustained signal. Upon PMA stimulation for 1 or 4 hrs, we find that total and phospho-PKCα expression is maintained at a higher proportion in the CD82OE cells when

compared to Palm-CD82OE cells (Fig.4.1I). Next, we investigated if the reduced PKCα expression upon activation is due to proteasomal degradation. Combining four hr of PMA with the proteasomal inhibitor, MG132, we find that PKCα expression is rescued to basal levels in control and Palm-CD82OE cells (Fig.4.1J). Collectively, these data suggest that CD82 scaffolding hyperstabilizes PKCα levels upon activation.

4.3.2 The CD82 scaffold regulates short-term PKCα membrane association.

One mechanism by which the CD82 scaffold could prolong PKCα activation is by stabilizing PKCα membrane recruitment. To visualize the molecular recruitment of PKCα to the plasma membrane upon activation, we performed single particle tracking (SPT) analysis. Using transiently transfected GFP-PKCα cells (Fig.4.2A-C) stimulated with PMA (Fig.4.2E-G), we analyzed the membrane track length or “dwell time” of GFP-PKCα, which we define as the time between the membrane appearance and disappearance of GFP-PKCα. Figures 4.2I-K display representative GFP-PKCα trajectories, which were generated by filtering and connecting localizations with the parameters described in the Methods section. A cumulative distribution plot of the GFP-PKCα track lengths indicates that the Palm-CD82OE cells have an increased proportion of short-lived GFP-PKCα tracks compared to control or CD82OE cells, suggesting a shortened PKCα dwell time (Fig.4.2M). We also quantified PKCα dwell time based on the average track length per cell analyzed ($n \geq 19$ cells) (Fig.4.2N) or per independently performed experiment ($n=3$ experiments) (Fig.4.2O), finding the same trend observed in our cumulative distribution plot. Interestingly, when analyzing GFP-PKCα dwell time in the CD82KD cells (Fig.4.2D,H,L), we are unable to detect a change in track length (Fig.4.2R), 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 PKCα.


A
Control
Control
GFP-PKCα
17.2%
Events
FL-1
C
Palm-CD82OE
Control
GFP-PKCα
27.8%
Events
FL-1
B
CD82OE
Control
GFP-PKCα
19.5%
Events
FL-1
D
CD82KD
Control
GFP-PKCα
21.7%
Events
FL-1
E
GFP-PKCα
Control
PMA
10 μm
F
GFP-PKCα
Control
PMA
10 μm
G
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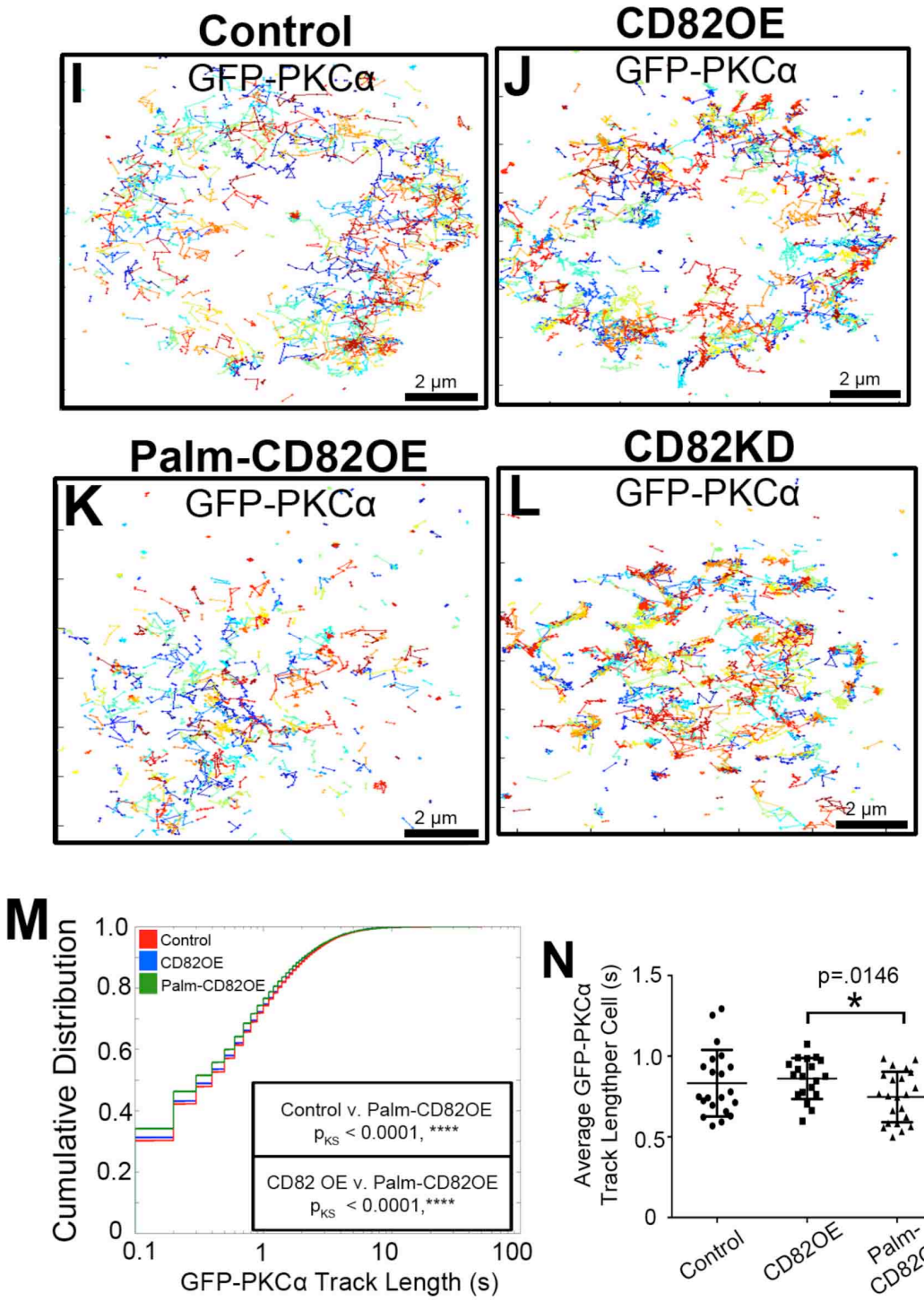
PMA
10 μm
H
GFP-PKCα
Control
PMA
10 μm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2 μm
CD82OE
J
GFP-PKCα
2 μm
Palm-CD82OE
K
GFP-PKCα
2 μm
CD82KD
L
GFP-PKCα
2 μm
M
Cumulative Distribution
1.0
0.8
0.6
0.4
0.2
0
Control
CD82OE
Palm-CD82OE
Control v. Palm-CD82OE
p_{KS} < 0.0001, ****
CD82 OE v. Palm-CD82OE
p_{KS} < 0.0001,****
0.1
1
10
100
GFP-PKCα Track Length (s)
N
Average GFP-PKCα Track Lengthper Cell (s)
1.5
1.0
0.5
0
p=.0146
*
Control
CD82OE
Palm-CD82OE

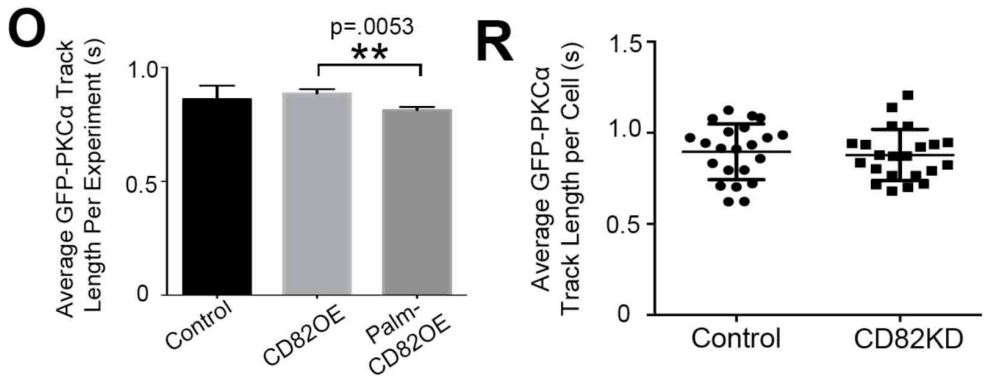
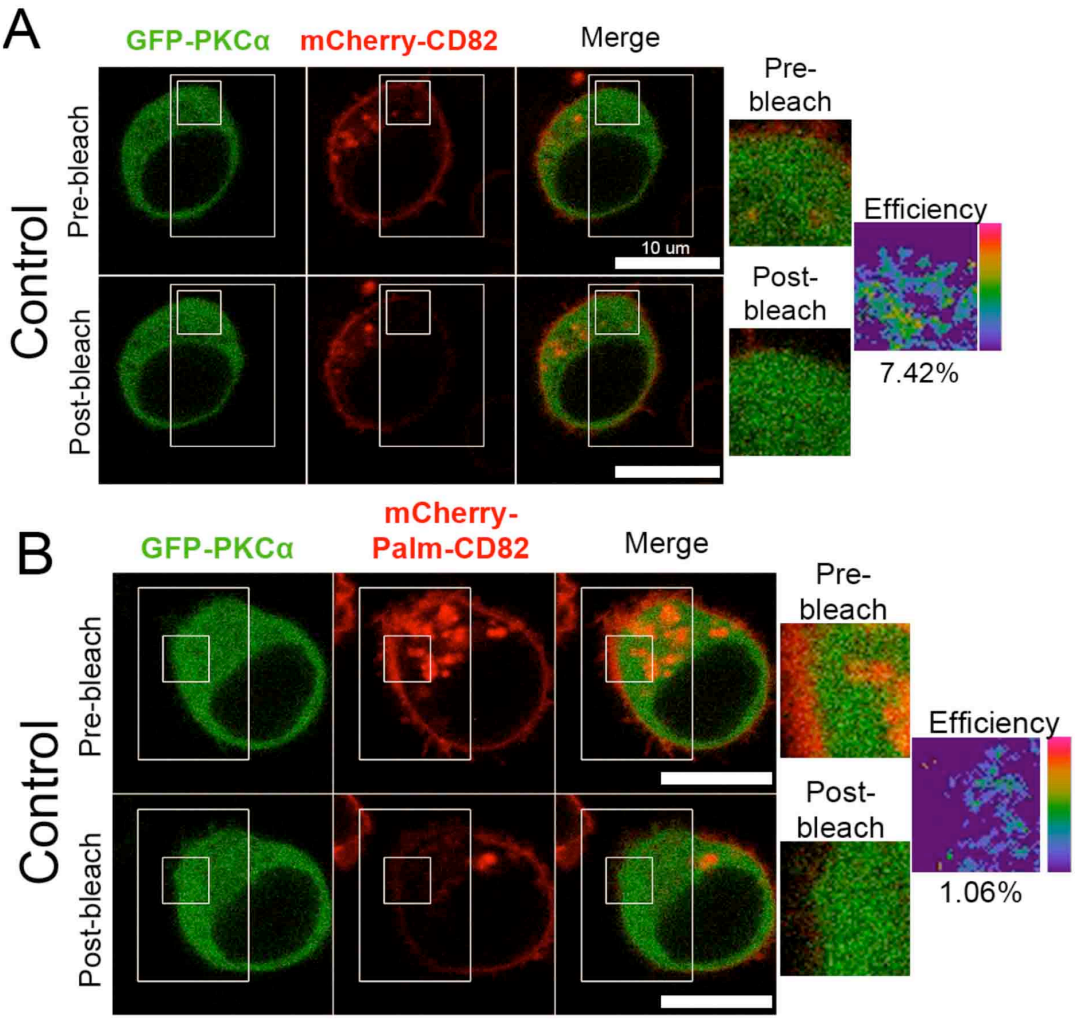


Figure 4.2 The CD82 scaffold regulates PKCα association with the membrane. (A-D) Flow cytometry analysis indicates the percentage of GFP-PKCα expression in transiently transfected cells. (E-H) Epifluorescence imaging of transfected cells showing GFP-PKCα localization +/- PMA. (I-L) PKCα trajectories from 600 frames of analyses are displayed. (M) Cumulative distribution plot of PKCα track length (n≥31227 tracks from n≥19 cells of each kind; the Kolmogorov-Smirnov test was used to compare cumulative distributions). (N) Average GFP-PKCα track length per cell and (O) per experiment (error bars indicate SD; n≥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).

4.3.3 PKCα is recruited to the CD82 scaffold upon stimulation.

An extensive series of immunoprecipitation studies demonstrated that upon PMA stimulation, PKCα interacts with CD82 (Zhang et al., 2001), although little is known about the dynamics of this interaction. Our SPT analyses suggest that CD82 palmitoylation may regulate the membrane stabilization of PKCα on a short time scale. However, we are particularly interested in whether CD82 scaffolding can stabilize long-lived PKCα membrane interactions, which could potentiate prolonged signal transduction. Using Förster resonance energy transfer (FRET), we measured the recruitment and retention of PKCα relative to the CD82 scaffold over time. FRET was measured by quantifying fluorescence intensity changes in the donor fluorophore (GFP-PKCα) after the acceptor (mCherry-CD82) was photobleached. CD82OE and Palm-CD82OE cells transiently transfected with GFP-PKCα were imaged under resting conditions and upon PMA stimulation for 5 mins or 1 hr to assess both short and long-term PKCα recruitment, respectively. Under resting conditions, we detect minimal FRET between CD82 and PKCα in both the CD82OE and Palm-CD82OE cells, although the CD82OE cells have higher basal FRET than the Palm-CD82OE cells (Fig.4.3A,B,G). Upon PMA stimulation, FRET is significantly increased in the CD82OE and Palm-CD82OE cells compared to resting cells (Fig.4.3C,D,G), indicating that PKCα 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.4.3E-G). These data suggest that disruption of the CD82 scaffold, in the case of the palmitoylation mutant, reduces the membrane association of PKCα with CD82. Together, these findings demonstrate that CD82 and PKCα have a prolonged membrane interaction that is hyperstabilized by overexpression of the CD82 scaffold.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Post-bleach
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Post-bleach
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Post-bleach
Efficiency
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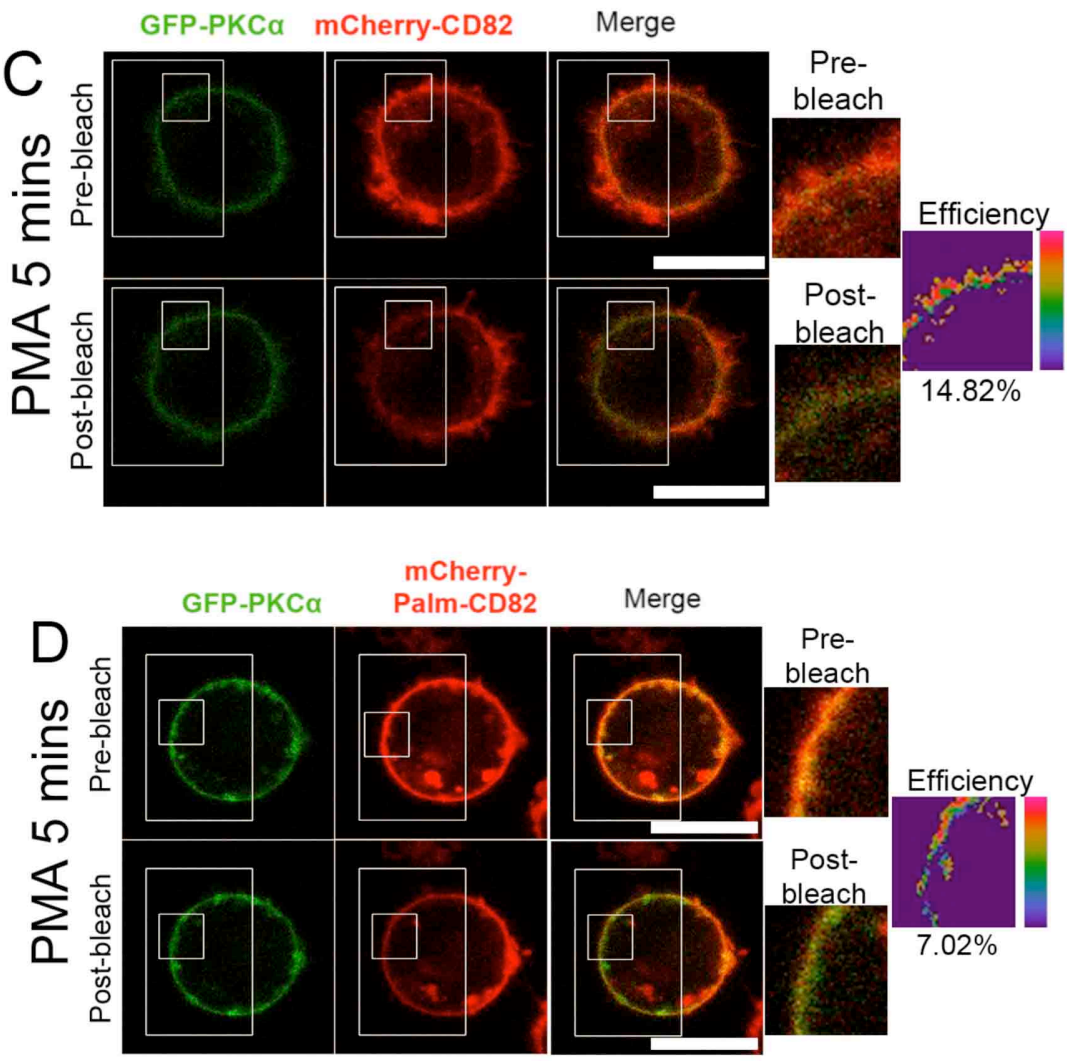
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Post-bleach
Pre-bleach
Post-bleach
Efficiency
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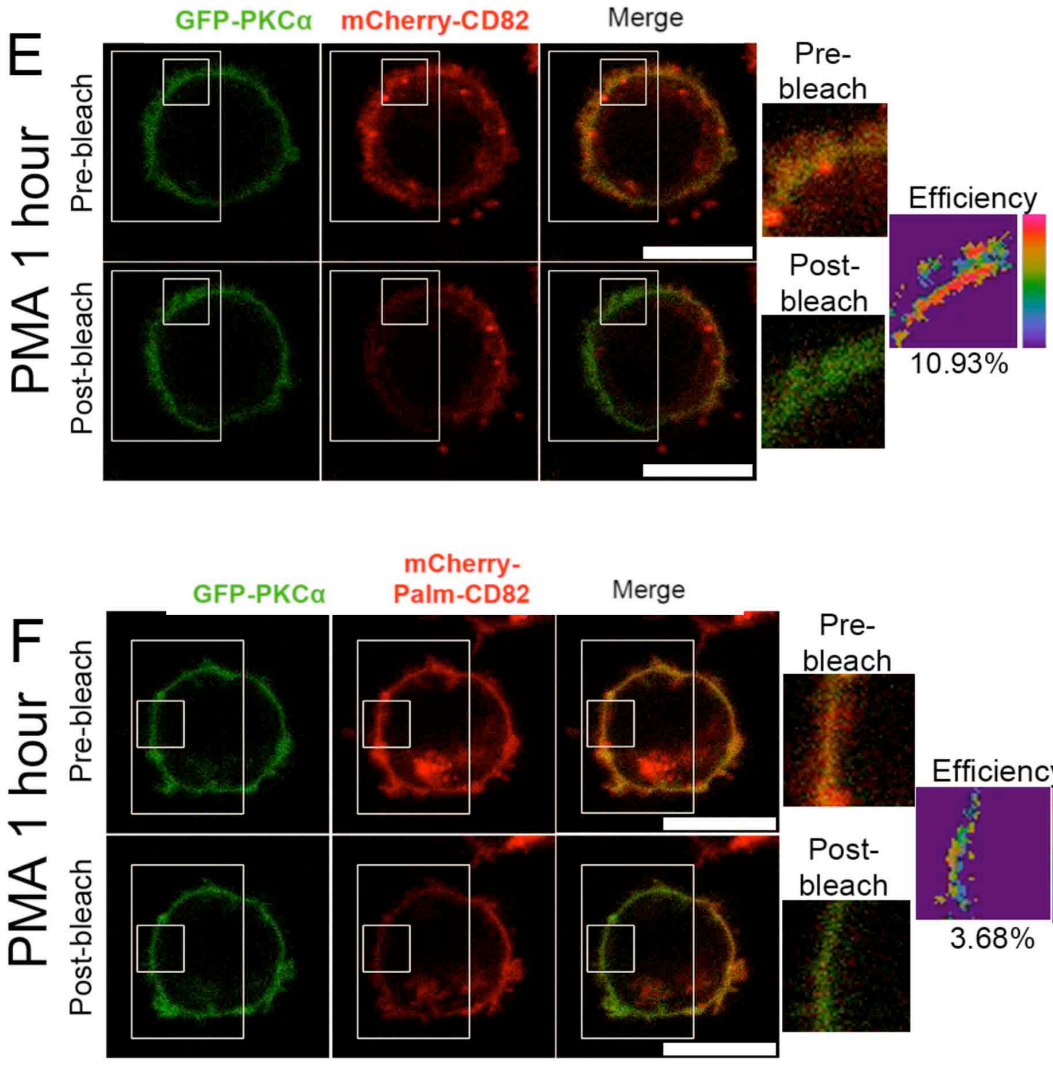
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Pre-bleach
Post-bleach
Efficiency
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3.68%

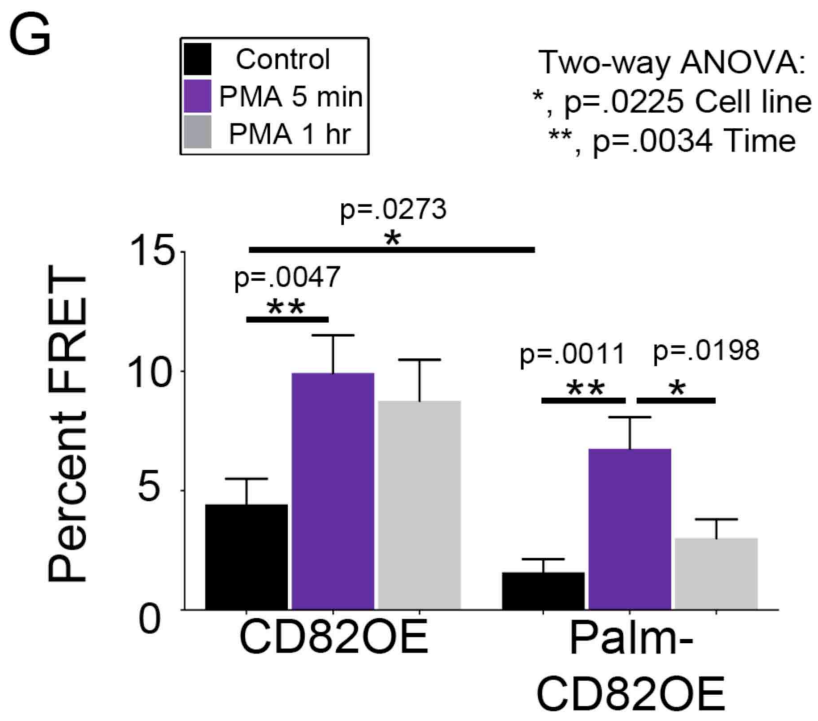


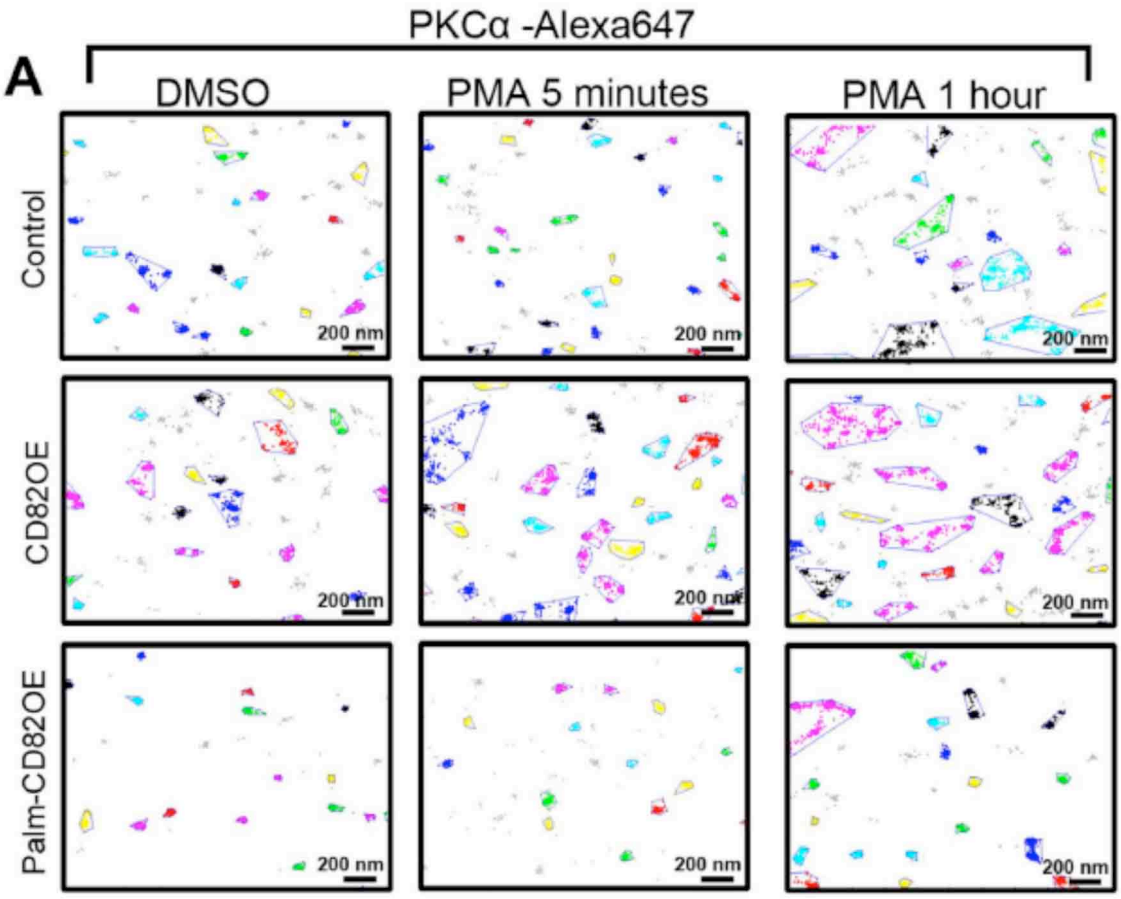
Figure 4.3 PKCα is stabilized by the CD82 scaffold. CD82OE or Palm-CD82OE KG1a cells were transfected with GFP-PKCα and imaged under (A-B) resting or upon PMA stimulation for (C-D) 5 mins or (E-F) 1 hr. (G) Percent FRET efficiencies were calculated in a region of interest per cell. (n=4 experiments, n≥21 cells per treatment, error bars indicate SEM, post-hoc unpaired t-test).

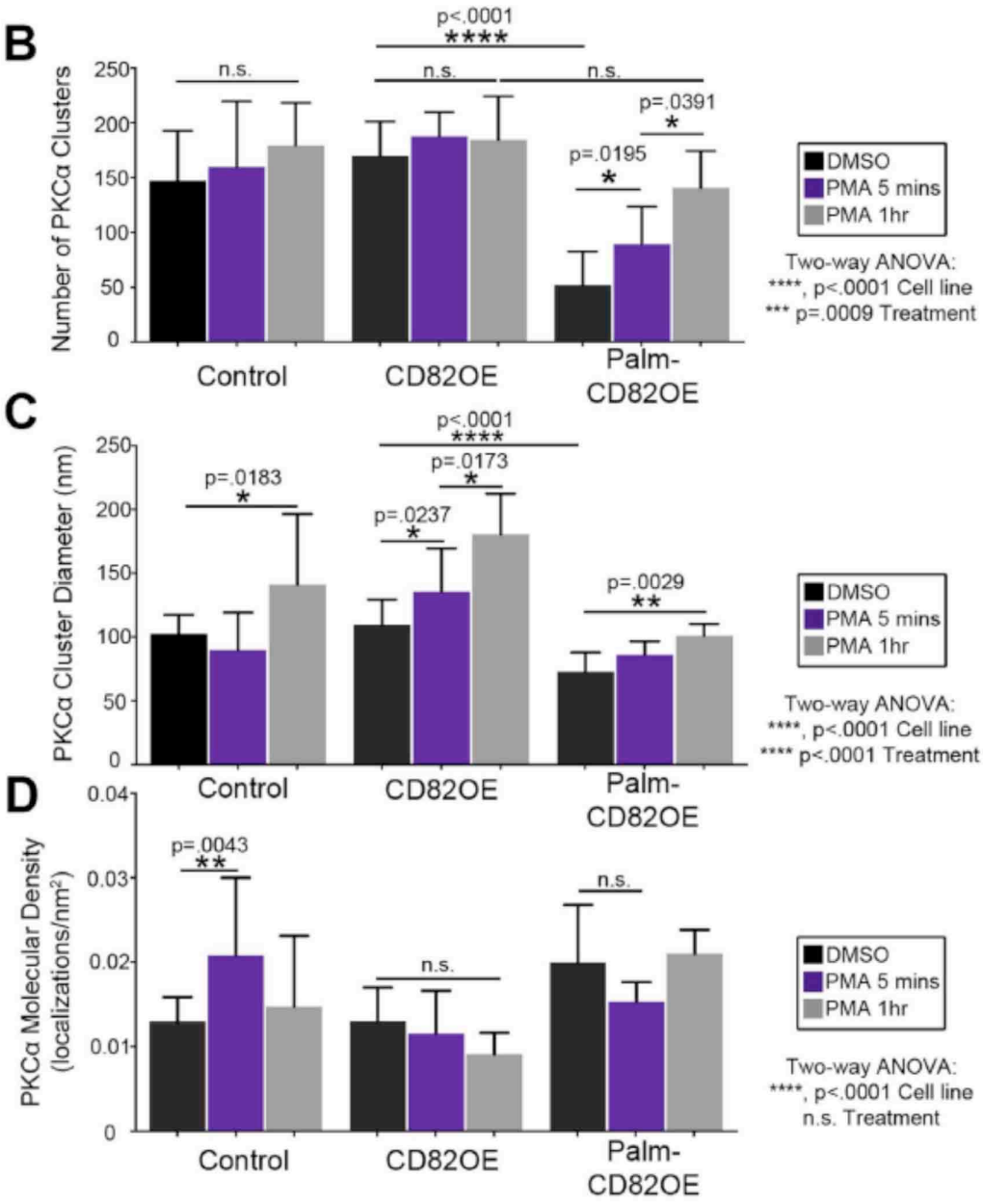
4.3.4 PKCα clustering at the membrane is controlled by the CD82 scaffold.

Tetraspanins can regulate the clustering of membrane proteins (Marjon et al., 2015; Termini et al., 2014; van Spriel et al., 2012). Interestingly, PKC has also been shown to oligomerize (Swanson et al., 2014) and aggregate upon activation(Huang, 1989). Therefore, we next wanted to determine how altered interactions between CD82 and PKCα described in our FRET studies could modulate PKCα clustering. Using the super-resolution imaging (SRI) technique, direct stochastic optical reconstruction microscopy (dSTORM), we resolved the molecular landscape of PKCα 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 regulators(Cebecauer et al., 2010). Therefore, we used the SRI data and quantified PKCα clustering with the density-based spatial clustering of applications with noise (DBSCAN) algorithm (Ester et al., 1996c) (Fig.4.4A). Under resting conditions, we detect a similar number of PKCα clusters between control and CD82OE cells, whereas the Palm-CD82OE cells display a significantly reduced number of PKCα clusters compared to control and CD82OE cells (Fig.4.4B). Next, upon PMA stimulation for 5 min or 1 hr, we again measure no significant change in the number of PKCα clusters in either the control or CD82OE cells. However, in the Palm-CD82OE cells, PMA stimulation results in a significant increase in PKCα cluster number (Fig.4.4B). In fact, upon PMA stimulation for 1 hr, the control, CD82OE and Palm-CD82OE cells all exhibit similar numbers of PKCα clusters (Fig.4.4B). These data suggest that while Palm-CD82OE cells have reduced PKCα clusters under basal conditions, PMA treatment stimulates a similar number of PKCα 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 transduction (Cebecauer et al., 2010). Therefore, we next addressed how CD82 scaffolding affects PKCα cluster size. Further analysis of the DBSCAN data indicates that


A
PKCα -Alexa647
DMSO
PMA 5 minutes
PMA 1 hour
Control
CD82OE
Palm-CD82OE
200 nm


B
Number of PKCα Clusters
250
200
150
100
50
0
p<.0001
****
n.s.
n.s.
n.s.
p=.0391
*
p=.0195
*
Control
CD82OE
Palm-
CD82OE
DMSO
PMA 5 mins
PMA 1hr
Two-way ANOVA:
****, p<.0001 Cell line
*** p=.0009 Treatment
C
PKCα Cluster Diameter (nm)
250
200
150
100
50
0
p<.0001
****
p=.0173
*
p=.0183
*
p=.0237
*
p=.0029
**
Control
CD82OE
Palm-
CD82OE
DMSO
PMA 5 mins
PMA 1hr
Two-way ANOVA:
****, p<.0001 Cell line
**** p<.0001 Treatment
D
PKCα Molecular Density
(localizations/nm²)
0.04
0.03
0.02
0.01
0
p=.0043
**
n.s.
n.s.
Control
CD82OE
Palm-
CD82OE
DMSO
PMA 5 mins
PMA 1hr
Two-way ANOVA:
****, p<.0001 Cell line
n.s. Treatment

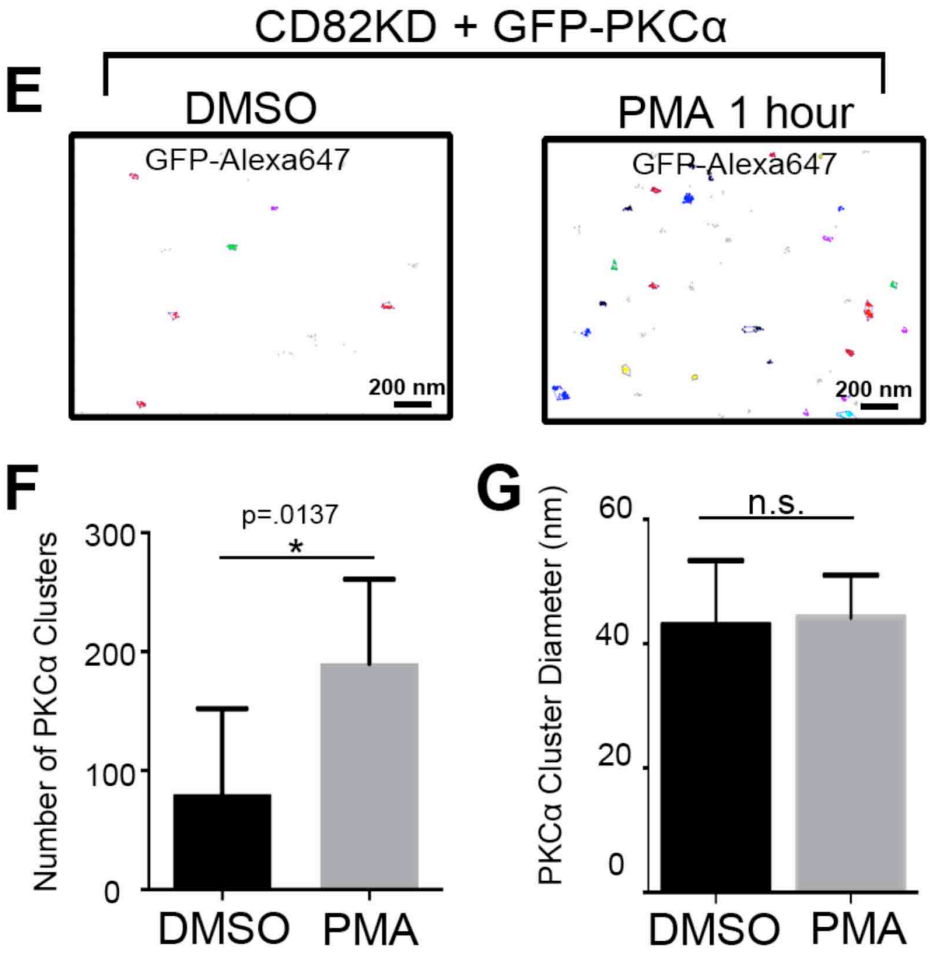


Figure 4.4 PKCα clustering at the membrane is controlled by the CD82 scaffold. Control, CD82OE and Palm-CD82OE KG1a cells were treated with DMSO, or PMA for 5 mins or 1 hr and imaged for PKCα (abcam, Y124; Invitrogen, rabbit-647) using dSTORM. (A) The DBSCAN algorithm was used to examine cluster organization within a subregion of the cells. Clustered localizations are indicated by color, whereas gray localizations did not meet the clustering parameters (ε=50nm, n=30 localizations). The DBSCAN algorithm was used to determine the (B) number of PKCα clusters, (C) PKCα cluster diameter, and (D) PKCα molecular density (n≥4 cells of each condition, error bars indicate SD, post-hoc unpaired t-test). CD82KD cells were transfected with GFP-PKCα and imaged using dSTORM. (E) PKCα clustering was quantified using the DBSCAN clustering algorithm in cells treated with DMSO or PMA (ε=50nm, n=10 localizations). (F) The number of clusters (n=7 cells, error bars indicate SD) and (G) the cluster diameter were quantified (n≥561 clusters, error bars indicate SEM, unpaired t-test).

under resting conditions, PKCα cluster diameter is similar between control and CD82OE cells, but is reduced in the Palm-CD82OE cells (Fig.4.4C). Upon PMA stimulation for 5 min, only the CD82OE cells exhibit an increase in PKCα cluster area. However, upon PMA stimulation for 1 hr, all the cells increase their PKCα cluster size with the CD82OE promoting even larger “superclusters” (Baddeley et al., 2009).

We also assessed how CD82 scaffolding modulates PKCα molecular density, or the number of PKCα localizations found per cluster area, because this is another mechanism by which PKCα may be recruited into clusters upon activation. Our data demonstrate that upon PMA stimulation for 5 mins, control cells display increased PKCα molecular density compared to resting conditions (Fig.4.4D). Meanwhile, the other cell lines exhibit similar PKCα molecular density upon resting or stimulated conditions. These data illustrate that CD82 concentration affects the means by which PKCα is initially recruited to the membrane. More specifically, in the case of the control cells, a lower concentration of CD82 results in PKCα becoming organized into densely packed clusters upon initial activation, whereas in the CD82OE cells, PKCα organizes into larger clusters (Fig.4.4C), while in the mutant Palm-CD82OE cells, PKCα is recruited into more clusters of the same size (Fig.4.4B,C). These data demonstrate that CD82 concentration and mutation regulate unique aspects of PKCα membrane clustering.

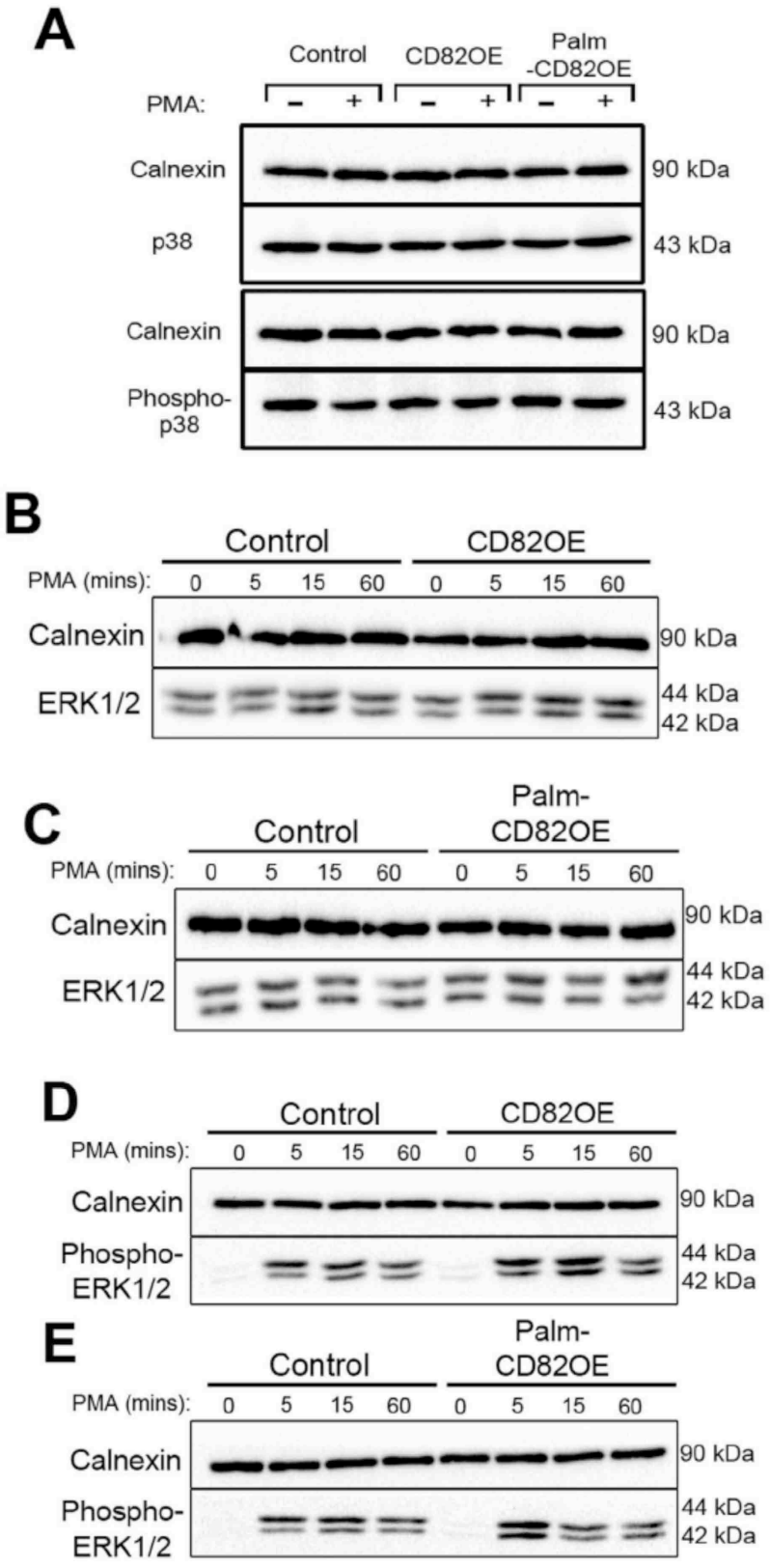
We also assessed PKCα clustering in the CD82KD cells by transiently transfecting in GFP-PKCα and performing SRI analyses (Fig.4.4E). Upon PMA stimulation for 1 hr, we measure an increase in the number of PKCα clusters, consistent with PKCα membrane translocation (Fig.4.4F). However, in contrast to the other cell lines, PKCα cluster area remains unchanged in the CD82KD cells following PMA activation (Fig.4.4G), suggesting that the CD82 scaffold is necessary to promote or stabilize the larger PKCα clusters measured following PMA stimulation. Combined, these data demonstrate that CD82 scaffolding significantly impacts PKCα cluster size.

4.3.5 CD82 modulates ERK1/2 activity downstream of PKCα stimulation.

The ability of PKCα to propagate a signal is dependent upon activation and sufficient membrane recruitment, which allows PKCα to phosphorylate a substrate and elicit a downstream response. Our findings suggest that CD82 stabilizes PKCα at the plasma membrane and promotes larger-scale clustering. We next examined how this stabilization and clustering affects PKCα–mediated signal propagation. One pathway that has been studied extensively with respect to PKCα 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 apoptosis (Milella et al., 2001). To determine how CD82 scaffolding affects PKCα-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.4.5A). Moreover, we find no change in total ERK1/2 expression between the cells (Fig.4.5B,C) and detect only minimal phospho-ERK1/2 expression in unstimulated cells (Fig.4.5D,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.4.5D-F). Interestingly, the CD82OE cells maintain significantly higher phospho-ERK expression upon 1 hr of PMA stimulation compared to Palm-CD82OE cells (Fig.4.5F). Similar results were observed using an alternative leukemia cell line (Suppl.Fig.S.4.2Q). These data demonstrate that CD82 scaffolding is critical for regulating the signaling kinetics of ERK1/2 downstream of PKCα activation.

4.3.6 CD82 regulates AML colony formation in a PKCα-dependent manner.

Finally, we wanted to determine how PKCα activation and ERK signaling affect the leukemia colony forming potential of AML cells. We treated cells with DMSO, PMA alone, or PMA in combination with the ERK1/2 inhibitor, FR180204.


A
Control
CD82OE
Palm
-CD82OE
PMA:
– + – + – +
Calnexin
90 kDa
p38
43 kDa
Calnexin
90 kDa
Phospho-
p38
43 kDa
B
Control
CD82OE
PMA (mins): 0 5 15 60 0 5 15 60
Calnexin
90 kDa
ERK1/2
44 kDa
42 kDa
C
Control
Palm-
CD82OE
PMA (mins): 0 5 15 60 0 5 15 60
Calnexin
90 kDa
ERK1/2
44 kDa
42 kDa
D
Control
CD82OE
PMA (mins): 0 5 15 60 0 5 15 60
Calnexin
90 kDa
Phospho-
ERK1/2
44 kDa
42 kDa
E
Control
Palm-
CD82OE
PMA (mins): 0 5 15 60 0 5 15 60
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90 kDa
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ERK1/2
44 kDa
42 kDa

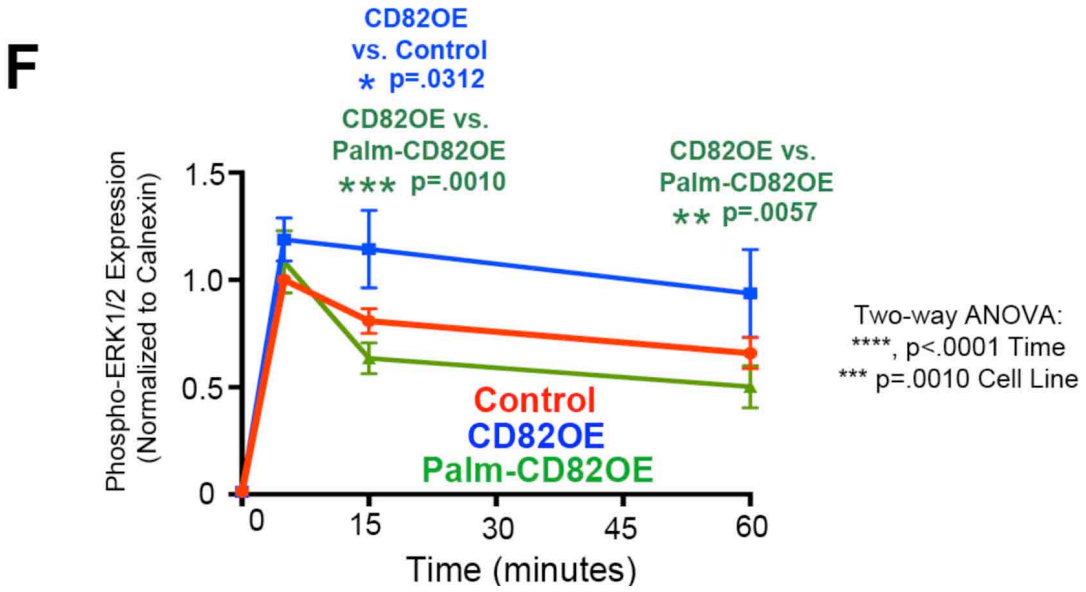


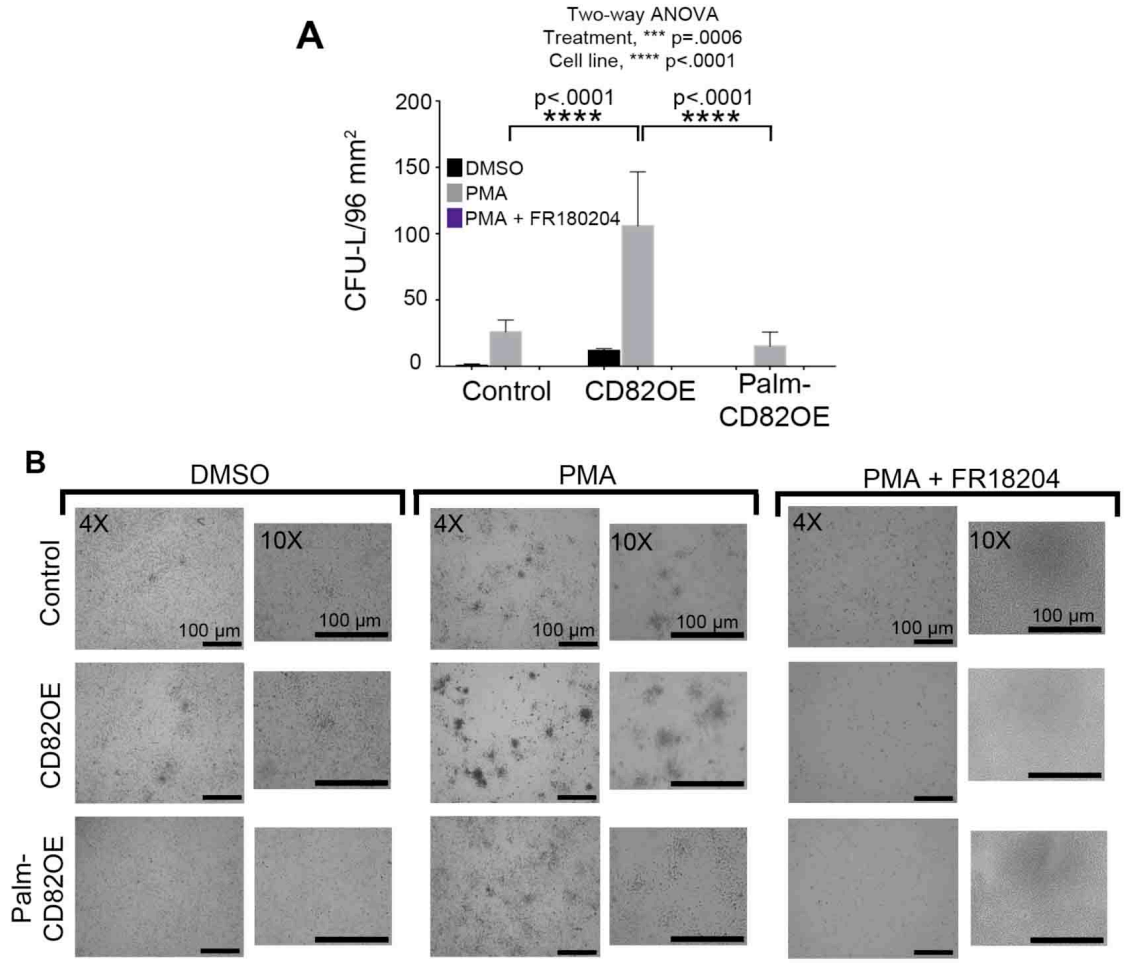
Figure 4.5 CD82 modulates ERK1/2 activity downstream of PKCα stimulation. (A) Control, CD82OE and Palm-CD82OE cells were treated with DMSO or PMA for 1 hr and analyzed by Western blot analysis for total (D13E1) and phospho-p38 (Thr180/Tyr182). Representative Western blot showing control and (B) CD82OE 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≥4 experiments, error bars depict SEM; post-hoc unpaired t-test).

Cells were then plated in MethoCult H4334 media for 14 days, after which, the leukemia colony-forming units (CFU-L) were counted via microscopy. Interestingly, following PMA treatment, we find that the CD82OE cells display more than four times as many CFU-L compared to control and Palm-CD82OE cells (Fig.4.6A,B). Interestingly, in all cells treated with PMA and the ERK1/2 inhibitor, colony growth was completely inhibited. These data suggest that the CD82 scaffold enhances PKCα signaling for controlling leukemia colony formation, which occurs through ERK1/2 signaling. From these collective data, we suggest the current model (Fig.4.6C) where the CD82 scaffold recruits and stabilizes PKCα in clusters, which sustains ERK1/2 signaling for the development of an aggressive leukemia phenotype.

4.4 Discussion

In this study, we provide new insights into how tetraspanins can serve as membrane scaffolds that control signal transduction in AML. As PKCα is a critical signaling hub for controlling AML cell proliferation and survival (Kornblau et al., 2006), we focused on identifying the properties of tetraspanins that contribute to aberrant PKCα signaling in AML. Numerous studies defined an interaction between PKCα and tetraspanins, but the mechanisms regulating this association and the downstream signaling consequences remain unclear. Our study describes a role for CD82 membrane organization in regulating PKCα expression, membrane stabilization and signaling.

Increased phospho-PKCα expression has been correlated with poor survival rates in AML patients (Kurinna et al., 2006), while elevated phospho-PKCα levels are correlated with increased AML cell viability(Zabkiewicz et al., 2014). Data from our study demonstrate that the overexpression of CD82 increases total and phospho-PKCα expression (Fig.4.1D). These findings are consistent with previous results where increased CD82 expression elevated PKCα phosphorylation (Wang et al., 2007b). Upon mutation of the palmitoylation sites within CD82, we detect decreased total and phospho-PKCα expression (Fig.4.1D) when compared to control or CD82OE cells, offering the interesting possibility that CD82 scaffolding modulates PKCα expression. A similar decrease


A
Two-way ANOVA
Treatment, *** p=.0006
Cell line, **** p<.0001
p<.0001
****
p<.0001
****
CFU-L/96 mm²
200
150
100
50
0
DMSO
PMA
PMA + FR180204
Control
CD82OE
Palm-CD82OE
B
DMSO
PMA
PMA + FR18204
Control
CD82OE
Palm-CD82OE
4X
10X
100 µm

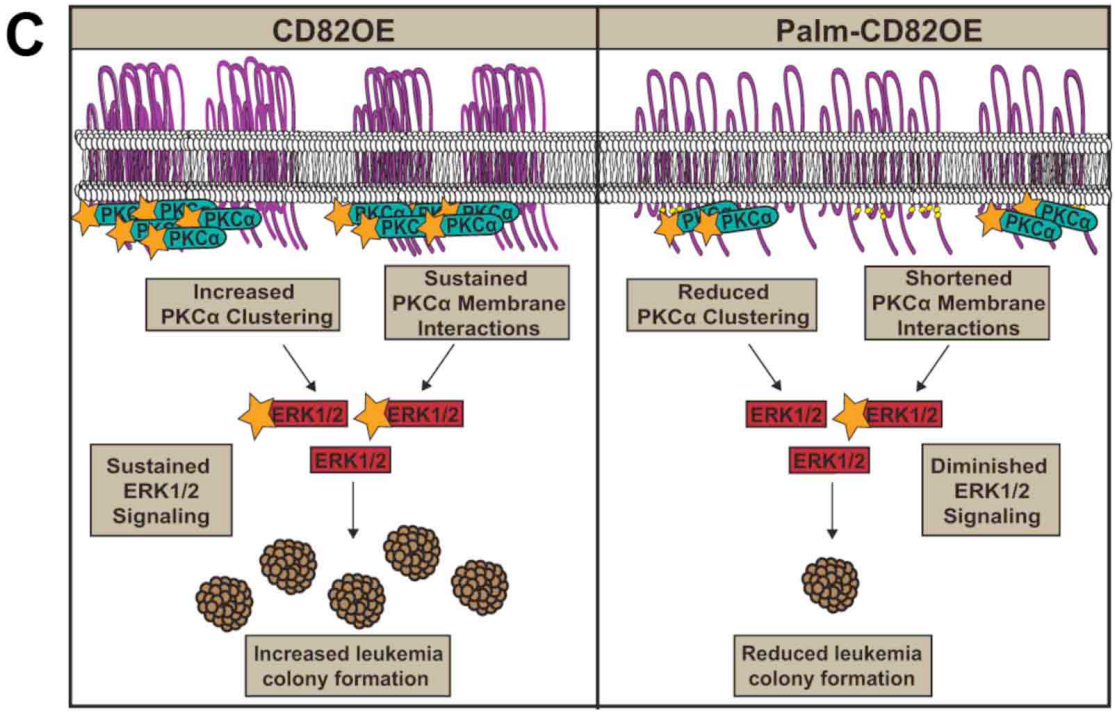


Figure 4.6. CD82 regulates AML colony formation in a PKCα-dependent manner. (A) and (B) Control, CD82OE and Palm-CD82OE cells grown in clonogenic assays in the presence of PMA alone (10ng/ml), or PMA+FR180204 (100μM), or equal volumes of DMSO and assessed after 14 days by microscopy for the number of leukemia colony-forming units per 96mm2 (n≥4 experiments, error bars indicate SD). (C) Proposed model whereby the scaffolding function of CD82 regulates the membrane clustering and stabilization of PKCα, which controls ERK1/2 signaling and AML colony forming potential.

in PKCα expression was observed in previous studies when CD82 expression was knocked down by siRNA (Wang et al., 2007b), further supporting the importance of the CD82 scaffold for maintaining PKCα expression. In this study, we find that PKCα transcript levels are similar between the control, CD82OE and Palm-CD82OE cells (Fig.4.1G), suggesting that changes in protein production are less likely to be responsible for the change in PKCα protein expression observed. However, the PKCα transcript levels of PKCα in the CD82KD cells are significantly decreased, suggesting that CD82 may have transcriptional control of PKCα in these cells, though the mechanism remains undiscovered. Our data make the case that CD82 scaffolding can dramatically increase PKCα expression, through the stabilization of PKCα at the plasma membrane.

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 PKCα phosphorylation of c-Cbl following HB-EGF activation, which led the authors to suggest that CD82 could in fact serve to recruit PKCα (Odintsova et al., 2013). Additionally, a described role for CD151 was to recruit PKCα into proximity with the α6β4 integrin, which significantly impacted tumor initiation and progression (Li et al., 2013a). Our SPT data suggests a decrease in PKCα membrane dwell time in the Palm-CD82OE cells (Fig.4.2M-O), indicating that disruption of the CD82 scaffold organization may shorten PKCα membrane interactions. Our PKCα tracking experiments used GFP, which has a relatively short fluorescent lifetime; as such, we detect sub-second PKCα track lengths. This may account for the modest change seen in PKCα track length in the Palm-CD82OE cells. Interestingly, the CD82OE cells also exhibit deceased levels of CD81 (Suppl.Fig.S.4.1B), which can also interact with PKC (Zhang et al., 2001). We hypothesize that this occurs in the CD82OE cells but not the Palm-CD82OE cells because the role of CD81 and CD82 has redundancy in the CD82OE cells, leading to a downregulation of CD81 in CD82OE cells. However, in the Palm-CD82OE cells, we hypothesize that the palmitoylation deficient form of CD82 may not be fully functional. Therefore, CD81 may serve a compensatory role for regulating PKCα dynamics and therefore, it's expression is needed in the Palm-

CD82OE cells. Moreover, the differential CD81 expression may mask a larger change in PKCα membrane stabilization as detected with SPT. Despite the experimental limitations, these data suggest that CD82 organization retains PKCα at the membrane.

Biochemical characterization of tetraspanins suggests that PKCα and PI4K may have distinct tetraspanin recruitment sites, indicating the potential for differential recruitment of signaling enzymes to specific tetraspanins (Zhang et al., 2001). One possible explanation for how decreased PKCα dwell time could occur is through diminished interactions with the CD82 signaling platform. Our FRET analyses (Fig.4.3) indicate that PKCα 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 PKCα 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 PKCα and CD82 at the membrane. Interestingly, PKCα can also be palmitoylated, which was shown to facilitate its membrane recruitment (Ford et al., 1998). Therefore, future studies will explore how PKCα palmitoylation contributes to the robust membrane interaction between CD82 and PKCα.

While tetraspanins have been described to regulate membrane protein clustering (Marjon et al., 2015; Termini et al., 2014; van Spriel et al., 2012), our study explores how tetraspanins modulate cytosolic protein clustering. Previous work has demonstrated that the number and size of Ras clusters contributes to the downstream response(Harding and Hancock, 2008; Tian et al., 2007). Moreover, increased expression of galectin-1, a Ras membrane scaffold, can enhance Ras-mediated signaling (Elad-Sfadia et al., 2002). The current study has uncovered a role for CD82 in regulating PKCα oligomerization, a concept that was hypothesized to have physiological signaling consequences (Swanson et al., 2014). In order to bypass the loss of PKCα expression that occurs in the cells after prolonged PMA stimulation (Fig.4.1I), we stimulated cells for just 5 mins and visualized PKCα. Our data demonstrate that the PKCα clusters in

CD82OE cells are significantly larger than both control and Palm-CD82OE cells upon 5 mins of stimulation (Fig.4.4C). It is important to take into account the ratio of surface CD82 to PKCα in our cell lines for interpretation of these data. By setting the control cells at a 1/1 ratio of CD82: PKCα, the CD82OE cells have a ratio of 2/2, while the Palm-CD82OE cells have a ratio of 2/0.5. Although the Palm-CD82OE cells have fourfold as much CD82 as PKCα, they are unable to generate larger PKCα clusters upon 5 mins of stimulation but exhibit an increase in the number of clusters (Fig.4.4B-C). We suggest that the excess of CD82 in the Palm-CD82OE cells serves as a platform to enhance the recruitment of PKCα to the membrane in new clusters. As the scaffolding capacity of CD82 is disrupted in the Palm-CD82OE cells, we hypothesize that PKCα is unable to assemble into larger clusters, but it is indeed recruited, consistent with our FRET data in Fig.4.3. Meanwhile, the control cells have half as much CD82 and PKC compared to the CD82OE cells and also do not display an increase in cluster size. Rather, the control cells have the same number of clusters, though the clusters become more densely packed (Fig.4.4D). Therefore, it appears that the amount of CD82 helps establish the ability for PKCα to organize into more clusters, while palmitoylation mutation of CD82 assists with the ability for PKCα to grow into larger clusters upon stimulation. Furthermore, these data support the concept that the CD82 scaffold can modulate the stoichiometry of signaling molecules recruited to a set number of signaling platforms, although at this time, the mechanism responsible for establishing the number of PKCα clusters remains unclear.

Aberrant activation of the ERK pathway is implicated in AML progression (Blume-Jensen and Hunter, 2001). Previous studies have shown that inhibiting MAPK signaling in AML can lead to increased apoptosis and reduced proliferation(James et al., 2003; Kerr et al., 2003; Lunghi et al., 2003; Milella et al., 2007). Additional studies have shown that the treatment of lymphoid cells with CD81 and CD9 antibodies modulated proliferation through alterations in the ERK1/2/MAPK pathway (Carloni et al., 2004; Hemler, 2005; Murayama et al., 2004). 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.4.5F). 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 PKCα in the CD82OE cells (Fig.4.1I) serves to stimulate and maintain the activation of ERK1/2. Conversely, we suggest that the reduced levels of PKCα seen in the Palm-CD82OE cells upon activation (Fig.4.1I) leads to a quick turnover of 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 transduction (Cebecauer et al., 2010). Our findings are consistent with this notion, demonstrating that increased PKCα "microcluster" formation seen in the CD82OE cells (Fig.4.4C) correlates with sustained ERK1/2 signaling. Our findings demonstrate 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 PKCα can significantly impact the prolonged downstream ERK1/2 effector signaling.

ERK activity has been linked to cell proliferation and leukemia chemoresistance (Steelman et al., 2004; Willard and Crouch, 2001). Additionally, CD82 expression was shown to be increased in the chemotherapy-resistant CD34(+)/CD38(-) cells in AML (Nishioka et al., 2015b). 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 PKCα stimulation. Interestingly, following PMA treatment, CD82OE cells generate an even greater increase in leukemia CFU formation, indicating that PKCα activation and downstream signaling regulate the aggressiveness of AML. Moreover, our data demonstrate that modifications in the CD82 scaffold can regulate ERK activation downstream of PKCα, which when inhibited with FR180204, significantly impacts leukemia CFU formation. Together, these data suggest that targeting CD82 scaffold may provide an alternative route towards regulating

PKCα and its downstream signaling response in AML. Tetraspanins are already being used in clinical trials for the treatment of chronic lymphocytic leukemia (Beckwith et al., 2015). 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.

4.5 Methods

4.5.1 Cell culture

The KG1a, K562 and U937 cell lines (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10%FBS, 2mM l-glutamine, 100u/ml penicillin, and 100µm/ml streptomycin. Cells were incubated at 37°C, 95% humidity, and 5%CO_2. For stimulation experiments, cells were treated with 10ng/ml of PMA alone (Sigma), or combined with FR180204 (Sigma) at 100µM or equivalent volumes of DMSO.

4.5.2 Plasmids/cell line generation

The mCherry-CD82 and mCherry-Palm-CD82 plasmids were constructed as previously described(Termini et al., 2014). 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 and kept under selection using 500ug/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-PKCα 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-PKCα according to the manufacturer's protocol (Amaxa, Lonza Group).

4.5.3 Western blotting

Western blots were performed as previously described (Termini et al., 2014). Antibodies used for Western blotting were purchased from Cell Signaling Technology as follows: calnexin (C5C9), PKCα (#2056, polyclonal), phospho-PKCα (Thr638), p42/44 (137F5), phospho-p42/44 (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.

4.5.4 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).

4.5.5 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: PKCα forward: 5' ATC CGC AGTGGA ATG AGT CCT TTA CAT 3', PKCα 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.

4.5.6 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; PKCα, 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 100M inverted microscope (LSM 510) system (Carl Zeiss, Jena, Germany) using an excitation wavelength of 488 or 633nm and a 63X/1.2 numerical aperture oil immersion objective. Image analysis was performed using the Zeiss LSM 510 software.

4.5.7 Super-resolution microscopy

Cells were plated on chamber slide wells that were treated with fibronectin (25μg/ml, Millipore). Cells were fixed with 4% paraformaldehyde and blocked/permeabilized (1%BSA/PBS/0.2%Tween). Cells were labeled with an anti-PKCα antibody (1:200, abcam, Y124), washed, and incubated with a goat anti-rabbit AlexaFluor647 secondary antibody (1:200; Invitrogen). Cells transfected with GFP-PKCα were labeled with an anti-GFP Alexa647 antibody (Biolegend, FM264G). Cells were washed post-label fixed with 4% paraformaldehyde. Cells were washed and imaged in dSTORM imaging buffer consisting of 50mM Tris, 10mM NaCl, 10% w/v glucose, 168.8 u/ml glucose oxidase (Sigma #G2133), 1404.0 U/ml catalase (Sigma #C9332), and 50mM 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 previously(Valley et al., 2015) that uses an inverted microscope (IX71, Olympus America Inc.). A 637nm laser (HL63133DG, Thorlabs) is coupled along with a 405nm laser (Crystal laser), into two mode fibers and focused onto the objective lens with a 1.45 NA (UAPON 150XTIRF, 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 ~20ms (50 frames/second) for a 256x256 pixel region. All of the instrumentation is controlled by custom-written software in Matlab

(MathWorks Inc.). For one color imaging, the 637nm and 405nm lasers were used concurrently. The 561nm 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 counts (Huang et al., 2011). 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. Clusters in Fig.4.4A-D were determined as having at least 30 localizations within a 50nm search radius, while clusters in Fig.4.4E-G only required 10 localizations. DBSCAN provides the number of clusters detected and their area. The equivalent cluster diameter represents the diameter of a circle with the same cluster area detected by DBSCAN. Molecular density is calculated as the number of localizations in a cluster divided by the cluster area.

4.5.8 Förster resonance energy transfer (FRET)

Stable KG1a cells were transfected with GFP-PKCα and plated on 25μg/μl 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 488nm (GFP) and 561nm (mCherry). Fluorescence from the 488nm channel was collected using a HyD1 detector and fluorescence from the 561nm channel was collected using the HyD SMD2 in standard mode. Photobleaching was performed at 100% 561nm laser power for 2 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. FRET efficiencies were calculated using the formula: Efficiency = $(Donor_{post\text{-}bleach} - Donor_{pre\text{-}bleach})/Donor_{post\text{-}bleach}$ where D is the fluorescence intensity in a plasma membrane

region of interest of fixed shape and size (3x7 ellipse). Analysis was performed using the Leica Application Suite AF Lite software.

4.5.9 Single particle tracking (SPT)

SPT was performed using the TIRF microscope optical setup as described in the "Super-resolution Microscopy" section. A 488nm 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 previously(Schwartz et al., 2015). 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 to use 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.

4.5.10 Leukemia colony-forming unit assay

100,000 KG1a cells were treated with PMA (10ng/ml) alone, PMA+FR180204 (100μM) 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.

4.5.11 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. (*<.05, **<.01, ***<.001, ****<.0001).

Chapter 5: Conclusions, significance and future directions

5.1 Conclusions

The studies described in this dissertation provide novel information regarding how tetraspanins propagate their control of cellular adhesion, homing and signaling. We have identified tetraspanin CD82 as a critical regulator of hematopoietic stem/progenitor cell adhesion as well as acute myeloid leukemia homing and signaling. Furthermore, our studies have used super-resolution imaging techniques to reveal the molecular landscape of membrane proteins, including CD82, the α4 integrin subunit, and N-cadherin.

In chapter 1, our data demonstrate that the palmitoylation of CD82 contributes to HSPC adhesion to ECM components. We determined that the overexpression of CD82 increases the surface expression of the α4 integrin subunit by reducing α4 internalization and increasing α4 recycling. We found that overexpressing a palmitoylation deficient version of CD82 does not increase HSPC adhesion, which led us to examine how CD82 palmitoylation regulates integrin organization. Our data demonstrate that the palmitoylation of CD82 contributes to the molecular packing, or density, of the α4 integrin subunit. We also determined that this increase occurs in a ligand-dependent manner. These data indicate that integrin organization should be considered as a means by which HSPC adhesion can be increased, with the ultimate goal of improving HSC transplantation.

In chapter 2, we took a closer examination of how CD82 contributes to AML interactions with the bone marrow microenvironment. With the use of primary AML patient samples, our data show that AML blasts with increased CD82 expression exhibit increased bone marrow homing. With the use of AML cell lines that overexpress either palmitoylation or glycosylation deficient versions of CD82, we identify a role for these post-translation modifications in mediating AML homing. Furthermore, our data show that palmitoylation mutation diminishes CD82 scaffolding, while glycosylation mutation enhances CD82 organization. We also examined how CD82 scaffolding modulates the clustering of N-cadherin. Our data demonstrate that palmitoylation mutation reduces the percentage of N-

cadherin localizations organized into clusters. These clusters are also reduced in size. Therefore, we propose that CD82 scaffolding regulates the organization of N-cadherin for the control of AML homing.

In chapter 3, we took a visual and quantitative examination into how PKCα interacts with CD82. Our data show that the expression and palmitoylation of CD82 are critical for maintaining PKCα expression. Meanwhile, increased CD82 expression can protect PKCα from degradation upon activation. Using single particle tracking, we determined that CD82 palmitoylation mutation diminishes individual PKCα membrane interactions. Furthermore, using FRET analyses, we find that wild-type and palmitoylation mutant forms of CD82 can interact with PKCα. However, this interaction is transient in our palmitoylation mutant cells. A closer examination into how CD82 regulates the oligomerization of PKCα upon activation shows that CD82 overexpression promotes PKCα organization into large clusters. Meanwhile, removal of the palmitoylation sites of CD82 significantly reduces PKCα cluster area. Using Western blot analyses, we determine that CD82 overexpression can enhance ERK1/2 signaling downstream of PKCα activation when compared to our palmitoylation mutant cells. We also examined how alterations in PKCα activation manifest in AML colony formation. Our data show that the overexpression of CD82 significantly enhances leukemia colony formation, whereas palmitoylation mutant cells form few leukemia colonies. Furthermore, colony formation downstream of PKCα activation can be attenuated with the treatment with an ERK1/2 inhibitor. These data demonstrate the critical role of ERK1/2 signaling downstream of PKCα in mediating the formation of an aggressive AML phenotype.

Collectively, these projects point to the need for further investigation regarding the suitability of CD82 and in particular the scaffolding of CD82, as molecular targets to enhance hematopoietic stem cell transplantation. Additionally, our findings demonstrate that the palmitoylation of CD82 may serve as a potential therapeutic target to dislodge AML cells from the bone marrow niche, while attenuating aberrant signaling.

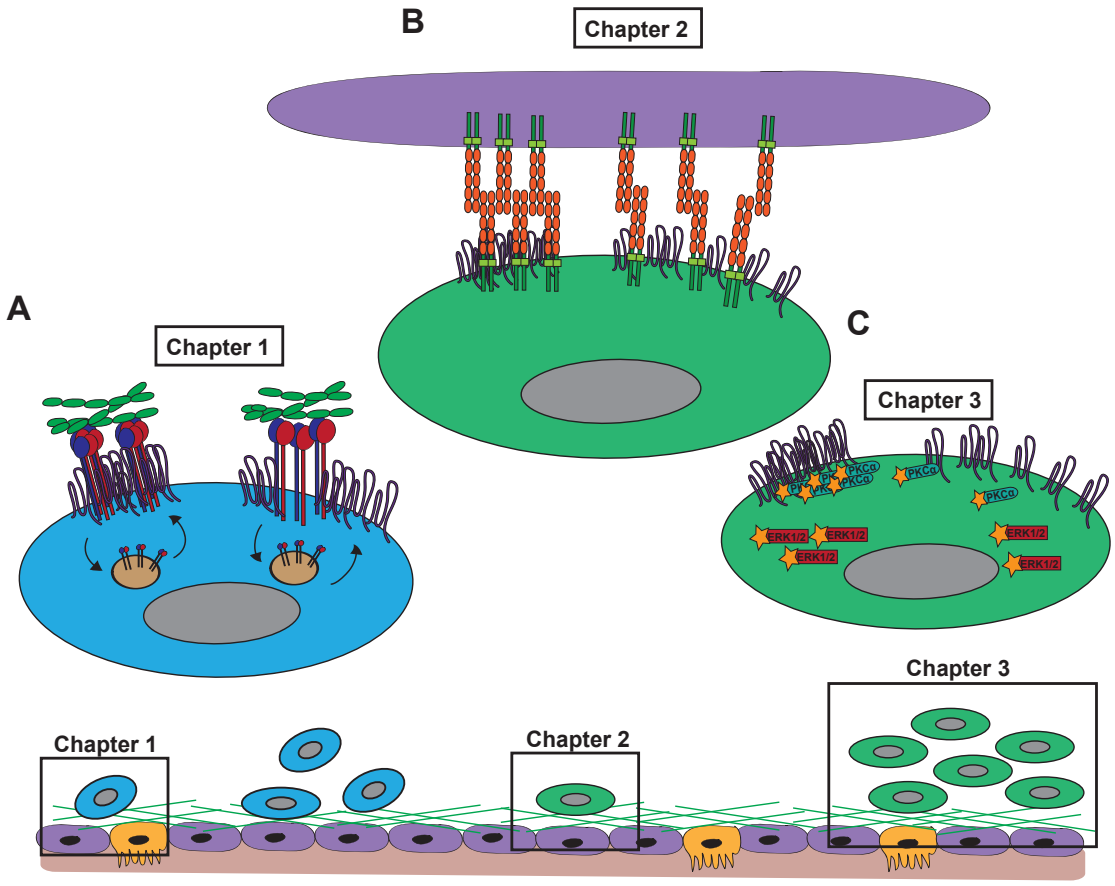


Figure 5.1: Conclusion and hypothesized models. (A) CD82 regulates hematopoietic stem/progenitor cell adhesion by promoting the tightly packed organization of the α4 integrin subunit in a ligand-dependent manner. The palmitoylation of CD82 is critical for promoting the molecular density of α4. (B) CD82 promotes AML bone marrow homing in a N-cadherin dependent manner. Mutation of the glycosylation sites within CD82 promotes the tight packing of N-cadherin, which enhances AML bone marrow homing. (C) CD82 serves as a signaling scaffold for the recruitment of PKCα, which promotes sustained signaling to enhance leukemia colony growth. The palmitoylation of CD82 is critical for this process, as mutation of the palmitoylation sites within CD82 disrupts PKCα-mediated signaling and colony growth.

5.2 Significance

Within the field of tetraspanin research, it is commonly accepted that tetraspanins can regulate integrin-mediated adhesion (Barreiro et al., 2005; Barreiro et al., 2008; Bassani and Cingolani, 2012; Feigelson et al., 2003; Gustafson-Wagner and Stipp, 2013; He et al., 2005; Lammerding et al., 2003). More specifically, tetraspanins can regulate integrin expression (He et al., 2005), avidity (Feigelson et al., 2003) as well as integrin-mediated signaling through FAK (Iwasaki et al., 2013; Jung et al., 2012; Yamada et al., 2008) for the control of cellular adhesion. However, few reports have examined how tetraspanins regulate integrin organization (van Spriel et al., 2012), which could also contribute to cellular adhesion. As such, in Chapter 2, we used single-molecule imaging techniques to determine how CD82 regulates integrin organization for the control of cellular adhesion. Previous studies have shown that integrin-mediated adhesion is optimal when integrin subunits are separated by just 58 nm, as compared with integrins separated by 73nm (Arnold et al., 2004). In line with such findings, we find that cells exhibit increased adhesion when α4 integrin subunits are more densely packed compared α4 integrin clusters that are more diffusely organized.

A recent report used super-resolution imaging to resolve the organization of tetraspanin enriched microdomains (Zuidscherwoude et al., 2015). Consistent with our data (Fig 2.4), the authors find that tetraspanins organize into clusters that are approximately 100nm in diameter. Biochemical studies have also shown that tetraspanin scaffolding regulates interactions between tetraspanins and integrins (Berditchevski et al., 2002; Yang et al., 2002), which may alter the webbing of TEMs. Consistent with these findings, we find that the overexpression of the palmitoylation-deficient form of CD82 significantly diminishes CD82 clustering (Fig. 2.4, Fig. 3.2). Although we did not examine the role of CD82 scaffolding in regulating the organization of other tetraspanins, we hypothesize that disruption in the CD82 scaffold impacts the organization of other tetraspanins and receptors within the plasma membrane.

As the plasma membrane is crowded with numerous membrane proteins, it is highly likely that altering the tetraspanin expression levels will affect the plasma membrane composition. For example, in our CD82OE cells, we found a decrease in CD81 expression (Fig.S4.1). This is likely to accommodate for the increase in CD82 and subsequent tetraspanin scaffolding within the membrane. Additionally, in our Palm-CD82OE cells, we find an increase in CD81 expression, which is likely an attempt to compensate for the disruption in TEM integrity that is caused by overexpression of the palmitoylation deficient form of CD82. Furthermore, we find that CD82 expression regardless of palmitoylation mutation increases α4 integrin surface expression (Fig. 2.2). As we found that CD82 and the α4 integrin subunit do not associate with one another on the plasma membrane (Fig.S2.4), we hypothesize that other tetraspanins, such as CD81, CD9 and CD151 may regulate the stabilization of the α4 integrin on the surface through protein-protein interactions.

We found that the CD82-mediated increase in α4 integrin expression occurs not through alterations in transcription, but rather through deceased α4 integrin internalization and increased protein recycling (Fig. 2.3). As tetraspanins have been shown to regulate the trafficking of several other proteins such as EGFR (Odintsova et al., 2000) and GPCRs (Xu et al., 2004), these data are consistent with the conventions within the field. Moreover, in our CD82OE and Palm-CD82OE cells we find an increased expression of the pro- and mature forms of the α4 integrin subunit (Fig. 2.2). It is not clear within the literature if the immature form of α4 can exist on the plasma membrane. As such, it is difficult to determine if the increase in the immature form of the α4 integrin subunit is due to alterations during protein production or the altered tetraspanin landscape seen on the plasma membrane. Therefore, these data question whether CD82-mediated alterations in α4 integrin expression are strictly due to changes in membrane stabilization or also result from alterations in α4 integrin production.

Although extensive biochemical analyses have shown that tetraspanins can interact with transmembrane proteins, our understanding of how tetraspanins regulate the organization of such receptors remains limited. Our work provides visual and quantitative evidence of how CD82 expression and scaffolding regulate the molecular organization of the α4 integrin subunit. Our report is the first of its kind to identify a role for tetraspanins in promoting tightly packed integrins for the control of cellular adhesion.

With regards to AML, previous reports have demonstrated that CD82 expression is increased in CD34(+)/CD38(-) chemotherapy resistant AML cells (Nishioka et al., 2013). Furthermore, the bone marrow has been demonstrated to offer this population of cells a protective microenvironment, thereby allowing cells to evade chemotherapeutic treatments (Meads et al., 2008). Therefore, understanding the interplay between CD82 and bone marrow interactions will clarify how CD82 offers AML cells selective resistance to therapies.

We find that AML blasts with increased CD82 expression have improved homing to the bone marrow. These findings are consistent with previous work that identifies a role for CD82 in regulating the bone marrow homing of HSCs (Larochelle et al., 2012). Previous work has shown that N-cadherin can regulate interactions between healthy hematopoietic stem cells and the bone marrow microenvironment (Calvi et al., 2003). As such, we aimed to determine if CD82 alters N-cadherin for the control of bone marrow homing. There is limited work focused on the role of tetraspanins in regulating N-cadherin. As such, our study is significant because it identifies a role for CD82 in regulating the organization of N-cadherin, which had not been previously demonstrated. Our data provide visual and quantitative evidence that CD82 can control the clustering of N-cadherin for the control of AML bone marrow homing. Furthermore, N-cadherin oligomerization has been shown to contribute to adhesion strengthening (Niessen and Gumbiner, 2002). Therefore, our data suggest that CD82 promotes the oligomerization of N-cadherin for the control of AML homing. As such, targeting CD82 may offer a means by which N-cadherin can be neutralized to dislodge AML cells from the bone marrow. Furthermore, we find that the

palmitoylation and N-linked glycosylation of CD82 are critical regulators of N-cadherin-mediated AML homing to the bone marrow. Therefore, manipulating the scaffolding capacity of CD82 may ultimately downregulate N-cadherin mediated AML adhesion within the niche thereby sensitizing AML cells to therapeutics.

Beyond the organization of N-cadherin, using immunoprecipitation analyses, we find that the N-linked glycosylation of CD82 controls the ability for CD82 to interact with N-cadherin. Moreover, mutation of the N-linked glycosylation sites within CD82 improves the association between CD82 and N-cadherin (Fig.3.3). We hypothesize that removing a bulky sugar moiety from the extracellular domain of CD82 may expose new sites available for N-cadherin to interact with CD82, thereby enhancing their association. Further analysis examining the precise sites of association will improve our understanding of how N-linked glycosylation regulates protein-protein interactions.

In Chapter 4, we examined the role of CD82 in regulating PKCα signaling in AML. Although previous work using immunoprecipitation assays identified an interaction between PKCα and CD9, CD81 and CD82, very little was known about what regulates this interaction and how this feeds into signaling dynamics (Zhang et al., 2001). Our work took advantage of single molecule and ensemble fluorescence imaging techniques to further elucidate the role of CD82 in mediating PKCα signaling. We determined that the scaffolding of CD82 is a critical regulator of the generation of stable PKCα-CD82 interactions. Additionally, we are the first to have resolved the molecular organization of PKCα using single-molecule imaging. A recent report from the Lipp lab demonstrated that PKCα-PKCα interactions are critical for PKCα to form higher order oligomers (Bonny et al., 2016). By using CD82KD cells, we demonstrate that activation of PKCα stimulates the recruitment of more PKCα localizations to the plasma membrane, but they are unable to organize into higher ordered structures. As such, our data suggest that the presence of CD82 is essential for PKCα molecules to effectively oligomerize.

As tetraspanins have also been demonstrated to regulate MAPK signaling for the control of tumor growth and cellular migration (Danglot et al., 2010;

Franco et al., 2010; Li et al., 2013b). However, the contribution of tetraspanin scaffolding to the regulation of MAPK signaling has yet to be examined. As such, we assessed the consequences of defective CD82 scaffolding with regards to ERK signaling. We find that CD82 scaffolding is a critical regulator of ERK1/2 activation downstream of PKCα signaling. Meanwhile, CD82 scaffolding is also a critical regulator of the formation of leukemia colonies downstream of PKCα and ERK1/2. Therefore, the scaffolding of CD82 generates a more aggressive disease phenotype.

5.2.1 Clinical Significance

The success of hematopoietic stem cell transplantation remains limited by the ability for HSPCs to reach the bone marrow microenvironment and make stable contact with niche components. As such, our work is significant for potential therapeutic options to enhance HSPC niche adhesion. For example, the enforced expression of CD82 in the HSPC population may serve as a means to alter integrin clustering for the control of HSPC homing/adhesion. Additionally, because we now know that the organization of integrins into tightly packed clusters is critical for HSPC adhesion, therapeutics that promote integrin clustering prior to transplantation may offer a means by which HSPC success can be improved.

Our work also suggests that CD82 may be a suitable target to ultimately downregulate N-cadherin mediated AML adhesion within the niche. As the niche offers a protective microenvironment allowing cells to evade treatment, disruption of AML adhesion may provide an opportunity to sensitize AML cells to therapeutics by mobilizing them out of the protective bone marrow niche into the blood for more effective targeting. Furthermore, we suggest that the disruption of the CD82 scaffold could serve as a means by which to attenuate PKC-mediated signaling. In particular, there is evidence that patient samples with high levels of activated PKCα have reduced susceptibility to chemotherapeutics (Milella et al., 2001; Ruvolo et al., 2011). Therefore, initial screening for PKCα expression and activation levels in a clinical setting could potentially identify patients who may be responsive to targeting the CD82 scaffold.

5.3 Future directions

There are still several unanswered questions about the means by which CD82 contributes to HSC homing. As homing is a complicated process, involving migration, extravasation, and adhesion, it remains to be seen whether CD82 regulates all or some of these processes through its control of integrin organization. For example, the α4 integrin subunit was shown to mediate transendothelial/stromal migration of CD34(+) cells in NSG mice (Peled et al., 2000). Therefore, it is quite possible that CD82 mediated alterations in α4 integrin organization could assist with HSC entry from the vasculature into the microenvironment in addition to isolated bone marrow adhesion. Future studies focused on the isolated role of CD82 in mediating HSC migration and extravasation through the vasculature will provide valuable mechanistic insight.

Our projects have focused primarily on the role of CD82 in mediating bone marrow interactions. However, it is highly likely that CD82 also contributes to adhesive and migratory interactions with other supporting niches, such as the vascular niche. As VCAM-1 is highly enriched in the vasculature and we have identified VCAM-1 mediated alterations in the α4 integrin subunit organization, we hypothesize that CD82 also contributes to HSC interactions with the vasculature. Furthermore, the role of integrin clustering in mediating HSPC migration has yet to be examined. It is quite possible that integrin clustering assists with the ability for integrins to be sequestered, relocated, and recycled quickly, which contributes to migration. Future studies focus on how CD82 regulates integrin clustering for the control of cellular migration and how alterations in integrin trafficking contribute to this process may provide insight into how integrins mediate HSPC-niche interactions.

The bone marrow niche is a complicated microenvironment with numerous cellular and extracellular components. We have yet to examine the role of CD82 in regulating HSC interactions with mesenchymal stem cells, CXCL12-abundant reticular cells, or adipose cells, all of which are found within the HSC niche. HSCs make unique interactions with these cells, which may occur in a CD82 dependent manner. As different cellular components deposit unique extracellular

matrices, we hypothesize that CD82 may regulate the organization of different integrin subunits based on ligand specificity. As such, a careful examination of the role of CD82 in regulating the organization of ligand specific in a ligand dependent manner will provide significant mechanistic insight into the role of tetraspanins in mediating molecular organization.

We have established that CD82 expression and post-translational modifications control AML interactions with the bone marrow through alterations in N-cadherin molecular organization. N-cadherin serves as a scaffold for the recruitment of β-catenin and p120. However, we do not yet know how alterations in N-cadherin organization regulate downstream signaling. Furthermore, N-cadherin serves to recruit intermediates that help establish stable attachments to the actin cytoskeleton. This could significantly impact the ability for cells to adhere or migration. As such, the role that N-cadherin organization plays in mediating cytoskeletal dynamics remains to be examined.

Although our data identify CD82 expression and post-translational modifications as regulators of AML homing, we have yet to examine the role this plays in longer-term disease states. For example, mutation of the CD82 glycosylation sites offers cells an improved ability to home to the bone marrow after 16 hours. We do not yet know how these cells will behave when long-term xenografts are performed. As β-catenin can mediate cellular proliferation and survival, there is a potential role for N-cadherin organization in regulating downstream signaling, which may further enhance AML-niche interactions.

We have also assessed the role for CD82 in regulating the spatial and temporal dynamics of PKCα. Our data show that disruption of the CD82 scaffold offers a unique means to attenuate PKCα signaling, and thereby diminish AML growth *in vitro*. Future studies should focus how other tetraspanins regulate PKCα signaling, as several have been shown to interaction with PKC isoforms. Furthermore, the role of CD82 in mediating PKCβI and PKCβII membrane interactions has yet to be examined. PKCβ can be deregulated in several disease pathologies beyond AML. Therefore, CD82 targeting may be suitable in clinical settings beyond leukemia.

Connections between integrins signaling and PKCα have been described, but the shared role that tetraspanins may have in regulating both of these classes of molecules has yet to be discovered. As such, we hypothesize that the link between integrins and PKCα signaling may be mediated in part by tetraspanins. Our studies have provided mechanistic insight regarding how CD82 palmitoylation is critical for sustained PKCα signaling. Work has shown that the β1 integrin subunit can be phosphorylated by PKCα, which may regulate integrin activation. Therefore, we hope that future studies will take a closer examination towards how CD82-mediated changes in PKCα signaling can regulate integrin activation and organization. These studies may provide even more evidence that CD82 and in particular the palmitoylation of CD82 should be targeted in AML.

Appendices

Appendix A: Abbreviations used

ADP – adenosine diphosphate

AML – acute myeloid leukemia

AMIDAS – adjacent to MIDAS

APP – amyloid precursor protein

BMP – bone morphogenic protein

CBF – core binding factor

CAR cells – CXCL12-abundant reticular cells

CCG – Cys-Cys-Gly amino acid motif within large tetraspanin loop

CD82KD-- KG1a knockdown cells created with CD82 shRNA

CD82OE – KG1a overexpressing cells that overexpress CD82 tagged with the mCherry fluorescent protein

CFSE – carboxyfluorescein succinimidyl ester

CFU – colony forming unit

CHO – Chinese hamster ovarian cell line

CLL – chronic lymphocytic leukemia

CMP – common myeloid progenitor

cPKCs – classical protein kinase Cs

CXCL12 – C-X-C motif ligand 12, also known as SDF-1

CXCR4- C-X-C chemokine receptor 4

DAG – diacylglycerol

dSTORM – direct stochastic optical reconstruction microscopy

DBSCAN – density-based spatial clustering of applications with noise

EC domain – Extracellular cadherin domain

EC1 – small extracellular loop of tetraspanins

EC2 – large extracellular loop of tetraspanins

ECM – extracellular matrix

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

ER – endoplasmic reticulum

ERK – extracellular signal-regulated kinases

E-selectin – Endothelial selectin

ETO – eight-twenty-one

FAK – focal adhesion kinase

FLT-3 – FMS-like tyrosine kinase 3

FRET - Förster resonance energy transfer

G-CSF – granulocyte-colony stimulating factor

GM-CSF – granulocyte-macrophage colony-stimulating factor

GPCR – G-protein-coupled receptor

GRAIL– gene related to anergy in lymphocytes

GSC – germline stem cell

GTP – guanosine triphosphate

GVHD – graft-versus-host disease

HA – hyaluronic acid

HGF – hepatocyte growth factor

HLA – human leukocyte antigen

HSPC – hematopoietic stem/progenitor cell

HSC – hematopoietic stem cell

ICAM-1 – intracellular adhesion molecule-1

ILK – integrin linked kinase

ITD – internal tandem duplication

LDV – leucine-aspartic acid-valine sequence

LFA-1 – leukocyte function-associated antigen

Lin (-) – lineage negative

LSC – leukemia stem cell

LT-HSPC – long-term hematopoietic stem/progenitor cell

MAPK – mitogen-activated protein kinase

MARCKS – myristoylated alanine-rich c-kinase receptor substrate

MLL – mixed-lineage leukemia

MIDAS – metal-ion dependent adhesion site

MPL – myeloproliferative leukemia

MPP – multipotent progenitor cell

MSC – mesenchymal stem cell

MSD – mean squared displacement

NCID – Notch intracellular domain

NSG – NOD scid gamma

Palm-CD82OE – KG1a overexpressing cells that overexpress palmitoylation deficient form of CD82 tagged with the mCherry fluorescent protein

PI – phosphatidylinositol

PI3K – phosphoinositide-3-kinase

PI4K – phosphatidylinositol 4-kinase

PI4P – phosphatidylinositol 4-phosphate

PLC-γ – Phospholipase C-γ

PKC – protein kinase C

PML – promyelocytic leukemia

PMA – phorbol 12-myristate 13-acetate

PSI – plexin-semaphorin-integrin

PTB – phosphotyrosine-binding

RACK – receptors for the activated C kinase

RARα – retinoic acid receptor alpha

RDS – retinal degeneration slow

RGD – arginine-glutamine-aspartic acid sequence

RTK – receptor tyrosine kinase

rtPCR – real time polymerase chain reaction

SAPK/JNK – stress-activated protein kinase/Jun amino-terminal kinase

SCF – stem cell factor

SDF-1 – stromal cell-derived factor-1, also known as CXCL12

SNO – N-cadherin+CD45-

SPT – single particle tracking

SRI – super-resolution imaging

STAT5 – signal transducer and activator of transcription 5

Tcf/Lef – T-cell factor/lymphoid enhancer factor

TEM – tetraspanin enriched microdomain

TGF – transforming growth factor

THPO – thrombopoietin

TIRF – total internal reflection fluorescence

TKI – tyrosine kinase inhibitor

TPA – Tetradecanoylphorbol-13-acetate

VAMP – vesicular associated membrane protein

VCAM-1 – vascular cell adhesion molecule-1

Appendix B: Supplemental methods

Real time PCR

Total RNA was isolated using TRIzol Reagent protocol for suspension cells (Life Technologies). cDNA was synthesized using manufacturer protocol for qScript cDNA SuperMix (Quanta Biosciences). Fast SYBR Green Master Mix (Applied Biosystems) was used to prepare the PCR reaction mix. The following primers were used for amplification: Human integrin α4 forward primer: 5'-CCTCCTTGGTCCTCATGTCAT-3', human integrin α4 reverse: 5'-CATGCGCAACATTCTGATCCT-3', human 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. Fold differences in expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Affinity binding assays

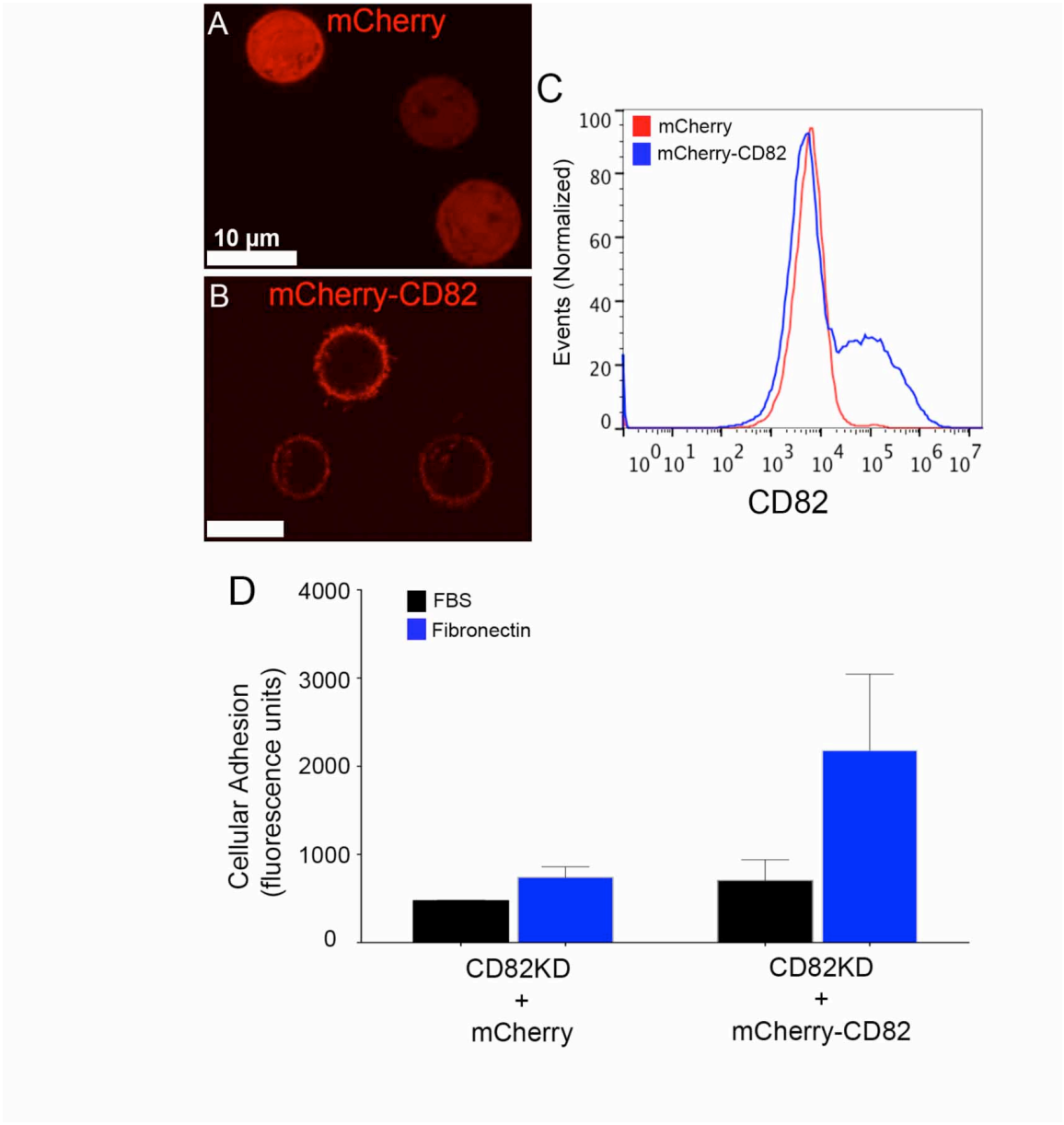
Cells were treated with either 0.1% DMSO or blocked with LDV (1 μM) and incubated for 30 min at 37°C. LDV-FITC (Tocris) at increasing concentrations (0nM, 0.25nM, 0.75nM, 2.5nM, 7.5nM, 25nM, 75nM, and 250nM) was then added in duplicate to eppendorf tubes containing 400 μL blocked or non-blocked cells, and the cells were incubated for an additional 30 min at 37°C with gentle shaking. Following centrifugation and resuspension in 200 μL media, blocked and non-blocked cells were assessed by flow cytometry to assess levels of specific ligand-integrin binding, as measured by mean fluorescence minus baseline (blocked). LDV-FITC concentration was plotted against mean channel fluorescence. The binding affinity was determined from the generated binding curve using the built-in one site specific binding (hyperbola) model in Prism.

Affinity dissociation "off-rate" assays

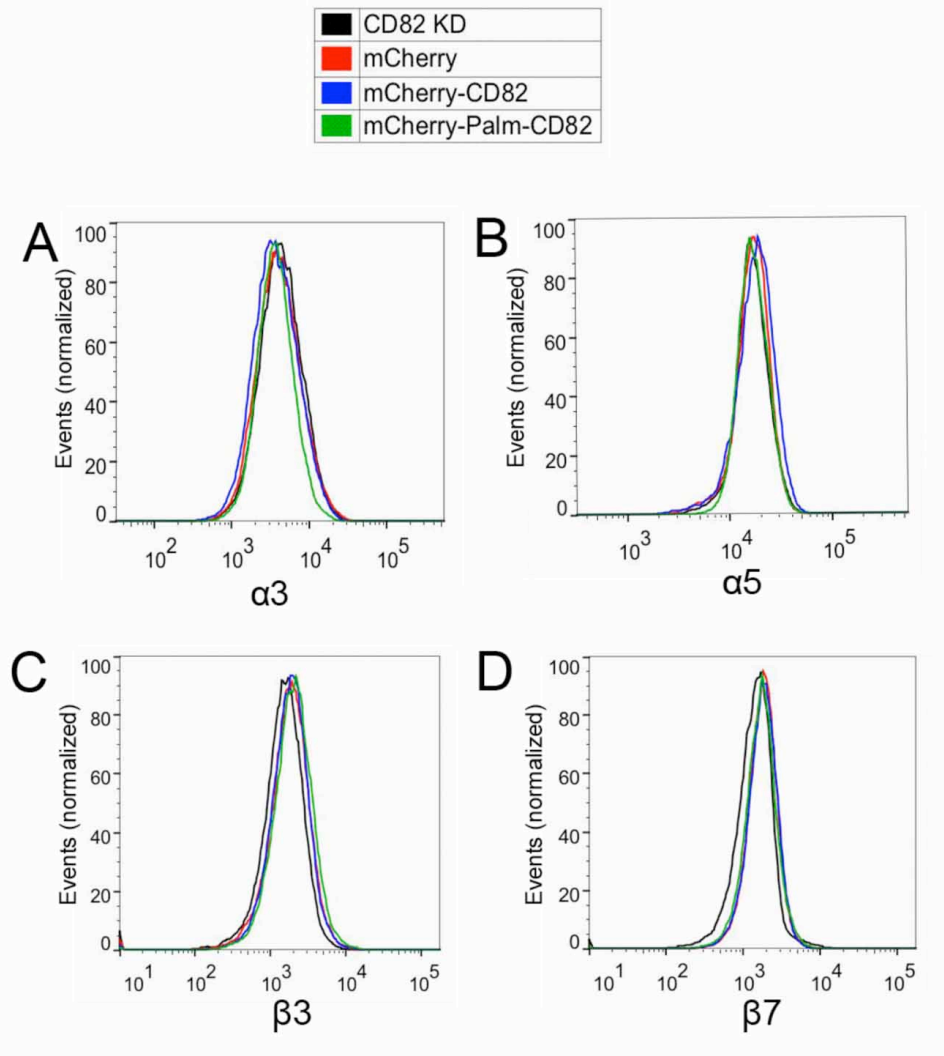
Cells were treated with either DMSO or blocked with LDV in media. A saturating LDV-FITC concentration of 75 nM was then added to 200 μL blocked or non-blocked cells in triplicate. Samples were continuously stirred with a 5x2 mm

magnetic stir bar, and real-time flow cytometry was used to assess the dissociation kinetics or “off-rate” of LDV-FITC upon addition of a saturating, competitive concentration of unlabeled LDV (1 μM), which was added 1 min after starting the measurements. The mean fluorescence readings were collected over a 6-min time period and were baseline-corrected and normalized to 1. The dissociation rate constant, k_{off}, was determined from the nonlinear fit using the dissociation – one phase exponential decay model in Prism.

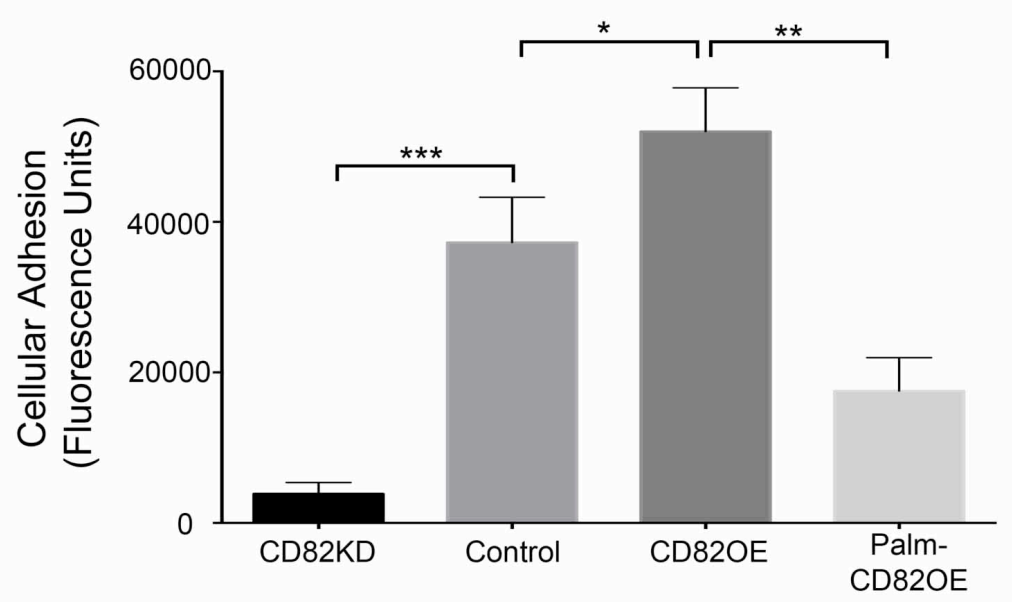
Appendix C: Chapter 2 supplemental figures



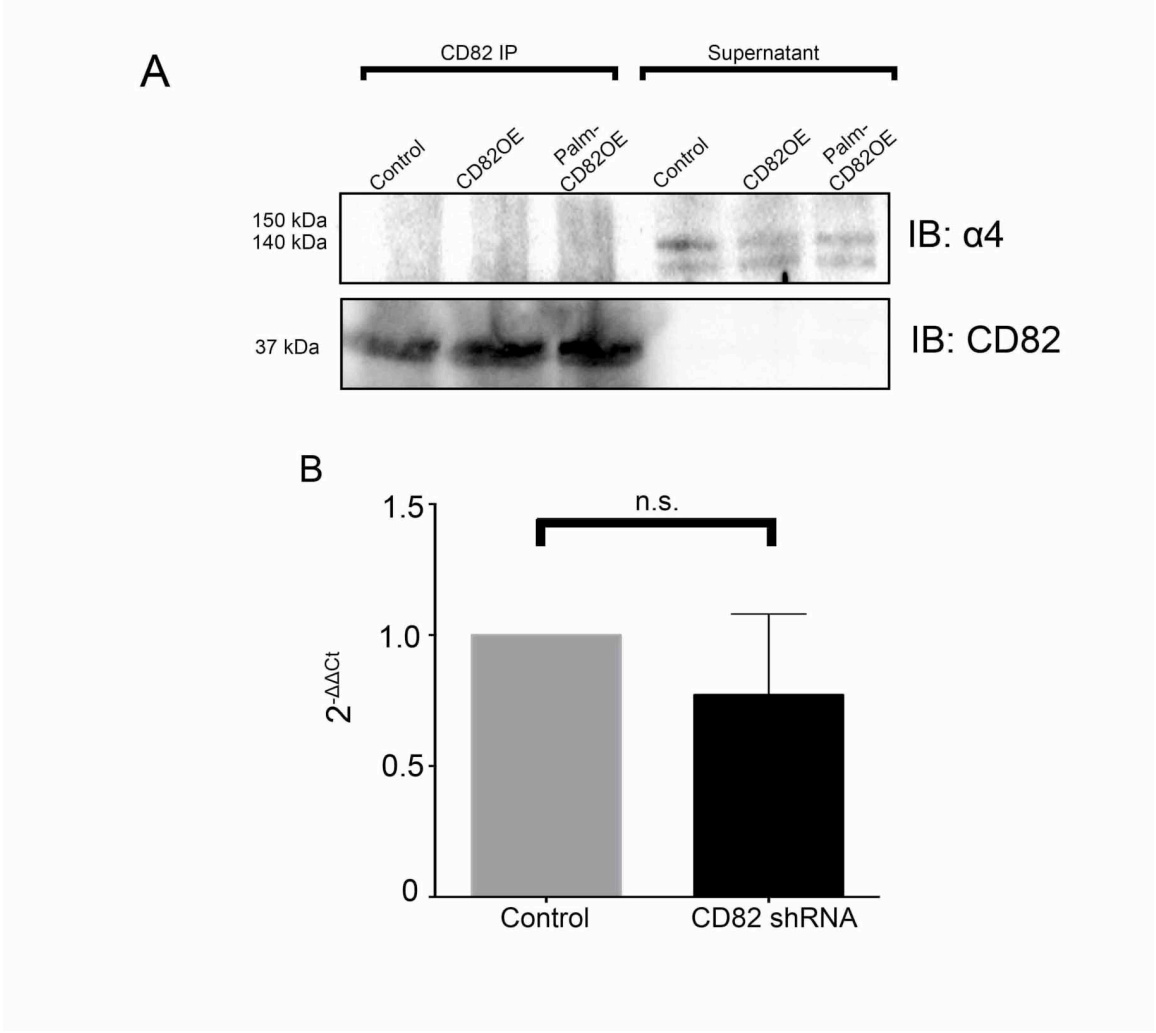
Supplemental figure S.2.1: CD82 re-expression rescues cellular adhesion to fibronectin. KG1a CD82KD cells were transiently transfected with (A) mCherry or (B) mCherry-CD82 constructs. (C) CD82 expression of transiently transfected cells was assessed using flow cytometry. (D) These cells were assessed for cellular adhesion to FBS and fibronectin using a fluorescence-based adhesion assay.



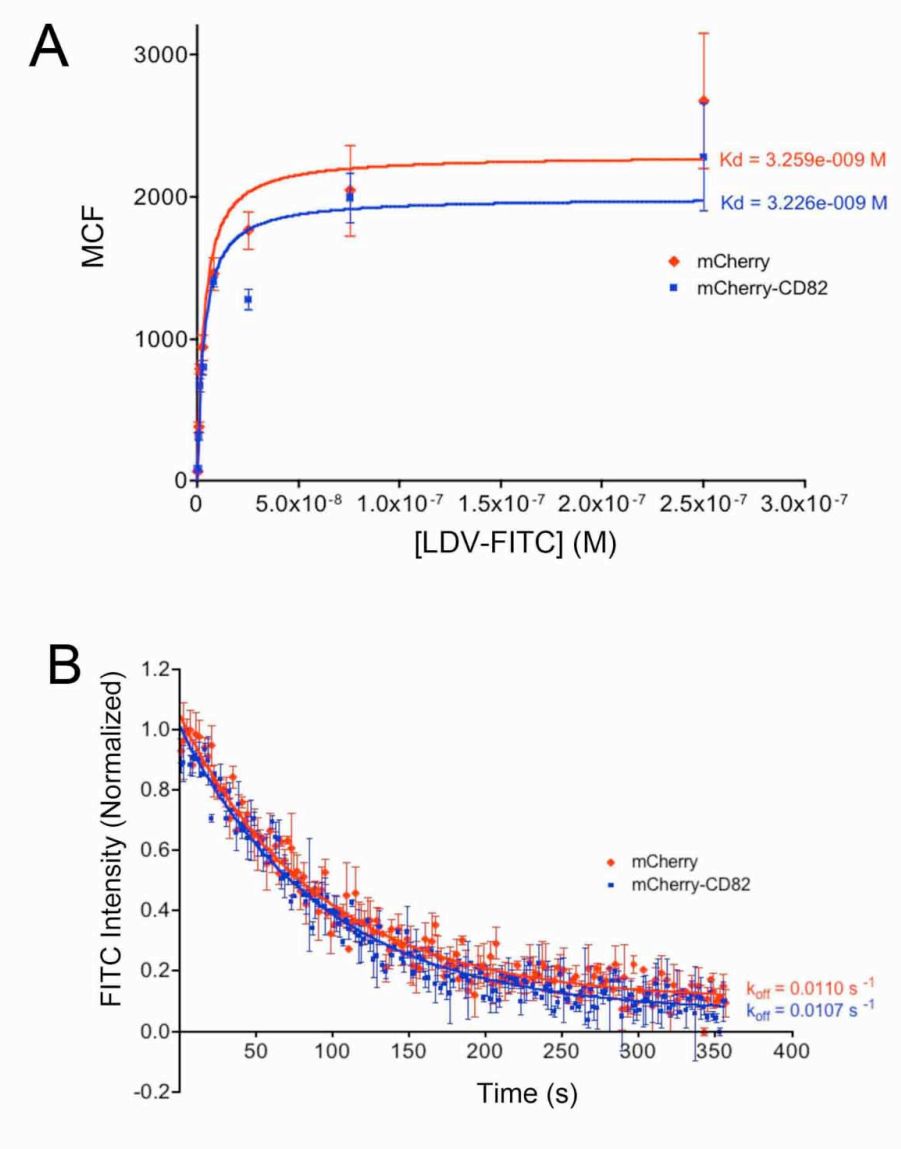
Supplemental figure S.2.2: The effect of CD82 overexpression on integrin surface expression. CD82 KD, control, CD82OE, and Palm-CD82OE cells were examined for surface expression of the (A) α3, (B) α5, (C) β3 and (D) β7 integrin subunits as assessed by flow cytometry analysis.



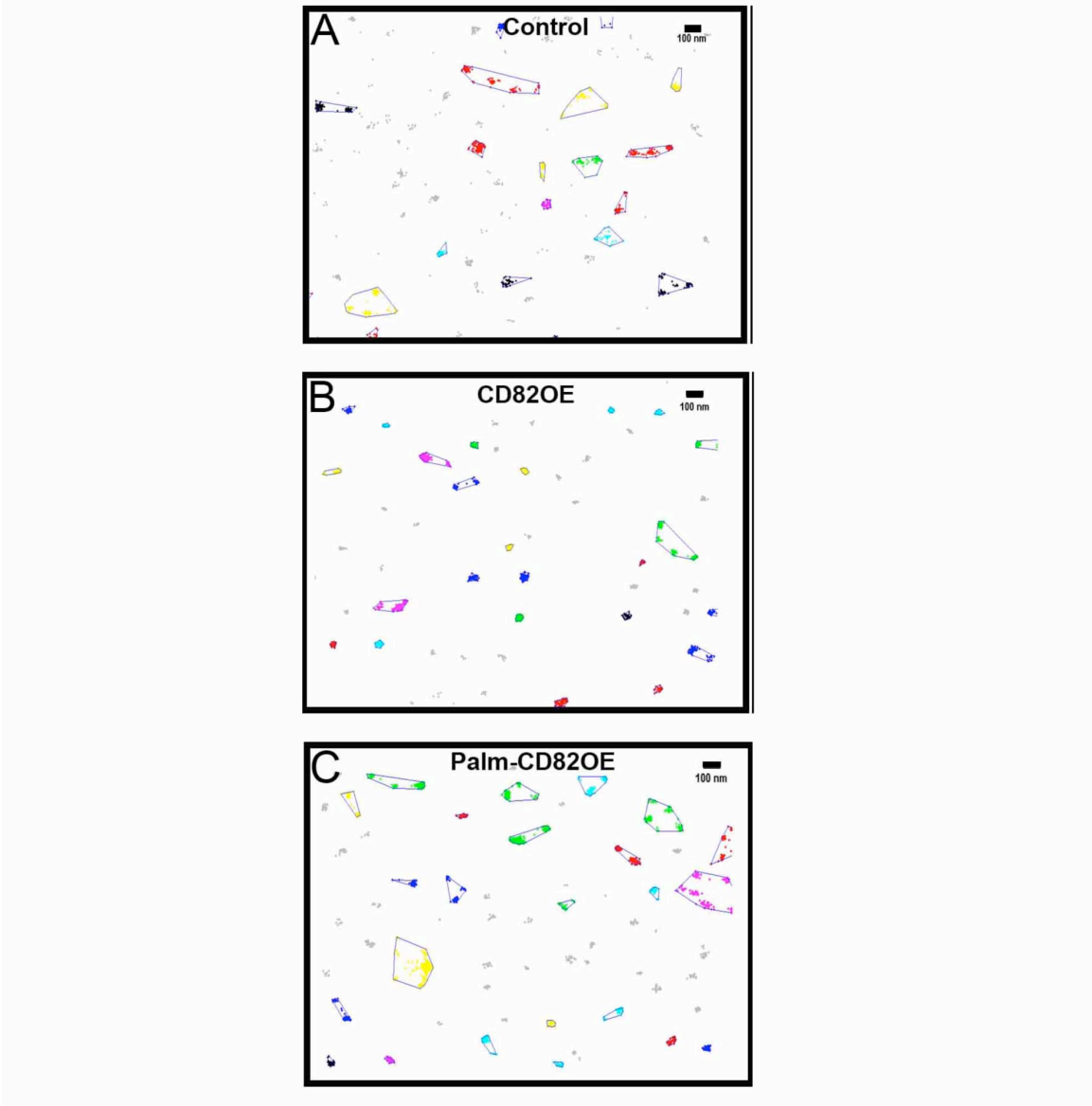
Supplemental figure S.2.3: CD82 Regulates cellular adhesion to VCAM-1. Cellular adhesion of CD82KD, WT, CD82OE and Palm-CD82OE KG1a cells was measured using fluorescence-based adhesion assay. Cells were plated on 10 µg/ml of recombinant VCAM-1.



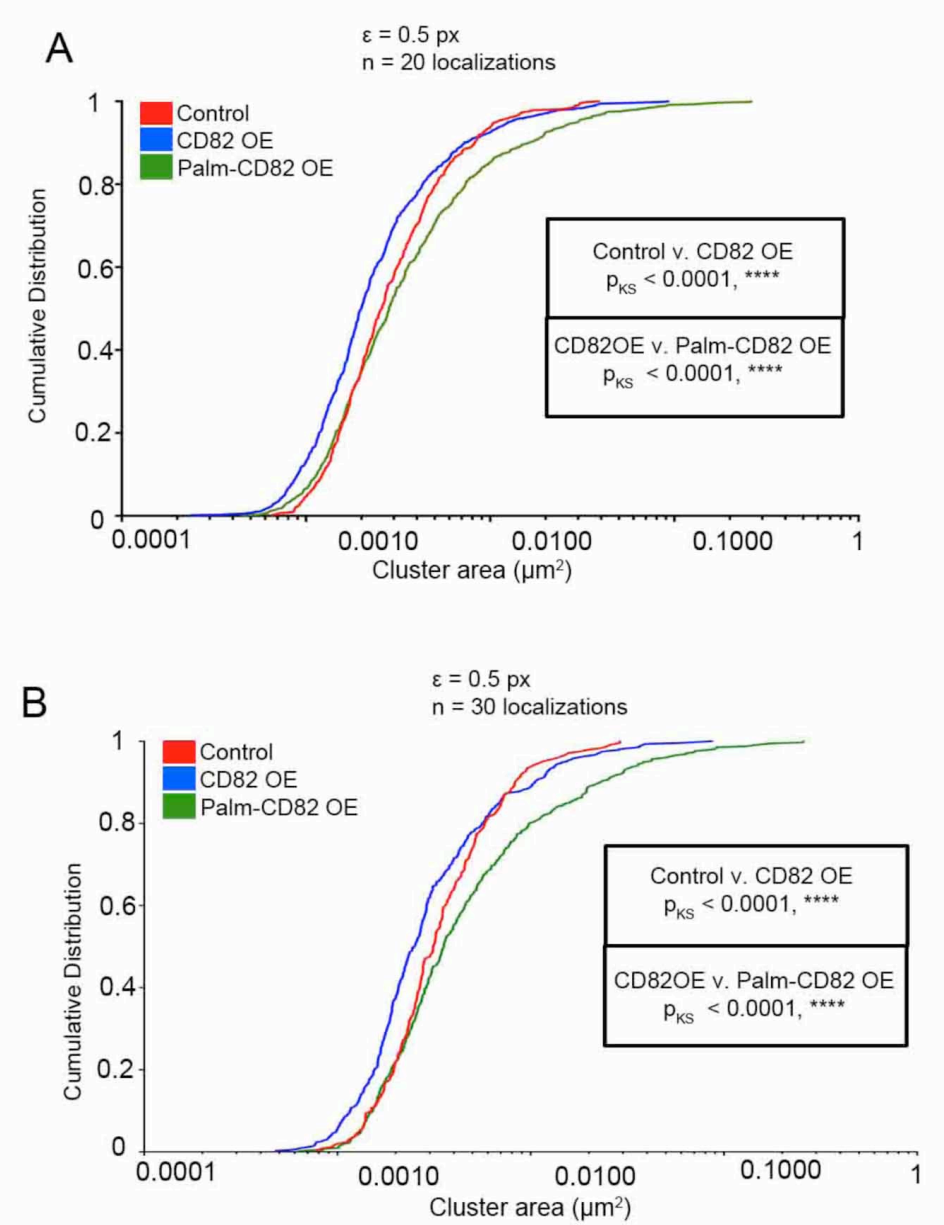
Supplemental figure S.2.4: CD82 regulation of the α4 integrin. (A) CD82 immunoprecipitation was performed with control, CD82OE, and Palm-CD82OE KG1a cells lysed in BRIJ O10. The immunoprecipitant and unbound supernatants were analyzed by Western blot using α4 and CD82 specific antibodies. (B) Real-time PCR was performed for the α4 subunit mRNA levels in control and CD82KD KG1a cells. The formula $2^{-\Delta\Delta Ct}$ indicates the relative gene expression level when compared to control cells as normalized to GAPDH. The data displayed represents the mean ± SD from three independent experiments.



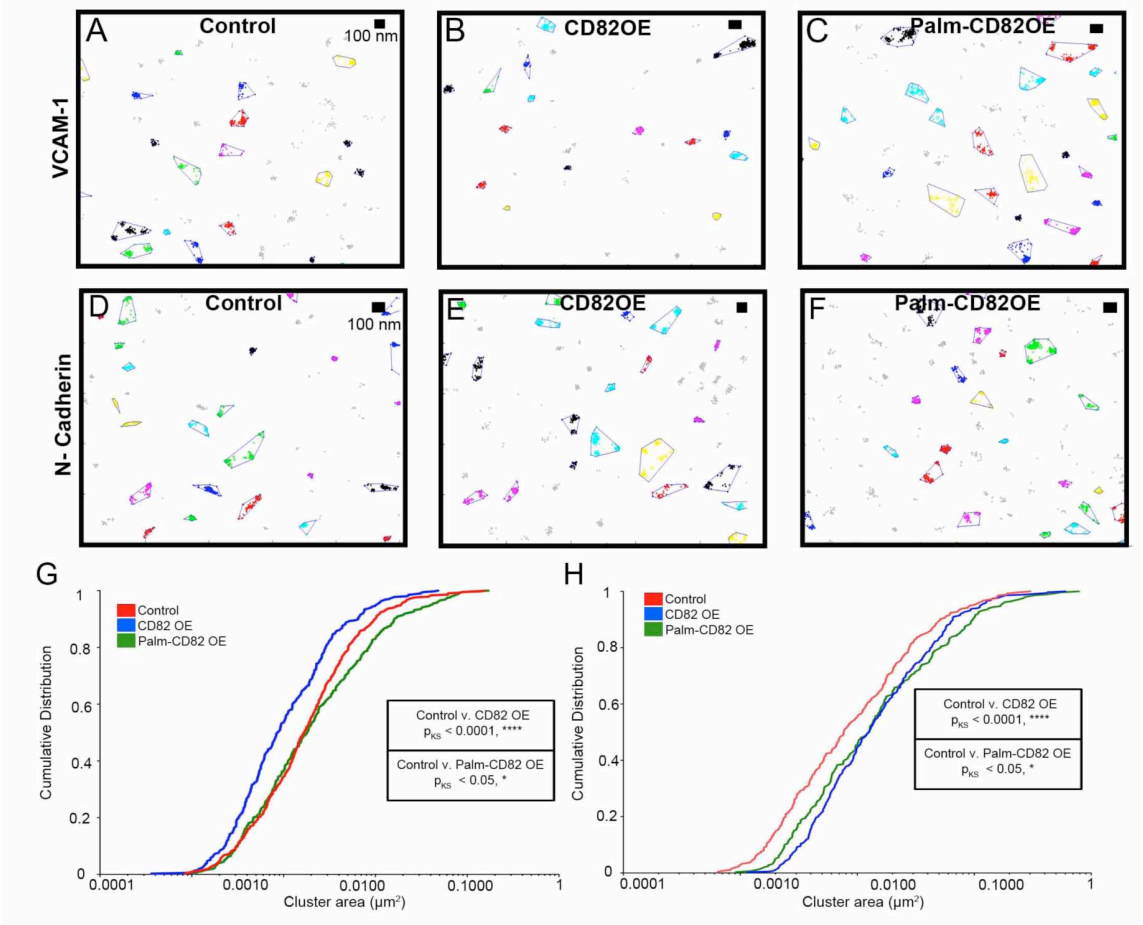
Supplemental figure S.2.5: The effect of CD82 expression on VLA4 affinity. (A) Affinity binding assay in which control and CD82OE cells were treated with either 0.1% DMSO or blocked with the α4β1-specific ligand, LDV (1μM) and then incubated with increasing concentrations of LDV-FITC (0nM – 250 nM). Blocked and nonblocked cells were analyzed by flow cytometry to assess levels of specific ligand-integrin binding, as measured by mean fluorescence minus baseline (blocked). The dissociation constant, K_d, was determined from the nonlinear fit. (B) The cells were treated with either DMSO or blocked with LDV and then incubated with an LDV-FITC concentration of 75nM. Real-time flow cytometry was used to analyze the dissociation kinetics or "off-rate" of LDV-FITC over the six-minute time-course upon addition of a saturating, competitive concentration of unlabeled LDV (1μM) at the 1-minute mark. The mean fluorescence readings were baseline-corrected and normalized to 1. The dissociation rate constant, K_{off}, was determined from the nonlinear fit.



Supplemental figure S.2.6: The DBSCAN algorithm detects small and large scale organization of α4 on the cell surface. The DBSCAN clustering algorithm was used to detect α4 clusters of various sizes on the cell surface of (A) control, (B) CD82OE, and (C) Palm-CD82OE cells plated on fibronectin. The DBSCAN parameters used were ε = 1 px, n = 30 localizations. Colored localizations denote localizations organized into a cluster and grey localizations indicate molecules not organized as they did not meet the DBSCAN parameters.

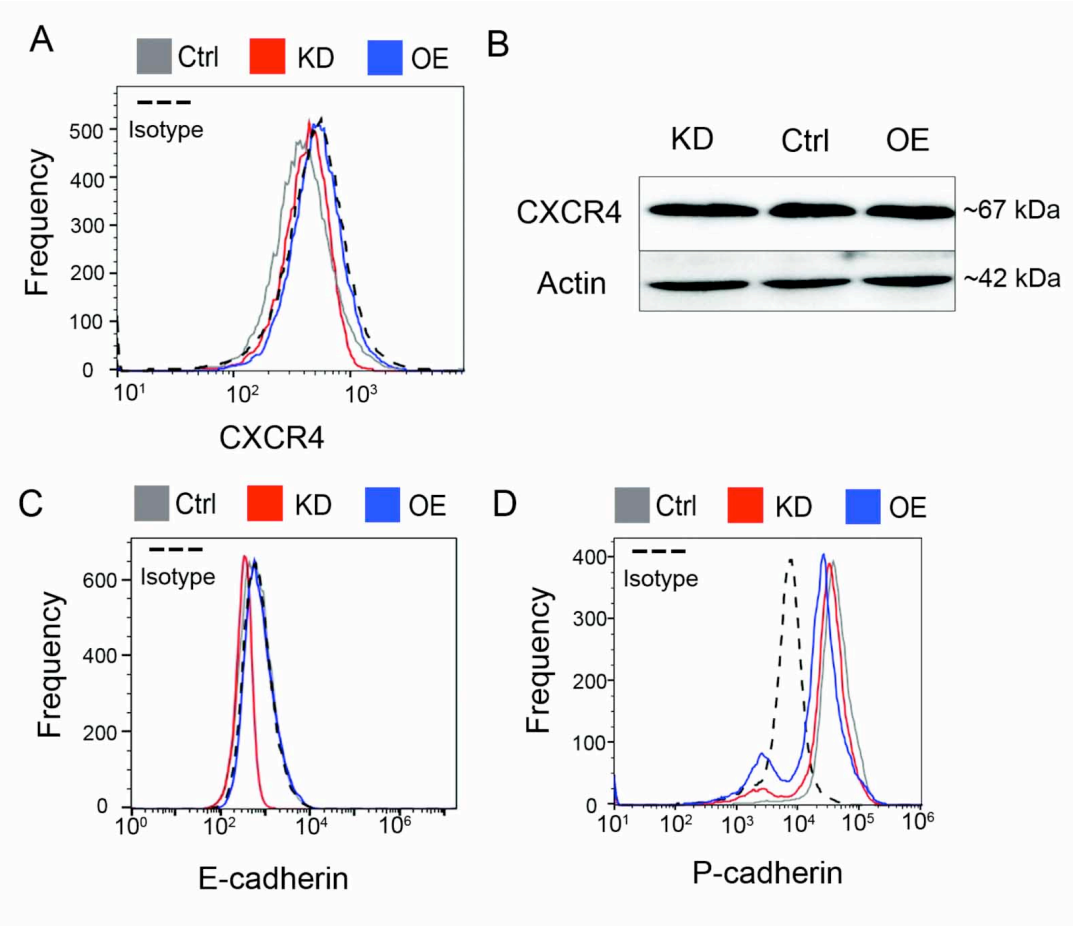


Supplemental figure S.2.7: Alteration of DBSCAN parameters does not change the distribution of clusters found using the DBSCAN algorithm. A 56 x 56 px region of the same four cells was analyzed using modified DBSCAN parameters of (A) ε = 0.5 px, n = 20 localizations and (B) ε = 0.5 px, n = 30 localizations. The clusters obtained were analyzed for their cumulative distribution curve and examined statistically using the Kolmogorov-Smirnov test.



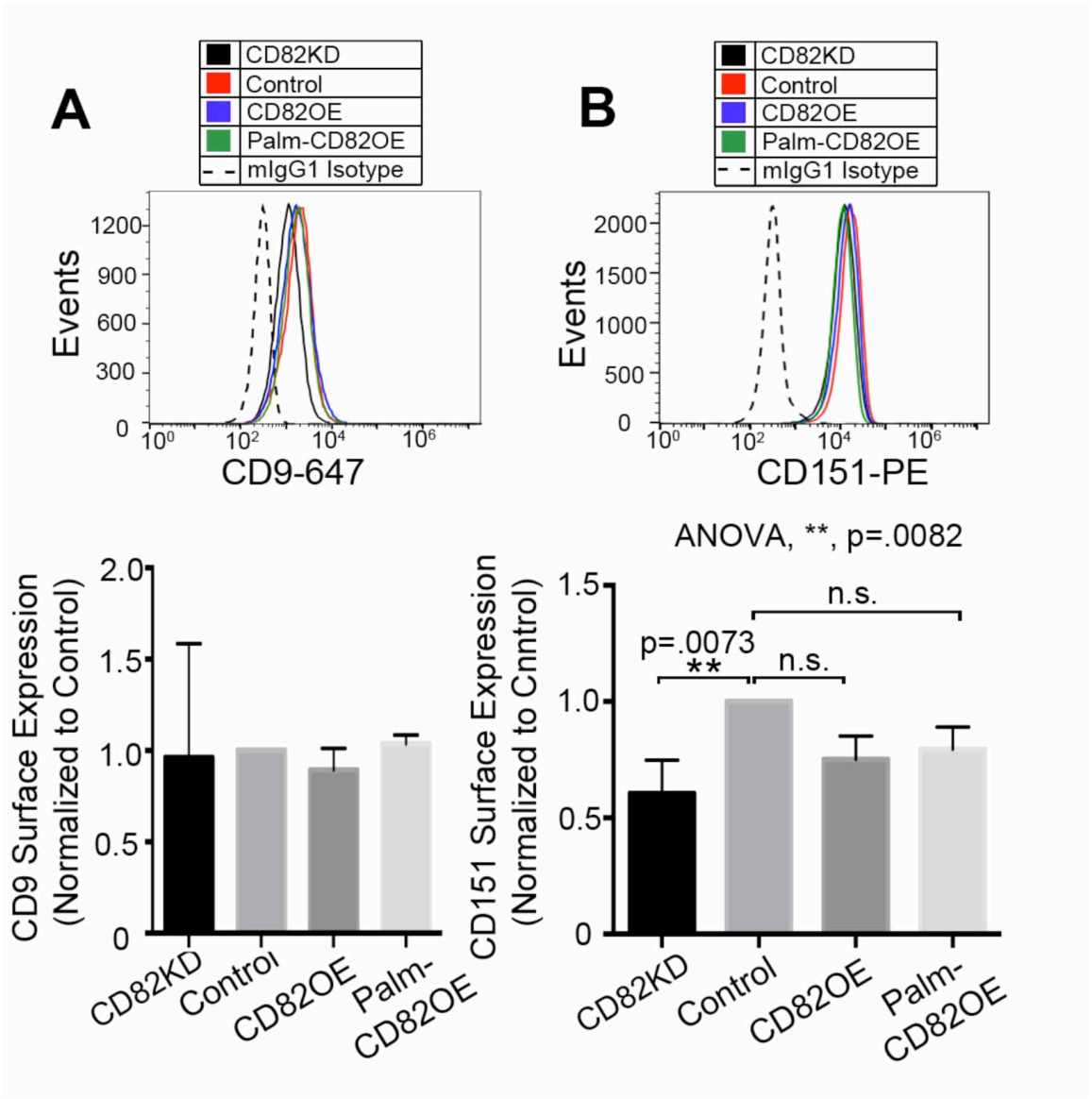
Supplemental figure S.2.8: The DBSCAN algorithm detects clusters of α4 in cells plated on N-cadherin and VCAM-1. The DBSCAN clustering algorithm was used to detect α4 clusters of various sizes on the cell surface of control, CD82OE, and Palm-CD82OE cells plated on VCAM-1 (A-C) and N-cadherin (D-F). The DBSCAN parameters used were ε = 1 px, n = 30 localizations. Colored localizations denote localizations organized into a cluster and grey localizations indicate molecules not organized as they did not meet the DBSCAN parameters. Cumulative distribution plot of the clusters compiled from n = 3 cells of each cell line plated on VCAM-1 (G) and N-cadherin (H), n > 250 clusters. Statistics were determined using the Kolmogorov-Smirnov test.

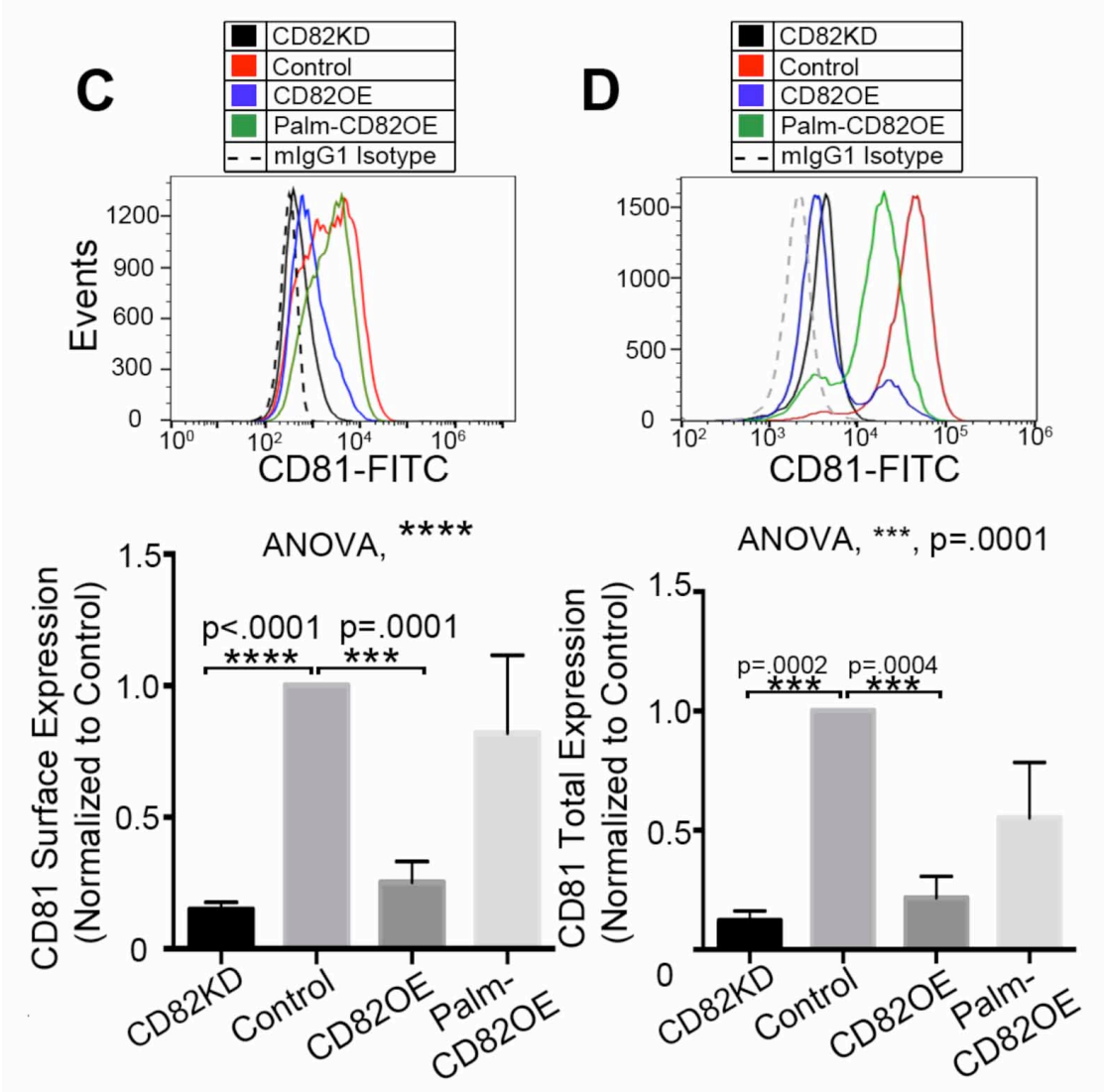
Appendix D: Chapter 3 supplemental figure



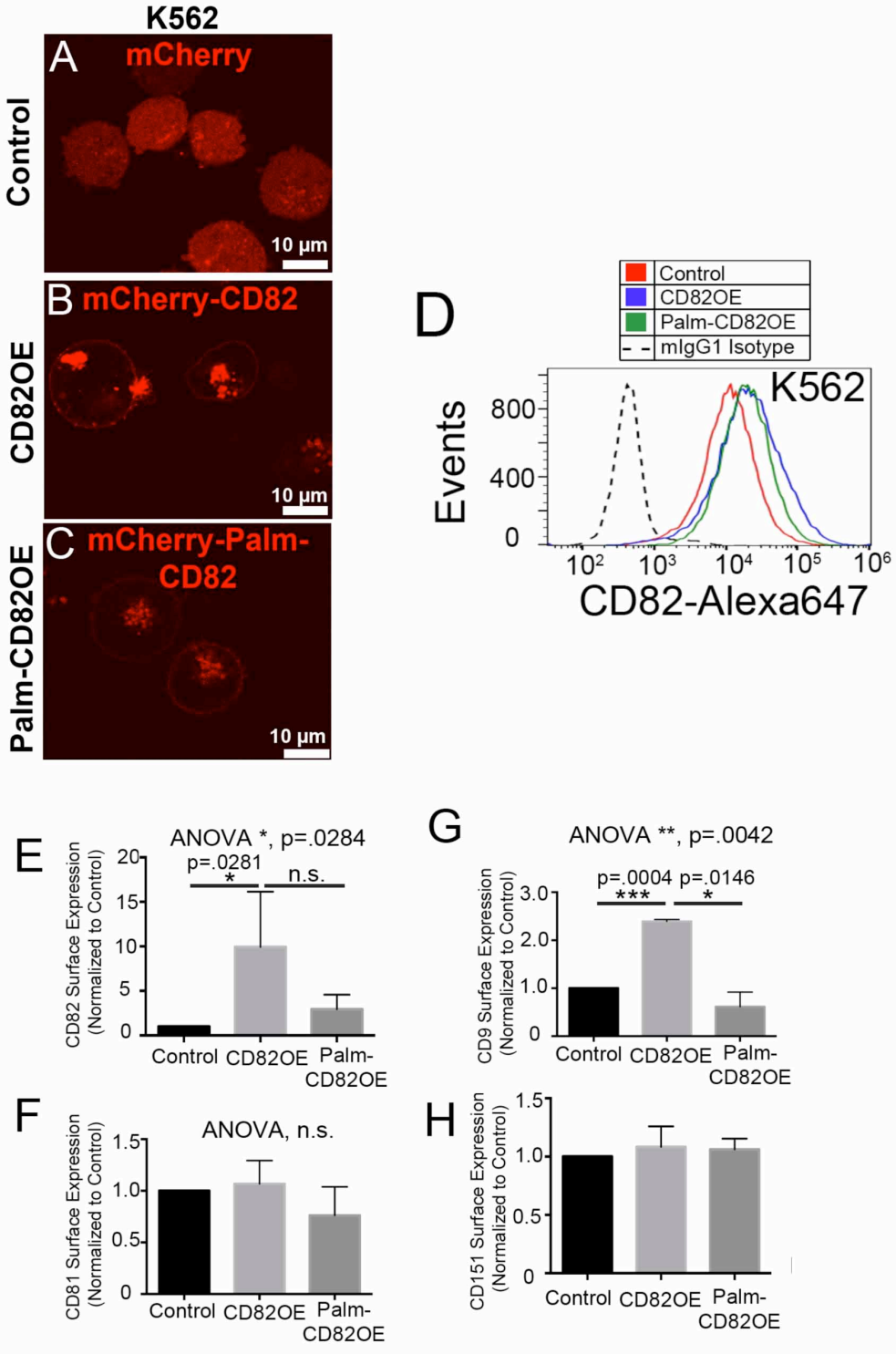
Supplemental Figure S.3.1. Flow cytometry analysis of (A) CXCR4 (Clone 12G5, BD Bioscience), (C) E-cadherin (clone 36/E-cadherin, BD Bioscience) and (D) P-cadherin (clone 56/P-cadherin) surface expression on Ctrl, CD82KD or CD82OE KG1a cells. (B) Western blot analysis of total CXCR4 protein expression (clone Ab-2 (1-14), Calbiochem) on Ctrl, CD82KD or CD82OE KG1a cells with actin (clone AC-74, Sigma) used as the loading control.

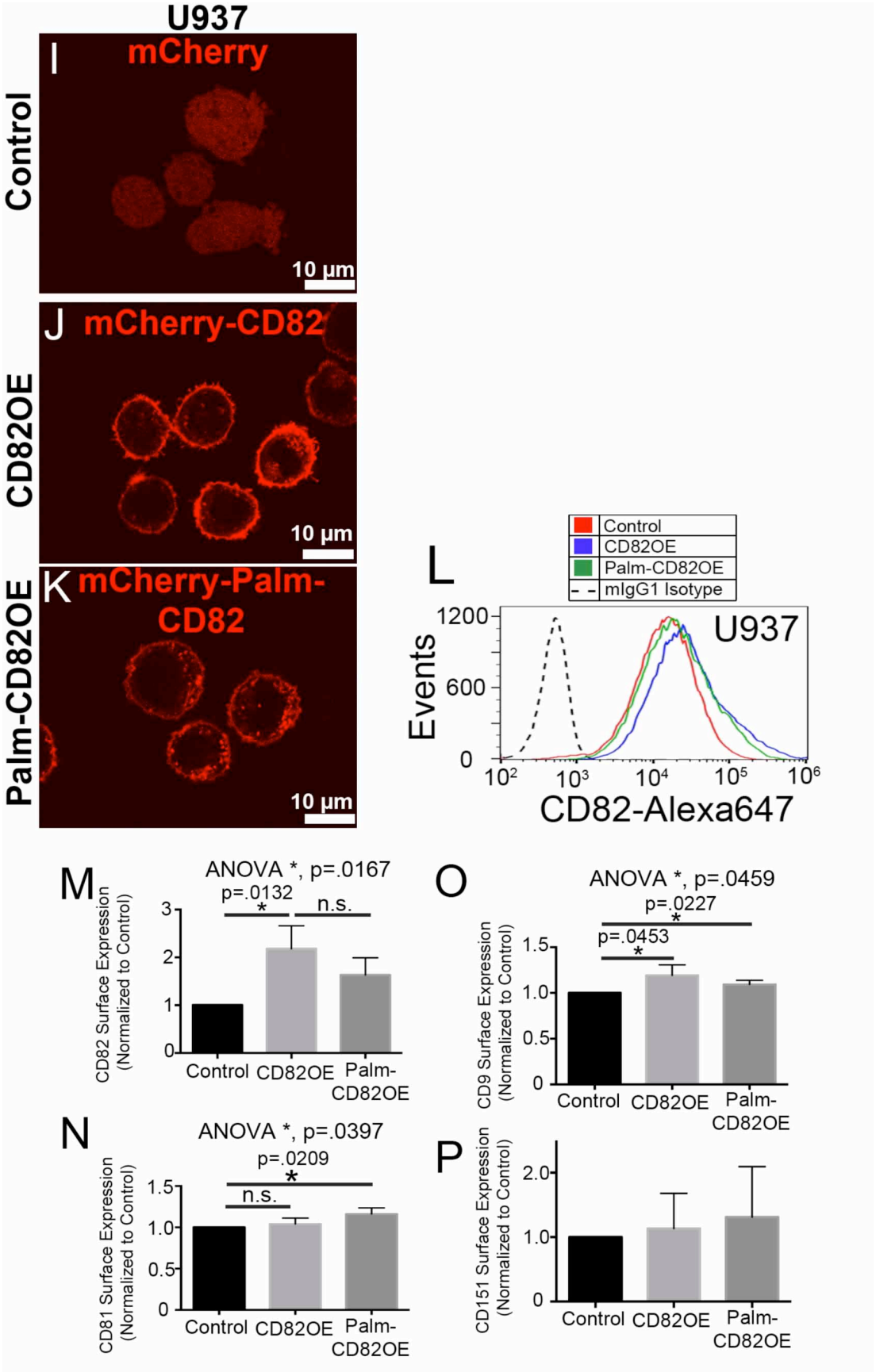
Appendix E: Chapter 4 supplemental figures

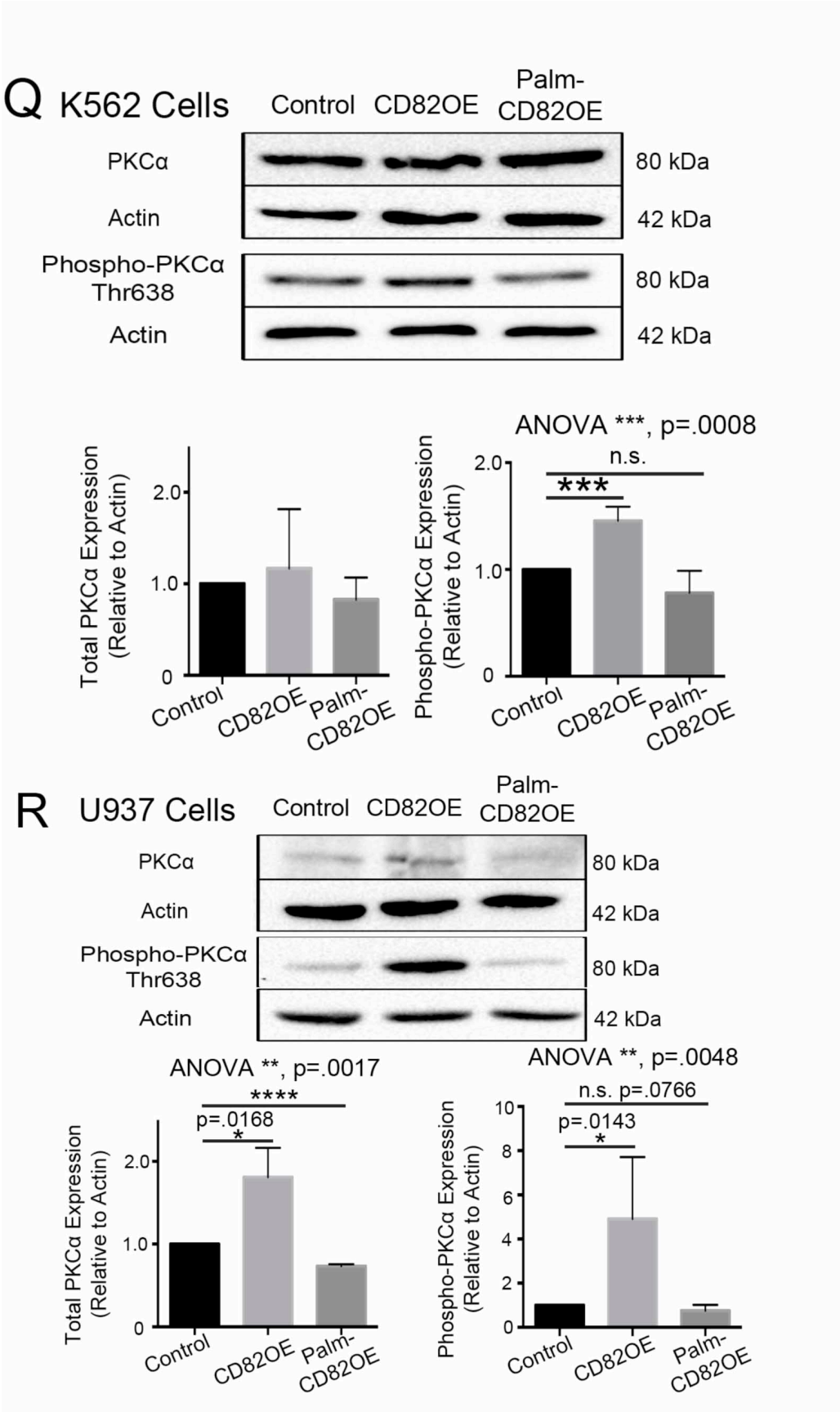


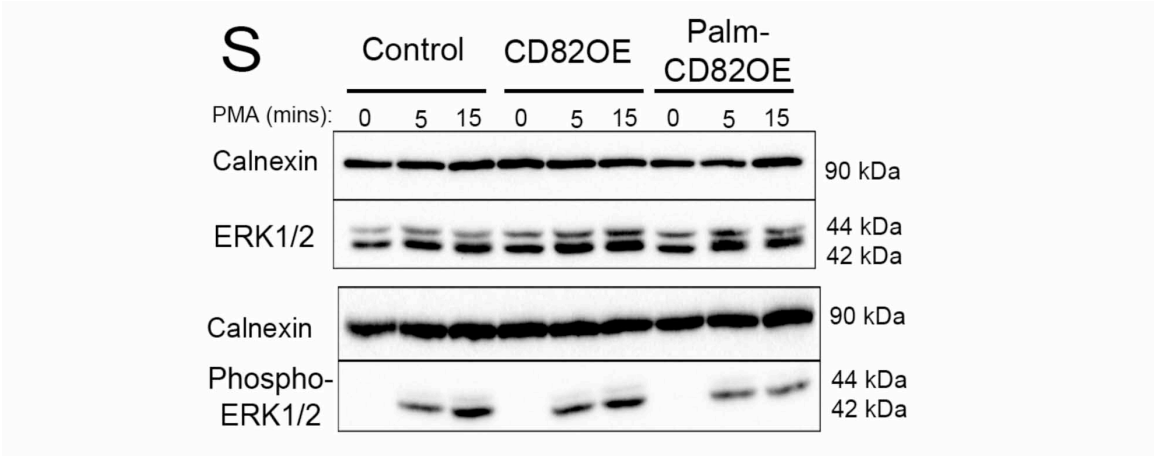


Supplemental figure S.4.1. **CD82 regulates tetraspanin expression levels.** Surface expression of tetraspanins assessed in stable KG1a cells using flow cytometry with antibodies specific to (A) CD9 (AbdSerotec, MM2/57), (B) CD151 (BD Biosciences, 14A2.H1), and (C) CD81 (Biolegend, 5A6). (D) Permeabilized cells were used to assess total CD81 expression. Quantification of normalized mean fluorescence intensity is depicted below histograms (n≥3 independent experiments, error bars denote SD).


K562
A
mCherry
Control
10 µm
B
mCherry-CD82
CD82OE
10 µm
C
mCherry-Palm-CD82
Palm-CD82OE
10 µm
D
Control
CD82OE
Palm-CD82OE
mIgG1 Isotype
K562
Events
800
400
0
10^2
10^3
10^4
10^5
10^6
CD82-Alexa647
E
ANOVA *, p=.0284
p=.0281
*
n.s.
CD82 Surface Expression (Normalized to Control)
20
15
10
5
0
Control
CD82OE
Palm-CD82OE
F
ANOVA, n.s.
CD81 Surface Expression (Normalized to Control)
1.5
1.0
0.5
0
Control
CD82OE
Palm-CD82OE
G
ANOVA **, p=.0042
p=.0004
***
p=.0146
*
CD9 Surface Expression (Normalized to Control)
3.0
2.0
1.0
0
Control
CD82OE
Palm-CD82OE
H
CD151 Surface Expression (Normalized to Control)
1.5
1.0
0.5
0
Control
CD82OE
Palm-CD82OE


U937
I
mCherry
Control
10 µm
J
mCherry-CD82
CD82OE
10 µm
K
mCherry-Palm-CD82
Palm-CD82OE
10 µm
L
Control
CD82OE
Palm-CD82OE
mIgG1 Isotype
U937
Events
1200
600
0
10^2
10^3
10^4
10^5
10^6
CD82-Alexa647
M
ANOVA *, p=.0167
p=.0132
*
n.s.
CD82 Surface Expression (Normalized to Control)
3
2
1
0
Control
CD82OE
Palm-CD82OE
N
ANOVA *, p=.0397
p=.0209
*
n.s.
CD81 Surface Expression (Normalized to Control)
1.5
1.0
0.5
0
Control
CD82OE
Palm-CD82OE
O
ANOVA *, p=.0459
p=.0227
*
p=.0453
*
CD9 Surface Expression (Normalized to Control)
1.5
1.0
0.5
0
Control
CD82OE
Palm-CD82OE
P
CD151 Surface Expression (Normalized to Control)
2.0
1.0
0
Control
CD82OE
Palm-CD82OE


Q K562 Cells
Control
CD82OE
Palm-CD82OE
PKCα
Actin
Phospho-PKCα Thr638
Actin
80 kDa
42 kDa
80 kDa
42 kDa
Total PKCα Expression (Relative to Actin)
2.0
1.0
0
Control
CD82OE
Palm-CD82OE
ANOVA ***, p=.0008
Phospho-PKCα Expression (Relative to Actin)
n.s.
***
2.0
1.0
0
Control
CD82OE
Palm-CD82OE
R U937 Cells
Control
CD82OE
Palm-CD82OE
PKCα
Actin
Phospho-PKCα Thr638
Actin
80 kDa
42 kDa
80 kDa
42 kDa
ANOVA **, p=.0017
****
p=.0168
*
Total PKCα Expression (Relative to Actin)
2.0
1.0
0
Control
CD82OE
Palm-CD82OE
ANOVA **, p=.0048
n.s. p=.0766
p=.0143
*
Phospho-PKCα Expression (Relative to Actin)
10
8
6
4
2
0
Control
CD82OE
Palm-CD82OE



Supplemental figure S.4.2. CD82 regulates PKCα expression and activation in other AML cell lines. (A-C) The mCherry, mCherry-CD82 or mCherry-Palm-CD82 constructs were stably expressed in K562 cells. K562 cells were analyzed for surface expression of (D-E) CD82 (ASL-24), (F) CD81 (5A6), (G) CD9 (MM2/57) and (H) CD151 (14A2.H1) (n≥3 experiments, error bars denote SD). (I-K) The mCherry, mCherry-CD82 or mCherry-Palm-CD82 constructs were stably expressed in U937 cells. U937 cells were analyzed for surface expression of (L-M) CD82 (ASL-24), (N) CD81 (5A6), (O) CD9 (MM2/57) and (P) CD151 (14A2.H1) (n≥3 experiments, error bars denote SD). (Q) Western blot analysis of stable K562 cells and densitometry was performed to quantify total and phospho-PKCα expression levels (n≥3 experiments, error bars denote SD). (R) Western blot analysis of stable U937 cells and densitometry was performed to quantify total and phospho-PKCα expression levels (n≥3 experiments, error bars denote SD). (S) U937 cells were treated with PMA for 0, 5, or 15 mins and analyzed for total and phospho-ERK expression.

REFERENCES

Abdul-Nabi, A.M., E.R. Yassin, N. Varghese, H. Deshmukh, and N.R. Yaseen. 2010. In vitro transformation of primary human CD34+ cells by AML fusion oncogenes: early gene expression profiling reveals possible drug target in AML. *PloS one*. 5:e12464.

Abe, M., T. Sugiura, M. Takahashi, K. Ishii, M. Shimoda, and K. Shirasuna. 2008. A novel function of CD82/KAI-1 on E-cadherin-mediated homophilic cellular adhesion of cancer cells. *Cancer Lett*. 266:163-170.

Aiuti, A., I.J. Webb, C. Bleul, T. Springer, and J.C. Gutierrez-Ramos. 1997. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *The Journal of experimental medicine*. 185:111-120.

Akashi, K., D. Traver, T. Miyamoto, and I.L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 404:193-197.

Albers, T., M. Maniak, E. Beitz, and J. von Bulow. 2016. The C Isoform of Dictyostelium Tetraspanins Localizes to the Contractile Vacuole and Contributes to Resistance against Osmotic Stress. *PloS one*. 11:e0162065.

Alharbi, R.A., R. Pettengell, H.S. Pandha, and R. Morgan. 2013. The role of HOX genes in normal hematopoiesis and acute leukemia. *Leukemia*. 27:1000-1008.

Alkan, S., Q. Huang, M. Ergin, M.F. Denning, S. Nand, T. Maududi, G.P. Paner, F. Ozpuyan, and K.F. Izban. 2005. Survival role of protein kinase C (PKC) in chronic lymphocytic leukemia and determination of isoform expression pattern and genes altered by PKC inhibition. *Am J Hematol*. 79:97-106.

Altin, J.G., and E.K. Sloan. 1997. The role of CD45 and CD45-associated molecules in T cell activation. *Immunol Cell Biol*. 75:430-445.

Anasetti, C., D. Amos, P.G. Beatty, F.R. Appelbaum, W. Bensinger, C.D. Buckner, R. Clift, K. Doney, P.J. Martin, E. Mickelson, and et al. 1989. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med*. 320:197-204.

Angst, B.D., C. Marcozzi, and A.I. Magee. 2001. The cadherin superfamily: diversity in form and function. *Journal of cell science*. 114:629-641.

Anzai, N., Y. Lee, B.S. Youn, S. Fukuda, Y.J. Kim, C. Mantel, M. Akashi, and H.E. Broxmeyer. 2002. C-kit associated with the transmembrane 4

superfamily proteins constitutes a functionally distinct subunit in human hematopoietic progenitors. *Blood*. 99:4413-4421.

Appelbaum, F.R. 2003. The current status of hematopoietic cell transplantation. *Annu Rev Med*. 54:491-512.

Appelbaum, F.R., J.M. Rowe, J. Radich, and J.E. Dick. 2001. Acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program*:62-86.

Ara, T., K. Tokoyoda, T. Sugiyama, T. Egawa, K. Kawabata, and T. Nagasawa. 2003. Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. *Immunity*. 19:257-267.

Arai, F., A. Hirao, M. Ohmura, H. Sato, S. Matsuoka, K. Takubo, K. Ito, G.Y. Koh, and T. Suda. 2004. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell*. 118:149-161.

Arnaout, M.A., S.L. Goodman, and J.P. Xiong. 2002. Coming to grips with integrin binding to ligands. *Curr Opin Cell Biol*. 14:641-651.

Arnaud, M.P., A. Vallee, G. Robert, J. Bonneau, C. Leroy, N. Varin-Blank, A.G. Rio, M.B. Troadec, M.D. Galibert, and V. Gandemer. 2015. CD9, a key actor in the dissemination of lymphoblastic leukemia, modulating CXCR4-mediated migration via RAC1 signaling. *Blood*. 126:1802-1812.

Arnold, M., E.A. Cavalcanti-Adam, R. Glass, J. Blummel, W. Eck, M. Kantlehner, H. Kessler, and J.P. Spatz. 2004. Activation of integrin function by nanopatterned adhesive interfaces. *Chemphyschem*. 5:383-388.

Arroyo, A.G., J.T. Yang, H. Rayburn, and R.O. Hynes. 1999. Alpha4 integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors in vivo. *Immunity*. 11:555-566.

Austin, T.W., G.P. Solar, F.C. Ziegler, L. Liem, and W. Matthews. 1997. A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. *Blood*. 89:3624-3635.

Avigdor, A., P. Goichberg, S. Shivtiel, A. Dar, A. Peled, S. Samira, O. Kollet, R. Hershkoviz, R. Alon, I. Hardan, H. Ben-Hur, D. Naor, A. Nagler, and T. Lapidot. 2004. CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. *Blood*. 103:2981-2989.

Baba, Y., K.P. Garrett, and P.W. Kincade. 2005. Constitutively active beta-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors. *Immunity*. 23:599-609.

Baba, Y., T. Yokota, H. Spits, K.P. Garrett, S. Hayashi, and P.W. Kincade. 2006. Constitutively active beta-catenin promotes expansion of multipotent hematopoietic progenitors in culture. *J Immunol*. 177:2294-2303.

Baddeley, D., I.D. Jayasinghe, L. Lam, S. Rossberger, M.B. Cannell, and C. Soeller. 2009. Optical single-channel resolution imaging of the ryanodine receptor distribution in rat cardiac myocytes. *Proceedings of the National Academy of Sciences of the United States of America*. 106:22275-22280.

Balaban, N.Q., U.S. Schwarz, D. Riveline, P. Goichberg, G. Tzur, I. Sabanay, D. Mahalu, S. Safran, A. Bershadsky, L. Addadi, and B. Geiger. 2001. Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. *Nat Cell Biol*. 3:466-472.

Ballen, K.K., E. Gluckman, and H.E. Broxmeyer. 2013. Umbilical cord blood transplantation: the first 25 years and beyond. *Blood*. 122:491-498.

Barczyk, M., S. Carracedo, and D. Gullberg. 2010. Integrins. *Cell Tissue Res*. 339:269-280.

Barragan, M., B. Bellosillo, C. Campas, D. Colomer, G. Pons, and J. Gil. 2002. Involvement of protein kinase C and phosphatidylinositol 3-kinase pathways in the survival of B-cell chronic lymphocytic leukemia cells. *Blood*. 99:2969-2976.

Barreiro, O., M. Yanez-Mo, M. Sala-Valdes, M.D. Gutierrez-Lopez, S. Ovalle, A. Higginbottom, P.N. Monk, C. Cabanas, and F. Sanchez-Madrid. 2005. Endothelial tetraspanin microdomains regulate leukocyte firm adhesion during extravasation. *Blood*. 105:2852-2861.

Barreiro, O., M. Zamai, M. Yanez-Mo, E. Tejera, P. Lopez-Romero, P.N. Monk, E. Gratton, V.R. Caiolfa, and F. Sanchez-Madrid. 2008. Endothelial adhesion receptors are recruited to adherent leukocytes by inclusion in preformed tetraspanin nanoplatforms. *J Cell Biol*. 183:527-542.

Baselga, J., J. Mendelsohn, Y.M. Kim, and A. Pandiella. 1996. Autocrine regulation of membrane transforming growth factor-alpha cleavage. *The Journal of biological chemistry*. 271:3279-3284.

Bassani, S., and L.A. Cingolani. 2012. Tetraspanins: Interactions and interplay with integrins. *Int J Biochem Cell Biol*. 44:703-708.

Baum, C.M., I.L. Weissman, A.S. Tsukamoto, A.M. Buckle, and B. Peault. 1992. Isolation of a candidate human hematopoietic stem-cell population. *Proceedings of the National Academy of Sciences of the United States of America*. 89:2804-2808.

Beckwith, K.A., J.C. Byrd, and N. Muthusamy. 2015. Tetraspanins as therapeutic targets in hematological malignancy: a concise review. *Frontiers in physiology*. 6:91.

Bendall, L.J., K. Kortlepel, and D.J. Gottlieb. 1993. Human acute myeloid leukemia cells bind to bone marrow stroma via a combination of beta-1 and beta-2 integrin mechanisms. *Blood*. 82:3125-3132.

Berditchevski, F. 2001. Complexes of tetraspanins with integrins: more than meets the eye. *Journal of cell science*. 114:4143-4151.

Berditchevski, F., and E. Odintsova. 1999. Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. *J Cell Biol*. 146:477-492.

Berditchevski, F., and E. Odintsova. 2007. Tetraspanins as regulators of protein trafficking. *Traffic*. 8:89-96.

Berditchevski, F., E. Odintsova, S. Sawada, and E. Gilbert. 2002. Expression of the palmitoylation-deficient CD151 weakens the association of alpha 3 beta 1 integrin with the tetraspanin-enriched microdomains and affects integrin-dependent signaling. *The Journal of biological chemistry*. 277:36991-37000.

Berditchevski, F., K.F. Tolias, K. Wong, C.L. Carpenter, and M.E. Hemler. 1997. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *The Journal of biological chemistry*. 272:2595-2598.

Berditchevski, F., M.M. Zutter, and M.E. Hemler. 1996. Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins). *Molecular biology of the cell*. 7:193-207.

Bergin, D.A., C.M. Greene, E.E. Sterchi, C. Kenna, P. Geraghty, A. Belaaouaj, C.C. Taggart, S.J. O'Neill, and N.G. McElvaney. 2008. Activation of the epidermal growth factor receptor (EGFR) by a novel metalloprotease pathway. *The Journal of biological chemistry*. 283:31736-31744.

Birchmeier, C., W. Birchmeier, E. Gherardi, and G.F. Vande Woude. 2003. Met, metastasis, motility and more. *Nature reviews. Molecular cell biology*. 4:915-925.

Bishop, A.L., and A. Hall. 2000. Rho GTPases and their effector proteins. *The Biochemical journal*. 348 Pt 2:241-255.

Blaskovic, S., M. Blanc, and F.G. van der Goot. 2013. What does S-palmitoylation do to membrane proteins? *FEBS J*. 280:2766-2774.

Blume-Jensen, P., and T. Hunter. 2001. Oncogenic kinase signalling. *Nature*. 411:355-365.

Boavida, L.C., P. Qin, M. Broz, J.D. Becker, and S. McCormick. 2013. Arabidopsis tetraspanins are confined to discrete expression domains and cell types in reproductive tissues and form homo- and heterodimers when expressed in yeast. *Plant Physiol*. 163:696-712.

Bocchietto, E., A. Guglielmetti, F. Silvagno, G. Taraboletti, G.P. Pescarmona, A. Mantovani, and F. Bussolino. 1993. Proliferative and migratory responses of murine microvascular endothelial cells to granulocyte-colony-stimulating factor. *J Cell Physiol*. 155:89-95.

Boissel, N., H. Leroy, B. Brethon, N. Philippe, S. de Botton, A. Auvrignon, E. Raffoux, T. Leblanc, X. Thomas, O. Hermine, B. Quesnel, A. Baruchel, G. Leverger, H. Dombret, C. Preudhomme, A. Acute Leukemia French, and G. Leucemies Aigues Myeloblastiques de l'Enfant Cooperative. 2006. Incidence and prognostic impact of c-Kit, FLT3, and Ras gene mutations in core binding factor acute myeloid leukemia (CBF-AML). *Leukemia*. 20:965-970.

Bonardi, F., F. Fusetti, P. Deelen, D. van Gosliga, E. Vellenga, and J.J. Schuringa. 2013. A proteomics and transcriptomics approach to identify leukemic stem cell (LSC) markers. *Molecular & cellular proteomics : MCP*. 12:626-637.

Bonig, H., D. Chudziak, G. Priestley, and T. Papayannopoulou. 2009a. Insights into the biology of mobilized hematopoietic stem/progenitor cells through innovative treatment schedules of the CXCR4 antagonist AMD3100. *Exp Hematol*. 37:402-415 e401.

Bonig, H., K.L. Watts, K.H. Chang, H.P. Kiem, and T. Papayannopoulou. 2009b. Concurrent blockade of alpha4-integrin and CXCR4 in hematopoietic stem/progenitor cell mobilization. *Stem cells*. 27:836-837.

Bonnet, D., and J.E. Dick. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 3:730-737.

Bonny, M., X. Hui, J. Schweizer, L. Kaestner, A. Zeug, K. Kruse, and P. Lipp. 2016. C2-domain mediated nano-cluster formation increases calcium signaling efficiency. *Sci Rep*. 6:36028.

Boots, A.W., M. Hristova, D.I. Kasahara, G.R. Haenen, A. Bast, and A. van der Vliet. 2009. ATP-mediated activation of the NADPH oxidase DUOX1 mediates airway epithelial responses to bacterial stimuli. *The Journal of biological chemistry*. 284:17858-17867.

Bos, J.L., M. Verlaan-de Vries, A.J. van der Eb, J.W. Janssen, R. Delwel, B. Lowenberg, and L.P. Colly. 1987. Mutations in N-ras predominate in acute myeloid leukemia. *Blood*. 69:1237-1241.

Boucheix, C., and E. Rubinstein. 2001. Tetraspanins. *Cellular and molecular life sciences : CMLS*. 58:1189-1205.

Bradstock, K.F., and D.J. Gottlieb. 1995. Interaction of acute leukemia cells with the bone marrow microenvironment: implications for control of minimal residual disease. *Leukemia & lymphoma*. 18:1-16.

Breshears, L.M., P.M. Schlievert, and M.L. Peterson. 2012. A disintegrin and metalloproteinase 17 (ADAM17) and epidermal growth factor receptor (EGFR) signaling drive the epithelial response to Staphylococcus aureus toxic shock syndrome toxin-1 (TSST-1). *The Journal of biological chemistry*. 287:32578-32587.

Bromberg, O., B.J. Frisch, J.M. Weber, R.L. Porter, R. Civitelli, and L.M. Calvi. 2012. Osteoblastic N-cadherin is not required for microenvironmental support and regulation of hematopoietic stem and progenitor cells. *Blood*. 120:303-313.

Bunse, S., S. Garg, S. Junek, D. Vogel, N. Ansari, E.H. Stelzer, and E. Schuman. 2013. Role of N-cadherin cis and trans interfaces in the dynamics of adherens junctions in living cells. *PloS one*. 8:e81517.

Burbach, B.J., R.B. Medeiros, K.L. Mueller, and Y. Shimizu. 2007. T-cell receptor signaling to integrins. *Immunological reviews*. 218:65-81.

Burchert, A., M. Notter, H. Dietrich Menssen, S. Schwartz, W. Knauf, A. Neubauer, and E. Thiel. 1999. CD82 (KAI1), a member of the tetraspan family, is expressed on early haemopoietic progenitor cells and up-regulated in distinct human leukaemias. *Br J Haematol*. 107:494-504.

Burger, J.A., A. Spoo, A. Dwenger, M. Burger, and D. Behringer. 2003. CXCR4 chemokine receptors (CD184) and alpha4beta1 integrins mediate spontaneous migration of human CD34+ progenitors and acute myeloid leukaemia cells beneath marrow stromal cells (pseudoemperipolesis). *Br J Haematol*. 122:579-589.

Burns, L.J., D.J. Weisdorf, T.E. DeFor, T.L. Repka, K.M. Ogle, C. Hummer, and J.S. Miller. 2000. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with interleukin 2 plus granulocyte colony-stimulating factor in patients with advanced breast cancer. *Exp Hematol*. 28:96-103.

Burroughs, L., M. Mielcarek, M.T. Little, G. Bridger, R. Macfarland, S. Fricker, J. Labrecque, B.M. Sandmaier, and R. Storb. 2005. Durable engraftment of

AMD3100-mobilized autologous and allogeneic peripheral-blood mononuclear cells in a canine transplantation model. *Blood*. 106:4002-4008.

Burtness, B.A., A. Psyrri, M. Rose, E. D'Andrea, C. Staugaard-Hahn, M. Henderson-Bakas, M.B. Clark, S. Mechanic, D. Krause, E. Snyder, R.B. Cooper, J. Abrantes, R. Corringham, A. Deisseroth, and D.L. Cooper. 1999. A phase I study of paclitaxel for mobilization of peripheral blood progenitor cells. *Bone Marrow Transplant*. 23:311-315.

Butler, J.M., D.J. Nolan, E.L. Vertes, B. Varnum-Finney, H. Kobayashi, A.T. Hooper, M. Seandel, K. Shido, I.A. White, M. Kobayashi, L. Witte, C. May, C. Shawber, Y. Kimura, J. Kitajewski, Z. Rosenwaks, I.D. Bernstein, and S. Rafii. 2010. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell*. 6:251-264.

Calderwood, D.A., Y. Fujioka, J.M. de Pereda, B. Garcia-Alvarez, T. Nakamoto, B. Margolis, C.J. McGlade, R.C. Liddington, and M.H. Ginsberg. 2003. Integrin beta cytoplasmic domain interactions with phosphotyrosine-binding domains: a structural prototype for diversity in integrin signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 100:2272-2277.

Calvi, L.M., G.B. Adams, K.W. Weibrecht, J.M. Weber, D.P. Olson, M.C. Knight, R.P. Martin, E. Schipani, P. Divieti, F.R. Bringhurst, L.A. Milner, H.M. Kronenberg, and D.T. Scadden. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*. 425:841-846.

Calvi, L.M., and D.C. Link. 2015. The hematopoietic stem cell niche in homeostasis and disease. *Blood*. 126:2443-2451.

Campbell, I.D., and M.J. Humphries. 2011. Integrin structure, activation, and interactions. *Cold Spring Harb Perspect Biol*. 3.

Carloni, V., A. Mazzocca, and K.S. Ravichandran. 2004. Tetraspanin CD81 is linked to ERK/MAPKinase signaling by Shc in liver tumor cells. *Oncogene*. 23:1566-1574.

Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *The Journal of biological chemistry*. 257:7847-7851.

Caswell, P.T., S. Vadrevu, and J.C. Norman. 2009. Integrins: masters and slaves of endocytic transport. *Nature reviews. Molecular cell biology*. 10:843-853.

Catlin, S.N., L. Busque, R.E. Gale, P. Guttorp, and J.L. Abkowitz. 2011. The replication rate of human hematopoietic stem cells in vivo. *Blood*. 117:4460-4466.

Cavallaro, U., and G. Christofori. 2004. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nature reviews. Cancer*. 4:118-132.

Cebecauer, M., M. Spitaler, A. Serge, and A.I. Magee. 2010. Signalling complexes and clusters: functional advantages and methodological hurdles. *Journal of cell science*. 123:309-320.

Chang, J.H., J.C. Pratt, S. Sawasdikosol, R. Kapeller, and S.J. Burakoff. 1998. The small GTP-binding protein Rho potentiates AP-1 transcription in T cells. *Mol Cell Biol*. 18:4986-4993.

Charrin, S., S. Jouannet, C. Boucheix, and E. Rubinstein. 2014. Tetraspanins at a glance. *Journal of cell science*. 127:3641-3648.

Charrin, S., F. le Naour, O. Silvie, P.E. Milhiet, C. Boucheix, and E. Rubinstein. 2009a. Lateral organization of membrane proteins: tetraspanins spin their web. *The Biochemical journal*. 420:133-154.

Charrin, S., F. le Naour, O. Silvie, P.E. Milhiet, C. Boucheix, and E. Rubinstein. 2009b. Lateral organization of membrane proteins: tetraspanins spin their web. *The Biochemical journal*. 420:133-154.

Charrin, S., S. Manie, M. Oualid, M. Billard, C. Boucheix, and E. Rubinstein. 2002. Differential stability of tetraspanin/tetraspanin interactions: role of palmitoylation. *FEBS Lett*. 516:139-144.

Chattopadhyay, N., Z. Wang, L.K. Ashman, S.M. Brady-Kalnay, and J.A. Kreidberg. 2003. alpha3beta1 integrin-CD151, a component of the cadherin-catenin complex, regulates PTPmu expression and cell-cell adhesion. *J Cell Biol*. 163:1351-1362.

Cherukuri, A., R.H. Carter, S. Brooks, W. Bornmann, R. Finn, C.S. Dowd, and S.K. Pierce. 2004. B cell signaling is regulated by induced palmitoylation of CD81. *The Journal of biological chemistry*. 279:31973-31982.

Chigaev, A., A. Waller, G.J. Zwartz, T. Buranda, and L.A. Sklar. 2007. Regulation of cell adhesion by affinity and conformational unbending of alpha4beta1 integrin. *J Immunol*. 178:6828-6839.

Chitaev, N.A., and S.M. Troyanovsky. 1998. Adhesive but not lateral E-cadherin complexes require calcium and catenins for their formation. *The Journal of cell biology*. 142:837-846.

Choi, C.K., M. Vicente-Manzanares, J. Zareno, L.A. Whitmore, A. Mogilner, and A.R. Horwitz. 2008. Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat Cell Biol*. 10:1039-1050.

Choi, U.J., B.K. Jee, Y. Lim, and K.H. Lee. 2009. KAI1/CD82 decreases Rac1 expression and cell proliferation through PI3K/Akt/mTOR pathway in H1299 lung carcinoma cells. *Cell Biochem Funct*. 2009:1.

Civin, C.I., and S.D. Gore. 1993. Antigenic analysis of hematopoiesis: a review. *J Hematother*. 2:137-144.

Clark, K.L., A. Oelke, M.E. Johnson, K.D. Eilert, P.C. Simpson, and S.C. Todd. 2004. CD81 associates with 14-3-3 in a redox-regulated palmitoylation-dependent manner. *The Journal of biological chemistry*. 279:19401-19406.

Clayton, E.L., S. Minogue, and M.G. Waugh. 2013. Mammalian phosphatidylinositol 4-kinases as modulators of membrane trafficking and lipid signaling networks. *Prog Lipid Res*. 52:294-304.

Clemens, M.J., I. Trayner, and J. Menaya. 1992. The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. *Journal of cell science*. 103 (Pt 4):881-887.

Clerk, A., and P.H. Sugden. 2001. Untangling the Web: specific signaling from PKC isoforms to MAPK cascades. *Circ Res*. 89:847-849.

Cluzel, C., F. Saltel, J. Lussi, F. Paulhe, B.A. Imhof, and B. Wehrle-Haller. 2005. The mechanisms and dynamics of (alpha)v(beta)3 integrin clustering in living cells. *J Cell Biol*. 171:383-392.

Collins, R.N. 2003. "Getting it on"--GDI displacement and small GTPase membrane recruitment. *Mol Cell*. 12:1064-1066.

Colmone, A., M. Amorim, A.L. Pontier, S. Wang, E. Jablonski, and D.A. Sipkins. 2008. Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science*. 322:1861-1865.

Conley, S.M., M.W. Stuck, and M.I. Naash. 2012. Structural and functional relationships between photoreceptor tetraspanins and other superfamily members. *Cellular and molecular life sciences : CMLS*. 69:1035-1047.

Coombs, C.C., M.S. Tallman, and R.L. Levine. 2016. Molecular therapy for acute myeloid leukaemia. *Nat Rev Clin Oncol*. 13:305-318.

Corbacioglu, S., M. Kilic, M.A. Westhoff, D. Reinhardt, S. Fulda, and K.M. Debatin. 2006. Newly identified c-KIT receptor tyrosine kinase ITD in

childhood AML induces ligand-independent growth and is responsive to a synergistic effect of imatinib and rapamycin. *Blood*. 108:3504-3513.

Corral, J., I. Lavenir, H. Impey, A.J. Warren, A. Forster, T.A. Larson, S. Bell, A.N. McKenzie, G. King, and T.H. Rabbitts. 1996. An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: a method to create fusion oncogenes. *Cell*. 85:853-861.

Coulombel, L., I. Auffray, M.H. Gaugler, and M. Rosemblatt. 1997. Expression and function of integrins on hematopoietic progenitor cells. *Acta Haematol*. 97:13-21.

Coussens, L., P.J. Parker, L. Rhee, T.L. Yang-Feng, E. Chen, M.D. Waterfield, U. Francke, and A. Ullrich. 1986. Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. *Science*. 233:859-866.

Craddock, C.F., B. Nakamoto, R.G. Andrews, G.V. Priestley, and T. Papayannopoulou. 1997. Antibodies to VLA4 integrin mobilize long-term repopulating cells and augment cytokine-induced mobilization in primates and mice. *Blood*. 90:4779-4788.

Crowe, D.T., H. Chiu, S. Fong, and I.L. Weissman. 1994. Regulation of the avidity of integrin alpha 4 beta 7 by the beta 7 cytoplasmic domain. *The Journal of biological chemistry*. 269:14411-14418.

Danglot, L., M. Chaineau, M. Dahan, M.C. Gendron, N. Boggetto, F. Perez, and T. Galli. 2010. Role of TI-VAMP and CD82 in EGFR cell-surface dynamics and signaling. *Journal of cell science*. 123:723-735.

Das, M., S. Subbayya Ithychanda, J. Qin, and E.F. Plow. 2014. Mechanisms of talin-dependent integrin signaling and crosstalk. *Biochim Biophys Acta*. 1838:579-588.

De Toni, F., C. Racaud-Sultan, G. Chicanne, V.M. Mas, C. Cariven, F. Mesange, J.P. Salles, C. Demur, M. Allouche, B. Payrastre, S. Manenti, and L. Ysebaert. 2006. A crosstalk between the Wnt and the adhesion-dependent signaling pathways governs the chemosensitivity of acute myeloid leukemia. *Oncogene*. 25:3113-3122.

De Waele, M., W. Renmans, K. Jochmans, R. Schots, P. Lacor, F. Trullemans, J. Otten, N. Balduck, K. Vander Gucht, B. Van Camp, and I. Van Riet. 1999. Different expression of adhesion molecules on CD34+ cells in AML and B-lineage ALL and their normal bone marrow counterparts. *Eur J Haematol*. 63:192-201.

de Winde, C.M., M. Zuidscherwoude, A. Vasaturo, A. van der Schaaf, C.G. Figdor, and A.B. van Spriel. 2015. Multispectral imaging reveals the tissue

distribution of tetraspanins in human lymphoid organs. *Histochem Cell Biol*. 144:133-146.

Deaglio, S., S. Aydin, T. Vaisitti, L. Bergui, and F. Malavasi. 2008. CD38 at the junction between prognostic marker and therapeutic target. *Trends Mol Med*. 14:210-218.

Delandre, C., T.R. Penabaz, A.L. Passarelli, S.K. Chapes, and R.J. Clem. 2009. Mutation of juxtamembrane cysteines in the tetraspanin CD81 affects palmitoylation and alters interaction with other proteins at the cell surface. *Exp Cell Res*. 315:1953-1963.

Dempsey, E.C., A.C. Newton, D. Mochly-Rosen, A.P. Fields, M.E. Reyland, P.A. Insel, and R.O. Messing. 2000. Protein kinase C isozymes and the regulation of diverse cell responses. *Am J Physiol Lung Cell Mol Physiol*. 279:L429-438.

Devine, S.M., N. Flomenberg, D.H. Vesole, J. Liesveld, D. Weisdorf, K. Badel, G. Calandra, and J.F. DiPersio. 2004. Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. *J Clin Oncol*. 22:1095-1102.

Devine, S.M., R. Vij, M. Rettig, L. Todt, K. McGlauchlen, N. Fisher, H. Devine, D.C. Link, G. Calandra, G. Bridger, P. Westervelt, and J.F. Dipersio. 2008. Rapid mobilization of functional donor hematopoietic cells without G-CSF using AMD3100, an antagonist of the CXCR4/SDF-1 interaction. *Blood*. 112:990-998.

Ding, L., T.L. Saunders, G. Enikolopov, and S.J. Morrison. 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*. 481:457-462.

Dohner, H., D.J. Weisdorf, and C.D. Bloomfield. 2015. Acute Myeloid Leukemia. *N Engl J Med*. 373:1136-1152.

Dominguez-Jimenez, C., P. Sanchez-Aparicio, J.P. Albar, and A. Garcia-Pardo. 1996. The alpha 4 beta 1 fibronectin ligands CS-1, Hep II, and RGD induce different intracellular events in B lymphoid cells. Comparison with the effects of the endothelial ligand VCAM-1. *Cell adhesion and communication*. 4:251-267.

Dong, J.T., P.W. Lamb, C.W. Rinker-Schaeffer, J. Vukanovic, T. Ichikawa, J.T. Isaacs, and J.C. Barrett. 1995. KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. *Science*. 268:884-886.

Dornier, E., F. Coumailleau, J.F. Ottavi, J. Moretti, C. Boucheix, P. Mauduit, F. Schweisguth, and E. Rubinstein. 2012. TspanC8 tetraspanins regulate

ADAM10/Kuzbanian trafficking and promote Notch activation in flies and mammals. *J Cell Biol*. 199:481-496.

Dosil, M., S. Wang, and I.R. Lemischka. 1993. Mitogenic signalling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. *Mol Cell Biol*. 13:6572-6585.

Downing, J.R., M. Higuchi, N. Lenny, and A.E. Yeoh. 2000. Alterations of the AML1 transcription factor in human leukemia. *Semin Cell Dev Biol*. 11:347-360.

Drexler, H.G. 1996. Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. *Leukemia*. 10:588-599.

Dwivedi, C., M. Dixit, and R.E. Hardy. 1988. Plasma sialyltransferase as a tumor marker. *Cancer detection and prevention*. 11:191-196.

Eisenberg, S., A.J. Laude, A.J. Beckett, C.J. Mageean, V. Aran, M. Hernandez-Valladares, Y.I. Henis, and I.A. Prior. 2013. The role of palmitoylation in regulating Ras localization and function. *Biochemical Society transactions*. 41:79-83.

Elad-Sfadia, G., R. Haklai, E. Ballan, H.J. Gabius, and Y. Kloog. 2002. Galectin-1 augments Ras activation and diverts Ras signals to Raf-1 at the expense of phosphoinositide 3-kinase. *The Journal of biological chemistry*. 277:37169-37175.

Emsley, J., C.G. Knight, R.W. Farndale, M.J. Barnes, and R.C. Liddington. 2000. Structural basis of collagen recognition by integrin alpha2beta1. *Cell*. 101:47-56.

Ester, M., H. Kreigel, J. Sander, and X. Xu. 1996a. A Density-Based Algorithm for Discovering Clusters in Large Spatial Databases with Noise. *Proceedings of 2nd International Conference on Knowledge Discovery and Data Mining*:226-231.

Ester, M., H. Kreigel, J. Sander, and X. Xu. 1996b. A Density-Based Algorithm for Discovering Clusters in Large Spatial Databases with Noise. *In* 2nd International Conference on Knowledge Discovery and Data Mining. 226-231.

Ester, M., H.-P. Kreigel, J. Sander, and X. Xu. 1996c. A density-based algorithm for discovering clusters in large spatial databases with noise. *In* Proceedings of the 2nd International Conference on Knowledge Discovery and Data Mining. AAAI Press, Menlo Park, CA. 226–231.

Estey, E., and H. Dohner. 2006. Acute myeloid leukaemia. *Lancet*. 368:1894-1907.

Fan, H., and R. Derynck. 1999. Ectodomain shedding of TGF-alpha and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. *EMBO J*. 18:6962-6972.

Farr, C.J., R.K. Saiki, H.A. Erlich, F. McCormick, and C.J. Marshall. 1988. Analysis of RAS gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes. *Proceedings of the National Academy of Sciences of the United States of America*. 85:1629-1633.

Feigelson, S.W., V. Grabovsky, R. Shamri, S. Levy, and R. Alon. 2003. The CD81 tetraspanin facilitates instantaneous leukocyte VLA-4 adhesion strengthening to vascular cell adhesion molecule 1 (VCAM-1) under shear flow. *The Journal of biological chemistry*. 278:51203-51212.

Feigelson, S.W., V. Grabovsky, E. Winter, L.L. Chen, R.B. Pepinsky, T. Yednock, D. Yablonski, R. Lobb, and R. Alon. 2001. The Src kinase p56(lck) up-regulates VLA-4 integrin affinity. Implications for rapid spontaneous and chemokine-triggered T cell adhesion to VCAM-1 and fibronectin. *The Journal of biological chemistry*. 276:13891-13901.

Fernandez, A., F. De Arriba, J. Rivera, I. Heras, V. Vicente, and M.L. Lozano. 2008. Successful mobilization of hematopoietic peripheral blood progenitor cells with paclitaxel-based chemotherapy as initial or salvage regimen in patients with hematologic malignancies. *Haematologica*. 93:1436-1438.

Flomenberg, N., S.M. Devine, J.F. Dipersio, J.L. Liesveld, J.M. McCarty, S.D. Rowley, D.H. Vesole, K. Badel, and G. Calandra. 2005. The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood*. 106:1867-1874.

Ford, D.A., C.C. Horner, and R.W. Gross. 1998. Protein kinase C acylation by palmitoyl coenzyme A facilitates its translocation to membranes. *Biochemistry*. 37:11953-11961.

Franco, M., C. Muratori, S. Corso, E. Tenaglia, A. Bertotti, L. Capparuccia, L. Trusolino, P.M. Comoglio, and L. Tamagnone. 2010. The tetraspanin CD151 is required for Met-dependent signaling and tumor cell growth. *The Journal of biological chemistry*. 285:38756-38764.

Fritsch, G., P. Buchinger, D. Printz, F.M. Fink, G. Mann, C. Peters, T. Wagner, A. Adler, and H. Gadner. 1993. Rapid discrimination of early CD34+ myeloid progenitors using CD45-RA analysis. *Blood*. 81:2301-2309.

Furuya, M., H. Kato, N. Nishimura, I. Ishiwata, H. Ikeda, R. Ito, T. Yoshiki, and H. Ishikura. 2005. Down-regulation of CD9 in human ovarian carcinoma cell might contribute to peritoneal dissemination: morphologic alteration and

reduced expression of beta1 integrin subsets. *Cancer research*. 65:2617-2625.

Galy, A., M. Travis, D. Cen, and B. Chen. 1995. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity*. 3:459-473.

Garcia-Frigola, C., F. Burgaya, L. de Lecea, and E. Soriano. 2001. Pattern of expression of the tetraspanin Tspan-5 during brain development in the mouse. *Mech Dev*. 106:207-212.

Geiger, B., A. Bershadsky, R. Pankov, and K.M. Yamada. 2001. Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nature reviews. Molecular cell biology*. 2:793-805.

Gibson, L.F. 2002. Survival of B lineage leukemic cells: signals from the bone marrow microenvironment. *Leukemia & lymphoma*. 43:19-27.

Gil, M.L., N. Vita, S. Lebel-Binay, B. Miloux, P. Chalon, M. Kaghad, C. Marchiol-Fournigault, H. Conjeaud, D. Caput, P. Ferrara, and et al. 1992. A member of the tetra spans transmembrane protein superfamily is recognized by a monoclonal antibody raised against an HLA class I-deficient, lymphokine-activated killer-susceptible, B lymphocyte line. Cloning and preliminary functional studies. *J Immunol*. 148:2826-2833.

Gillette, J.M., and J. Lippincott-Schwartz. 2009. Hematopoietic progenitor cells regulate their niche microenvironment through a novel mechanism of cell-cell communication. *Commun Integr Biol*. 2:305-307.

Gilliland, D.G., and J.D. Griffin. 2002. The roles of FLT3 in hematopoiesis and leukemia. *Blood*. 100:1532-1542.

Goemans, B.F., C.M. Zwaan, M. Miller, M. Zimmermann, A. Harlow, S. Meshinchi, A.H. Loonen, K. Hahlen, D. Reinhardt, U. Creutzig, G.J. Kaspers, and M.C. Heinrich. 2005. Mutations in KIT and RAS are frequent events in pediatric core-binding factor acute myeloid leukemia. *Leukemia*. 19:1536-1542.

Gould, R.J., M.A. Polokoff, P.A. Friedman, T.F. Huang, J.C. Holt, J.J. Cook, and S. Niewiarowski. 1990. Disintegrins: a family of integrin inhibitory proteins from viper venoms. *Proc Soc Exp Biol Med*. 195:168-171.

Graf, L., S. Heimfeld, and B. Torok-Storb. 2001. Comparison of gene expression in CD34+ cells from bone marrow and G-CSF-mobilized peripheral blood by high-density oligonucleotide array analysis. *Biol Blood Marrow Transplant*. 7:486-494.

Green, C.E., D.N. Pearson, R.T. Camphausen, D.E. Staunton, and S.I. Simon. 2004. Shear-dependent capping of L-selectin and P-selectin glycoprotein ligand 1 by E-selectin signals activation of high-avidity beta2-integrin on neutrophils. *J Immunol*. 172:7780-7790.

Greenbaum, A.M., L.D. Revollo, J.R. Woloszynek, R. Civitelli, and D.C. Link. 2012. N-cadherin in osteolineage cells is not required for maintenance of hematopoietic stem cells. *Blood*. 120:295-302.

Greenwald, E.C., J.M. Redden, K.L. Dodge-Kafka, and J.J. Saucerman. 2014. Scaffold state switching amplifies, accelerates, and insulates protein kinase C signaling. *The Journal of biological chemistry*. 289:2353-2360.

Gribben, J.G., A.S. Freedman, D. Neuberg, D.C. Roy, K.W. Blake, S.D. Woo, M.L. Grossbard, S.N. Rabinowe, F. Coral, G.J. Freeman, and et al. 1991. Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. *N Engl J Med*. 325:1525-1533.

Grignani, F., P.F. Ferrucci, U. Testa, G. Talamo, M. Fagioli, M. Alcalay, A. Mencarelli, F. Grignani, C. Peschle, I. Nicoletti, and et al. 1993. The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. *Cell*. 74:423-431.

Griner, E.M., and M.G. Kazanietz. 2007. Protein kinase C and other diacylglycerol effectors in cancer. *Nature reviews. Cancer*. 7:281-294.

Grunwald, M.R., and M.J. Levis. 2013. FLT3 inhibitors for acute myeloid leukemia: a review of their efficacy and mechanisms of resistance. *Int J Hematol*. 97:683-694.

Guo, W., J.L. Lasky, 3rd, and H. Wu. 2006. Cancer stem cells. *Pediatr Res*. 59:59R-64R.

Gustafson-Wagner, E., and C.S. Stipp. 2013. The CD9/CD81 tetraspanin complex and tetraspanin CD151 regulate alpha3beta1 integrin-dependent tumor cell behaviors by overlapping but distinct mechanisms. *PloS one*. 8:e61834.

Gutcher, I., P.R. Webb, and N.G. Anderson. 2003. The isoform-specific regulation of apoptosis by protein kinase C. *Cellular and molecular life sciences : CMLS*. 60:1061-1070.

Guzman, M.L., and J.N. Allan. 2014. Concise review: Leukemia stem cells in personalized medicine. *Stem Cells*. 32:844-851.

Guzman, M.L., X. Li, C.A. Corbett, R.M. Rossi, T. Bushnell, J.L. Liesveld, J. Hebert, F. Young, and C.T. Jordan. 2007. Rapid and selective death of leukemia stem and progenitor cells induced by the compound 4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5 dione (TDZD-8). *Blood*. 110:4436-4444.

Hackney, J.A., P. Charbord, B.P. Brunk, C.J. Stoeckert, I.R. Lemischka, and K.A. Moore. 2002. A molecular profile of a hematopoietic stem cell niche. *Proceedings of the National Academy of Sciences of the United States of America*. 99:13061-13066.

Haining, E.J., J. Yang, R.L. Bailey, K. Khan, R. Collier, S. Tsai, S.P. Watson, J. Frampton, P. Garcia, and M.G. Tomlinson. 2012. The TspanC8 subgroup of tetraspanins interacts with A disintegrin and metalloprotease 10 (ADAM10) and regulates its maturation and cell surface expression. *The Journal of biological chemistry*. 287:39753-39765.

Halova, I., and P. Draber. 2016. Tetraspanins and Transmembrane Adaptor Proteins As Plasma Membrane Organizers-Mast Cell Case. *Front Cell Dev Biol*. 4:43.

Han, J., K. Reddig, and H.S. Li. 2007. Prolonged G(q) activity triggers fly rhodopsin endocytosis and degradation, and reduces photoreceptor sensitivity. *EMBO J*. 26:4966-4973.

Han, S.Y., M. Lee, Y.K. Hong, S. Hwang, G. Choi, Y.S. Suh, S.H. Park, S. Lee, S.H. Lee, J. Chung, S.H. Baek, and K.S. Cho. 2012. Tsp66E, the Drosophila KAI1 homologue, and Tsp74F function to regulate ovarian follicle cell and wing development by stabilizing integrin localization. *FEBS Lett*. 586:4031-4037.

Hansra, G., F. Bornancin, R. Whelan, B.A. Hemmings, and P.J. Parker. 1996. 12-O-Tetradecanoylphorbol-13-acetate-induced dephosphorylation of protein kinase Calpha correlates with the presence of a membrane-associated protein phosphatase 2A heterotrimer. *The Journal of biological chemistry*. 271:32785-32788.

Hao, Q.L., F.T. Thiemann, D. Petersen, E.M. Smogorzewska, and G.M. Crooks. 1996. Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood*. 88:3306-3313.

Harburger, D.S., and D.A. Calderwood. 2009. Integrin signalling at a glance. *Journal of cell science*. 122:159-163.

Harding, A., and J.F. Hancock. 2008. Ras nanoclusters: combining digital and analog signaling. *Cell cycle*. 7:127-134.

Harris, T.J., and U. Tepass. 2010. Adherens junctions: from molecules to morphogenesis. *Nature reviews. Molecular cell biology*. 11:502-514.

Harrison, O.J., X. Jin, S. Hong, F. Bahna, G. Ahlsen, J. Brasch, Y. Wu, J. Vendome, K. Felsovalyi, C.M. Hampton, R.B. Troyanovsky, A. Ben-Shaul, J. Frank, S.M. Troyanovsky, L. Shapiro, and B. Honig. 2011. The extracellular architecture of adherens junctions revealed by crystal structures of type I cadherins. *Structure*. 19:244-256.

Hart, S.M., and L. Foroni. 2002. Core binding factor genes and human leukemia. *Haematologica*. 87:1307-1323.

Hartwig, J.H., S. Kung, T. Kovacsovics, P.A. Janmey, L.C. Cantley, T.P. Stossel, and A. Toker. 1996. D3 phosphoinositides and outside-in integrin signaling by glycoprotein IIb-IIIa mediate platelet actin assembly and filopodial extension induced by phorbol 12-myristate 13-acetate. *The Journal of biological chemistry*. 271:32986-32993.

Hartwig, J.H., M. Thelen, A. Rosen, P.A. Janmey, A.C. Nairn, and A. Aderem. 1992. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature*. 356:618-622.

Hartz, B., T. Volkmann, S. Irle, C. Loechelt, A. Neubauer, and C. Brendel. 2011. alpha4 integrin levels on mobilized peripheral blood stem cells predict rapidity of engraftment in patients receiving autologous stem cell transplantation. *Blood*. 118:2362-2365.

Hatta, K., A. Nose, A. Nagafuchi, and M. Takeichi. 1988. Cloning and expression of cDNA encoding a neural calcium-dependent cell adhesion molecule: its identity in the cadherin gene family. *J Cell Biol*. 106:873-881.

He, B., L. Liu, G.A. Cook, S. Grgurevich, L.K. Jennings, and X.A. Zhang. 2005. Tetraspanin CD82 attenuates cellular morphogenesis through down-regulating integrin alpha6-mediated cell adhesion. *The Journal of biological chemistry*. 280:3346-3354.

Heilemann, M., S. van de Linde, M. Schuttpelz, R. Kasper, B. Seefeldt, A. Mukherjee, P. Tinnefeld, and M. Sauer. 2008. Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes. *Angew Chem Int Ed Engl*. 47:6172-6176.

Heimbrook, D.C., and A. Oliff. 1998. Therapeutic intervention and signaling. *Curr Opin Cell Biol*. 10:284-288.

Hemler, M.E. 2005. Tetraspanin functions and associated microdomains. *Nature reviews. Molecular cell biology*. 6:801-811.

Hemler, M.E. 2008a. Targeting of tetraspanin propteins--potential benefits and strategies. *Nat Rev Drug Discov*. 7:747-758.

Hemler, M.E. 2008b. Targeting of tetraspanin proteins--potential benefits and strategies. *Nature reviews. Drug discovery*. 7:747-758.

Hemler, M.E. 2013. Tetraspanin proteins promote multiple cancer stages. *Nature reviews. Cancer*. 14:49-60.

Henig, I., and T. Zuckerman. 2014. Hematopoietic stem cell transplantation-50 years of evolution and future perspectives. *Rambam Maimonides Med J*. 5:e0028.

Herget, T., S.A. Oehrlein, D.J. Pappin, E. Rozengurt, and P.J. Parker. 1995. The myristoylated alanine-rich C-kinase substrate (MARCKS) is sequentially phosphorylated by conventional, novel and atypical isotypes of protein kinase C. *Eur J Biochem*. 233:448-457.

Herr, M.J., S.E. Mabry, and L.K. Jennings. 2014. Tetraspanin CD9 regulates cell contraction and actin arrangement via RhoA in human vascular smooth muscle cells. *PloS one*. 9:e106999.

Hinkle, C.L., M.J. Mohan, P. Lin, N. Yeung, F. Rasmussen, M.E. Milla, and M.L. Moss. 2003. Multiple metalloproteinases process protransforming growth factor-alpha (proTGF-alpha). *Biochemistry*. 42:2127-2136.

Honda, S., H. Shirotani-Ikejima, S. Tadokoro, Y. Maeda, T. Kinoshita, Y. Tomiyama, and T. Miyata. 2009. Integrin-linked kinase associated with integrin activation. *Blood*. 113:5304-5313.

Hong, I.K., D.I. Jeoung, K.S. Ha, Y.M. Kim, and H. Lee. 2012. Tetraspanin CD151 stimulates adhesion-dependent activation of Ras, Rac, and Cdc42 by facilitating molecular association between beta1 integrins and small GTPases. *The Journal of biological chemistry*. 287:32027-32039.

Hong, S., R.B. Troyanovsky, and S.M. Troyanovsky. 2013. Binding to F-actin guides cadherin cluster assembly, stability, and movement. *The Journal of cell biology*. 201:131-143.

Hoque, M., C. Rentero, R. Cairns, F. Tebar, C. Enrich, and T. Grewal. 2014. Annexins - scaffolds modulating PKC localization and signaling. *Cellular signalling*. 26:1213-1225.

Horton, S.J., and B.J. Huntly. 2012. Recent advances in acute myeloid leukemia stem cell biology. *Haematologica*. 97:966-974.

Huang, F., S.L. Schwartz, J.M. Byars, and K.A. Lidke. 2011. Simultaneous multiple-emitter fitting for single molecule super-resolution imaging. *Biomed Opt Express*. 2:1377-1393.

Huang, K.P. 1989. The mechanism of protein kinase C activation. *Trends Neurosci*. 12:425-432.

Huang, S., and L.W. Terstappen. 1994. Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38- hematopoietic stem cells. *Blood*. 83:1515-1526.

Huang, S., S. Yuan, M. Dong, J. Su, C. Yu, Y. Shen, X. Xie, Y. Yu, X. Yu, S. Chen, S. Zhang, P. Pontarotti, and A. Xu. 2005. The phylogenetic analysis of tetraspanins projects the evolution of cell-cell interactions from unicellular to multicellular organisms. *Genomics*. 86:674-684.

Hughes, P.E., and M. Pfaff. 1998. Integrin affinity modulation. *Trends Cell Biol*. 8:359-364.

Humphries, J.D., A. Byron, and M.J. Humphries. 2006. Integrin ligands at a glance. *Journal of cell science*. 119:3901-3903.

Humphries, M.J., E.J. Symonds, and A.P. Mould. 2003. Mapping functional residues onto integrin crystal structures. *Curr Opin Struct Biol*. 13:236-243.

Ikeda, H., Y. Kanakura, T. Tamaki, A. Kuriu, H. Kitayama, J. Ishikawa, Y. Kanayama, T. Yonezawa, S. Tarui, and J.D. Griffin. 1991. Expression and functional role of the proto-oncogene c-kit in acute myeloblastic leukemia cells. *Blood*. 78:2962-2968.

Imai, T., K. Fukudome, S. Takagi, M. Nagira, M. Furuse, N. Fukuhara, M. Nishimura, Y. Hinuma, and O. Yoshie. 1992. C33 antigen recognized by monoclonal antibodies inhibitory to human T cell leukemia virus type 1-induced syncytium formation is a member of a new family of transmembrane proteins including CD9, CD37, CD53, and CD63. *J Immunol*. 149:2879-2886.

Imhof, I., W.J. Gasper, and R. Derynck. 2008. Association of tetraspanin CD9 with transmembrane TGF{alpha} confers alterations in cell-surface presentation of TGF{alpha} and cytoskeletal organization. *Journal of cell science*. 121:2265-2274.

Inoue, O., K. Suzuki-Inoue, W.L. Dean, J. Frampton, and S.P. Watson. 2003. Integrin alpha2beta1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCgamma2. *J Cell Biol*. 160:769-780.

Inuzuka, H., S. Miyatani, and M. Takeichi. 1991. R-cadherin: a novel Ca(2+)-dependent cell-cell adhesion molecule expressed in the retina. *Neuron*. 7:69-79.

Isberg, R.R., and G. Tran Van Nhieu. 1994. Binding and internalization of microorganisms by integrin receptors. *Trends Microbiol*. 2:10-14.

Ishikawa, F., S. Yoshida, Y. Saito, A. Hijikata, H. Kitamura, S. Tanaka, R. Nakamura, T. Tanaka, H. Tomiyama, N. Saito, M. Fukata, T. Miyamoto, B. Lyons, K. Ohshima, N. Uchida, S. Taniguchi, O. Ohara, K. Akashi, M. Harada, and L.D. Shultz. 2007. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol*. 25:1315-1321.

Ivanova, N.B., J.T. Dimos, C. Schaniel, J.A. Hackney, K.A. Moore, and I.R. Lemischka. 2002. A stem cell molecular signature. *Science*. 298:601-604.

Iwasaki, T., Y. Takeda, K. Maruyama, Y. Yokosaki, K. Tsujino, S. Tetsumoto, H. Kuhara, K. Nakanishi, Y. Otani, Y. Jin, S. Kohmo, H. Hirata, R. Takahashi, M. Suzuki, K. Inoue, I. Nagatomo, S. Goya, T. Kijima, T. Kumagai, I. Tachibana, I. Kawase, and A. Kumanogoh. 2013. Deletion of tetraspanin CD9 diminishes lymphangiogenesis in vivo and in vitro. *The Journal of biological chemistry*. 288:2118-2131.

Jackson, D.Y., C. Quan, D.R. Artis, T. Rawson, B. Blackburn, M. Struble, G. Fitzgerald, K. Chan, S. Mullins, J.P. Burnier, W.J. Fairbrother, K. Clark, M. Berisini, H. Chui, M. Renz, S. Jones, and S. Fong. 1997. Potent alpha 4 beta 1 peptide antagonists as potential anti-inflammatory agents. *Journal of medicinal chemistry*. 40:3359-3368.

Jacobson, L.O. 1952. Evidence for a humoral factor (or factors) concerned in recovery from radiation injury: a review. *Cancer research*. 12:315-325.

Jacobson, L.O., E.L. Simmons, E.K. Marks, and J.H. Eldredge. 1951. Recovery from radiation injury. *Science*. 113:510-511.

James, J.A., M.A. Smith, E.L. Court, C. Yip, Y. Ching, C. Willson, and J.G. Smith. 2003. An investigation of the effects of the MEK inhibitor U0126 on apoptosis in acute leukemia. *Hematol J*. 4:427-432.

Jarikji, Z., L.D. Horb, F. Shariff, C.A. Mandato, K.W. Cho, and M.E. Horb. 2009. The tetraspanin Tm4sf3 is localized to the ventral pancreas and regulates fusion of the dorsal and ventral pancreatic buds. *Development*. 136:1791-1800.

Jee, B.K., J.Y. Lee, Y. Lim, K.H. Lee, and Y.H. Jo. 2007. Effect of KAI1/CD82 on the beta1 integrin maturation in highly migratory carcinoma cells. *Biochem Biophys Res Commun*. 359:703-708.

Jin, L., K.J. Hope, Q. Zhai, F. Smadja-Joffe, and J.E. Dick. 2006. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nature medicine*. 12:1167-1174.

Joazeiro, C.A., S.S. Wing, H. Huang, J.D. Leverson, T. Hunter, and Y.C. Liu. 1999. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science*. 286:309-312.

Johnson, D.B., K.S. Smalley, and J.A. Sosman. 2014. Molecular pathways: targeting NRAS in melanoma and acute myelogenous leukemia. *Clin Cancer Res*. 20:4186-4192.

Johnson, M.S., N. Lu, K. Denessiouk, J. Heino, and D. Gullberg. 2009. Integrins during evolution: evolutionary trees and model organisms. *Biochimica et biophysica acta*. 1788:779-789.

Jordan, C.T. 2002. Unique molecular and cellular features of acute myelogenous leukemia stem cells. *Leukemia*. 16:559-562.

Jordan, C.T., M.L. Guzman, and M. Noble. 2006. Cancer stem cells. *N Engl J Med*. 355:1253-1261.

Jun, J.E., I. Rubio, and J.P. Roose. 2013. Regulation of ras exchange factors and cellular localization of ras activation by lipid messengers in T cells. *Front Immunol*. 4:239.

Jung, O., S. Choi, S.B. Jang, S.A. Lee, S.T. Lim, Y.J. Choi, H.J. Kim, D.H. Kim, T.K. Kwak, H. Kim, M. Kang, M.S. Lee, S.Y. Park, J. Ryu, D. Jeong, H.K. Cheong, H.J. Kim, K.H. Park, B.J. Lee, D.D. Schlaepfer, and J.W. Lee. 2012. Tetraspan TM4SF5-dependent direct activation of FAK and metastatic potential of hepatocarcinoma cells. *Journal of cell science*. 125:5960-5973.

Jung, S.M., and M. Moroi. 2001. Platelet collagen receptor integrin alpha2beta1 activation involves differential participation of ADP-receptor subtypes P2Y1 and P2Y12 but not intracellular calcium change. *Eur J Biochem*. 268:3513-3522.

Jura, N., N.F. Endres, K. Engel, S. Deindl, R. Das, M.H. Lamers, D.E. Wemmer, X. Zhang, and J. Kuriyan. 2009. Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment. *Cell*. 137:1293-1307.

Kaiser, H.J., A. Orlowski, T. Rog, T.K. Nyholm, W. Chai, T. Feizi, D. Lingwood, I. Vattulainen, and K. Simons. 2011. Lateral sorting in model membranes by cholesterol-mediated hydrophobic matching. *Proceedings of the National Academy of Sciences of the United States of America*. 108:16628-16633.

Kaji, K., S. Oda, S. Miyazaki, and A. Kudo. 2002. Infertility of CD9-deficient mouse eggs is reversed by mouse CD9, human CD9, or mouse CD81; polyadenylated mRNA injection developed for molecular analysis of sperm-egg fusion. *Dev Biol*. 247:327-334.

Kaji, K., S. Oda, T. Shikano, T. Ohnuki, Y. Uematsu, J. Sakagami, N. Tada, S. Miyazaki, and A. Kudo. 2000. The gamete fusion process is defective in eggs of Cd9-deficient mice. *Nat Genet*. 24:279-282.

Kedzierski, W., D. Bok, and G.H. Travis. 1999. Transgenic analysis of rds/peripherin N-glycosylation: effect on dimerization, interaction with rom1, and rescue of the rds null phenotype. *J Neurochem*. 72:430-438.

Kemler, R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends in genetics : TIG*. 9:317-321.

Kenny, P.A., and M.J. Bissell. 2007. Targeting TACE-dependent EGFR ligand shedding in breast cancer. *J Clin Invest*. 117:337-345.

Kerr, A.H., J.A. James, M.A. Smith, C. Willson, E.L. Court, and J.G. Smith. 2003. An investigation of the MEK/ERK inhibitor U0126 in acute myeloid leukemia. *Ann N Y Acad Sci*. 1010:86-89.

Kiel, M.J., M. Acar, G.L. Radice, and S.J. Morrison. 2009. Hematopoietic stem cells do not depend on N-cadherin to regulate their maintenance. *Cell Stem Cell*. 4:170-179.

Kiel, M.J., G.L. Radice, and S.J. Morrison. 2007. Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. *Cell Stem Cell*. 1:204-217.

Kiessling, L.L., J.E. Gestwicki, and L.E. Strong. 2006. Synthetic multivalent ligands as probes of signal transduction. *Angew Chem Int Ed Engl*. 45:2348-2368.

Kiger, A.A., H. White-Cooper, and M.T. Fuller. 2000. Somatic support cells restrict germline stem cell self-renewal and promote differentiation. *Nature*. 407:750-754.

Kim, I., W. Pan, S.A. Jones, Y. Zhang, X. Zhuang, and D. Wu. 2013. Clathrin and AP2 are required for PtdIns(4,5)P2-mediated formation of LRP6 signalosomes. *J Cell Biol*. 200:419-428.

Kim, M., C.V. Carman, and T.A. Springer. 2003. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science*. 301:1720-1725.

Kimble, J.E., and J.G. White. 1981. On the control of germ cell development in Caenorhabditis elegans. *Dev Biol*. 81:208-219.

Kinashi, T. 2005. Intracellular signalling controlling integrin activation in lymphocytes. *Nat Rev Immunol*. 5:546-559.

Kirk, R.I., M.R. Sanderson, and K.M. Lerea. 2000. Threonine phosphorylation of the beta 3 integrin cytoplasmic tail, at a site recognized by PDK1 and Akt/PKB in vitro, regulates Shc binding. *The Journal of biological chemistry*. 275:30901-30906.

Kirstetter, P., K. Anderson, B.T. Porse, S.E. Jacobsen, and C. Nerlov. 2006. Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nature immunology*. 7:1048-1056.

Kiselyov, V.V., A. Kochoyan, F.M. Poulsen, E. Bock, and V. Berezin. 2006. Elucidation of the mechanism of the regulatory function of the Ig1 module of the fibroblast growth factor receptor 1. *Protein Sci*. 15:2318-2322.

Kitadokoro, K., D. Bordo, G. Galli, R. Petracca, F. Falugi, S. Abrignani, G. Grandi, and M. Bolognesi. 2001. CD81 extracellular domain 3D structure: insight into the tetraspanin superfamily structural motifs. *EMBO J*. 20:12-18.

Klein, G. 1995. The extracellular matrix of the hematopoietic microenvironment. *Experientia*. 51:914-926.

Kobilka, B.K. 2007. G protein coupled receptor structure and activation. *Biochim Biophys Acta*. 1768:794-807.

Koch, A.W., D. Bozic, O. Pertz, and J. Engel. 1999. Homophilic adhesion by cadherins. *Curr Opin Struct Biol*. 9:275-281.

Kolanus, W., and B. Seed. 1997. Integrins and inside-out signal transduction: converging signals from PKC and PIP3. *Curr Opin Cell Biol*. 9:725-731.

Kolch, W., G. Heidecker, G. Kochs, R. Hummel, H. Vahidi, H. Mischak, G. Finkenzeller, D. Marme, and U.R. Rapp. 1993. Protein kinase C alpha activates RAF-1 by direct phosphorylation. *Nature*. 364:249-252.

Kollet, O., A. Spiegel, A. Peled, I. Petit, T. Byk, R. Hershkoviz, E. Guetta, G. Barkai, A. Nagler, and T. Lapidot. 2001. Rapid and efficient homing of human CD34(+)CD38(-/low)CXCR4(+) stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2m(null) mice. *Blood*. 97:3283-3291.

Komoriya, A., L.J. Green, M. Mervic, S.S. Yamada, K.M. Yamada, and M.J. Humphries. 1991. The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. *The Journal of biological chemistry*. 266:15075-15079.

Konopleva, M., S. Konoplev, W. Hu, A.Y. Zaritskey, B.V. Afanasiev, and M. Andreeff. 2002. Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia*. 16:1713-1724.

Koo, L.Y., D.J. Irvine, A.M. Mayes, D.A. Lauffenburger, and L.G. Griffith. 2002. Co-regulation of cell adhesion by nanoscale RGD organization and mechanical stimulus. *Journal of cell science*. 115:1423-1433.

Kopan, R. 2012. Notch signaling. *Cold Spring Harb Perspect Biol*. 4.

Kopp, H.G., S.T. Avecilla, A.T. Hooper, and S. Rafii. 2005. The bone marrow vascular niche: home of HSC differentiation and mobilization. *Physiology (Bethesda)*. 20:349-356.

Kornblau, S.M., Y.H. Qiu, B.N. Bekele, J.S. Cade, X. Zhou, D. Harris, C.E. Jackson, Z. Estrov, and M. Andreeff. 2006. Studying the right cell in acute myelogenous leukemia: dynamic changes of apoptosis and signal transduction pathway protein expression in chemotherapy resistant ex-vivo selected "survivor cells". *Cell cycle*. 5:2769-2777.

Kotha, J., C. Longhurst, W. Appling, and L.K. Jennings. 2008. Tetraspanin CD9 regulates beta 1 integrin activation and enhances cell motility to fibronectin via a PI-3 kinase-dependent pathway. *Exp Cell Res*. 314:1811-1822.

Kovalenko, O.V., X. Yang, T.V. Kolesnikova, and M.E. Hemler. 2004. Evidence for specific tetraspanin homodimers: inhibition of palmitoylation makes cysteine residues available for cross-linking. *The Biochemical journal*. 377:407-417.

Krauss, K., and P. Altevogt. 1999. Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. *The Journal of biological chemistry*. 274:36921-36927.

Krebsbach, P.H., S.A. Kuznetsov, P. Bianco, and P.G. Robey. 1999. Bone marrow stromal cells: characterization and clinical application. *Crit Rev Oral Biol Med*. 10:165-181.

Kuendgen, A., and U. Germing. 2009. Emerging treatment strategies for acute myeloid leukemia (AML) in the elderly. *Cancer Treat Rev*. 35:97-120.

Kuhne, M.R., T. Mulvey, B. Belanger, S. Chen, C. Pan, C. Chong, F. Cao, W. Niekro, T. Kempe, K.A. Henning, L.J. Cohen, A.J. Korman, and P.M. Cardarelli. 2013. BMS-936564/MDX-1338: a fully human anti-CXCR4 antibody induces apoptosis in vitro and shows antitumor activity in vivo in hematologic malignancies. *Clin Cancer Res*. 19:357-366.

Kurinna, S., M. Konopleva, S.L. Palla, W. Chen, S. Kornblau, R. Contractor, X. Deng, W.S. May, M. Andreeff, and P.P. Ruvolo. 2006. Bcl2

phosphorylation and active PKC alpha are associated with poor survival in AML. *Leukemia*. 20:1316-1319.

Kwon, H.Y., J. Bajaj, T. Ito, A. Blevins, T. Konuma, J. Weeks, N.K. Lytle, C.S. Koechlein, D. Rizzieri, C. Chuah, V.G. Oehler, R. Sasik, G. Hardiman, and T. Reya. 2015. Tetraspanin 3 Is Required for the Development and Propagation of Acute Myelogenous Leukemia. *Cell Stem Cell*. 17:152-164.

Lammerding, J., A.R. Kazarov, H. Huang, R.T. Lee, and M.E. Hemler. 2003. Tetraspanin CD151 regulates alpha6beta1 integrin adhesion strengthening. *Proceedings of the National Academy of Sciences of the United States of America*. 100:7616-7621.

Lane, T.A., A.D. Ho, A. Bashey, S. Peterson, D. Young, and P. Law. 1999. Mobilization of blood-derived stem and progenitor cells in normal subjects by granulocyte-macrophage- and granulocyte-colony-stimulating factors. *Transfusion*. 39:39-47.

Lanzkron, S.M., M.I. Collector, and S.J. Sharkis. 1999. Homing of long-term and short-term engrafting cells in vivo. *Ann N Y Acad Sci*. 872:48-54; discussion 54-46.

Lapidot, T., C. Sirard, J. Vormoor, B. Murdoch, T. Hoang, J. Caceres-Cortes, M. Minden, B. Paterson, M.A. Caligiuri, and J.E. Dick. 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*. 367:645-648.

Larochelle, A., J.M. Gillette, R. Desmond, B. Ichwan, A. Cantilena, A. Cerf, A.J. Barrett, A.S. Wayne, J. Lippincott-Schwartz, and C.E. Dunbar. 2012. Bone marrow homing and engraftment of human hematopoietic stem and progenitor cells is mediated by a polarized membrane domain. *Blood*. 119:1848-1855.

Larochelle, A., A. Krouse, M. Metzger, D. Orlic, R.E. Donahue, S. Fricker, G. Bridger, C.E. Dunbar, and P. Hematti. 2006. AMD3100 mobilizes hematopoietic stem cells with long-term repopulating capacity in nonhuman primates. *Blood*. 107:3772-3778.

Larson, P. 1989. Paul Larson, COMT, president, Association of Technical Personnel in Ophthalmology. Interview by Norma Garber. *J Ophthalmic Nurs Technol*. 8:71-72.

Laudanna, C., J.Y. Kim, G. Constantin, and E. Butcher. 2002. Rapid leukocyte integrin activation by chemokines. *Immunological reviews*. 186:37-46.

Le Naour, F., E. Rubinstein, C. Jasmin, M. Prenant, and C. Boucheix. 2000. Severely reduced female fertility in CD9-deficient mice. *Science*. 287:319-321.

Lebel-Binay, S., M.L. Gil, C. Lagaudriere, B. Miloux, C. Marchiol-Fournigault, A. Quillet-Mary, M. Lopez, D. Fradelizi, and H. Conjeaud. 1994. Further characterization of CD82/IA4 antigen (type III surface protein): an activation/differentiation marker of mononuclear cells. *Cellular immunology*. 154:468-483.

Lee, H.W., L. Smith, G.R. Pettit, A. Vinitsky, and J.B. Smith. 1996. Ubiquitination of protein kinase C-alpha and degradation by the proteasome. *The Journal of biological chemistry*. 271:20973-20976.

Lee, J.O., L.A. Bankston, M.A. Arnaout, and R.C. Liddington. 1995. Two conformations of the integrin A-domain (I-domain): a pathway for activation? *Structure*. 3:1333-1340.

Legate, K.R., and R. Fassler. 2009. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. *Journal of cell science*. 122:187-198.

Legate, K.R., S.A. Wickstrom, and R. Fassler. 2009. Genetic and cell biological analysis of integrin outside-in signaling. *Genes Dev*. 23:397-418.

Lennartsson, J., and L. Ronnstrand. 2012. Stem cell factor receptor/c-Kit: from basic science to clinical implications. *Physiol Rev*. 92:1619-1649.

Lerea, K.M., A.Y. Venjara, S.C. Olson, and M.R. Kelly. 2007. Threonine phosphorylation of integrin beta3 in calyculin A-treated platelets is selectively sensitive to 5'-iodotubercidin. *Biochim Biophys Acta*. 1773:185-191.

Leung, K.T., K.Y. Chan, P.C. Ng, T.K. Lau, W.M. Chiu, K.S. Tsang, C.K. Li, C.K. Kong, and K. Li. 2011. The tetraspanin CD9 regulates migration, adhesion, and homing of human cord blood CD34+ hematopoietic stem and progenitor cells. *Blood*. 117:1840-1850.

Levy, S., V.Q. Nguyen, M.L. Andria, and S. Takahashi. 1991. Structure and membrane topology of TAPA-1. *The Journal of biological chemistry*. 266:14597-14602.

Levy, S., and T. Shoham. 2005. The tetraspanin web modulates immune-signalling complexes. *Nat Rev Immunol*. 5:136-148.

Li, M.Q., X.F. Hou, J. Shao, C.L. Tang, and D.J. Li. 2010. The DSC-expressed CD82 controls the invasiveness of trophoblast cells via integrinbeta1/MAPK/MAPK3/1 signaling pathway in human first-trimester pregnancy. *Biology of reproduction*. 82:968-979.

Li, P., and L.I. Zon. 2010. Resolving the controversy about N-cadherin and hematopoietic stem cells. *Cell Stem Cell*. 6:199-202.

Li, Q., X.H. Yang, F. Xu, C. Sharma, H.X. Wang, K. Knoblich, I. Rabinovitz, S.R. Granter, and M.E. Hemler. 2013a. Tetraspanin CD151 plays a key role in skin squamous cell carcinoma. *Oncogene*. 32:1772-1783.

Li, Y., X. Huang, J. Zhang, Y. Li, and K. Ma. 2013b. Synergistic inhibition of cell migration by tetraspanin CD82 and gangliosides occurs via the EGFR or cMet-activated Pl3K/Akt signalling pathway. *Int J Biochem Cell Biol*. 45:2349-2358.

Lineberry, N., L. Su, L. Soares, and C.G. Fathman. 2008. The single subunit transmembrane E3 ligase gene related to anergy in lymphocytes (GRAIL) captures and then ubiquitinates transmembrane proteins across the cell membrane. *The Journal of biological chemistry*. 283:28497-28505.

Little, K.D., M.E. Hemler, and C.S. Stipp. 2004. Dynamic regulation of a GPCR-tetraspanin-G protein complex on intact cells: central role of CD81 in facilitating GPR56-Galpha q/11 association. *Molecular biology of the cell*. 15:2375-2387.

Liu, F., J. Poursine-Laurent, and D.C. Link. 2000. Expression of the G-CSF receptor on hematopoietic progenitor cells is not required for their mobilization by G-CSF. *Blood*. 95:3025-3031.

Liu, L., B. He, W.M. Liu, D. Zhou, J.V. Cox, and X.A. Zhang. 2007. Tetraspanin CD151 promotes cell migration by regulating integrin trafficking. *The Journal of biological chemistry*. 282:31631-31642.

Liu, W.M., F. Zhang, S. Moshiach, B. Zhou, C. Huang, K. Srinivasan, S. Khurana, Y. Zheng, J.M. Lahti, and X.A. Zhang. 2012. Tetraspanin CD82 inhibits protrusion and retraction in cell movement by attenuating the plasma membrane-dependent actin organization. *PloS one*. 7:e51797.

Liu, X., T.M. Kapoor, J.K. Chen, and M. Huse. 2013a. Diacylglycerol promotes centrosome polarization in T cells via reciprocal localization of dynein and myosin II. *Proceedings of the National Academy of Sciences of the United States of America*. 110:11976-11981.

Liu, Z., F. Tian, X. Feng, Y. He, P. Jiang, J. Li, F. Guo, X. Zhao, H. Chang, and S. Wang. 2013b. LPS increases MUC5AC by TACE/TGF-alpha/EGFR pathway in human intrahepatic biliary epithelial cell. *Biomed Res Int*. 2013:165715.

Longley, B.J., M.J. Reguera, and Y. Ma. 2001. Classes of c-KIT activating mutations: proposed mechanisms of action and implications for disease classification and therapy. *Leuk Res*. 25:571-576.

Lorenz, E., C. Congdon, and D. Uphoff. 1952. Modification of acute irradiation injury in mice and guinea-pigs by bone marrow injections. *Radiology*. 58:863-877.

Lorenz, E., D. Uphoff, T.R. Reid, and E. Shelton. 1951. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst*. 12:197-201.

Losordo, D.W., T.D. Henry, C. Davidson, J. Sup Lee, M.A. Costa, T. Bass, F. Mendelsohn, F.D. Fortuin, C.J. Pepine, J.H. Traverse, D. Amrani, B.M. Ewenstein, N. Riedel, K. Story, K. Barker, T.J. Povsic, R.A. Harrington, and R.A. Schatz. 2011. Intramyocardial, autologous CD34+ cell therapy for refractory angina. *Circ Res*. 109:428-436.

Lowenberg, B., J.R. Downing, and A. Burnett. 1999. Acute myeloid leukemia. *N Engl J Med*. 341:1051-1062.

Lu, C.F., and T.A. Springer. 1997. The alpha subunit cytoplasmic domain regulates the assembly and adhesiveness of integrin lymphocyte function-associated antigen-1. *J Immunol*. 159:268-278.

Lucas, M., and V. Sanchez-Margalet. 1995. Protein kinase C involvement in apoptosis. *Gen Pharmacol*. 26:881-887.

Lund, A.H., and M. van Lohuizen. 2002. RUNX: a trilogy of cancer genes. *Cancer Cell*. 1:213-215.

Lunghi, P., A. Tabilio, P.P. Dall'Aglio, E. Ridolo, C. Carlo-Stella, P.G. Pelicci, and A. Bonati. 2003. Downmodulation of ERK activity inhibits the proliferation and induces the apoptosis of primary acute myelogenous leukemia blasts. *Leukemia*. 17:1783-1793.

Luo, B.H., T.A. Springer, and J. Takagi. 2004. A specific interface between integrin transmembrane helices and affinity for ligand. *PLoS Biol*. 2:e153.

Machida, U., M. Kami, and H. Hirai. 1999. Hematopoietic stem-cell transplantation for acute leukemia. *The New England journal of medicine*. 340:810; author reply 811-812.

Mackay, H.J., and C.J. Twelves. 2007. Targeting the protein kinase C family: are we there yet? *Nature reviews. Cancer*. 7:554-562.

Maecker, H.T., S.C. Todd, and S. Levy. 1997. The tetraspanin superfamily: molecular facilitators. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 11:428-442.

Maheshwari, G., G. Brown, D.A. Lauffenburger, A. Wells, and L.G. Griffith. 2000. Cell adhesion and motility depend on nanoscale RGD clustering. *Journal of cell science*. 113 (Pt 10):1677-1686.

Majeti, R., C.Y. Park, and I.L. Weissman. 2007. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell*. 1:635-645.

Malhotra, S., Y. Baba, K.P. Garrett, F.J. Staal, R. Gerstein, and P.W. Kincade. 2008. Contrasting responses of lymphoid progenitors to canonical and noncanonical Wnt signals. *J Immunol*. 181:3955-3964.

Malik, F.A., A.J. Sanders, and W.G. Jiang. 2009. KAI-1/CD82, the molecule and clinical implication in cancer and cancer metastasis. *Histology and histopathology*. 24:519-530.

Mammen, M., S.-K. Choi, G. Wihitesides, and 1998. 1998. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew Chem Int Ed*. 37.

Mancini, S.J., N. Mantei, A. Dumortier, U. Suter, H.R. MacDonald, and F. Radtke. 2005. Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood*. 105:2340-2342.

Mannion, B.A., F. Berditchevski, S.K. Kraeft, L.B. Chen, and M.E. Hemler. 1996. Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associated with integrin alpha 4 beta 1 (CD49d/CD29). *J Immunol*. 157:2039-2047.

Mansouri, A., N. Spurr, P.N. Goodfellow, and R. Kemler. 1988. Characterization and chromosomal localization of the gene encoding the human cell adhesion molecule uvomorulin. *Differentiation*. 38:67-71.

Marjon, K.D., C.M. Termini, K.L. Karlen, C. Saito-Reis, C.E. Soria, K.A. Lidke, and J.M. Gillette. 2015. Tetraspanin CD82 regulates bone marrow homing of acute myeloid leukemia by modulating the molecular organization of N-cadherin. *Oncogene*.

Matsunaga, M., K. Hatta, and M. Takeichi. 1988. Role of N-cadherin cell adhesion molecules in the histogenesis of neural retina. *Neuron*. 1:289-295.

Matsunaga, T., F. Fukai, S. Miura, Y. Nakane, T. Owaki, H. Kodama, M. Tanaka, T. Nagaya, R. Takimoto, T. Takayama, and Y. Niitsu. 2008. Combination therapy of an anticancer drug with the FNIII14 peptide of fibronectin effectively overcomes cell adhesion-mediated drug resistance of acute myelogenous leukemia. *Leukemia*. 22:353-360.

Matsunaga, T., N. Takemoto, T. Sato, R. Takimoto, I. Tanaka, A. Fujimi, T. Akiyama, H. Kuroda, Y. Kawano, M. Kobune, J. Kato, Y. Hirayama, S. Sakamaki, K. Kohda, K. Miyake, and Y. Niitsu. 2003. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. *Nat Med*. 9:1158-1165.

Matthews, A.L., P.J. Noy, J.S. Reyat, and M.G. Tomlinson. 2016. Regulation of A disintegrin and metalloproteinase (ADAM) family sheddases ADAM10 and ADAM17: The emerging role of tetraspanins and rhomboids. *Platelets*:1-9.

Mattila, P.K., C. Feest, D. Depoil, B. Treanor, B. Montaner, K.L. Otipoby, R. Carter, L.B. Justement, A. Bruckbauer, and F.D. Batista. 2013. The actin and tetraspanin networks organize receptor nanoclusters to regulate B cell receptor-mediated signaling. *Immunity*. 38:461-474.

Mazo, I.B., S. Massberg, and U.H. von Andrian. 2011. Hematopoietic stem and progenitor cell trafficking. *Trends Immunol*. 32:493-503.

Mazurov, D., G. Heidecker, and D. Derse. 2007. The inner loop of tetraspanins CD82 and CD81 mediates interactions with human T cell lymphotrophic virus type 1 Gag protein. *The Journal of biological chemistry*. 282:3896-3903.

Meads, M.B., L.A. Hazlehurst, and W.S. Dalton. 2008. The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. *Clin Cancer Res*. 14:2519-2526.

Melnikov, S., and R. Sagi-Eisenberg. 2009. Down-regulating protein kinase C alpha: functional cooperation between the proteasome and the endocytic system. *Cellular signalling*. 21:1607-1619.

Mendez-Ferrer, S., T.V. Michurina, F. Ferraro, A.R. Mazloom, B.D. Macarthur, S.A. Lira, D.T. Scadden, A. Ma'ayan, G.N. Enikolopov, and P.S. Frenette. 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*. 466:829-834.

Metzelaar, M.J., P.L. Wijngaard, P.J. Peters, J.J. Sixma, H.K. Nieuwenhuis, and H.C. Clevers. 1991. CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. *The Journal of biological chemistry*. 266:3239-3245.

Milella, M., M. Konopleva, C.M. Precupanu, Y. Tabe, M.R. Ricciardi, C. Gregorj, S.J. Collins, B.Z. Carter, C. D'Angelo, M.T. Petrucci, R. Foa, F. Cognetti, A. Tafuri, and M. Andreeff. 2007. MEK blockade converts AML differentiating response to retinoids into extensive apoptosis. *Blood*. 109:2121-2129.

Milella, M., S.M. Kornblau, Z. Estrov, B.Z. Carter, H. Lapillonne, D. Harris, M. Konopleva, S. Zhao, E. Estey, and M. Andreeff. 2001. Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. *J Clin Invest*. 108:851-859.

Miller, J.S., V. McCullar, M. Punzel, I.R. Lemischka, and K.A. Moore. 1999. Single adult human CD34(+)/Lin-/CD38(-) progenitors give rise to natural killer cells, B-lineage cells, dendritic cells, and myeloid cells. *Blood*. 93:96-106.

Minder, P., E. Bayha, C. Becker-Pauly, and E.E. Sterchi. 2012. Meprinalpha transactivates the epidermal growth factor receptor (EGFR) via ligand shedding, thereby enhancing colorectal cancer cell proliferation and migration. *The Journal of biological chemistry*. 287:35201-35211.

Miranti, C.K. 2009. Controlling cell surface dynamics and signaling: how CD82/KAI1 suppresses metastasis. *Cellular signalling*. 21:196-211.

Miyado, K., G. Yamada, S. Yamada, H. Hasuwa, Y. Nakamura, F. Ryu, K. Suzuki, K. Kosai, K. Inoue, A. Ogura, M. Okabe, and E. Mekada. 2000. Requirement of CD9 on the egg plasma membrane for fertilization. *Science*. 287:321-324.

Miyake, K., I.L. Weissman, J.S. Greenberger, and P.W. Kincade. 1991. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J Exp Med*. 173:599-607.

Mochizuki, S., and Y. Okada. 2007. ADAMs in cancer cell proliferation and progression. *Cancer science*. 98:621-628.

Molineux, G., A. Migdalska, M. Szmitkowski, K. Zsebo, and T.M. Dexter. 1991. The effects on hematopoiesis of recombinant stem cell factor (ligand for c-kit) administered in vivo to mice either alone or in combination with granulocyte colony-stimulating factor. *Blood*. 78:961-966.

Moral, Z., K. Dong, Y. Wei, H. Sterling, H. Deng, S. Ali, R. Gu, X.Y. Huang, S.C. Hebert, G. Giebisch, and W.H. Wang. 2001. Regulation of ROMK1 channels by protein-tyrosine kinase and -tyrosine phosphatase. *The Journal of biological chemistry*. 276:7156-7163.

Morrison, S.J., and A.C. Spradling. 2008. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell*. 132:598-611.

Mould, A.P., A. Komoriya, K.M. Yamada, and M.J. Humphries. 1991. The CS5 peptide is a second site in the IIICS region of fibronectin recognized by the integrin alpha 4 beta 1. Inhibition of alpha 4 beta 1 function by RGD peptide homologues. *The Journal of biological chemistry*. 266:3579-3585.

Murayama, Y., J. Miyagawa, K. Oritani, H. Yoshida, K. Yamamoto, O. Kishida, T. Miyazaki, S. Tsutsui, T. Kiyohara, Y. Miyazaki, S. Higashiyama, Y. Matsuzawa, and Y. Shinomura. 2004. CD9-mediated activation of the p46 Shc isoform leads to apoptosis in cancer cells. *Journal of cell science*. 117:3379-3388.

Murayama, Y., Y. Shinomura, K. Oritani, J. Miyagawa, H. Yoshida, M. Nishida, F. Katsube, M. Shiraga, T. Miyazaki, T. Nakamoto, S. Tsutsui, S. Tamura, S. Higashiyama, I. Shimomura, and N. Hayashi. 2008. The tetraspanin CD9 modulates epidermal growth factor receptor signaling in cancer cells. *J Cell Physiol*. 216:135-143.

Nakao, M., S. Yokota, T. Iwai, H. Kaneko, S. Horiike, K. Kashima, Y. Sonoda, T. Fujimoto, and S. Misawa. 1996. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia*. 10:1911-1918.

Nakashima, S. 2002. Protein kinase C alpha (PKC alpha): regulation and biological function. *Journal of biochemistry*. 132:669-675.

Navarro, M.N., and D.A. Cantrell. 2014. Serine-threonine kinases in TCR signaling. *Nature immunology*. 15:808-814.

Naveiras, O., V. Nardi, P.L. Wenzel, P.V. Hauschka, F. Fahey, and G.Q. Daley. 2009. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature*. 460:259-263.

Nelson, W.J. 2008. Regulation of cell-cell adhesion by the cadherin-catenin complex. *Biochemical Society transactions*. 36:149-155.

Nemerow, G.R., and D.A. Cheresh. 2002. Herpesvirus hijacks an integrin. *Nat Cell Biol*. 4:E69-71.

Nervi, B., P. Ramirez, M.P. Rettig, G.L. Uy, M.S. Holt, J.K. Ritchey, J.L. Prior, D. Piwnica-Worms, G. Bridger, T.J. Ley, and J.F. DiPersio. 2009. Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. *Blood*. 113:6206-6214.

Newton, A.C. 1995. Protein kinase C: structure, function, and regulation. *The Journal of biological chemistry*. 270:28495-28498.

Newton, A.C. 2010. Protein kinase C: poised to signal. *Am J Physiol Endocrinol Metab*. 298:E395-402.

Nielsen, J.S., and K.M. McNagny. 2008. Novel functions of the CD34 family. *Journal of cell science*. 121:3683-3692.

Niessen, C.M., and B.M. Gumbiner. 2002. Cadherin-mediated cell sorting not determined by binding or adhesion specificity. *J Cell Biol*. 156:389-399.

Niessen, C.M., D. Leckband, and A.S. Yap. 2011. Tissue organization by cadherin adhesion molecules: dynamic molecular and cellular mechanisms of morphogenetic regulation. *Physiol Rev*. 91:691-731.

Nilsson, S.K., M.E. Debatis, M.S. Dooner, J.A. Madri, P.J. Quesenberry, and P.S. Becker. 1998. Immunofluorescence characterization of key extracellular matrix proteins in murine bone marrow in situ. *J Histochem Cytochem*. 46:371-377.

Nilsson, S.K., H.M. Johnston, G.A. Whitty, B. Williams, R.J. Webb, D.T. Denhardt, I. Bertoncello, L.J. Bendall, P.J. Simmons, and D.N. Haylock. 2005. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood*. 106:1232-1239.

Nishioka, C., T. Ikezoe, M. Furihata, J. Yang, S. Serada, T. Naka, A. Nobumoto, S. Kataoka, M. Tsuda, K. Udaka, and A. Yokoyama. 2013. CD34(+)/CD38(-) acute myelogenous leukemia cells aberrantly express CD82 which regulates adhesion and survival of leukemia stem cells. *Int J Cancer*. 132:2006-2019.

Nishioka, C., T. Ikezoe, A. Takeuchi, A. Nobumoto, M. Tsuda, and A. Yokoyama. 2015a. The novel function of CD82 and its impact on BCL2L12 via AKT/STAT5 signal pathway in acute myelogenous leukemia cells. *Leukemia*. 29:2296-2306.

Nishioka, C., T. Ikezoe, J. Yang, A. Nobumoto, S. Kataoka, M. Tsuda, K. Udaka, and A. Yokoyama. 2014. CD82 regulates STAT5/IL-10 and supports survival of acute myelogenous leukemia cells. *Int J Cancer*. 134:55-64.

Nishioka, C., T. Ikezoe, J. Yang, and A. Yokoyama. 2015b. Tetraspanin Family Member, CD82, Regulates Expression of EZH2 via Inactivation of p38 MAPK Signaling in Leukemia Cells. *PloS one*. 10:e0125017.

Nishioka, C., T. Ikezoe, and A. Yokoyama. 2015c. Blockade of CD82 by a monoclonal antibody potentiates anti-leukemia effects of AraC in vivo. *Cancer Med*. 4:1426-1431.

Nishiuchi, R., N. Sanzen, S. Nada, Y. Sumida, Y. Wada, M. Okada, J. Takagi, H. Hasegawa, and K. Sekiguchi. 2005. Potentiation of the ligand-binding activity of integrin alpha3beta1 via association with tetraspanin CD151. *Proceedings of the National Academy of Sciences of the United States of America*. 102:1939-1944.

Nollet, F., P. Kools, and F. van Roy. 2000. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J Mol Biol*. 299:551-572.

Nose, A., A. Nagafuchi, and M. Takeichi. 1987. Isolation of placental cadherin cDNA: identification of a novel gene family of cell-cell adhesion molecules. *EMBO J*. 6:3655-3661.

Novitskaya, V., H. Romanska, R. Kordek, P. Potemski, R. Kusinska, M. Parsons, E. Odintsova, and F. Berditchevski. 2014. Integrin alpha3beta1-CD151 complex regulates dimerization of ErbB2 via RhoA. *Oncogene*. 33:2779-2789.

Nowak, G. 2002. Protein kinase C-alpha and ERK1/2 mediate mitochondrial dysfunction, decreases in active Na+ transport, and cisplatin-induced apoptosis in renal cells. *The Journal of biological chemistry*. 277:43377-43388.

Nowell, P.C., L.J. Cole, J.G. Habermeyer, and P.L. Roan. 1956. Growth and continued function of rat marrow cells in x-radiated mice. *Cancer research*. 16:258-261.

O'Toole, T.E., Y. Katagiri, R.J. Faull, K. Peter, R. Tamura, V. Quaranta, J.C. Loftus, S.J. Shattil, and M.H. Ginsberg. 1994. Integrin cytoplasmic domains mediate inside-out signal transduction. *J Cell Biol*. 124:1047-1059.

O'Toole, T.E., J. Ylanne, and B.M. Culley. 1995. Regulation of integrin affinity states through an NPXY motif in the beta subunit cytoplasmic domain. *The Journal of biological chemistry*. 270:8553-8558.

Odintsova, E., T.D. Butters, E. Monti, H. Sprong, G. van Meer, and F. Berditchevski. 2006. Gangliosides play an important role in the organization of CD82-enriched microdomains. *The Biochemical journal*. 400:315-325.

Odintsova, E., T. Sugiura, and F. Berditchevski. 2000. Attenuation of EGF receptor signaling by a metastasis suppressor, the tetraspanin CD82/KAI-1. *Current biology : CB*. 10:1009-1012.

Odintsova, E., G. van Niel, H. Conjeaud, G. Raposo, R. Iwamoto, E. Mekada, and F. Berditchevski. 2013. Metastasis suppressor tetraspanin CD82/KAI1 regulates ubiquitylation of epidermal growth factor receptor. *The Journal of biological chemistry*. 288:26323-26334.

Odintsova, E., J. Voortman, E. Gilbert, and F. Berditchevski. 2003. Tetraspanin CD82 regulates compartmentalisation and ligand-induced dimerization of EGFR. *Journal of cell science*. 116:4557-4566.

Oka, N., M. Yamamoto, C. Schwencke, J. Kawabe, T. Ebina, S. Ohno, J. Couet, M.P. Lisanti, and Y. Ishikawa. 1997. Caveolin interaction with protein kinase C. Isoenzyme-dependent regulation of kinase activity by the

caveolin scaffolding domain peptide. *The Journal of biological chemistry*. 272:33416-33421.

Okuda, T., J. van Deursen, S.W. Hiebert, G. Grosveld, and J.R. Downing. 1996. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*. 84:321-330.

Ono, M., K. Handa, D.A. Withers, and S. Hakomori. 1999. Motility inhibition and apoptosis are induced by metastasis-suppressing gene product CD82 and its analogue CD9, with concurrent glycosylation. *Cancer research*. 59:2335-2339.

Oostendorp, R.A., S. Ghaffari, and C.J. Eaves. 2000. Kinetics of in vivo homing and recruitment into cycle of hematopoietic cells are organ-specific but CD44-independent. *Bone Marrow Transplant*. 26:599-566.

Organ, S.L., and M.S. Tsao. 2011. An overview of the c-MET signaling pathway. *Ther Adv Med Oncol*. 3:S7-S19.

Orr, J.W., L.M. Keranen, and A.C. Newton. 1992. Reversible exposure of the pseudosubstrate domain of protein kinase C by phosphatidylserine and diacylglycerol. *The Journal of biological chemistry*. 267:15263-15266.

Oshevski, S., M.C. Le Bousse-Kerdiles, D. Clay, Z. Levashova, N. Debili, N. Vitral, C. Jasmin, and M. Castagna. 1999. Differential expression of protein kinase C isoform transcripts in human hematopoietic progenitors undergoing differentiation. *Biochem Biophys Res Commun*. 263:603-609.

Oxley, C.L., N.J. Anthis, E.D. Lowe, I. Vakonakis, I.D. Campbell, and K.L. Wegener. 2008. An integrin phosphorylation switch: the effect of beta3 integrin tail phosphorylation on Dok1 and talin binding. *The Journal of biological chemistry*. 283:5420-5426.

Oxvig, C., and T.A. Springer. 1998. Experimental support for a beta-propeller domain in integrin alpha-subunits and a calcium binding site on its lower surface. *Proceedings of the National Academy of Sciences of the United States of America*. 95:4870-4875.

Pampori, N., T. Hato, D.G. Stupack, S. Aidoudi, D.A. Cheresh, G.R. Nemerow, and S.J. Shattil. 1999. Mechanisms and consequences of affinity modulation of integrin alpha(V)beta(3) detected with a novel patch-engineered monovalent ligand. *The Journal of biological chemistry*. 274:21609-21616.

Pandiella, A., and J. Massague. 1991. Cleavage of the membrane precursor for transforming growth factor alpha is a regulated process. *Proceedings of*

the National Academy of Sciences of the United States of America. 88:1726-1730.

Papaemmanuil, E., M. Gerstung, L. Bullinger, V.I. Gaidzik, P. Paschka, N.D. Roberts, N.E. Potter, M. Heuser, F. Thol, N. Bolli, G. Gundem, P. Van Loo, I. Martincorena, P. Ganly, L. Mudie, S. McLaren, S. O'Meara, K. Raine, D.R. Jones, J.W. Teague, A.P. Butler, M.F. Greaves, A. Ganser, K. Dohner, R.F. Schlenk, H. Dohner, and P.J. Campbell. 2016. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med*. 374:2209-2221.

Papayannopoulou, T., C. Craddock, B. Nakamoto, G.V. Priestley, and N.S. Wolf. 1995. The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. *Proceedings of the National Academy of Sciences of the United States of America*. 92:9647-9651.

Papayannopoulou, T., and B. Nakamoto. 1993. Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. *Proceedings of the National Academy of Sciences of the United States of America*. 90:9374-9378.

Park, S.H., H.S. Chi, S.K. Min, B.G. Park, S. Jang, and C.J. Park. 2011. Prognostic impact of c-KIT mutations in core binding factor acute myeloid leukemia. *Leuk Res*. 35:1376-1383.

Parker, P.J., L. Coussens, N. Totty, L. Rhee, S. Young, E. Chen, S. Stabel, M.D. Waterfield, and A. Ullrich. 1986. The complete primary structure of protein kinase C--the major phorbol ester receptor. *Science*. 233:853-859.

Paschka, P., G. Marcucci, A.S. Ruppert, K. Mrozek, H. Chen, R.A. Kittles, T. Vukosavljevic, D. Perrotti, J.W. Vardiman, A.J. Carroll, J.E. Kolitz, R.A. Larson, C.D. Bloomfield, Cancer, and B. Leukemia Group. 2006. Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. *J Clin Oncol*. 24:3904-3911.

Peled, A., O. Kollet, T. Ponomaryov, I. Petit, S. Franitza, V. Grabovsky, M.M. Slav, A. Nagler, O. Lider, R. Alon, D. Zipori, and T. Lapidot. 2000. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood*. 95:3289-3296.

Peschon, J.J., J.L. Slack, P. Reddy, K.L. Stocking, S.W. Sunnarborg, D.C. Lee, W.E. Russell, B.J. Castner, R.S. Johnson, J.N. Fitzner, R.W. Boyce, N. Nelson, C.J. Kozlosky, M.F. Wolfson, C.T. Rauch, D.P. Cerretti, R.J. Paxton, C.J. March, and R.A. Black. 1998. An essential role for

ectodomain shedding in mammalian development. *Science*. 282:1281-1284.

Petit, I., M. Szyper-Kravitz, A. Nagler, M. Lahav, A. Peled, L. Habler, T. Ponomaryov, R.S. Taichman, F. Arenzana-Seisdedos, N. Fujii, J. Sandbank, D. Zipori, and T. Lapidot. 2002. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nature immunology*. 3:687-694.

Pierce, A., C.M. Heyworth, S.E. Nicholls, E. Spooncer, T.M. Dexter, J.M. Lord, P.J. Owen-Lynch, G. Wark, and A.D. Whetton. 1998. An activated protein kinase C alpha gives a differentiation signal for hematopoietic progenitor cells and mimicks macrophage colony-stimulating factor-stimulated signaling events. *J Cell Biol*. 140:1511-1518.

Plow, E.F., T.A. Haas, L. Zhang, J. Loftus, and J.W. Smith. 2000. Ligand binding to integrins. *The Journal of biological chemistry*. 275:21785-21788.

Prox, J., M. Willenbrock, S. Weber, T. Lehmann, D. Schmidt-Arras, R. Schwanbeck, P. Saftig, and M. Schwake. 2012. Tetraspanin15 regulates cellular trafficking and activity of the ectodomain sheddase ADAM10. *Cellular and molecular life sciences : CMLS*. 69:2919-2932.

Qian, H., K. Tryggvason, S.E. Jacobsen, and M. Ekblom. 2006. Contribution of alpha6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with alpha4 integrins. *Blood*. 107:3503-3510.

Qiu, S., Y. Jia, H. Xing, T. Yu, J. Yu, P. Yu, K. Tang, Z. Tian, H. Wang, Y. Mi, Q. Rao, M. Wang, and J. Wang. 2014. N-Cadherin and Tie2 positive CD34(+)CD38(-)CD123(+) leukemic stem cell populations can develop acute myeloid leukemia more effectively in NOD/SCID mice. *Leuk Res*. 38:632-637.

Rabinovitz, I., L. Tsomo, and A.M. Mercurio. 2004. Protein kinase C-alpha phosphorylation of specific serines in the connecting segment of the beta 4 integrin regulates the dynamics of type II hemidesmosomes. *Mol Cell Biol*. 24:4351-4360.

Ramirez, P., M.P. Rettig, G.L. Uy, E. Deych, M.S. Holt, J.K. Ritchey, and J.F. DiPersio. 2009. BIO5192, a small molecule inhibitor of VLA-4, mobilizes hematopoietic stem and progenitor cells. *Blood*. 114:1340-1343.

Rattis, F.M., C. Voermans, and T. Reya. 2004. Wnt signaling in the stem cell niche. *Curr Opin Hematol*. 11:88-94.

Redig, A.J., and L.C. Platanias. 2008. Protein kinase C signalling in leukemia. *Leukemia & lymphoma*. 49:1255-1262.

Renstrom, J., M. Kroger, C. Peschel, and R.A. Oostendorp. 2010. How the niche regulates hematopoietic stem cells. *Chem Biol Interact*. 184:7-15.

Reuter, C.W., M.A. Morgan, and L. Bergmann. 2000. Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies? *Blood*. 96:1655-1669.

Reya, T., and H. Clevers. 2005. Wnt signalling in stem cells and cancer. *Nature*. 434:843-850.

Reya, T., A.W. Duncan, L. Ailles, J. Domen, D.C. Scherer, K. Willert, L. Hintz, R. Nusse, and I.L. Weissman. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature*. 423:409-414.

Reya, T., M. O'Riordan, R. Okamura, E. Devaney, K. Willert, R. Nusse, and R. Grosschedl. 2000. Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. *Immunity*. 13:15-24.

Rieu, P., T. Ueda, I. Haruta, C.P. Sharma, and M.A. Arnaout. 1994. The A-domain of beta 2 integrin CR3 (CD11b/CD18) is a receptor for the hookworm-derived neutrophil adhesion inhibitor NIF. *J Cell Biol*. 127:2081-2091.

Roca-Cusachs, P., N.C. Gauthier, A. Del Rio, and M.P. Sheetz. 2009. Clustering of alpha(5)beta(1) integrins determines adhesion strength whereas alpha(v)beta(3) and talin enable mechanotransduction. *Proceedings of the National Academy of Sciences of the United States of America*. 106:16245-16250.

Rocha, V., J. Cornish, E.L. Sievers, A. Filipovich, F. Locatelli, C. Peters, M. Remberger, G. Michel, W. Arcese, S. Dallorso, K. Tiedemann, A. Busca, K.W. Chan, S. Kato, J. Ortega, M. Vowels, A. Zander, G. Souillet, A. Oakill, A. Woolfrey, A.L. Pay, A. Green, F. Garnier, I. Ionescu, P. Wernet, G. Sirchia, P. Rubinstein, S. Chevret, and E. Gluckman. 2001. Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. *Blood*. 97:2962-2971.

Rocha-Perugini, V., J.M. Gonzalez-Granado, E. Tejera, S. Lopez-Martin, M. Yanez-Mo, and F. Sanchez-Madrid. 2014. Tetraspanins CD9 and CD151 at the immune synapse support T-cell integrin signaling. *European journal of immunology*. 44:1967-1975.

Ron, D., and D. Mochly-Rosen. 1995. An autoregulatory region in protein kinase C: the pseudoanchoring site. *Proceedings of the National Academy of Sciences of the United States of America*. 92:492-496.

Rosnet, O., H.J. Buhring, S. Marchetto, I. Rappold, C. Lavagna, D. Sainty, C. Arnoulet, C. Chabannon, L. Kanz, C. Hannum, and D. Birnbaum. 1996.

Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. *Leukemia*. 10:238-248.

Rosse, C., M. Linch, S. Kermorgant, A.J. Cameron, K. Boeckeler, and P.J. Parker. 2010. PKC and the control of localized signal dynamics. *Nature reviews. Molecular cell biology*. 11:103-112.

Rubinstein, E., F. Le Naour, M. Billard, M. Prenant, and C. Boucheix. 1994. CD9 antigen is an accessory subunit of the VLA integrin complexes. *European journal of immunology*. 24:3005-3013.

Ruiz-Garcia, A., S. Lopez-Lopez, J.J. Garcia-Ramirez, V. Baladron, M.J. Ruiz-Hidalgo, L. Lopez-Sanz, A. Ballesteros, J. Laborda, E.M. Monsalve, and M.J. Diaz-Guerra. 2016. The Tetraspanin TSPAN33 Controls TLR-Triggered Macrophage Activation through Modulation of NOTCH Signaling. *J Immunol*.

Ruoslahti, E. 1996. RGD and other recognition sequences for integrins. *Annual review of cell and developmental biology*. 12:697-715.

Ruseva, Z., P.X. Geiger, P. Hutzler, M. Kotzsch, B. Luber, M. Schmitt, E. Gross, and U. Reuning. 2009. Tumor suppressor KAI1 affects integrin alphavbeta3-mediated ovarian cancer cell adhesion, motility, and proliferation. *Exp Cell Res*. 315:1759-1771.

Ruvolo, P.P., L. Zhou, J.C. Watt, V.R. Ruvolo, J.K. Burks, T. Jiffar, S. Kornblau, M. Konopleva, and M. Andreeff. 2011. Targeting PKC-mediated signal transduction pathways using enzastaurin to promote apoptosis in acute myeloid leukemia-derived cell lines and blast cells. *Journal of cellular biochemistry*. 112:1696-1707.

Ryan, P.E., G.C. Davies, M.M. Nau, and S. Lipkowitz. 2006. Regulating the regulator: negative regulation of Cbl ubiquitin ligases. *Trends in biochemical sciences*. 31:79-88.

Sachs, N., N. Claessen, J. Aten, M. Kreft, G.J. Teske, A. Koeman, C.J. Zuurbier, H. Janssen, and A. Sonnenberg. 2012. Blood pressure influences end-stage renal disease of Cd151 knockout mice. *J Clin Invest*. 122:348-358.

Sadej, R., H. Romanska, D. Kavanagh, G. Baldwin, T. Takahashi, N. Kalia, and F. Berditchevski. 2010. Tetraspanin CD151 regulates transforming growth factor beta signaling: implication in tumor metastasis. *Cancer research*. 70:6059-6070.

Samokhvalov, I.M., N.I. Samokhvalova, and S. Nishikawa. 2007. Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. *Nature*. 446:1056-1061.

Scheller, M., J. Huelsken, F. Rosenbauer, M.M. Taketo, W. Birchmeier, D.G. Tenen, and A. Leutz. 2006. Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nature immunology*. 7:1037-1047.

Schlaepfer, D.D., C.R. Hauck, and D.J. Sieg. 1999. Signaling through focal adhesion kinase. *Prog Biophys Mol Biol*. 71:435-478.

Schlessinger, J. 2000. Cell signaling by receptor tyrosine kinases. *Cell*. 103:211-225.

Schlessinger, J., and A. Ullrich. 1992. Growth factor signaling by receptor tyrosine kinases. *Neuron*. 9:383-391.

Schofield, R. 1978. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*. 4:7-25.

Schonwasser, D.C., R.M. Marais, C.J. Marshall, and P.J. Parker. 1998. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol Cell Biol*. 18:790-798.

Schwartz, M.A. 2001. Integrin signaling revisited. *Trends Cell Biol*. 11:466-470.

Schwartz, S.L., Q. Yan, C.A. Telmer, K.A. Lidke, M.P. Bruchez, and D.S. Lidke. 2015. Fluorogen-activating proteins provide tunable labeling densities for tracking FcepsilonRI independent of IgE. *ACS chemical biology*. 10:539-546.

Scott, L.M., G.V. Priestley, and T. Papayannopoulou. 2003. Deletion of alpha4 integrins from adult hematopoietic cells reveals roles in homeostasis, regeneration, and homing. *Mol Cell Biol*. 23:9349-9360.

Scott, M.A., J.F. Apperley, D.M. Bloxham, H.K. Jestice, S. John, R.E. Marcus, and M.Y. Gordon. 1997. Biological properties of peripheral blood progenitor cells mobilized by cyclophosphamide and granulocyte colony-stimulating factor. *Br J Haematol*. 97:474-480.

Seales, E.C., G.A. Jurado, A. Singhal, and S.L. Bellis. 2003. Ras oncogene directs expression of a differentially sialylated, functionally altered beta1 integrin. *Oncogene*. 22:7137-7145.

Seals, D.F., and S.A. Courtneidge. 2003. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes Dev*. 17:7-30.

Seigneuret, M. 2006. Complete predicted three-dimensional structure of the facilitator transmembrane protein and hepatitis C virus receptor CD81:

conserved and variable structural domains in the tetraspanin superfamily. *Biophys J*. 90:212-227.

Seigneuret, M., A. Delaguillaumie, C. Lagaudriere-Gesbert, and H. Conjeaud. 2001. Structure of the tetraspanin main extracellular domain. A partially conserved fold with a structurally variable domain insertion. *The Journal of biological chemistry*. 276:40055-40064.

Seita, J., and I.L. Weissman. 2010. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med*. 2:640-653.

Selhuber-Unkel, C., M. Lopez-Garcia, H. Kessler, and J.P. Spatz. 2008. Cooperativity in adhesion cluster formation during initial cell adhesion. *Biophys J*. 95:5424-5431.

Sengupta, P., T. Jovanovic-Talisman, D. Skoko, M. Renz, S.L. Veatch, and J. Lippincott-Schwartz. 2011. Probing protein heterogeneity in the plasma membrane using PALM and pair correlation analysis. *Nat Methods*. 8:969-975.

Senn, H.P., C. Tran-Thang, A. Wodnar-Filipowicz, J. Jiricny, M. Fopp, A. Gratwohl, E. Signer, W. Weber, and C. Moroni. 1988. Mutation analysis of the N-ras proto-oncogene in active and remission phase of human acute leukemias. *Int J Cancer*. 41:59-64.

Serru, V., F. Le Naour, M. Billard, D.O. Azorsa, F. Lanza, C. Boucheix, and E. Rubinstein. 1999. Selective tetraspan-integrin complexes (CD81/alpha4beta1, CD151/alpha3beta1, CD151/alpha6beta1) under conditions disrupting tetraspan interactions. *The Biochemical journal*. 340 (Pt 1):103-111.

Seubert, B., H. Cui, N. Simonavicius, K. Honert, S. Schafer, U. Reuning, M. Heikenwalder, B. Mari, and A. Kruger. 2015. Tetraspanin CD63 acts as a pro-metastatic factor via beta-catenin stabilization. *Int J Cancer*. 136:2304-2315.

Shapiro, L., and W.I. Weis. 2009. Structure and biochemistry of cadherins and catenins. *Cold Spring Harb Perspect Biol*. 1:a003053.

Shen, B., M.K. Delaney, and X. Du. 2012. Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. *Curr Opin Cell Biol*. 24:600-606.

Shi, W., H. Fan, L. Shum, and R. Derynck. 2000. The tetraspanin CD9 associates with transmembrane TGF-alpha and regulates TGF-alpha-induced EGF receptor activation and cell proliferation. *J Cell Biol*. 148:591-602.

Shigeta, M., N. Sanzen, M. Ozawa, J. Gu, H. Hasegawa, and K. Sekiguchi. 2003. CD151 regulates epithelial cell-cell adhesion through PKC- and Cdc42-dependent actin cytoskeletal reorganization. *J Cell Biol*. 163:165-176.

Shimoyama, Y., T. Yoshida, M. Terada, Y. Shimosato, O. Abe, and S. Hirohashi. 1989. Molecular cloning of a human Ca2+-dependent cell-cell adhesion molecule homologous to mouse placental cadherin: its low expression in human placental tissues. *J Cell Biol*. 109:1787-1794.

Singh, B., and R.J. Coffey. 2014. From wavy hair to naked proteins: the role of transforming growth factor alpha in health and disease. *Semin Cell Dev Biol*. 28:12-21.

Sipkins, D.A., X. Wei, J.W. Wu, J.M. Runnels, D. Cote, T.K. Means, A.D. Luster, D.T. Scadden, and C.P. Lin. 2005. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature*. 435:969-973.

Sison, E.A., E. McIntyre, D. Magoon, and P. Brown. 2013. Dynamic chemotherapy-induced upregulation of CXCR4 expression: a mechanism of therapeutic resistance in pediatric AML. *Mol Cancer Res*. 11:1004-1016.

Sit, S.T., and E. Manser. 2011. Rho GTPases and their role in organizing the actin cytoskeleton. *Journal of cell science*. 124:679-683.

Slupsky, J.R., J.G. Seehafer, S.C. Tang, A. Masellis-Smith, and A.R. Shaw. 1989. Evidence that monoclonal antibodies against CD9 antigen induce specific association between CD9 and the platelet glycoprotein IIb-IIIa complex. *The Journal of biological chemistry*. 264:12289-12293.

Smith, L.H., T. Makinodan, and C.C. Congdon. 1957. Circulating rat platelets in lethally x-radiated mice given rat bone marrow. *Cancer research*. 17:367-369.

Sohn, S.K., J.G. Kim, K.W. Seo, Y.S. Chae, J.T. Jung, J.S. Suh, and K.B. Lee. 2002. GM-CSF-based mobilization effect in normal healthy donors for allogeneic peripheral blood stem cell transplantation. *Bone Marrow Transplant*. 30:81-86.

Spangrude, G.J., S. Heimfeld, and I.L. Weissman. 1988. Purification and characterization of mouse hematopoietic stem cells. *Science*. 241:58-62.

Sridhar, S.C., and C.K. Miranti. 2006. Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. *Oncogene*. 25:2367-2378.

Staal, F.J., and T.C. Luis. 2010. Wnt signaling in hematopoiesis: crucial factors for self-renewal, proliferation, and cell fate decisions. *Journal of cellular biochemistry*. 109:844-849.

Stawowy, P., C. Margeta, F. Blaschke, C. Lindschau, C. Spencer-Hansch, M. Leitges, G. Biagini, E. Fleck, and K. Graf. 2005. Protein kinase C epsilon mediates angiotensin II-induced activation of beta1-integrins in cardiac fibroblasts. *Cardiovasc Res*. 67:50-59.

Steelman, L.S., S.C. Pohnert, J.G. Shelton, R.A. Franklin, F.E. Bertrand, and J.A. McCubrey. 2004. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia*. 18:189-218.

Steinberg, S.F. 2008. Structural basis of protein kinase C isoform function. *Physiol Rev*. 88:1341-1378.

Stier, S., T. Cheng, D. Dombkowski, N. Carlesso, and D.T. Scadden. 2002. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood*. 99:2369-2378.

Stipp, C.S. 2010. Laminin-binding integrins and their tetraspanin partners as potential antimetastatic targets. *Expert Rev Mol Med*. 12:e3.

Stipp, C.S., and M.E. Hemler. 2000. Transmembrane-4-superfamily proteins CD151 and CD81 associate with alpha 3 beta 1 integrin, and selectively contribute to alpha 3 beta 1-dependent neurite outgrowth. *Journal of cell science*. 113 (Pt 11):1871-1882.

Stipp, C.S., D. Orlicky, and M.E. Hemler. 2001. FPRP, a major, highly stoichiometric, highly specific CD81- and CD9-associated protein. *The Journal of biological chemistry*. 276:4853-4862.

Stirewalt, D.L., and J.P. Radich. 2003. The role of FLT3 in haematopoietic malignancies. *Nature reviews. Cancer*. 3:650-665.

Stuck, M.W., S.M. Conley, and M.I. Naash. 2012. Defects in the outer limiting membrane are associated with rosette development in the Nrl-/- retina. *PloS one*. 7:e32484.

Stuck, M.W., S.M. Conley, and M.I. Naash. 2015. Retinal Degeneration Slow (RDS) Glycosylation Plays a Role in Cone Function and in the Regulation of RDS.ROM-1 Protein Complex Formation. *The Journal of biological chemistry*. 290:27901-27913.

Sugiyama, T., H. Kohara, M. Noda, and T. Nagasawa. 2006. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity*. 25:977-988.

Sulzmaier, F.J., C. Jean, and D.D. Schlaepfer. 2014. FAK in cancer: mechanistic findings and clinical applications. *Nature reviews. Cancer*. 14:598-610.

Suzuki, J., S. Yamasaki, J. Wu, G.A. Koretzky, and T. Saito. 2007. The actin cloud induced by LFA-1-mediated outside-in signals lowers the threshold for T-cell activation. *Blood*. 109:168-175.

Swanson, C.J., M. Ritt, W. Wang, M.J. Lang, A. Narayan, J.J. Tesmer, M. Westfall, and S. Sivaramakrishnan. 2014. Conserved modular domains team up to latch-open active protein kinase Calpha. *The Journal of biological chemistry*. 289:17812-17829.

Swindall, A.F., A.I. Londono-Joshi, M.J. Schultz, N. Fineberg, D.J. Buchsbaum, and S.L. Bellis. 2013. ST6Gal-I protein expression is upregulated in human epithelial tumors and correlates with stem cell markers in normal tissues and colon cancer cell lines. *Cancer research*. 73:2368-2378.

Tadokoro, S., S.J. Shattil, K. Eto, V. Tai, R.C. Liddington, J.M. de Pereda, M.H. Ginsberg, and D.A. Calderwood. 2003. Talin binding to integrin beta tails: a final common step in integrin activation. *Science*. 302:103-106.

Taichman, R., M. Reilly, R. Verma, K. Ehrenman, and S. Emerson. 2001. Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors. *Br J Haematol*. 112:438-448.

Taichman, R.S., and S.G. Emerson. 1994. Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. *J Exp Med*. 179:1677-1682.

Takada, Y., X. Ye, and S. Simon. 2007. The integrins. *Genome Biol*. 8:215.

Takahashi, M., T. Sugiura, M. Abe, K. Ishii, and K. Shirasuna. 2007. Regulation of c-Met signaling by the tetraspanin KAI-1/CD82 affects cancer cell migration. *Int J Cancer*. 121:1919-1929.

Takeichi, M. 1995. Morphogenetic roles of classic cadherins. *Current opinion in cell biology*. 7:619-627.

Tallman, M.S., D.G. Gilliland, and J.M. Rowe. 2005. Drug therapy for acute myeloid leukemia. *Blood*. 106:1154-1163.

Taniguchi, H., T. Toyoshima, K. Fukao, and H. Nakauchi. 1996. Presence of hematopoietic stem cells in the adult liver. *Nat Med*. 2:198-203.

Taussig, D.C., D.J. Pearce, C. Simpson, A.Z. Rohatiner, T.A. Lister, G. Kelly, J.L. Luongo, G.A. Danet-Desnoyers, and D. Bonnet. 2005. Hematopoietic

stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood*. 106:4086-4092.

Tavassoli, M. 1981. Structure and function of sinusoidal endothelium of bone marrow. *Prog Clin Biol Res*. 59B:249-256.

Teixido, J., M.E. Hemler, J.S. Greenberger, and P. Anklesaria. 1992. Role of beta 1 and beta 2 integrins in the adhesion of human CD34hi stem cells to bone marrow stroma. *J Clin Invest*. 90:358-367.

Tejera, E., V. Rocha-Perugini, S. Lopez-Martin, D. Perez-Hernandez, A.I. Bachir, A.R. Horwitz, J. Vazquez, F. Sanchez-Madrid, and M. Yanez-Mo. 2013. CD81 regulates cell migration through its association with Rac GTPase. *Molecular biology of the cell*. 24:261-273.

Tenen, D.G. 2003. Disruption of differentiation in human cancer: AML shows the way. *Nature reviews. Cancer*. 3:89-101.

ter Huurne, M., C.G. Figdor, and R. Torensma. 2010. Hematopoietic stem cells are coordinated by the molecular cues of the endosteal niche. *Stem Cells Dev*. 19:1131-1141.

Termini, C.M., M.L. Cotter, K.D. Marjon, T. Buranda, K.A. Lidke, and J.M. Gillette. 2014. The membrane scaffold CD82 regulates cell adhesion by altering alpha4 integrin stability and molecular density. *Molecular biology of the cell*. 25:1560-1573.

Termini, C.M., and J.M. Gillette. 2017. Tetraspanins Function as Regulators of Cellular Signaling. *Frontiers in Cell and Developmental Biology*. 5.

Termini, C.M., K.A. Lidke, and J.M. Gillette. 2016. Tetraspanin CD82 Regulates the Spatiotemporal Dynamics of PKCalpha in Acute Myeloid Leukemia. *Sci Rep*. 6:29859.

Thoren, L.A., K. Liuba, D. Bryder, J.M. Nygren, C.T. Jensen, H. Qian, J. Antonchuk, and S.E. Jacobsen. 2008. Kit regulates maintenance of quiescent hematopoietic stem cells. *J Immunol*. 180:2045-2053.

Thorne, B.A., and G.D. Plowman. 1994. The heparin-binding domain of amphiregulin necessitates the precursor pro-region for growth factor secretion. *Mol Cell Biol*. 14:1635-1646.

Tian, T., A. Harding, K. Inder, S. Plowman, R.G. Parton, and J.F. Hancock. 2007. Plasma membrane nanoswitches generate high-fidelity Ras signal transduction. *Nat Cell Biol*. 9:905-914.

Till, J.E., and C.E. Mc. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res*. 14:213-222.

Tomas, A., C.E. Futter, and E.R. Eden. 2014. EGF receptor trafficking: consequences for signaling and cancer. *Trends Cell Biol*. 24:26-34.

Tomlinson, M.G., A.F. Williams, and M.D. Wright. 1993. Epitope mapping of anti-rat CD53 monoclonal antibodies. Implications for the membrane orientation of the Transmembrane 4 Superfamily. *European journal of immunology*. 23:136-140.

Tsai, F.D., and M.R. Philips. 2012. Rac1 gets fattier. *EMBO J*. 31:517-518.

Tsai, Y.C., and A.M. Weissman. 2011. Dissecting the diverse functions of the metastasis suppressor CD82/KAI1. *FEBS Lett*. 585:3166-3173.

Tsukamoto, S., M. Takeuchi, T. Kawaguchi, E. Togasaki, A. Yamazaki, Y. Sugita, T. Muto, S. Sakai, Y. Takeda, C. Ohwada, E. Sakaida, N. Shimizu, K. Nishii, M. Jiang, K. Yokote, H. Bujo, and C. Nakaseko. 2014. Tetraspanin CD9 modulates ADAM17-mediated shedding of LR11 in leukocytes. *Exp Mol Med*. 46:e89.

Tuteja, N. 2009. Signaling through G protein coupled receptors. *Plant Signal Behav*. 4:942-947.

Uchtmann, K., E.R. Park, A. Bergsma, J. Segula, M.J. Edick, and C.K. Miranti. 2015. Homozygous loss of mouse tetraspanin CD82 enhances integrin alphaIIbbeta3 expression and clot retraction in platelets. *Exp Cell Res*. 339:261-269.

Valenta, T., G. Hausmann, and K. Basler. 2012. The many faces and functions of beta-catenin. *EMBO J*. 31:2714-2736.

Valley, C.C., S. Liu, D.S. Lidke, and K.A. Lidke. 2015. Sequential superresolution imaging of multiple targets using a single fluorophore. *PloS one*. 10:e0123941.

Van Den Berg, D.J., A.K. Sharma, E. Bruno, and R. Hoffman. 1998. Role of members of the Wnt gene family in human hematopoiesis. *Blood*. 92:3189-3202.

van den Dries, K., S.L. Schwartz, J. Byars, M.B. Meddens, M. Bolomini-Vittori, D.S. Lidke, C.G. Figdor, K.A. Lidke, and A. Cambi. 2013. Dual-color superresolution microscopy reveals nanoscale organization of mechanosensory podosomes. *Molecular biology of the cell*. 24:2112-2123.

van Kooyk, Y., and C.G. Figdor. 2000. Avidity regulation of integrins: the driving force in leukocyte adhesion. *Curr Opin Cell Biol*. 12:542-547.

van Kooyk, Y., P. Weder, K. Heije, and C.G. Figdor. 1994. Extracellular Ca2+ modulates leukocyte function-associated antigen-1 cell surface distribution

on T lymphocytes and consequently affects cell adhesion. *J Cell Biol*. 124:1061-1070.

van Spriel, A.B. 2011. Tetraspanins in the humoral immune response. *Biochemical Society transactions*. 39:512-517.

van Spriel, A.B., S. de Keijzer, A. van der Schaaf, K.H. Gartlan, M. Sofi, A. Light, P.C. Linssen, J.B. Boezeman, M. Zuidscherwoude, I. Reinieren-Beeren, A. Cambi, F. Mackay, D.M. Tarlinton, C.G. Figdor, and M.D. Wright. 2012. The tetraspanin CD37 orchestrates the alpha(4)beta(1) integrin-Akt signaling axis and supports long-lived plasma cell survival. *Sci Signal*. 5:ra82.

Varga-Szabo, D., I. Pleines, and B. Nieswandt. 2008. Cell adhesion mechanisms in platelets. *Arterioscler Thromb Vasc Biol*. 28:403-412.

Varnum-Finney, B., L. Xu, C. Brashem-Stein, C. Nourigat, D. Flowers, S. Bakkour, W.S. Pear, and I.D. Bernstein. 2000. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat Med*. 6:1278-1281.

Veatch, S.L., B.B. Machta, S.A. Shelby, E.N. Chiang, D.A. Holowka, and B.A. Baird. 2012. Correlation functions quantify super-resolution images and estimate apparent clustering due to over-counting. *PloS one*. 7:e31457.

Verma, U.N., B. van den Blink, R. Pillai, J. Chawla, A. Mazumder, H.B. Herscowitz, and K.R. Meehan. 1999. Paclitaxel vs cyclophosphamide in peripheral blood stem cell mobilization: comparative studies in a murine model. *Exp Hematol*. 27:553-560.

Vermeulen, M., F. Le Pesteur, M.C. Gagnerault, J.Y. Mary, F. Sainteny, and F. Lepault. 1998. Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. *Blood*. 92:894-900.

Waldron, R.T., O. Rey, T. Iglesias, T. Tugal, D. Cantrell, and E. Rozengurt. 2001. Activation loop Ser744 and Ser748 in protein kinase D are transphosphorylated in vivo. *The Journal of biological chemistry*. 276:32606-32615.

Walter, R.B., T.A. Alonzo, R.B. Gerbing, P.A. Ho, F.O. Smith, S.C. Raimondi, B.A. Hirsch, A.S. Gamis, J.L. Franklin, C.A. Hurwitz, M.R. Loken, and S. Meshinchi. 2010a. High expression of the very late antigen-4 integrin independently predicts reduced risk of relapse and improved outcome in pediatric acute myeloid leukemia: a report from the children's oncology group. *J Clin Oncol*. 28:2831-2838.

Walter, R.B., H.M. Kantarjian, X. Huang, S.A. Pierce, Z. Sun, H.M. Gundacker, F. Ravandi, S.H. Faderl, M.S. Tallman, F.R. Appelbaum, and E.H. Estey.

2010b. Effect of complete remission and responses less than complete remission on survival in acute myeloid leukemia: a combined Eastern Cooperative Oncology Group, Southwest Oncology Group, and M. D. Anderson Cancer Center Study. *J Clin Oncol*. 28:1766-1771.

Wang, H., W. Zhang, J. Zhao, L. Zhang, M. Liu, G. Yan, J. Yao, H. Yu, and P. Yang. 2012a. N-Glycosylation pattern of recombinant human CD82 (KAI1), a tumor-associated membrane protein. *J Proteomics*. 75:1375-1385.

Wang, J.F., I.W. Park, and J.E. Groopman. 2000. Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. *Blood*. 95:2505-2513.

Wang, X.Q., Q. Yan, P. Sun, J.W. Liu, L. Go, and S.M. McDaniel. 2007a. Suppression of epidermal growth factor receptor signaling by protein kinase C activation requires CD82, caveolin-1, and ganglioside. *Cancer research*. 67:9986-9995.

Wang, X.Q., Q. Yan, P. Sun, J.W. Liu, L. Go, S.M. McDaniel, and A.S. Paller. 2007b. Suppression of epidermal growth factor receptor signaling by protein kinase C-alpha activation requires CD82, caveolin-1, and ganglioside. *Cancer research*. 67:9986-9995.

Wang, Y., X. Tong, E.S. Omoregie, W. Liu, S. Meng, and X. Ye. 2012b. Tetraspanin 6 (TSPAN6) negatively regulates retinoic acid-inducible gene I-like receptor-mediated immune signaling in a ubiquitination-dependent manner. *The Journal of biological chemistry*. 287:34626-34634.

Wang, Y., Y. Wang, H. Zhang, Y. Gao, C. Huang, A. Zhou, Y. Zhou, and Y. Li. 2016. Sequential posttranslational modifications regulate PKC degradation. *Molecular biology of the cell*. 27:410-420.

Watanabe, T., Y. Kawano, S. Kanamaru, T. Onishi, S. Kaneko, Y. Wakata, R. Nakagawa, A. Makimoto, Y. Kuroda, Y. Takaue, and J.E. Talmadge. 1999. Endogenous interleukin-8 (IL-8) surge in granulocyte colony-stimulating factor-induced peripheral blood stem cell mobilization. *Blood*. 93:1157-1163.

Wayner, E.A., A. Garcia-Pardo, M.J. Humphries, J.A. McDonald, and W.G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J Cell Biol*. 109:1321-1330.

Wee, J.L., K.E. Schulze, E.L. Jones, L. Yeung, Q. Cheng, C.F. Pereira, A. Costin, G. Ramm, A.B. van Spriel, M.J. Hickey, and M.D. Wright. 2015.

Tetraspanin CD37 Regulates beta2 Integrin-Mediated Adhesion and Migration in Neutrophils. *J Immunol*. 195:5770-5779.

Wegener, K.L., and I.D. Campbell. 2008. Transmembrane and cytoplasmic domains in integrin activation and protein-protein interactions (review). *Mol Membr Biol*. 25:376-387.

Wehrle-Haller, B. 2012. Assembly and disassembly of cell matrix adhesions. *Curr Opin Cell Biol*. 24:569-581.

White, A., P.W. Lamb, and J.C. Barrett. 1998. Frequent downregulation of the KAI1(CD82) metastasis suppressor protein in human cancer cell lines. *Oncogene*. 16:3143-3149.

Wight, T.N., M.G. Kinsella, A. Keating, and J.W. Singer. 1986. Proteoglycans in human long-term bone marrow cultures: biochemical and ultrastructural analyses. *Blood*. 67:1333-1343.

Willard, F.S., and M.F. Crouch. 2001. MEK, ERK, and p90RSK are present on mitotic tubulin in Swiss 3T3 cells: a role for the MAP kinase pathway in regulating mitotic exit. *Cellular signalling*. 13:653-664.

Willert, K., J.D. Brown, E. Danenberg, A.W. Duncan, I.L. Weissman, T. Reya, J.R. Yates, 3rd, and R. Nusse. 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature*. 423:448-452.

Williams, D.A., M. Rios, C. Stephens, and V.P. Patel. 1991. Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. *Nature*. 352:438-441.

Winkler, I.G., V. Barbier, B. Nowlan, R.N. Jacobsen, C.E. Forristal, J.T. Patton, J.L. Magnani, and J.P. Levesque. 2012. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat Med*. 18:1651-1657.

Winterwood, N.E., A. Varzavand, M.N. Meland, L.K. Ashman, and C.S. Stipp. 2006. A critical role for tetraspanin CD151 in alpha3beta1 and alpha6beta4 integrin-dependent tumor cell functions on laminin-5. *Molecular biology of the cell*. 17:2707-2721.

Wright, M.D., J. Ni, and G.B. Rudy. 2000. The L6 membrane proteins--a new four-transmembrane superfamily. *Protein Sci*. 9:1594-1600.

Wu, A.M., J.E. Till, L. Siminovitch, and E.A. McCulloch. 1968. Cytological evidence for a relationship between normal hemotopoietic colony-forming cells and cells of the lymphoid system. *J Exp Med*. 127:455-464.

Xie, T., and A.C. Spradling. 2000. A niche maintaining germ line stem cells in the Drosophila ovary. *Science*. 290:328-330.

Xiong, J.P., T. Stehle, B. Diefenbach, R. Zhang, R. Dunker, D.L. Scott, A. Joachimiak, S.L. Goodman, and M.A. Arnaout. 2001. Crystal structure of the extracellular segment of integrin alpha Vbeta3. *Science*. 294:339-345.

Xu, C., Y.H. Zhang, M. Thangavel, M.M. Richardson, L. Liu, B. Zhou, Y. Zheng, R.S. Ostrom, and X.A. Zhang. 2009a. CD82 endocytosis and cholesterol-dependent reorganization of tetraspanin webs and lipid rafts. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 23:3273-3288.

Xu, D., C. Sharma, and M.E. Hemler. 2009b. Tetraspanin12 regulates ADAM10-dependent cleavage of amyloid precursor protein. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 23:3674-3681.

Xu, H., S.J. Lee, E. Suzuki, K.D. Dugan, A. Stoddard, H.S. Li, L.A. Chodosh, and C. Montell. 2004. A lysosomal tetraspanin associated with retinal degeneration identified via a genome-wide screen. *EMBO J*. 23:811-822.

Xu, L., and R.O. Hynes. 2007. GPR56 and TG2: possible roles in suppression of tumor growth by the microenvironment. *Cell cycle*. 6:160-165.

Xu, Y., J. Zhuo, Y. Duan, B. Shi, X. Chen, X. Zhang, L. Xiao, J. Lou, R. Huang, Q. Zhang, X. Du, M. Li, D. Wang, and D. Shi. 2014. Construction of protein profile classification model and screening of proteomic signature of acute leukemia. *International journal of clinical and experimental pathology*. 7:5569-5581.

Yamada, M., Y. Sumida, A. Fujibayashi, K. Fukaguchi, N. Sanzen, R. Nishiuchi, and K. Sekiguchi. 2008. The tetraspanin CD151 regulates cell morphology and intracellular signaling on laminin-511. *FEBS J*. 275:3335-3351.

Yamaguchi, M., K. Ikebuchi, F. Hirayama, N. Sato, Y. Mogi, J. Ohkawara, Y. Yoshikawa, K. Sawada, T. Koike, and S. Sekiguchi. 1998. Different adhesive characteristics and VLA-4 expression of CD34(+) progenitors in G0/G1 versus S+G2/M phases of the cell cycle. *Blood*. 92:842-848.

Yanez-Mo, M., A. Alfranca, C. Cabanas, M. Marazuela, R. Tejedor, M.A. Ursa, L.K. Ashman, M.O. de Landazuri, and F. Sanchez-Madrid. 1998. Regulation of endothelial cell motility by complexes of tetraspan molecules CD81/TAPA-1 and CD151/PETA-3 with alpha3 beta1 integrin localized at endothelial lateral junctions. *J Cell Biol*. 141:791-804.

Yanez-Mo, M., O. Barreiro, M. Gordon-Alonso, M. Sala-Valdes, and F. Sanchez-Madrid. 2009. Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. *Trends Cell Biol*. 19:434-446.

Yanez-Mo, M., F. Sanchez-Madrid, and C. Cabanas. 2011. Membrane proteases and tetraspanins. *Biochemical Society transactions*. 39:541-546.

Yanez-Mo, M., R. Tejedor, P. Rousselle, and F. Sanchez -Madrid. 2001. Tetraspanins in intercellular adhesion of polarized epithelial cells: spatial and functional relationship to integrins and cadherins. *Journal of cell science*. 114:577-587.

Yang, X., C. Claas, S.K. Kraeft, L.B. Chen, Z. Wang, J.A. Kreidberg, and M.E. Hemler. 2002. Palmitoylation of tetraspanin proteins: modulation of CD151 lateral interactions, subcellular distribution, and integrin-dependent cell morphology. *Molecular biology of the cell*. 13:767-781.

Yang, X., O.V. Kovalenko, W. Tang, C. Claas, C.S. Stipp, and M.E. Hemler. 2004. Palmitoylation supports assembly and function of integrin-tetraspanin complexes. *J Cell Biol*. 167:1231-1240.

Yap, A.S., W.M. Brieher, and B.M. Gumbiner. 1997a. Molecular and functional analysis of cadherin-based adherens junctions. *Annual review of cell and developmental biology*. 13:119-146.

Yap, A.S., W.M. Brieher, M. Pruschy, and B.M. Gumbiner. 1997b. Lateral clustering of the adhesive ectodomain: a fundamental determinant of cadherin function. *Current biology : CB*. 7:308-315.

Yauch, R.L., D.P. Felsenfeld, S.K. Kraeft, L.B. Chen, M.P. Sheetz, and M.E. Hemler. 1997. Mutational evidence for control of cell adhesion through integrin diffusion/clustering, independent of ligand binding. *J Exp Med*. 186:1347-1355.

Yauch, R.L., and M.E. Hemler. 2000. Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase. *The Biochemical journal*. 351 Pt 3:629-637.

Ye, F., C. Kim, and M.H. Ginsberg. 2011. Molecular mechanism of inside-out integrin regulation. *J Thromb Haemost*. 9 Suppl 1:20-25.

Yoshida, T., Y. Kawano, K. Sato, Y. Ando, J. Aoki, Y. Miura, J. Komano, Y. Tanaka, and Y. Koyanagi. 2008. A CD63 mutant inhibits T-cell tropic human immunodeficiency virus type 1 entry by disrupting CXCR4 trafficking to the plasma membrane. *Traffic*. 9:540-558.

Yoshihara, H., F. Arai, K. Hosokawa, T. Hagiwara, K. Takubo, Y. Nakamura, Y. Gomei, H. Iwasaki, S. Matsuoka, K. Miyamoto, H. Miyazaki, T. Takahashi,

and T. Suda. 2007. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell*. 1:685-697.

Yu, Z., T.G. Pestell, M.P. Lisanti, and R.G. Pestell. 2012. Cancer stem cells. *Int J Biochem Cell Biol*. 44:2144-2151.

Zabkiewicz, J., L. Pearn, R.K. Hills, R.G. Morgan, A. Tonks, A.K. Burnett, and R.L. Darley. 2014. The PDK1 master kinase is over-expressed in acute myeloid leukemia and promotes PKC-mediated survival of leukemic blasts. *Haematologica*. 99:858-864.

Zaidel-Bar, R., S. Itzkovitz, A. Ma'ayan, R. Iyengar, and B. Geiger. 2007. Functional atlas of the integrin adhesome. *Nat Cell Biol*. 9:858-867.

Zaitseva, L., M.Y. Murray, M.S. Shafat, M.J. Lawes, D.J. MacEwan, K.M. Bowles, and S.A. Rushworth. 2014. Ibrutinib inhibits SDF1/CXCR4 mediated migration in AML. *Oncotarget*. 5:9930-9938.

Zeng, Z., I.J. Samudio, M. Munsell, J. An, Z. Huang, E. Estey, M. Andreeff, and M. Konopleva. 2006. Inhibition of CXCR4 with the novel RCP168 peptide overcomes stroma-mediated chemoresistance in chronic and acute leukemias. *Mol Cancer Ther*. 5:3113-3121.

Zeng, Z., Y.X. Shi, I.J. Samudio, R.Y. Wang, X. Ling, O. Frolova, M. Levis, J.B. Rubin, R.R. Negrin, E.H. Estey, S. Konoplev, M. Andreeff, and M. Konopleva. 2009. Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. *Blood*. 113:6215-6224.

Zhang, B., M. Li, T. McDonald, T.L. Holyoake, R.T. Moon, D. Campana, L. Shultz, and R. Bhatia. 2013. Microenvironmental protection of CML stem and progenitor cells from tyrosine kinase inhibitors through N-cadherin and Wnt-beta-catenin signaling. *Blood*. 121:1824-1838.

Zhang, F., J.E. Michaelson, S. Moshiach, N. Sachs, W. Zhao, Y. Sun, A. Sonnenberg, J.M. Lahti, H. Huang, and X.A. Zhang. 2011. Tetraspanin CD151 maintains vascular stability by balancing the forces of cell adhesion and cytoskeletal tension. *Blood*. 118:4274-4284.

Zhang, H., J.M. Casasnovas, M. Jin, J.H. Liu, C.G. Gahmberg, T.A. Springer, and J.H. Wang. 2008. An unusual allosteric mobility of the C-terminal helix of a high-affinity alphaL integrin I domain variant bound to ICAM-5. *Mol Cell*. 31:432-437.

Zhang, J., K. Leiderman, J.R. Pfeiffer, B.S. Wilson, J.M. Oliver, and S.L. Steinberg. 2006. Characterizing the topography of membrane receptors

and signaling molecules from spatial patterns obtained using nanometer-scale electron-dense probes and electron microscopy. *Micron*. 37:14-34.

Zhang, J., and L. Li. 2008. Stem cell niche: microenvironment and beyond. *The Journal of biological chemistry*. 283:9499-9503.

Zhang, J., C. Niu, L. Ye, H. Huang, X. He, W.G. Tong, J. Ross, J. Haug, T. Johnson, J.Q. Feng, S. Harris, L.M. Wiedemann, Y. Mishina, and L. Li. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*. 425:836-841.

Zhang, X.A., A.L. Bontrager, and M.E. Hemler. 2001. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. *The Journal of biological chemistry*. 276:25005-25013.

Zhang, Y., S. Sivasankar, W.J. Nelson, and S. Chu. 2009. Resolving cadherin interactions and binding cooperativity at the single-molecule level. *Proceedings of the National Academy of Sciences of the United States of America*. 106:109-114.

Zhi, L., M. Wang, Q. Rao, F. Yu, Y. Mi, and J. Wang. 2010. Enrichment of N-Cadherin and Tie2-bearing CD34+/CD38-/CD123+ leukemic stem cells by chemotherapy-resistance. *Cancer Lett*. 296:65-73.

Zhou, B., L. Liu, M. Reddivari, and X.A. Zhang. 2004. The palmitoylation of metastasis suppressor KAI1/CD82 is important for its motility- and invasiveness-inhibitory activity. *Cancer research*. 64:7455-7463.

Ziegler, W.H., A.R. Gingras, D.R. Critchley, and J. Emsley. 2008. Integrin connections to the cytoskeleton through talin and vinculin. *Biochemical Society transactions*. 36:235-239.

Zimmerman, B., B. Kelly, B.J. McMillan, T.C. Seegar, R.O. Dror, A.C. Kruse, and S.C. Blacklow. 2016. Crystal Structure of a Full-Length Human Tetraspanin Reveals a Cholesterol-Binding Pocket. *Cell*. 167:1041-1051 e1011.

Zohren, F., D. Toutzaris, V. Klarner, H.P. Hartung, B. Kieseier, and R. Haas. 2008. The monoclonal anti-VLA-4 antibody natalizumab mobilizes CD34+ hematopoietic progenitor cells in humans. *Blood*. 111:3893-3895.

Zoller, M. 2009. Tetraspanins: push and pull in suppressing and promoting metastasis. *Nature reviews. Cancer*. 9:40-55.

Zuidscherwoude, M., F. Gottfert, V.M. Dunlock, C.G. Figdor, G. van den Bogaart, and A.B. van Spriel. 2015. The tetraspanin web revisited by super-resolution microscopy. *Sci Rep*. 5:12201.