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PLAC1 affects cell to cell communication by interacting with the desmosome complex

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Abstract

Introduction—X-linked PLAC1 is highly expressed in placenta during embryogenesis, and when ablated in mice, causes aberrant placental cell layer organization. It is also highly expressed in many types of cancer cell-lines. Although it has been shown that it promotes AKT phosphorylation in cancer cells, the exact mechanism by which it influences placental layer differentiation is unclear.

Methods—To investigate the mechanism of action of PLAC1 we did cell fractionation and immunoprecipitation of the protein and Mass Spectrometry analysis to identify its interaction partners. The associated proteins were directly tested for interactions by co-transfection with PLAC1 and immunoprecipitation. Mutations in the ZP-N domain of PLAC1 were introduced to assess its involvement in the interactions.

Results—We provide evidence that Desmoglein-2 (DSG2), a component of the membrane-associated desmosomal complex, directly interacts with PLAC1. Mutations of cysteines in ZP-N domain disrupt the interaction between PLAC1 and DSG-2.

Discussion—Because desmosomes are responsible for establishing lateral cell-cell junctions, we suggest that direct interaction with the lateral junction protein complex may be implicated in the PLAC1 effects on cell-cell interactions, and thereby on the layer structure of the placenta.

Introduction:

X-linked PLAC1 is a placental protein whose expression is elevated during development specifically in placenta, in the trophoblast giant cells and the labyrinthine layer derived from the trophoblast lineage [1]. Deletion of Plac1 led to placental defects in mice, with placental hyperplasia and junctional zone encroachment into the labyrinthine layer. Furthermore, in crosses between mutant and wild-type Plac1, poor male progeny recovery was observed when the knock-out allele was maternally inherited, and led to significant loss of homozygous KO females when mutant alleles were derived from both parents [2].

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Confirming and extending the observations of Jackman et al. [2], Plac1 has been shown not to be essential for implantation, but maternally expressed Plac1 is central to post implantation embryonic development [3]. When Muto et al. [3] then restored Plac1 expression in ES cells by lentiviral transduction into blastocysts from X—X— females mated with wild type males and transplanted the blastocysts into pseudo-pregnant mice; the hyperplastic junctional zone persisted and a normal phenotype was not restored. Thus, fetal expression of PLAC1 is also probably necessary for proper placental regulation and survival of progeny.

Although PLAC1 is undetectable in adult somatic tissues, it is upregulated in a wide variety of cancer cells [4–6]. The regulation of its expression is complex: we have previously shown that *PLAC1* is transcribed from two promoters P1 and P2 [7] and Tp53 acts as a repressor at P1 promoter while pRB in combination with NCOA2 as a cofactor stimulates *PLAC1* P1 promoter in the presence of RXR and LXR [8]. Further features of complex expression were reported by Massabal et al., [9], who suggested that FGF-7 stimulates PLAC1 mRNA and FGF-7 along with its receptor FGFR-2b mediates PLAC1 stimulation via MAP kinase and the PI-3 kinase-dependent signaling pathway. In results that suggested a possible role for PLAC1 in some cancers, Koslowski et al., [4] showed that silencing of PLAC1 expression in breast cancer cells led to loss of invasiveness, thus making it an attractive potential target in breast cancer cell-lines. They also showed that inhibition of PLAC1 expression led to loss or reduction of AKT phosphorylation and inhibition of Cyclin-D1. Extending these observations Roldán et al. [10] showed that PLAC1 interacts with FGF7 and its receptor FGFR2IIIb to form a trimeric complex, and signaling via FGFR2IIIb activates AKT phosphorylation.

In spite of these clues to its expression and function, how PLAC1 knockout leads to placental hyperplasia and hydrocephalus during embryonic development in knock-out mice [11] has remained obscure. As an approach to study its effects, we have asked whether there are likely interactions of PLAC1 with other cellular protein components. To identify and characterize any direct interacting partners we carried out immunoprecipitation using an antigen tagged-PLAC1 expressed in BeWo cells – a placental derived cell-line. Among the immunoprecipitated proteins we found consistent and pronounced enrichment of desmosomal proteins. We have further characterized those interactions and discuss their possible implications.

Methods and Materials:

Materials

Human placental choriocarcinoma cell line BeWo (CCL-98), JEG3 (CCL-HTB36), JAR (CCL-144) and SV40 T-antigen transformed human embryonic kidney cell line 293 (CCL-1573) were obtained from ATCC (Manassas, VA, USA) and cultured in the recommended medium with 10% fetal bovine serum (FBS) in 5% CO2/95% air at 37°C according to the protocol from ATCC. All media were purchased from Invitrogen. Anti-PLAC1 (SC-365919), anti-DSG2 (SC-20115), anti-Myc (SC-40) and anti-Actin (SC-8432) were purchased from Santa Cruz; anti-V5 antibody from Thermofisher (46-0705); anti-GFP (632460), anti-mCherry (632543) from Takara Bio Inc.

Plasmids and Primers

PLAC1 fusion constructions, and DSG2-Myc constructions.

PLAC1 coding sequence was amplified from cDNA library [12] and cloned into pcDNA3.1-V5-His vector (Thermofisher) using the primers listed below. PLAC1-F: 5' CCACCATGAAAGTTTTTAAGTTCATAGGACT and PLAC1-R: 5' TTTCATGGACCCAATCATATCATCTG

PLAC1-EGFP plasmid was constructed using following primers to amplify Plac1 cDNA from PCR. The PCR products were ligated into Topo pcDNA3.1 vector. The resulting construct was cut with HindIII and Age1, followed by the ligation of this Hind3 and Age 1 fragment to pEGFP-N1 vector to form pCMV-PLAC1-EGFP. The primers used for this construction were as follows:

Forward primer:

5' AAGCTTAAGCTTCCACCATGAAAGTTTTTAAGTTCATAGGACT

Reverse primer:

5' ACCGGTACCGGTGCCATGGACCCAATCATATCATCTG

Following primers were used for the construction of PLAC1 mutations:

PLAC1-M1-F: GTCCAATGACTGTGCTGGCCTCCATAGACTGGTTCATG

PLAC1-M1-R: CATGAACCAGTCTATGGAGGCCAGCACAGTCATTGGAC,

PLAC1-M2-F: CCTACCGTGTTACTGAAGCTGGCATCAGGGCCAAAGC

PLAC1-M2-R: GCTTTGGCCCTGATGCCAGCTTCAGTAACACGGTAGG. The amplified products were cloned into pcDNA3.1-V5/His vector and mutations were verified by sequencing the resulting plasmids.

Construction of DSG2-N and -C-terminal plasmids.

The plasmids pCMV-Desmoglein2-Myc (#32232) and pCMV-Desmoglein2-mCherry (#36991) were purchased from Addgene.

To construct N-DSG-2 and C-DSG2 expression vectors, pCMV-DSG2-Myc was digested with BamHI and Acc65I, which generated two bands. The N-terminus1884bp (coding for amino acids 1-633) was ligated to pcDNA-Myc/His vector, resulting in pcDNA-DSG2-N-Myc plasmid, and the C-terminus 1478bp (amino acids 634-1117) including Myc-tag and stop codon was ligated to pEGFP-vector resulting in pEGFP-DSG2C-Myc.

Transient transfections

cDNA plasmid constructs were transfected into BeWo cells by Lipofectamine 2000 Plus reagent (Invitrogen). 72 hours later, BeWo cells were lysed in RIPA buffer. After centrifugation at 13,000rpm for 10 minutes at 4°C. Protein expression was determined by SDS-page gel and Western Blot.

Immunoprecipitation and Western blot

The cells were solubilized in RIPA buffer with cOmplete™ ULTRA Tablets (Roche 05892791001) on ice for 15 min then centrifuged at 4°C for 15 min at 13,000 rpm. After centrifugation, supernatants were transferred to fresh tubes and incubated with antibody at 4°C for overnight. The immunocomplexes were washed four times with lysis Buffer and analyzed by SDS-PAGE. For Western blot, the membrane was blocked with 5% nonfat milk at room temperature for one hour and incubated with antibody at 4°C overnight. Blots were washed with TBS buffer for five times prior to incubation with the peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (GE health) as required for 45 minutes and washed again in TBS buffer following second antibody incubation. The immunoreactive bands were detected with the enhanced chemiluminescence reagent (Amersham) by exposure to GenHunter Blue film.

Cell fractionation

BeWo cells were transient transfected with PLAC1-V5. After 72 hrs, cells were harvested washed in PBS and fractionated with Pierce kit (cat# 78840). 11mg/well protein was loaded on an SDS-PAGE gel. Proteins were transferred to PVDF membrane and incubated with anti-V5 (1:3000) antibody at 4°C overnight. Membranes were washed for 15 minutes at room temperature (RT) for 4 times with TBS with 1% triton. Respective Anti-mouse HRP in relation to the antibody used (1:10000) was added and incubated at room temperature for 45 minutes and washed for 15’ with TBS, at RT for 4 times. The membranes were exposed to GenHunter Blue film.

Immunoprecipitation and Mass Spectrometry

BeWo cells were transfected with PLAC1-V5 or control vector. 72 hours later, cells were solubilized in IP buffer (Tris 50mM, pH 7.4, NaCl 150mM, $MgCl_2$ 2 mM, EDTA 2mM. 0.1%Triton X-100 and 0.1%Igepal CA-630) on the ice for 15 minutes and passed through 26-gauge needle for three times to disperse large aggregates. The lysates were centrifuged at 13,000 at 4C for 15 minutes and the supernatants were incubated with goat anti-V5 agarose beads (S190-119, Bethyl lab) at 4°C, overnight. The beads were washed X5 with IP buffer at 4C, 15 minutes each, on a rotator. The final IP complexes were eluted by 0.5N NH4OH with 0.5mM EDTA and dried under vacuum. Mass Spectrometry was performed at Mass Spectrometry and Proteomics facility, John Hopkins School of Medicine.

Results:

Endogenous Plac1 multimerizes to form higher order complexes:

PLAC1 is naturally expressed in BeWo, Jeg3, or Jar cells, all placentally derived, and is abundantly expressed in Cos7 cells transfected with a *PLAC1* expression vector. All of these cell types were grown up and proteins were extracted (see Methods) and analyzed by Western blotting, probing with an anti-human PLAC1 antibody (see Methods for this and other antibody details). The results showed immune reactivity to high molecular weight forms corresponding to dimeric, tetrameric and higher order complexes, with a much weaker band at the expected size for the monomeric form of PLAC1, suggesting that most of it

exists in higher order structure in the extracts (Figure 1A). Such forms might be expected, because the ZP-N domain in Plac1 has been previously shown to be sufficient to form dimers [13].

The multimeric complexes of PLAC1 were not dissociated by sodium dodecyl sulfate, as assessed in gel electrophoresis, suggesting a possible covalent linkage. For comparison, BeWo cells were transfected with V5-epitope tagged PLAC1 and Western blots were probed with either anti-PLAC1 or anti-V5 antibodies. Again, anti-PLAC1 antibody detected the expected higher molecular weight V5-tagged protein along with the higher order complexes (Figure 1B). Notably, in the extracts, the anti-V5 antibody showed a predominantly monomeric form in the soluble protein fraction; the multimeric complexes were seen in the insoluble fraction of protein recovered as a pellet from the extract by centrifugation at low speed (Figure 1C).

PLAC1 localizes to membrane and cytoskeletal fractions:

To investigate its subcellular localization, the PLAC1 coding sequence tagged with V5-epitope (PLAC1-V5) was transfected into BeWo cells. Cellular proteins were then recovered and separated (see Methods) into cytoplasmic, nuclear, membrane, cytoskeletal and insoluble pellet fractions. The fractions were then analyzed by SDS polyacrylamide gel electrophoresis and Western blotting analysis. Figure 2 shows that probing with anti-V5 antibody, a small amount of PLAC1 was seen in the nuclear fraction, but it was largely distributed among the cytoplasmic, membrane, and insoluble cytoskeletal fractions, with a major portion associated with the insoluble membrane and cytoskeleton.

We further fractionated the soluble fraction from V5-tagged PLAC1 from BeWo cells on a Superose 6B column (Supplementary Figure 1) and found that the majority of the “free” soluble protein centered around an apparent molecular weight of 29KD for the V5- tagged fusion protein (slightly larger than the calculated 26 kD molecular weight of PLAC1due to V5 tag); but again a significant fraction was distributed between higher molecular weight range of 66 kD and 400 kD, suggesting that the protein likely exists in higher molecular weight complexes (Figure 1A) including forms corresponding to dimeric, tetrameric and larger.

To look more directly at its localization in vivo, an immunofluorescence image of BeWo cells transfected with a PLAC1-EGFP fusion [see Methods] is shown in Figure 3. EGFP and Anti-PLAC1 antibody staining colocalize in transfected cells, with intense labeling around the cell membrane and cytoplasm. These findings thus reinforce the observation that the protein is largely associated with membrane or possibly membrane-associated cytoskeleton.

Identification of PLAC1 interacting proteins

Immunoprecipitation and Mass Spectrometry: PLAC1 fused with V5 tag (PLAC1-V5) was transfected into BeWo cells which were then grown for 72 hours. Cell extracts were then prepared and immuno-precipitated using anti-V5 agarose beads. The agarose beads were washed (see Methods) and recovered proteins were analyzed by Mass Spectrometry. A parallel immunoprecipitation was carried out in the same manner with anti-V5 antibody and

non-transfected cells. Supplementary Figure 2 shows the eluate from anti-V5 agarose beads probed with anti-PLAC1 antibody. Lanes 1 and 2 show input extract for immunoprecipitation without and with transfection, respectively. Lanes 3 and 4 show the supernatant fraction of soluble proteins from cell extract after anti-V5 precipitation, showing the presence of endogenous PLAC1 and a trace amount of PLAC1-V5 respectively, both detected by anti-PLAC1 antibody. The eluate from immunoprecipitation with anti V5-agarose beads does not contain PLAC1 protein in a control extract from cells with no transfection (Lane 5). By contrast, Lane 6 shows the immunoprecipitated PLAC1-V5 protein at a level comparable to the input extract in Lane 2.

The immunoprecipitated proteins were then analyzed by Mass Spectrometry. Among the 102 peptides identified were those specific for several components of the desmosome complex. Supplementary Table 1 lists desmosomal proteins recovered from the analysis that were enriched compared to control after immunoprecipitation with IgG.

The desmosome, a multiprotein complex that establishes lateral cellular junctions between cells, is comprised of 5 proteins: desmoglein, desmocollin, desmoplakin, plakoglobin, and plakophilin. Mass Spectrometric analysis identified 4 of the 5 components of the desmosome – all but plakophilin -- with peptide enrichment over control IP for 2, 4, 7, and 4 peptides respectively. Because the desmosomal proteins interact with one another, some interactions might be indirect. We therefore further tested several of these proteins for their interaction with PLAC1 directly by co-transfection with PLAC1-V5 and immunoprecipitation. We found clear interaction with DSG2, as follows.

Confirmation of interactions with desmosome complex:

We expressed DSG2 expression plasmids tagged with Myc-tag (DSG2-Myc, Addgene plasmid #32232), by co-transfection with PLAC1-V5 in COS7 cells. Cell extracts were immunoprecipitated (IP) with anti-V5 agarose beads and the IP products analyzed by Western blotting following their separation on an SDS gel. Probing an anti-V5 IP with anti-Myc antibody showed that PLAC1-V5 had precipitated along with DSG2-Myc (Figure 4A, Lane 2). Figure 4A, Lane 1 shows the expected lack of DSG-2 after control cotransfection with PLAC1-V5 and a vector without DSG-2-Myc. To confirm whether there was an endogenous interaction between DSG2 and PLAC1, BeWo cell extracts were immunoprecipitated with IgG or anti-DSG2 antibody. The IP was analyzed by Western blot and probed with anti PLAC1 antibody (Figure 4B), which revealed the presence of PLAC1. Thus, the results confirmed endogenous interaction between PLAC1 and DSG2 in BeWo cells.

Although other desmosome components were seen in the immunoprecipitate, upon testing by co-transfections of Desmocollin, Desmoplakin, Plakoglobin or Plakophilin individually with PLAC1-V5, as described above with DSG-2, we found no evidence for any direct interactions (results not shown). We suggest that because desmosomes exist as a multiprotein complex the other components associated with DSG-2 were carried along during immunoprecipitation and thus observed in Mass Spectrometry analysis.

Mutations in the ZP-N domain abolish interaction with DSG2:

PLAC1 contains a ZP-N domain that is essential for dimerization [13]. Based on the crystal structure of the ZP-N domain of ZP3 protein, a classic ZP-N domain containing protein, conserved cysteine residues 1 and 4 and cysteines 2–3 interact with each other to create the secondary and tertiary structural integrity of the protein fold [14]. For reference, the amino-acid comparisons between human and mouse PLAC1 are shown in Supplementary Figure 3 with the 4 cysteine positions and ZP-domains highlighted. We mutated cysteines 1(Mut1) and 3 (Mut2) independently to alanine and also constructed PLAC1 with both mutations (Mut1+2), tagged the mutant constructs with the V5-epitope, and expressed the mutant proteins by transfection into BeWo cells. Figure 5, Panel A, at the bottom, shows that the mutant proteins are expressed in BeWo cells and are detected by probing with anti-V5 antibody. The Western blot analysis of IP with anti-V5 antibody and probed with anti-DSG2 antibody then shows (Figure 5, Panel A, top) that while WT-PLAC1 interaction with DSG2 is robust, the mutated protein species fail to interact with DSG2. We infer that the interaction of PLAC1 with DSG2 requires an intact ZP-N domain, and disrupting either of the cysteine interactions is enough to abolish this interaction.

PLAC1 Interacts with DSG-2 C-terminal fragment:

There is evidence that DSG2 exists in cells both in an intact form and in two fragments, an N-terminal cadherin domain portion cleaved off by metalloproteases and shed from the cells and a C-terminal domain released into the cell cytoplasm [15]. We wanted to determine if PLAC1 interacts with the C or N-terminal domain of the cleaved product. Both N-terminal cadherin domain and C-terminal fragment were cloned, with a Myc-tag at their C-terminal end and expressed in HEK293 cells co-transfected with PLAC1-V5. Figure 5, Panel B shows the expression and detection of Myc-tagged full length, N-terminal, and C-terminal DSG2 proteins visualized by probing a Western blot with anti-Myc antibody. The same blot was then stripped and probed with an anti-V5 antibody, showing the PLAC1-V5 expression level, Figure 5, Panel C. Figure 5, Panel D shows that immunoprecipitation with anti-V5 coimmunoprecipitates both full length DSG2 and its cleaved C-terminal product of DSG-2, suggesting that PLAC1 interacts strongly and specifically with the C-terminal portion of DSG2 but not with the N-terminal fragment.

Immunofluorescent colocalization of PLAC1 and DSG2:

To visualize their interaction *in vivo*, PLAC1 was tagged with EGFP and DSG2 with mCherry (Addgene #36991). These constructs were co-transfected into BeWo cells. The cells were fixed after 72 hours and visualized by fluorescence light microscopy as well as confocal microscopy. Supplementary Figure 4b shows images of cells with dual fluorescence at 40X from light fluorescent microscope. Cells transfected with control EGFP vector lacking PLAC1 shows diffuse green fluorescence throughout the cell (Supplementary Figure 4A, Panel 3), and DSG-2-mCherry shows localization along the membrane (Supplementary Figure 4A, Panel 2). However, when EGFP is fused to the PLAC1, the PLAC1-EGFP signal is more limited to the periphery (Supplementary Figure. 4B, Panel C). The confocal image in Figure 6 then again shows colocalization of PLAC1-EGFP (Panel B) and DSG2-mCherry (Panel A) and EGFP (Panel B) in the merged image in Panel C. The

arrows in Figure 6, Panel C highlight the positions of co-localization of the two fluorescent proteins in cells that were cotransfected with both plasmids. An analysis along the line drawn along the yellow spot (colocalization spot) as indicated in Figure 6, Panel C, shows the graphical output of overlap of green and red signals, Figure 6, Panel D. These results further confirm the Western blot analysis results showing interaction between DSG2 and PLAC1.

Discussion:

Our results show that the majority of PLAC1 is present in an insoluble membrane fraction in placental derived cell-lines, confirmed by immunofluorescence localization of EGFP tagged PLAC1 expressed in BeWo cells. Results based on Mass-spectrometric analysis of IP of V5-tagged PLAC1 constructs gave us a list of proteins that could potentially interact with PLAC1, notably including desmosomal proteins. Direct test of enriched desmosomal genes with immunoprecipitation confirmed an interaction with desmosomal gene DSG2. Further evidence of their interaction was provided when fluorescent protein tagged PLAC1 and DSG2 proteins showed co-localization at the cell periphery. Additionally, we have demonstrated that PLAC1 interacts with the C-terminal fragment DSG2, which projects from the intact protein into the cytoplasm. Mutations disrupting the secondary structure of the protein in the N-terminal cysteines led to failure of PLAC1 interaction with DSG2, reinforcing the specificity of their interaction.

DSG2 is one of the components of the multiprotein complex, desmosomes, that span the membrane. It has 4 cadherin ectodomains that establish lateral cell contacts. Desmosomes are especially predominant in the epidermal layer, heart, and in the epithelial cells that line, for example, the uterine epithelial layer and intestine [16, 17]. Desmosomes sense the cellular environment and undergo modifications leading to shedding of the extracellular cadherin domain, cleaved by metalloproteases, and the internalization of the DSG2 cytoplasmic domain [15]. DSG2 also acts as a receptor for adenovirus serotypes 3,7, 11, and 14, and its interaction triggers activation of PI3 kinase and MAP/ERK1,2 kinases, which are key kinases involved in the epithelial to mesenchymal transition. Thus, the desmosomes - through their sensing of the cellular environment and subsequent cleavage - communicate external signals to the internal cell milieu [15].

Dsg2 knockout mice are embryonic lethal [18], and Overmiller et al.[19] showed that the cleaved C-terminal fragment of DSG2 is exported in extracellular vesicles from squamous cell carcinoma cells. If PLAC1 interacts with the DSG2 component of desmosomes, it might then be secreted as cargo in extracellular vesicles and act at a distant site as well as locally. But a major phenotype of loss of Plac1 in mouse is local hyperplastic placenta, with invasion of the junctional zone into the labyrinthine layer [2, 3] when the mutant gene is inherited from female mice. These findings have suggested that while Plac1is expressed at early stages from the paternal chromosome, there may be a switch to maternal transcription following paternal chromosome inactivation [3].

Invasion of the junctional zone is clearly the result of overgrowth. Such overgrowth would require a weakening or loss of cell-cell contact as part of the process. It is well-known that

desmosomes are important in maintaining lateral cell-cell contact, although their molecular and physiological dynamics are not known in detail. Given that PLAC1 interacts with DSG2, a desmosomal component, it seems a likely route for it to influence the nature of cell-cell interactions. Our further finding that it interacts with the internal C-terminal portion of DSG2 suggests that it could promote the integrity of the cadherin moiety in tissues such as placenta, which could then favor normal placental layer differentiation. If so, the ablation of PLAC1 could then lead to increased loss of the cadherin moiety and thereby promote the observed overgrowth of the junctional layer. A similar effect can also be envisioned in the invasive nature of breast cancer cells when PLAC1 is upregulated and might even be involved in the etiology of hydrocephalus in Plac1 knockout mice [11]. These conjectures may be testable by further study of the extent and kinetics of cleavage of DSG2 in the presence and absence of PLAC1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

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- PLAC1 interacts with Desmogelin-2 (DSG-2), a component of desmosomes
- Specifically interacts with c-terminal cleaved fragment of DSG-2
- Integrity of ZP-N domain of PLAC1 is essential for this interaction
- Mutations of Cysteines in ZP-N domain disrupt interaction with DSG-2

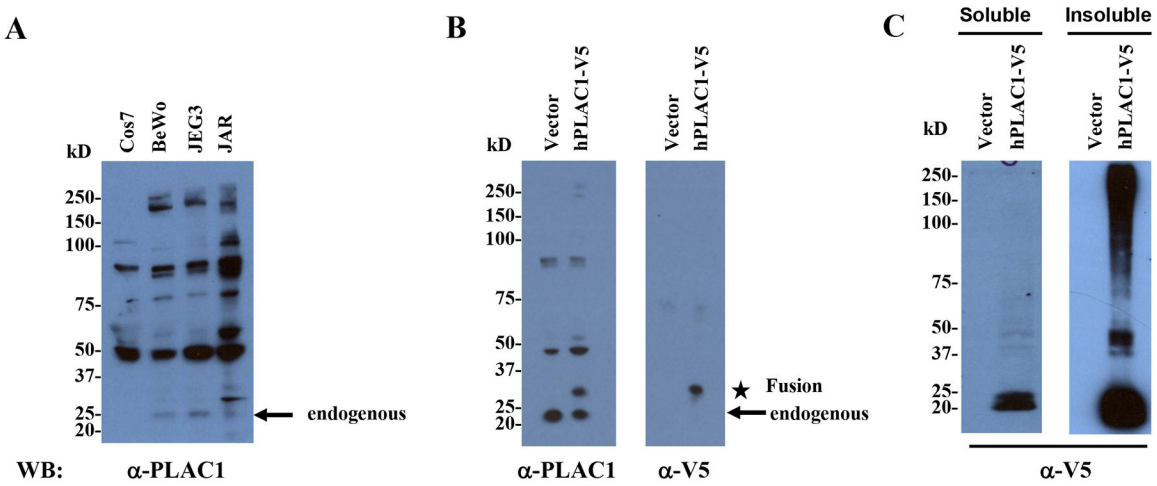

Figure 1: Western blots showing Anti-PLAC1 and anti-V5 probing of endogenous and V5-tagged PLAC1.

Panel A shows Western blot probing of cell extracts from Cos7, BeWo, JEG3 and JAR cells with anti-PLAC1 antibody (SC3659198). The antibody detects polymeric forms of the protein. Panel B shows Western blot of cell extracts from BeWo cells transfected with either the empty vector or V5-epitope fused to c-terminal end of PLAC1 (left image). PLAC1 Antibody identifies both the endogenous and epitope fused PLAC1 migrating as a higher molecular weight band. Anti-V5 probing shows just the fusion protein (Right image). Panel C shows the distribution of V5 tagged PLAC1 in soluble and insoluble cell- fractions, with the latter in large excess.

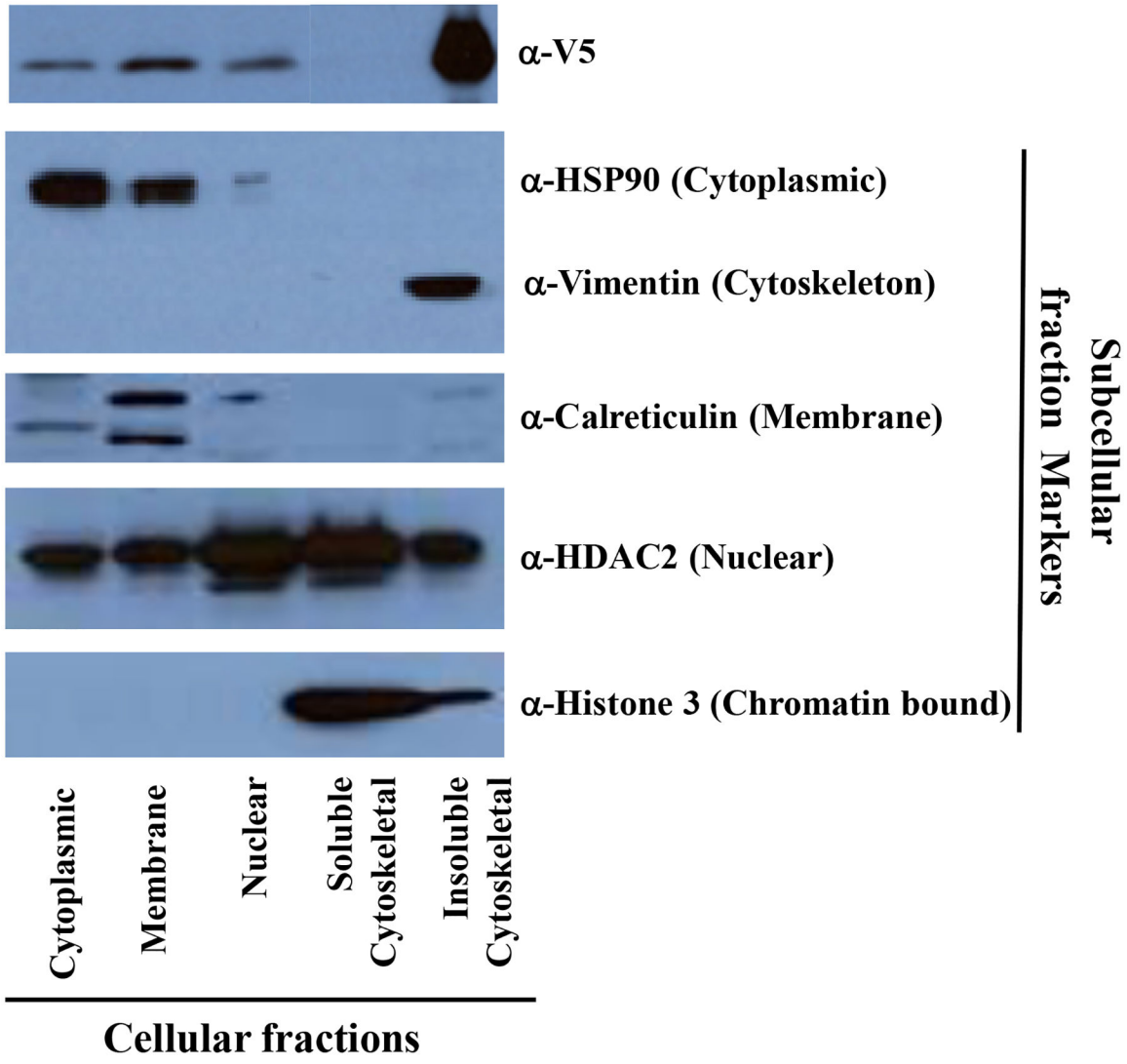

Figure 2: Cell Fractionation to localize PLAC1.

Extracts of BeWo cells transfected with V5-tagged PLAC1 were fractionated using the Pierce cell fractionation protocol to yield cytoplasmic, membrane, nuclear, and soluble and insoluble cytoskeletal fractions. Top panel shows Western blot probed with anti-V5 and distribution of V5-tagged PLAC1 in cytoplasmic, membrane and nuclear fractions and an intense staining band in the insoluble cytoskeletal fraction. Cellular compartment markers in panels below identify different cellular fractions.

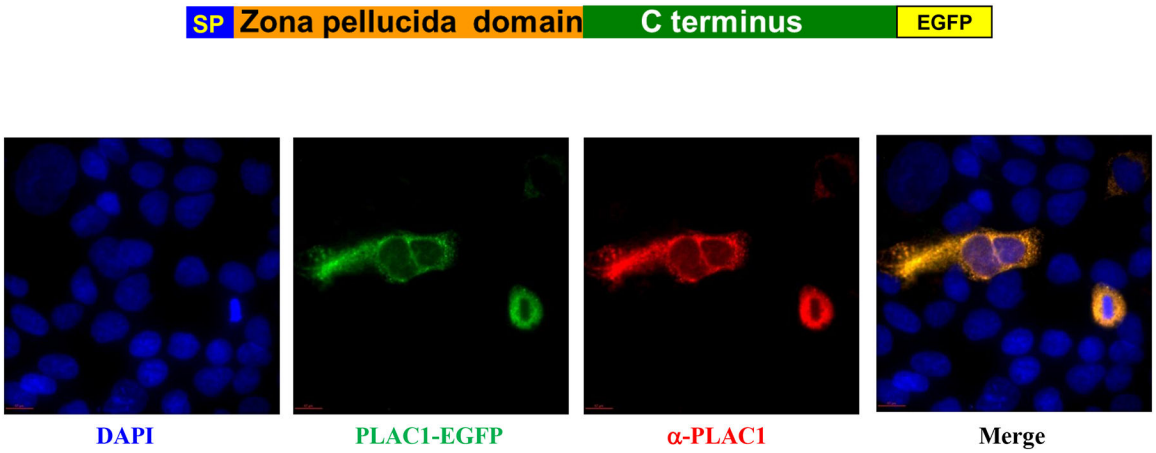

Figure 3: Immunofluorescence localization of PLAC1.

BeWo cells were transfected with EGFP tagged PLAC1 and stained with anti-PLAC1 antibody (Red). At the top, the schematic of the fusion construct is illustrated. The panels below from left, show DAPI stain, EGFP fluorescence, Anti-PLAC1 stain and the merged image respectively.

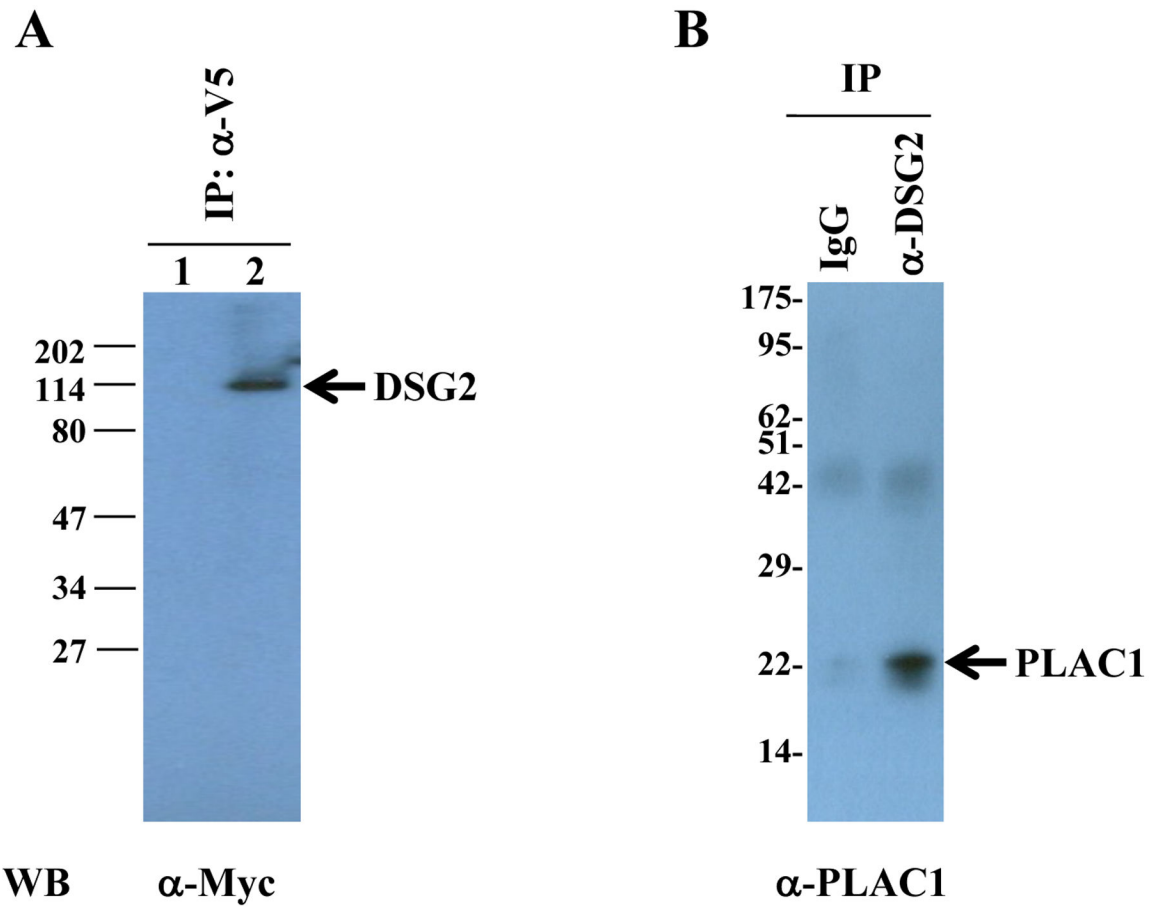

Figure 4: Western blots analyzing immunoprecipitations from cells to show interaction of DSG-2 and PLAC1.

Panel A. Cos-7 cells co-transfected with DSG-2-Myc and PLAC1-V5 and immuno-precipitated with anti-V5 agarose beads and tested for the presence of DSG2- by probing western blots with anti-Myc antibody. Lane 1 shows immunoprecipitation after cotransfection of PLAC1-V5 and control vector without DSG2; Lane 2 shows immunoprecipitation following cotransfection with both PLAC1-V5 and DSG-2-Myc. Panel B. Immunoprecipitation from BeWo cells with IgG in Lane 1 and anti-DSG2 in lane 2; the immunoprecipitated was then analyzed on SDS gel and probed with anti-PLAC1.

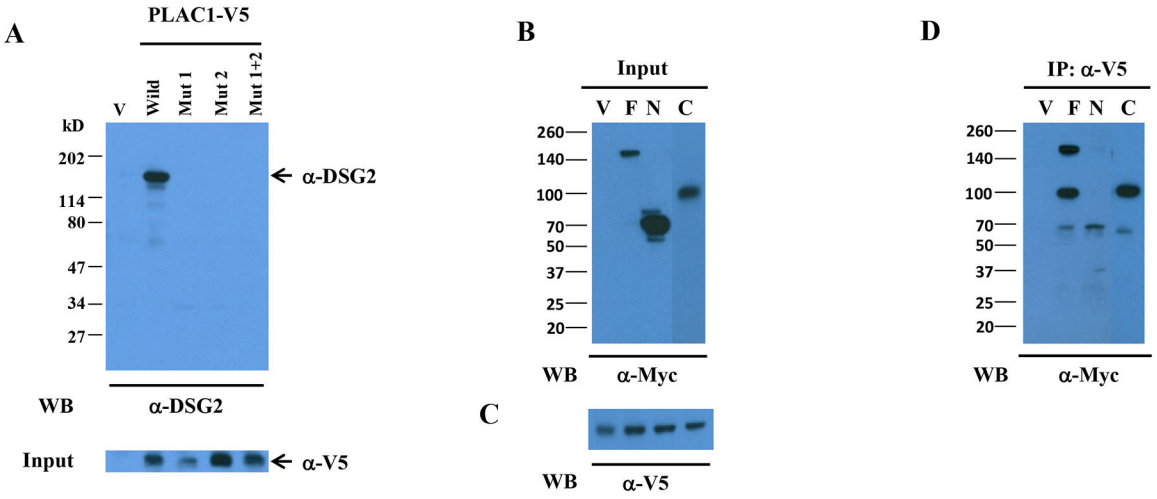

Figure 5: Expression of wild-type and PLAC1 with.cysteine mutations and their interaction with full length, N- and C-terminal DSG2 constructs.

Panel A. BeWo cells were transfected with wild-type PLAC1 and mutated constructs Mut1, Mut2 and Mut 1+2. Top Panel is a Western blot probed with anti-DSG2 of an immunoprecipitation with anti-V5 agarose beads separated on an SDS gel. Only wild type PLAC1 brings down DSG-2 while the mutant forms fail to interact with DSG2. Bottom panel shows the expression of wild-type and mutant forms of PLAC1-V5. Panel B. HEK 293 cells were co-transfected with Empty vector (V), Myc-tagged full length (F), N-terminal fragment (N) or C-terminal Fragment (C) of DSG2 along with PLAC1-V5 expression vector. Western blot probed with anti-Myc antibody showing the expression of full length and truncated versions of DSG2. Panel C shows anti-V5 probing to detect expression of PLAC1-V5 in the same cells. Panel D is a Western blot of an immunoprecipitation with anti-V5 antibody. Full length DSG-2(F) is immunoprecipitated along with the endogenously processed C-terminal DSG-2 fragment. Lane marked ‘C’ shows immunoprecipitation of C-terminal fragment, whereas lane marked ‘N’, shows that N-terminal terminal fragment fails to interact with PLAC1

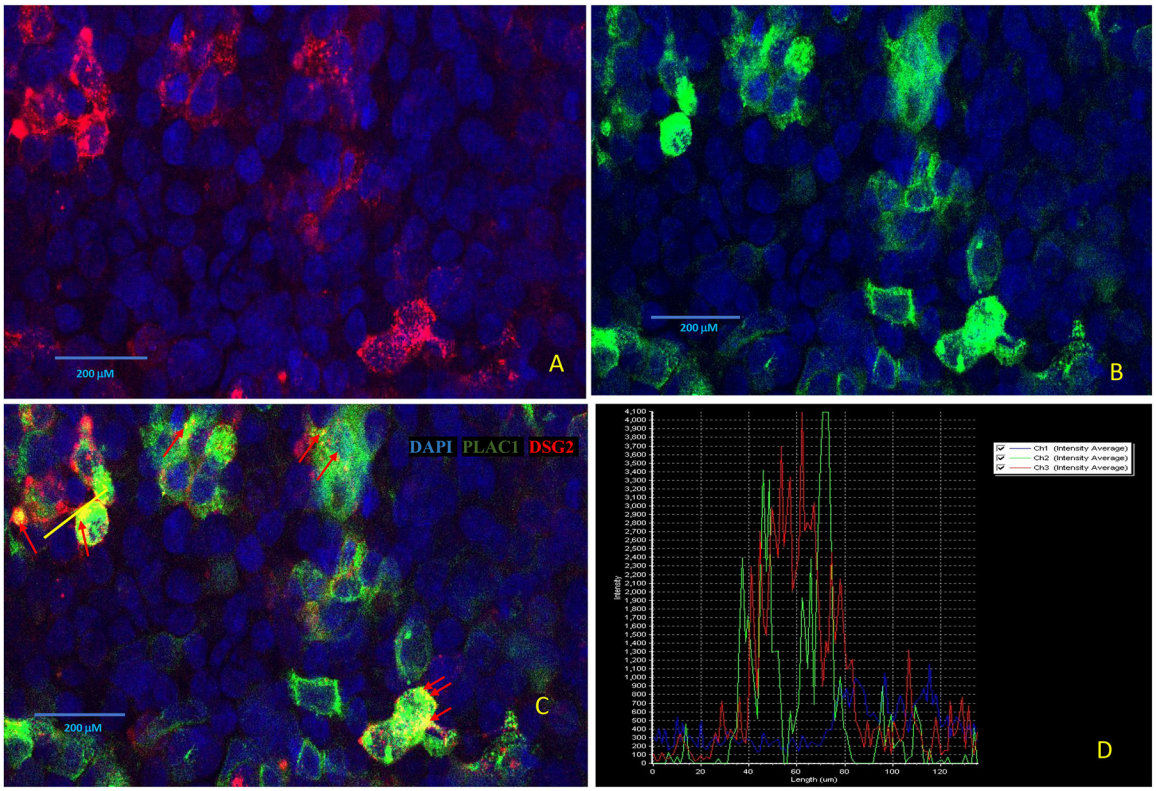

Figure 6: Confocal images of Immunofluorescence colocalization.

BeWo cells transfected with DSG2-mCherry and PLAC1-EGFP were visualized under confocal microscope. Panel A, shows DAPI stained nuclei and mCherry fused DSG2 in red. Panel B shows DAPI stained nuclei and PLAC1-EGFP. Panel C shows merged image. Yellow region in cells transfected with both plasmids (red arrows) indicate co-localization of PLAC1and DSG2. Panel D shows the overlap between the red and green signals in a graphic form along the line drawn in yellow in Panel C through the region of colocalization.