The Effects of CD82 Palmitoylation on the Metabolic Pathways of EGFR and c-Met in Breast Cancer Cells and their Molecular Mechanisms

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Research article

Keywords: Tetraspanin ,CD82, Palmitoylation, EGFR ,Rab11a

Posted Date: June 7th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-532321/v1

Abstract

Background: As a tumor metastasis suppressor, tetraspanin CD82 is reduced in many malignant tumors and often affects the composition of tumor microenvironment by changing the heterogeneity of cell membrane. EGFR or c-Met signaling pathway can regulate the metastasis ability of tumor cells and participate in the formation of tetraspanin web. The study of CD82 palmitoylation modification and metabolic pathway of tumor related molecules in tumor cells is still incomplete. This article focuses on studying the expression and distribution of EGFR and c-Met in cancer cells as well as related metabolic pathways and their molecular mechanisms after studying different palmitoylation site mutations.

Methods: Western blot and immunofluorescence methods were used to detect the distribution of EGFR in breast cancer MDA-MB-231 cells after different CD82 palmitoylation site mutations. Then use the immunoprecipitation method to determine the interaction relationship between the molecules and the molecular mechanism.

Results: We found that when CD82 combined with palmitoylation mutation at Cys5+Cys74 can enhance the internalization of EGFR, but has no effect on the expression and location of c-Met. When CD82 is combined with palmitoylation mutation at the Cys5+Cys74 site, with the assistance of tubulin, EGFR is internalized and strengthened by direct binding to CD82 and a large number of localizations on the recycling endosome. By forming the EGFR/CD82/Rab11a/FIP2 complex, it is metabolized through the circulation pathway, and re-expression of EGFR and CD82 on the cell membrane.

Conclusions: From our results, we can demonatrate that CD82 palmitoylation mutation can change the distribution of EGFR in breast cancer cells, which may provide new ideas for breast cancer treatment.

Introduction

The tetraspanin CD82 belongs to the family of tetraspanins and is a small molecular membrane protein with four transmembrane regions. CD82 is encoded by the KAI1 gene, as a recognized tumor suppressor factor, it is widely distributed in various normal tissues. In addition to four transmembrane regions, CD82 also contains an extracellular small loop (EC1), an extracellular macroloop (EC2), and an intracellular small loop. In the variable region of EC2, there are sites that can bind to other proteins. This structural feature helps the formation of the subsequent tetraspanin network. In the CD82 transmembrane domain, there are three highly conserved polar residues that can interact with the transmembrane domains of other tetraspanins, so CD82 can connect to other types of tetraspanins to form compounds with specific functions. In addition, CD82 can also directly or indirectly bind to signal molecules, such as integrins, epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (c-Met), and G protein-coupled receptors, to form microdomains enriched with tetraspanins on the cell membrane.

EGFR is one of the main members of the ErbB family, which has a regulatory role in embryonic development, tissue differentiation, and tumorigenesis and development[1]. Studies have shown that in solid tumors, the overexpression of EGFR is usually related to the increase in the secretion of homologous

ligands, which leads to the chronic activation of EGFR. When EGFR has not been activated, CD82 can down-regulate EGFR expression by regulating internalization kinetics. CD82 can also cooperate with vesicle-associated membrane protein and actin to change the signal transduction pathway of EGFR[2, 3]. c-Met is also a receptor tyrosine kinase, which is mainly expressed in epithelial and endothelial cells[4]. As the only high-affinity receptor for hepatocyte growth factor, c-Met has shown a trend of overexpression in breast cancer, pancreatic cancer, gastric cancer and other tumors. Both EGFR and c-Met are tumor metastasis-related receptors, which are involved in the regulation of tumor cell metastasis and have been extensively studied. CD82 can directly inhibit the expression of c-Met, and reduce cell invasion and growth by weakening the signal interaction between c-Met and integrin [5–7].

Palmitoylation modification is an important and more common post-translational modification process of proteins. At present, the most widely studied process is S-palmitoylation, that is ,16 carbon palmitate to cysteine (Cys) residues of proteins. Lipid bonds on palmitoyl groups can bind to Cys residues and affect protein expression and function[8, 9]. The palmitoylation mutation of the tetraspanin protein can promote the formation of the tetraspanin protein network, which is conducive to the biological function of the tetraspanin protein-enriched microdomain. The palmitoylation of CD82 can regulate the biological characteristics of tumor cells[10], but its specific molecular mechanism remains to be studied. CD82 contains five cysteine residues in the proximal membrane region: Cys5, Cys74, Cys83, Cys251 and Cys253. So far, the relationship between CD82 palmitoylation mutation and EGFR and c-Met has not clear theoretical mechanism for its expression and localization changes in tumor cells and specific metabolic pathways. Therefore, our experiment will compare the distribution and metabolic pathways of EGFR and c-Met by different palmitoylation mutants of CD82, and explore the corresponding molecular mechanisms.

In our experiment, three sites of Cys5, Cys74, and Cys83 in the cytoplasmic proximal membrane area were selected for single point (Cys5, Cys74, Cys83), double point (Cys5 + Cys74, Cys74 + Cys83, Cys5 + Cys83) and three point mutation (Cys5 + Cys74 + Cys83). The CD82 palmitoylation mutant was constructed, as shown in Fig. 1.

Materials And Methods

Cell culture and transfection

Breast cancer MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium(DMEM) basic culture media supplemented with 10% fetal bovine serum (FBS), in 5% CO_2. The cell lines used in our experiments are free of mycoplasma infections. HighGene transfection reagent was used for the transfection of MDA-MB-231 cells. The cells were inoculated on 6-well plates and ready for transfection when the cell density reached 70% to 90%. Three micrograms of plasmid were added to 200 microliters of serum-free opti-MEM medium, mixed well, then 6 microliters HighGene transfection reagent were added and mixed well. The mixture was evenly dripped into 6-well plates and mixed. After 4 to 6 hours of

transfection, replace the complete medium with 10% fetal bovine serum (FBS), continue to cultivate 24-48 hours before the follow-up experiment.

Protein extraction and western blotting

Using Membrane and Cytosol Protein Extraction Kit clustering cell total proteins into cytomembrance protein and cytoplasm protein. Extract total protein using whole protein extraction kit. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and were transferred to PVDF membranes, then blocking in QuickBlock™ for 10 min and incubation of primary antibodies(1:1000) overnight at 4°C. Incubate the secondary antibody(1:10000) for 1h at room temperature. The Odyssey fluorescence scanning imaging system was used for protein detection

Co-immunoprecipitation

After extracting fresh protein samples, use Protein A+G Agarose and appropriate primary antibodies for co-immunoprecipitation, rotate slowly and shake well, overnight at 4°C. After washing with PBS, isolate the protein by immunoblotting and incubate the other primary antibody to be tested.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde (PFA) 15 minutes and 1% NP-40 for 20 minutes at room temperature, PBS wash three times. Blocked by 5% Normal Goat Serum for 30 minutes at 37°C. Incubated with the primary antibodies overnight at 4°C, primary antibodies(1:100) were diluted by blocking buffer. Incubated with fluorophore-conjugated secondary antibodies(1:50), which diluted by PBST bufferfor 1 h. Dyed the nucleus and packaged cells cover glasses with Antifade Mounting Medium with DAPI. Imaging was performed either with a Leica Tcs sp8 laser scanning confocal microscope or a Leica DM4B positive fluorescence microscope.

Statistical analysis

GraphPad Prism 5 software was used to create the figures and One-way analysis of variance(ANOVA) was used for comparison between multiple groups. $P<0.05$ is considered to be statistically different.

Results

After different palmitoylation sites of CD82, the expression and localization of EGFR, CD82 and c-Met in the cells

Separate and extract the cytoplasmic and membrane proteins of breast cancer MDA-MB-231 cells mutated at different palmitoylation sites of CD82. Western blot results showed that in cytoplasmic proteins, when CD82 palmitoylated Cys5+Cys74 sites were combined with mutations, the expression of CD82 in the cytoplasm increased, and at the same time, the expression of EGFR in the cytoplasm also increased. However, the expression of c-Met in the cytoplasmic protein did not differ significantly between different CD82 palmitoylation mutant groups, as shown in Figure 2A. In the cell membrane protein, CD82 palmitoylation Cys5+Cys74 site combined mutation, the expression of CD82 on the cell membrane decreased, and the expression of EGFR on the cell membrane also showed a decreasing trend. There is still no significant difference in the expression of c-Met among the different mutant groups in the membrane protein, as shown in Figure 2B.

The expression of CD82 and EGFR in the total protein after mutations at different palmitoylation sites of CD82

The above experiments have confirmed that the mutation of different palmitoylation sites of CD82 has no significant effect on the expression and location of c-Met, so follow-up experiments will focus on the expression and metabolism of CD82 and EGFR. The immunoblotting method was further used to detect the expression of CD82 in the total protein after the mutation of different palmitoylation sites of CD82. The immunoblotting results showed that when the Cys74+Cys83 two-point mutation, CD82 expressed the most in the total protein, which was significantly different from the wild-type WT group and the normal control N group. When Cys83 single-point mutation or Cys5+Cys74+Cys83 three-point combined mutation, CD82 expression in total protein is slightly lower than Cys74+Cys83 two-point mutation, which is significantly different from the normal control N group, while Cys5+Cys74 site when combined with mutations, CD82 has no difference in total protein from group N, as shown in Figure 3A. However, the expression level of EGFR in the total protein did not differ significantly between the groups, as shown in Figure 3B.

Immunofluorescence method to detect the co-localization of CD82 and EGFR with Lamp1, Rab7a and Rab11a, respectively

The previous results showed that when the Cys5+Cys74 sites were combined with mutations, the expression of EGFR and CD82 in the cytoplasm increased, and there was a tendency to transfer into the cell. The metabolic pathways of CD82 and EGFR in the cytoplasm after the combined mutation of CD82 palmitoylated Cys5+Cys74 sites were explored, and EGFR and CD82 were detected with Lamp1 (lysosomal marker) and Rab11a (recycling endosome marker) by immunofluorescence method. In order to prevent the destruction of these endosomes, 1% NP-40, a milder permeabilizing agent, was used for cell permeabilization. The immunofluorescence results showed that the co-localization of CD82 and

Rab11a was the most obvious, as shown in Figure 4A. The degree of co-localization of EGFR and Rab11a is also higher than that of Lamp1 and Rab7a, as shown in Figure 4B.

Co-immunoprecipitation method detects the interaction between CD82 and Lamp1, Rab7a and Rab11a after mutations at different palmitoylation sites of CD82

After mutation of the different palmitoylation sites of CD82, CD82 was immunoprecipitated with Lamp1, Rab7a and Rab11a in each mutant. The immunoblotting results showed that in the IP group, Rab11a had protein expression, but Lamp1 and Rab7a did not. In the Input control group, there are protein expressions, as shown in Figure 3C. It proves that CD82 and Rab11a can be directly combined, and the two have a direct interaction relationship, but it cannot be directly combined with Lamp1 and Rab7a, and there is no direct interaction relationship.

Co-immunoprecipitation method to detect the interaction between EGFR and CD82, Lamp1, Rab7a and Rab11a after the combined mutation of CD82 palmitoylated Cys5+Cys74 sites

In order to explore the molecular mechanism of the enhanced internalization of EGFR into the cytoplasm when the CD82 palmitoylation Cys5+Cys74 sites are combined with mutations, the interaction between EGFR and CD82 was detected by immunoprecipitation method. The results of immunoblotting showed that EGFR can directly bind to CD82, as shown in Figure 3E. Similarly, CD82 was used to reversely verify the binding to EGFR, and the results showed that CD82 can directly bind to EGFR, as shown in Figure 3D. At the same time, the results of immunoblotting showed that EGFR could not directly bind to Lamp1, Rab7a and Rab11a, as shown in Figure 3E.

Detection of CD82 palmitoylated Cys5+Cys74 sites combined with mutations to regulate the metabolic pathway of EGFR through cell metabolism pathway inhibitors

Cycloheximide (CHX) is a protein translation inhibitor, which can inhibit the process of protein translation and thereby inhibit protein expression. Monensin (Mon) is a protein transport inhibitor that can inhibit the transport process from endosomes to cell membranes, and it is also an inhibitor of circulating bodies. Chloroquine (CQ) is a lysosomal inhibitor, and MG132 is a proteasome inhibitor. Western blotting showed that when CHX (300ug/ml, 8h) was added, the expression of EGFR would decrease; when Mon was

added, the expression of EGFR resumed and increased; when CQ and MG132 were added, the expression of EGFR increased, but there was no difference , shown in Figure5A. The cells were treated with the same drug concentration, and immunofluorescence experiments were performed. A certain number of cells were counted in each group to calculate the average fluorescence intensity of EGFR. The results showed that when the cells were treated with Mon, the average fluorescence intensity of EGFR increased significantly, as shown in Figure 5B.

Detection of CD82 palmitoylated Cys5+Cys74 sites combined with mutations to regulate the metabolic pathway of EGFR through tubulin inhibitors

In this experiment, Nocodazole was selected to inhibit the aggregation of microtubules. When CD82 palmitoylated Cys5+Cys74 sites are combined with mutations, immunofluorescence results can clearly show that EGFR is expressed in the cytoplasm in the experimental group treated with Nocodazole (20μg/ml, 4min) compared with the control group without Nocodazole. Decrease, the expression on the cell membrane increases, as shown in Figure 5C. Therefore, it can be proved that the internalization process of EGFR needs the assistance of tubulin.

Co-immunoprecipitation method to detect the interaction between FIP2 and Rab11a after the combined mutation of CD82 palmitoylated Cys5+Cys74 sites

FIP2 is a member of the Rab11 interaction family. In the CD82 palmitoylated Cys5+Cys74 site combined mutation group, WT group and N control group, the interaction between FIP2 and Rab11a was detected by immunoprecipitation method. The results of western blotting showed that in the IP group, FIP2 can directly bind to Rab11a, and at the same time, FIP2 can also directly bind to CD82; in the Input control group, CD82 palmitoylated Cys5+Cys74 site combined mutation group, WT group and N The control group all had protein expression, as shown in Figure 5D.

Discussion

The tetraspanin CD82 can regulate the occurrence, development and metastasis of most tumors, and has the effect of inhibiting tumor metastasis[11-13]. CD82 can also regulate the distribution of proteins on the cell membrane by interacting with other molecules. When the protein undergoes palmitoylation modification, the protein located in the cytoplasm has the ability to anchor on the cell membrane, and the protein located on the cell membrane will promote the signal transport process and stably maintain normal physiological functions[3, 14]. Palmitoylation contributes to the formation of the tetraspanin protein network and cell signal transduction[15, 16]. Studies have shown[17] that when the palmitoylation

mutation of CD82 occurs, it can promote the recombination of dynein in the microdomain and also affect the formation of cadherin binding protein complex. CD82 palmitoylation mutation can change the composition of the cell membrane and stabilize the expression and activation of protein kinase C. When palmitoylation is inhibited, the stability of protein kinase C on the membrane is reduced, and the activation of the ERK1/2 signaling pathway is also affected weakened[18].

The EGFR signaling pathway is regulated by many factors, and different ligands can transmit different signals. CD82 can weaken the EGF/EGFR induction signal and inhibit tumor metastasis and spread. Studies have shown that CD82 can regulate the ligand-induced ubiquitination of EGFR[19], but before EGFR is activated, CD82 can regulate it[20]. CD82 can also reduce the formation of EGFR dimers, but the mechanism is still unclear [21]. In epithelial tumor cells, CD82 can promote the endocytosis and desensitization effect of EGFR. In gastric cancer cells, CD82 can be inhibited by miR-197, which increases the activated EGFR and enhances tumor cell invasion and metastasis [22].

Up to now, the effect of CD82 palmitoylation mutation on the expression, location and metabolism of EGFR and c-Met in breast cancer cells remains to be further studied. In this experiment, by constructing different CD82 palmitoylation mutants to explore the influence of different site mutations on the expression, distribution and metabolic pathways of EGFR and c-Met. When the Cys5+Cys74 site of CD82 is combined with palmitoylation mutation, the internalization ability of EGFR is strengthened, and more EGFR cannot be stably expressed on the cell membrane, but transferred from the cell membrane to the cytoplasm. CD82 palmitoylation mutations cannot affect the expression and localization of c-Met, which may also indicate that CD82 has regulation of c-Met in tumor cells requires the participation of other post-translational modifications or the assistance of other signaling molecules, which are still to be studied in the future. Therefore, in the follow-up, we will mainly discuss the interaction relationship and molecular mechanism of the combined palmitoylation mutation at Cys5+Cys74 of CD82 and EGFR.

We further researched and found that there was no significant difference in the expression of EGFR in the total protein of different CD82 palmitoylation mutants. This shows that palmitoylation mutations at different sites of CD82 cannot change the total content of EGFR in cells, but can change its distribution position in cells. At the same time, for CD82 itself, when the Cys5+Cys74 site has a combined palmitoylation mutation, CD82 also changes its own distribution. It has no effect on the amount of self-expression.

Therefore, the following conjectures can be put forward. These internalized EGFR and CD82 may be (1) decomposed through certain metabolic pathways, such as lysosomal metabolic pathways. (2) Circulate metabolism through the circulatory pathway. (3) Metabolism through late endosomal pathway. Lamp1, Rab11a, and Rab7a are lysosomal markers, recycling endosome markers, and advanced endosomal markers respectively [23]. After the Cys5+Cys74 site of CD82 combined with palmitoylation mutation, most of the CD82 and EGFR with enhanced internalization ability were located in the recycling endosome. That is to say, most of them are metabolized through the circulatory pathway, and a small part of CD82 and EGFR are metabolized through the lysosomal pathway.

Studies have pointed out that in small cell lung cancer A549, primary keratinocytes and human breast epithelial cells, CD82 can directly bind to EGFR and inhibit EGF-induced cell migration. CD82 does not affect the activation of EGFR, but the signal induced by EGF will be desensitized faster, and the process of weakening of this signal may be related to endocytosis [24]. In this experiment, when CD82 palmitoylation Cys5+Cys74 sites are combined with mutations, the enhancement of EGFR internalization is also related to the direct binding of EGFR and CD82, and EGFR and CD82 are transferred into the cytoplasm in the form of direct interaction.

Endosomes can be transported along microtubules with the assistance of dynein. Studies have shown that endosomes containing CD133 can be recruited to the centrosome area through a dynein-based transport system [25]. In this experiment, to further verify the specific molecular mechanism of EGFR internalization into the cytoplasm after the Cys5+Cys74 site of CD82 combined with palmitoylation mutations and metabolization through the circulatory pathway, the tubulin inhibitor Nocodazole was used to inhibit the aggregation of tubulin. Observe the expression of EGFR. From the immunofluorescence results, it can be seen that when Nocodazole is added, the expression of EGFR in the cytoplasm is reduced, and most of them are located on the cell membrane, and the internalization ability is weakened. Therefore, it can be inferred that after the Cys5+Cys74 site of CD82 is combined with palmitoylation mutation, EGFR is internalized under the binding of CD82, and this process also requires the assistance of tubulin.

Monensin is a polyether antibiotic that can change the pH of the environment by adjusting the Na/H converter, destroy the structure of related proteins, and inhibit the transport process of circulating endosomes to the cell membrane[26], Actinomycetes Ketone is a protein production inhibitor, which can inhibit the production of most proteins. Chloroquine is a selective inhibitor of lysosomes, and MG132 is an inhibitor of proteasomes [23]. In order to further verify that the Cys5+Cys74 site of CD82 combined with palmitoylation mutations, EGFR is internalized and then metabolized through the circulatory pathway. In this experiment, monensin was selected as an inhibitor of the circulatory pathway. Inhibit the circulation pathway and observe the changes in the expression of EGFR. At the same time, cycloheximide was selected to inhibit the production of EGFR, and chloroquine was compared with MG132 as a control group. The results of immunoblotting and immunofluorescence show that when monensin is added, the effect of cycloheximide on the reduction of EGFR expression can be restored to a large extent. That is to say, when the circulatory pathway is inhibited, the metabolism of EGFR is blocked at this time, which reversely verifies that EGFR is metabolized through the circulatory pathway.

Rab11a belongs to the small molecule GTPase family Rab11 subfamily member [27], and can be used as a marker protein of the recycling endosome. The role of Rab11a in cancer progression has also been extensively studied. Studies have shown that Rab11a can promote the activation of the Wnt signaling pathway and further enhance the invasion ability of pancreatic cancer[28]. Rab11a has many effect factors, which play a regulatory role in different signal pathways. The most important one is Rab11-FIPs. Rab11-FIPs can be divided into three subtypes. The first type of subtype includes: FIP2, Rab coupling protein (RCP) and Rab11 interacting protein (Rip11), the second type of subtype includes: FIP3 and FIP4,

and the third type of subtype: FIP1 [29]. The first subtypes often specifically bind to Rab14 in a GTP-dependent manner. FIP2 is one of the subfamily members of Rab11-FIPs, and plays an important regulatory role in the process of molecular recycling of the cell surface [30, 31]. Rab11a can recruit myosin Vb and cytoplasmic dynein through the effectors FIP2 and FIP3 [32]. Insulin is a C-type lectin receptor. As a cargo molecule, it can be recovered through the endosomal circulation pathway in the Rab11a-positive membranous region. Studies have shown that the myosin Vb/Rab11a/Rab11-FIP2 signaling pathway is involved in the endosomal recovery process of islet protein. In the late stage of recovery, it can assist the connection and fusion of islet protein and plasma membrane.

When the Cys5+Cys74 sites of CD82 were combined with palmitoylation mutations, CD82 and Rab11a were immunoprecipitated with FIP2 respectively. The results confirmed that FIP2 can directly bind to Rab11a and CD82. Therefore, it can be inferred that CD82, Rab11a and FIP2 can form a complex to assist the re-recovery of EGFR on the cell membrane. At the same time, CD82 will also be expressed on the cell membrane again, as shown in Figure 6.

However, this experiment only explored the metabolic pathways of EGFR after the Cys5+Cys74 sites of CD82 combined with palmitoylation mutations. The metabolic pathways of other mutants and the mechanism of CD82 internalization enhancement are still unclear. Whether the EGFR and CD82 located in the lysosome are completely degraded remains to be verified.

Conclusion

In summary, when the Cys5 + Cys74 site of CD82 is combined with palmitoylation mutation, EGFR is transferred from the cell membrane to the cytoplasm under the mediation of CD82, and is located on the recycling endosome under the co-transportation of tubulin. Furthermore, by forming an EGFR/CD82/Rab11a/FIP2 complex, EGFR and CD82 are transported and recovered to the cell membrane for re-expression. The study of the metabolic pathways and mechanisms of CD82 and tumor metastasis-related factors in breast cancer cells will help to further understand the mechanism of breast cancer formation and metastasis, and provide ideas for more precise targeted therapy.

Declarations

Consent for publication

All authors gave consent for the publication of the manuscript in Breast Cancer Research.

Acknowledgements

Not applicable

Funding

This study was support by grants from the Scientific Research Fund of Education Department of Liaoning Province(grant no. LZ2019008) and the National Natural Science Foundation of China (grant no. 81601619).

Conflict of interest

The authors declare no conflict of interest.

Ethical Approval and Consent to participate

Not applicable

Availability of supporting data

All data generated or analysed during this study are included in this published article

Authors' contributions

Conceived and designed the experiments: YL and WZ; Performed the experiments: JB and ML; Analyzed the data: JB, SH, and MZ; Contributed reagents/materials/analysis tools: YZ andYL. All authors read and approved the final manuscript

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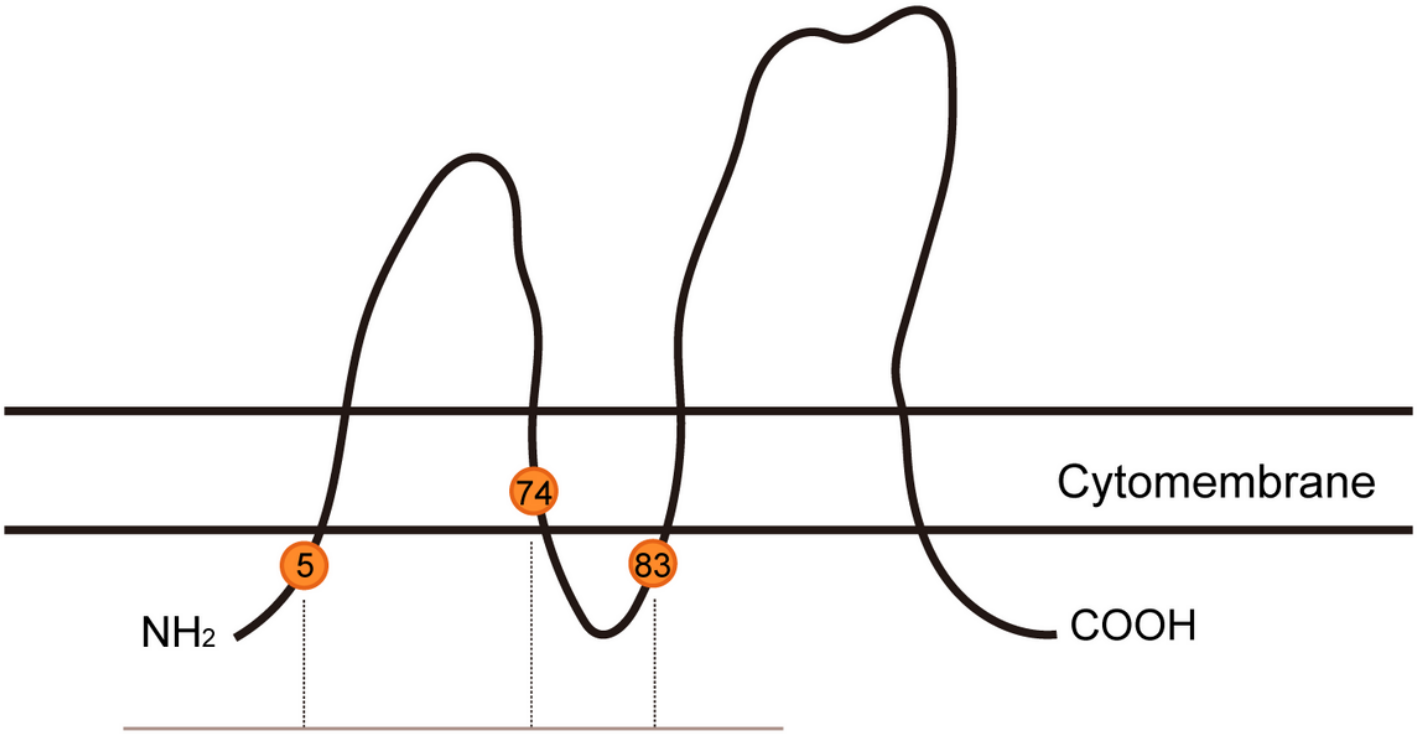
Figures



	Cys5	Cys74	Cys83
1	+	-	-
2	-	+	-
3	-	-	+
4	+	+	-
5	-	+	+
6	+	-	+
7	+	+	+

Figure 1

Schematic diagram of mutations at different palmitoylation sites of CD82. In the tetraspanin CD82 molecule, there are Cys5, Cys74 and Cys83 palmitoylation mutation sites near the membrane area of the cell membrane. The single-point, double-point, and three-point combined mutations used in this experiment are represented by 1 to 7, respectively, "+" represents palmitoylation mutation, and "-" represents no palmitoylation mutation.

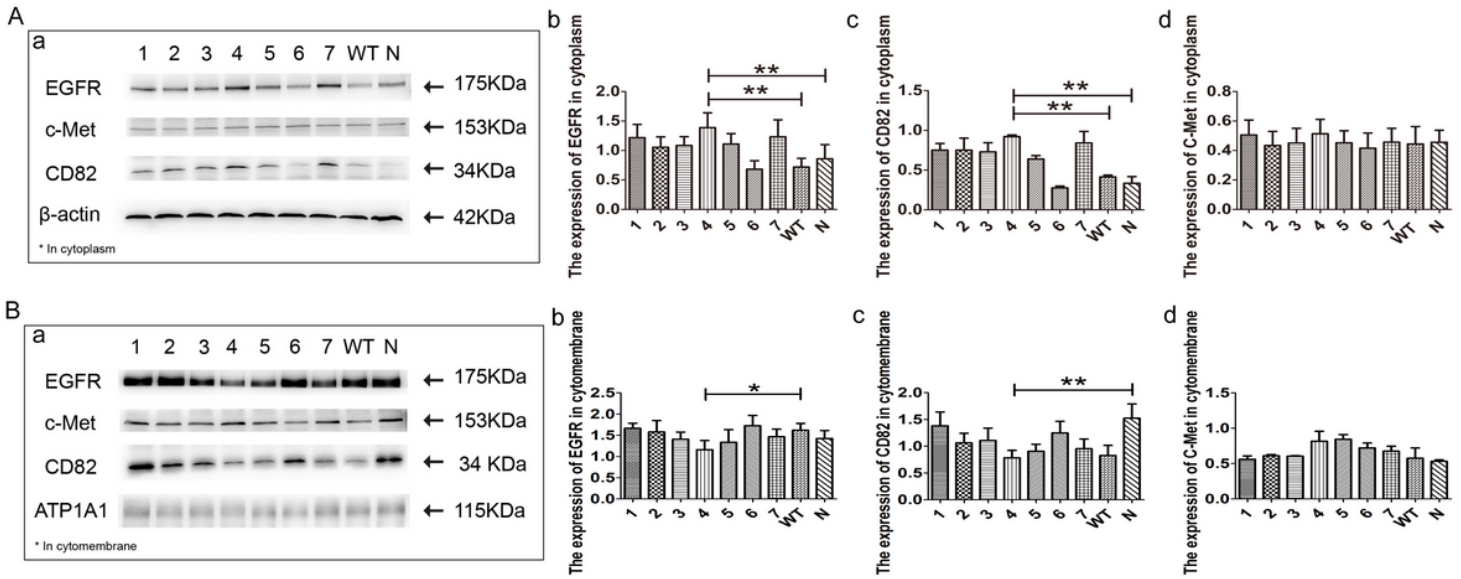


Figure 2

A: Western blot method to detect the expression of EGFR, CD82 and c-Met in the cytoplasmic protein after mutations at different palmitoylation sites of CD82. B: Western blot method to detect the expression of EGFR, CD82 and c-Met in the membrane protein after mutations at different palmitoylation sites of CD82.

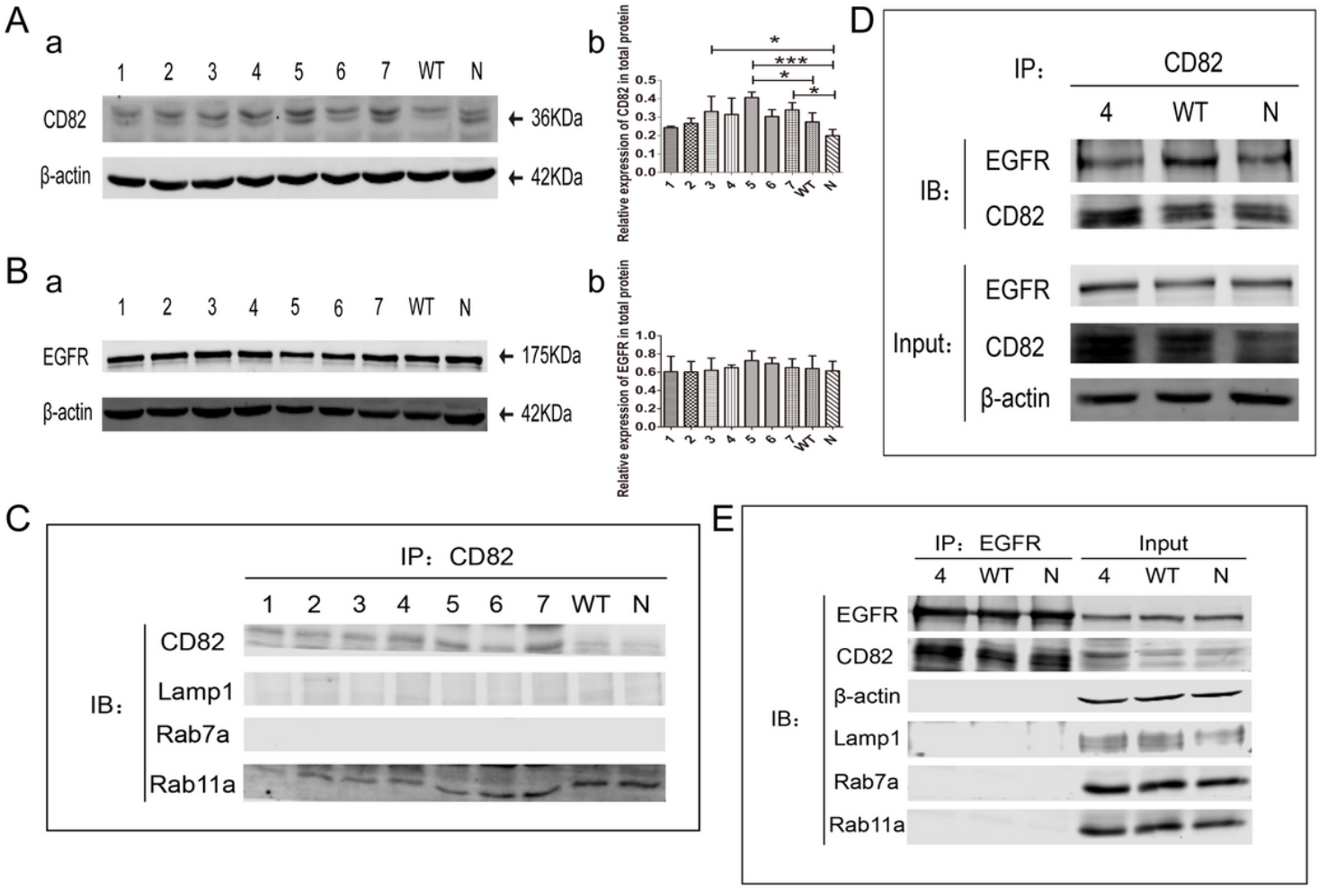


Figure 3

A: The expression of CD82 in the total protein after being mutated at different palmitoylation sites of CD82. B: The expression of EGFR in the total protein after mutations at different palmitoylation sites of CD82. C: After the mutation of different palmitoylation sites of CD82, the immunoblotting method shows the physical interaction relationship between CD82 and Lamp1, Rab7a and Rab11a. D: CD82 can interact directly with EGFR in the CD82 palmitoylation Cys5+Cys74 site combined mutation group, WT group and N control group, and protein expression is also found in the Input group. E: EGFR in the CD82 palmitoylation Cys5+Cys74 site combined mutation group, WT group and N control group, the results of the IP group showed that EGFR can directly bind to CD82, but not to Lamp1, Rab7a and Rab11a. In the Input group, CD82 palmitoylated Cys5+Cys74 site combined mutation group, WT group and N control group all had protein expression.

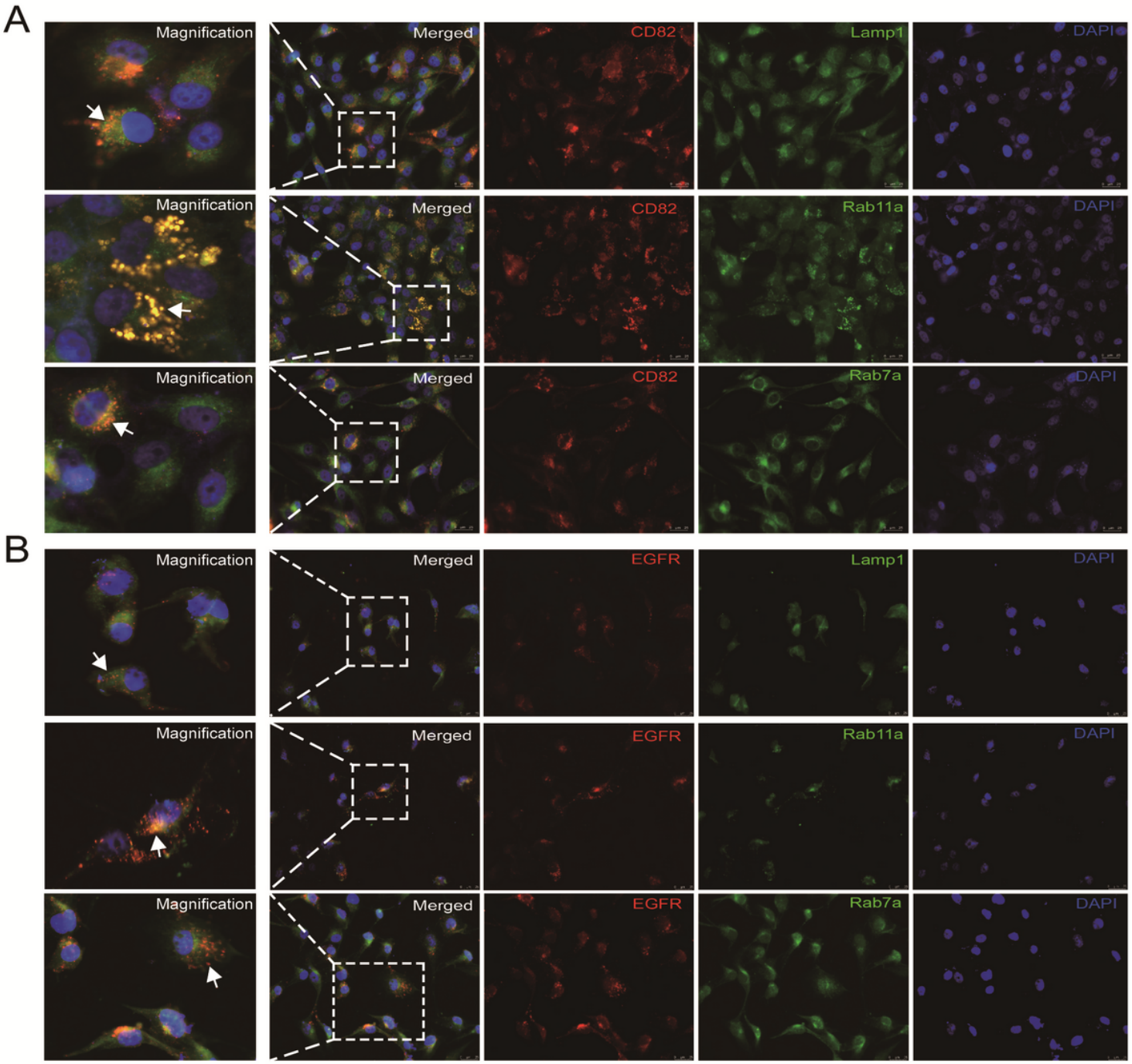


Figure 4

When CD82 palmitoylation Cys5+Cys74 site joint mutation, A: CD82 and Lamp1, Rab11a and Rab7a co-localization situation. B: Co-localization of EGFR with Lamp1, Rab11a and Rab7a. The scale bar is 25μm.

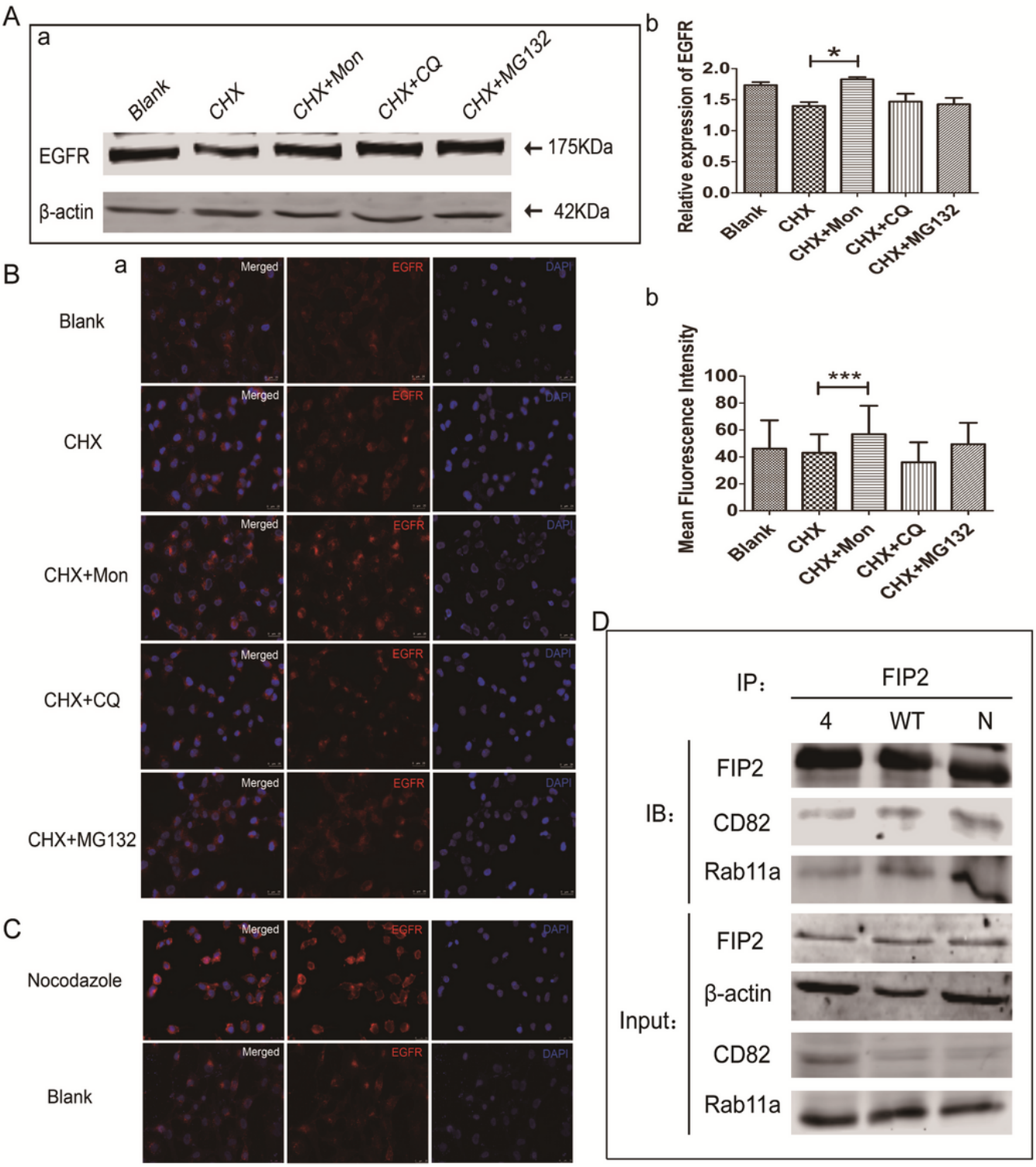


Figure 5

A: CD82 palmitoylation Cys5+Cys74 site after combined mutation, Western blotting method to detect the expression of EGFR in the total protein after adding different inhibitors. B: When CD82 palmitoylation Cys5+Cys74 sites are combined with mutations, immunofluorescence method to detect the expression of EGFR after adding different inhibitors. The scale bar is 25μm. C: After the combined mutation of CD82 palmitoylated Cys5+Cys74 sites, the tubulin inhibitor Nocodazole (20μg/ml, 4min) was added to detect

the expression of EGFR. The first row of the figure is the Nocodazole group, the second row is the control group, and the scale bar is 25μm. D: FIP2 in the CD82 palmitoylation Cys5+Cys74 site combined mutation group, WT group and N control group, the results of the IP group show that FIP2 can directly bind to Rab11a and CD82. In the Input group, CD82 palmitoylated Cys5+Cys74 site combined mutation group, WT group and N control group all had protein expression.

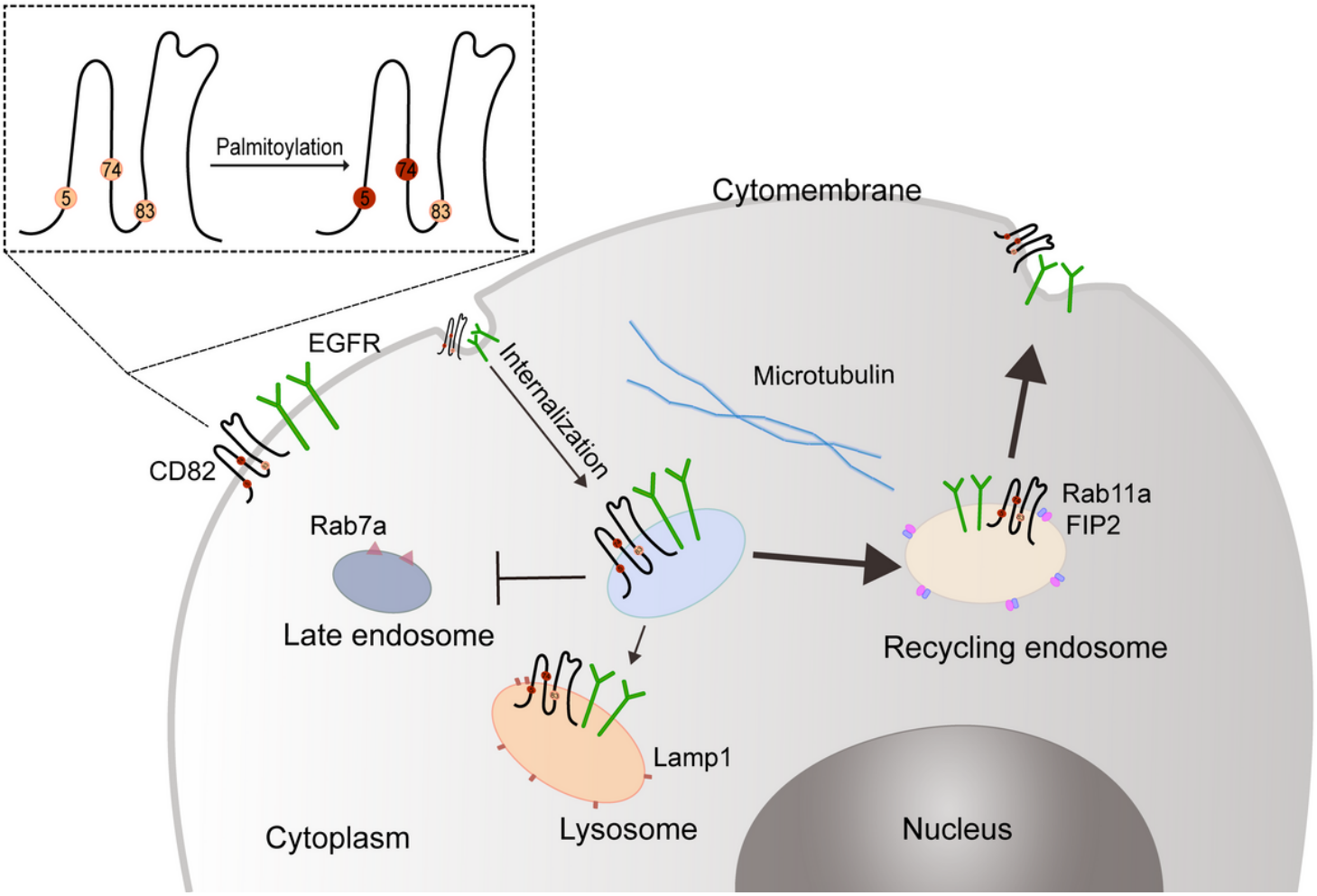


Figure 6

The mechanism diagram of the effect of the tetraspanin CD82 palmitoylation mutation on the expression and localization of EGFR. When the Cys5+Cys74 site of CD82 is combined with palmitoylation mutation, the internalization ability of EGFR is enhanced. After internalization, most of EGFR and CD82 enter the recycling endosome in a direct binding manner. This process requires the assistance of tubulin. CD82 can directly bind with Rab11a and FIP2 to form an EGFR/CD82/Rab11a/FIP2 complex, which is metabolized by the circulatory pathway to re-express EGFR and CD82 on the cell membrane surface. The remaining small part of the EGFR/CD82 complex is degraded in the lysosome, and this metabolic process does not go through the late endosomal pathway.