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CLMP-mediated regulation of intestinal homeostasis in IBD

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The intestinal mucosa is composed of a single layer of epithelial cells that forms a selective physical barrier allowing the passage of nutrients and solutes while protecting the body against external antigens. The impairment of the intestinal barrier function is well-appreciated to negatively contribute to the pathogenesis of inflammatory bowel disease. Barrier properties are due to intercellular junction proteins and among these proteins are found the members of the Cortical Thymocyte marker in Xenopus (CTX) family. CAR-Like Membrane Protein (CLMP) belongs to the CTX family, however, its function in the intestine has been poorly investigated. The global aim of our project is to characterize the role of CLMP in the intestinal mucosal homeostasis and inflammatory diseases. During this first year of the research period, we studied the role of CLMP in intestinal epithelial cell proliferation, migration and barrier function under normal conditions. Our results show that CLMP is concentrated at contacts between adjacent epithelial cells in adult mouse colon and human intestinal epithelial cell lines (IECs). CLMP overexpression in IECs decreases cell proliferation and prevents the growth of subcutaneous xenograft tumors in Rag1-/- mice. Interestingly, CLMP overexpression in IECs enhances cell-cell interactions and promotes epithelial wound healing after injury. In contrast, CLMP down-regulation increases cell proliferation and diminishes epithelial barrier function. In conclusion, our findings strongly suggest that CLMP plays a key role in regulation of several aspects of the mucosal epithelial homeostasis including barrier properties, cell proliferation and wound repair.
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1. Introduction

The intestine is lined by a single layer of epithelial cells that forms a selective-permeable barrier permitting the uptake of luminal nutrients while protecting the body against external noxious substances and pathogens. Intestinal mucosal homeostasis requires coordinated proliferation of epithelial progenitors at the base of the crypt, their migration and differentiation along the crypt-luminal axis, followed by shedding at the luminal surface. During this complex process which lasts 5-7 days, cell-cell cohesion and barrier function is maintained. Importantly, impairment of mucosal homeostasis has been shown to lead to compromised epithelial permeability which has been linked to the pathogenesis of inflammatory bowel disease (IBD).

At the molecular level, the intestinal barrier is dependent on a collection of proteins that form the apical junctional complex (AJC) that seals the apical-most region of the paracellular space between adjacent epithelial cells. Among the transmembrane protein proteins of the AJC involved in regulation of intestinal barrier function are members of the Cortical Thymocyte marker for Xenopus (CTX) family including Junctional Adhesion Molecule-A (JAM-A), Coxsackie and Adenovirus Receptor (CAR) and CAR-Like Membrane Protein (CLMP) (1, 2). Unlike JAM-A and CAR, CLMP function has been poorly investigated. Recently, mutations in CLMP have been found in patients with Congenital Short Bowel syndrome, a disease characterized by a shortening of the small intestine and impaired intestinal absorptive function suggesting a role for CLMP in the embryonic development (3); however its function in the adult intestine is largely unknown.

The aim of our project is to characterize the role of CLMP in intestinal mucosal homeostasis and inflammatory bowel disease. This knowledge will help to better understand the molecular interactions that control intestinal barrier function and are linked to the pathogenesis of IBD. Furthermore, these studies may help to develop new therapeutics to either facilitate the delivery of drugs through the mucosal barrier or for the treatment of chronic inflammatory gut diseases.

2. Keywords

CAR-Like Membrane Protein, intestinal mucosal homeostasis, cell proliferation, epithelial barrier function
3. Overall Project Summary

The major aim of the first year of this research project was to study the role of CLMP in the regulation of intestinal epithelial cells proliferation, cell migration and barrier function. For first goal, we generated stable single clones of intestinal epithelial cell lines (IECs) with overexpression or down-regulation of CLMP protein as exemplified in figure 1. For the over-expression of CLMP, the human full-length CLMP coding sequence was cloned into pLEX-MCS lentiviral vector. Human intestinal epithelial cell lines which lack endogenous expression of CLMP (such as SKCO15 and SW480 cells) were transduced with pLEX MCS-CLMP lentivirus or an empty virus as control. For the knock-down of CLMP, Caco-2 cells that express endogenous CLMP were infected with a non-silencing shRNA (control) or shRNA vector against CLMP gene. In both case (overexpression or knock-down of CLMP), virus-infected cells were selected with 2mg/mL of puromycin and single clones were obtained by limiting dilution.

![Figure 1: CLMP overexpression and knockdown in intestinal epithelial cell lines. Expression profile of CLMP by flow cytometry. SKCO15 (A) and SW480 cells (B) were infected with lentivirus expressing human full length CLMP (OE) or an empty virus (control). Caco-2 cells (C) were infected with a non-silencing shRNA vector (NS) or a shRNA against CLMP (KD). Cells were incubated with a primary antibody against CLMP (black line) or isotype control antibody (grey filled line). Primary antibodies were detected with PE-labeled secondary antibody and analyzed with a BD FACSCanto II cytometer.](image-url)
- **Task 1.1A (PI: Nusrat; months 1-4):** In vitro studies on CLMP control of intestinal epithelial cells (IEC) homeostasis using assays of proliferation, cell migration and apoptosis with knockdown and overexpressing cell lines.

- To investigate the role of CLMP in epithelial cell proliferation, we evaluated the incorporation of EdU (a modified thymidine analogue) into newly synthesized DNA, in cells that overexpress or knock-down for CLMP versus control cells. Cells were seeded at subconfluent density (10^5 cells) on collagen coated glass coverslips. EdU was added in the culture medium for 45 min at different time point following the seeding of the cells. After the incubation time, cells were fixed with formalin and the EdU was detected with fluorescently labeled Alexa Fluor 488 in accordance with the manufacturer’s recommendations (Click-it EdU, Invitrogen). Cells were stained with Topro-3 iodide for the detection of total nuclei and the amount of EdU-fluorescent labeling proliferating cells was quantified. As shown in figure 2A, the overexpression of CLMP in SKCO15 and SW480 cells significantly decreased cell proliferation at day 1 following the seeding. In contrast, CLMP knock-down increased Caco-2 cell proliferation. We next analyzed the expression level of c-myc and phosphorylated histone H3 which have been well-described in the literature to promote cell proliferation. We found that CLMP overexpression decreased the expression of c-myc and phosphorylated histone H3 (figure 2B). Consequently, these findings confirmed the results obtained with an EdU incorporation assay and strongly suggest that CLMP regulates cell proliferation.

- We further examined whether CLMP expression may affect cell death. For this purpose we analyzed the expression level of nuclear cleaved Poly (ADP-ribose) polymerase (cleaved PARP) which is a target of caspases and a marker of cells apoptosis. We found that PARP is less subjected to cleavage in cells overexpressing CLMP than control cells suggesting that CLMP overexpression may protect the cells from apoptosis.

- One major aspect of the intestinal mucosa is its ability to repair after injury in a process called wound healing by collective migration of the epithelial cells. We assessed the putative role of CLMP on wound closure *in vitro*. As exemplified in figure 2C, overexpression of CLMP in SKCO15 cells increased the migration of cell monolayers after scratch wounding. This result suggests that CLMP is involved in epithelial wound healing after injury. Altogether, we found that the level of expression of CLMP regulates intestinal cell proliferation, survival and migration. These findings show that CLMP plays a role in regulation of many aspects of intestinal epithelial homeostasis.
Task 1.1B (PI: Parkos; months 1-3):

CLMP regulation of intestinal epithelial cells barrier properties

CLMP is structurally related to Coxsackie and adenovirus receptor (CAR) which has been reported to be involved in intestinal barrier function (4). Thus, we investigated the role of CLMP on the regulation of paracellular permeability. For this purpose, cells were plated at confluent density on semi-permeable filters coated with collagen and transepithelial resistance (TER) was measured at different time points up to 5 days after cells seeding. We found that overexpression of CLMP significantly enhanced the TER of SW480 cell monolayers (figure 3A). In contrast, Caco-2 cell monolayers depleted for CLMP failed to develop high TERs (figure 3B) and were leakier than control cells as shown by the increase of paracellular permeability.

Figure 2: CLMP controls cell proliferation, apoptosis and migration (Task 1.1A). (A) Cell proliferation was measured by evaluating EdU incorporation (Click-IT EdU, Invitrogen) in cells overexpressing CLMP (SKCO15/CLMP OE, SW480/CLMP OE), knockdown cells (Caco-2 KD) or control cells (control, NS). At day 1 or day 2 after seeding, cells were incubated with EdU for 45 min, then samples were processed to detect incorporated EdU by fluorescence microscopy and total nuclei were stained with Topro-3 iodide. The percentage of proliferating cells was quantified as a ratio of EdU-positive nuclei to total nuclei. Results are means ± S.E.M. (n=20 images) of a representative three independents experiments. (B) Western blot analysis of the level of cell proliferation markers (c-myc, phospho-histone H3) or apoptosis (cleaved PARP) in cells overexpressing CLMP (+) and control cells (-) at day 1 after seeding. (C) Confluent SKCO15 monolayers were subjected to wound healing by introduction of a single linear scratch wound. Wound widths were monitored at day 0 and day 3 with an optic microscope. ***P<0.001, **P<0.01 t-test.
permeability to fluorescein isothiocyanate (FITC)-labeled dextrans (4kDa) (figure 3C). These results strongly suggest that CLMP participates in the regulation of the intestinal epithelial barrier.

We next studied whether CLMP is involved in the formation of epithelial tight junctions. We used a calcium switch assay to study the assembly of intercellular junctions. T84 cells were grown at confluence on semi-permeable filters coated with collagen until they reach a TER value about 1400 ohm.cm². Cells were incubated with 2mM EGTA in Hank's Balanced Salt Solution (HBSS) for 30 min to induce a quick loss of intercellular contacts by chelation of extracellular calcium. Then, complete cell culture media with calcium was added to allow the recovery of intercellular junctions. During the recovery period, anti-CLMP or isotype control antibodies were added. We found that anti-CLMP antibodies delayed the re-assembly of intercellular contacts after calcium switch, suggesting that CLMP supports tight junction formation (figure 3D). We further confirmed the role of CLMP on cell-cell cohesion using a dispase-based dissociation assay. As shown in figure 3E, the overexpression of CLMP on both, SKCO15 and HT29 cells strengthened cell-cell adhesion, as evidence by a reduction of cell monolayer fragmentations after dispase treatment when compared to control cells lacking CLMP.

Figure 3: CLMP regulates barrier properties and cell-cell cohesion (Task 1.1B). (A,B,C) Cells were seeded at high density (200,000 cells) on collagen-coated transwell filters. (A,B) transepithelial resistance (TER) measurements were determined using an EVOM volt-ohmmeter for 5 days. (C) Permeability to fluorescein isothiocyanate (FITC)-dextrans (4 KDa) was quantified at day 4. FITC-dextrans at a final concentration of 0.5 mg/mL was added to the upper chamber. After 2 hours of incubation, the fluorescent content in the basal chamber was measured using a fluorescence plate reader. Results are means ± S.E.M. (n=3), ***P<0.001, t-test. (D) T84 cells were grown to confluence and subjected to a calcium switch assay. Cells were incubated with 2mM EGTA in HBSS minus for 30 min, then switched to complete medium supplemented with a blocking antibody against CLMP (CLMP Ab) or isotype control antibody. Results are means ± S.E.M. (n=3), ***P<0.001, t-test. (E) Cell confluent monolayers were subjected to dispase-based dissociation assays by incubation with 5mg/ml dispase in HBSS+ for 20 min at 37°C. Monolayers were then rotated on an orbital shaker (250 rpm) to induce fragmentation. Images show one well of a six-well plate of a representative experiment (n=3) performed in triplicate.
Task 1.1C (PI: Nusrat; months 4-8): CLMP growth and polarity in cell cultures using knockdown and overexpression cell lines.

We have been delayed with the delivery of this task because we faced some issues in finding commercially available antibodies against CLMP which are appropriate for immunolabeling and fluorescence microscopy. Recently, we have successfully identified two antibodies which will be used for this task that we plan the completion in four months and the results will be presented in the next project report. We have performed immunolabeling endogenous CLMP in mouse colon OCT-embedded frozen sections (figure 4A) and Caco-2 cells (figure 4B). CLMP is detected along the intercellular junctions and co-localized with both, the tight junction protein ZO-1 and the adherens junction protein, E-cadherin. Using of SKCO15 cells, transiently transfected with a vector expressing CLMP, we found that CLMP is concentrated mainly at contacts between adjacent cells expressing CLMP (figure 4C). Altogether, these observations suggest that CLMP is a junctional protein with lateral distribution and may engage in homophilic interactions in trans. Currently, we are studying the effect of CLMP (knock-down or overexpression), on cell polarity by analyzing subcellular localization of polarity complex (Par3, aPKC) and tight junction proteins (claudins, occludin, JAM-A, CAR and ZO proteins).

Figure 4: CLMP is a enriched at the intercellular junctions (Task 1.1C). Images represent the immunofluorescence staining of CLMP (green), ZO-1 or E-cadherin (red) and nuclei (blue). (A) Frozen tissue sections of wild-type mouse colon (C57BL/6 strain). (B) Confluent monolayer of caco-2 cells expressing endogenous CLMP. Arrows show the orthogonal XZ section used for the projection image. (C) Confluent SKCO15 cells transiently transfected with a vector expressing human CLMP or empty vector. (+) indicates CLMP positive cells. CLMP is accumulated at cell-cell contacts between adjacent cells expressing CLMP.
- Task 1.1D (PI: Nusrat; months 8-12): CLMP control of epithelial growth in Xenografts.

To analyze the putative role of CLMP in proliferative capacity of epithelial cells and tumor formation in vivo, we performed xenograft experiments with Rag 1-/- mice as recipients. We injected subcutaneously $10^6$ SKCO15 cells overexpressing CLMP or control cells into twelve-week-old Rag 1-/- mice (8 mice per condition). Tumor growth was monitored every day by palpation and mice were sacrificed at day 21 post-injection when tumors reached $\approx 2$ cm in size. As shown in figure 5, the overexpression of CLMP prevented tumor growth. Indeed, tumors developed in 100 percent in mice injected with control cells lacking CLMP, while only 1 mouse that was injected with cells overexpressing CLMP produced a small tumor. Altogether figures 2A and 5 provide evidence that CLMP expression controls epithelial cells proliferation in vitro and in vivo. We are currently performing xenografts with Caco-2 CLMP knockdown cells versus controls in order to study whether the depletion of CLMP may increase the incidence of tumor growth in vivo.

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**Figure 5: CLMP controls in vivo xenograft tumor growth (Task 1.1D).** Twelve-week-old male Rag1-/- mice were injected subcutaneously with $1.10^5$ SKCO15 cells overexpressing CLMP versus control cells. Animals were killed at 21 days post injection and tumors were harvested then measured. All animal experiments were performed in accordance with protocols approved by the Emory University IACUC.
• **Task 1.2A (PI: Parkos; months 3-7):** CLMP co-association with tight junction (TJ) proteins by immunoprecipitation, immunofluorescence labeling and confocal microscopy.

CLMP is structurally related to Junctional adhesion molecule -A and CAR which have been reported to be localized at TJs and interact with TJ-associated proteins such as ZO-1 (2). As mentioned above, we found endogenous CLMP co-distributed at the plasma membrane with ZO-1 in mouse colon tissue and Caco-2 cells (figure 4). It is tempting to speculate that CLMP may physically interact with TJ-associated proteins. Because, TJ complexes have been reported to be associated with lipid rafts, we investigated whether CLMP is present in these plasma membrane microdomains. We performed a sucrose gradient ultracentrifugation (5 - 30%) to separate lipid rafts by flotation (5). Protein fractions were resolved on SDS-PAGE gel and lipid raft-associated proteins such as flotilin-1 and desmoglein-2 were used as markers for Western blotting (figure 6A). The majority of CLMP proteins was not found in lipid rafts, however at higher exposure we detected CLMP in flotilin-1 and desmoglein-2 rich fractions. We conclude that CLMP localized in two pools in the lateral cell membrane and may interact with TJ proteins in lipid rafts.

![Figure 6: CLMP co-association with tight junction proteins (Task 1.2A).](image)

(A) Isolation of membrane rafts microdomains from Caco-2 cells. Cells were lysed with 1.5% Triton X-100 lysis buffer, then lysates were adjusted to a final concentration of 40% sucrose (w/w) and separated in a linear 5%-30% sucrose gradient by ultracentrifugation. 0.5 ml fractions were collected and analyzed. (A, upper part) Profiles of light scattering at 600 nm and membrane alkaline phosphatase activity of sucrose gradient fractions. (A, lower part) Western blot analysis of sucrose gradient fractions. Flotilin-1 and desmoglein-2 are used as markers of lipid raft-associated proteins. (B) Interaction between CLMP and ZO-1 by Proximity ligation assay (Sigma-Aldrich) in Caco-2 cells grown on transwell filters for 10 days. PLA signals were obtained for the antibody combinations: anti-CLMP+ Ig control or anti-CLMP+anti-ZO-1. The samples were analyzed by immunofluorescence microscopy with actin staining as a control of the cell monolayer integrity.
We further studied the interaction between CLMP and the TJ-associated protein ZO-1 using proximity ligation assays. This technique is a sensitive method for detecting protein-protein interactions (closer than 40 nm) visualized as individual fluorescent dots. We detected intensive PLA signals between endogenous CLMP and ZO-1 in Caco-2 cells, at intercellular contacts when cells were incubated with specific antibodies against both, CLMP and ZO-1 (figure 6B). In contrast, no fluorescent dots were detected with the antibody combination containing anti-CLMP and isotype control. We are currently focused on optimizing co-immunoprecipitation protocols to study the physical interaction between CLMP and TJ-associated protein. We are testing several detergent-based lysis buffers to solubilize cellular contents and maintain the integrity of CLMP-associated protein complexes.

- **Task 1.2B (PI: Parkos; months 7-12):** Mass spectrometry of CLMP immunoprecipitates to identify associated proteins and verification by immunofluorescence and western blot of immunoprecipitates.

As we have just discussed above in task 1.2A, we are currently working on optimizing co-immunoprecipitation protocols to study the physical interaction between CLMP and associated partners. In figure 7, we provide an example of coomassie blue stained proteins co-precipitated with CLMP and separated by SDS-PAGE. We used HT29 cells overexpressing CLMP versus control cells lysed with 1% Brij97 buffer (mild non-ionic detergent). With this condition, CLMP is found slightly enriched by immunoprecipitation but we did not distinguish clear protein bands for putative partners. For mass spectrometry analysis, we are actively testing several methods to improve the immunoprecipitation of CLMP and its partners.

![Figure 7: Coomassie blue stained gel of proteins co-immunoprecipitated with CLMP (Task 1.2B).](image-url)

HT29 cells overexpressing CLMP (+) or control cells (-) were lysed in 1% Brij97 lysis buffer. Cell lysates were subjected to immunoprecipitation using a polyclonal anti-CLMP antibody. The elute immunoprecipitates complexes were resolved by 10% SDS-PAGE gel and proteins were visualized with Coomassie blue staining.
- Task 1.5A (PI: Parkos; months 1-18): Generation of mice with inducible targeted deletion of CLMP.

Since CLMP has been reported to be critical in embryonic gut development (3), the global CLMP gene loss in mice would be problematic to study the role of CLMP in adult intestine. To circumvent this issue, we generated transgenic mice with CLMP gene flanked by loxP sites that will allow conditional tissue specific deletion of CLMP when such animal are mated with tamoxifen-tissue specific promoter gene-Cre<sup>+</sup> animal. For the study of CLMP in intestinal mucosa, these animals will be crossed with tamoxifen inducible-villin Cre<sup>+</sup> animal to generate CLMP knock-down specifically in intestinal epithelial cells (Figure 8 A-B). We have generated CLMP homozygous flox/flox animals from heterozygous flox/wt animals as shown in Figure 8C. CLMP flox/flox animals will be crossed with mice expressing tamoxifen-dependent Cre recombinase expression under villin-1 promoter. The production of CLMP specific knock-down in intestinal epithelial cells will be an important tool to study CLMP function in vivo, in mucosal epithelial homeostasis and inflammatory diseases.

Figure 8: Generation of mice with inducible targeted deletion of CLMP (Task 1.5A) Schematic of the generation of conditional CLMP mouse. (A) Original schematic of the floxed exon 3 CLMP allele (AW557819) with conditional potential from EUCOMM. These ES cells were transfected with a Flip recombinase expressing plasmid to excise the LacZ/neomycin cassette and generate conditional CLMP knock-out ES cells. (B) Schematic for generation of tamoxifen inducible conditional CLMP knockout mice from homozygous CLMP floxed mice that were created from the conditional CLMP ES cells. (Frt = flip recombinase target sequence, LoxP = Cre recombinase target sequence). (C) Genotyping of CLMP Flox/Flox mice. To identify floxed CLMP allele from the wild type CLMP allele, genomic DNA was isolated from tail snips and subjected to PCR using CLMP primers. For identification of the floxed animal PCR was run using CLMP uni R1 primer (5'-CTTCTTTGTTGCGATGTTAGACAC-3') with an Es cell vector specific primer BactP flox gen 2F (5'-CTGAGTCGGCATCCATGG-3'). PCR with the CLMP uni R1+BactP flox gen 2F primer amplifies a 137 bp product in the CLMP floxed animal. To identify the presence of the wild type CLMP allele PCR was done using the CLMP uni R1 primer with the CLMP WT F3 primer (5'-GTAGAAAATCCCTACGCTGCC-3'). This PCR results in the amplification of a 177bp product if the CLMP wild type allele is present.
4. Key Research Accomplishments

At the end of this first year our key accomplishments are:
- Generate stable human intestinal epithelial cell lines (IECs) knock-down or overexpressing CLMP in order to study in vitro the role of CLMP in intestinal epithelial cells homeostasis.
- Generate homozygous CLMP flox/flox mice for the production of tamoxifen inducible knock-out of CLMP in intestinal epithelial cells.
- Identify CLMP as a transmembrane protein localized at intercellular contacts between adjacent cells in mouse colon, Caco-2 and SKCO15 cell lines. CLMP co-localizes with both, the tight junction protein, ZO-1 as well as the adherens protein, E-cadherin. Interestingly, CLMP presents two pools at plasma membrane and co-sediments with lipid raft-associated proteins
- Show the involvement of CLMP in epithelial barrier properties. CLMP overexpression enhances cell-cell cohesion and barrier function in vitro. In contrast, CLMP down-regulation impairs barrier function and increases paracellular permeability to fluorescein dextrans (4kDa).
- Measure the effect of CLMP on cell proliferation in vitro and in vivo. CLMP overexpression in IECs decreased cell proliferation while its down-regulation increased cell proliferation. In vivo, CLMP overexpression in IECs prevented xenograft tumor growth in Rag-1-/- mice.

5. Conclusion

CLMP is a transmembrane glycoprotein of the immunoglobulin superfamily (IgSF) structurally related to Coxsackie and adenovirus receptor and has been reported to play a role in intestine development, however its role on the adult intestinal under normal and pathological conditions remains unclear. During this first year of the research project, we focused on deciphering the role of CLMP in normal intestinal epithelial cells. We have accumulated evidence that CLMP plays a role in several aspects of intestinal epithelial cells homeostasis such as cell proliferation, migration and barrier function. For the second year of this study, we will work on the identification of CLMP-associated partners and CLMP-dependent signaling pathways. We will also assess the role of CLMP in vivo in mucosal homeostasis with the conditional CLMP knock-out mice. Interestingly, we recently found that CLMP may facilitate the recruitment of polymorphonuclear leukocytes (PMN) in vitro in response to bacterial formyl peptides such as fMLF. As inflammatory bowel disease (IBD) is characterized by uncontrolled infiltration of immune cells including PMN, we will study the role of CLMP in the recruitment of leukocytes in vitro and in vivo in response to various inflammatory signals.

The overarching goal of this project is to characterize the role of CLMP in regulation of intestinal homeostasis under normal conditions and under conditions of pathological inflammation. We believe that our findings will contribute to a better understanding of the molecules involved in mucosal barrier properties and in the pathogenesis of IBD, a requisite for the development of new therapeutic strategies.
Given the high impact of IBD on health and medical management of both military recruits and veterans, studies directed at understanding the pathogenesis and treatment of IBD comprising ulcerative colitis and Crohn's disease are timely, warranted and justified.


Anny-Claude Luissint (PhD) did a presentation at the Annual 2014 Meeting of the American Society for Investigative Pathology (ASIP) in conjunction with Experimental Biology 2014 at the San Diego (April 26-30, 2014). The title of the abstract was: CAR-like membrane protein negatively regulates the intestinal barrier and promotes neutrophil transepithelial migration (60.6) FASEB J April 2014 28:60.6

7. Inventions, Patents and Licenses

Nothing to report

8. Reportable Outcomes

Nothing to report

9. Other Achievements

Nothing to report

10. References


11. Appendices

None