CCDC11 Acts as a Scaffold to Assemble the ESCRT Membrane-Scission Machinery at Viral Budding Sites for HIV-1 Release: Identifying a Novel Therapeutic Strategy for Antiviral Therapy

Research Plan

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a) Rationale

Acquired ImmunoDeficiency Syndrome (AIDS) is a chronic, often life-threatening disease caused by infection of the Human Immunodeficiency Virus (HIV). Through damaging the immune system, HIV reduces the body's ability to fight the organisms that cause the disease. Since the beginning of the HIV/AIDS epidemic, more than 70 million people have been infected with HIV, and over 35 million have died of this disease. Each year, almost 2 million additional individuals are infected or diagnosed with AIDS. However, molecular and cellular mechanisms of viral infections are highly complex and not fully understood.

In an effort to discover potential targets for antiviral therapy against HIV and other harmful viruses, we previously researched on whether a ciliary protein named Coiled-Coil Domain-Containing (CCDC11) plays a role in HIV-1 budding. CCDC11 has previously been shown to be involved in ciliogenesis and cytokinesis. During cytokinesis, it recruits the Endosomal Sorting Complex Required for Transport III (ESCRT-III) membrane scission machinery to the midbody to mediate the physical separation of two dividing daughter cells. More importantly, ESCRT-III is also integral to the budding process of HIV-1 and many other viruses. Consistent with this, we found that CCDC11 plays a key role in HIV-1 production using CCDC11-kockout Human Embryonic Kidney (HEK) 293T cells. However, the molecular functions of CCDC11 in viral budding remain unknown.

To further extend the role of CCDC11 in the viral budding process, we plan to knockout CCDC11 in human cervical cancer HeLa cells, which is a widely used model for HIV research, using the CRISPR-Cas9 technology. This will be verified by sequencing, western blotting, and immunofluorescence microscopy. We will identify multiple independent CCDC11-knockout HeLa cell lines. In order to determine the effect of CCDC11 knockout on HIV-1 budding, we will transfect negative control and CCDC11-knockout HeLa cells with a plasmid for HIV-1 WT-Gag structural protein or mutant P7L-Gag (a negative control). We will use the Enzyme-Linked ImmunoSorbent Assay (ELISA) p24 capture assay to assess HIV-1 release by measuring the relative concentration of p24 viral proteins in cell culture media.

To explore the molecular mechanisms of CCDC11 functions in viral budding, we plan to do immunofluorescence staining for HIV-1 Gag and CCDC11 co-transfected in HeLa cells to evaluate if they colocalize at the plasma membrane. In addition, we will perform co-localization assays in mammalian cultured cells using immunofluorescence staining to examine if CCDC11 colocalize with any of ESCRT components including TSG101, ALIX, CHarged Multivesicular Body Protein (CHMP) 2A, CHMP4B, CHMP6, and CHMP8. Furthermore, we will use co-immunoprecipitation (Co-IP) assays to test whether CCDC11 binds to any of the above ESCRT components.

We anticipate that our experiments will provide valuable information regarding novel antiviral drug design and therapies.

b) Hypothesis

Recent studies indicate that CCDC11 is required for cytokinesis during cell division through recruitment of the ESCRT-III component CHarged Multivesicular Body Protein 2A (CHMP2A) to the midbody. More recently, we found that CCDC11 plays a key role in the production of HIV-1. Since ESCRT-III is also known to be important for HIV-1 budding, we hypothesize that CCDC11

promotes viral budding from the plasma membrane of host cells via direct recruitment of ESCRT components such as TSG101, ALIX, CHMP2A, and CHMP4B to the sites of viral budding.

Research Questions:

1. Does loss of CCDC11 compromise HIV-1 budding in other cell types in addition to HEK293T cells?
2. Can we document if there is a defect in HIV-1 release from the surface of CCDC11-knockout cells, or in another word, if there is HIV-1 particle accumulation on the surface of CCDC11-knockout cells?
3. Does CCDC11 colocalize with TSG101, ALIX, and ESCRT-III components such as CHMP2A and CHMP4B, which are important for viral budding?
4. Does CCDC11 colocalize with the HIV-1 Gag structural protein at the sites of viral budding on cell membranes? Is the recruitment of the ESCRT-1 component TSG101, ALIX, or the ESCRT-III components CHMP2A and CHMP4B to the sites of viral budding reduced in CCDC11-knockout cells?
5. Does CCDC11 physically interact with TSG101, ALIX, and/or the ESCRT-III components CHMP2A and CHMP4B?

Engineering Goals:

First, we aim at successfully generating CCDC11-knockout cell lines using human cervical cancer HeLa cells. Next, we will evaluate their possible defects in cytokinesis/cell division. Then, we will investigate the effects of CCDC11 depletion on HIV-1 release and explore the molecular mechanisms related to CCDC11 functions in HIV-1 budding. Our overall goal is to determine if CCDC11 is commonly required for viral budding in different cell types in addition to HEK293T cells and gain insight into the molecular functions of CCDC11 in the viral budding process.

Expected Outcomes:

We expect that CCDC11-knockout HeLa cells are most likely viable and defective in cell division. We also expect that the efficiency of HIV-1 release into culture media is significantly lower from CCDC11-knockout HeLa cells. CCDC11 may form a stable complex with some of ESCRT components. Taken together, our project will support the hypothesis that CCDC11 is important for HIV-1 budding from infected cells, and mechanistically, it ultimately facilitate the recruitment of the ESCRT-III membrane scission machinery to the site of viral budding at plasma membranes.

c) Procedure, Risk and Safety, Data Analysis

I. Procedure:

Bacterial transformation:

CCDC11-related plasmids will be transformed into DH5alpha *E. coli* competent cells to amplify plasmid DNAs. The Qiagen Plasmid Plus Midi Kit will be used to purify the plasmid DNAs.

Cell culture:

HEK293T (CRL-3216), HeLa (CCL-2), and U2OS (HTB-96) cells will be purchased from American Type Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS) with 100 U/ml penicillin-streptomycin at 37 °C in a 5% CO_2 incubator.

Transient transfection:

The day before transfection, cells will be trypsinized and counted. $2X10^5$ cells will be seeded into 6-well tissue culture plates with 2 ml DMEM and 10% fetal bovine serum (FBS) each well. After incubating for 18-24 hrs, the cells should be at 70-90% confluence at the time of transfection. 2.5 µg total DNA will be transfected using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol.

Generation of CCDC11-knockout HeLa cells using the CRISPR/CAS9 system:

HeLa cells will be transfected with pSpCas9(BB)-2A-Puro (PX459) V2.0 containing non-targeting or targeting human CCDC11 gRNA using Lipofectamine 3000. Transfected cells will be selected in the presence of puromycin (2.5 ug/ml). Two different gRNAs targeting CCDC11 will be used: gRNA-1, GCGGTTTGGCACCGTACAGC, gRNA-2, AGCGGTTTGGCACCGTACAG. Following 1-2 weeks of continued culture, when transfected cells have formed multiple single colonies, the colonies will be isolated and further grown for experiments. To determine CCDC11 mutations, genomic DNA will be isolated from the cell colonies, and PCR fragments encompassing the gRNA sequence will be generated and used for sequencing.

Western blotting:

Wild-type (WT) and CCDC11-knockout HeLa cells will be harvested and lysed in the Radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Nonidet P-40 [NP-40], 0.5% Sodium deoxycholate, 0.1% SDS) with protease cocktail inhibitors (Roche) added before use, and the cell lysates will be sonicated to disrupt cellular membranes and release the cell contents. The lysates will be centrifuged at 13000-rpm and supernatant will be collected and added 5xSDS sample buffer followed by denaturing the proteins at 95 °C for 3 mins. The samples will be loaded into a 10% SDS-Polyacrylamide gel electrophoresis (PAGE) and the gel will be run for 2 hrs at 90 V. Proteins will be transferred from the gel onto the Nitrocellulose membrane (0.45um) with the power supply running at 16V for 14 hrs. The membrane will then be washed with Tris buffer saline with 0.1% Tween (TBST) and blocked for 1 hr at room temperature in 5% skim milk. Primary antibody CCDC11 will be added and allowed to incubate for 1 hr. The membrane will be washed 3 times for 15 mins each with TBST. Secondary antibodies conjugated with horseradish peroxidase will be added and allowed to incubate for 1 hr at room temperature. Antibodies will then be removed and the membrane will be washed with TBST 3 times for 15 mins each. Chemiluminescent substrates (SuperSignal™ West Pico Stable Peroxide Solution and SuperSignal™ West Pico Luminol Enhancer Solution, ThermoFisher Scientific) will be used to detect the protein followed by film exposure to visualize the specific protein transferred on the membrane.

Immunofluorescence staining:

HeLa cells will be grown on glass coverslips in a 12-well plate 24-48 hrs. The cells will then be washed and fixed with cold 50% methanol/50% acetone. The fixed cells will be incubated for one hr with 300 µl of blocking buffer containing 5% goat serum in diluent solution (2% bovine serum albumin [BSA] and 0.2% Triton X-100 in PBS). The blocking buffer will be discarded, and the cells will be incubated at 4 °C overnight with 300 µl of the diluent solution containing the following primary antibodies: rabbit anti-CCDC11 (Sigma-Aldrich, HPA040595, 1:300), mouse anti-acetylated alpha-tubulin (Sigma-Aldrich, T7451, 1:500), rabbit anti-Flag (Sigma-Aldrich, F7425, 1:500), mouse anti-HA (Proteintech, 66066-1-Ig), rabbit anti-GFP (Proteintech, 50430-2-AP, 1:500), mouse anti-Flag M2 (Sigma-Aldrich, F3165, 1:500). Subsequently, the cells will be washed and incubated for one hr with appropriate anti-rabbit or anti-mouse IgG secondary antibodies conjugated with DyLight 488 and DyLight 549 (Vector Laboratories). The cells will then be washed, counterstained with 4',6-diamidino-2-phenylindole (DAPI), used as a nuclear

counterstain, for two mins at room temperature, washed again, and mounted onto a glass slide using Fluoromount-G (SouthernBiotech) for microscopic analyses.

Quantification of Gag p24 release using ELISA:

The HIV-1 Gag protein p24 (MW, 24 kDa) is a structural protein of the HIV-1 capsid. It is initially synthesized as a polyprotein precursor, which is necessary and sufficient for the assembly and production of non-infectious, virus-like particles (VLPs) in the absence of other viral proteins or packageable viral RNA. Therefore, detectable p24 in cell culture supernatants is strongly correlated to released VLPs. The antigen capture enzyme-linked immunosorbent assay (ELISA) is currently the most common method used to assess viral replication both *in vivo* and *in vitro* for quantitation of the HIV-1 Gag p24 protein. We plan to utilize the HIV-1 p24 Capture ELISA kit (Immuno DX LLC) to measure the accumulation of Gag p24 in culture supernatants by reading optical densities (O. D.) at a wavelength of 450 nm using a microplate reader to monitor the effects of CCDC11 on HIV-1 budding.

CCDC11-deficient HeLa cells and control cells will be transfected in triplicate with plasmid bearing HIV-1 WT-Gag p24 or P7L-Gag. P7L-Gag is a p24 mutant containing a single amino acid substitution that impairs TSG101 binding during viral budding. TSG101 is a component of ESCRT-I, which functions in a sequential manner with ESCRT-0,-II, and -III in a series of membrane fusion and budding events. An expression plasmid for Renilla luciferase (pRL-TK) will be co-transfected with WT- or P7L-Gag to normalize transfection frequency. 24-48 hrs following DNA transfection, tissue culture media and cells will be harvested. The tissue culture media will be clarified by centrifugation to remove cellular debris and used for ELISA according to manufacturer's instructions. The cells will be lysed using RIPA buffer with sonication and used for measuring Renilla luciferase activities by the Dual-Luciferase Reporter Assay System (Promega) and a Berthold luminometer (Berthold Technologies). The value of Gag p24 production will be normalized by the value of Renilla luciferase activity from each sample. P7L-Gag release will be compared with the WT-Gag release, and the effects of CCDC11 depletion on WT-Gag and P7L-Gag release will be evaluated. One-way ANOVA will be used for statistical analysis, and *$p<0.05$ will be considered statistically significant. As an alternative method to monitor Gag p24 expression and release, we will perform western blotting using cell culture supernatants and lysates, followed by densitometric measurements of Gag bands on the immunoblots using the NIH ImageJ software.

Structured Illumination Microscopy (SIM):

For SIM imaging, HeLa or U2OS cells will be co-transfected with Flag-CCDC11 and HA-tagged CHMPs and immunostained with anti-Flag antibody for CCDC11 (red) and anti-HA antibody for CHMPs (green). Nuclei will be visualized by DAPI. Specimens will be analyzed by an N-SIM (Nikon) with a 100¡Ñ/1.49 NA objective. SIM takes 15 images with different illumination patterns, and the raw images are reconstructed into a single super-resolution image. It produces 8-fold higher resolution than the conventional confocal microscopy.

Co-immunoprecipitation (Co-IP) assays to investigate whether CCDC11 physically interacts with the ESCRT-III components CHMP2A, CHMP4B, CHMP6, and CHMP8.

Flag-tagged CCDC11 will be co-transfected with HA-tagged CHMP2A, CHMP4B, CHMP6, or CHMP8 into HEK293T cells, and cell lysates from each transfection will be immunoprecipitated with anti-Flag antibody (mouse, Sigma-Aldrich) and detected with anti-HA antibody (rat, Sigma-Aldrich) for the CHMPs. Cell lysates will also be immunoblotted with anti-Flag antibody for CCDC11 and anti-HA antibody for CHMPs to confirm stable protein expression.

II. Risks and Safety:

1) Human subjects: N/A

2) Vertebrate animals: N/A

3) Potentially hazardous biological agents (PHBA):
Human embryonic kidney (HEK) 293T (CRL-3216), human cervical epithelial HeLa (CCL-2), and human osteosarcoma U2OS (HTB-96) cells will be purchased from American Type Culture Collection (ATCC) and handled in a biological safety cabinet under proper supervision. Appropriate safety equipment will be used including gloves, goggles, and lab coats. This requires biosafety level 2 practices and containment facilities. DH5alpha *E. coli* cells are classified as biosafety level 1. Personal safety equipment should be worn at all times when handling. Tissue culture and bacterial wastes including culture media will be treated with bleach (a final concentration of >10%) for 30 mins and discarded into the laboratory sink. The sink will be thoroughly rinsed after waste discharge.

4) Hazardous chemicals/activities/devices:
Safety precautions must be taken when handling chemicals, and appropriate protective attire will be used at all times. These include gloves, goggles, lab coats, close-toed shoes, and long pants. Infectious/hazardous wastes will be properly disposed in designated containers. Reagents will be handled with care as described below.

Phosphate-buffered saline (PBS):
It may cause irritation to the eyes and skin through contact. If ingested, PBS may cause irritation to the digestive tract. If inhaled, it may cause irritation to the respiratory tract. If one's eye comes in contact with PBS, one can use water to flush out the chemical. In the case of skin irritation, the chemical can be washed off with water. In the case of ingestion, one may rinse the mouth with water.

Dulbecco's modified eagle medium (DMEM):
This is a common medum for tissue culture cells. It may cause irritation to the eyes, skin, mucous membranes, and respiratory tract if inhaled. If eye irritation occurs, one must wash their eye thoroughly with water and seek medical assistance. In the case of skin irritation, the affected area must be washed thoroughly with a large amount of soap and water. If swallowed, one must wash their mouth with water.

Penicillin-streptomycin antibiotics:
It may cause allergic skin reactions, eye irritations, and breathing difficulties. If skin contact occurs, immediately wash off with water for at least 15 mins. If eye contact occurs, rinse immediately with water, particularly under the eyelids, for 15 mins. If ingested, call a poison control or a physician immediately. If inhaled, immediately move to fresh air. Give artificial respiration if necessary and call poison control or a physician immediately.

Ampicillin antibiotic:
It is a potential skin and eye irritant. Prolonged exposure may cause damage to organs. Proper protective equipment should be used when handling.

10% fetal bovine serum (FBS):
It is used to supplement tissue culture media to stimulate cell growth. It may cause irritation if inhaled, swallowed, or comes in contact with the skin and eyes. Eye protection and appropriate clothing should be used when handling.

Puromycin:
Puromycin is an aminonucleoside antibiotic produced by *Streptomyces alboniger*. It specifically inhibits peptidyl transfer on both prokaryotic and eukaryotic ribosomes. This antibiotic inhibits the growth of Gram-positive bacteria and various animal and insect cells.

Tris-buffered saline (TBS):
It is a serious eye irritant. It may also be dangerous if inhaled or swallowed. Eye protection and proper gear should be worn.

Acrylamide:
It can permeate skin and irritate eyes. If eye contact occurs, flush eyes immediately with water for 15 mins. If skin contact occurs, flush affected area with water for 15 mins. If inhaled, get fresh air and if needed, give artificial respiration. If ingested, seek medical attention.

4',6-diamidino-2-phenylindole (DAPI):
It may cause skin and eye irritation. It may also be harmful if swallowed or inhaled. DAPI is a carcinogen or potential carcinogen as it binds to regions in DNA. In the case of eye contact, flush eyes out with water. In the case of skin contact, thoroughly wash and cover the contaminated area with an emollient. It is important to avoid prolonged exposure. If ingested, wash out the mouth with water, and if inhaled, move to an area with fresh air.

Qiagen Plasmid Plus Midi Kit buffers:
These buffers (**Lysis buffer P1 containing RNaseA, Precipitation buffer S3, and Elution buffer EB**) are proprietary components and therefore have no associated concentrations. They can cause serious skin and eye irritation upon exposure. Protective gloves, goggles, and lab coats should be worn. Hands should be thoroughly washed after handling

Methanol:
Methanol is toxic when ingested. It may also cause serious skin and eye irritation when exposed. Protective gears should be worn at all times. It is flammable and should be stored in a flammable storage cabinet.

Acetone:
Acetone may cause skin and eye irritation upon exposure. Protective gloves, goggles, and lab coats should be worn at all times. It is flammable and should be stored in a flammable storage cabinet.

TE buffer:
This is a Tris-based buffer and can cause skin and eye irritations. If eye contact occurs, flush eyes immediately with water for 15 mins. If skin contact occurs, flush affected area with water for 15 mins. If inhaled, get fresh air. Seek medical attention. If ingested, do not induce vomiting.

Lysogeny Broth (LB) and lipofectamine:
LB is a nutritional medium for bacteria. Lipofectamine are cationic lipids used for transient transfection of tissue culture cells. These are not hazardous but potentially irritate skin and eye upon exposure.

III. Data Analysis:

1) Immunofluorescence microscopy: Immunostained HeLa and U2OS cells will be viewed under a Leica DM6000B epifluorescence microscope, a Leica SP8X confocal microscope, or a Nikon SIM. The subcellular localization of related proteins will be carefully evaluated.

2) Western blotting: Protein bands will be visualized using the enhanced chemiluminescent (ECL) method and analyzed based on their molecular weights and intensities. Densitometric measurements of bands on the immunoblots corresponding to the protein of interest will be analyzed using the NIH ImageJ software.

3) ELISA: Effects of CCDC11 on the budding of HIV-1 virus-like particles (VLPs) will be measured based on a standard curve prepared from several different concentrations of recombinant HIV-1 p24 protein.

4) Quantification analysis: The standard errors of the mean will be calculated using the Excel software and error bars will be shown in results graph. Statistical significance will be assessed using the GraphPad Prism software.

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No Addendums Exist