INTRODUCTION

Coiled-Coil Domain-Containing 11 (CCDC11) was initially discovered as a gene mutated in human laterality disorder patients suffering from heterotaxy and situs inversus (1). Made up of 514 amino acids, CCDC11 harbors three coiled-coil domains. Four unique human CCDC11 mutations have been reported to date (1-4). Further studies showed that CCDC11 plays an important role in the establishment of left-right lateral asymmetry (2-5). Interestingly, in animals, the left-right asymmetry of internal organs is determined during early embryogenesis through the function of cilia, small hair-like structures that are present on the surface of many cell types (6-7). Cilia are evolutionarily conserved microtubule-based organelles that protrude from the apical cell surface to perform diverse biological functions (7-9). They are present on most cells of the human body and play crucial roles in mechanosensation, photoreception, intracellular signaling, and fluid movement. Cilia in the embryonic node break left-right symmetry by rotating clockwise to generate leftward fluid flow. Genetic defects in the structure and function of cilia are associated with a range of disorders termed ciliopathies including organ laterality defects, polydactyly (extra digits), and polycystic kidney disease (7-9).

Consistent with CCDC11’s role as an essential factor for ciliogenesis, CCDC11 localizes to centrosomes, centriolar satellites, and cilia (2,4). depletion of CCDC11 from mammalian cultured cells or zebrafish and frog embryos leads to abnormal cilia, resulting in left-right patterning defects (2-5). Surprisingly, more recently, it was reported that CCDC11 is also important for cell division (10). CCDC11 has been shown to localize to the middle region between two dividing daughter cells, called the midbody, in different human cell lines. Knockdown of CCDC11 protein in human cultured cells using small interfering RNA (siRNA) blocks cell division, resulting in abnormal cells with two or more nuclei. Multinucleation is a hallmark of cell division defects due to the failure of cytokinesis, the final phase of mitosis (10). The Endosomal Sorting Com-

SUMMARY

Acquired immunodeficiency syndrome (AIDS) is a life-threatening condition caused by the human immunodeficiency virus (HIV). By damaging the immune system, HIV compromises the body’s ability to fight infectious and non-infectious diseases. To discover potential targets for antiviral therapies, we explored the role of Coiled-Coil Domain-Containing 11 (CCDC11) in HIV-1 budding. CCDC11 serves an important role in cytokinesis by recruiting Endosomal Sorting Complex Required for Transport III (ESCRT-III) membrane scission machinery to the midbody of the cell in preparation for the separation into two daughter cells. ESCRT-III has also been implicated in the budding process of HIV and other enveloped viruses. Therefore, we hypothesized that CCDC11 is required for viral budding. To investigate this, we performed ELISAs (enzyme-linked immunosorbent assays) to detect the HIV-1 Gag capsid protein p24 as a measure of viral particle release. We found that ectopic expression of CCDC11 in human embryonic kidney (HEK) 293T cells significantly increased the production of HIV-1 particles in culture media. To rigorously test our hypothesis, we established CCDC11-knockout HEK293T cells using CRISPR-Cas9 technology. CCDC11 knockout was verified by DNA sequencing, western blotting, and immunofluorescence staining. We found that knockdown of CCDC11 markedly decreased the production of HIV-1 particles. The defective viral production in CCDC11-knockout cells was partially restored when wild-type CCDC11 was re-expressed. Collectively, our data suggested that CCDC11 is critical for efficient HIV-1 budding. Considering that CCDC11 expression is generally low in the majority of adult human tissues, CCDC11 might be a viable target for antiviral therapeutics without major side effects.

Investigating the role of the novel ESCRT-III recruitment factor CCDC11 in HIV budding: a potential target for antiviral therapy

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plex Required for Transport III (ESCRT-III) machinery acts in membrane deformation and scission in different biological processes (11-13). At the end of cytokinesis, ESCRT-III is recruited to the midbody to cleave the intercellular membrane bridge between nascent daughter cells in a process known as abscission (11-13). Importantly, CCDC11 facilitates the midbody recruitment of Charged Multivesicular Body Protein 2A (CHMP2A), a core subunit of the ESCRT-III complex (10). At present, the precise molecular roles of CCDC11 in ciliogenesis and cytokinesis are largely unknown.

ESCRT-III has been shown to play an important role for the budding of viral particles from the cell surface (14-17). Viral buds, small protrusions at the plasma membrane, are initially induced by the assembly of the major HIV-1 structural polyprotein Gag. During final stages of membrane remodeling, the ESCRT machinery is recruited to the sites of viral budding to complete membrane scission and release of mature viral particles from the host cell. ESCRT consists of four distinct subcomplexes (ESCRT-0, -I, -II, and -III) and associated proteins such as Vacuolar Protein Sorting 4 (Vps4) and the ALG2-Interacting protein X (ALIX) (14,17-18). These ESCRTs are recruited sequentially and transiently to Gag assembly sites. At the final step of viral budding, ESCRT-III, which is composed of CHMP family members, forms spiral filaments that facilitate the constriction of membrane necks to promote viral release. In addition to cytokinetic abscission and viral budding, ESCRTs are implicated in a variety of cellular processes requiring an internal membrane fission event including the formation of multivesicular bodies (MVBs), plasma and nuclear membrane repair, neuronal pruning, and autophagy (19-20).

CCDC11 has been reported to be involved in ciliogenesis and cytokinesis, but its potential role in other biological processes is currently unknown. Intriguingly, T cells, the physiological hosts for HIV-1, lack cilia but have been suggested to repurpose components of the ciliary machinery for the assembly and function of the immunological synapse even in the absence of a primary cilium (21-22). Since the ESCRT-III machinery functions in viral budding, we set out to test our hypothesis that CCDC11 is necessary for the recruitment of ESCRT-III to the sites of viral budding at the plasma membrane, thereby promoting the release of viral particles from the cell surface.

RESULTS

**Ectopic expression of CCDC11 robustly promotes the production of HIV particles**

Since ESCRT-III directly participates in the budding process of several different enveloped viruses, and because CCDC11 is required for ESCRT-III recruitment to the midbody, we explored the possibility that CCDC11 promotes the release of HIV particles from cells. To test this possibility, we first investigated the effect of CCDC11 overexpression on the production of HIV-1 particles. We transiently transfected wild-type (WT) HEK293T cells with a plasmid encoding the entire HIV-1 genome except for the envelope (HIV-1 pNL4-3-ΔEnv) and increasing amounts of DNA encoding CCDC11. The pNL4-3-ΔEnv plasmid lacks the envelope protein and thus produces noninfectious HIV-1 particles. As a control, we transfected cells with pNL4-3-ΔEnv harboring a proline-to-leucine substitution in the gag gene that impairs Gag binding to Tsg101 (23). Tsg101 is a component of ESCRT-I that directly binds the P-TAP motif in Gag to promote the recruitment of the ESCRT-III complex. Twenty-four hours after transfection, we harvested culture media and performed an enzyme-linked immunosorbent assay (ELISA) for HIV-1 Gag-related capsid protein p24 to evaluate viral particle release. We detected a significant level of p24 antigen in the media from cells transfected with WT pNL4-3 (Figure 1). In contrast, we found much lower p24 levels in the media of P7L-pNL4-3-expressing cells, as expected, due to defective recruitment of Tsg101 to the viral budding sites (4.6-fold decrease, p<0.0001). Intriguingly, coexpression of WT-pNL4-3 with increasing amounts of CCDC11 robustly enhanced p24 antigen levels in a dose-dependent fashion (8.9-fold increase from 0 to 2 µg of the CCDC11 plasmid, p<0.00001). These results suggested that CCDC11 promotes the budding of HIV-1 particles from the cell surface into the culture media in HEK293T cells.

**Characterization of CCDC11-knockout HEK293T cells**

To generate CCDC11-knockout cells using the CRISPR/Cas9 system (24-25), we chose human embryonic kidney (HEK) 293T cells since these cells are widely used, show
high transfection efficiency, and have the ability to form cilia, albeit at low efficiency. We designed two short guide RNAs (gRNAs) against the 5' region of the human CCDC11 coding sequence (gRNA-1 and -2) as described in Materials and Methods. gRNAs escort Cas9 nucleases to specific target genomic sites and generate double-strand breaks, which are repaired by error-prone non-homologous end joining (NHEJ), thereby inducing short insertions and deletions (indels). The pSpCas9(9B)-2A-Puro (PX459) V2.0 expression vector (24) containing CCDC11 gRNA-1 or -2 was transiently transfected into HEK293T cells and grown in the presence of puromycin. Multiple single colonies were selected and further expanded. The empty vector was also transfected to establish negative control cell clones.

To validate CCDC11 knockout, we prepared total cell lysates from two control (V1 and V2) and five CCDC11-knockout candidate clones (g1-1, g1-4, g1-8, g2-2, and g2-9) and subjected these samples to western blot analysis using an antibody against CCDC11 (Figure 2). As expected, a single band at the size of about 62 kDa for CCDC11 in control V1 and V2 lanes. Although g1-8 from gRNA-1-transfected cells and g2-2 and g2-9 from gRNA-2-transfected cells lacked the endogenous protein, we performed subsequent short and long PCR to verify the presence of the endogenous protein (Figure 2). We reasoned that g1-4 cells might contain a deletion or insertion. As expected from the presence of the endogenous protein (Figure 2), the g2-9 cell line retained significant CCDC11 expression, no CCDC11 expression was detected in cells transfected with g1-1 and g1-4 cell clone, even though this clone showed no CCDC11 expression. We reasoned that g1-4 cells might contain a deletion or insertion larger than the 403-bp CCDC11 genomic region that we analyzed. We therefore attempted to sequence a longer PCR product (1081 bp) and found a 481-bp deletion (Figure 3C). It is largely possible that a third mutation could be an even larger deletion or insertion. As expected from the presence of the endogenous protein (Figure 2), the g2-9 cell line contained two in-frame deletions in addition to one frameshift deletion. Based on these results, we selected g1-1 and g1-4 for further studies below.

To further confirm that g1-1 and g1-4 CCDC11-knockout cells lacked the endogenous protein, we performed immunofluorescence staining. In control V1 cells, CCDC11 localized to acetylated α-tubulin (A-tub)-marked centrioles (arrows), whereas CCDC11 was undetectable at centrioles in g1-1 and g1-4 cells (Figure 4A). In control V1 cells undergoing cytokinesis, clear CCDC11 fluorescence signals were detected at the midbody between the A-tub-positive microtubule bundles, while CCDC11 signals were missing in CCDC11-knockout cells (Figure 4B). In agreement with its critical role in cytokinesis (10), CCDC11-knockout HEK293T cells exhibited a moderate growth defect with the appearance of some multinucleated cells (data not shown). These data demonstrate that these CCDC11-knockout cells are indeed devoid of the endogenous protein.

CCDC11 has been reported to function in ciliogenesis, and knockdown of CCDC11 using siRNA in cultured cells or
morpholino oligos in zebrafish embryos results in a significant reduction in the number of cilia (2). A significant decrease in ciliary numbers was also observed in skin fibroblasts from a CCDC11 mutant patient with situs inversus totalis (5). To investigate whether a complete loss of CCDC11 is associated with defective ciliogenesis in HEK293T cells, we cultured control V1 and g1-1 and g1-4 CCDC11-knockout cell lines to confluence and serum-starved for 24 hours to induce ciliogenesis. We then performed immunofluorescence staining for CCDC11 and the ciliary marker A-tub. Many control V1 cells possessed fully elongated cilia (arrows), and CCDC11 was clearly detectable at the base of each cilium (Figure 5). However, neither cilia nor CCDC11 were observed in g1-1 or g1-4 CCDC11-knockout cells. Taken together, these data confirm that the CCDC11-knockout cells lack endogenous CCDC11 and further support the important role of CCDC11 in ciliogenesis.

CCDC11 is required for efficient HIV particle production

Having established CCDC11-knockout cell lines, we asked if loss of CCDC11 influences the production of HIV-1 particles. Transfection of the WT pNL4-3-ΔEnv plasmid into control V1 cells increased p24 antigen levels in the culture media 11.0-fold compared to untransfected V1 background levels (Figure 6A). Expression of the P7L-pNL4-3-ΔEnv plasmid again resulted in lower p24 levels in the media. Significantly, transfection of the WT plasmid into g1-1 and g1-4 CCDC11-knockout cells yielded low p24 antigen levels near the background level (g1-1, 9.2-fold decrease, p<0.0001; g1-4, 13.8-fold decrease, p<0.0001 compared to the V1 control). Consistent with expression of endogenous CCDC11 protein, g2-9 cells transfected with the WT plasmid produced a p24 level comparable to that of control V1 cells transfected with the WT plasmid. These findings supported our hypothesis that CCDC11 is required for the efficient production of HIV-1 particles.

Next, we performed a rescue experiment by reintroducing CCDC11 into the CCDC11-knockout cells. Transient transfection of a CCDC11 expression plasmid into g1-1 and g1-4 CCDC11-knockout cells effectively increased p24 antigen levels in the culture media in a dose-dependent manner (g1-1, 11.1-fold increase from 0 to 2 µg of the CCDC11 plasmid, p<0.0001; g1-4, 12.0-fold increase, p<0.0001) (Figure 6B). Taken together, these results demonstrate...
that CCDC11 plays a crucial role in the production of HIV-1 particles, most likely by acting during the viral assembly and/or budding process.

Discussion

During cell division, CCDC11 is required for the efficient midbody recruitment of CHMP2A, a core component of the ESCRT-III membrane scission complex, at the terminal stage of cytokinesis (10). Since ESCRT-III has also been shown to function in viral budding (14-17), we hypothesized that CCDC11 also plays a critical role in this process. To examine this possibility, we established CCDC11-knockout HEK293T cells. We chose HEK293T cells since these cells are widely used, show high transfection efficiency, and have the ability to form cilia, albeit at low efficiency. To our knowledge, our study is the first report of CCDC11-knockout mammalian cell culture models. We demonstrated that the CCDC11-knockout cell lines completely lack the endogenous protein when examined by western blotting, immunofluorescence staining, or sequencing analysis. This protein had not been previously linked to HIV-1 replication but, remarkably, appeared to play a crucial role in production of HIV-1 particles in HEK293T cells, as CCDC11-knockout cells failed to produce viral particles. The defect was rescued by ectopic CCDC11 expression, providing direct evidence, for the first time, that the protein is required for productive assembly (Figure 6). Moreover, expression of exogenous CCDC11 robustly augmented HIV-1 particle production (Figure 1). This finding suggested that CCDC11 might be a limiting factor in the viral assembly and/or budding process.

Based on our findings, we proposed the following model for the potential role of CCDC11 in HIV-1 budding: We hypothesized that, in wild-type cells, CCDC11 is initially recruited to sites of viral budding at the plasma membrane (Figure 7). By analogy to its role in cytokinesis, CCDC11 then engages in the recruitment of the ESCRT-III complex, thereby promoting membrane deformation, bud scission and viral release. In contrast, in the absence of CCDC11, the targeting of ESCRT-III is diminished, leading to inefficient viral release.

CCDC11 has been shown to localize to centrioles, centriolar satellites, and cilia, and CCDC11 is required for ciliogenesis in multiple different experimental systems (2,5). However, a conflicting report demonstrated that CCDC11 does not overtly influence ciliary numbers or morphology (4). All of these previous studies employed either gene knockdown approaches or skin fibroblasts from a patient with situs inversus totalis harboring a CCDC11 mutation that expresses a truncated protein (amino acids 1-349) (2,4-5). Using our CCDC11-knockout HEK293T cells, which completely lack the protein, we found that CCDC11 was indeed necessary for ciliogenesis (Figure 5). Whether the viral function of CCDC11 is related to its role in ciliogenesis is currently unknown.

What is the underlying molecular mechanism of CCDC11 function in the production of HIV-1 particles? Although several possibilities exist, we favored the idea that CCDC11 facilitates the recruitment of ESCRT-III components to the site of viral budding, possibly via direct protein-protein interactions. At least two candidate interacting partners exist: During virion formation at the plasma membrane, Tsg101 is recruited to the PTAP motif in the HIV-1 Gag p6 protein, where it facilitates recruitment of the ESCRT-III complex (14,26). The ESCRT adaptor protein ALIX, although typically considered auxiliary, could play an important role in egress from cell types that
exhibit weak PTAP-dependence (27). ALIX physically binds to the LYPXnL motif of Gag p6, as well as to the nucleocapsid domain in Gag. ALIX then recruits CHMP4B, a component of ESCRT-III, to virus assembly sites (28-29). Since we observed that CCDC11-knockout cells exhibited dramatically reduced viral production (Figure 6A), it is possible that CCDC11 is involved in the Tsg101 pathway or in both Tsg101 and ALIX pathways. At this point, however, we cannot rule out the possibility that CCDC11 regulates other aspects of viral production, for instance, the stability and intracellular trafficking of viral proteins. Future investigations will examine CCDC11 localization and colocalization with ESCRT components at viral budding sites, as well as protein-protein interactions between CCDC11 and various ESCRT components. Future experiments could also test for non-ESCRT interactors through proteomic analysis. Additionally, it would be particularly important to assess if CCDC11 is expressed in T cells or induced upon HIV infection as T lymphocytes are the primary target for HIV.

In summary, we have established the first CCDC11-knockout mammalian cell models. Our results demonstrated that CCDC11 is crucial for ciliogenesis and, notably, HIV-1 production. We envision that CCDC11-knockout cells will be a useful tool for studies of CCDC11 functions in various biological processes such as ciliogenesis, cytokinesis, and viral budding. Interestingly, CCDC11 expression is generally low in the majority of adult human tissues in comparison to the abundant expression of Tsg101 and ALIX (Human Protein Atlas, http://www.proteinatlas.org). Possibly, due to its restricted expression, targeted inhibition of CCDC11 function by small molecules may be beneficial as an anti-HIV therapy without major side effects and could be used in conjunction with existing anti-HIV medications.

MATERIALS AND METHODS

Plasmids and bacterial transformation

Expression plasmids for flag-tagged human CCDC11 and for HIV-1 pNL4-3-ΔEnv have been described previously (15). All plasmids were transformed into DH5α E. coli competent cells (New England Biolabs), and a Plasmid Plus Midi Kit (Qiagen) was used to purify the plasmid DNAs.

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were purchased from American Type Culture Collection (ATCC, CRL-3216) and grown in Dulbecco’s Modified Eagle Medium (DMEM) and 10% fetal bovine serum with 100 U/ml penicillin-streptomycin at 37 °C in a 5% CO₂ incubator. Cells were transfected with Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s instructions.

Generation of CCDC11-knockout HEK293T cells using the CRISPR/Cas9 system

To generate CCDC11-knockout cells using the CRISPR/Cas9 system (24-25), two short guide RNAs (gRNAs) against the 5’ region of the human CCDC11 coding sequence (gRNA-1 and -2) were designed using the CRISPR design tool (http://crispr.mit.edu): gRNA-1, 5’-GCGGTTTGGCACCGTACAGC-3’; gRNA-2, 5’-AGCGGTTTGGCACCGTACAG-3’. HEK293T cells were transfected with pSpCas9(BB)-2A-Puro (PX459) V2.0 (24) (Addgene plasmid 62988) containing a human CCDC11 gRNA using Lipofectamine 3000. The empty vector was transfected as a negative control. Transfected cells were selected in the presence of puromycin (2.5 µg/ml). Following 1-2 weeks of culture, multiple single colonies were isolated and further grown for experiments.

To determine CCDC11-knockout mutations, genomic DNA was isolated from the cell colonies, and PCR fragments encompassing the gRNA sequence were generated using GoTaq DNA polymerase (Promega) and used for sequencing at the Genomics Core Facility at the Stony Brook University. The primers used to amplify CCDC11 genomic fragments were: 5’-ATGGTGACCAGACCGACTTC-3’ and 5’-GGTGAGCGACCTTACCC-3’ (403-bp amplicon), 5’-GACAGAGCGAGACCTTGTCT-3’ and 5’-GGTGAGCGACCTTACCC-3’ (1081-bp amplicon).

Figure 7. Proposed molecular mechanisms for CCDC11 functions during HIV-1 budding. Left: In wild-type cells, CCDC11 facilitates the recruitment of the ESCRT-III membrane scission complex to the sites of viral budding at the plasma membrane, thereby promoting viral release. Right: In CCDC11-knockout cells, the recruitment of ESCRT-III is diminished, leading to inefficient viral release.
Western blotting

WT and CCDC11-knockout HEK293T cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate, and 0.1% SDS) with protease cocktail inhibitors (Roche) added before use, followed by sonication. The lysates were centrifuged at 13,000 rpm, and supernatant was collected and mixed with 5X SDS sample buffer, followed by denaturing the proteins at 95 °C for 3 minutes. The samples were loaded onto a 10% SDS-polyacrylamide gel and the gel was run for 2 hours at 90 V. Proteins were transferred from the gel onto a nitrocellulose membrane (0.45 μm) (Bio-Rad) with a power supply running for 14 hours at 16 V. The membrane was then washed with Tris-buffered saline with 0.1% Tween 20 (TBST) and blocked in 5% skim milk for 1 hour at room temperature. Rabbit anti-CCDC11 primary antibody (Sigma-Aldrich, HPA040595, 1:500) or mouse anti-GAPDH antibody (Proteintech, 60004-1-Ig, 1:5,000) was added, followed by incubation for 1 hour. The membrane was washed 3 times for 15 minutes each with TBST. Secondary antibodies conjugated with horseradish peroxidase were added and incubated for 1 hour at room temperature. Antibodies were then removed, and the membrane was washed with TBST 3 times for 15 minutes each. SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used to detect the proteins of interest on X-ray films.

Immunofluorescence staining

HEK293T cells were grown on glass coverslips in a 12-well plate for 24-48 hours. The cells were then washed and fixed with cold 50% methanol/50% acetone. The fixed cells were incubated for 1 hour in 300 μl of blocking buffer containing 5% goat serum in diluent solution (2% bovine serum albumin and 0.2% Triton X-100 in PBS). The blocking buffer was discarded, and the cells were incubated at 4 °C overnight in 300 μl of the diluent solution containing the following primary antibodies: rabbit anti-CCDC11 (Sigma-Aldrich, HPA040595, 1:300) and mouse anti-acetylated α-tubulin (Sigma-Aldrich, T7451, 1:300). Subsequently, cells were washed and incubated for 1 hour with appropriate anti-rabbit or anti-mouse IgG secondary antibodies conjugated with DyLight 488 and DyLight 549 (Vector Laboratories). The cells were washed, counterstained with 4',6-diamidino-2-phenylindole (DAPI), used as a nuclear counterstain, for two minutes at room temperature, washed again, and mounted onto a glass slide using Fluoromount-G (Southern Biotech) for microscopic analyses.

Quantification of HIV particle release using p24 ELISA

The day before transfection, cells were trypsinized and split in triplicate into 6-well plates with 2 ml DMEM culture media. After 18-24 hours, when cells reached 70-90% confluency, either 3 μg total DNA (Figures 1 and 6B) or 2 μg total DNA (Figure 6A) were transfected into each well using Lipofectamine 3000 following the manufacturer’s protocol. The total amount of plasmid DNA was adjusted using an empty vector if appropriate. An expression plasmid for Renilla luciferase (pRL-TK) (50 ng/well) was cotransfected to normalize transfection efficiency.

After 30 hours of transfection, culture media were harvested and clarified by centrifugation to remove cellular debris. We utilized the HIV-1 p24 Capture ELISA kit (ImmunoDX, LLC) to measure the levels of Gag p24 in the culture media by reading the optical densities (OD) at a wavelength of 450 nm using a microplate reader. The cells were lysed using 1X passive lysis buffer (Promega) with sonication and used to measure Renilla luciferase activities by the Dual-Luciferase Reporter Assay System (Promega) and a Berthold luminometer (Berthold Technologies). For normalization, the ELISA OD values of Gag p24 were divided by the values of Renilla luciferase activities to account for transfection efficiency.

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