HIV-1 Vpr hijacks EDD-DYRK2-DDB1DCAF1 to disrupt centrosome homeostasis

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Viruses are pathogens that infect all life forms and reproduce inside living cells. To do so, they must be able to counteract and evade immune defenses, as well as utilize cellular machinery from the host for their own replication. HIV type 1 (HIV-1) subverts DNA repair regulatory proteins and induces G2/M arrest. The preintegration complex of HIV-1 is known to traffic along microtubules and accumulate near the microtubule-organizing center. The centrosome is the major microtubule-organizing center in most eukaryotic cells, but precisely how HIV-1 impinges on centrosome biology remains poorly understood. We report here that the HIV-1 accessory protein viral protein R (Vpr) localized to the centrosome through binding to DCAF1, forming a complex with the ubiquitin ligase EDD-DYRK2-DDB1DCAF1 and Cep78, a resident centrosomal protein previously shown to inhibit EDD-DYRK2-DDB1DCAF1. Vpr did not affect ubiquitination of Cep78. Rather, it enhanced ubiquitination of an EDD-DYRK2-DDB1DCAF1 substrate, CP110, leading to its degradation, an effect that could be overcome by Cep78 expression. The down-regulation of CP110 and elongation of centrioles provoked by Vpr were independent of G2/M arrest. Infection of T lymphocytes with HIV-1, but not with HIV-1 lacking Vpr, promoted CP110 degradation and centriole elongation. Elongated centrioles recruited more γ-tubulin to the centrosome, resulting in increased microtubule nucleation. Our results suggest that Vpr is targeted to the centrosome where it hijacks a ubiquitin ligase, disrupting organelle homeostasis, which may contribute to HIV-1 pathogenesis.

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2 The abbreviations used are: Vpr, viral protein R; TERT, telomerase reverse transcriptase; HA, hemagglutinin; Ub, ubiquitin; DDR, DNA damage response; DAPI, 4’,6-diamidino-2-phenylindole; ROI, region of interest.
The centrosome is the major microtubule-organizing center in most eukaryotic cells and acts as a central hub for coordinating a multitude of cellular events. Various molecules and cargos are known to transit through this organelle (42). The viral core of HIV-1 disassembles upon entry into the host cells, and the resulting preintegration complex traffics along micro-
tubules and accumulates near the microtubule-organizing center (43–46). Another study reports that HIV-1 subviral particles accumulate at the centrosome under resting T-cells through an unknown mechanism, and infection resumes upon stimulation (47). Interestingly, Vpr has been observed to disrupt certain protein interactions at the centrosome (48) and induce centrosome amplification and multipolar spindle formation (49, 50), suggesting that this viral protein is capable of exerting an effect on the centrosome either directly or indirectly. Despite these observations, the extent to which Vpr modulates different aspects of centrosome biology and the underlying mechanisms have not been studied in detail.

Results

Vpr binds to Cep78 and EDD-DYRK2-DDB1DCAF1 and localizes to the centrosome

We recently demonstrated that Cep78 forms a complex with EDD-DYRK2-DDB1DCAF1 through DCAF1 (37). Given that Vpr is known to associate with DCAF1 (15, 25), we first asked whether Vpr and Cep78 interact. Endogenous Cep78 and DCAF1 co-immunoprecipitated with HA-Vpr (Fig. 1, A and B). When DCAF1 was depleted with siRNA, very little Cep78 was detected in Vpr immunoprecipitates (Fig. 1A). Moreover, endogenous DCAF1 and Cep78 bound to WT Vpr, but neither protein interacted with a Vpr mutant refractory to DCAF1 binding (Vpr(Q65R)) (Fig. 1B). Thus, Vpr likely associates with Cep78 through DCAF1, results that are consistent with the findings that the Vpr- and Cep78-binding sites of DCAF1 are nonoverlapping. Vpr binds to the WD40 domain of Cep78 (37).
DYRK2-DDB1DCAF1 but not to CRL4DCAF1, which is expected (37), and to Vpr (Fig. 1C). WT Vpr co-localized with endogenous Cep78 in ~20–25% of transfected HeLa cells, indicating that this viral protein is targeted to the centrosome in some contexts (Fig. 2, A and B). In contrast, Vpr(Q65R) did not exhibit centrosomal localization (Fig. 2, A and B). Taken together, these data suggest that Vpr engages in a complex with EDD-DYRK2-DDB1DCAF1 and Cep78 at the centrosome through binding to DCAF1.

Vpr hijacks EDD-DYRK2-DDB1DCAF1 to enhance ubiquitination and degradation of CP110

To explore the relevance of Vpr binding to EDD-DYRK2-DDB1DCAF1 and Cep78, we tested whether Vpr might promote ubiquitination of proteins at the centrosome. The ubiquitination levels of Cep78, an inhibitor and nonsubstrate of EDD-DYRK2-DDB1DCAF1 (37), remained the same upon Vpr expression (Fig. 3A). Likewise, centrosomal localization and steady-state levels of Cep78 were not altered by WT Vpr or Vpr(Q65R) (Figs. 2, A and C, and 3, A–C). On the contrary, ubiquitination of CP110, a known centrosomal EDD-DYRK2-DDB1DCAF1 substrate (37), became greatly enhanced by Vpr (Fig. 3D). This was accompanied by a decrease in CP110 protein levels (Fig. 3, D–H) and a loss of centrosomal CP110 staining by immunofluorescence (Fig. 4, A and B). Notably, diminished levels of CP110 were specifically induced by WT Vpr but not Vpr(Q65R) mutant (Figs. 3, G and H, and 4, A and B) and could be rescued by the addition of a proteasome inhibitor, MG132 (Fig. 5, A and B), or deletion of DCAF1 (Fig. 5, C and D). Furthermore, co-expression of Cep78 drastically reduced ubiquitination of CP110 (Fig. 3D) and restored endogenous CP110 to WT levels (Figs. 3, E and F, and 8, A and B). These data indicate that Vpr subverts centrosomal EDD-DYRK2-DDB1DCAF1 to accelerate ubiquitination and proteasomal degradation of a native substrate, CP110; and these effects can be counteracted by overexpression of Cep78.

Vpr induces centriole elongation through CP110 degradation

Previously, it has been shown that depletion of CP110 induces the formation of overlong or elongated centrioles, represented by γ-tubulin filaments, in nonciliated or poorly ciliated cells including HeLa (38–41). This phenotype can also be recapitulated by CP110 loss resulting from ablation of Cep78 or overexpression of EDD-DYRK2-DDB1DCAF1 (37). To further substantiate our observations that Vpr enhances degradation of CP110, we found that WT Vpr provokes centriole elongation, whereas Vpr(Q65R) mutant cannot (Fig. 4, A and C). Of note, WT Vpr also induced centrosome amplification (>2 γ-tubulin foci, Fig. 4, A and D), consistent with a previous report (50), but this phenotype is unlikely to be a consequence of CP110 loss because excessive

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Figure 4. Vpr induces CP110 loss, centriole elongation, and centrosome amplification. A, HeLa cells transfected with plasmid expressing HA, HA-Vpr(WT), or HA-Vpr(Q65R) were processed for immunofluorescence and stained with antibodies against HA (green) and CP110 or γ-tubulin (red). DNA was stained with DAPI (blue). Scale bar, 2 μm. B, the percentage of HA-expressing cells with no centrosomal CP110 staining was determined. C and D, the percentage of HA-expressing cells with elongated centrioles (γ-tubulin filaments) (C) or centrosome amplification (>2 γ-tubulin dots) (D) was determined. For B–D, at least 100 cells were scored for each condition in each experiment, and the mean (thick open line) and standard error (bar) of three independent experiments (E) are shown in the graph. *, p < 0.01.

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Vpr usurps EDD-DYRK2-DDB1DCAF1 at the centrosome

J. Biol. Chem. (2018) 293(24) 9448–9460 9451
CP110, rather than loss of CP110, drives centrosome amplification (52).

**Vpr-induced CP110 degradation and centriole elongation are independent of G<sub>2</sub>/M arrest**

A recent study shows that Vpr associates with the SLX4 complex to induce chromosomal instability, triggering DNA damage response (DDR) and cell cycle arrest at the G<sub>2</sub>/M phase (20), although this is in debate (53). Coincidentally, CP110 has been documented to undergo ubiquitination by SCF<sup> cyclin F</sup> and EDD-DYRK2-DDB1<sup>DCAF1</sup> (37, 52), and subsequently proteasomal degradation, in G<sub>2</sub>/M. Thus, we sought to address whether down-regulation of CP110 induced by Vpr is due to prolonged G<sub>2</sub>/M arrest. For this purpose, we utilized a well-characterized Vpr mutant, Vpr(R80A), which, in contrast to Vpr(Q65R), can bind to DCAF1 but is unable to provoke G<sub>2</sub>/M arrest (Ref. 54, 55 and Fig. 6A). Similar to WT Vpr, Vpr(R80A) was detected at the centrosome in ~20–25% of the transfected cells (Fig. 6, B and C). Next, we investigated the consequences of expressing Vpr(R80A) on CP110 and its effect on centriole length. WT Vpr and Vpr(R80A) were equally able to enhance CP110 ubiquitination (Fig. 6D), causing a diminution of CP110 levels (Fig. 6, E and F) and immunostaining at the centrosome (Fig. 7, A and B). Furthermore, WT Vpr and Vpr(R80A) induced γ-tubulin filament formation to similar extent (Fig. 7A, C). The down-regulation of CP110 provoked by WT Vpr or Vpr(R80A) was rescued by ectopic expression of Cep78 (Fig. 8). Remarkably, unlike WT Vpr, Vpr(R80A) did not induce centrosome amplification (Fig. 7, A and D). These data suggest that CP110 down-regulation and centriole elongation could be attributed to the subversion of EDD-DYRK2-DDB1<sup>DCAF1</sup> rather than to G<sub>2</sub>/M arrest. On the contrary, the other phenotype caused by Vpr, namely centrosome amplification, depends on cell cycle arrest at the G<sub>2</sub>/M phase.

**Vpr induces CP110 degradation and centriole elongation in infected T-cells**

We have thus far shown that Vpr induces the loss of CP110 in two model cell lines, HEK293 and HeLa. However, it remains unknown whether this accessory protein could trigger the same response in CD4<sup>+</sup> T lymphocytes that HIV-1 normally infects. To interrogate the relationship between Vpr and CP110 in a

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**Figure 5. Vpr-induced proteasomal degradation of CP110 occurs in a DCAF1-dependent manner.** A, HEK293 cells transfected with plasmid expressing HA or HA-Vpr(WT) were treated with or without 10 μM MG132 for 6 h. Lysates were Western-blotted with the indicated antibodies. α-Tubulin was used as loading control. B, normalized CP110 protein level. The mean (thick open line) and standard error (bar) of three independent experiments (E) are shown in the graph. *, p < 0.01; ns, nonsignificant. C, HEK293 cells were transfected with nonspecific (NS) or DCAF1 siRNA followed by plasmid expressing HA or HA-Vpr(WT). Lysates were Western-blotted with the indicated antibodies. α-Tubulin was used as loading control. D, normalized CP110 protein level. The mean (thick open line) and standard error (bar) of three independent experiments (E) are shown in the graph. *, p < 0.01.
more physiologically relevant cell line, we infected CD4+ MT4 T-cells, which are highly susceptible to and permissive for infection with HIV-1. We found that a significant percentage of cells infected with WT HIV-1 (HIV-1 Vpr+) exhibit CP110 loss and centriole elongation (Fig. 9A–C) in addition to centrosome amplification (Fig. 9A, D). In contrast, very few mock-infected cells or cells infected with HIV-1 lacking Vpr (HIV-1 Vpr–) possess these phenotypes (Fig. 9). These results indicate that HIV-1 can also induce CP110 degradation and centriole elongation in T-cells in a Vpr-dependent manner.

Elongated centrioles enhance microtubule nucleation

To shed light on the net effects of down-regulating CP110 for Vpr, we studied how elongated centrioles might influence centrosome function. When a centriole becomes abnormally long, the surrounding pericentriolar material becomes distorted into the shape of a filament (38–41). Given that γ-tubulin present in the pericentriolar material plays a crucial role in microtubule nucleation, we addressed the question of whether elongated centrioles might alter nucleation. We quantified the staining area occupied by γ-tubulin and found that it is substantially bigger in CP110-depleted or Vpr-expressing cells than in control cells (Fig. 10A–C). γ-Tubulin staining intensity was likewise higher upon depletion of CP110 or expression of Vpr (Fig. 10A, B, and D). Next, we performed microtubule regrowth assays following microtubule depolymerization with nocodazole. Shortly after the removal of the nocodazole, control cells nucleated an aster of microtubules emanating from the centrosome (Fig. 10A and B). With time, the aster enlarged, signifying an increase in the length and number of microtubules (Fig. 10A and B, 1'). Strikingly, cells depleted of CP110 or expressing Vpr formed a bigger aster and nucleated more microtubules at comparable time points (Fig. 10A and B, 1' and 5', and E). In contrast, no gross microtubule-anchoring defects were observed (Fig. 10A and B, 45'). Together, these data strongly sug-
gest that elongated centrioles have the capacity to recruit more γ-tubulin, resulting in enhanced microtubule nucleation.

Discussion

In this work, we sought to obtain molecular insights into how HIV-1 Vpr exploits host machinery at the centrosome. Although an intimate connection exists among HIV-1, Vpr, and centrosomes (42, 47–50, 56), the extent to which Vpr orchestrates its effects on this organelle remains poorly understood. Our data show that Vpr associates with a resident centrosomal protein, Cep78, through DCAF1 and that it localizes to the centrosome by engaging in a complex with the ubiquitin ligase EDD-DYRK2-DDB1DCAF1 and Cep78. Because Vpr and Vpr(R80A) localize to the centrosome with similar efficiency and EDD-DYRK2-DDB1DCAF1 components are known to be present at this organelle throughout the cell cycle (37), it seems likely that centrosomal localization of Vpr is independent of G2/M arrest. Vpr is able to hijack EDD-DYRK2-DDB1DCAF1, accelerating the ubiquitination and degradation of a native centrosomal substrate, CP110. Down-regulation of CP110 triggers the formation of abnormally long centrioles, which recruit excess γ-tubulin, and as a consequence, the nucleation of cytoplasmic microtubules becomes greatly enhanced. In addition, Vpr provokes other centrosome anomalies such as amplification (Ref. 50 and this study), indicating that proteins involved in the regulation of organelle copy number might also be affected.

It would therefore be interesting to identify novel Vpr-interacting partners and/or EDD-DYRK2-DDB1DCAF1 substrates and to test whether any of these might be responsible for the centrosome amplification phenotype.

Although Vpr triggers centriole elongation and centrosome amplification, it is clear that these phenotypes occur through two distinct mechanisms. We show that centriole elongation as a result of Vpr-mediated CP110 loss is independent of G2/M arrest, whereas centrosome amplification necessitates G2/M arrest. How then does Vpr-induced G2/M arrest result in centrosome amplification? It is reported that Vpr targets DNA repair factors such as HLTF and UNG (33, 35) for degradation and inappropriately activates the SLX4 complex in the nucleus (20), conditions that could contribute to replication stress and the induction of DDR (57). The DDR protein ataxia telangiectasia-mutated (ATM) and Rad3-related protein (ATR), once activated, initiates downstream signaling cascades that involve activation of checkpoint kinase 1 (CHK1) and inhibition of cell division cycle 25C (CDC25C) and cyclin B/cyclin-dependent kinase 1 (CDK1), ultimately leading to G2/M arrest (58). Curiously, other studies have shown that DNA damage alone is sufficient to induce centrosome amplification (59), and several DDR proteins, such as ATM, ATR, and CHK1, are found in the nucleus and at the centrosome (60). Although the precise functions of DDR proteins at the centrosome await future investi-
gation, it is plausible that a DDR signal originating from the nucleus impinges on the centrosome through the DDR proteins, causing amplification to occur.

What are the benefits that HIV-1 might receive by hijacking EDD-DYRK2-DDB1DCAF1 at the centrosome? The regulation of microtubule dynamics and microtubule-associated proteins such as end-binding proteins and motor proteins is an important facet of the HIV-1 replication cycle. For example, HIV-1 promotes the formation of stable microtubules, an event crucial for early infection and translocation of the viral core in the cytoplasm en route to the nucleus (45). Intact microtubules are needed to facilitate HIV-1 uncoating, and disruption of microtubules by nocodazole impairs this process (46). In macrophages, HIV-1 Vpr perturbs the localization of end-binding protein 1 (EB1) to impair the maturation of phagosomes, leading to defects in innate immunity (61). Moreover, HIV-1 Tat can promote or hinder microtubule stability in a context-dependent fashion (62–64). Thus, it is clear that HIV-1 employs different strategies to remodel the host microtubule network during infection. Further studies will be needed to decipher how CP110 loss, elongated centrioles, and enhanced microtubule nucleation provoked by Vpr might affect various stages of HIV-1 infection.

One interesting finding from our studies is that Cep78 counteracts the effects of Vpr on CP110, raising the possibility that it might have antiviral properties. It would therefore be interesting to test whether this protein might safeguard the centrosome to inhibit viral infection.

**Experimental procedures**

**Cell culture and plasmids**

HeLa, HEK293, and HEK293T cells were grown in DMEM (Wisent Inc., 319-005-CL) supplemented with 5% fetal bovine serum (Wisent Inc, 080150) at 37 °C in a humidified 5% CO2 atmosphere. MT4 T-cells were grown in RPMI 1640 (Wisent Inc., 350-000-CL) supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO2 atmosphere. The following proteins were expressed from plasmids in mammalian cells: HA-Ub (65), pCBF-FLAG-Cep78 (37), pEGFP-C1-Cep78 (37), pCBF-FLAG-Cep78 (66), pQBI25, SVCVM-HA-Vpr, SVCVM-HA-Vpr(Q65R), and SVCVM-HA-Vpr(R80A) (55).
Viral production and infection

Infectious GFP-marked HIV-1 NL4.3 or NL4.3ΔVpr viruses were generated by calcium phosphate transfection of HEK293T cells. Virus-containing supernatants were recovered 2 days post-transfection, clarified, pelleted by ultracentrifugation, and titrated by analyzing the percentage of GFP-positive MT4 T-cells using flow cytometry. MT4 T-cells were infected with the different GFP-expressing NL4.3 viruses at a multiplicity of infection of 0.75. Three days post-infection, cells were plated on coated coverslips and processed for immunofluorescence.

Antibodies

Antibodies used in this study included anti-CP110 (Bethyl Laboratories, A301-344A), anti-Cep78 (Bethyl Laboratories, A301-799A and IRCM6), anti-DCAF1 (Proteintech, 11612-1-AP), anti-EDD (Bethyl Laboratories, A300-573A), anti-DDB1 (Bethyl Laboratories, A300-462A), anti-cullin 4A (Bethyl Laboratories, A300-739A), anti-GFP (Roche, 11814460001), anti-HA (Santa Cruz Biotechnology, sc-7392, and Novus Biologicals, NB600-362), anti-FLAG (Sigma-Aldrich, F7425 and F3165), anti-α-tubulin (Sigma-Aldrich, T5168), anti-γ-tubulin (Sigma-Aldrich, T3559 and T6557), and anti-DYRK2 (Abcam, ab37912). The anti-p24 monoclonal antibodies were produced from hybridomas 31-90-25 (HB9725) obtained from the American Type Culture Collection.

RNAi and transient expression of recombinant proteins

For RNAi, synthetic siRNA for nonspecific (NS) control, DCAF1, and CP110 were as described previously (29, 67, 68) and purchased from GE Dharmacon. Transfection of siRNA into HEK293 or HeLa cells was performed using siIMPORTER (Milipore, 64-101) per the manufacturer’s instructions, and cells were processed for immunoprecipitation, immunoblotting, or immunofluorescence at 72 h post-transfection. For expression of recombinant proteins, expression vector(s) was/were transfected into HEK293 cells using calcium phosphate or HeLa cells using polyethylenimine, and the cells were processed at 72 h post-transfection. For experiments involving both RNAi and recombinant protein expression, HEK293 cells were transfected with siRNA followed by transfection of expression vector 24 h later. Cells were processed 72 h after siRNA transfection. Optimal knockdown and recombinant protein expression were achieved at 72 and 48–72 h, respectively, post-transfection.

Immunoprecipitation, immunoblotting, and immunofluorescence

Immunoprecipitation, immunoblotting, and immunofluorescence were performed as described (67, 68). Cells were lysed in a lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM EDTA/pH 8, 0.1% NP-40, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg of aprotinin, 10 mM NaF, 50 mM β-glycerophosphate, and 10%
glycerol) at 4 °C for 30 min. The extracted proteins were recovered in the supernatant after centrifugation at 16,000 g. For immunoblotting, 100 µg of extract was used as input. For immunoprecipitation, 2 mg of extract was incubated with anti-FLAG-(Sigma-Aldrich, A2220) or anti-HA–agarose (Sigma-Aldrich, A2095) beads at 4 °C for 2 h. The beads were washed three times with a lysis buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotted with primary antibodies and horseradish peroxidase–conjugated secondary antibodies (Rockland Inc, 610-703-002 and 611-7302). For indirect immunofluorescence, cells were fixed with cold methanol and permeabilized with 1% Triton X-100/PBS. Slides were blocked with 3% BSA in 0.1% Triton X-100/PBS prior to incubation with primary antibodies. Secondary antibodies used were Cy3 (Jackson Immunoresearch Laboratories, 711-165-151 and 715-165-152), Alexa Fluor 488 (Jackson Immunoresearch Laboratories, 711-605-152), DyLight 649 (Jackson Immunoresearch Laboratories, 715-495-151), or Alexa 488 (Thermo Fisher Scientific, A11008, A11055, and A11001)–conjugated donkey anti-mouse, anti-goat, or anti-rabbit IgG. Cells might also be stained with DAPI (Molecular Probes, D3571), and slides were mounted, observed, and photographed using a Leitz DMRB (Leica) microscope (×100, NA 1.3) equipped with a Retiga EXi cooled camera.

**In vivo ubiquitination assay**

*In vivo* ubiquitination assays were performed as described (37, 69). Briefly, HEK293 cells were transfected with various plasmids including HA-Ub. Cells were lysed at 72 h post-transfection, and the desired protein was immunoprecipitated with 1% SDS (Bio Basic Inc., SB0485) to prevent noncovalently linked binding partners from co-immunoprecipitating with the desired protein. After extensive washing, the bound proteins...
were analyzed by SDS-PAGE and immunoblotting with an anti-HA antibody.

**Microtubule regrowth assay**

Cells were treated with 10 μM nocodazole (Sigma–Aldrich, M1404) for 1 h at 4 °C. After washing the cells several times with cold medium, they were placed in a prewarmed medium at 37 °C. Cells were fixed at various time points (0, 1, 5, and 45 min) after reaching 37 °C and processed for immunofluorescence.

**Cell cycle analysis**

HEK293T cells were co-transfected with plasmids expressing GFP (pQBI25) and WT or mutant HA-Vpr. At 48 h post-transfection, cells were fixed, permeabilized, and stained with propidium iodide as described (55). Cell cycle analysis was performed on the GFP+ population by flow cytometry (BD FACSCalibur, Becton Dickinson). The ModFit mathematical model (ModFit LT v4.1.7, Verity Software House) was used to enumerate the proportions of cells in G1 and G2/M phases.

**Quantitation of γ-tubulin staining area and intensity**

A region of interest (ROI) was drawn around γ-tubulin, which marks the centrosome, and the area of the ROI was calculated using Velocity 6 (PerkinElmer). The area of the ROI was then used to measure the fluorescence intensity of γ-tubulin, again using Velocity 6. Image conditions were identical in all cases, and none of the images were saturated, as confirmed by the pixel intensity range.

**Quantitation of cytoplasmic microtubules**

The number of microtubules emanated from the centrosome at 0 min after microtubule regrowth was subtracted from that at 1 min after regrowth and presented as microtubules nucleated/min.

**Quantitation of Western blotting**

Protein bands from Western blotting films were quantitated with ImageJ. Different film exposure lengths were used to prevent saturation. Quantitation was normalized with respect to the loading control.

**Data and statistical analysis**

Each experiment was conducted three times. The statistical significance of the difference between two means was calculated using a two-tailed Student’s t test. Differences were considered significant at p < 0.01.

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