



HAL
open science

CDC50A plays a key role in the uptake of the anticancer drug perifosine in human carcinoma cells

Francisco Muñoz-Martínez, Cristina Torres, Santiago Castanys, Francisco Gamarro

► **To cite this version:**

Francisco Muñoz-Martínez, Cristina Torres, Santiago Castanys, Francisco Gamarro. CDC50A plays a key role in the uptake of the anticancer drug perifosine in human carcinoma cells. *Biochemical Pharmacology*, 2010, 80 (6), pp.793. 10.1016/j.bcp.2010.05.017 . hal-00608936

HAL Id: hal-00608936

<https://hal.science/hal-00608936>

Submitted on 16 Jul 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: CDC50A plays a key role in the uptake of the anticancer drug perifosine in human carcinoma cells

Authors: Francisco Muñoz-Martínez, Cristina Torres, Santiago Castanys, Francisco Gamarro



PII: S0006-2952(10)00356-4
DOI: doi:10.1016/j.bcp.2010.05.017
Reference: BCP 10572

To appear in: *BCP*

Received date: 10-2-2010
Revised date: 13-5-2010
Accepted date: 17-5-2010

Please cite this article as: Muñoz-Martínez F, Torres C, Castanys S, Gamarro F, CDC50A plays a key role in the uptake of the anticancer drug perifosine in human carcinoma cells, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2010.05.017

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

TITLE:

CDC50A plays a key role in the uptake of the anticancer drug perifosine in human carcinoma cells

AUTHOR NAMES AND AFFILIATION:

Francisco Muñoz-Martínez ^{a, 1}, Cristina Torres ^a, Santiago Castanys ^{a, #} and

Francisco Gamarro ^{a, #}

^aInstituto de Parasitología y Biomedicina “López-Neyra”, Consejo Superior de Investigaciones Científicas. Parque Tecnológico de Ciencias de la Salud, Avenida del Conocimiento s/n, 18100 Armilla, Granada. Spain.

[#]Corresponding author. Mailing address of Francisco Gamarro: Instituto de Parasitología y Biomedicina López-Neyra, CSIC, Parque Tecnológico de Ciencias de la Salud, Avda. del Conocimiento s/n, 18100 Armilla, Granada, Spain. Phone: 34 958 181667. Fax: 34 958 181632. E-mail: gamarro@ipb.csic.es. For Santiago Castanys, phone: +34 958 181666; fax: +34 958 181632, e-mail: castanys@ipb.csic.es

¹Present address: Estación Experimental del Zaidín (CSIC), Profesor Albareda, 1. Granada. 18008. SPAIN

ABSTRACT:

1
2 Functional aminophospholipid translocases are composed of at least two
3
4 proteins: an alpha subunit from the P4 subfamily of P-type ATPases and a beta
5
6 subunit from the CDC50-Lem3p family. Over-expression and knockdown of the
7
8 human beta subunit CDC50A in KB cells enhanced and decreased, respectively, the
9
10 uptake of both fluorescent aminophospholipid analogues and the anticancer alkyl-
11
12 phospholipid perifosine. Confocal microscopy showed that CDC50A-V5 was
13
14 localized at the endoplasmic reticulum and the Golgi complex of both KB (perifosine-
15
16 sensitive) and KB PER-R (perifosine-resistant, alkyl-phospholipid uptake deficient)
17
18 cells, but was only widely distributed in the early and late endosomes in KB cells.
19
20 Biotinylation of cell surface proteins allowed CDC50A-V5 to be detected in the
21
22 plasma membrane of KB cells but not in KB PER-R cells, thereby suggesting a
23
24 defect in CDC50A trafficking that could explain the inability of KB PER-R to uptake
25
26 perifosine. Over-expression of CDC50A in HeLa and HEK293T cells did not increase
27
28 uptake, since the protein was retained at the endoplasmic reticulum and Golgi.
29
30 However, when CDC50A was co-expressed with the P4-ATPase Atp8b1, the two
31
32 proteins co-localized at the plasma membrane and the uptake of
33
34 aminophospholipids and perifosine increased strikingly in both cell lines. These
35
36 findings suggest that CDC50A plays a key role in perifosine uptake in human cells,
37
38 presumably by forming a functional plasma membrane translocator in combination
39
40 with a P4-ATPase.
41
42
43
44
45
46
47
48
49
50
51
52

53 **KEYWORDS:** perifosine uptake, CDC50A, Atp8b1, P4-ATPase, aminophospholipid
54
55 translocase, resistance to perifosine, cancer chemotherapy.
56
57
58
59
60
61
62
63
64
65

1. INTRODUCTION

1
2
3 Perifosine is an oral alkyl-phospholipid (ALP) Akt inhibitor that is currently being
4
5 tested in phase II clinical trials. Unlike most kinase inhibitors, which target the
6
7 adenosine triphosphate-binding region, perifosine targets the pleckstrin homology
8
9 domain of Akt, thereby preventing its translocation to the plasma membrane [1].
10
11 Single-agent activity with perifosine has been observed in sarcoma and
12
13 Waldenström macroglobulinemia patients [2]. However, the disappointing response
14
15 rates of common solid tumors to perifosine as a single agent [3-6] have diminished
16
17 expectations and prompted further investigation into its mechanism of action.
18
19
20
21

22 The ability to identify patients likely to respond to targeted therapeutics would
23
24 decrease the size, duration, and cost of clinical trials, thus resulting in more efficient
25
26 translation to improved patient outcomes. In this sense, Hennessy and co-workers
27
28 [7] reported a strong correlation between proportional modulation of PI3K pathway
29
30 biomarkers and quantified perifosine efficacy, thereby suggesting that the early
31
32 measurements of such biomarkers will optimize the selection of responsive patients
33
34 and guide perifosine dosage during cancer treatment. Furthermore, the identification
35
36 of other potential biomarkers for perifosine efficacy in cancer patients would allow
37
38 those patients most likely to respond to perifosine, either alone or in combination
39
40 with other therapies, to be selected.
41
42
43
44
45

46 ALP uptake is a prerequisite for triggering the intracellular events that finally
47
48 cause cell death in different cellular systems, including yeasts [8, 9], the protozoan
49
50 parasite *Leishmania donovani* [10] and cancer cells [11]. Indeed, a clear correlation
51
52 between ALP uptake and apoptosis induction has been reported previously [12].
53
54 Two pathways for the entry of ALP into cancer cells, namely raft-mediated
55
56 endocytosis in lymphoma cells [13] and translocator-mediated uptake in carcinoma
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

cells [14-16], have been described. A clearer insight into the molecular determinants of perifosine entry into cancer cells would therefore be of enormous importance for the identification of additional biomarkers for the efficacy of perifosine as an anticancer drug.

Data from our previous work [14] strongly supported the hypothesis that a putative aminophospholipid translocase (APLT; P-type ATPase P4 subfamily) could be responsible for the uptake of ALPs in human KB carcinoma cells. Indeed, the plasma membrane P4-ATPase homologues of yeast and *Leishmania* have been shown to play a key role in the uptake of miltefosine and edelfosine [9, 17] in combination with a second protein from the Cdc50p-Lem3p family that functions as a beta subunit [8, 10, 18, 19]. As fourteen putative APLTs [20] and only three beta subunits (CDC50A, B and C) [21] have been identified in humans, we decided to try to elucidate the role of these human beta subunits as functional elements of the putative plasma membrane perifosine translocator (and other related ALPs) in cancer cells, and found that CDC50A plays a key role in the uptake of perifosine in human cancer cells, presumably by forming a functional plasma membrane translocator in combination with a P4-ATPase.

2. MATERIALS AND METHODS

2.1. Chemicals. Perifosine (octadecyl-[*N,N*-dimethyl-piperidinio-4-yl]-phosphate), and [¹⁴C]-perifosine (30.9 mCi/mmol) were provided by Zentaris GmbH (Frankfurt, Germany). Lipofectamine 2000, Anti-V5 monoclonal antibody, Alexa Fluor 633-conjugated anti-mouse IgG, pEF6-V5/His and pcDNA3.1-HYGRO vectors, FITC-concanavalin A, BODIPY-lactosylceramide, and Alexa Fluor 488-labeled dextran

10,000 were purchased from Invitrogen (Prat de Llobregat, Barcelona, Spain). Pre-designed ON-TARGETplus siRNA for human *CDC50A* and *CDC50B* were purchased from Dharmacon (Lafayette, CO). Fluorescent (NBD-labeled) phospholipid analogues 1-palmitoyl-2-{6-[(7-nitro-2-(1,3-benzoxadiazol)-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphoethanolamine (NBD-PE), -phosphoserine (NBD-PS) and -phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Anti-rabbit IgG monoclonal antibody, monoclonal anti- α -tubulin antibody, and the rabbit polyclonal antibodies anti-*myc*, anti-calnexin, anti-GM130, anti-TGN46, anti-Rab5 and anti-Rab7, and MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich (Madrid, Spain). Fugene HD, High Pure RNA isolation kit and Expand High Fidelity PCR System were purchased from Roche (San Cugat del Vallés, Barcelona, Spain), and the High-capacity cDNA Reverse transcription kit plus RNase inhibitor from Applied Biosystems (Alcobendas, Madrid, Spain). The Perfectprep[®] plasmid isolation kit from Eppendorf Ibérica (San Sebastian de los Reyes, Madrid, Spain) was used to isolate purified plasmids. QuickChange II XL Site-directed Mutagenesis kit was purchased from Stratagene-Cultek SLU (Madrid, Spain), and the pGEM-T Easy vector from Promega Biotech Ibérica (Alcobendas, Madrid, Spain). All other chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

2.2. Cell lines and cultures. The mammalian cell lines described in the present work were grown at 37 °C in a humidified atmosphere and 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM Glutamax I, 1:50 Micokill AB solution 50X (PAA Laboratories GmbH) and 250 U/ml penicillin G plus 250 µg/ml streptomycin sulphate (Invitrogen), unless indicated otherwise. The following cells were used: murine fibroblast embryo NIH

1 3T3 cells (ATCC number CCL-1658); chinese hamster ovary CHO-K1 cells (ATCC
2 number CCL-61; cultured in F-12K medium); canine kidney MDCKII cells [22];
3
4 monkey epithelial kidney Vero and COS-7 cells (ATCC CRL-1586 and CRL-1651);
5
6 human epidermal carcinoma KB cells (wild-type) (ATCC number CCL-17) and its
7
8 perifosine-resistant counterparts KB PER-R clone 4, 8 and 10 [14]; human breast
9
10 carcinoma MCF-7 and MDA-MB-23 cells [23, 24]; human cervix epithelial
11
12 adenocarcinoma HeLa cells (ATCC CCL-2); human epithelial embryonic kidney
13
14 HEK-293T cells (ATCC CRL-11268); human pancreas carcinoma AsPC1 and
15
16 MiaPaca2 cells (ATCC CRL-1682 and CRL-1420); human 2008 ovary cells [25],
17
18 kindly provided by Prof. Piet Borst (Division of Molecular Biology, The Netherlands
19
20 Cancer Institute, Amsterdam, The Netherlands); and human osteosarcoma HOS
21
22 cells (ATCC CRL-1543).
23
24
25
26
27
28

29 **2.3. MTT survival assays, [¹⁴C]-perifosine uptake, accumulation of** 30 **fluorescent phospholipid analogues and measurement of endocytosis rates.** 31 32

33 Unless otherwise indicated, these four different assays were performed as described
34
35 in our previous work [14], with the only modification that, in the MTT-based
36
37 colorimetric assay to determine perifosine sensitivity, the cell lines were incubated
38
39 with the anticancer drug for only 2 h instead of 24 h before adding MTT to the culture
40
41 medium. This modification allowed us to better link the observed cytotoxicity with the
42
43 amount of perifosine incorporated in a short time by each cell line, and therefore to
44
45 establish whether cell death actually correlated with intracellular accumulation of
46
47 perifosine.
48
49
50
51
52
53

54 **2.4. Isolation of *CDC50A*, *CDC50B* and *Atp8b1*, and DNA constructs.** The 55 56 human cDNAs of *CDC50A* (GenBank accession no. NM_018247, longer 57 58 transcription variant 1) and *CDC50B* (GenBank accession no. NM_001017970) 59 60 61 62 63 64 65

1 genes, which code for the putative beta subunits of the human APLT(s) (P-type
2 ATPase P4 subfamily), were isolated by RT-PCR with the High Capacity cDNA
3 Reverse transcription kit plus RNase inhibitor (Applied Biosystems), using total RNA
4 obtained from KB cells with the High Pure RNA isolation kit (Roche) as template;
5 [primers: CDC50A-F (5'-ACCATGGCGATGAACTATAACG), and CDC50A-R (5'-
6 TTAAATGGTAATGTCAGCTGTATTAC); CDC50B-F (5'-
7 ACCATGACCTGGAGCGCCACGGC) and CDC50B-R (5'-
8 TCACTCCTCGTCGTCGTCCTGG)]. The resulting PCR products were cloned into
9 the pEF6-V5/His TOPO TA expression vector from Invitrogen, in frame with the C-
10 terminal V5 epitope and the polyhistidine (6 x His) tag. The cDNA of *Atp8b1*
11 (GenBank accession no. **BC117946**), the murine orthologue of the human gene that
12 encodes for the putative APLT ATP8B1 (95% identity), was purchased from Source
13 BioScience Geneservice (Cambridge, UK) as a full open-reading frame (ORF)
14 cloned into pCR-BluntII-TOPO vector. The construct was isolated and purified using
15 the Perfectprep[®] plasmid isolation kit (Eppendorf), and the ORF subsequently
16 amplified using the Expand High Fidelity PCR System with the primers: 8b1-F (5'-
17 ACCATGGGCACAGAAAGAGACTCG); 8b1-R (5'-
18 TCACAGATCCTCTTCAGAGATGAGTTTCTGCTCGCTCTCCACTGTGCGCCGGTA
19), (*myc*-tag coding sequence underlined). The resulting PCR product was cloned into
20 pGEMT-Easy from Promega. The nucleotide sequences of all cloned cDNAs were
21 determined automatically, as described previously [26], and compared against their
22 respective records annotated in GenBank. Unexpectedly, after repeating PCR and
23 isolating new clones, we found that the supplier had provided us with a mutant
24 variant of the *Atp8b1* gene (A1954G, which resulted in the amino acid mutation
25 R652G). Since we were concerned about the possible undesired effects that this
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

mutation could cause in the phenotypic function of the resulting protein, we decided to fix it by using the QuickChange II XL Site-directed Mutagenesis kit from Stratagene using the primers (fixing point-mutation is underlined): F*Atp8b1*-mut (5'-AAGTGAGACTCTCAGGACCCTGTGCCTGTG); R*Atp8b1*-mut (5'-CACAGGCACAGGGTCCTGAGAGTCTCACTTG). Once this annoying problem had been resolved, we re-amplified the *Atp8b1* ORF with the primers F*Atp8b1*-NheI (5'-TTATTGCTAGCACCATGGGCACAGAAAGAGACTCG); R*Atp8b1*-BamHI (5'-TTATTGGATCCTCAAGATCTCTTCAGAGATGAG), and cloned the resulting PCR product as an NheI/BamHI fragment into the pcDNA3.1-HYGRO expression vector.

2.5. Cell transfection. KB Wt, KB PER-R, HeLa and HEK-293T cells were stably transfected with pEF6-*CDC50A-V5* and pEF6-*CDC50B-V5* using Lipofectamine 2000 following the manufacturer's protocol. The stable transfectants were selected with 10 µg/ml blasticidin in only one week, and the expression levels of both proteins readily visualized by Western blotting or indirect immunofluorescence (IFI) under a microscope using monoclonal anti-V5 1:5000 from Invitrogen. Untransfected and *CDC50A-V5*-transfected KB Wt and KB PER-R cells were refractory to both stable and transient transfection with the pcDNA3.1-HYGRO-*Atp8b1-myc* construct using either Lipofectamine 2000 or Fugene HD, as judged by the fact that no *Atp8b1-myc* expression was observed, either by Western blot or by IFI using several anti-myc antibodies. In contrast, HeLa and HEK-293T cells could be transiently transfected with *CDC50A-V5* alone or in combination with *Atp8b1-myc*, with the best results being obtained using Fugene HD.

2.6. siRNA-based *CDC50A* and *CDC50B* knockdown. Human *CDC50A* and *CDC50B* were targeted using SMARTpool siRNA oligonucleotides (Dharmacon),

1 following a previously described procedure [14], at concentrations of 1, 10 and 100
2 nM of siRNA oligonucleotides in order to monitor the concentration dependency of
3 the gene silencing.
4
5
6
7
8

9 **2.7. Biotinylation of cell surface proteins.** KB Wt and PER-R cells were
10 washed with PBS and incubated on ice with 1 mM EZ-Link Sulfo-NHS-SS-biotin
11 (Thermo Scientific Pierce, Madrid, Spain) in 1 ml PBS for 2 h at 4 °C. The
12 biotinylation reaction was quenched by washing the cells three times with 50 mM
13 Tris-HCl, pH 7.4, then cells were incubated on ice for 30 min in 100 µl lysis buffer
14 (50 mM Tris- HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40) plus a protease inhibitor
15 cocktail (Sigma-Aldrich). The cell lysate was clarified by centrifugation at 21,000 x g
16 for 10 min at 4 °C, and the supernatant incubated for 2 h at 4 °C in continuous
17 agitation with 100 µl of packed streptavidin-agarose beads (Thermo Scientific
18 Pierce) pre-washed with lysis buffer. The agarose beads were then washed three
19 times with 1 ml of PBS + 1 M NaCl and two times with 1 ml of PBS, and the
20 biotinylated proteins eluted in 30 µl of 2 x Laemmli buffer.
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40

41 **2.8. Immunoblotting.** Protein samples were fractionated by SDS-PAGE under
42 standard conditions and electrotransferred onto Immobilon P membranes (Millipore,
43 Bedford, MA). Immunodetection of the V5-tagged beta subunits and Atp8b1-myc
44 was performed with 1:5000 monoclonal anti-V5 and 1:1000 anti-myc polyclonal
45 antibody in buffer A (PBS containing 0.01% Tween 20 and 0.1% BSA). After
46 washing, the membranes were incubated with horseradish peroxidase-conjugated
47 secondary rabbit anti-mouse or goat anti-rabbit (1:5000) immunoglobulin G (Dako,
48 Barcelona, Spain) in buffer A. Immunodetection of α -tubulin for sample normalization
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 was performed with monoclonal anti- α -tubulin antibody (Sigma-Aldrich) at 1:5000.
2 Signals were detected using either the ECL or the ECL Plus chemiluminescent
3 substrate (Thermo Scientific Pierce).
4
5
6
7
8
9

10 **2.9. Immunofluorescence and confocal microscopy.** Cells were seeded onto
11 cover slip circles (diameter: 12 mm; thickness: 0.13-0.16 mm, from Menzsel-
12 Gläser) in 24-well plates (Thermo Scientific Nunc, Madrid, Spain) and cultured
13 under standard conditions. On the day of the experiment, the cells were washed
14 three times with PBS and fixed with 2% para-formaldehyde in PBS for 20 min at
15 room temperature. After quenching with 10 mM Tris-HCl pH 7.4 and washing twice
16 with the same buffer, the cells were permeabilized with a 0.1% solution of Triton X-
17 100 detergent (Sigma-Aldrich) in PBS for 10 min at room temperature. They were
18 then washed with PBS and incubated for 30 min with PBS 1% BSA to block the non-
19 specific binding of antibodies. The primary antibodies anti-V5 (1:5000), anti-myc
20 (1:1000), anti-calnexin (1:200), anti-GM130 (1:2500), anti-TGN46 (1:200), anti-Rab5
21 (1:200) and anti-Rab7 (1:200) were incubated at 37 °C in a humidified atmosphere
22 for 1-2 h in PBS 1% BSA. The cells were then rinsed three times with PBS and
23 treated with the secondary antibodies FITC-conjugated anti-rabbit IgG antibody
24 (1:1000-1:2000) from Sigma-Aldrich, and AlexaFluor 633-conjugated anti-mouse IgG
25 antibody (1:500) from Invitrogen in PBS 1% BSA. After the labelling procedure was
26 complete, the cells were washed three times with PBS and mounted onto glass
27 slides using Prolong Gold mounting medium with DAPI (Invitrogen).
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52

53 Confocal images were acquired using a Leica TCS-SP5 confocal scanning laser
54 microscope equipped with Ar and He-Ne lasers and connected to a Leica DMIRBE
55 inverted epi-fluorescence microscope. The following filter settings were used: DAPI
56
57
58
59
60
61
62
63
64
65

1 (blue): excitation at 405 nm (405 diode laser, UV), emission at 409-469 nm; FITC
2 (green): excitation at 458 nm (argon laser, 20% power), emission at 494-603 nm;
3
4 Alexa Fluor 633 (red): excitation at 633nm (633 HeNe laser, 20% power), emission
5 at 648-692 nm. Serial fluorescence images were obtained using the 63x objective
6
7 and processed and assembled with LAS AF Leica confocal software. Image analysis
8
9 and subcellular colocalization fluorograms were generated and analyzed using the
10
11 Leica confocal software package.
12
13
14
15
16
17
18

19 **2.10. Semi-quantitative RT-PCRs.** Semi-quantitative RT-PCRs were performed
20 using the total cDNAs synthesized above as templates to establish the endogenous
21 expression of *CDC50A* and *ATP8B1* genes in KB, HeLa and HEK293T cells.
22
23 Standard PCRs were performed using serial dilutions of total cDNAs as templates
24 and highly specific primers [ATP8B1 forward (5'-CAG ACT GCA TAC GAG GAT
25 TGG); ATP8B1 reverse (5'-CCT TCA ACC GCT TGC GAT G); CDC50A forward (5'-
26 CTA TAA CGC GAA GGA TGA AGT G); CDC50A reverse (5'- TCC ATA ATA CAT
27 AAA CAC GTT GCC); β -actin forward (5'-CCT CAT GAA GAT CCT CAC CG); β -
28 actin reverse (5'-CAG TGA TCT CCT TCT GCA TCC)] to amplify internal 631-, 357-
29 and 660-bp sequences from the *ATP8B1*, *CDC50A* and β -actin cDNAs, respectively.
30
31 The PCR conditions for each step were as follows: Initial denaturation (95 °C, 5 min);
32
33 35 cycles (denaturation: 92 °C, 30 s; annealing: 52 °C, 45 s; extension: 72 °C, 45 s);
34
35 final extension: 72 °C, 3 min. The resulting PCR products were run in 0.8% agarose
36
37 gels stained with ethidium bromide and visualized in a UV transilluminator.
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

RESULTS

3.1. Perifosine and NBD-phosphatidylethanolamine (NBD-PE) uptake are linked processes in KB cell lines

In our previous work we generated a perifosine-resistant line (designated as KB PER-R clone 10) from the perifosine-hypersensitive KB Wt cells (with an EC_{50} of less than 1 μ M for 24 h perifosine exposure). Along with a high resistance to perifosine (grows suitably at 20 μ M perifosine), this cell line showed significantly decreased perifosine uptake, a low rate of NBD-aminophospholipid translocation through the plasma membrane, a relatively high exposure of endogenous phosphatidylserine in the cell surface, and lacked NEM- and orthovanadate-sensitive, plasma-membrane-associated ATPase activity [14]. Similar perifosine-resistant clones generated in our laboratory from KB Wt cells (clones 4 and 8) showed intermediate levels of perifosine uptake with respect to KB cells and KB PER-R clone10 cells, along with intermediate rates of NBD-PE translocation through the plasma membrane, and were only moderately resistant to perifosine compared with clone 10 (Fig. 1). This correlation between perifosine uptake and NBD-PE translocation therefore suggested that, as proposed previously [14], a link could exist between both processes in KB cells. We also tested the uptake rates of BODIPY-lactosylceramide (raft-dependent endocytosis marker) and Alexa Fluor 488-dextran 10,000 (fluid phase endocytosis marker) in a subset of fourteen cell lines, and found no significant correlation upon comparing their rates of perifosine uptake (Fig. 2).

3.2. CDC50A is implicated in both perifosine and fluorescent aminophospholipid analogues uptake in KB cells

1
2
3
4
5 In our previous work we proposed that a putative APLT could be responsible for
6
7 the internalization of both ALPs and aminophospholipids through the plasma
8
9 membrane in human KB carcinoma cells. APLTs seems to be a functional complex
10
11 of at least two proteins (alpha and beta subunits) [10, 18, 19], and since fourteen
12
13 different putative alpha but only three presumed beta subunits (CDC50A, B and C)
14
15 have been described in humans, we decided to start our study of the potential
16
17 implication of the latter proteins on perifosine uptake in mammalian cells. Since
18
19 *CDC50C* transcripts have been found to terminate aberrantly in humans [27], only
20
21 *CDC50A* and *B* were likely to play a putative role in perifosine uptake. We therefore
22
23 generated KB cells expressing *CDC50A-V5* or *CDC50B-V5* (Fig. 3A) and found that,
24
25 whereas NBD-aminophospholipid uptake was increased by about 50% when either
26
27 of these proteins was over-expressed, perifosine uptake was only boosted when
28
29 *CDC50A* was over-expressed (Fig. 3B). No significant increase of NBD-PC uptake
30
31 was detected in KB cells over-expressing *CDC50A-V5* or *CDC50B-V5*. Interestingly,
32
33 over-expression of *CDC50A-V5* in KB PER-R clone10 cells did not increase the rate
34
35 of perifosine or aminophospholipid uptake (data not shown). Moreover, knock-down
36
37 of *CDC50A* and *CDC50B* reduced perifosine and NBD-aminophospholipid uptake to
38
39 around 40% of the normal rate when *CDC50A* expression was lowered, whereas
40
41 *CDC50B* silencing only affected NBD-aminophospholipid uptake (Fig. 4A). No
42
43 significant reduction of NBD-PC was detected after the knock-down experiments.
44
45 However, since we had no suitable antibody for monitoring the expression levels of
46
47 endogenous *CDC50A* in KB cells, we also performed the same experiments in
48
49 *CDC50A-V5*-transfected KB cells, thereby allowing us to monitor *CDC50A-V5*
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 expression levels by Western blot (Fig. 4B). This study showed that lower CDC50A-
2 V5 levels correlated with lower rates of perifosine and NBD-aminophospholipid
3 uptake (Fig. 4C).
4
5
6
7
8

9 **3.3. CDC50A subcellular localization in KB Wt vs. KB PER-R clone 10 cells**

10 Cdc50-Lem3p proteins play a role as both molecular chaperones needed for the
11 correct folding and export of the alpha subunit P4-ATPase from the endoplasmic
12 reticulum (ER) [28, 29], and also as catalytically relevant, integral components of the
13 phospholipid translocation machinery [30]. Since we have found previously that
14 human CDC50A appears to play a role in perifosine uptake in KB cells, we decided
15 to establish the cellular localization of this protein. Thus, we performed indirect
16 immunofluorescence of V5-tagged CDC50A expressed in KB cells and then
17 visualized the samples by confocal microscopy. It is apparent from Fig. 5, which
18 shows the scattered intracellular distribution and co-localization with several markers
19 of different intracellular membrane compartments, that CDC50A-V5 is a membrane
20 protein that is distributed via the endocytic and exocytic pathways in KB cells.
21 However, we cannot conclude from this figure whether CDC50A-V5 reaches the
22 plasma membrane or not. A comparison of KB and KB PER-R clone10 cells, both of
23 which express similar levels of CDC50A-V5, as monitored by Western blot (not
24 shown), highlighted a striking difference in the intracellular localization of CDC50A-
25 V5. Thus, this protein was expressed well at the ER level, and was also exported to
26 the CIS-Golgi and TRANS-Golgi network (TGN) in both cell lines, whereas its
27 presence in early and late endosomes was significantly lower in KB PER-R cells
28 than in KB cells, thereby suggesting that CDC50A-V5 distribution via the pathways
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 connecting TGN, early endosomes, the plasma membrane and late endosomes is
2 hindered in KB PER-R cells.
3

4 As we were unable to ascertain the presence of CDC50A at the plasma
5 membrane of KB cells by confocal microscopy, we biotinylated the cell surface
6 proteins in *CDC50A-V5*-transfected KB and KB PER-R cells in parallel. Fig. 6 shows
7 that most of the CDC50A-V5 is intracellular (unbound fraction to streptavidin-
8 agarose beads) in KB cells, as also determined by confocal microscopy, but that a
9 detectable amount of CDC50A-V5 reaches the plasma membrane as well. In
10 contrast, no CDC50A-V5 was observable in the fraction of biotinylated cell surface
11 proteins in KB PER-R clone10 cells, although its intracellular levels were
12 comparable to those for KB cells. We used α -tubulin as a reference to compare the
13 amount of CDC50A-V5 expressed in both cell types and as a control of cell
14 membrane integrity throughout the entire biotinylation process. Only cell surface
15 proteins were biotinylated, since α -tubulin is only present in the unbound fraction.
16 The inability of CDC50A-V5 to reach the plasma membrane in KB PER-R cells is in
17 agreement with its defective membrane trafficking, as suggested previously by
18 confocal microscopy, and further supports our hypothesis that perifosine uptake is
19 very low in this cell line due to the lack of a P4-ATPase APLT at the plasma
20 membrane.
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

51 **3.4. CDC50A and Atp8b1 associate to exit the ER and traffic to the plasma** 52 **membrane in HeLa and HEK293T cells, where they form a functional perifosine** 53 **and aminophospholipid translocator** 54

55 In light of the functional involvement of CDC50A in perifosine uptake in KB cells,
56 we decided to investigate whether CDC50A was also involved in perifosine uptake in
57
58
59
60
61
62
63
64
65

1 other cell lines. Thus, we expressed CDC50A-V5 in HeLa and HEK-293T cells,
2 although with disappointing results: no increase was observed in perifosine or NBD-
3 phospholipid uptake in either of these cell lines (see Fig. 8B). However, this finding
4 does not allow us to state that CDC50A plays no role in perifosine uptake in this
5 case, as CDC50A may lack the appropriate alpha subunit partner in these cell lines.
6 To test this hypothesis, we performed semi-quantitative RT-PCR to compare the
7 mRNA expression levels of *CDC50A* and *ATP8B1* (the best studied P4-ATPase [31],
8 which was likely to be responsible for aminophospholipid translocation in these cell
9 lines). This study showed (Fig. 7) that the endogenous expression levels of *CDC50A*
10 were similar in all three cell lines, and that *ATP8B1* was expressed at similar levels
11 in KB and HEK-293T cells, and at even higher levels in HeLa cells (Fig. 7). This
12 finding suggests that a P4-ATPase other than ATP8B1 is likely to be the alpha
13 subunit responsible for the APLT activity observed in KB, HeLa and HEK293T cells.
14 At this point, instead of simply determining the expression levels of the remaining
15 thirteen putative human alpha subunits, we adopted a more straightforward
16 approach involving the simultaneous transfection of *CDC50A-V5* and murine *Atp8b1*
17 (a well-studied P4-ATPase, 95% homologous to its human counterpart [34]) as a C-
18 terminal myc-tagged protein in HeLa and HEK-293T cells (Fig. 8A). Interestingly, we
19 found that the rates of perifosine uptake in HeLa and HEK-293T cells increased by
20 60% and 130%, respectively (Fig. 8B). The uptake of NBD-aminophospholipids (but
21 not NBD-PC) was also similarly boosted in both cell lines (Fig. 8B). Confocal
22 microscopy studies revealed that CDC50A-V5 was retained in the intracellular
23 membrane compartments in HeLa cells when over-expressed alone, whereas when
24 it was co-expressed with *Atp8b1* in the same cells (Fig. 9), both proteins co-localized
25 at the plasma membrane, in a similar manner to that reported for the pair ATP8B1 +
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 CDC50A [29]. Co-localization of these two proteins was also observed when co-
2 expressed in HEK-293T cells (data not shown).
3
4
5
6

7 **4. DISCUSSION**

8
9 A detailed knowledge of the molecular mechanism of action of anticancer drugs
10 will help to identify those patients who are most likely to respond to a given
11 treatment based on such drugs, to overcome drug resistance phenomena and to
12 improve the final treatment outcome. Indeed, there seems to be a general
13 consensus amongst researchers regarding the manner in which perifosine causes
14 cell death in several different types of cancer cells. However, some discrepancies
15 have arisen concerning how perifosine enters cancer cells, and what previous steps
16 are required for the drug to reach its intracellular targets and trigger cell death. Thus,
17 it has been proposed that ALP enters cells either by raft-dependent endocytosis [13,
18 32, 33] or by transporter-mediated translocation of perifosine through the plasma
19 membrane [14, 15, 16].
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

36 We firmly support the latter hypothesis, and in this present work, which is a
37 continuation of a previous study [14], we present strong evidence to suggest that
38 perifosine enters cancer cells via APLT(s) at the plasma membrane, and that
39 CDC50A plays a key role in this process. Thus, we have found a positive correlation
40 between perifosine uptake and NBD-aminophospholipid translocation (a well-
41 established marker for aminophospholipid translocase activity) in several KB clones
42 obtained in our laboratory. Furthermore, we found no correlation whatsoever
43 between perifosine uptake and endocytosis rates in a subset of fourteen cell lines,
44 which is in stark contrast with previous works where the role of raft-dependent
45 endocytosis in perifosine uptake has been proposed [13, 32, 33]. This discrepancy
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

may, however, only be apparent if we consider that only adherent cell lines, but no leukemia or lymphoma cells, were used in the present study, whereas the authors who proposed the raft-dependent hypothesis for perifosine uptake have recently published a further study [15] where they discriminate between lymphoma and carcinoma cells on the basis of their prevalent perifosine-uptake mechanism (raft-dependent endocytosis in the former, and a plasma membrane translocator in the latter). It is also possible that both mechanisms of perifosine uptake could co-exist in the same cell, with their relative importance varying with cell type. Thus, for example, KB cells accumulate perifosine very rapidly, and independently of rafts endocytosis, due to their high APLT activity [14, 15], whereas HeLa cells accumulate perifosine five times slower as they exhibit lower APLT activity. In this case, the slow drug uptake would depend mainly on endocytosis, as suggested previously [33].

We also found that modulating the CDC50A expression levels in KB cells markedly affected the rate of perifosine and aminophospholipid uptake, thereby suggesting that a putative APLT is responsible for translocation of both types of molecules through the plasma membrane. It also appears that the CDC50A beta subunit should be the limiting factor for the APLT complex in KB cells, where it is distributed throughout the endo-membranous compartments, trafficking from the ER to the plasma membrane of KB cells. In contrast, CDC50A exports from the ER only to the TGN in KB PER-R clone10 cells and does not reach the cell surface, thereby suggesting that these perifosine-resistant cells harbor a defect that results in the incorrect trafficking of CDC50A at this point of the exocytic pathway. Since CDC50A must couple with a P4-ATPase for correct trafficking of the full APLT complex to the plasma membrane [29], the observed traffic defect of CDC50A in KB PER-R cells could explain the inability of this cell line to translocate perifosine. In contrast to KB

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

cells, over-expression of CDC50A in the human cell lines HeLa and HEK-293T did not modulate perifosine uptake, thus suggesting that, in this case, the alpha subunit rather than CDC50A may be the limiting component of the putative APLT. Human ATP8B1 is a P4-ATPase with APLT activity when complexed with CDC50A, although the expression levels of both *CDC50A* and *ATP8B1* in HeLa and HEK-293T cells were found to be very similar to those in KB. Therefore, if we assume that no differential regulation of the APLT complex occurs in each cell line, and that the overall APLT activity depends mainly on the relative expression levels of its two components, it seems likely that ATP8B1 is not the alpha subunit responsible for the different APLT activities measured in these cell lines. However, we found that the uptake of perifosine and NBD-aminophospholipids (but not NBD-PC) increased remarkably when both HeLa and HEK-293T cells were co-transfected with *Atp8b1-myc* and *CDC50A-V5*. Moreover, CDC50A-V5 remained intracellular when over-expressed alone, but when co-expressed with *Atp8b1* both proteins exported from the ER and co-localized at the plasma membrane, as is the case with human ATP8B1 + CDC50A [29]. CDC50A may be coupled with a P4 ATPase to drive perifosine and aminophospholipids uptake and could contribute to the transport specificity of the complex.

Taken together, the results of this work strongly support the role of an APLT as the means of entry of the anticancer drug perifosine through the plasma membrane in adherent carcinoma cells. Such an APLT in humans is most likely formed by the beta subunit CDC50A plus a P4-ATPase alpha subunit(s) whose identity remains to be established.

ACKNOWLEDGEMENTS

1
2 This work was supported by “Plan Andaluz de Investigación, Proyecto de
3
4 Excelencia (CTS 187)”, Junta de Andalucía. We acknowledge Aeterna Zentaris
5
6 (Frankfurt, Germany) for the financial support and for providing the perifosine and
7
8 [¹⁴C]-perifosine used in this study. We want to thank Carlos Castillo Pérez for his
9
10 help in the fixing and cloning of *Atp8b1* cDNA and Dr. Luis Carvalho for his technical
11
12 assistance in RT-PCR experiments.
13
14
15
16
17
18

REFERENCES

- 19
20
21 [1] Kondapaka SB, Singh SS, Dasmahapatra GP, Sausville EA, Roy KK.
22
23 Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. *Mol*
24
25 *Cancer Ther* 2003;2:1093-103.
26
27
28 [2] Gills JJ, Dennis PA. Perifosine: update on a novel Akt inhibitor. *Curr Oncol*
29
30 *Rep* 2009;11:102-10.
31
32
33 [3] Chee KG, Longmate J, Quinn DI, Chatta G, Pinski J, Twardowski P, et al. The
34
35 AKT inhibitor perifosine in biochemically recurrent prostate cancer: a phase II
36
37 California/Pittsburgh cancer consortium trial. *Clin Genitourin Cancer*
38
39 *2007;5:433-7.*
40
41
42 [4] Leighl NB, Dent S, Clemons M, Vandenberg TA, Tozer R, Warr DG, et al. A
43
44 Phase 2 study of perifosine in advanced or metastatic breast cancer. *Breast*
45
46 *Cancer Res Treat* 2008;108:87-92.
47
48
49 [5] Argiris A, Cohen E, Karrison T, Esparaz B, Mauer A, Ansari R, et al. A phase
50
51 II trial of perifosine, an oral alkylphospholipid, in recurrent or metastatic head
52
53 and neck cancer. *Cancer Biol Ther* 2006;5:766-70.
54
55
56
57
58
59
60
61
62
63
64
65

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [6] Knowling M, Blackstein M, Tozer R, Bramwell V, Dancey J, Dore N, et al. A phase II study of perifosine (D-21226) in patients with previously untreated metastatic or locally advanced soft tissue sarcoma: A National Cancer Institute of Canada Clinical Trials Group trial. *Invest New Drugs* 2006;24:435-9.
- [7] Hennessy BT, Lu Y, Poradosu E, Yu Q, Yu S, Hall H, et al. Pharmacodynamic markers of perifosine efficacy. *Clin Cancer Res* 2007;13:7421-31.
- [8] Hanson PK, Malone L, Birchmore JL, Nichols JW. Lem3p is essential for the uptake and potency of alkylphosphocholine drugs, edelfosine and miltefosine. *J Biol Chem* 2003;278:36041-50.
- [9] Riekhof WR, Voelker DR. The yeast plasma membrane P4-ATPases are major transporters for lysophospholipids. *Biochim Biophys Acta* 2009;1791:620-7.
- [10] Pérez-Victoria FJ, Sanchez-Cañete MP, Castanys S, Gamarro F. Phospholipid translocation and miltefosine potency require both *L. donovani* miltefosine transporter and the new protein LdRos3 in *Leishmania* parasites. *J Biol Chem* 2006;281:23766-75.
- [11] Gajate C, Fonteriz RI, Cabaner C, Álvarez-Noves G, Álvarez-Rodríguez Y, Modolell M, et al. Intracellular triggering of Fas, independently of FasL, as a new mechanism of antitumor ether lipid-induced apoptosis. *Int J Cancer* 2000;85:674-82.
- [12] Mollinedo F, Fernández-Luna JL, Gajate C, Martín-Martín B, Benito A, Martínez-Dalmau R, et al. Selective induction of apoptosis in cancer cells by the ether lipid ET-18-OCH₃ (Edelfosine): molecular structure requirements,

- cellular uptake, and protection by Bcl-2 and Bcl-X(L). *Cancer Res* 1997;57:1320-8.
- [13] van der Luit AH, Vink SR, Klarenbeek JB, Perrissoud D, Solary E, Verheij M, et al. A new class of anticancer alkylphospholipids uses lipid rafts as membrane gateways to induce apoptosis in lymphoma cells. *Mol Cancer Ther* 2007;6:2337-45.
- [14] Muñoz-Martínez F, Torres C, Castanys S, Gamarro F. The anti-tumor alkylphospholipid perifosine is internalized by an ATP-dependent translocase activity across the plasma membrane of human KB carcinoma cells. *Biochim Biophys Acta* 2008;1778:530-40.
- [15] Vink SR, van der Luit AH, Klarenbeek JB, Verheij M, van Blitterswijk WJ. Lipid rafts and metabolic energy differentially determine uptake of anti-cancer alkylphospholipids in lymphoma versus carcinoma cells. *Biochem Pharmacol* 2007;74:1456-65.
- [16] Menez C, Buyse M, Farinotti R, Barratt G. Inward translocation of the phospholipid analogue miltefosine across Caco-2 cell membranes exhibits characteristics of a carrier-mediated process. *Lipids* 2007;42:229-40.
- [17] Pérez-Victoria FJ, Gamarro F, Ouellette M, Castanys S. Functional cloning of the miltefosine transporter. A novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. *J Biol Chem* 2003;278:49965-71.
- [18] Sanchez-Cañete MP, Carvalho L, Pérez-Victoria FJ, Gamarro F, Castanys S. Low plasma membrane expression of the miltefosine transport complex renders *Leishmania braziliensis* refractory to the drug. *Antimicrob Agents Chemother* 2009;53:1305-13.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [19] Saito K, Fujimura-Kamada K, Furuta N, Kato U, Umeda M, Tanaka K. Cdc50p, a protein required for polarized growth, associates with the Drs2p P-type ATPase implicated in phospholipid translocation in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2004;15:3418-32.
- [20] Folmer DE, Elferink RP, Paulusma CC. P4 ATPases - lipid flippases and their role in disease. *Biochim Biophys Acta* 2009;1791:628-35.
- [21] Katoh Y, Katoh M. Identification and characterization of CDC50A, CDC50B and CDC50C genes in silico. *Oncol Rep* 2004;12:939-43.
- [22] Evers R, Cnubben NH, Wijnholds J, van Deemter L, van Bladeren PJ, Borst P. Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1. *FEBS Lett* 1997;419:112-6.
- [23] Cailleau R, Young R, Olive M, Reeves WJ, Jr. Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst* 1974;53:661-74.
- [24] Soule HD, Vazquez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* 1973;51:1409-16.
- [25] Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, et al. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. *Cancer Res* 1999;59:2532-5.
- [26] Lario A, González A, Dorado G. Automated laser-induced fluorescence DNA sequencing: equalizing signal-to-noise ratios significantly enhances overall performance. *Anal Biochem* 1997;247:30-3.
- [27] Osada N, Hashimoto K, Hirai M, Kusuda J. Aberrant termination of reproduction-related TMEM30C transcripts in the hominoids. *Gene* 2007;392:151-6.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [28] Puts CF, Holthuis JC. Mechanism and significance of P4 ATPase-catalyzed lipid transport: lessons from a Na⁺/K⁺-pump. *Biochim Biophys Acta* 2009;1791:603-11.
- [29] Paulusma CC, Folmer DE, Ho-Mok KS, de Waart DR, Hilarius PM, Verhoeven AJ, et al. ATP8B1 requires an accessory protein for endoplasmic reticulum exit and plasma membrane lipid flippase activity. *Hepatology* 2008;47:268-78.
- [30] Lenoir G, Williamson P, Puts CF, Holthuis JC. Cdc50p plays a vital role in the ATPase reaction cycle of the putative aminophospholipid transporter Drs2p. *J Biol Chem* 2009;284:17956-67.
- [31] Paulusma CC, Oude Elferink RP. The type 4 subfamily of P-type ATPases, putative aminophospholipid translocases with a role in human disease. *Biochim Biophys Acta* 2005;1741:11-24.
- [32] van der Luit AH, Budde M, Ruurs P, Verheij M, van Blitterswijk WJ. Alkyl-lysophospholipid accumulates in lipid rafts and induces apoptosis via raft-dependent endocytosis and inhibition of phosphatidylcholine synthesis. *J Biol Chem* 2002;277:39541-7.
- [33] Van Der Luit AH, Budde M, Verheij M, Van Blitterswijk WJ. Different modes of internalization of apoptotic alkyl-lysophospholipid and cell-rescuing lysophosphatidylcholine. *Biochem J* 2003;374:747-53.
- [34] Paulusma CC, Groen A, Kunne C, Ho-Mok KS, Spijkerboer AL, Rudi de Waart D, et al. Atp8b1 deficiency in mice reduces resistance of the canalicular membrane to hydrophobic bile salts and impairs bile salt transport. *Hepatology* 2006;44:195-204.

FIGURE LEGENDS

1
2
3
4
5 **Figure 1.** Correlation between perifosine sensitivity and ^{14}C -perifosine and NBD-PE
6 uptake in KB Wt cells and different KB PER-R clones. Perifosine sensitivity (EC_{50}
7 values) was determined by MTT assay after incubation for 2 h with different drug
8 concentrations. Uptake of $1\ \mu\text{M}$ [^{14}C]-perifosine ($0.03\ \mu\text{Ci}/\text{mL}$) and $2\ \mu\text{M}$ NBD-PE was
9 performed at $37\ ^\circ\text{C}$ for 1h and $10\ ^\circ\text{C}$ for 20 min, respectively, as described previously
10 [14]. Data were normalized to those measured in KB Wt, which were considered as
11 100%. The values shown are the average \pm SD of three to six different experiments
12 performed in triplicate.
13
14
15
16
17
18
19
20
21
22
23
24
25
26

27 **Figure 2.** Comparative analysis of perifosine and endocytosis markers in different
28 representative cell lines. Perifosine-uptake experiments were performed as described in
29 the legend to Fig. 1. Uptake of the endocytic markers BODIPY-Lactosyl-ceramide
30 (LacCER, $0.5\ \mu\text{M}$) and Alexa488-labelled dextran 10 KD (Dextran 10 KDa, $0.25\ \text{mg}/\text{mL}$)
31 was performed for 7 min at $37\ ^\circ\text{C}$ and analyzed by flow cytometry as described
32 previously [14]. Bars represent the average \pm S.D. of three different experiments
33 performed in duplicate. Data were normalized to those measured in KB Wt cells, which
34 were considered as 100%.
35
36
37
38
39
40
41
42
43
44
45
46
47
48

49 **Figure 3.** Uptake of perifosine and NBD-phospholipids in KB Wt cells over-
50 expressing CDC50A or CDC50B. **A)** Western blot analysis. KB Wt cells were
51 transfected with pEF6-V5 vector containing the corresponding gene in phase with a C-
52 terminal V5-tag. Expression levels were determined using anti-V5 monoclonal antibody.
53 A blot using anti- α -tubulin was performed in parallel as control of protein loading. **B)**
54
55
56
57
58
59
60
61
62
63
64
65

1 Uptake experiments. Uptake of perifosine and NBD-PE, -PS and -PC was performed as
2 described in the legend to Fig. 1. This is a representative experiment performed twice in
3
4 triplicate \pm S.D. from two different transfections. Data were normalized to those
5
6 measured in KB Wt cells (perifosine uptake: 3082 ± 151 pmol/mg protein; NBD-
7
8 phospholipids uptake expressed in flow cytometry mean channel values after
9
10 subtracting the cell autofluorescence: NBD-PE: 558 ± 57 ; NBD-PS: 497 ± 63 ; NBD-PC:
11
12 463 ± 84), which were considered as 100%.
13
14
15
16
17
18

19 **Figure 4.** Silencing of CDC50A and CDC50B in KB Wt cells. **A)** Uptake of NBD-
20
21 phospholipids and perifosine. KB Wt was transfected with 100 nM specific SMARTpool
22
23 CDC50A or CDC50B siRNA oligonucleotides and the uptake of [14 C]-perifosine and
24
25 NBD-PE and -PC determined as described in the legend to Figure 1. The figure shows
26
27 a representative experiment performed in triplicate \pm SD of four independent
28
29 experiments. Data were normalized to that measured in DharmaFECT 1 treated
30
31 (transfectant agent without siRNAs) KB Wt cells (perifosine uptake: 3450 ± 96 pmol/mg
32
33 protein; NBD-phospholipids uptake expressed in flow cytometry mean channel values
34
35 after subtracting the cell autofluorescence: NBD-PE: 597 ± 28 ; NBD-PC: 513 ± 49),
36
37 which was considered as 100%. **B)** Western blot showing the increasing reduction of
38
39 CDC50A-V5 expression in CDC50A-V5-transfected KB Wt cells at increasing
40
41 concentrations of the specific siRNA. A blot using anti- α -tubulin was performed in
42
43 parallel as control of protein loading. **C)** The same as in A) but targeting-only CDC50A-
44
45 V5 transfected cells with 1, 10 or 100 nM specific CDC50A siRNA oligonucleotides and
46
47 determination of [14 C]-perifosine and NBD-PE, -PS and -PC uptake. The figure shows a
48
49 representative experiment performed in triplicate \pm SD of five independent experiments.
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

The absolute uptake values measured in KB Wt cells with Dharmafect 1 in this set of experiments were: perifosine: 3657 ± 162 pmol/mg protein; NBD-phospholipids uptake expressed in flow cytometry mean channel values after subtracting the cell autofluorescence: NBD-PE: 609 ± 71 ; NBD-PS: 505 ± 63 ; NBD-PC: 521 ± 66 .

Figure 5. CDC50A subcellular localization by confocal microscopy analysis. KB Wt and KB PER-R clone 10 cells transfected with CDC50A-V5 were fixed and immunostained as described in section 2.9. Different protein markers specific for each subcellular compartment (green) were used to track the presence of CDC50A-V5 (red) in each of them (yellow pixels denote the co-localization of CDC50A-V5). The pictures are representative of at least three different microscopic fields per sample of fixed and immunostained cells. Three different cell samples from different days and different transfection batches were processed for each cell line and visualized under the microscope, in order to confirm that the intracellular distribution of CDC50A-V5 was not an artefact of the transfection procedure, cell batch or culture conditions.

Figure 6. Cell-surface expression of CDC50A. KB Wt and KB PER-R clone10 cells transfected with CDC50A-V5 were biotinylated as described in section 2.7. Samples were subjected to SDS-PAGE and immunoblotted with anti-V5 antibodies. Immunoblot of α -tubulin, a typical cytoplasmic protein, was used as control of plasma membrane integrity. The unbound fraction represents proteins that could not be biotinylated with the EZ-Link NHS-Biotin reagent, since the labeling was performed in whole cells with intact plasma membrane, and only surface proteins could be labeled (avidin-bound fraction).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure 7. *CDC50A* and *ATP8B1* mRNA levels in different cell lines. Semi-quantitative RT-PCRs showing the relative mRNA levels of *CDC50A*, *ATP8B1* and β -actin (control) in KB, HeLa and HEK-293T human cell lines were performed as described in section 2.10. Aliquots of the undiluted (1) and 10-fold diluted (1:10) cDNAs were used as template in the PCR reactions.

Figure 8. Uptake of NBD-phospholipids and perifosine in cells over-expressing both *CDC50A* and *Atp8b1*. **A)** Western blot showing the over-expression of *CDC50A-V5* and *Atp8b1-myc* in HeLa and HEK-293T cells. A blot using anti- α -tubulin was performed in parallel as control of protein loading. **B)** Effect of the co-expression of the two proteins on the accumulation of NBD-PC, -PE, -PS and ^{14}C -perifosine. Uptake experiments were performed as described in the legend to Fig. 1. The histograms represent the average \pm S.D. of two different experiments performed in triplicate. The absolute uptake values measured in HeLa cells were: perifosine: 726 ± 123 pmol/mg protein; NBD-PE: 313 ± 29 ; NBD-PS: 272 ± 17 ; NBD-PC: 313 ± 45 . In HEK293T cells were: perifosine: 877 ± 27 pmol/mg protein; NBD-PE: 446 ± 48 ; NBD-PS: 340 ± 53 ; NBD-PC: 403 ± 81 . NBD-phospholipids uptake expressed in flow cytometry mean channel values after subtracting the cell autofluorescence

Figure 9. Confocal microscopy analysis of the co-expression of *CDC50A* and *Atp8b1* in human cancer cells. HeLa cells were co-transfected with *CDC50A-V5* and *Atp8b1-myc*, fixed and immunostained with anti-V5 (red) and anti-myc (green) antibodies as described in section 2.9. Arrowheads indicate cells co-expressing both proteins, which also co-localize at the cell surface.

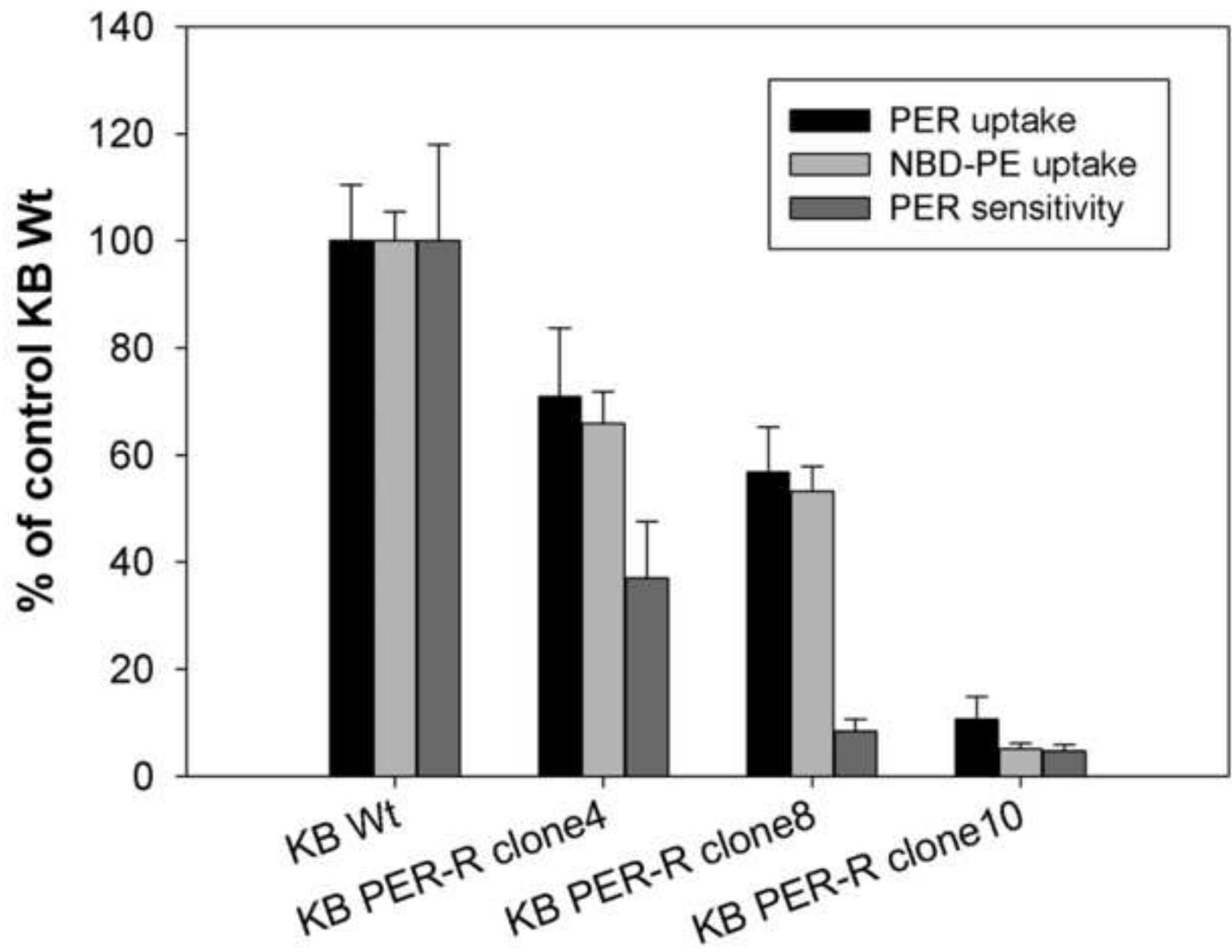
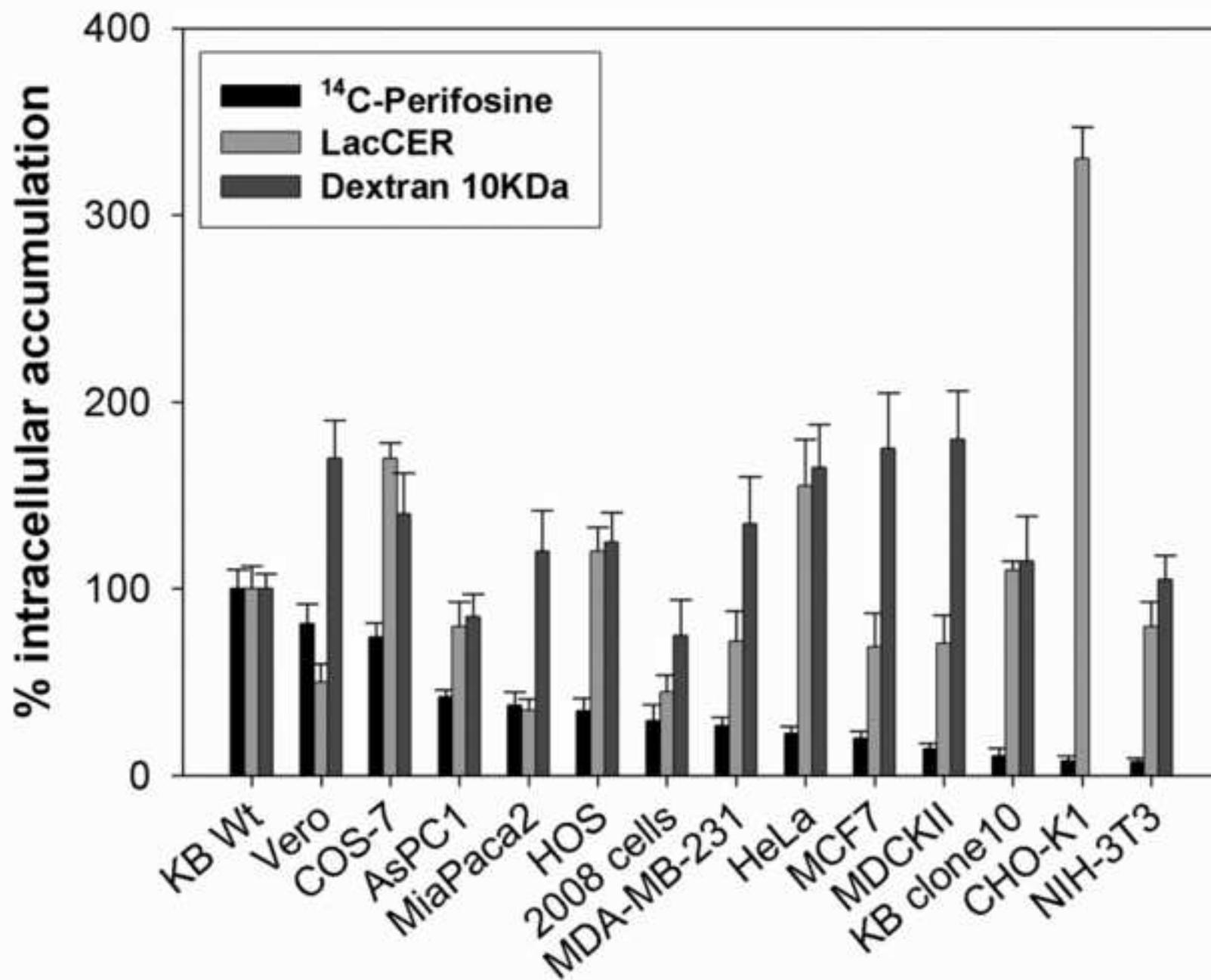
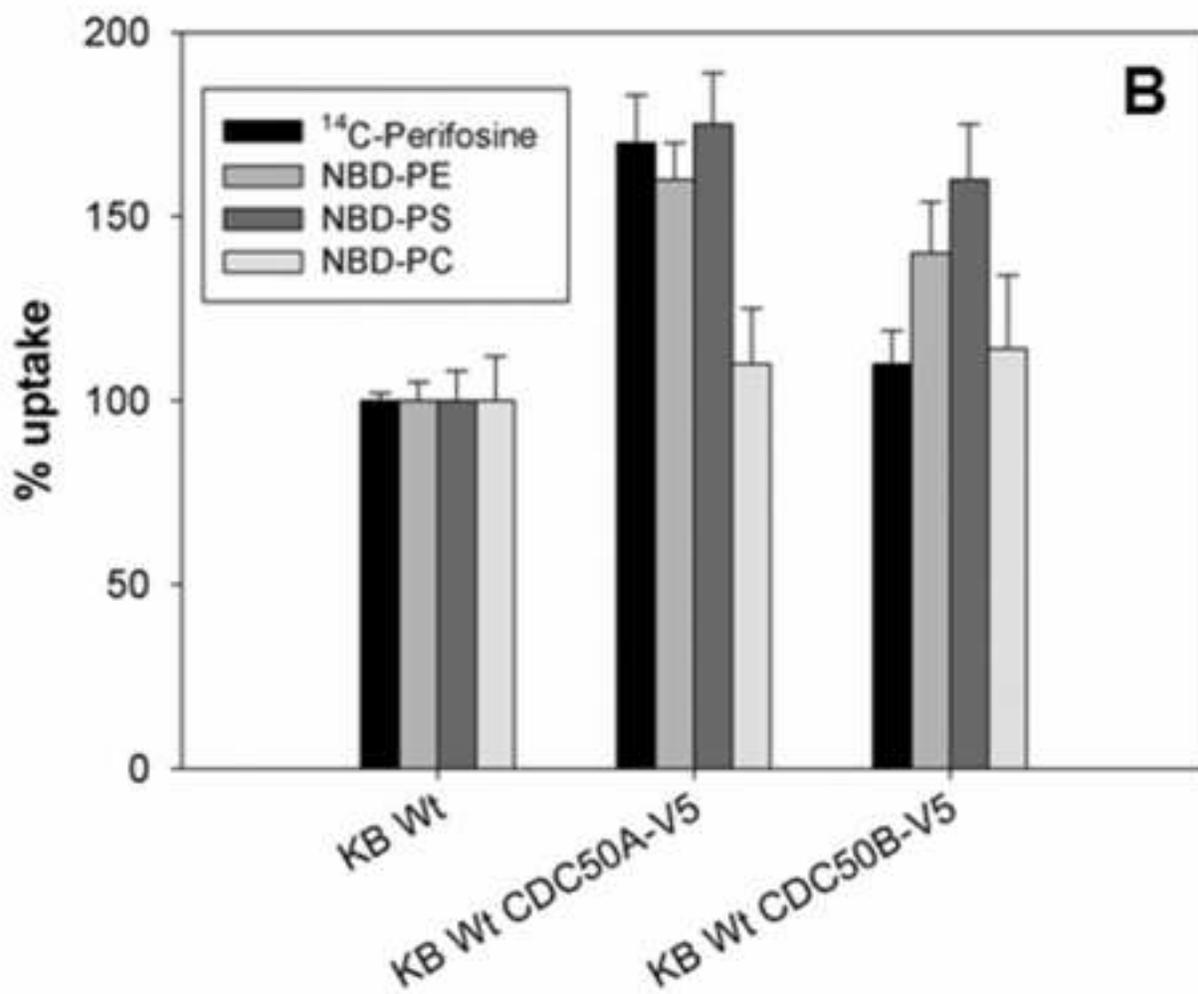
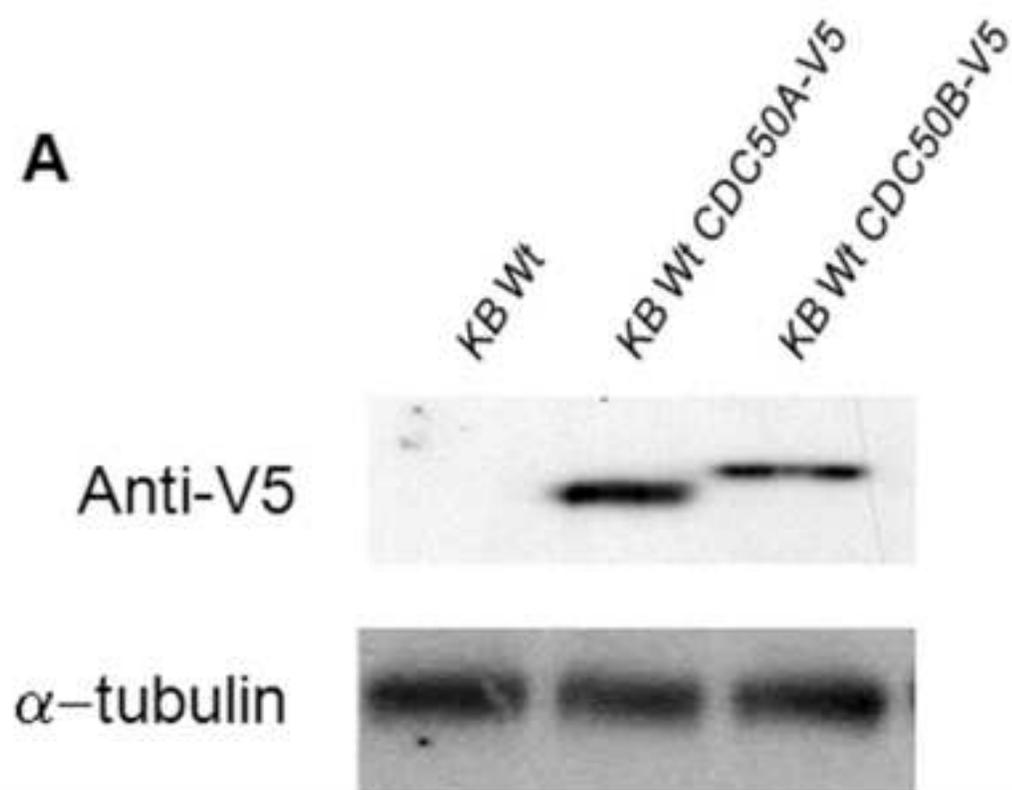


Figure 2



A

Manuscript

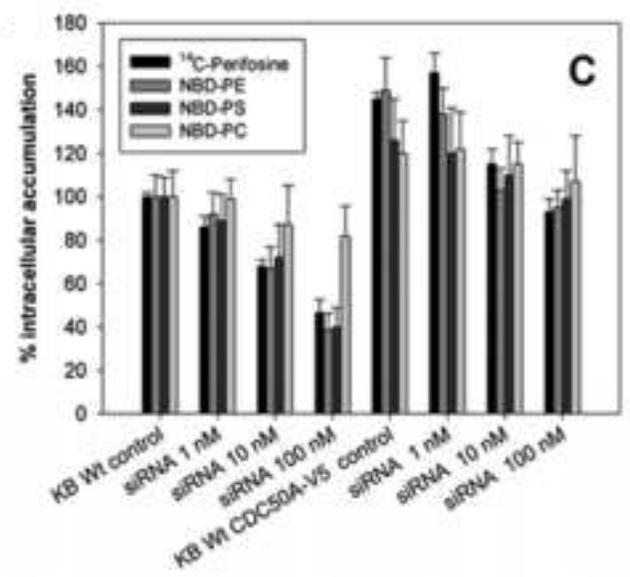
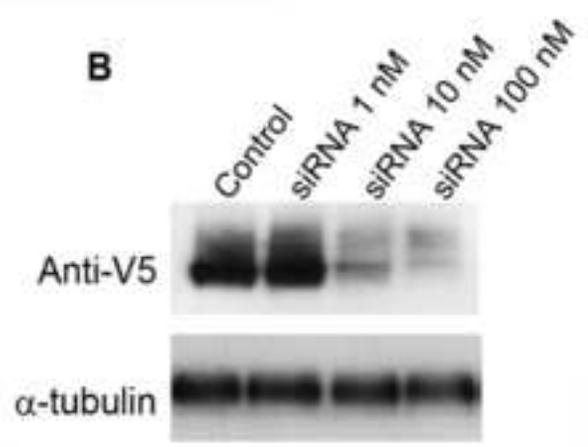
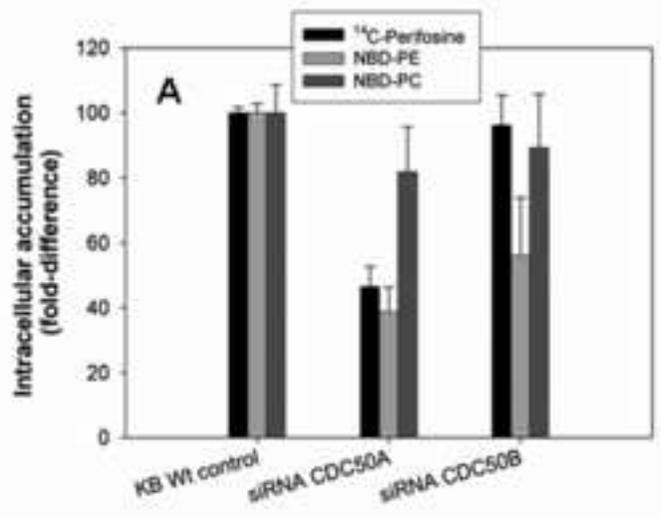


Figure 5

Manuscript

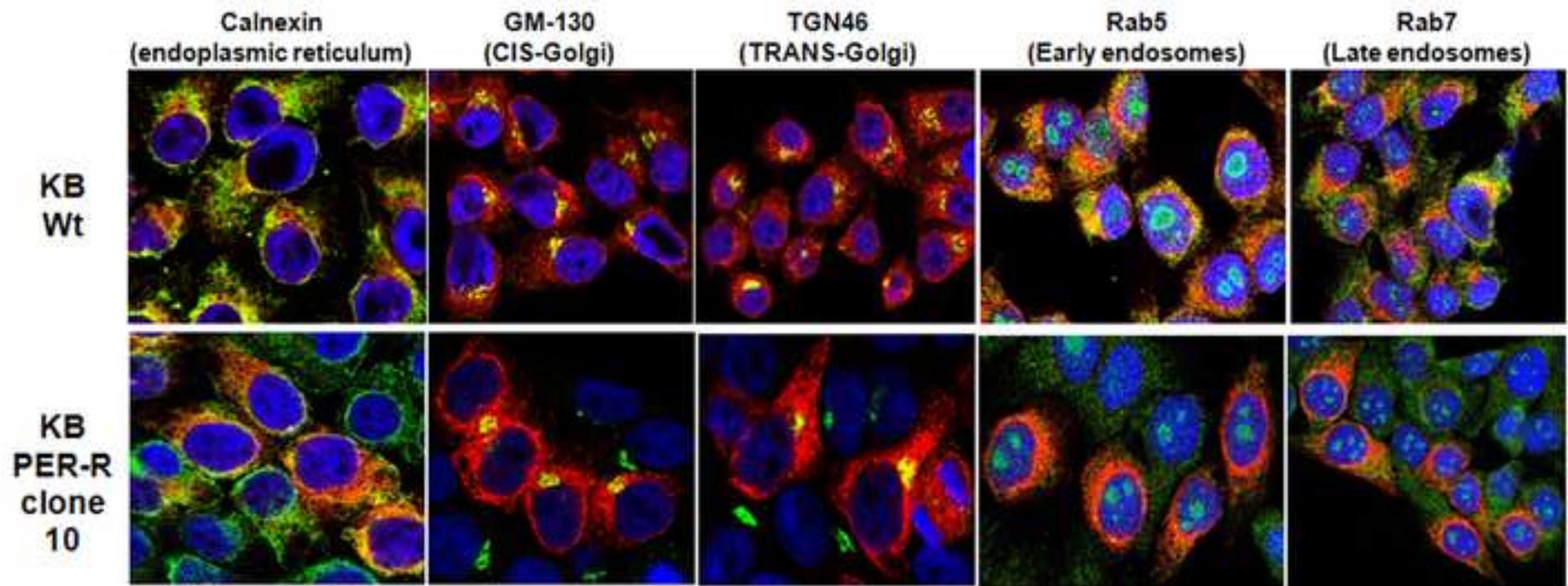
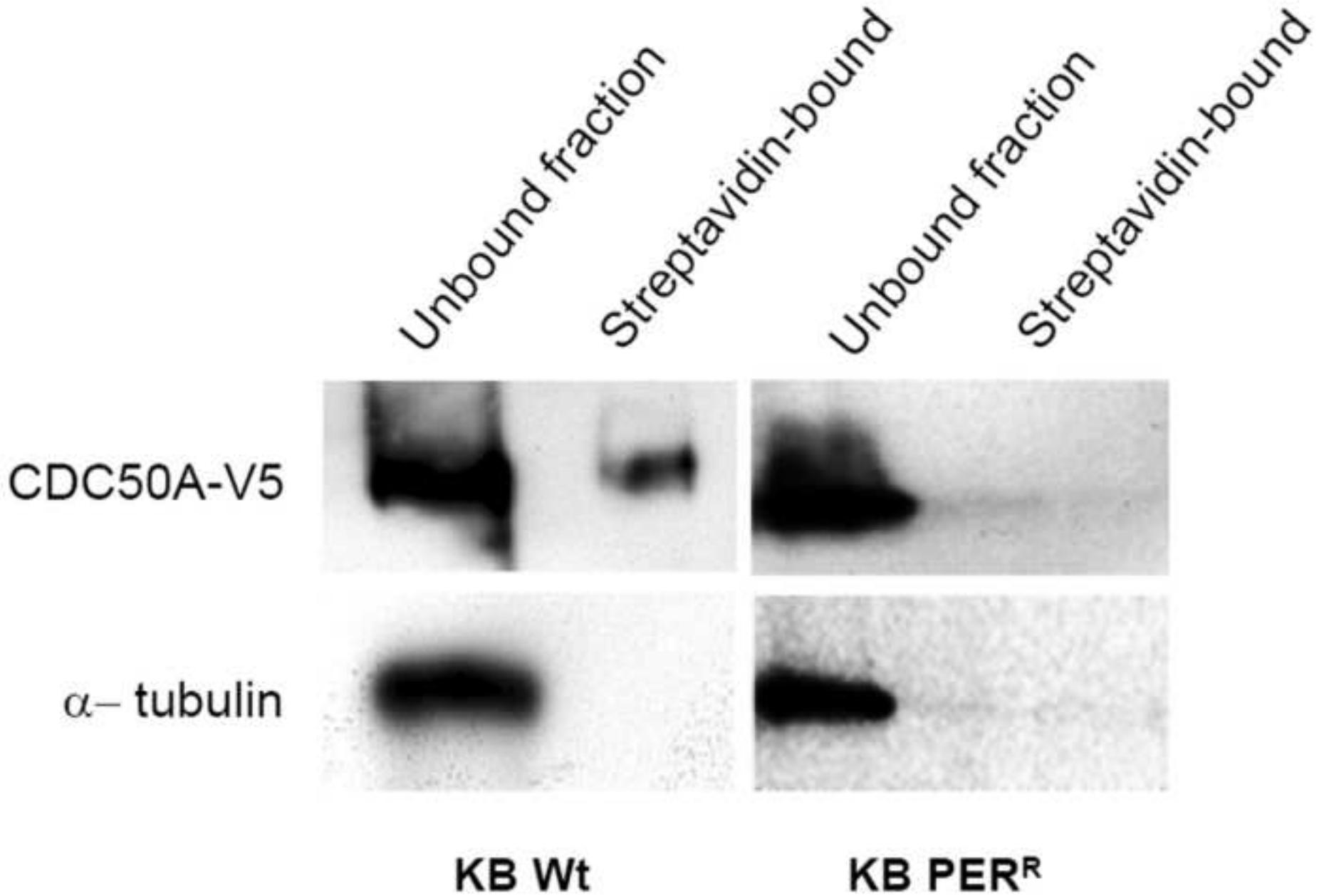
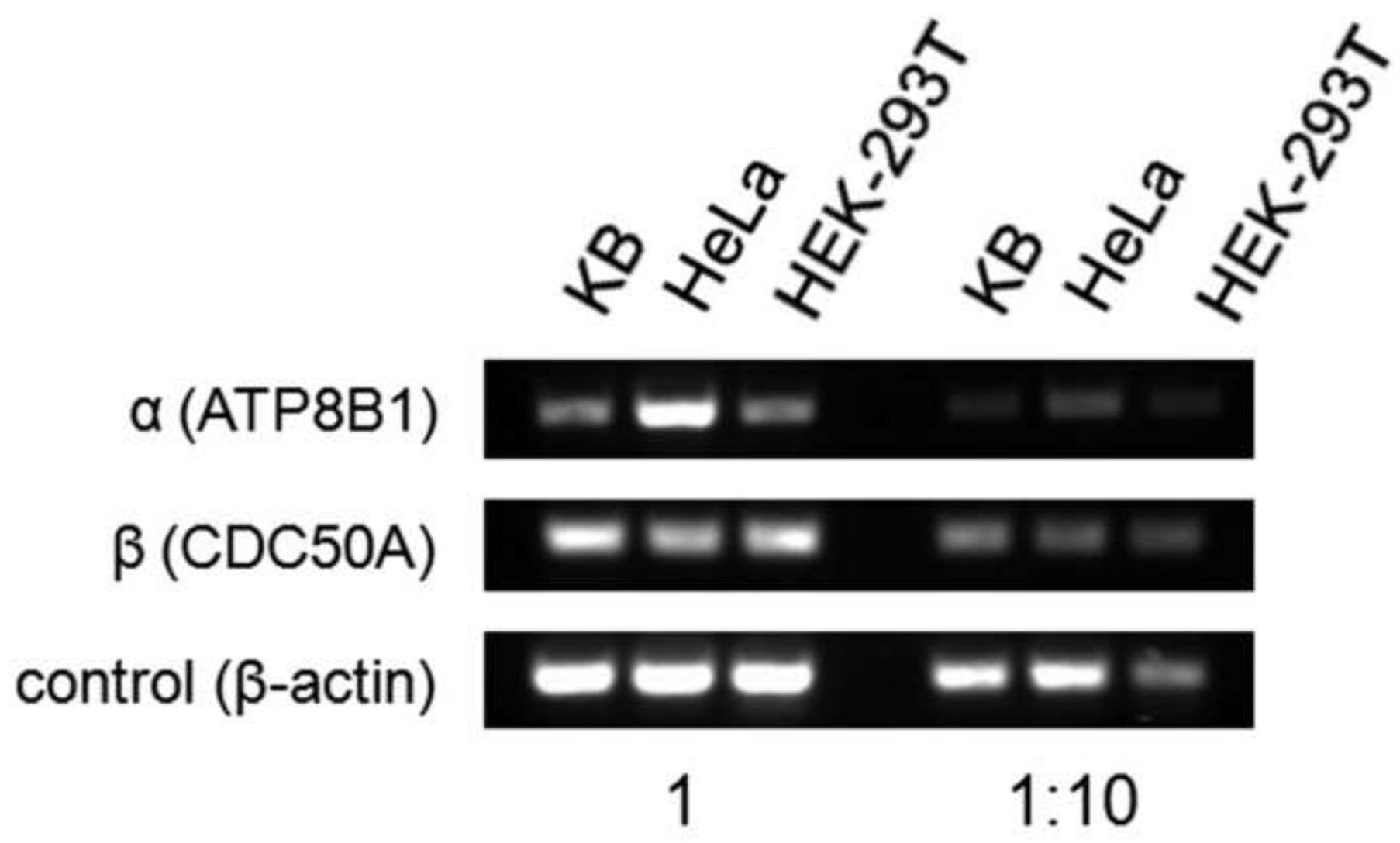


Figure 6

trip



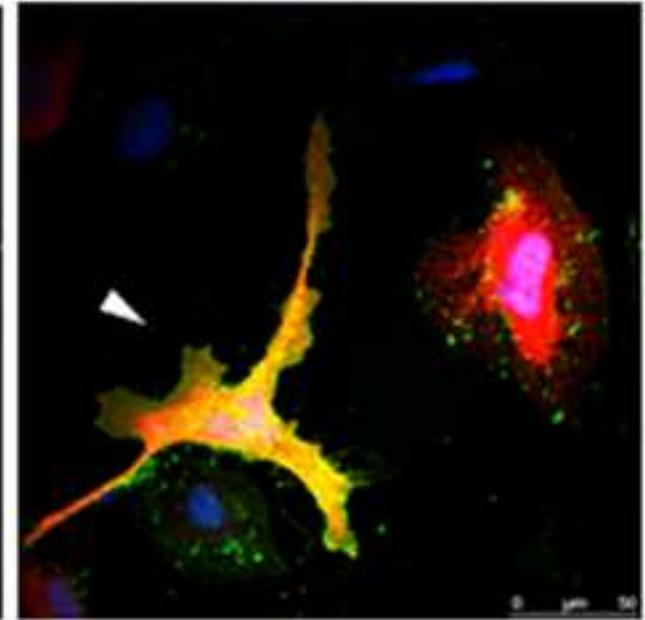
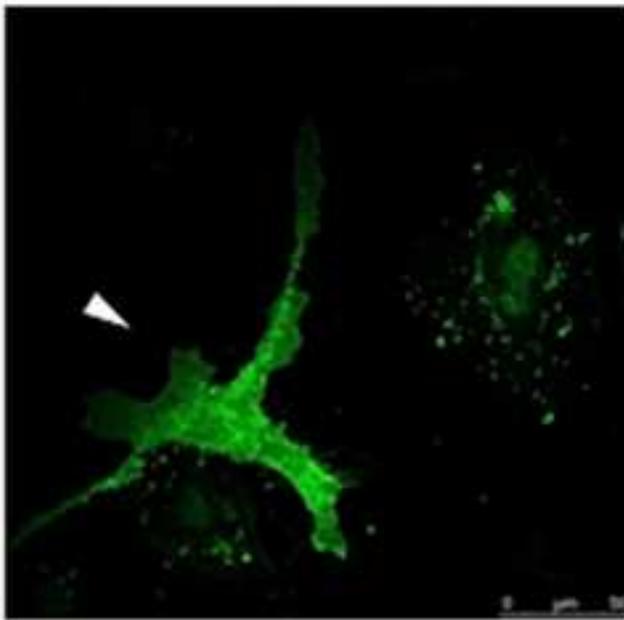
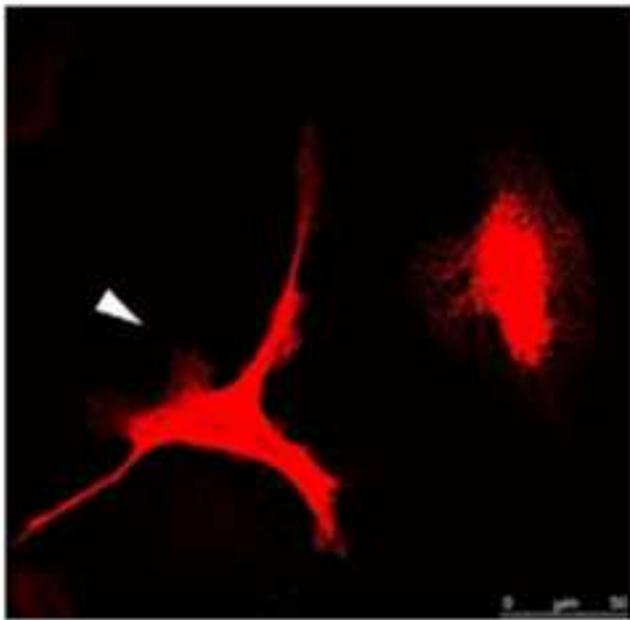
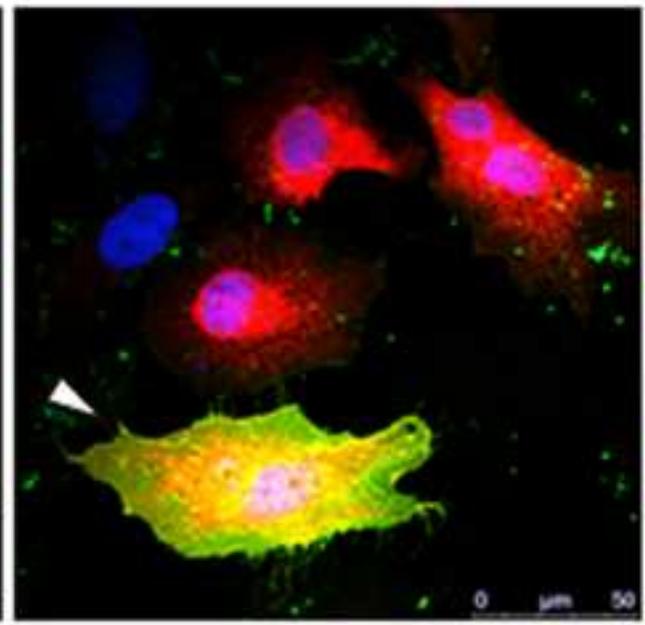
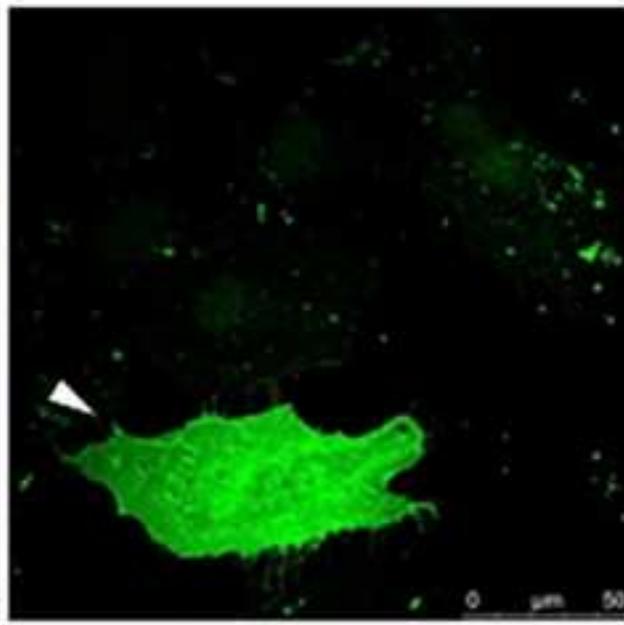
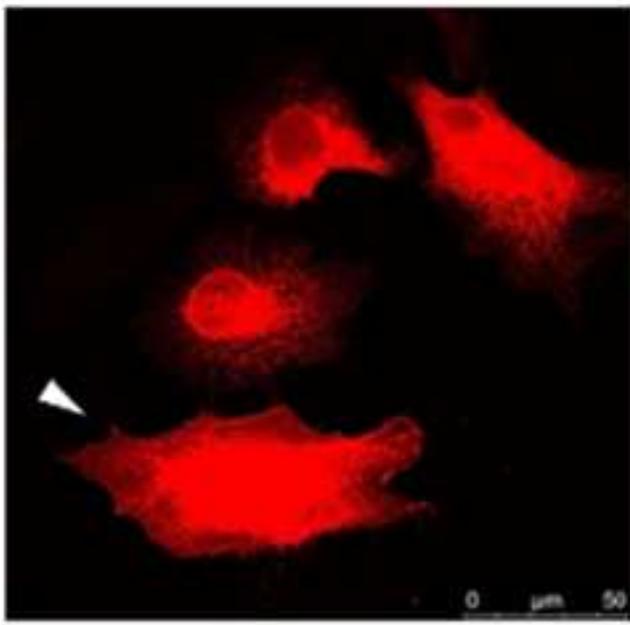
scrip



CDC50A-V5

ATP8B1-myc

MERGE



Manuscript

