Supplemental Materials

Molecular Biology of the Cell

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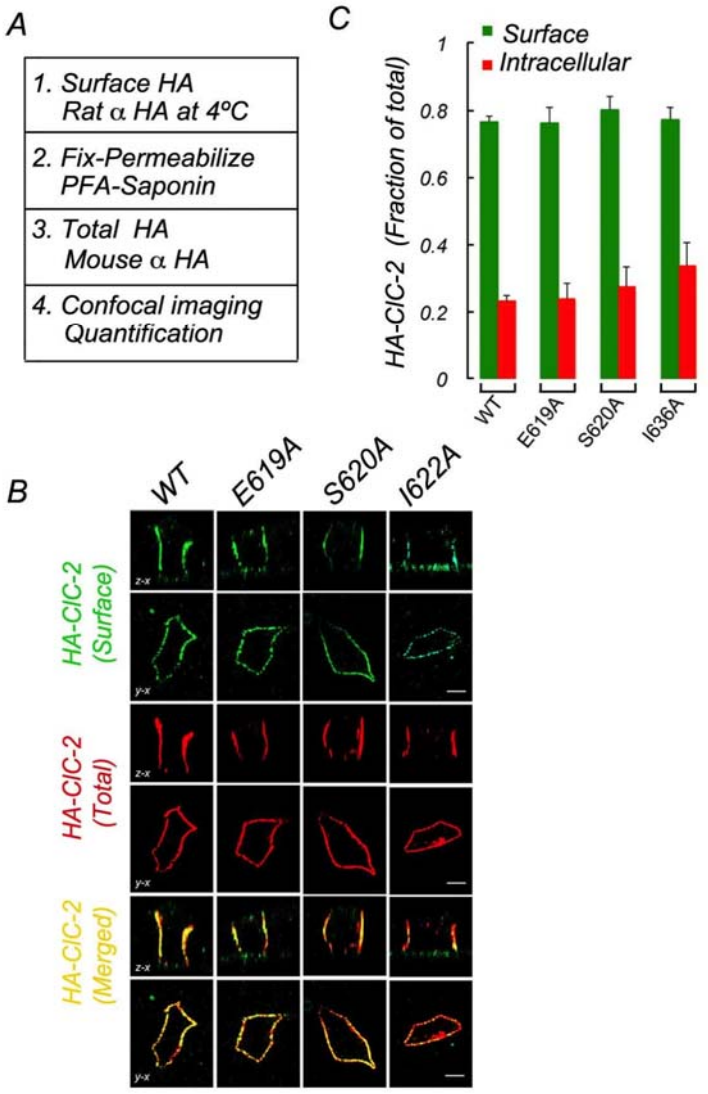
Supplemental Table S1: Surface distribution of HA-ClC-2 mutants in MDCK cells(statistical analysis of data corresponding to Figure 3).

		Surface			Intracellular		
HA-ClC-2	*N*	*Mean*	*STDEV*	*SEM*	*Mean*	*STDEV*	*SEM*
WT	*6*	*0.795*	*0.076*	*0.031*	*0.205*	*0.076*	*0.031*
L623A	*15*	*0.288*	*0.105*	*0.027*	*0.712*	*0.105*	*0.027*
L624A	*12*	*0.517*	*0.085*	*0.025*	*0.483*	*0.085*	*0.025*
L635A	*13*	*0.736*	*0.092*	*0.025*	*0.264*	*0.092*	*0.025*
L636A	*12*	*0.638*	*0.175*	*0.051*	*0.362*	*0.175*	*0.051*

	Bonferroni	
Pair	***Surface***	***Intracellular***
L623A vs WT	*1.84E-11*	*1.84E-11*
L624A vs WT	*1.16E-04*	*1.16E-04*
L624A vs L623A	*4.12E-05*	*4.12E-05*
L635A vs WT	*1*	*1*
L635A vs L623A	*3.22E-13*	*3.22E-13*
L635A vs L624A	*1.54E-04*	*1.54E-04*
L636A vs WT	*0.08462*	*0.08462*
L636A vs L623A	*1.91E-09*	*1.91E-09*
L636A vs L624A	*0.12682*	*0.12682*
L636A vs L635A	*0.38061*	*0.38061*
ANOVA	*1.13E-14*	*1.13E-14*

Data obtained from N regions of interest (ROI) from experiments as shown in figure 3 were analyzed with Origin9.1 statistical software using one-way ANOVA and further analyzed for multiple hypothesis testing using Bonferroni's correction. Numbers in red font represent statistically significant results.

Supplemental Figure S2: Sorting role of ESMI^{623}LL non-leucineresidues.



Mutants of HA-ClC-2 with single-residue replacement of non leucine residues in the ESMI^{623}LL motif were transiently expressed in MDCK cells and analyzed after 4 days in culture.

A) Scheme of sequential immunolabeling of HA-ClC-2 detected with mouse and rat antibodies to its luminal HA-epitope, before and after permeabilization, respectively (See "Materials and Methods" for details).

B) Surface (green) and total (red) immunolabeling of HA-ClC-2 in fully polarized cells. Note that alanine mutagenesis of ^{619}E, ^{620}S and ^{622}I did not affect the basolateral distribution of HA-ClC-2.

C)Bars represent surface and intracellular fractions of HA-ClC-2, calculated from confocal images (see Material and Methods for details). No statistical difference was observed in any of the residues mutated. Detailed statistical analysis is shown in supplemental Table S3.

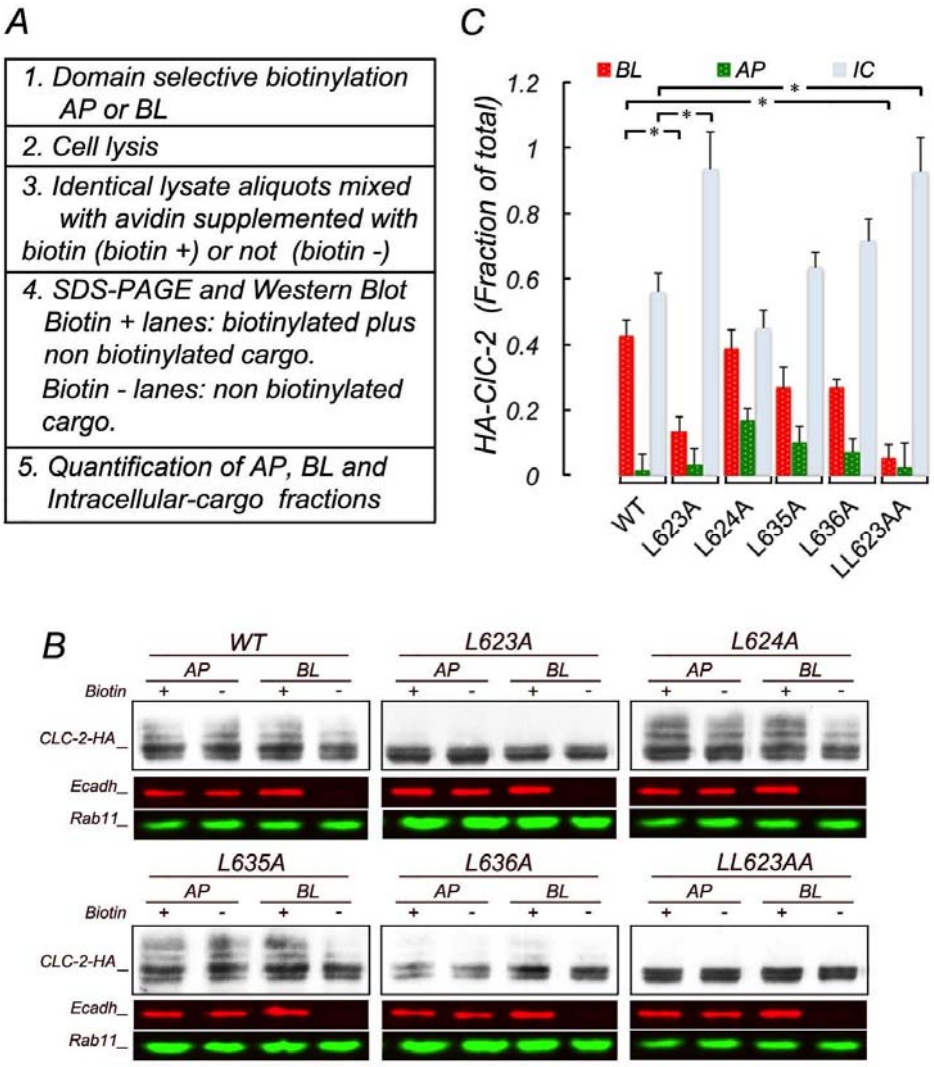
Supplemental Table S3: Surface and intracellular distributionof WT and mutant HA-ClC-2 with alanine substitutions in ESMILL (statistical analysis of data in Supplemantal Figure S2).

		Surface		*Intracellular*	
HA-ClC-2	*N*	*Mean*	*SEM*	*Mean*	*SEM*
WT	6	*0.76561*	*0.0176*	*0.23439*	*0.0176*
E619A	9	*0.76105*	*0.04615*	*0.23895*	*0.04615*
S620A	8	*0.80269*	*0.03823*	*0.27438*	*0.06159*
I622A	*8*	*0.77276*	*0.03707*	*0.33743*	*0.0684*

	Bonferroni Test	
Pair	*Surface*	*Intracellular*
E619A vs WT	*1*	*1*
S620A vs WT	*1*	*1*
S620A vs E619A	*1*	*1*
I622A vs WT	*1*	*1*
I622A vs E619A	*1*	*1*
I622A vs S620A	*1*	*1*
ANOVA	*0.23624*	*0.743*

Data obtained from N regions of interest (ROI) from experiments as shown in supplementary Figure S2 were analyzed with Origin9.1 statistical software using one-way ANOVA and further analyzed for multiple hypothesis testing using Bonferroni's correction. No statistically significant results were observed.

Supplemental Figure S4: Trafficking roles of leucine residues in ESMI^{623}LL and QVVA^{635}LL using a biochemical assay.



A panel of lentiviruses encoding full length ClC-2 with single leucine to alanine substitutions in ESMI^{623}LL and QVVA^{635}LL was used to study the basolateral sorting signal role of these dileucine motifs.

A) Protocol used to quantify steady state apical (AP), basolateral (BL) and intracellular (IC) fractions of various mutants of HA-ClC-2 in polarized MDCK cells using a SBAS assay.Briefly, MDCK cells expressing WT or mutant HA-ClC-2, transduced via lentiviruses, are biotinylated from the apical or basolateral sides and lysed. The lysates are incubated with avidin in the absence (biotin –) or presence (biotin +) of biotin. The apical and basolateral fractions of WT and mutant HA-ClC-2 are calculated from the difference in band intensities between biotin + and biotin - samples, according to the equation [(biotin +) – (biotin –)] / (biotin +).

B) Western blot of WT and mutant HA-ClC-2 processed as described in A. E-cadherin is shown as a positive control: its reduction in the BL biotin - lanes reflects its normal basolateral surface localization. Rab-11, a cytoplasmic protein, is shown as a negative control; as expected, no differences between biotin(+) and biotin(–) lanes were observed, reflecting its intracellular distribution.

C) Bars represent AP, BL and IC fractions of WT and mutant HA-ClC-2, calculated fom western blot data derived from seven independent experiments (see statistical analysis in supplemental Table S5). Consistently with the quantitative immunolabeling assay shown in Figure 3, the data indicate that WT HA-ClC-2 [WT] is expressed predominantly on the basolateral PM with similar sizes of surface and intracellular pools. In contrast, HA-ClC-2[^{623}L/A] and the double mutant HA-ClC-2[^{623}LL/AA] show a dramatic reduction in the surface pool and increase in the intracellular pool. The differences observed with the other mutants were not statistically significant. These biochemical experiments, together with the quantitative immunolocalization experiments in Fig. 3 support the conclusion that ESMI^{623}LL is the authentic basolateral sorting signal of ClC-2.

Statistical analysis is shown in supplemental Table S5

Supplemental Table S5: Surface distribution of HA-ClC-2 mutants in MDCK cells (statistical analysis of data corresponding to Supplemental Figure S4).

		APICAL		BASOLATERAL		INTRACELLULAR	
HA-ClC-2	N	Mean	SEM	Mean	SEM	Mean	SEM
WT	7	0.015	0.046	0.425	0.045	0.561	0.054
L623A	7	0.033	0.046	0.134	0.040	0.935	0.103
L624A	7	0.165	0.036	0.386	0.054	0.450	0.051
L635A	7	0.099	0.046	0.268	0.060	0.633	0.046
L635A	7	0.099	0.046	0.270	0.038	0.714	0.063
LL623AA	7	0.022	0.070	0.051	0.041	0.927	0.094

		Bonferroni		
Pair		AP	BL	IC
L623A	WT	1	0.0015	0.01114
L624A	WT	0.56223	1	1
L624A	L623A	0.98772	0.00835	4.41E-04
L635A	WT	1	0.35715	1
L635A	L623A	1	0.77275	0.07826
L635A	L624A	1	1	1
L636A	WT	1	0.38183	1
L636A	L623A	1	0.72647	0.53864
L636A	L624A	1	1	0.20163
L636A	L635A	1	1	1
LL623AA	WT	1	3.24E-05	0.014
LL623AA	L623A	1	1	1
LL623AA	L624A	0.70997	1.96E-04	5.64E-04
LL623AA	L635A	1	0.03543	0.09648
LL623AA	L636A	1	0.03276	0.64365
ANOVA		2.36E-01	9.33E-06	6.55E-05

Data for statistical analysis were obtained from two independent experiments (one triplicate, one quadruplicate, N=7) and analyzed with Origin9.1 statistical software using one-way ANOVA; the data were further analyzed for multiple hypothesis testing using Bonferroni's correction. Numbers in red font represent statistically significant results

Supplemental Table S6: Intracellular distribution of HA-ClC-2 mutants in MDCK cells (statistical analysis).

		RE			TGN		
HA-ClC-2	**N**	**Mean**	**STDEV**	**SEM**	**Mean**	**STDEV**	**SEM**
WT	*15*	*0.3127*	*0.1281*	*0.0331*	*0.0528*	*0.0369*	*0.0095*
L623A	*22*	*0.3152*	*0.0909*	*0.0194*	*0.1334*	*0.0829*	*0.0177*
L624A	*13*	*0.3638*	*0.1198*	*0.0332*	*0.0472*	*0.0264*	*0.0073*
L635A	*6*	*0.2795*	*0.1058*	*0.0432*	*0.0538*	*0.0438*	*0.0179*
L636A	*3*	*0.2510*	*0.0031*	*0.0018*	*0.0345*	*0.0199*	*0.0115*

Bonferroni		
Pair	**RE**	**TGN**
L623A vs WT	*1*	*0.00121*
L624A vs WT	*1*	*1*
L635A vs WT	*1*	*1*
L636A vs WT	*1*	*1*
L624A vs L623A	*1*	*8.61E-04*
L635A vs L623A	*1*	*0.04349*
L636A vs L623A	*1*	*0.07743*
L635A vs L624A	*1*	*1*
L636A vs L624A	*1*	*1*
L636A vs L635A	*1*	*1*
ANOVA	*0.3793*	*9.19E-05*

Wild type and mutants of HA-CLC-2 made by alanine substitution of single leucine in ESMI^{623}LLor QVVA^{635}LL motifs, were transiently expressed in MDCK cells.

Steady state distribution of HA-ClC-2 within TGN or recycling compartment (RE) was determined by confocal immunofluorecence after permeabilization with a rat anti-HA in combination with either rabbit or mouse antibodies to TGN38 or TfR, respectively. The extent of HA-ClC-2 co-localization values, expressed as the fraction of total HA-ClC-2 co-localizing with TGN38 or TfR were analyzed by one-way ANOVA to obtained mean, standard deviation, and p values obtained were subjected to multiple hypothesis testing using Bonferroni's (see statistical analyses in Material and Methods). A statistically significant increase in the TGN pool was found for HA-ClC-2[^{623}L/A] but not for other mutants. The recycling endosome (RE) pools of HA-ClC-2 [WT] and other mutants of ClC2-HA were not significantly changed.

Supplemental Table S7.Surface distribution of HA-ClC-2 in MDCK cells knocked-down for clathrin adaptors (statistical analysis of data corresponding to figure 4).

		Surface			***Intracellular***		
HA-ClC-2	*N*	*Mean*	*STDEV*	*SEM*	*Mean*	*STDEV*	*SEM*
WT	8	*0.588*	*0.115*	*0.041*	*0.412*	*0.115*	*0.041*
A-KD	8	*0.530*	*0.074*	*0.026*	*0.470*	*0.074*	*0.026*
B-KD	8	*0.501*	*0.135*	*0.048*	*0.499*	*0.135*	*0.048*
AB-KD	11	*0.287*	*0.112*	*0.034*	*0.713*	*0.112*	*0.034*

Bonferroni

Pair	*Surface*	*Intracellular*
A-KD WT	*1*	*1*
B-KD WT	*0.782329*	*0.782329*
B-KD A-KD	*1*	*1*
AB-KD WT	*1.24E-05*	*1.24E-05*
AB-KD A-KD	*3.02E-04*	*3.02E-04*
AB-KD B-KD	*1.46E-03*	*1.46E-03*
ANOVA	*6.85E-06*	*6.85E-06*

Data obtained from N regions of interest (ROI) from experiments as shown in Figure 4 were analyzed with Origin9.1 statistical software using one-way ANOVA and further analyzed for multiple hypothesis testing using Bonferroni's correction. Statistically significant results are shown in red font.

Supplemental Table 8: Surface distribution of p75-ClC-2ct in clathrin adaptor knock down MDCK cells(statistical analysis of data corresponding to figure 5).

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		Apical			***Basolateral***		
p75-ClC-2ct	*N*	*Mean*	*STDEV*	*SEM*	*Mean*	*STDEV*	*SEM*
WT	*12*	*0.029*	*0.021*	*0.006*	*0.436*	*0.089*	*0.026*
A-KD	*8*	*0.023*	*0.015*	*0.005*	*0.528*	*0.078*	*0.028*
B-KD	*12*	*0.044*	*0.033*	*0.010*	*0.486*	*0.058*	*0.017*
AB-KD	*12*	*0.162*	*0.080*	*0.023*	*0.339*	*0.088*	*0.025*

	Bonferroni	
Pair	*Apical*	*Basolateral*
A-KD vs WT	*1*	*0.08936*
B-KD vs WT	*1*	*0.79695*
AB-KD vs WT	*1.37E-07*	*2.81E-02*
A-KD vs B-KD	*1*	*1*
A-KD vs AB-KD	*5.92E-07*	*3.46E-05*
B-KD vs AB-KD	*1.67E-06*	*3.13E-04*
ANOVA	*1.20E-08*	*1.93E-05*

\Data obtained from N regions of interest (ROI) from experiments as shown in Figure 5 were analyzed with Origin9.1 statistical software using one-way ANOVA and further analyzed for multiple hypothesis testing using Bonferroni's correction. Statistically s