Inventory of supplemental information

Figure S1, related to Figure 1, shows clathrin light chain a is less change in NSCLC.
Figure S2, Related to Figure 2, shows differential rates of CME, CCP initiation and maturation in single CLCa vs CLCb expressing A549 cells.
Figure S3, related to Figure3, shows depletion of CLCb impairs EGFR trafficking and Western blotting of Dyn1 and Dyn2 in single CLCs and NSCLC cells.
Figure S4, related to Figures 4, shows CLCb up-regulation increased Akt/GSK3β phosphorylation dependent on Dyn1.
Figure S5, related to Figure 5, shows APPL1 is required for enhanced Akt/GSK3β phosphorylation in swCLCb H1299 cells.
Figure S6, related to Figure 6, shows switch of CLC isoform expression levels alters cancer cell behavior.
Figure S7, related to Figure 7, shows the effect of CLCa, Dyn2, and Dyn3 expression levels on NSCLC patient survival.

Supplemental Figures and Legends

Figure S1. Related to Figure 1.

Figure S1. Clathrin light chain a is less change in NSCLC.

(A) Protein levels of CLCa in NCI 60 cell lines (http://129.187.44.58:7070/NCI60/protein/show/3942). (B) Images of low and high immunohistochemical (IHC) of CLCa levels in representative tumor tissues. Scale bar, 50μm. Quantification of CLCa expression according to low and high IHC levels in normal (N, n=15), stage I tumor (I, n=23), stage II tumor (II, n=14), stage III tumor (III, n=13) and metastatic tumor (M, n=10). (C) Relative gene transcript intensity (Z score) of CLCa and CLCb in NCI 60 (http://genome-www.stanford.edu/nci60/) NSCLC cell lines.

Figure S2. Related to Figure 2.

Figure S2. Differential rates of CME, CCP initiation and maturation in single CLCa vs CLCb expressing A549 cells.

(A) Representative western blot of CLCs and CHC in parental A549 cells and single CLC A549 cells engineered to overexpressing saturating levels of either CLCa (sCLCa) or CLCb (sCLCb). (B) Endocytosis and recycling of TfnR were measured in parental, sCLCa, and sCLCb A549 cells. Percentage of internalized TfnR was calculated relative to the initial surface TfnR. Percentage of recycled biotinylated-Tfn was calculated relative to the initial loading (10 min). (D) Endocytosis and recycling of biotinylated-EGF (20 ng/ml) were measured in parental, sCLCa, and sCLCb A549 cells. Percentage of internalized biotinylated-EGF was calculated relative to the initial surface bound. Percentage of recycled biotinylated-EGF was calculated relative to the initial loading (10 min). Data in (B) and (C) were presented as mean±SEM, n=3. The statistical significance was analyzed by Student's t-test. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$. (D), (E) Representative western blot of CLCs and CHC in GFP-sCLCa and GFP-sCLCb H1299 and A549 cells. GFP-sCLCa H1299 and A549 cells were generated by retroviral over-expression of GFP-CLCa in single CLCa cells. GFP- sCLCb H1299 and A549 cells were generated by retroviral over-expression of GFP-CLCb in single CLCb cells. (F) Average CCP lifetime distribution in GFP-sCLCa and GFP-sCLCb A549 cells. (G) Initiation density of bona fide CCP in GFP-sCLCa and GFP-sCLCb A549 cells. CCP initiation rate (GFP-sCLCb A549: 0.122±0.007 CCP/μm^2/min; GFP-sCLCa A549: 0.075±0.003 CCP/μm^2/min).

Figure S3. Related to Figure3.

Depletion of CLCb impairs EGFR trafficking and Western blotting of Dyn1 and Dyn2 in single CLCs and NSCLC cells.

(A) Dyn1 expression is repressed in sCLCa cells and up-regulated in sCLCb cells relative to parental H1299 or A549 cells. (B) Western blot of phosphorylated Dyn1 (pSer 774), Dyn1, Dyn2, phosphorylated Akt (pSer 473) and Akt in H1299, A549, H226, H522, and EKVX cells. Bottom panel, commassie blue stained gel of 1 μg of recombinant Dyn1 and Dyn2 with 0.5 and 1 μg of BSA. reDyn, recombinant dynamin protein. According to the blots, we estimated that the ratio of Dyn1:Dyn2 levels is ~1:6 in H1299 cells, 5:1 in A549 cells, 1.5:1 in H226 cells, 1:3 in H522 cells, and 2:1 in EKVX cells. The amount of phosphorylated (i.e. inactive) Dyn1 is highest in the two cancer cell lines that express the highest levels of Dyn1 (i.e. A549 and EKVX cells).

Figure S4. Related to Figures 4.

CLCb up-regulation increased Akt/GSK3β phosphorylation dependent on Dyn1.

(A) Representative western blot of CLCs, Dyn1, and Dyn2 in Dyn1 KO and Dyn1 KO/swCLCb H1299 cells. (B) Quantification result of Akt (Ser473), ERK, GSK3β (Ser9) and TSC2 phosphorylation in EGF (20ng/ml) treated parental, swCLCb, Dyn1 KO and Dyn1 KO/swCLCb H1299 cells. Akt and GSK3β results for H1299 and swCLCb cells are also displayed in Fig 4A. (C) Representative western blots of phosphorylated- and total Akt, ERK, GSK3β and TSC2 in EGF (20ng/ml) treated parental, swCLCb, Dyn1 KO and Dyn1 KO/swCLCb H1299 cells. (D) Surface levels of EGFR in parental, swCLCb, Dyn1 KO and Dyn1 KO/swCLCb H1299 cells. Data in (B) and (D) were presented as mean±SEM, n=3. The statistical significance was analyzed by Student's t-test. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$.

Figure S5. Related to Figure 5.

APPL1 is required for enhanced Akt/GSK3β phosphorylation in swCLCb H1299 cells.

(A) Representative western blot of APPL1 in parental, swCLCb, Dyn1 KO, and Dyn1 KO/swCLCb H1299 cells. (B) Fluorescence intensity quantification of APPL1-positive endosomes in parental, swCLCb, Dyn1 KO, and Dyn1 KO/swCLCb H1299 cells. (C) Representative western blot of APPL1 knockdown efficiency in parental and

swCLCb H1299 cells. (D) Representative western blots of total and phosphorylated Akt (Ser473) and GSK3β (Ser9) in control and APPL1 siRNA treated parental and swCLCb H1299 cells. Representative immunofluorescence staining images and quantification of APPL1-positive endosomes in control and CLCb siRNA-treated H226 cells (E) and EKVX cells (F). Scale bar, 10 μm. Values in (B), (E) and (F) are presented as mean±SEM, n=20 cells. The statistical significance was analyzed by Student's t-test. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$. ns, non-significant.

Figure S6. Related to Figure 6.
Switch of CLC isoform expression levels alters cancer cell behavior.
(A) Bright field microscope images of parental, sCLCa and sCLCb H1299 cells. Scale bar, 100 μm. (B) 2D trajectory plots showing directionality in EGF (20 ng/ml) treated parental, swCLCb, Dyn1 KO, and Dyn1 KO/swCLCb H1299 cells. Data was presented as mean±SEM, n=60. (C) Representative left lungs, which were injected 8 weeks earlier with parental or swCLCb H1299 cells were embedded in paraffin, sectioned into 4 μm layers and then stained with hematoxylin and eosin (H&E) for histological analysis. Scale bar, 100 μm. Yellow arrow, tumor site.

Figure S7. Related to Figure 7.
The effect of CLCa, Dyn2, and Dyn3 expression levels on NSCLC patient survival.
Kaplan-Meier survival analysis of NSCLC patients was performed in CLCa, Dyn2, and Dyn3 high and low expression cohorts (A) and pre-stratifying NSCLC patients according to tumor stages was performed in CLCb (B) and Dyn1 (C) high and low expression cohorts.

Figure S1

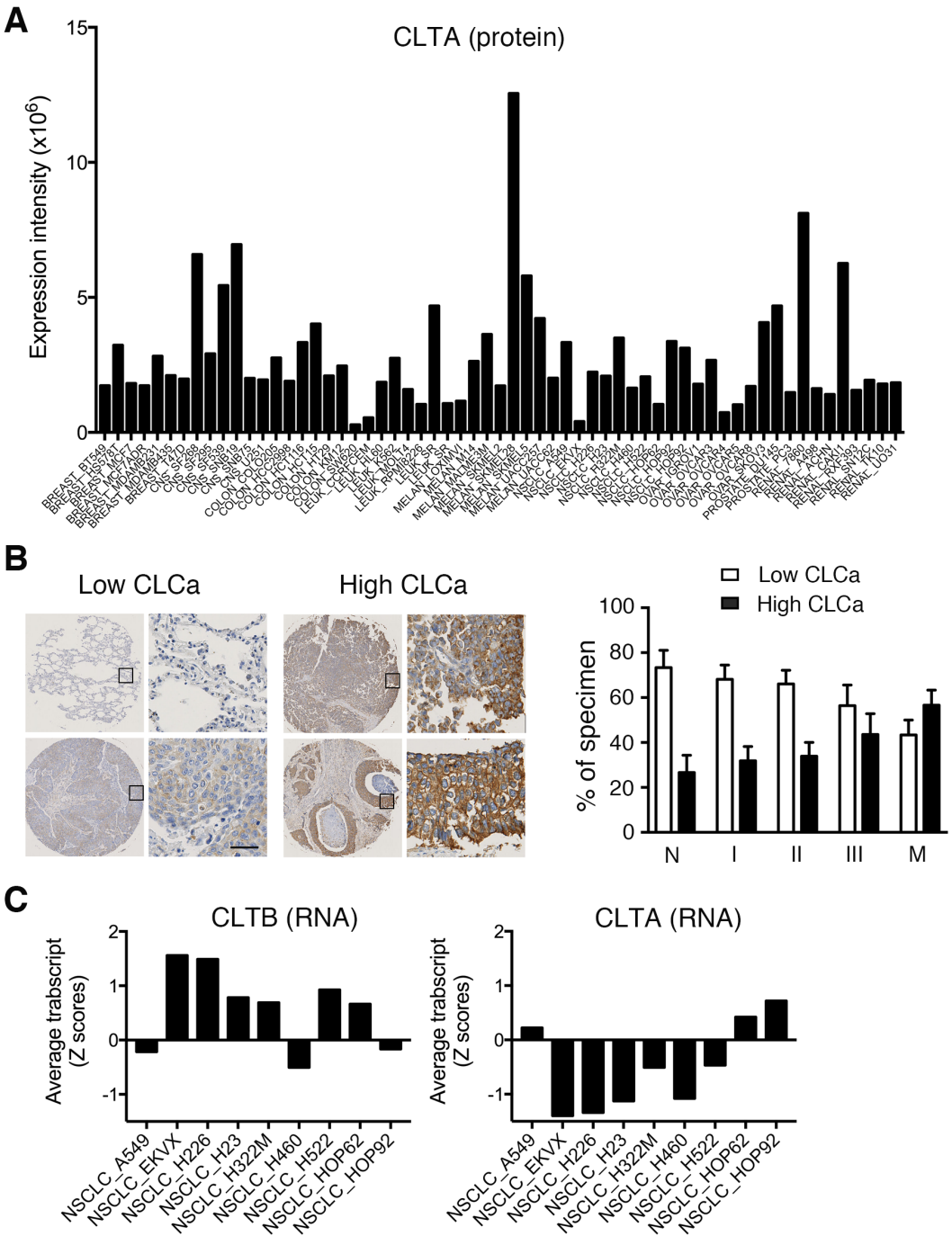

A
CLTA (protein)
Expression intensity (x10^6)
15
10
5
0
B
Low CLCa
High CLCa
Low CLCa
High CLCa
% of specimen
100
80
60
40
20
0
N
I
II
III
M
C
CLTB (RNA)
CLTA (RNA)
Average trabscript (Z scores)
2
1
0
-1
NSCLC_A549
NSCLC_EKVX
NSCLC_H226
NSCLC_H23
NSCLC_H322M
NSCLC_H460
NSCLC_H522
NSCLC_HOP62
NSCLC_HOP92

Figure S2.

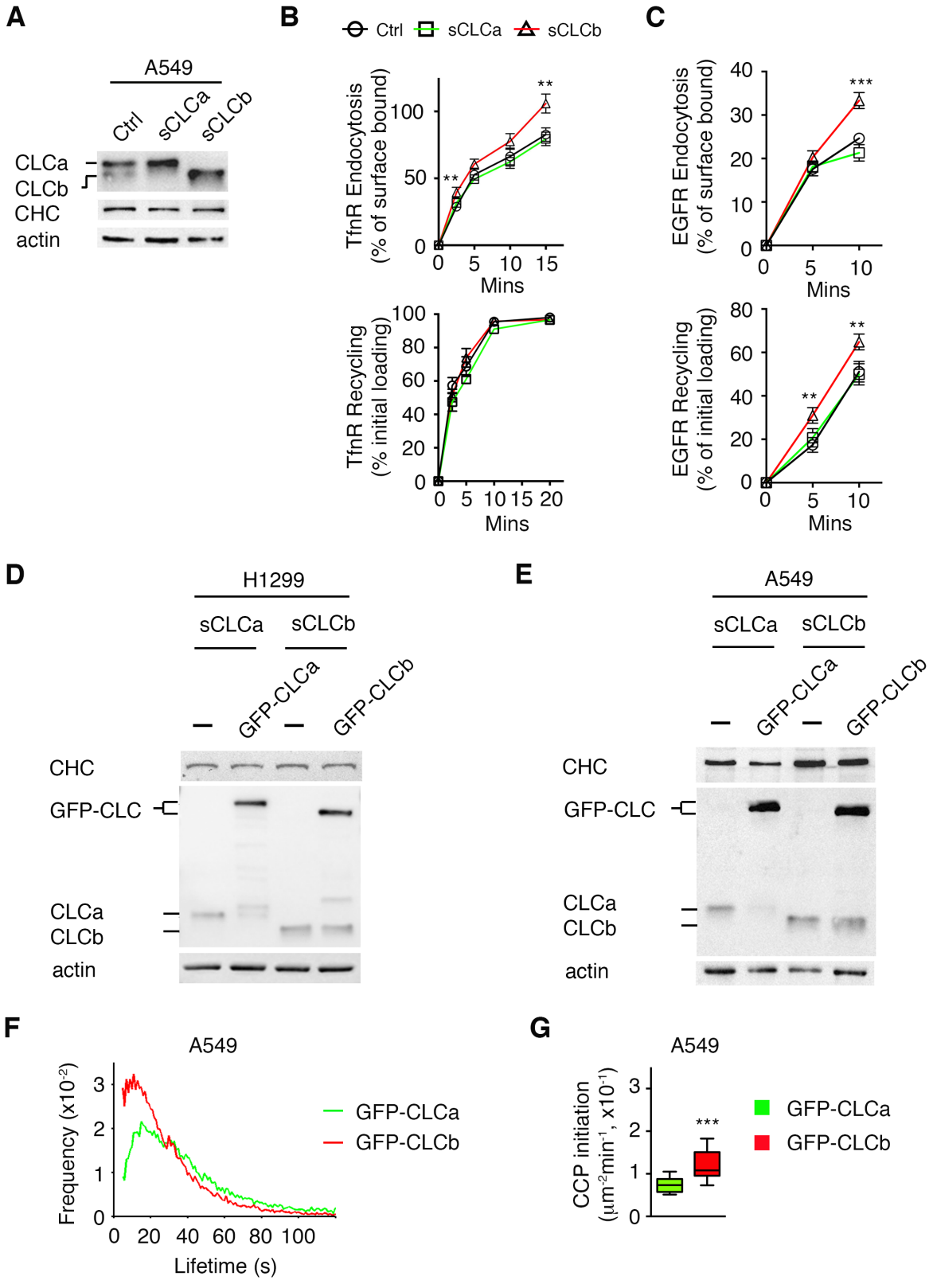

A
A549
Ctrl
sCLCa
sCLCb
CLCa
CLCb
CHC
actin
B
Ctrl
sCLCa
sCLCb
TfnR Endocytosis (% of surface bound)
Mins
TfnR Recycling (% initial loading)
C
EGFR Endocytosis (% of surface bound)
EGFR Recycling (% of initial loading)
D
H1299
sCLCa
sCLCb
GFP-CLCa
GFP-CLCb
CHC
GFP-CLC
CLCa
CLCb
actin
E
A549
F
A549
Frequency (x10-2)
Lifetime (s)
GFP-CLCa
GFP-CLCb
G
A549
CCP initiation (µm-2min-1, x10-1)
GFP-CLCa
GFP-CLCb

Figure S3.

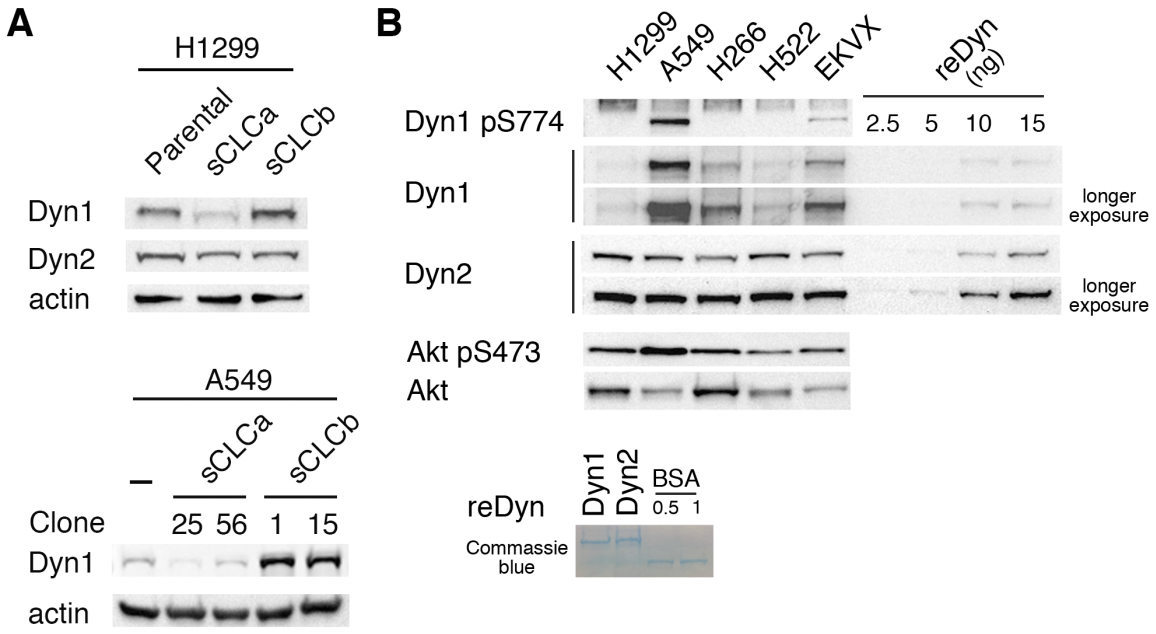

A
H1299
Parental
sCLCa
sCLCb
Dyn1
Dyn2
actin
A549
–
sCLCa
sCLCb
Clone
25 56 1 15
Dyn1
actin
B
H1299
A549
H266
H522
EKVX
reDyn (ng)
2.5 5 10 15
Dyn1 pS774
Dyn1
longer exposure
Dyn2
longer exposure
Akt pS473
Akt
reDyn
Dyn1
Dyn2
BSA
0.5 1
Commassie blue

Figure S4.

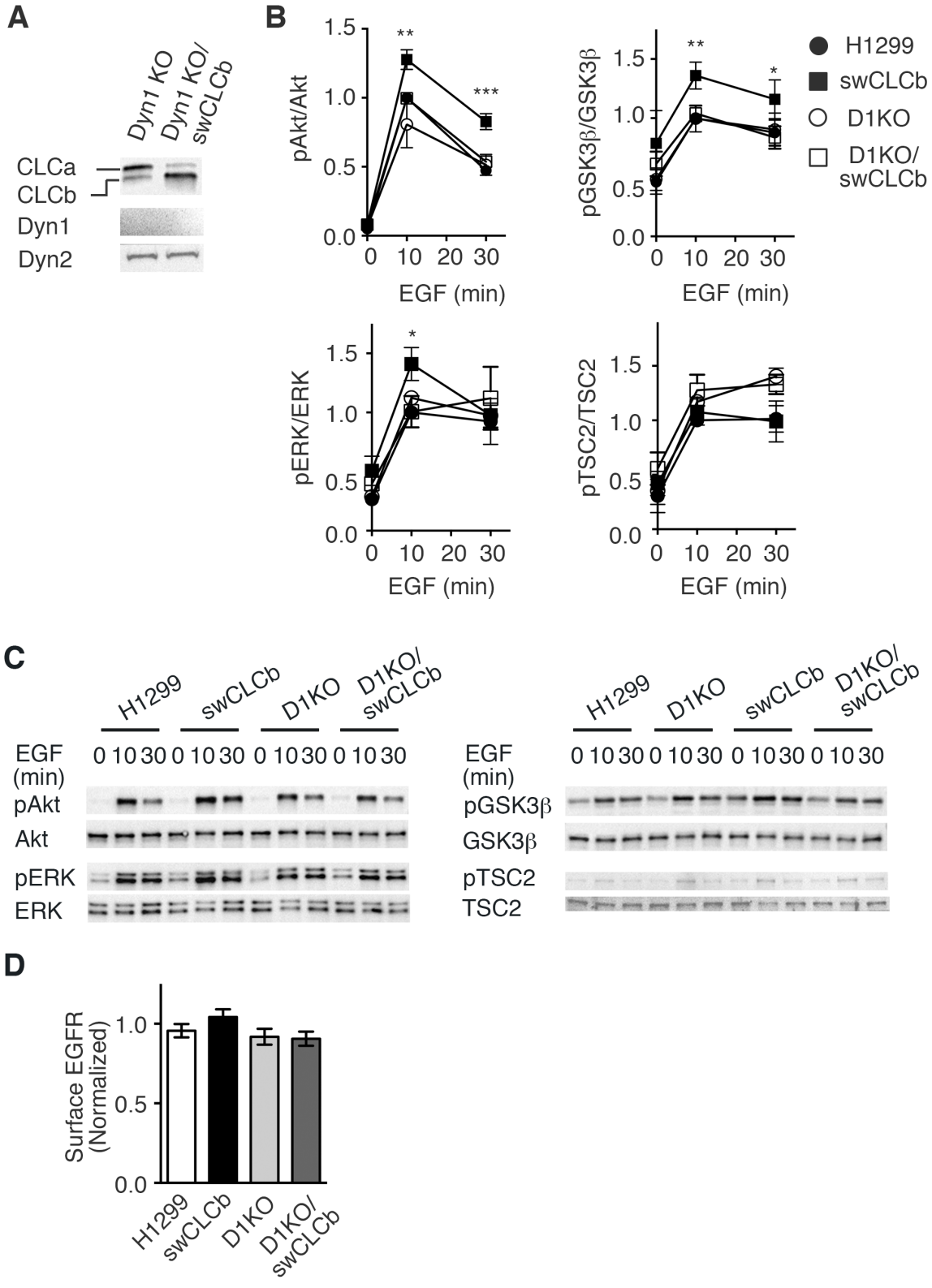

A
Dyn1 KO
Dyn1 KO/ swCLCb
CLCa
CLCb
Dyn1
Dyn2
B
pAkt/Akt
pGSK3β/GSK3β
pERK/ERK
pTSC2/TSC2
EGF (min)
H1299
swCLCb
D1KO
D1KO/ swCLCb
C
H1299
swCLCb
D1KO
D1KO/ swCLCb
EGF (min)
0 10 30 0 10 30 0 10 30 0 10 30
pAkt
Akt
pERK
ERK
pGSK3β
GSK3β
pTSC2
TSC2
D
Surface EGFR (Normalized)
H1299
swCLCb
D1KO
D1KO/ swCLCb

Figure S5.

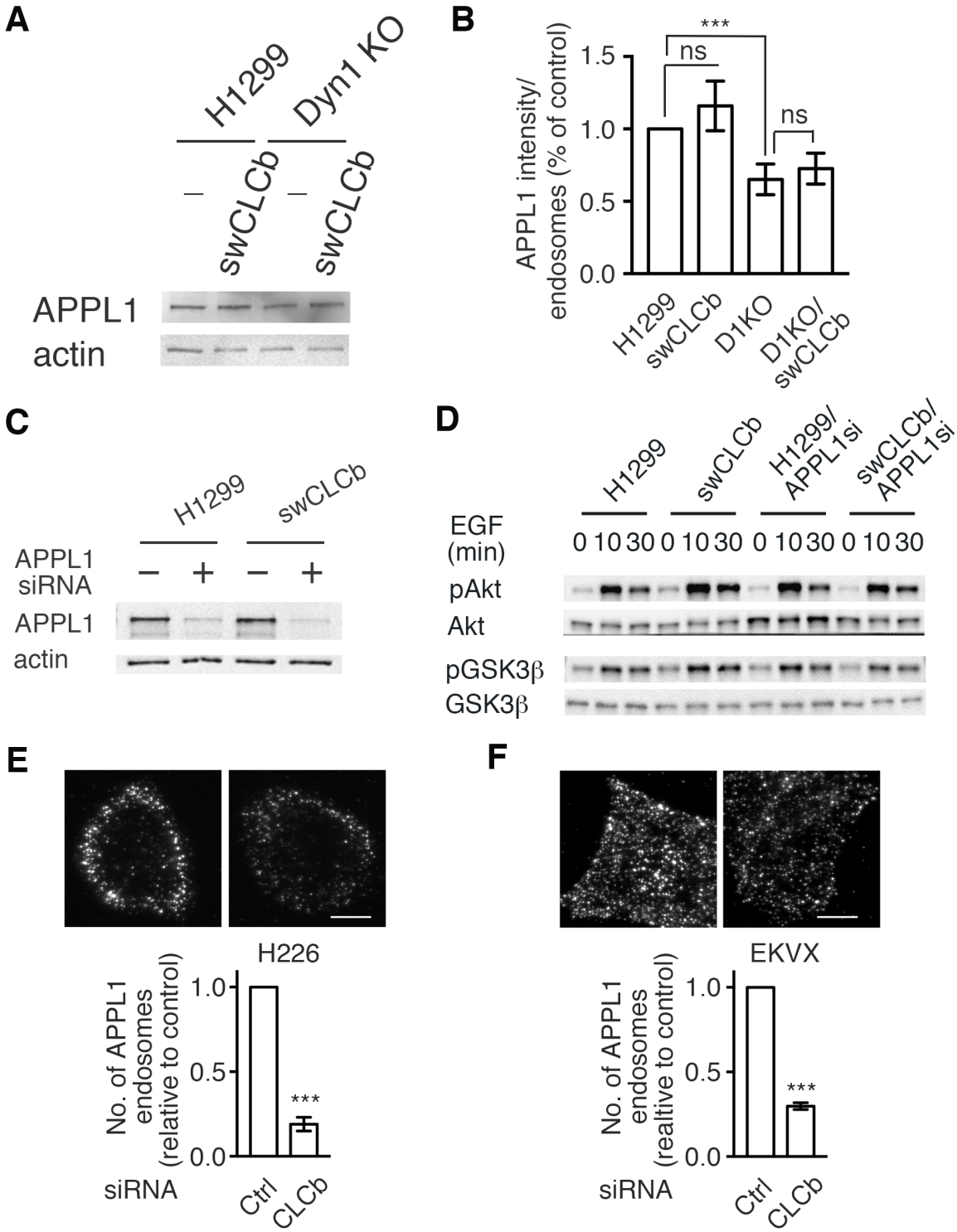

A
H1299
Dyn1 KO
–
swCLCb
–
swCLCb
APPL1
actin
B
APPL1 intensity/
endosomes (% of control)
1.5
1.0
0.5
0.0
***
ns
ns
H1299
swCLCb
D1KO
D1KO/
swCLCb
C
H1299
swCLCb
APPL1
siRNA
– + – +
APPL1
actin
D
H1299
swCLCb
H1299/
APPL1si
swCLCb/
APPL1si
EGF
(min)
0 10 30 0 10 30 0 10 30 0 10 30
pAkt
Akt
pGSK3β
GSK3β
E
H226
No. of APPL1
endosomes
(relative to control)
1.0
0.5
0.0
***
siRNA
Ctrl
CLCb
F
EKVX
No. of APPL1
endosomes
(realtive to control)
1.0
0.5
0.0
***
siRNA
Ctrl
CLCb

Figure S6.

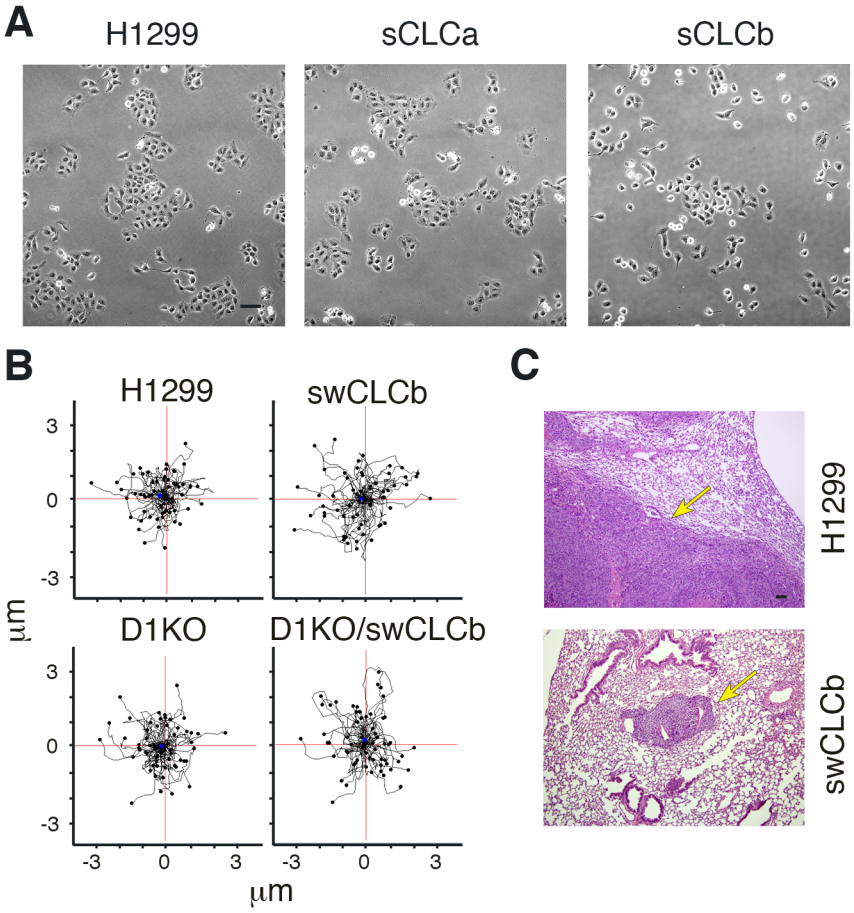
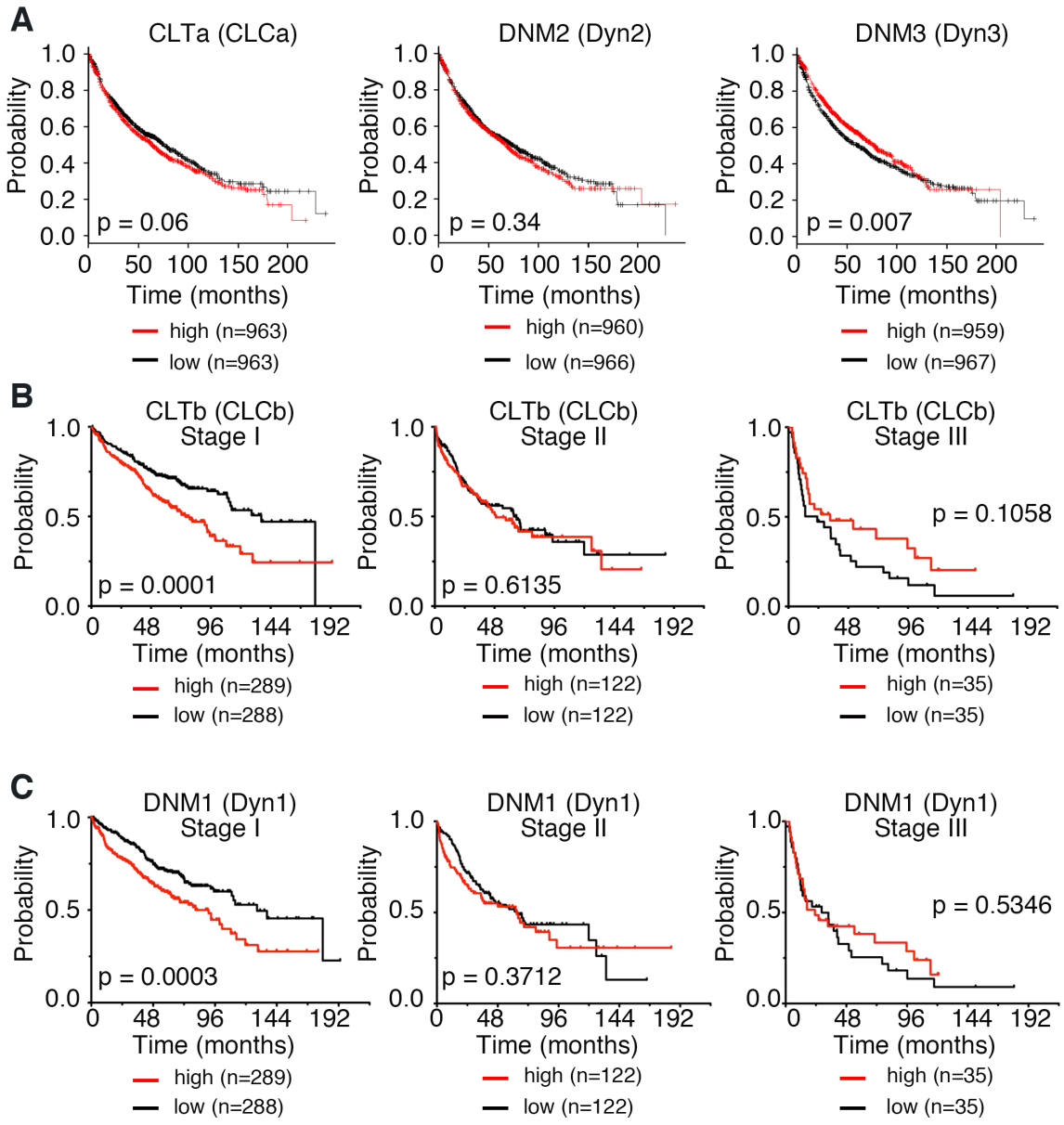

A
H1299
sCLCa
sCLCb
B
H1299
swCLCb
D1KO
D1KO/swCLCb
3
0
-3
μm
C
H1299
swCLCb

Figure S7.


A
CLTa (CLCa)
DNM2 (Dyn2)
DNM3 (Dyn3)
Probability
Time (months)
p = 0.06
p = 0.34
p = 0.007
high (n=963)
low (n=963)
high (n=960)
low (n=966)
high (n=959)
low (n=967)
B
CLTb (CLCb) Stage I
CLTb (CLCb) Stage II
CLTb (CLCb) Stage III
p = 0.0001
p = 0.6135
p = 0.1058
high (n=289)
low (n=288)
high (n=122)
low (n=122)
high (n=35)
low (n=35)
C
DNM1 (Dyn1) Stage I
DNM1 (Dyn1) Stage II
DNM1 (Dyn1) Stage III
p = 0.0003
p = 0.3712
p = 0.5346
high (n=289)
low (n=288)
high (n=122)
low (n=122)
high (n=35)
low (n=35)