# Constitutive Internalization and Recycling of the Delta opioid Receptor

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August 8, 2023

## Abstract

The complex and dynamic interplay between internalization, anterograde transport, recycling and degradation determines the density of functional G protein-coupled receptors (GPCRs) at the cell surface and, consequently, the magnitude of their associated physiological responses. As opposed to most members of the GPCR superfamily, the delta opioid receptor (DOP) is only weakly expressed at the neuronal plasma membrane, thus representing a critical limitation for its use as a therapeutic target. Although DOP appears as a promising candidate for the development of better-tolerated analgesics, the molecular and cellular mechanisms underlying the regulation of its cell surface expression remain poorly characterized. This work investigates the constitutive ( *i.e.* ligand-independent) trafficking of DOP, an understudied cellular process potentially involved in the control of plasma membrane-localized receptors. In HEK293 cells stably expressing Flag-tagged DOP, we first confirmed that this GPCR is constitutively internalized through a clathrin-dependent and b-arrestin-independent mechanism. Immunofluorescence experiments with selected Rab protein isoforms indicated that internalized DOP was mainly colocalized with the early endosome marker Rab5, as well as the rapid recycling endosome marker Rab4. Co-transfection with Rab5 dominant-negative mutant inhibited the intracellular distribution of the receptor, indicating that its constitutive endocytosis is Rab5-dependent. DOP cell surface expression and ligand-induced signaling were also significantly reduced following Rab4-specific DsiRNA treatments, suggesting a role for this small GTPase in the regulation of DOP constitutive recycling. Mapping of the major region of interaction between DOP and both Rabs revealed that Rab4 binds the third intracellular loop of DOP, whereas Rab5 seems to preferentially interact with the distal region of the C-terminal end of DOP. Altogether, these results show for the first time that DOP constitutive internalization and recycling are critical to maintain its cell surface bioavailability and responsiveness to agonists.

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# GRAPHICAL ABSTRACT

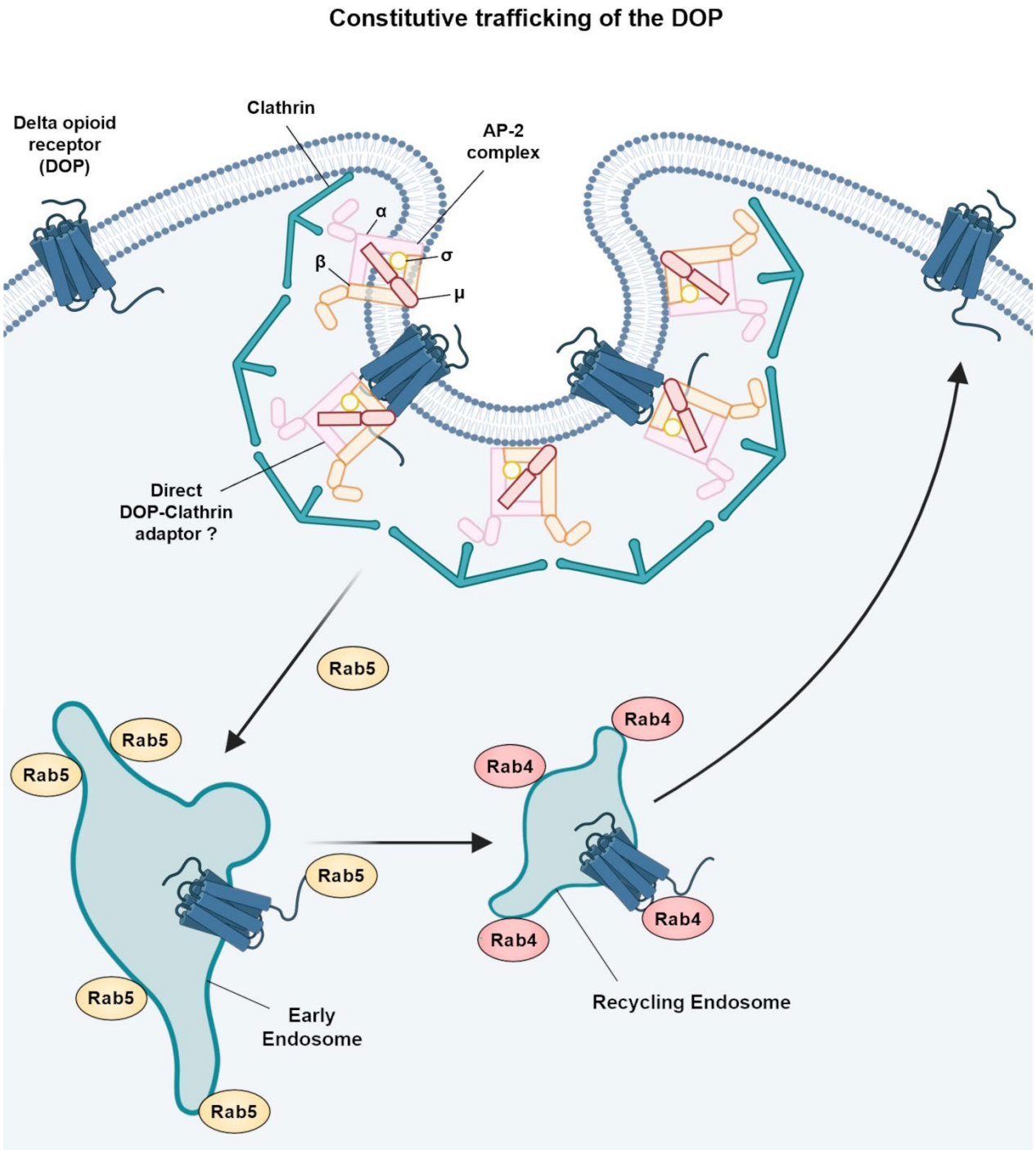


**FIGURE 1**

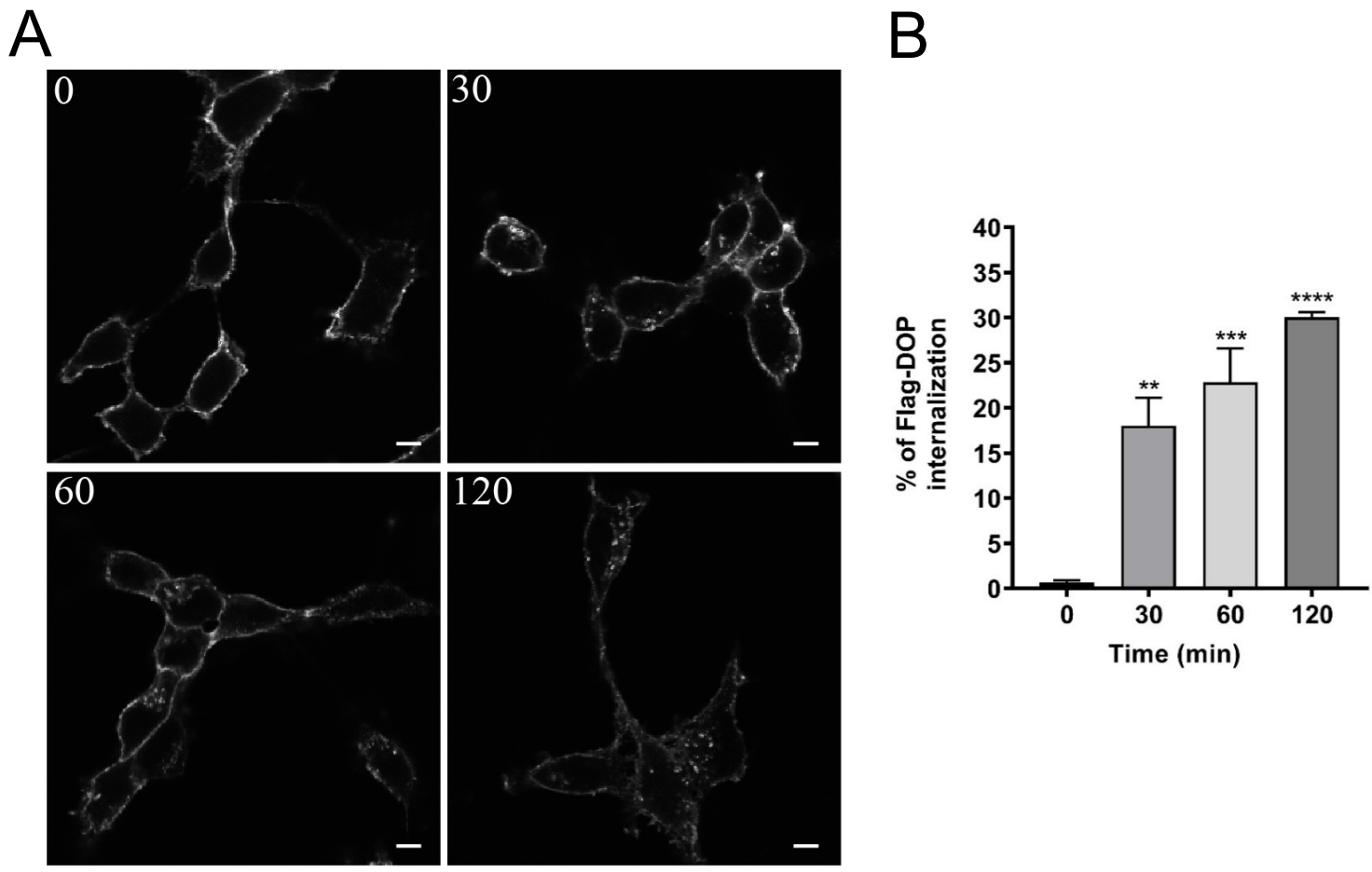

A
0
30
60
120
B
% of Flag-DOP internalization
40
35
30
25
20
15
10
5
0
**
***
****
0
30
60
120
Time (min)

**FIGURE 2**

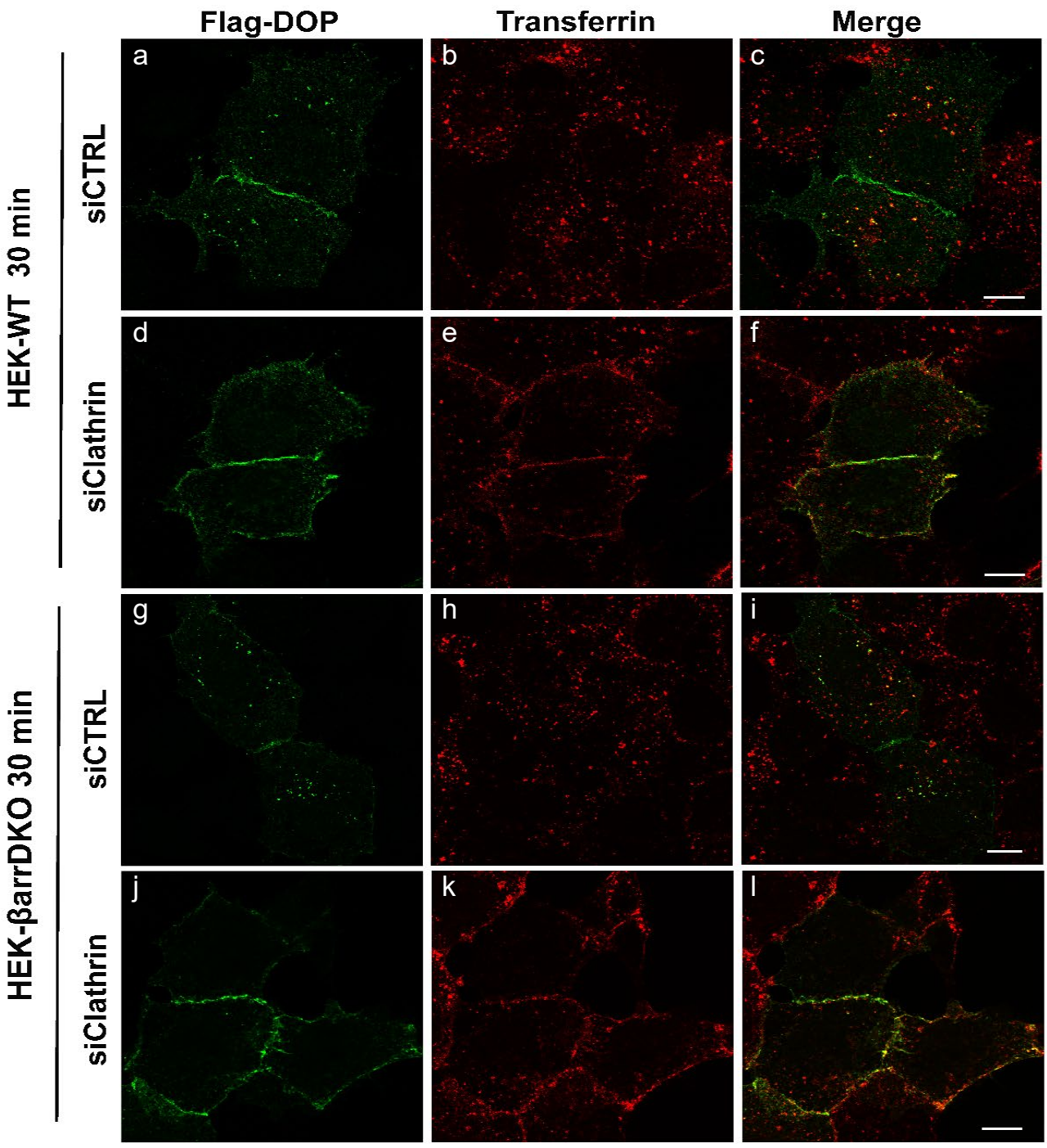

Flag-DOP
Transferrin
Merge
HEK-WT 30 min
siCTRL
siClathrin
HEK-βarrDKO 30 min
siCTRL
siClathrin
a
b
c
d
e
f
g
h
i
j
k
l

**FIGURE 3**

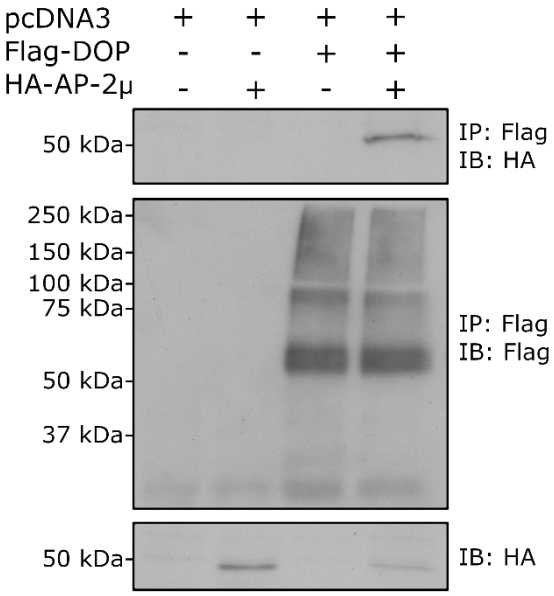
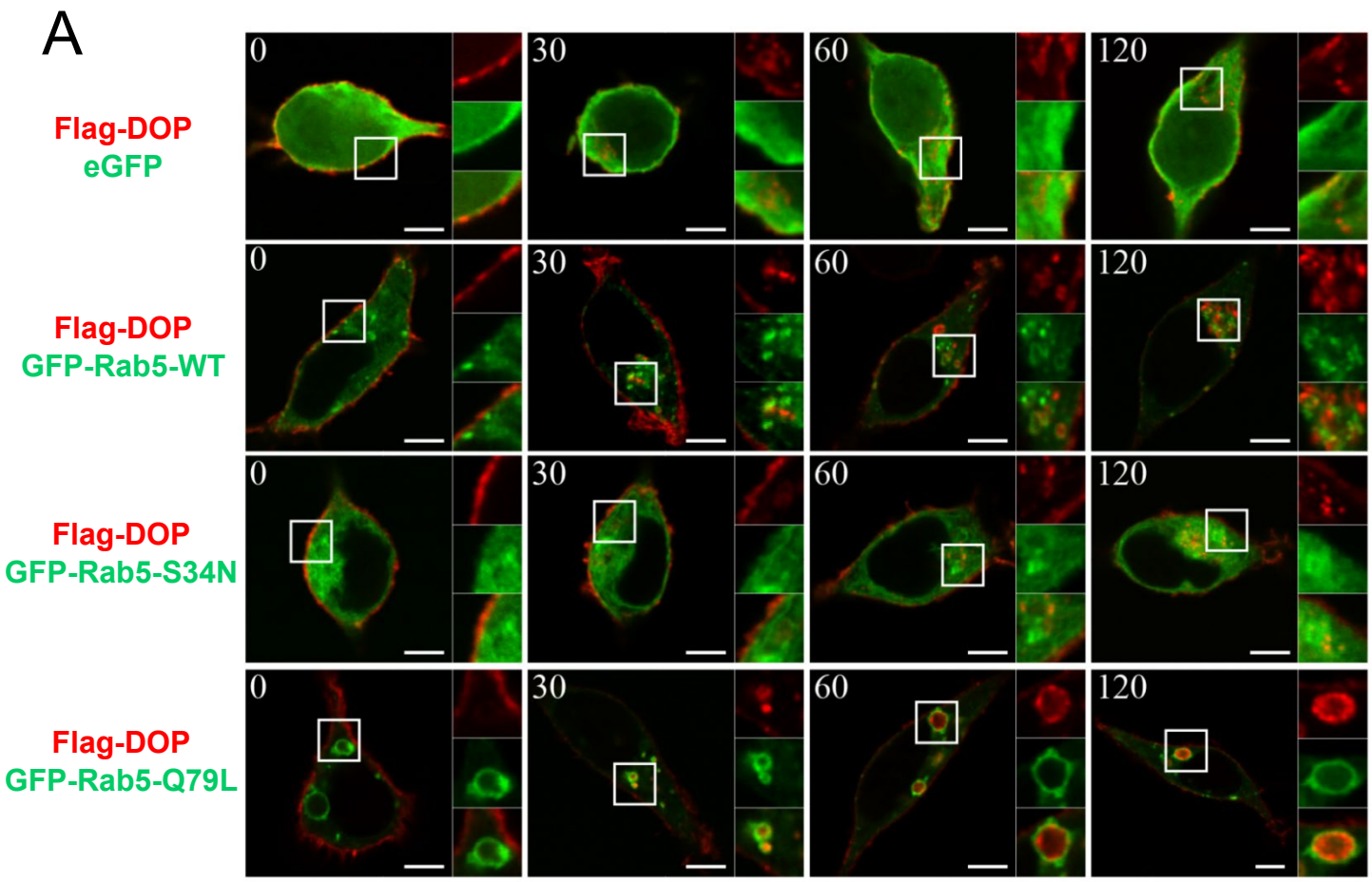
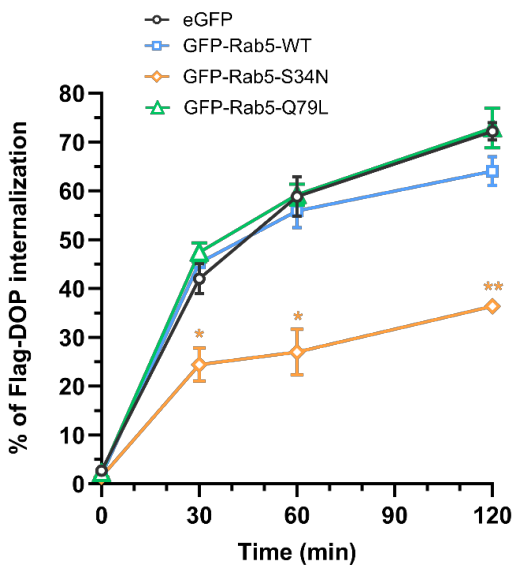

pcDNA3 + + + +
Flag-DOP - - + +
HA-AP-2µ - + - +
50 kDa
IP: Flag
IB: HA
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
IP: Flag
IB: Flag
50 kDa
IB: HA

**FIGURE 4**


A
Flag-DOP
eGFP
Flag-DOP
GFP-Rab5-WT
Flag-DOP
GFP-Rab5-S34N
Flag-DOP
GFP-Rab5-Q79L
0
30
60
120


B
eGFP
GFP-Rab5-WT
GFP-Rab5-S34N
GFP-Rab5-Q79L
% of Flag-DOP internalization
Time (min)
0
10
20
30
40
50
60
70
80
0
30
60
90
120
*
*
**

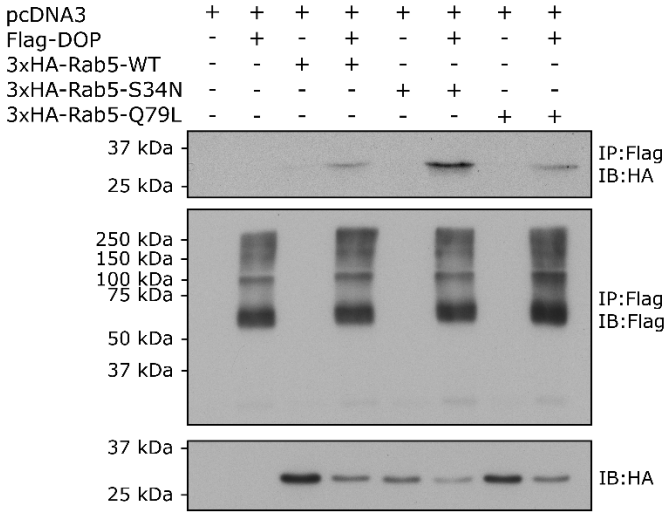
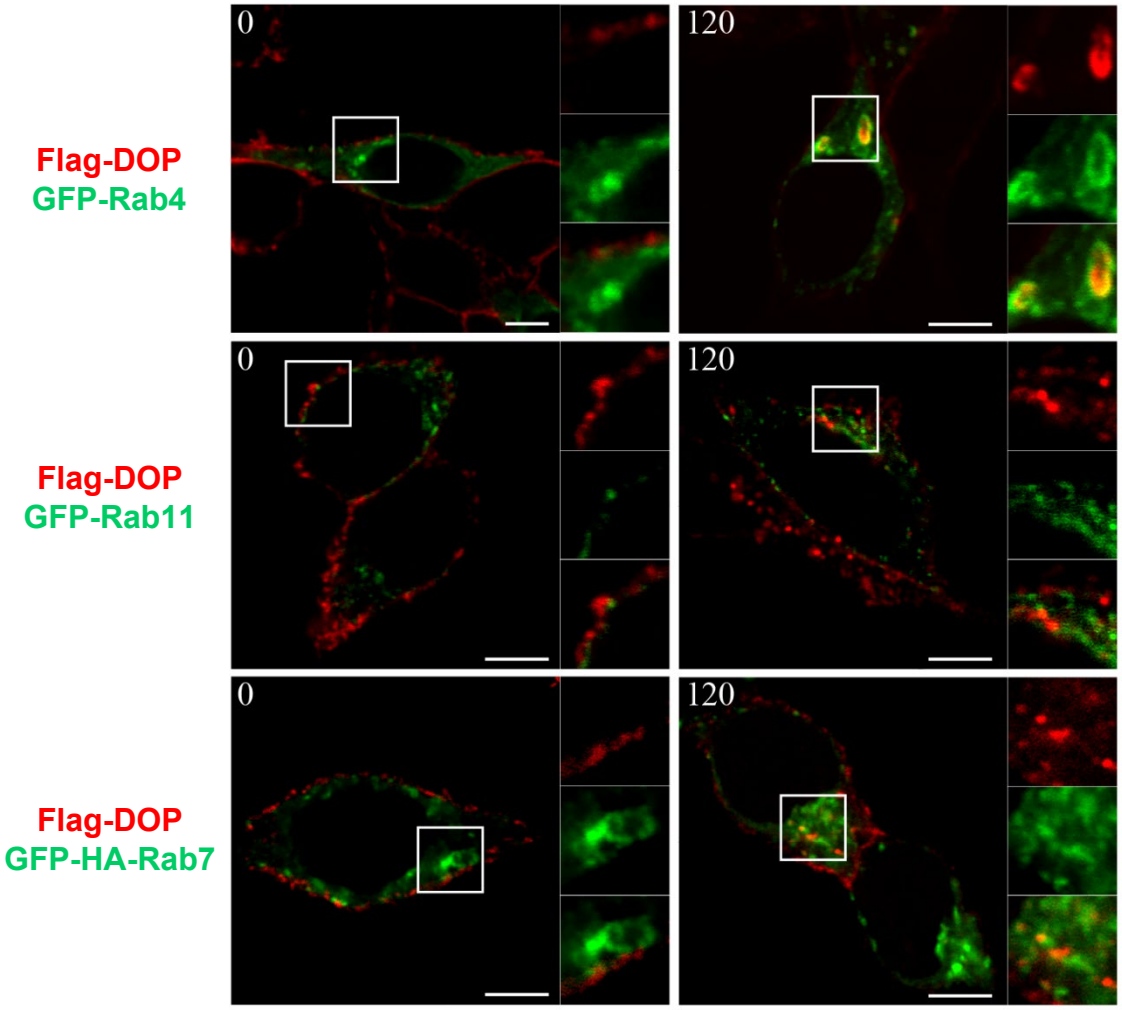
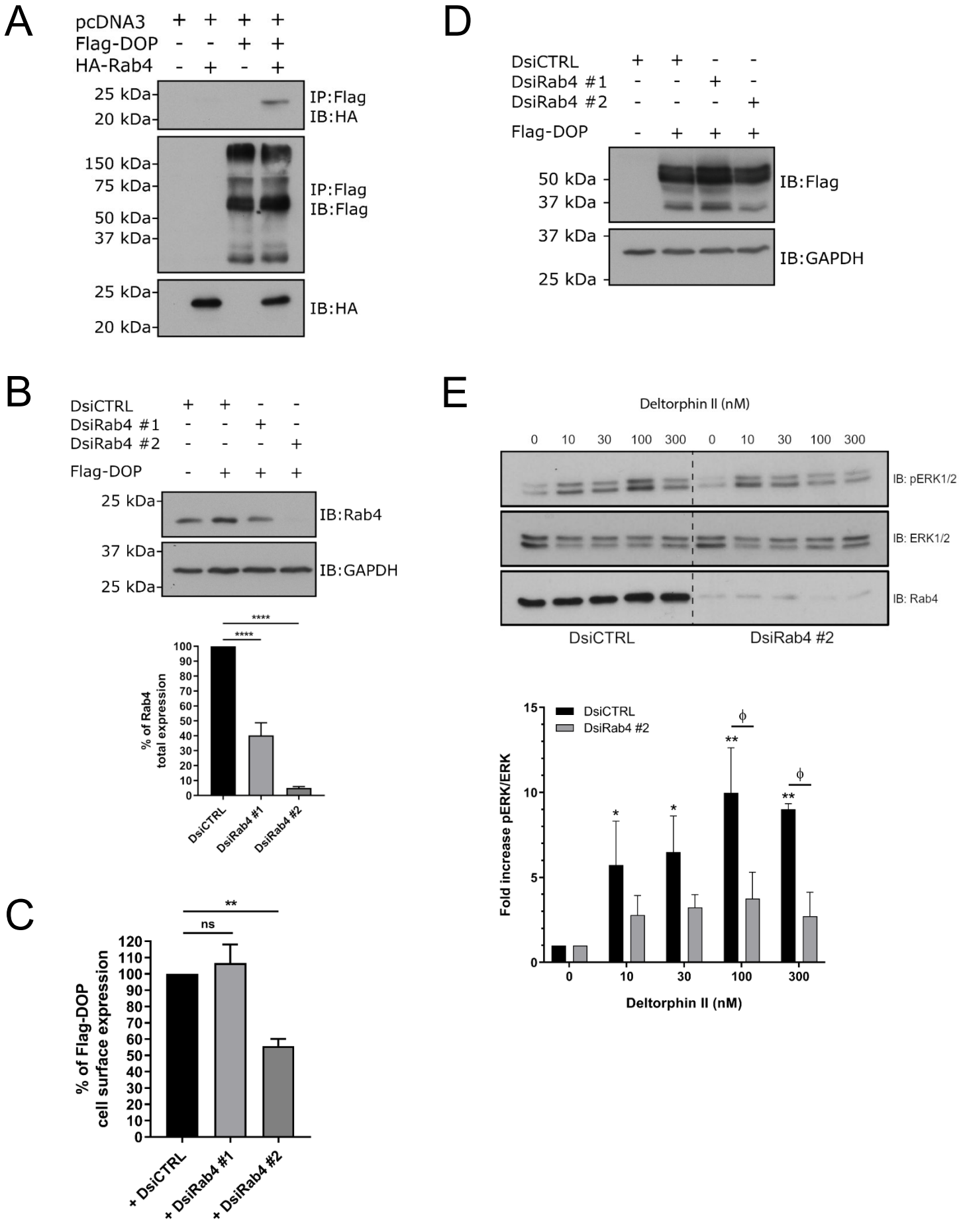

C
pcDNA3
Flag-DOP
3xHA-Rab5-WT
3xHA-Rab5-S34N
3xHA-Rab5-Q79L
37 kDa
25 kDa
IP:Flag
IB:HA
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
IP:Flag
IB:Flag
37 kDa
25 kDa
IB:HA

**FIGURE 5**

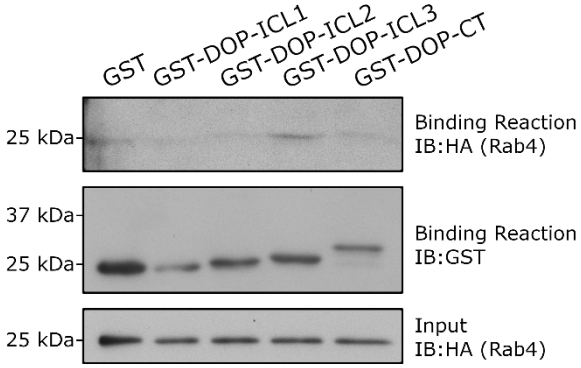

Flag-DOP
GFP-Rab4
0
120
Flag-DOP
GFP-Rab11
0
120
Flag-DOP
GFP-HA-Rab7
0
120

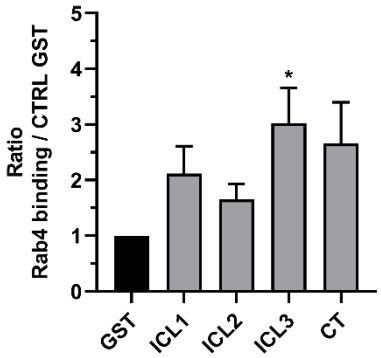
# FIGURE 6


A
pcDNA3 + + + +
Flag-DOP - - + +
HA-Rab4 - + - +
25 kDa
20 kDa
IP:Flag
IB:HA
150 kDa
75 kDa
50 kDa
37 kDa
IP:Flag
IB:Flag
25 kDa
20 kDa
IB:HA
B
DsiCTRL + + - -
DsiRab4 #1 - - + -
DsiRab4 #2 - - - +
Flag-DOP - + + +
25 kDa
IB:Rab4
37 kDa
25 kDa
IB:GAPDH
****
****
% of Rab4 total expression
100
90
80
70
60
50
40
30
20
10
0
DsiCTRL
DsiRab4 #1
DsiRab4 #2
C
**
ns
% of Flag-DOP cell surface expression
120
110
100
90
80
70
60
50
40
30
20
10
0
+ DsiCTRL
+ DsiRab4 #1
+ DsiRab4 #2
D
DsiCTRL + + - -
DsiRab4 #1 - - + -
DsiRab4 #2 - - - +
Flag-DOP - + + +
50 kDa
37 kDa
IB:Flag
37 kDa
25 kDa
IB:GAPDH
E
Deltorphin II (nM)
0 10 30 100 300 0 10 30 100 300
IB: pERK1/2
IB: ERK1/2
IB: Rab4
DsiCTRL
DsiRab4 #2
DsiCTRL
DsiRab4 #2
Fold increase pERK/ERK
15
10
5
0
*
*
**
**
ϕ
ϕ
0
10
30
100
300
Deltorphin II (nM)

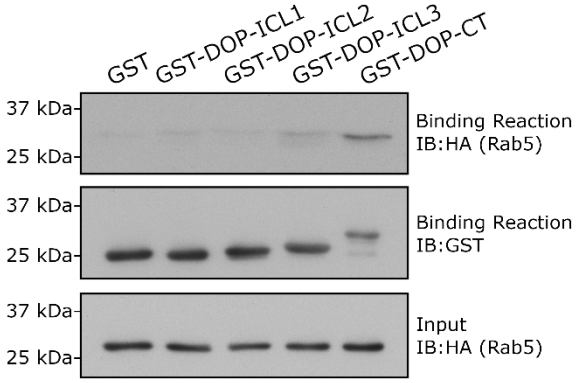
# FIGURE 7

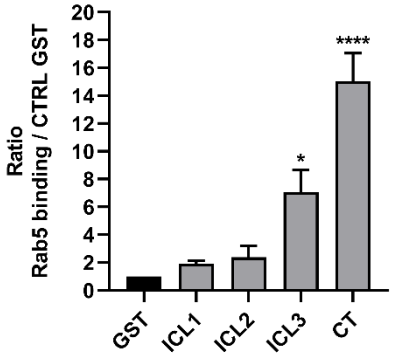
A


GST
GST-DOP-ICL1
GST-DOP-ICL2
GST-DOP-ICL3
GST-DOP-CT
25 kDa
Binding Reaction
IB:HA (Rab4)
37 kDa
25 kDa
Binding Reaction
IB:GST
25 kDa
Input
IB:HA (Rab4)

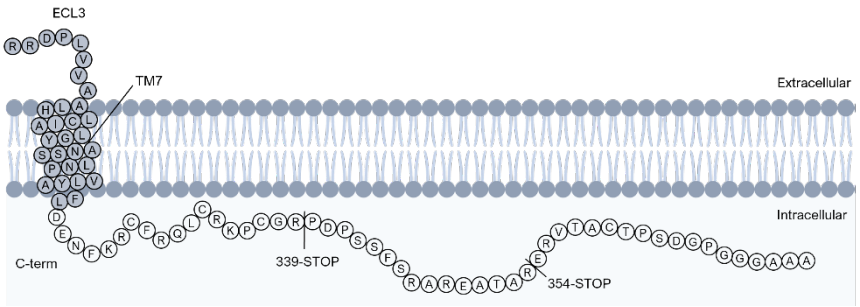

Ratio
Rab4 binding / CTRL GST
0
1
2
3
4
5
*
GST
ICL1
ICL2
ICL3
CT

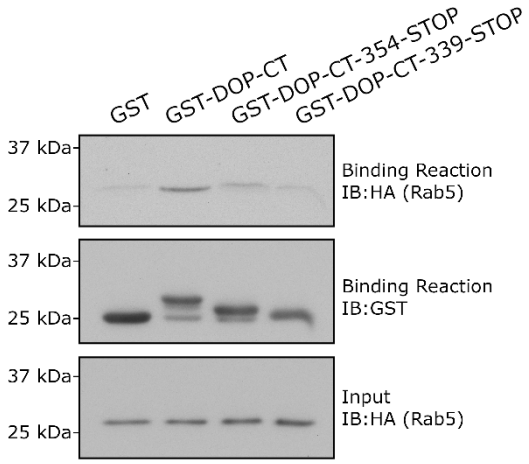
B


GST
GST-DOP-ICL1
GST-DOP-ICL2
GST-DOP-ICL3
GST-DOP-CT
37 kDa
25 kDa
Binding Reaction
IB:HA (Rab5)
37 kDa
25 kDa
Binding Reaction
IB:GST
37 kDa
25 kDa
Input
IB:HA (Rab5)


Ratio
Rab5 binding / CTRL GST
0
2
4
6
8
10
12
14
16
18
20
*
****
GST
ICL1
ICL2
ICL3
CT

C


ECL3
TM7
Extracellular
Intracellular
C-term
339-STOP
354-STOP


GST
GST-DOP-CT
GST-DOP-CT-354-STOP
GST-DOP-CT-339-STOP
37 kDa
25 kDa
Binding Reaction
IB:HA (Rab5)
37 kDa
25 kDa
Binding Reaction
IB:GST
37 kDa
25 kDa
Input
IB:HA (Rab5)

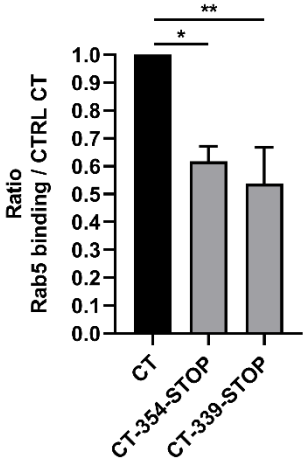

Ratio
Rab5 binding / CTRL CT
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
*
**
CT
CT-354-STOP
CT-339-STOP

**FIGURE S1**

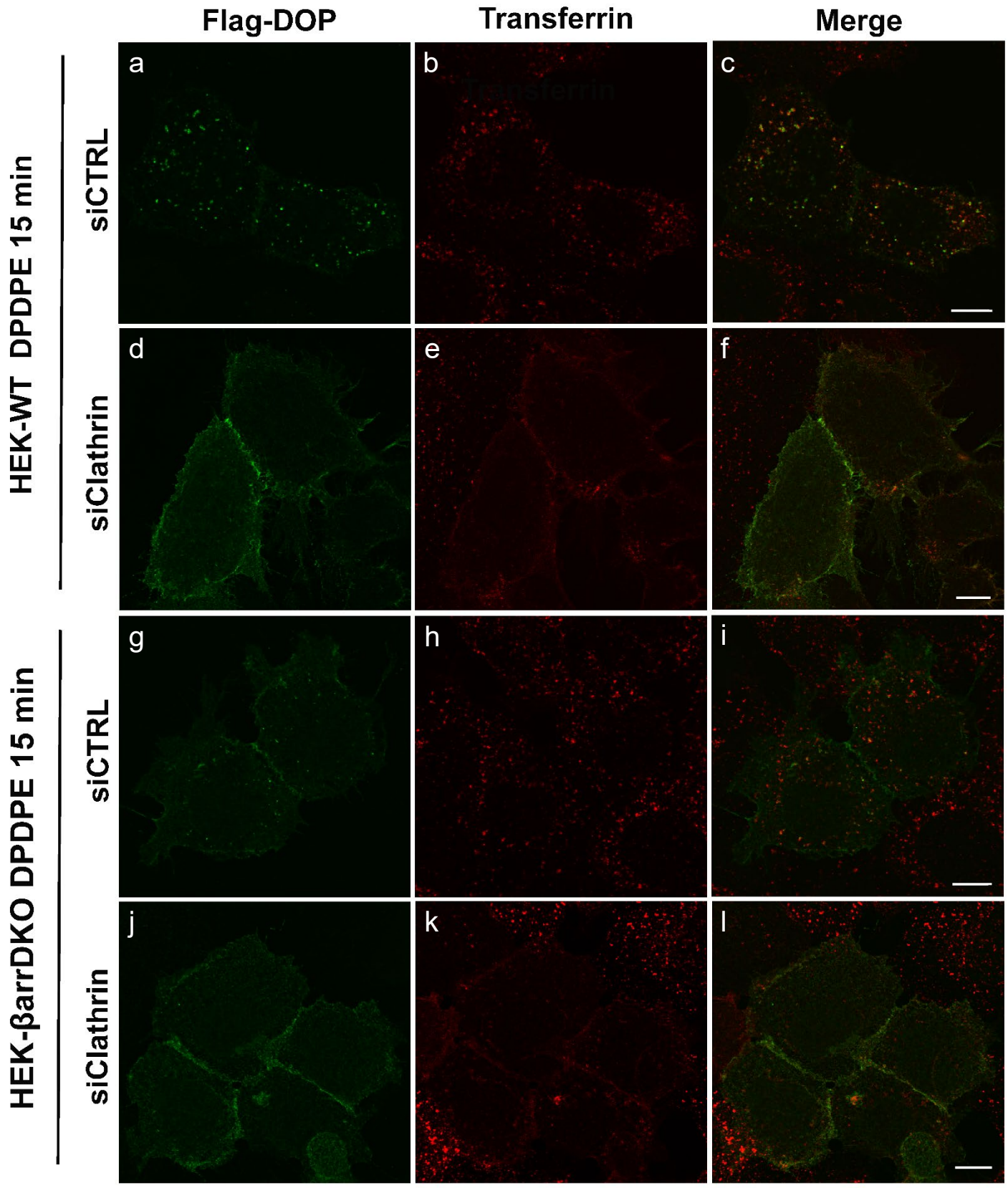
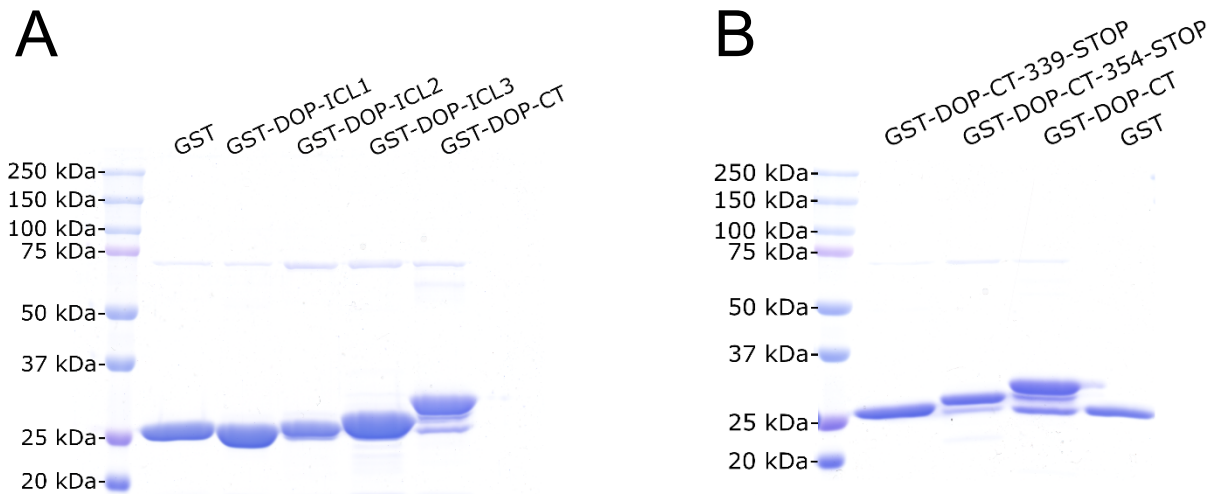

Flag-DOP
Transferrin
Merge
HEK-WT DPDPE 15 min
siCTRL
siClathrin
HEK-βarrDKO DPDPE 15 min
siCTRL
siClathrin
a
b
c
d
e
f
g
h
i
j
k
l

**FIGURE S2**


A
GST
GST-DOP-ICL1
GST-DOP-ICL2
GST-DOP-ICL3
GST-DOP-CT
250 kDa-
150 kDa-
100 kDa-
75 kDa-
50 kDa-
37 kDa-
25 kDa-
20 kDa-
B
GST-DOP-CT-339-STOP
GST-DOP-CT-354-STOP
GST-DOP-CT
GST
250 kDa-
150 kDa-
100 kDa-
75 kDa-
50 kDa-
37 kDa-
25 kDa-
20 kDa-

# TABLE S1 – siRNA and DsiRNA sequences

| **DsiRNAs** | **Duplex Sequences** |
|---|---|
| DsiRNA-Rab4 #1 | 5’- rArCrArArArArUrCrGrArArUrCrArGrGrUrGrArGrCrUrGGA -3’<br>5’- rUrCrCrArGrCrUrCrArCrCrUrGrArUrUrCrGrArUrUrUrUrGrUrUrA -3’ |
| DsiRNA-Rab4 #2 | 5’- rUrUrArUrCrUrUrUrUrGrArArCrCrArArArUrUrCrCrArCrUrCrCrUrA -3’<br>5’- rGrGrArGrUrGrGrArArUrUrUrGrGrUrUrCrArArArGrArUAA -3’ |
| **siRNA** | **Sequences** |
| siClathrin | |