GST Pull-down and Immunoprecipitation Assays

Expression of wild-type and mutant ORP9L ORP9S in CHO Tet-on cells were induced with doxycycline (1μg/ml). After 24 h, cells were harvested in PBS, collected by centrifugation and solubilized in PBS containing 0.5% TX-100 (v/v), 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 200 nM okadaic acid and protease inhibitors. The supernatant was collected after centrifugation for 15 min at 10,000xg, and incubated with GST or GST-VAP (1μM) at 25°C for 45 minutes. Glutathione-Sepharose was added for 30 minutes with constant shaking, the beads were washed three times with PBS and 0.1% TX-100 (v/v) and bound ORP9 was detected by SDS-PAGE and immunoblotting.

For immunoprecipitation experiments, cells were cultured and Triton X-100 lysates prepared as described as above. A VAP antibody was incubated with lysates for 2 h at 25°C with constant mixing, after which Protein A-Sepharose was added for an additional 30 min. Sepharose beads were collected by centrifugation, rinsed 3 times in PBS with 0.1% TX-100 (v/v) PBS, and bound ORP9 was detected by SDS-PAGE and immunoblotting.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Interaction of ORP9 mutants with VAP. (A and B) CHO-tet cells inducibly expressing wild-type or mutant ORP9L or ORP9S were cultured in the presence or absence of doxycycline (Dox) for 24 h. Cells were harvested, detergent extracts prepared and incubated with GST-VAP. Complexes were isolated on glutathione-Sepharose, resolved by SDS-PAGE and immunoblotting with an ORP9-

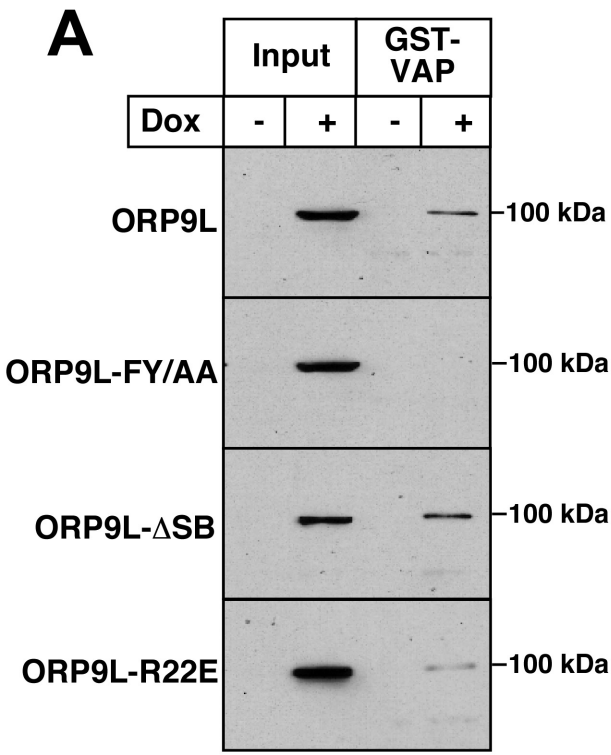
specific antibody. Input lane represents 10% of total extract used in the pull-down. (C and D). Detergent extracts of cells were prepared as described above and incubated with a VAP antibody (IP). Complexes were isolated on Protein A-Sepharose, resolved by SDS-PAGE and immunoblotted (IB) for V5-tagged ORP9L or ORP9S. Input lane represents 10% of total extract used in the immunoprecipitation.

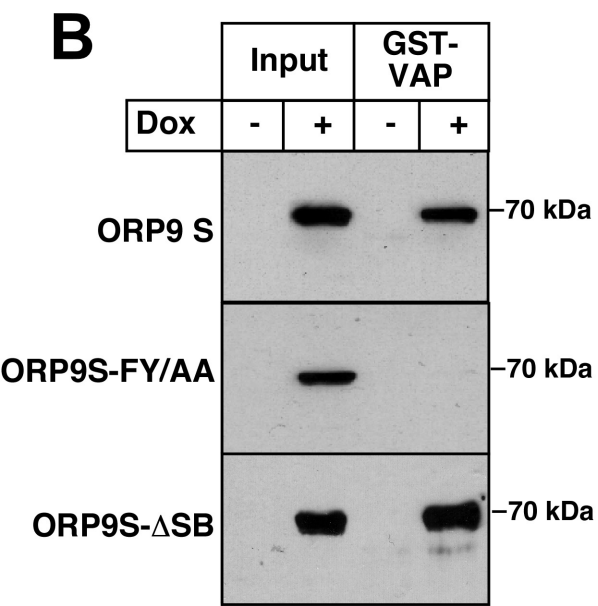
Supplemental Figure 2. Expression of ORP9S causes reversible growth arrest of CHO cells. CHO cells stably expressing the indicted ORP9S proteins under the control of the Tet repressor were cultured for 12 h prior to the addition of doxycycline (Dox, 1 μg/ml) (●) or solvent control (■) at time 0. At the indicated time points, cells were harvested by trypsin digestion, counted, and expression of V5-tagged ORP9S and actin was determined by immunoblotting as described in Materials and Methods. (A) Cell growth following induction of ORP9S expression. (B) Growth of cells expressing ORP9S and uninduced control was done as described in panel A. Following induction with Dox for 36 h, cells received Dox-free media (indicated by upward arrow) for a further 48 h (▲). Results are from a representative experiment. (C) Cell growth following induction of ORP9S-FY/AA expression compared to uninduced controls. (D) Cell growth following induction of ORP9S-ΔSB expression compared to uninduced controls. (E) Equivalent amounts of total cell extracts from Tet-on cells expressing ORP9L or ORP9S were immunoblotted with an ORP9 polyclonal antibody to evaluate the level of overexpression relative to endogenous ORP9L. Results in panels A, C and D are the means and standard error of 3 experiments.

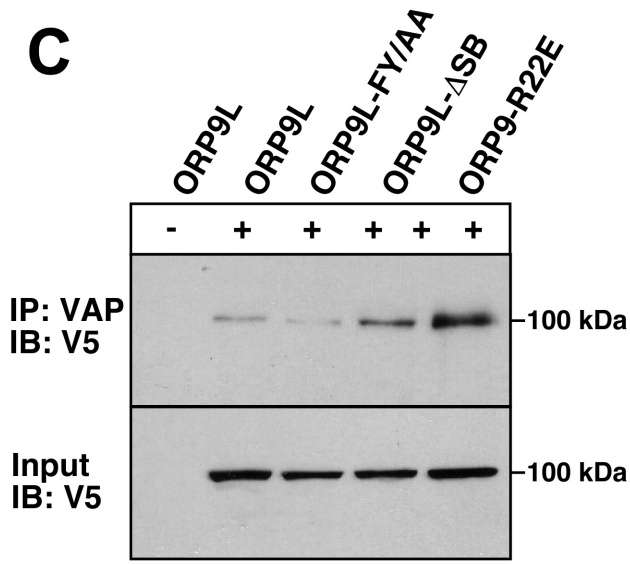
Supplemental Figure 3. Effect of ORP9L expression of cell growth. The proliferation of CHO-tet cells expressing ORP9L (A), ORP9L-FY/AA (B), ORP9L-ΔSB (C) and ORP9L-R22E (D) was measured in the presence and absence of doxycycline as described the legend to supplemental Figure 2. Results are the mean and SEM of 3 experiments.

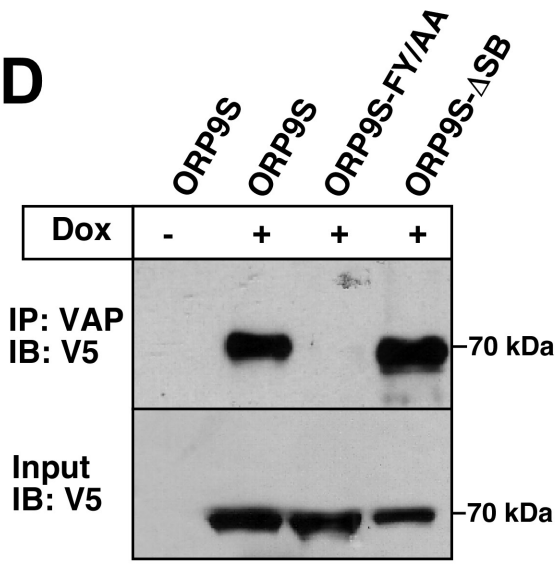
Supplemental Figure 4. Effect of enforced expression of ORP9L and ORP9S on cholesterol distribution, content and synthesis. CHO cells were transiently transfected with siNT or siORP9L for 48 (A) or expression of ORP9L or ORP9S was induced in CHO Tet-on cells for 24 h with doxycycline (Dox, 1 μg/ml). (A) Cells were pulse-labelled with [^{3}H]acetate (50 μCi/ml) for 20 min and incorporation into cholesterol was quantified after extraction and separation by thin-layer chromatography. Results are the mean of 2 experiments. (B and D) CHO inducibly expressing ORP9L or ORP9S were cultured in medium containing 5% FCS or LPDS and cholesterol was visualized with filipin. Fluorescence was quantified as described in Materials and Methods and results are expressed as mean and SEM to 3 separate experiments that evaluated 10-12 fields containing 20-30 cells each. (C and E) The total (T) and unesterified (U) cholesterol content of CHO cell cultured in medium containing 5% FCS or LPDS and inducibly expressing ORP9L and ORP9S was quantified as described in Materials and Methods. Results are the mean and SEM of 3 experiments.

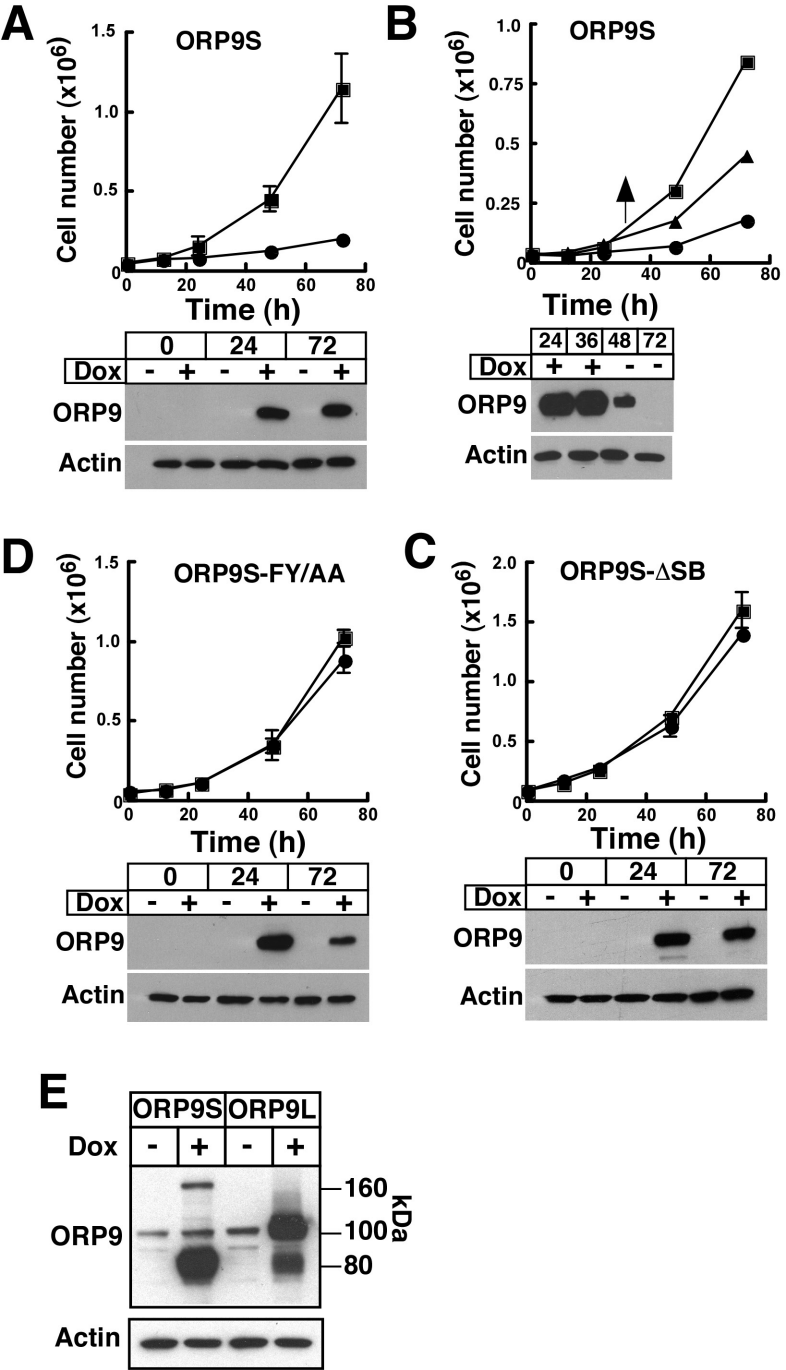
Supplemental Figure 1

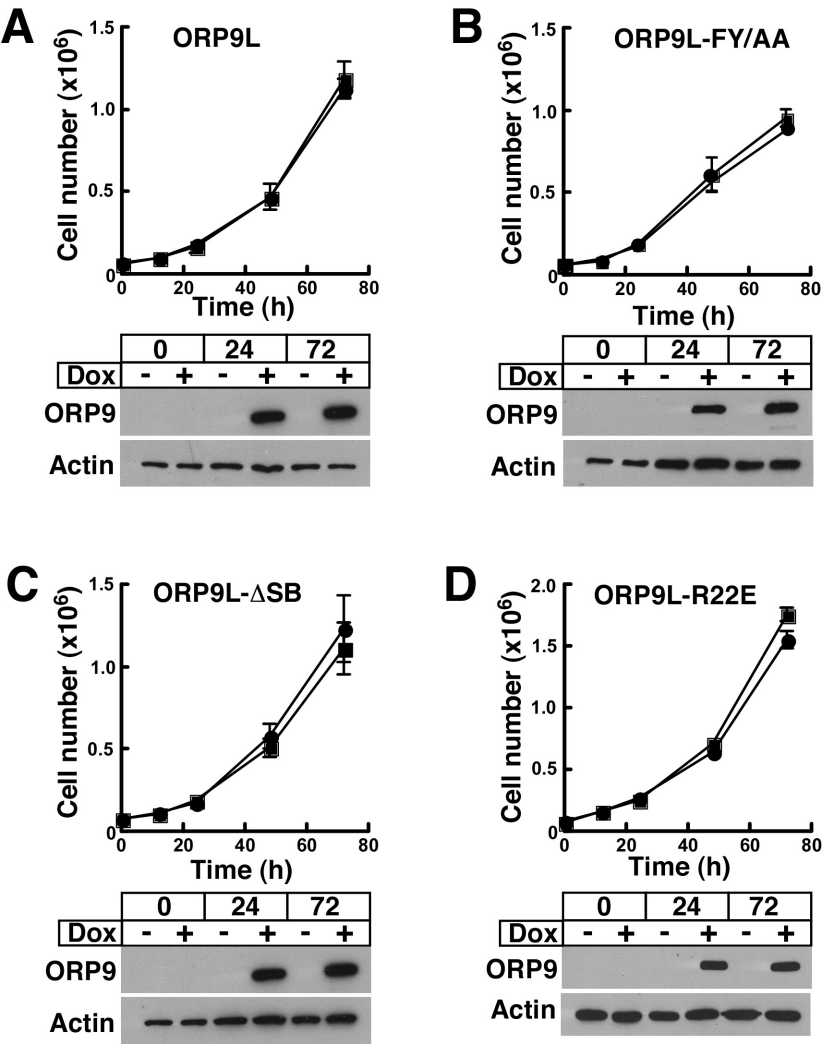


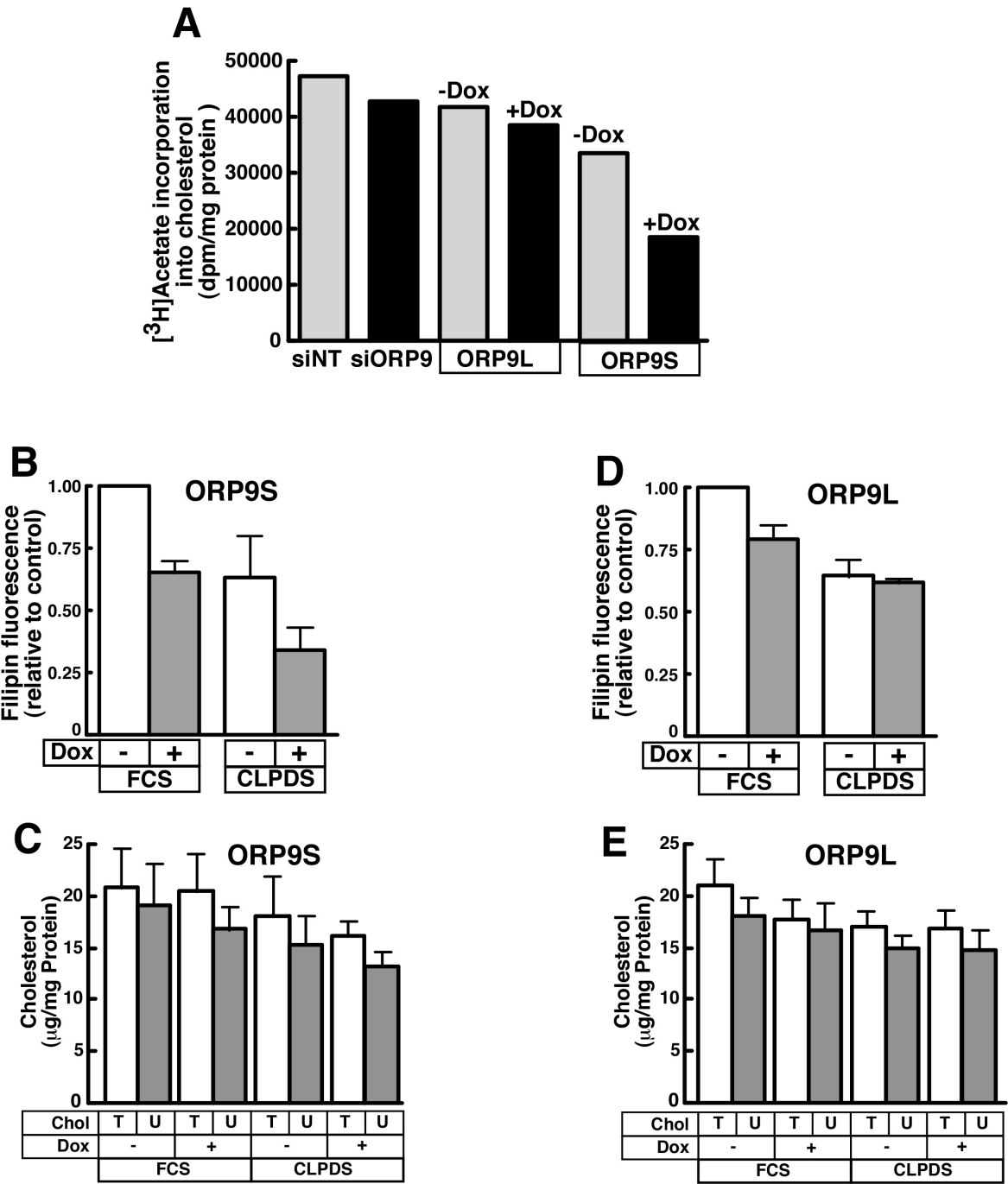







A
ORP9S
Cell number (x10^6)
Time (h)
0
24
72
Dox
ORP9
Actin
B
ORP9S
Cell number (x10^6)
Time (h)
24
36
48
72
Dox
ORP9
Actin
D
ORP9S-FY/AA
Cell number (x10^6)
Time (h)
Dox
ORP9
Actin
C
ORP9S-ΔSB
Cell number (x10^6)
Time (h)
Dox
ORP9
Actin
E
ORP9S
ORP9L
Dox
ORP9
160
100
80
kDa
Actin


A
ORP9L
Cell number (x10^6)
Time (h)
0
24
72
Dox
ORP9
Actin
B
ORP9L-FY/AA
C
ORP9L-ΔSB
D
ORP9L-R22E


A
[3H]Acetate incorporation into cholesterol (dpm/mg protein)
50000
40000
30000
20000
10000
0
-Dox
+Dox
-Dox
+Dox
siNT
siORP9
ORP9L
ORP9S
B
ORP9S
Filipin fluorescence (relative to control)
1.00
0.75
0.50
0.25
0
Dox
-
+
-
+
FCS
CLPDS
D
ORP9L
Filipin fluorescence (relative to control)
1.00
0.75
0.50
0.25
0
Dox
-
+
-
+
FCS
CLPDS
C
ORP9S
Cholesterol (μg/mg Protein)
25
20
15
10
5
0
Chol
T
U
T
U
T
U
T
U
Dox
-
+
-
+
FCS
CLPDS
E
ORP9L
Cholesterol (μg/mg Protein)
25
20
15
10
5
0
Chol
T
U
T
U
T
U
T
U
Dox
-
+
-
+
FCS
CLPDS