SUPPLEMENTAL FIGURES

Supplemental Fig. S1**. Immunocytochemical analysis of the internalization of extracellular PCSK9 and its GOF mutants.** HepG2 cells were incubated for 0 (S1A), 30 (S1B) or 60 (S1C) min at 4°C with condition media of HEK293 cells overexpressing either (**a**) PCSK9-V5, or (**b**) the prosegment deletant Δ33-58-PCSK9-V5. Following fixation and permeabilization of the cells, surface binding and internalization of PCSK9 and the mutated PCSK9 constructs as well as the localization of LDLR were detected by immunofluorescence with anti-V5 (*red*) and anti-LDLR antibodies (*green*). Arrows and arrowheads point to the cell surface labeling and the intracellular localization of PCSK9 and LDLR, respectively. Scale bars = 10μM.

Supplemental Fig.S2. **Time dependent effect of extracellular PCSK9 on the cell surface LDLR levels of HuH7 and HepG2 cells.** (**A**) FACS analysis of HuH7 and HepG2 cells for cell surface levels of LDLR following incubation of 0.7 μg/ml of PCSK9 secreted from HEK293 cells overexpressing it (quantitated by ELISA) for various times from 30-240 min. The right panels show that if the HuH7 cells are washed with fresh medium lacking PCSK9 following the first 30 min, the effect of PCSK9 is completely abrogated, suggesting that a continuous presence of PCSK9 is needed to see its time dependent effect on LDLR. (**B**) Analysis of the data show the presence of two phases of LDLR decrease, an initial rapid phase faster in HepG2 cells, followed by a slower linear one similar in both cell lines (the best fit linear equation is depicted).

Supplemental Fig.S3. **The pH dependence of PCSK9 activity is not regulated by the acidic stretch encompassing aa 31-58.** Various PCSK9 forms were pre-incubated at the indicated pHs for 1h, neutralized, and then incubated with HuH7 cells. FACS analysis of cell surface LDLR expression is shown compared to untreated cells. These data are representative of 2 independent experiments.

Supplemental Fig.S4. **PCSK9 does not cleave *in vitro* soluble LDLR at any pH.** ^{35}S-labeled soluble LDLR was obtained from the media of HEK293 cells overexpressing the ectodomain of LDLR-V5 (aa 1-540) and pulsed for 3h with ^{35}S-[Met + Cys]. The media were incubated at 37°C for 3h at pHs 7.5, 6.5, 6.0, 5.5 and 4.5 in the presence (+) or absence (-) of ~0.7 μg/ml of untagged PCSK9 secreted from HEK293 cells overexpressing it (quantitated by ELISA) or at pH 6.5 with 10 μg/ml purified PCSK9 (Figure 1A). All media were then neutralized to pH 7.5 and the immunoprecipitated with mAb-V5 and separated by 8% SDS-PAGE, the gel dried and then autoradiographed. The migration positions of the less glycosylated and fully glycosylated LDLR (10) are shown.

Supplemental Fig.S5. **Oligomerization of the cleaved forms of PCSK9 at pH 5.5.** SDS-PAGE analysis of the ^{35}S-labeled PCSK9 and its derivatives obtained from the media of HEK293 cells pulse labeled for 4h with ^{35}S-(Met+Cys). The samples were incubated for 1h at either pH 7.2 or 5.5 followed by neutralization, immunoprecipitation with mAb-V5 and then boiling in SDS buffer in the presence (+SH) or absence (-SH) of β-mercaptoethanol. With the exception of the CHRD, notice the propensity of the cleaved forms at pH 5.5 to oligomerize.

Supplemental Fig.S6. **Inability of furin to generate *in vitro* PCSK9 (153-Nx).** HEK293 cells were co-transfected with proCSK9-Prosegment (encoding V5-31-152) and either PCSK9-ΔPro-V5 or PCSK9 (H226A)-ΔPro-V5. The overnight media of these cells were incubated at either pH 7.2 or 5.5 for 1h at 37°C and the later neutralized to pH 7.2. In each case the media (200 ng of PCSK9) were incubated overnight in absence or presence of furin (5 units, Sigma). The media were then immunoprecipitated with Ab-03. The latter were separated by 8% SDS-PAGE and WB with Ab-03. Only the region representing the apparent molecular masses of 20-3.5 kDa is shown, together with the position of V5-Pro 31-152, PCSK9 (153-Nx), PCSK9 (153-217) and PCSK9 (153-198?). The question mark emphasizes the hypothetical cleavage site at Arg_{199} followed by carboxypeptidase action.

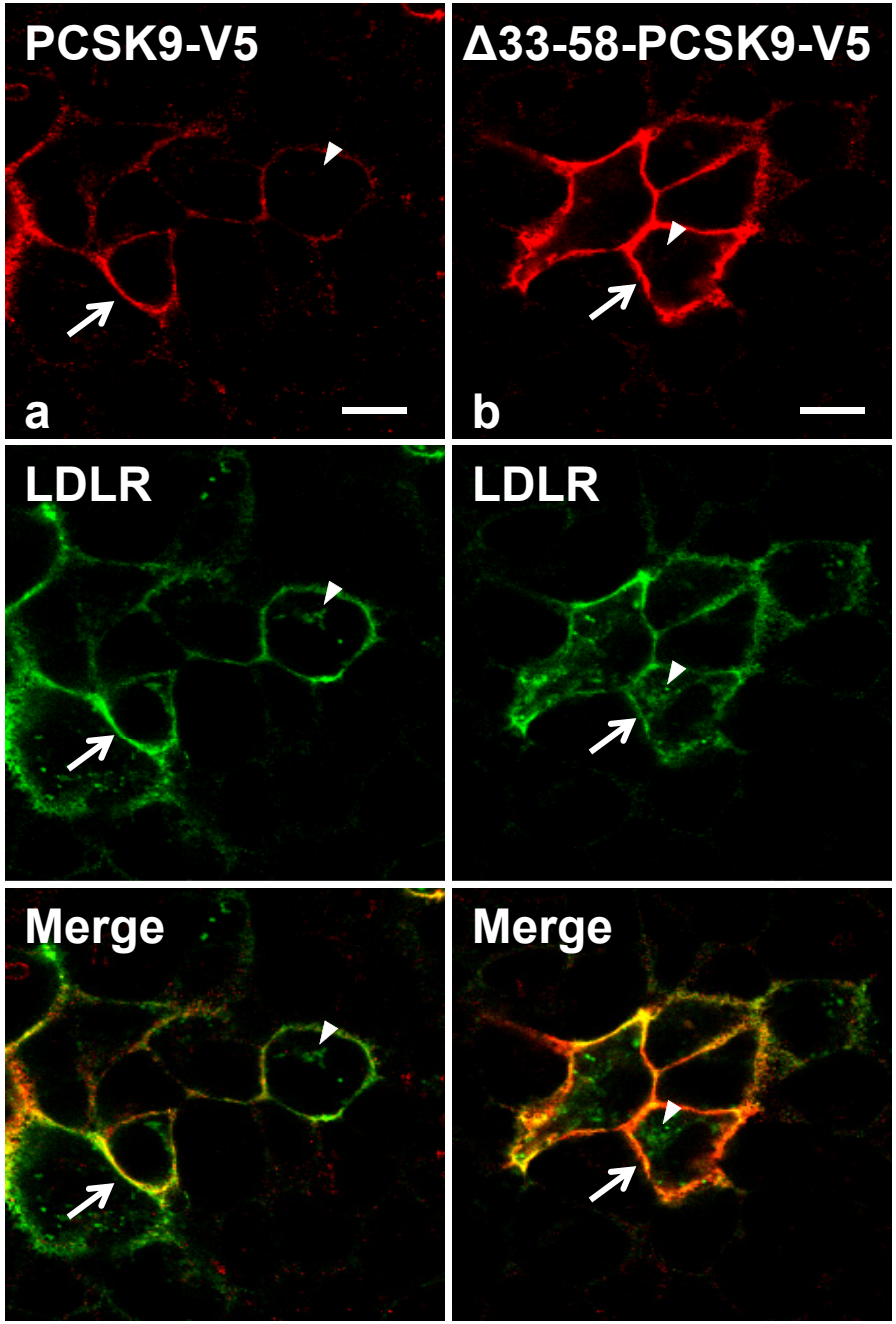


Figure S1A

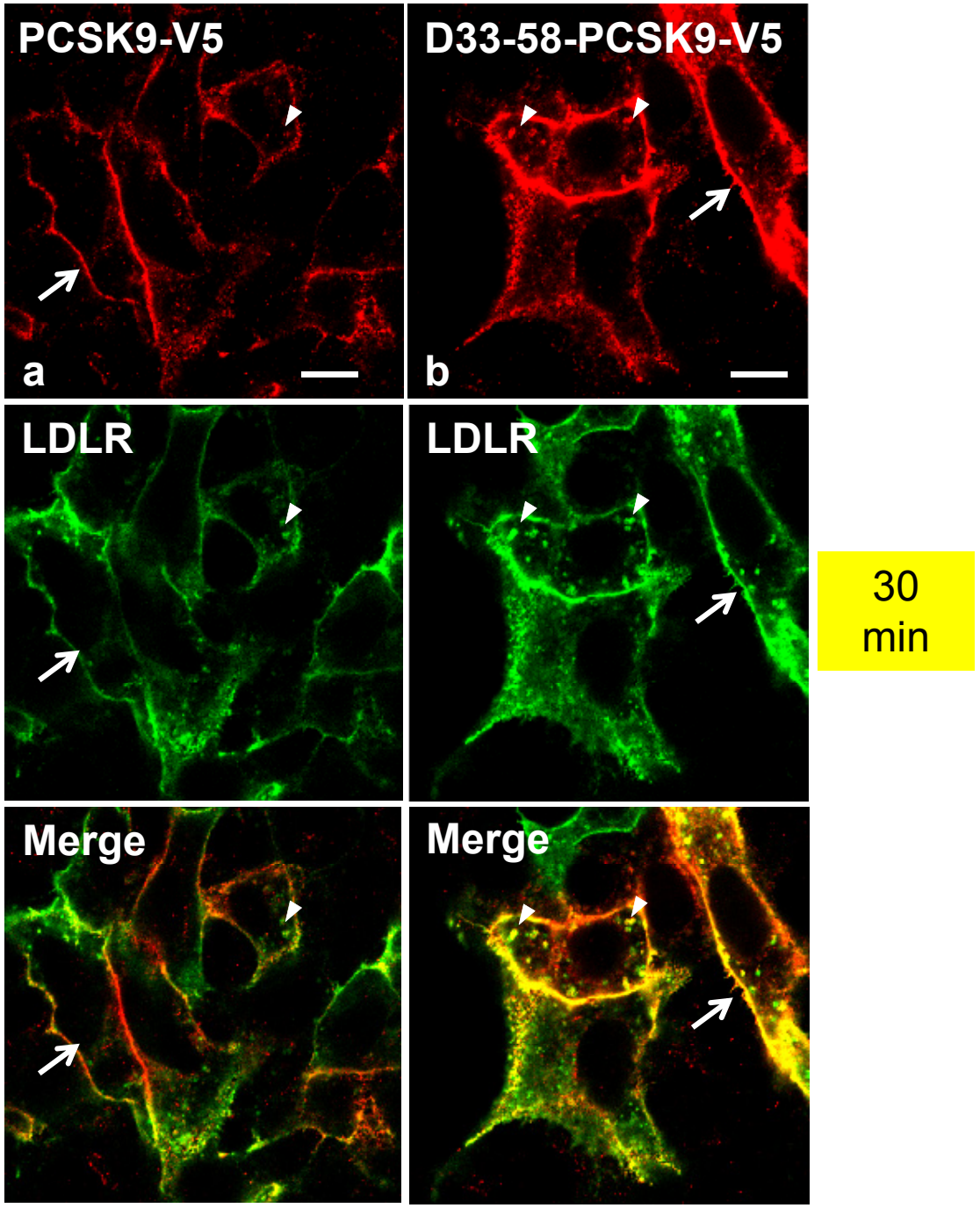


Figure S1B

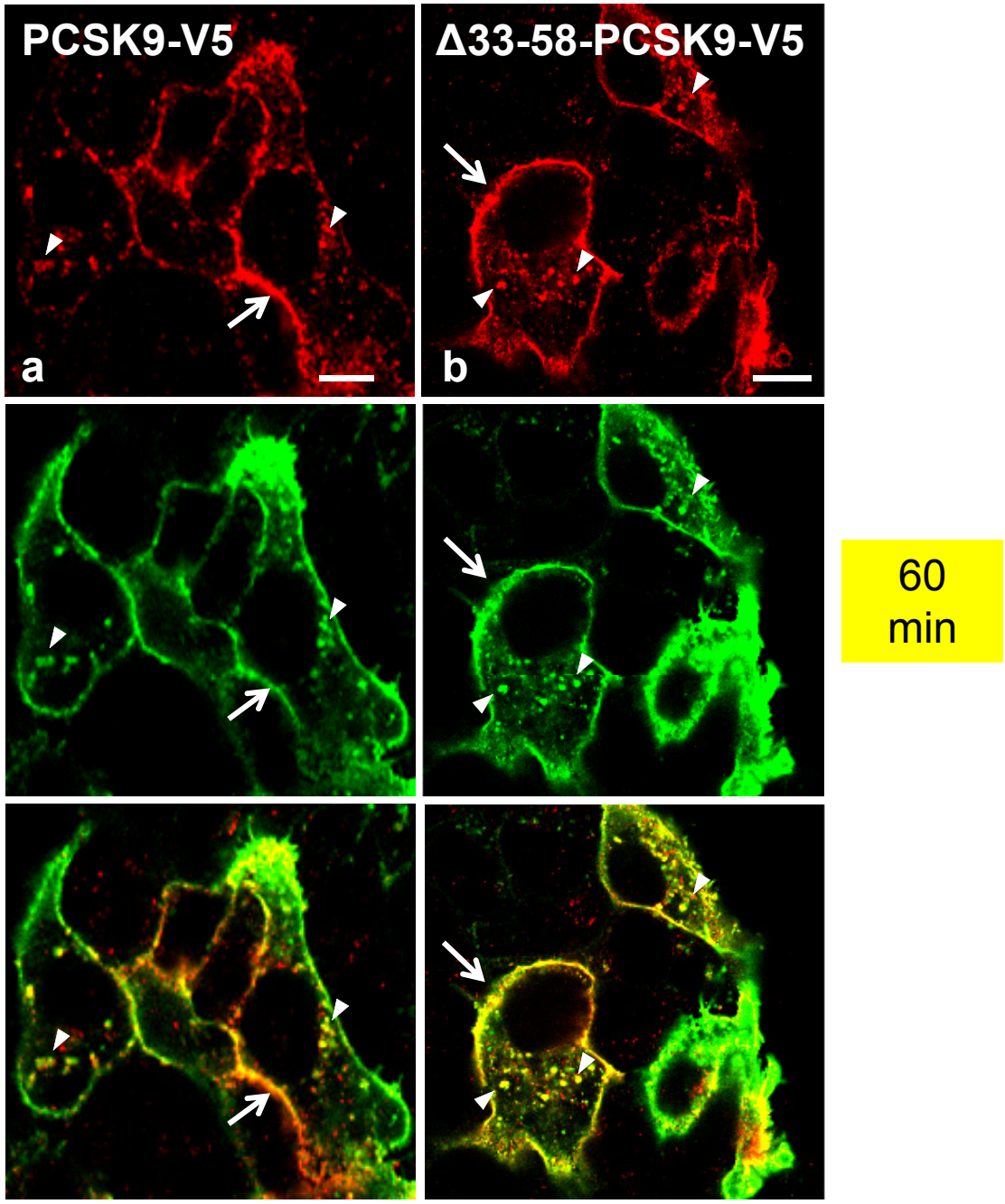


Figure S1C

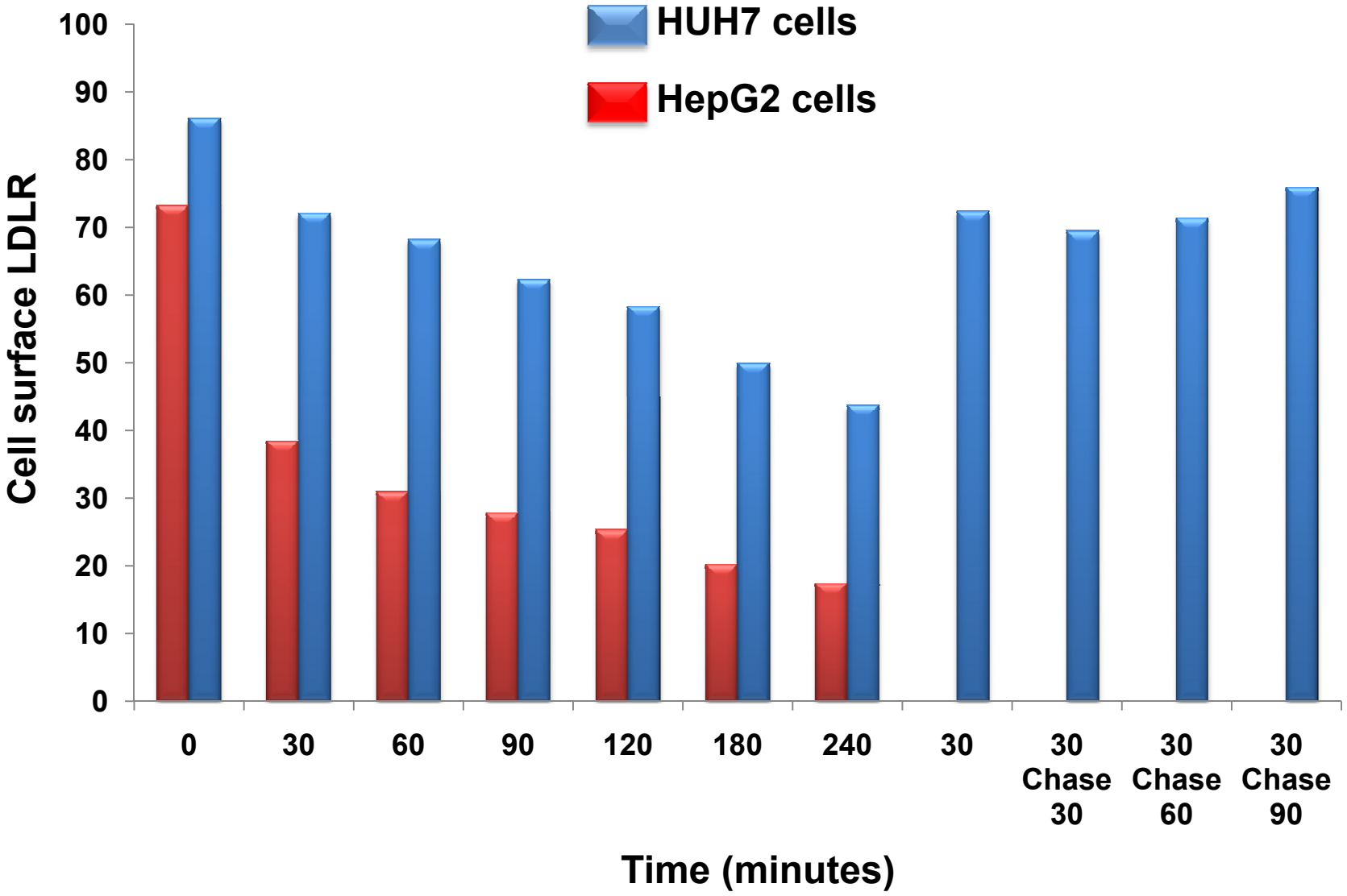
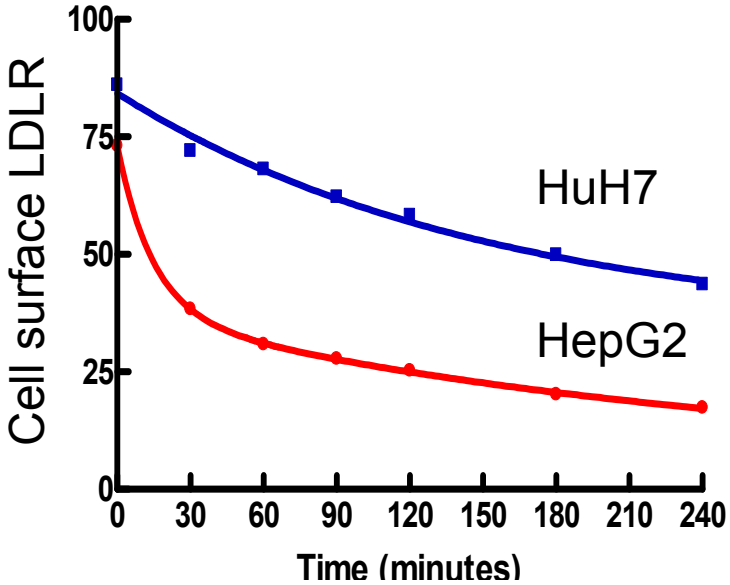

HUH7 cells
HepG2 cells
Cell surface LDLR
100
90
80
70
60
50
40
30
20
10
0
0
30
60
90
120
180
240
30
30 Chase 30
30 Chase 60
30 Chase 90
Time (minutes)

Figure S2A


Cell surface LDLR
100
75
50
25
0
0
30
60
90
120
150
180
210
240
Time (minutes)
HuH7
HepG2

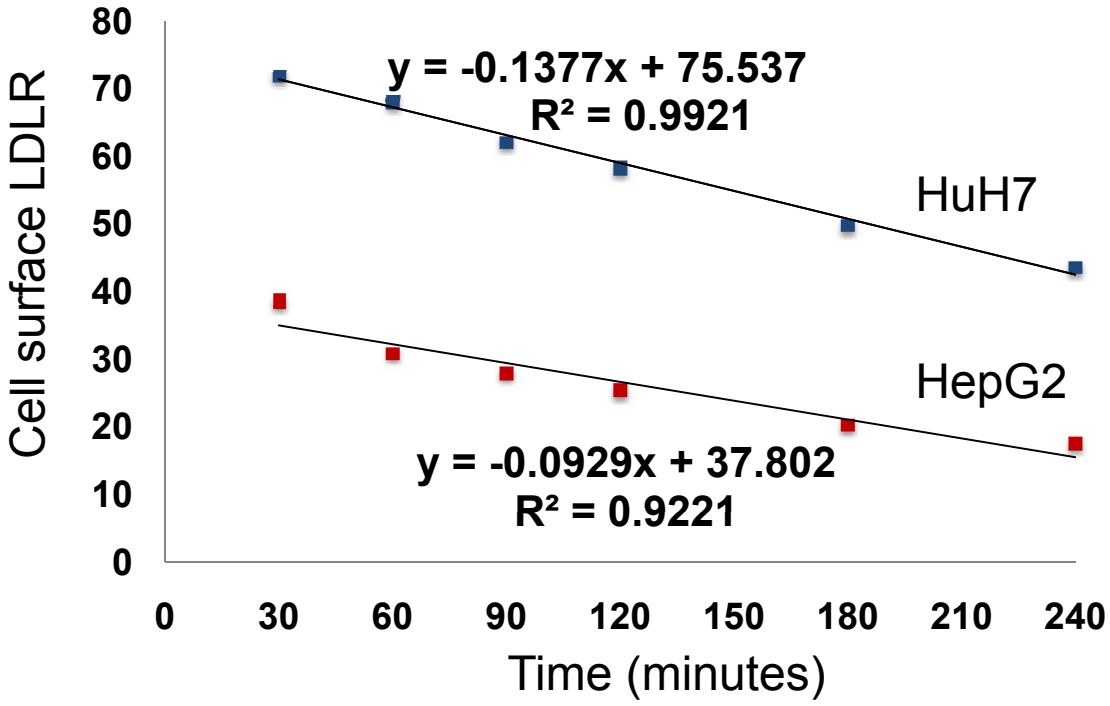

Cell surface LDLR
80
70
60
50
40
30
20
10
0
y = -0.1377x + 75.537
R² = 0.9921
HuH7
HepG2
y = -0.0929x + 37.802
R² = 0.9221
0
30
60
90
120
150
180
210
240
Time (minutes)

Figure S2B

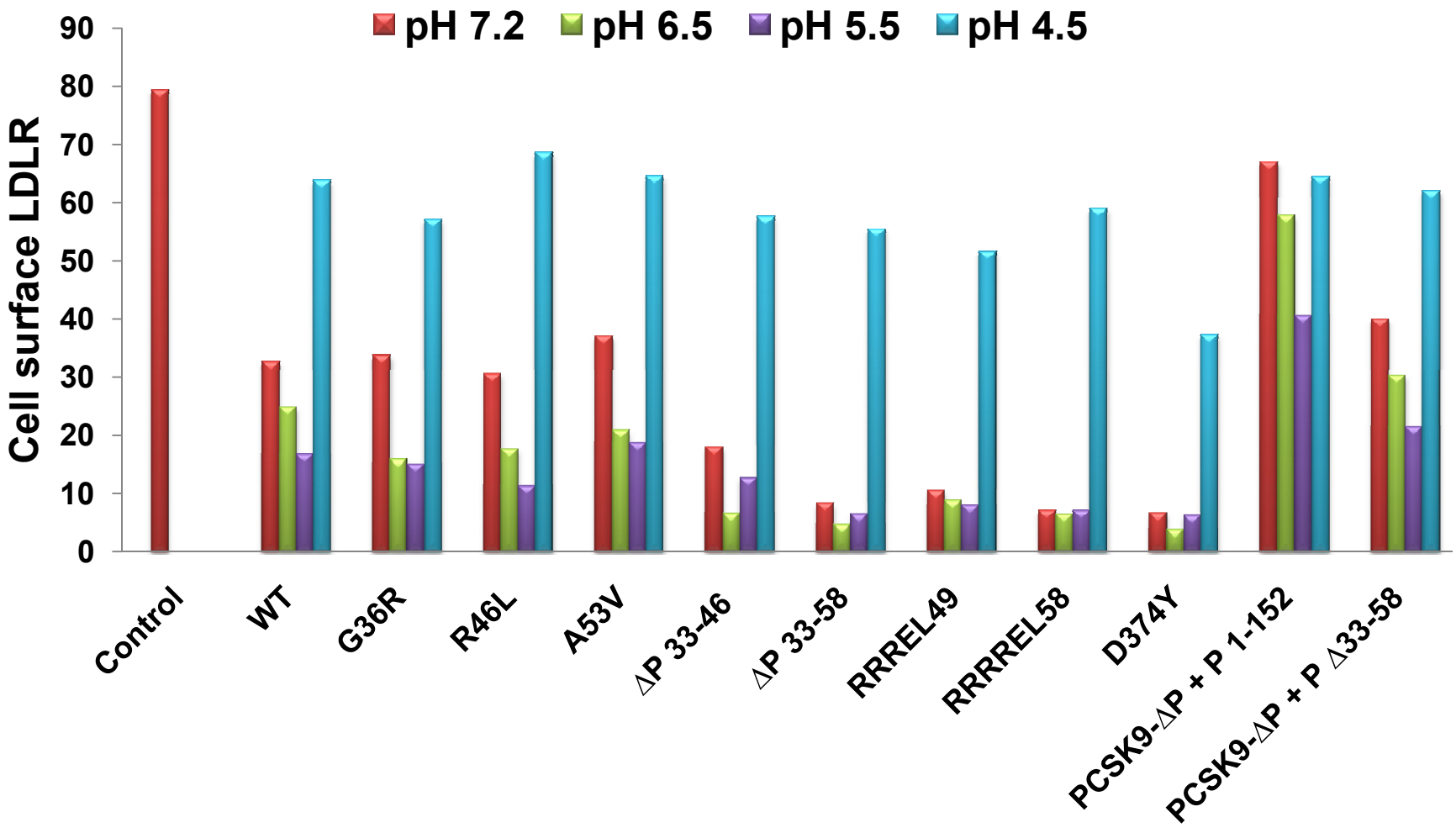

pH 7.2
pH 6.5
pH 5.5
pH 4.5
Cell surface LDLR
90
80
70
60
50
40
30
20
10
0
Control
WT
G36R
R46L
A53V
ΔP 33-46
ΔP 33-58
RRREL49
RRRREL58
D374Y
PCSK9-ΔP + P 1-152
PCSK9-ΔP + P Δ33-58

Figure S3

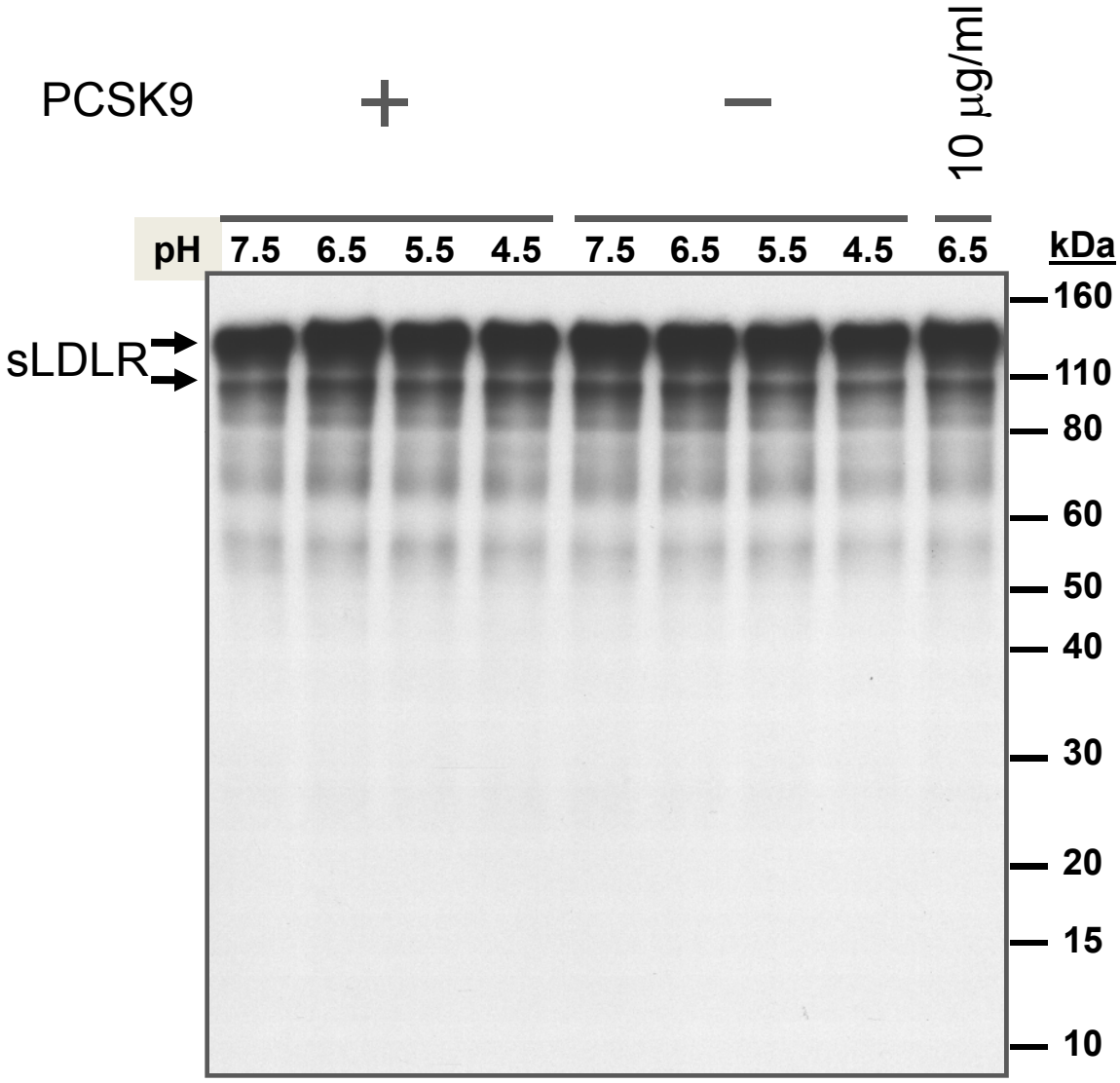

PCSK9
+
−
10 µg/ml
pH
7.5
6.5
5.5
4.5
7.5
6.5
5.5
4.5
6.5
kDa
160
110
80
60
50
40
30
20
15
10
sLDLR

Figure S4

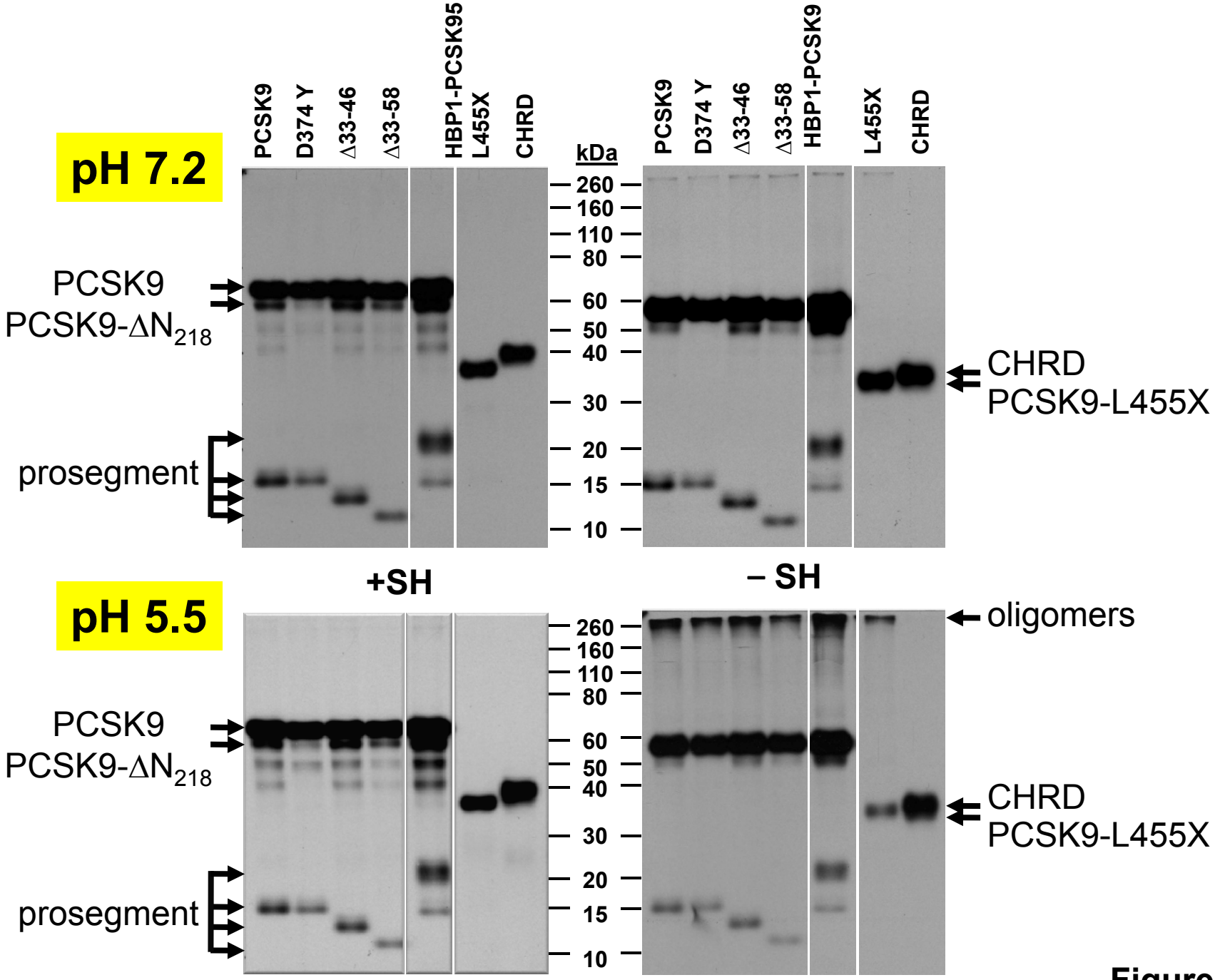

pH 7.2
PCSK9
D374 Y
Δ33-46
Δ33-58
HBP1-PCSK95
L455X
CHRD
kDa
260
160
110
80
60
50
40
30
20
15
10
PCSK9
PCSK9-ΔN218
prosegment
CHRD
PCSK9-L455X
+SH
– SH
pH 5.5
oligomers

Figure S5

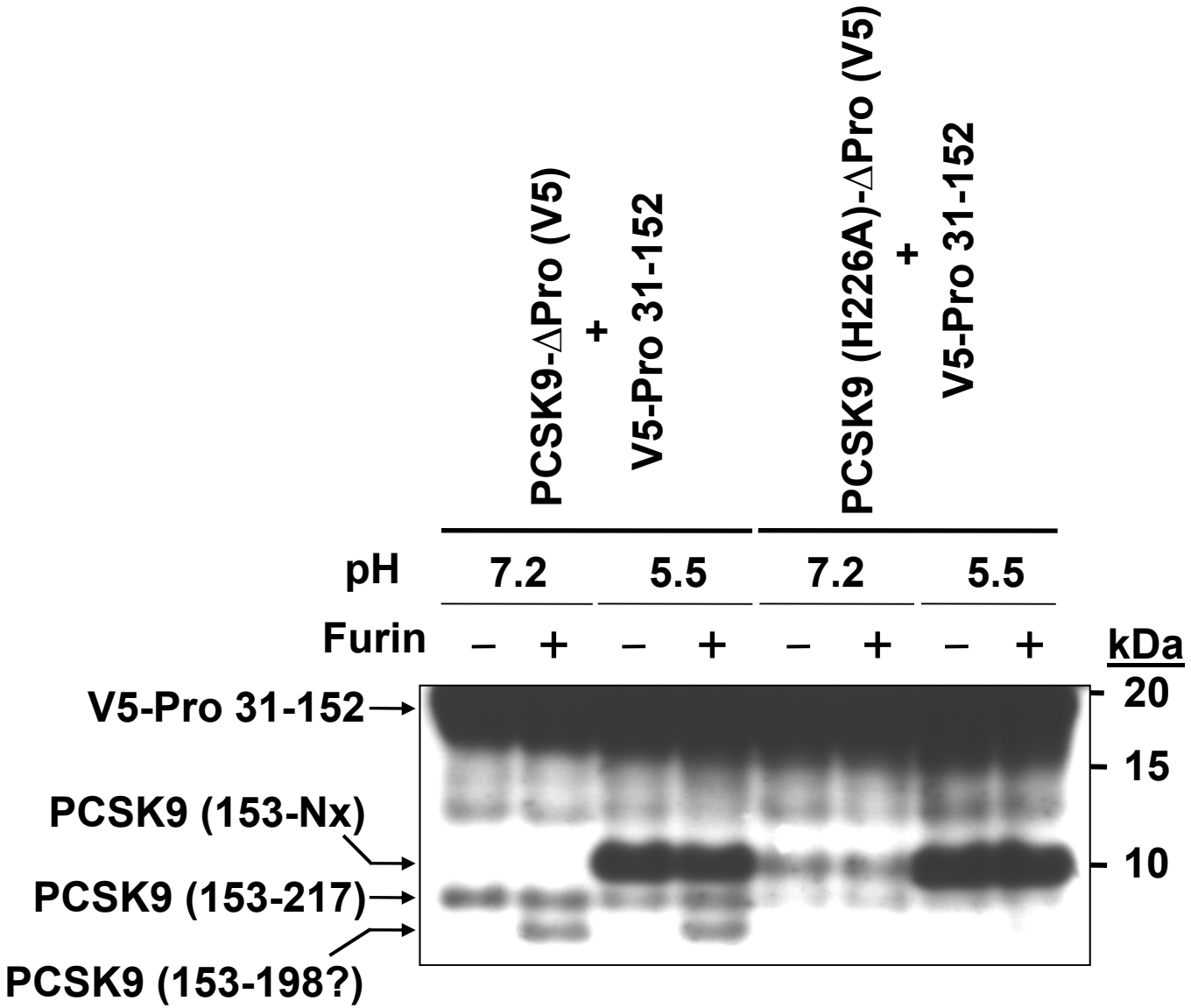

PCSK9-ΔPro (V5)
+
V5-Pro 31-152
PCSK9 (H226A)-ΔPro (V5)
+
V5-Pro 31-152
pH
7.2
5.5
7.2
5.5
Furin
– + – + – + – +
kDa
20
15
10
V5-Pro 31-152
PCSK9 (153-Nx)
PCSK9 (153-217)
PCSK9 (153-198?)

Figure S6