SUPPLEMENTAL MATERIAL

An FTS/Hook/p107^{FHIP} complex interacts with and promotes endosomal clustering by the Homotypic Vacuolar Protein Sorting (HOPS) Complex

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Contents: Supplemental Figures S1-S7.

Figure Legends

Figure S1. Characterization of the Hook/FTS interaction.

Sequence alignment of human and Drosophila Hook proteins, depicting the predicted coiled-coil region (indicated by green “C”) and the C-terminal helical region (indicated by red “H”. The indicated sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and displayed using Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

Figure S2. Characterization of the Hook/FTS interaction.

(A) Anti-Flag immune complexes were prepared from HEK293T cell extracts or from HEK293T/Flag-HA-FTS cells and these complexes along with crude lysates subjected to immunoblotting using the indicated antibodies. FTS was found to interact with Hook1, Hook2, and Hook3, confirming the results of mass spectrometry.

(B) Anti-FTS antibodies immunoprecipitate Hook1 and Hook3. Lysates from HEK293T cells were subjected to immunoprecipitation using the available FTS antibodies which IP only poorly. Nevertheless, Hook1 and Hook3 were detected in these immune complexes (lane 3) but not in control complexes (lane 2).

(C) FTS protects Hook1$^{657-728}$ from degradation via the proteasome. The indicated Hook1 proteins were expressed alone or in combination with GST-FTS. Prior to harvesting, cells were incubated with MG132 (20 μM, 8 h) prior to analysis of lystates by immunoblotting. As expected, Hook1$^{657-728}$ was readily detected in extracts from cells expressing GST-FTS independent of the presence of MG132. In contrast, Hook1$^{657-728}$ was absent when FTS was not co-expressed but was present under these conditions when MG132 was added. These data indicate that FTS protects this C-terminal fragment of Hook1 from turnover through the proteasome.

(D) Immunoblotting of extracts demonstrates depletion of the indicated proteins by RNAi. Cells were transfected with the indicated siRNAs and after 72 h, extracts were subjected to immunoblotting with the indicated antibodies.

Figure S3. Multi-sequence alignment of p107^{FHIP} and related proteins.

(A) The indicated sequences were aligned using ClustalW and displayed using Boxshade.

(B) Analysis of p107^{FHIP} for coiled-coil regions using the Coils Server at www.ch.embnet.org/software/COILS_form.html.

Figure S4. Depletion of FTS reduces the ability of Vps18 to promote late endosome/lysosome clustering.

(A) HeLa cells were transfected with control siRNA or siRNA targeting the indicated gene. After 48 h, cells were transfected with GFP-Vps18 and 60 h later, late endosomal/lysosomal clusters were examined immunofluorescence using anti-LAMP1 antibodies in conjunction with detection with Alexa598 conjugated secondary antibodies (red). GFP-Vps18 was identified by GFP fluorescence. In order to determine the integrated intensity for LAMP1 within clusters, a threshold (+ Threshold) was applied such that the maximal pixel signal was in the linear range. In the absence of threshold (- Threshold), individual vesicles not present within clusters can be seen in cells wherein the indicated gene was targeted for depletion. Two independent siRNAs were used for each gene.

Figure S5. The frequency of cells displaying LAMP1 staining intensity greater than 10,000 pixels within the GFP-Vps18 cluster is reduced upon depletion of FTS, Hook, and p107^{FHIP} proteins.

(A) HeLa cells were transfected with control siRNA or siRNA targeting the indicated gene. After 48 h, cells were transfected with GFP-Vps18 and 60 h later, late endosomal/lysosomal clusters were examined immunofluorescence using anti-LAMP1 antibodies in conjunction with detection with Alexa598 conjugated secondary antibodies (red). GFP-Vps18 was identified by GFP fluorescence. In order to determine the integrated intensity for LAMP1 within clusters, a threshold (+ Threshold) was applied such that the maximal pixel signal was in the linear range. In the absence of threshold (- Threshold), individual vesicles not present within clusters can be seen in cells wherein

the indicated gene was targeted for depletion. The cumulative number of cells displaying the indicated pixel intensity is shown. Two independent siRNAs were used for each gene.

Figure S6. Overexpression of FTS or Hook proteins induces late endosome/lysosome clustering.

(A) HeLa cells were transfected with plasmids expressing GFP, GFP-FTS, Flag-Hook1, Flag-Hook2, or Flag-Hook3. After 60 h, cells were fixed and stained with Lamp1 antibodies as described in Figure 6 legends.

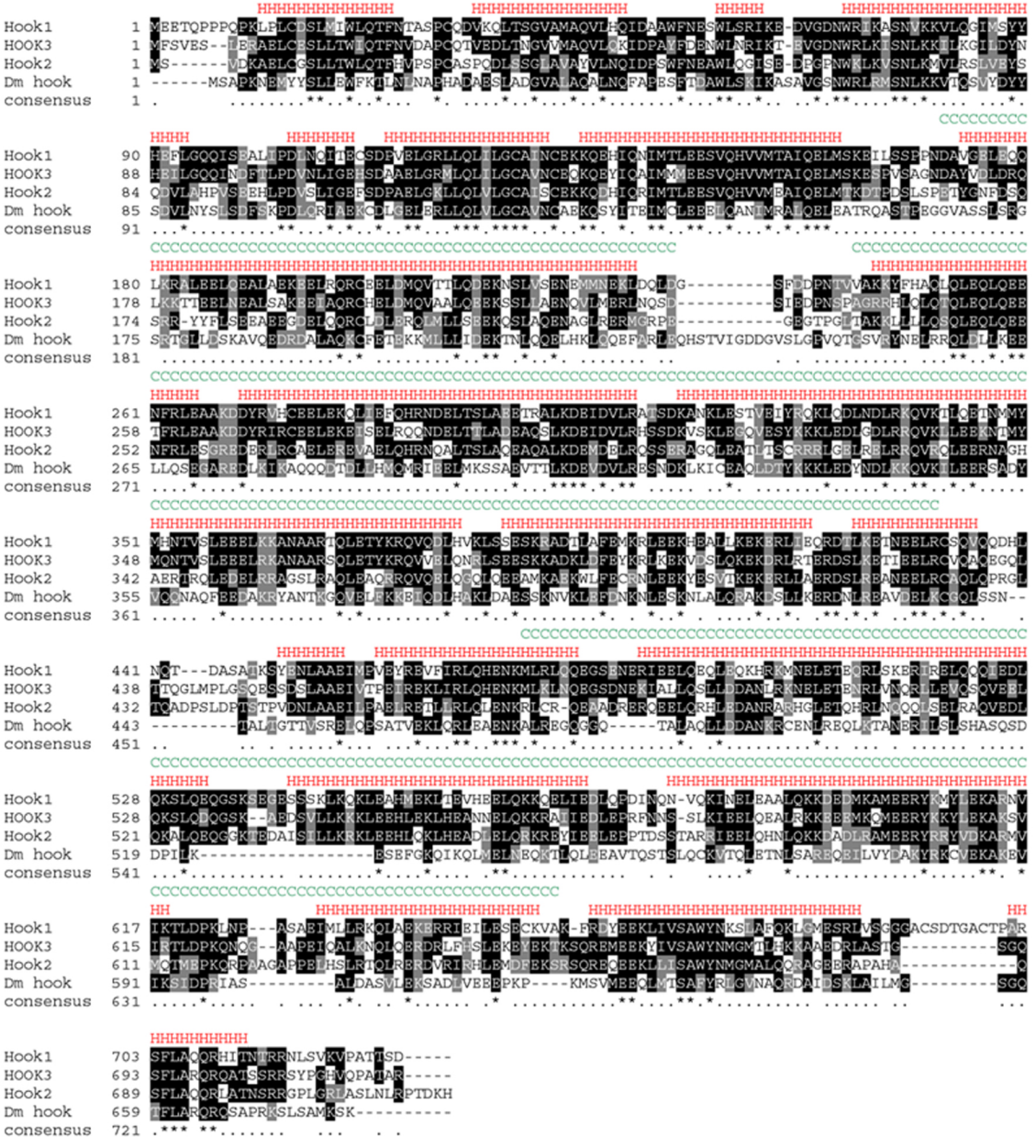
(B) Percentages of transfected cells displaying clear Lamp1 clusters were counted. The mean +/- SEM of two independent experiments is indicated.

Figure S7. Components of the FHF complex are required for efficient decay of internalized EGF.

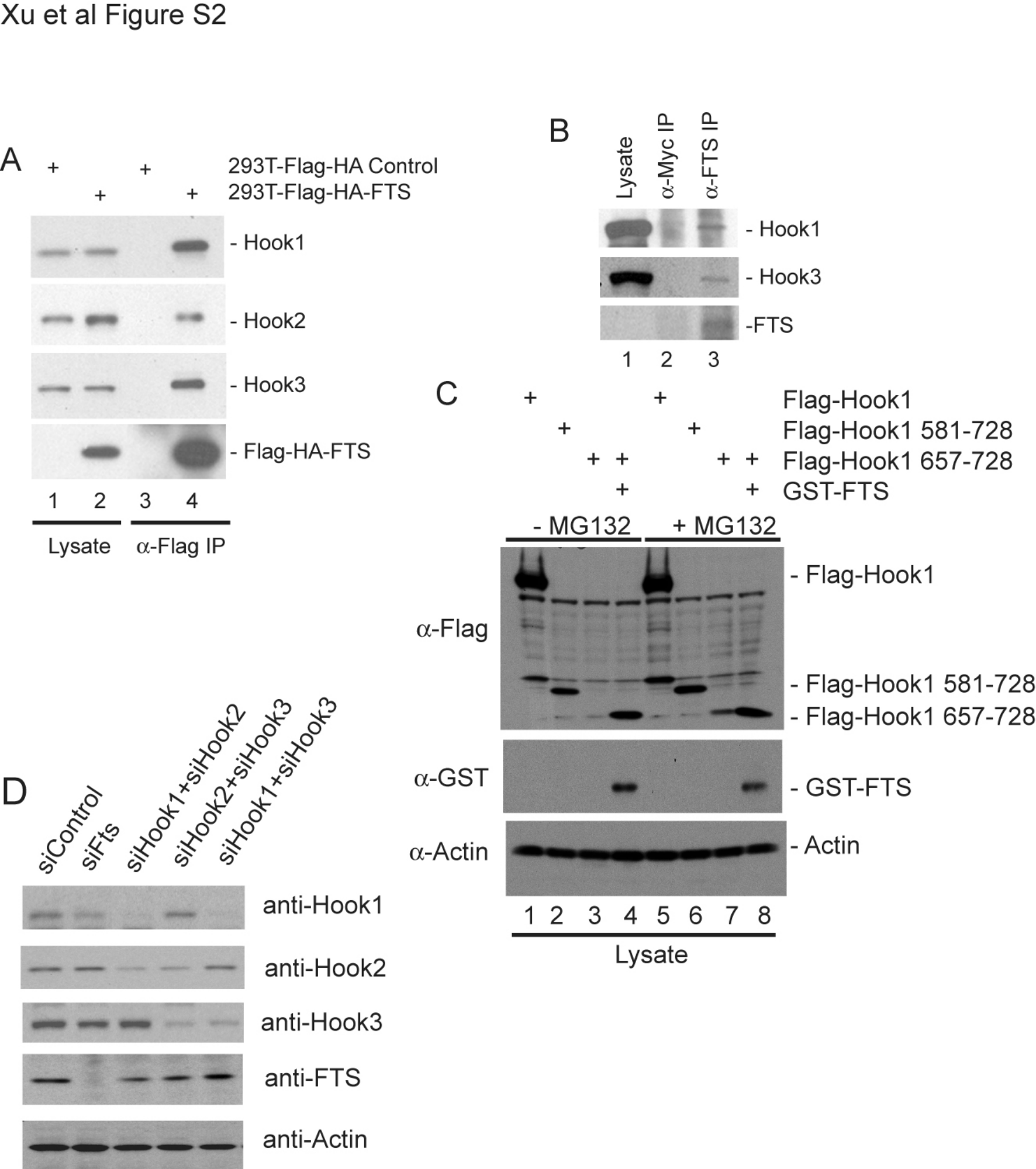
(A-E) Kinetics of Rhodamine-EGF decay in cells lacking FHF complex components. HeLa cells were transfected with the indicated siRNA and EGF decay measured as follows using the previously published assay [J. Cell Biol. 157, 91-101 (2002)]: Hela cells were serum-starved for 2 hours and treated with 500 ng/ml texas red conjugated EGF (EGF-TR) on ice. Cells were left on ice for 15min and then transferred to 19.5^{o}C to initiate the internalization and incubated for 1 hour prior to washing the cells with PBS. Cells were then transferred to 37^{o}C to initiate EGF transit through the endocytic system. At the indicated times, cells were imaged using fluorescence microscopy at the same

settings. Threshold was set using cells not treated with Rhodamine-EGF and cells with at least two Rhodamine-positive punta that exceed the threshold were counted as EGF positive using Metamorph software. In panels B and C, the indicated siRNA-resistant expressed vectors were transfected and transfected cells (based on GFP) counted. (A-D) The percentage of EGF positive cells over total cell numbers were plotted. (E) Images of control, siFTS, and siFTS/FTS rescue vector cells over the timecourse are shown.

Xu et al, 2008 Figure S1



Xu et al Figure S2



Xu et al, 2008 Figure S3A

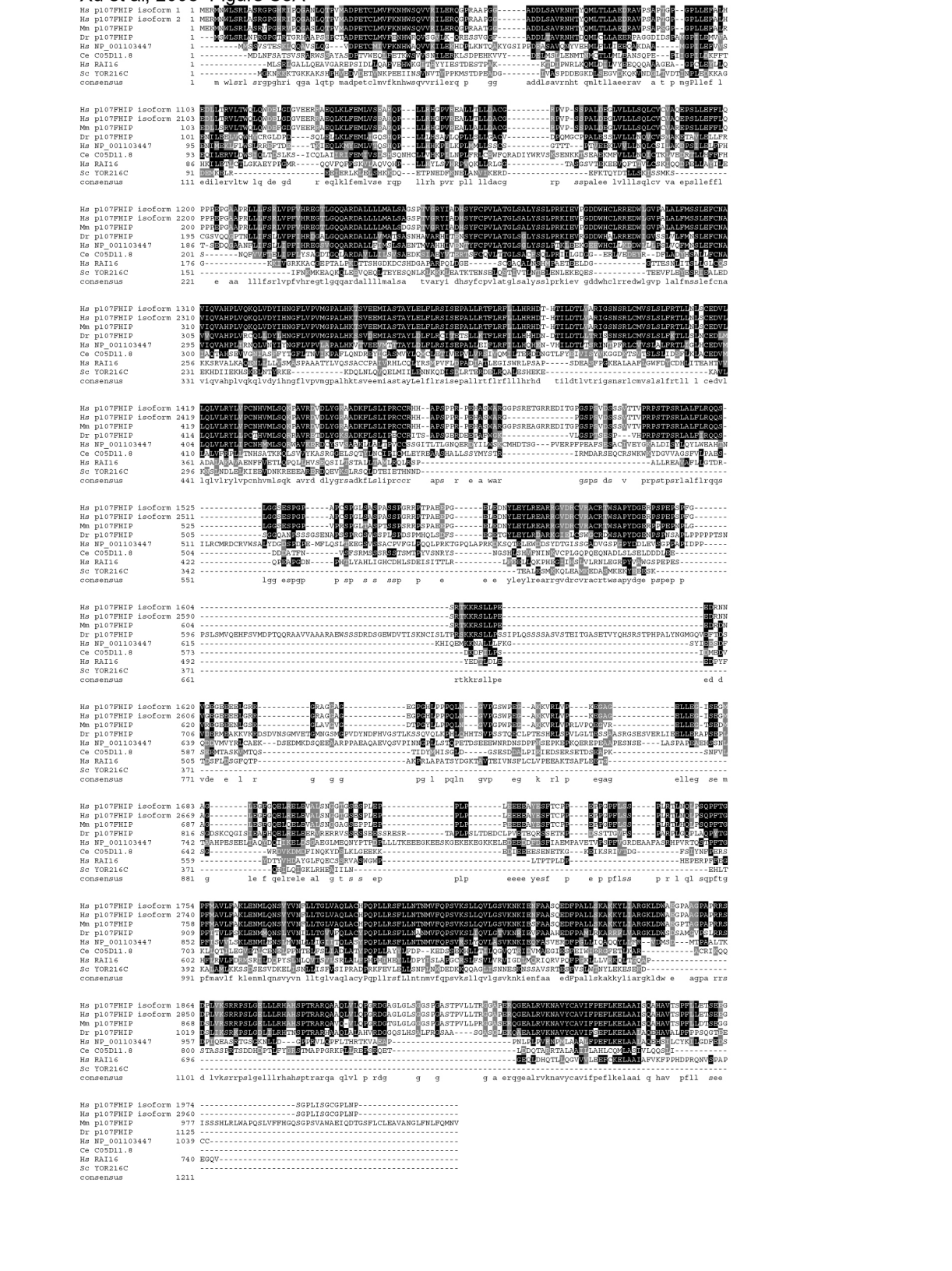
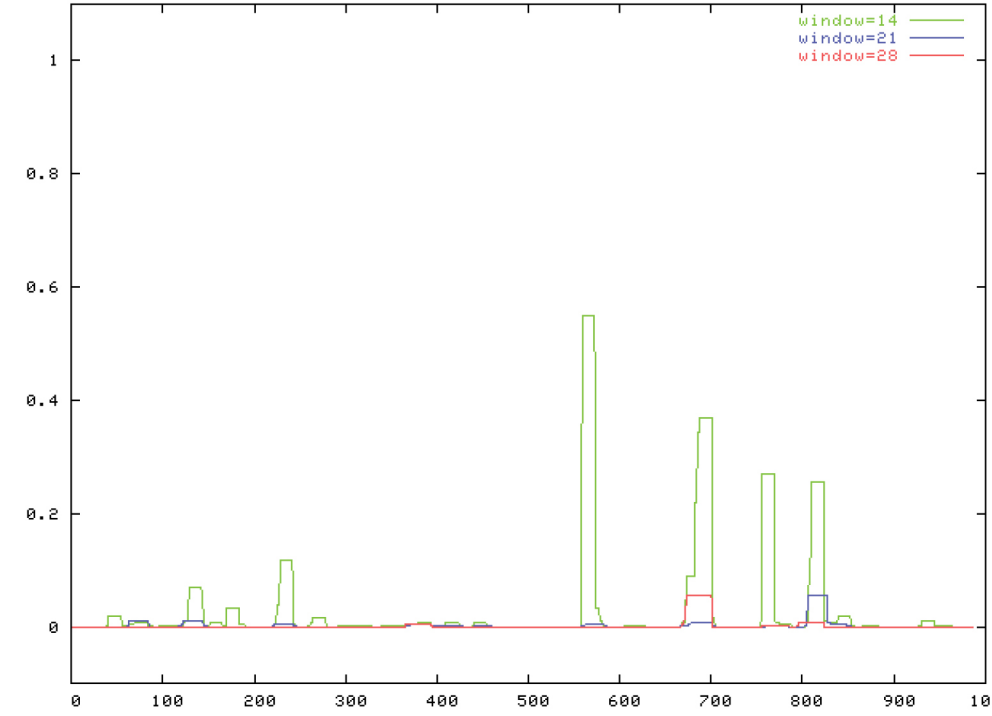
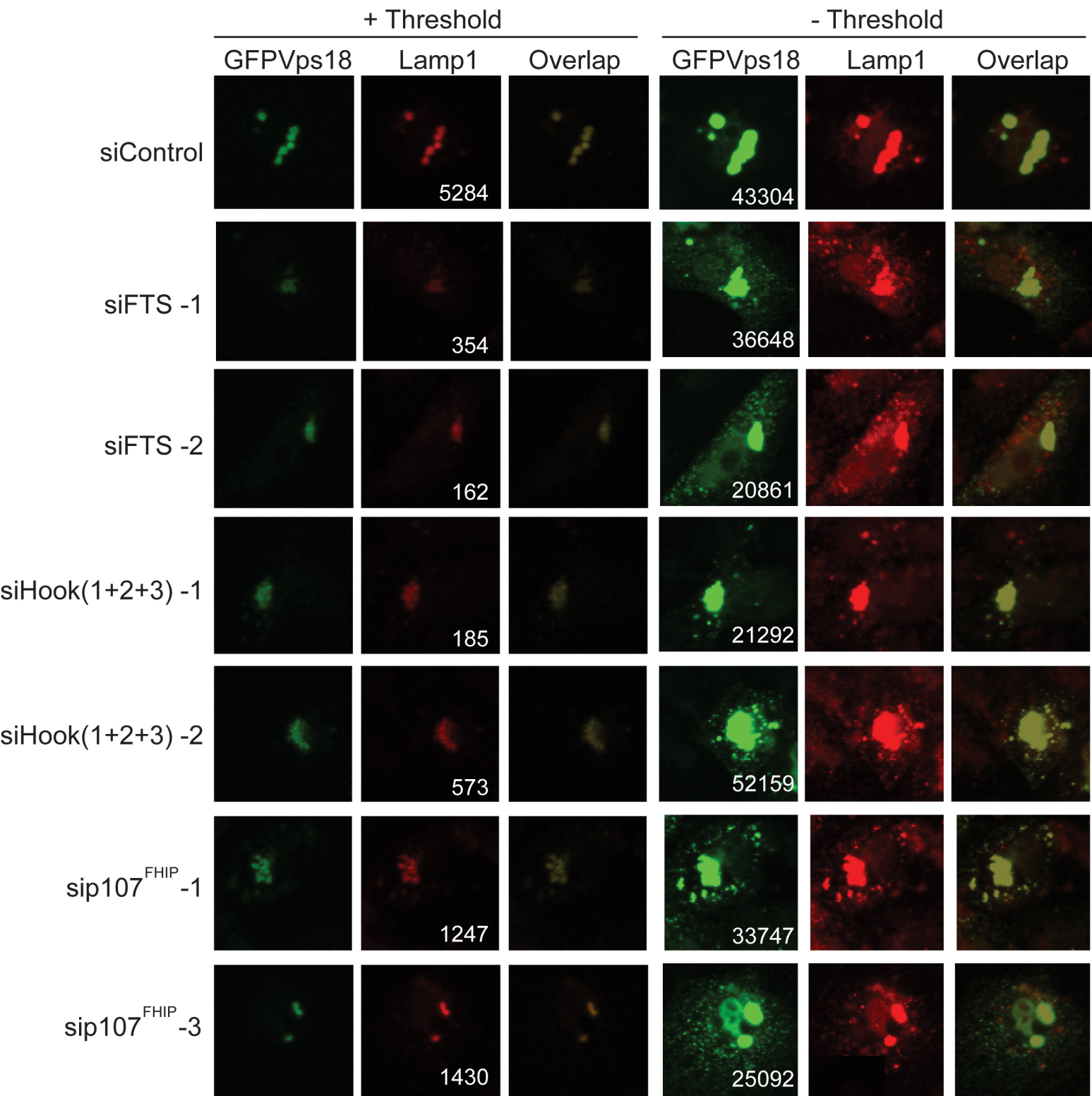


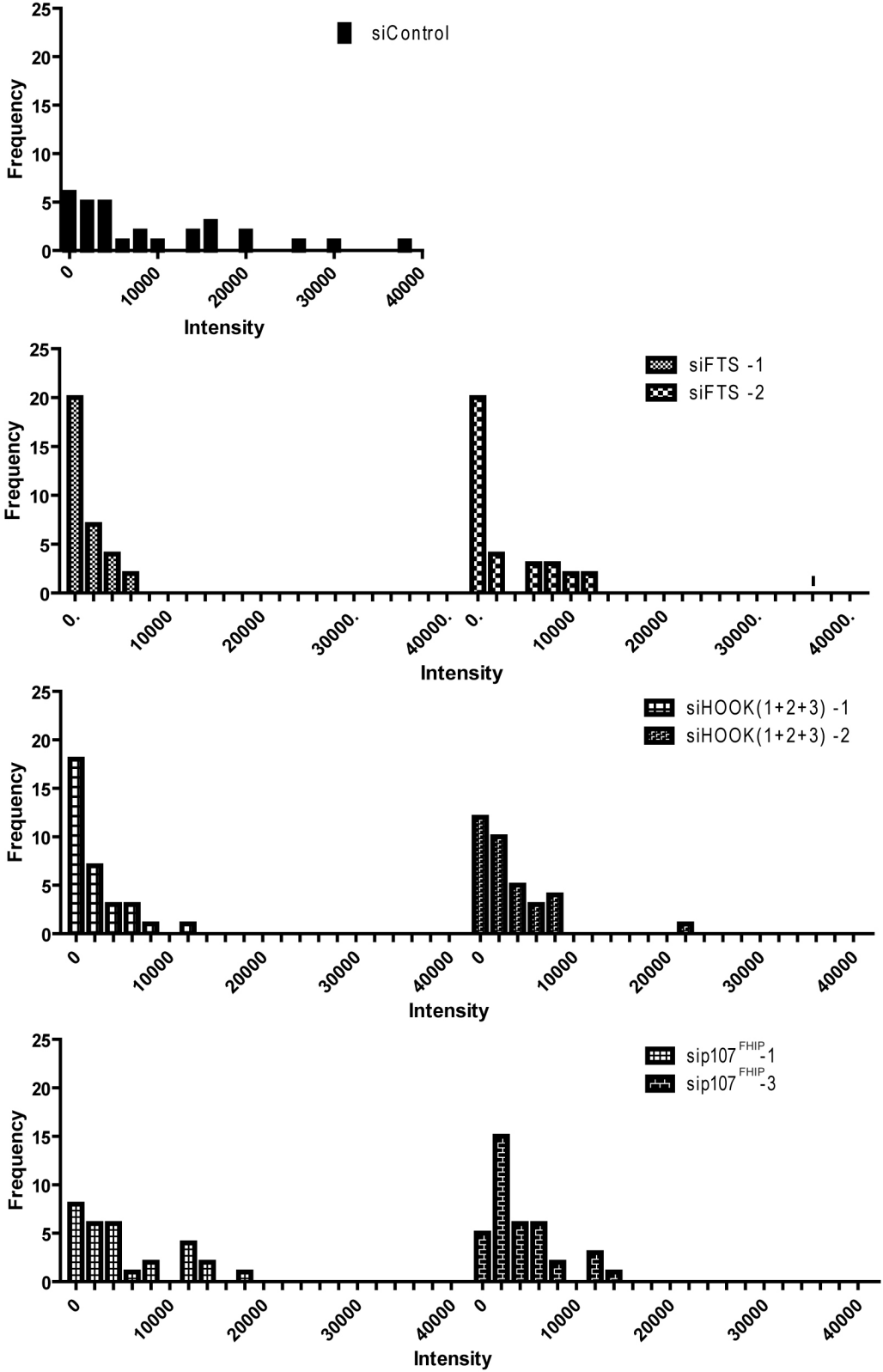
Figure S3B Analysis of p107FHIP for Coiled-coils



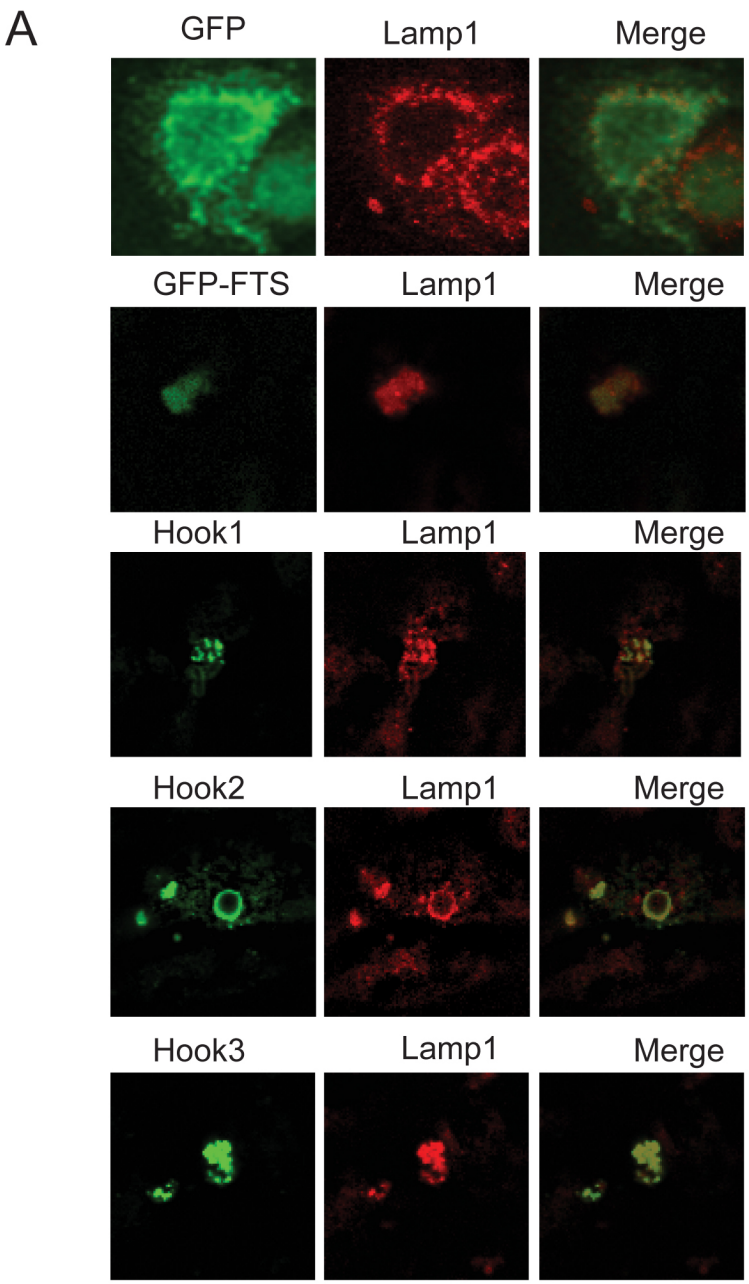
Xu et al, 2008 Supplemental Figure S4.

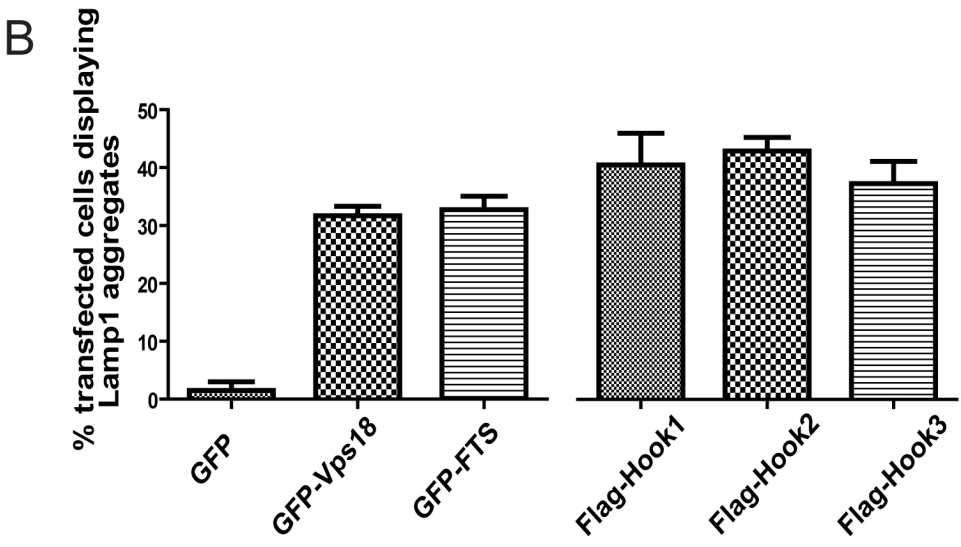


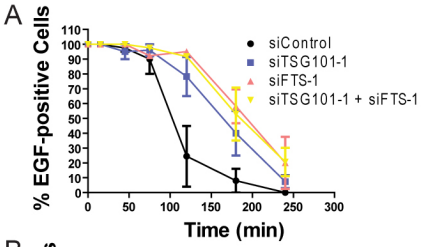
Xu et al., 2008 Figure S5



Xu et al., 2008 Figure S6



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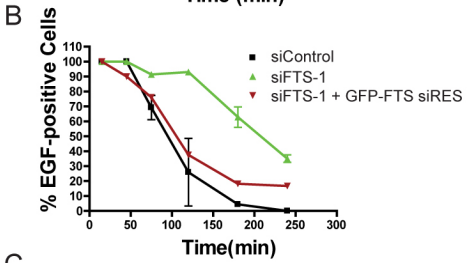
% EGF-positive Cells
Time (min)
siControl
siTSG101-1
siFTS-1
siTSG101-1 + siFTS-1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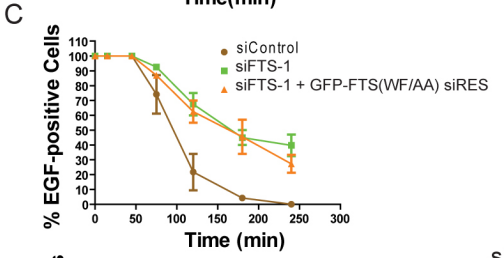
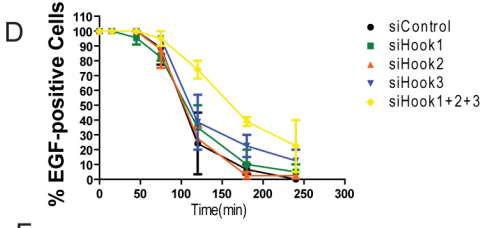
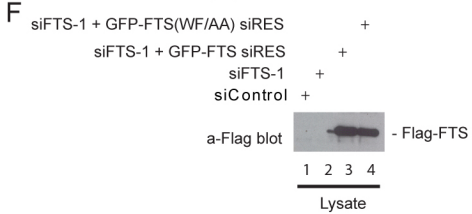
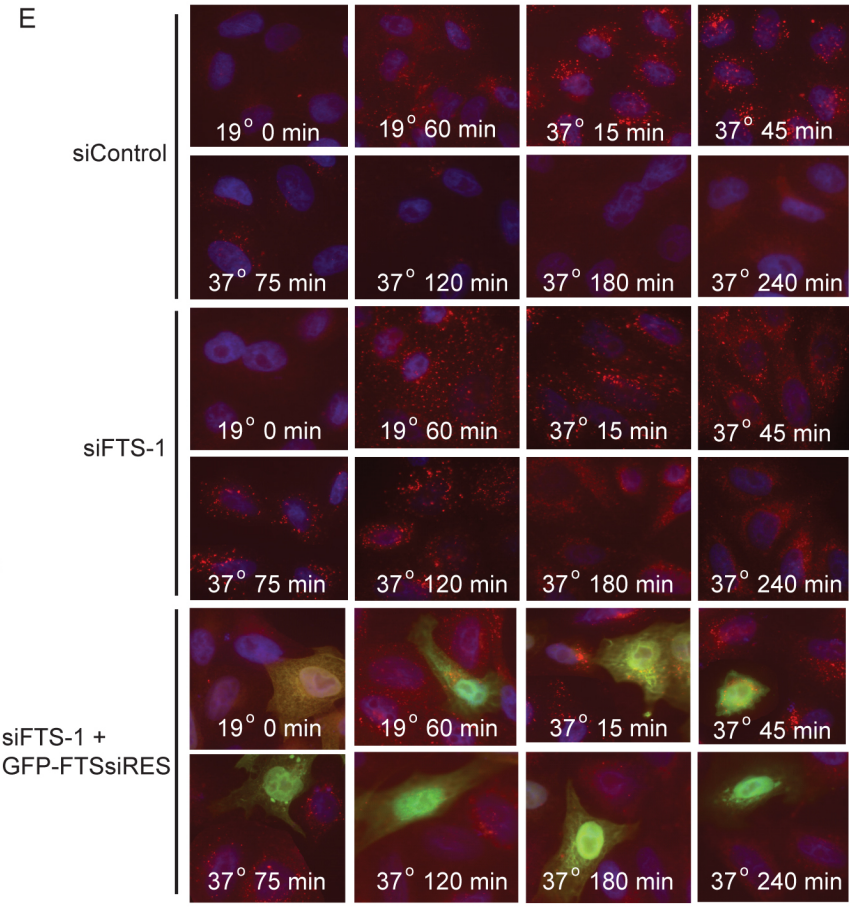
% EGF-positive Cells
Time(min)
siControl
siFTS-1
siFTS-1 + GFP-FTS siRES


C
% EGF-positive Cells
Time (min)
siControl
siFTS-1
siFTS-1 + GFP-FTS(WF/AA) siRES


D
% EGF-positive Cells
Time(min)
siControl
siHook1
siHook2
siHook3
siHook1+2+3


F
siFTS-1 + GFP-FTS(WF/AA) siRES +
siFTS-1 + GFP-FTS siRES +
siFTS-1 +
siControl +
a-Flag blot
- Flag-FTS
1 2 3 4
Lysate


E
siControl
siFTS-1
siFTS-1 +
GFP-FTSsiRES
19° 0 min
19° 60 min
37° 15 min
37° 45 min
37° 75 min
37° 120 min
37° 180 min
37° 240 min