Supplemental data for: Functional characterization of putative cilia genes by high-content analysis

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Supplemental Figure 1. Efficiency of *Ift88* knockdown.

A) S12 cells exhibit robust ciliation, which proved difficult to abolish by *Ift88* knockdown compared to 3T3 or IMCD3 cells. S12 (top) and 3T3 (middle) cells transfected with NTC (left) or *Ift88* (right) siRNAs and treated for 30 min with Hh were methanol fixed and stained for cilia (acetylated tubulin, green), Gli3 (red) and nuclei (DAPI, blue). Cilia with Gli3 at the tips were readily detectable in both control S12 and 3T3 cells, but in si*Ift88*-treated cells there were few detectable cilia in 3T3 cells, while S12 cells retained most cilia but failed to accumulate Gli3 at their tips. IMCD3 HTR6-GFP cells (bottom) were similarly transfected, but stained with anti-acetylated tubulin in the red channel, with HTR6-GFP fluorescence in the green channel. As with 3T3 cells, IMCD3 cilia mostly disappeared (together with the HTR6-GFP cargo) upon *Ift88* knockdown. Insets show two-fold magnifications of a representative cilium (left to right: merge, Ac-Tub, cargo). Scale bar is 10 μm; arrows indicate Gli3 at cilia tips in the insets; percentage ciliation is shown in each corner.

B) IFT88 Western blot of the four cell lines tested for *Ift88* depletion. The indicated cell lines were transfected with non-targeting control (NTC), murine *Ift88* (m88) or human (h88, for hTERT-RPE1 cells) *IFT88* siRNAs as in (A), then lysed in RIPA buffer and Western blotted with rabbit anti-IFT88 (ProteinTech 11797-1-AP at 1:4000) and loading control mouse anti-tubulin (clone 1A2, Sigma T9028, at 1:10,000), followed by HRP-conjugated secondary antibodies and ChemiGlow and ECL luminescence detection, respectively.

C) Quantitation of IFT88 knockdown. Western blot quantitation of the same samples used in (B), normalizing IFT88 to the tubulin loading control (using multiple exposures of the same blot) and plotting as a percentage of the siNTC. IFT88

protein was depleted by at least 85-98% in the murine cell lines, and slightly less well (~80%) in the human RPE1 cells.

D) Comparison of IFT88 and IFT46-GFP knockdown efficiency. 3T3 wild type or IFT46-GFP 3T3 stables were transfected as in (B) with NTC and either *Ift88* or *Ift46* siRNAs respectively. This enabled comparison of endogenous IFT88 with IFT46-GFP knockdown by Western blotting with anti-GFP antibodies, since no commercial antibodies to IFT46 are available. Blot shown is representative of 2 experiments.

E) Quantitation of the Western blot in (D), performed as in (C). Both proteins were efficiently depleted, more so for endogenous IFT88 (99%) than the stably expressed IFT46-GFP (87%).

Supplemental Figure 2. Hedgehog signaling controls.

A) Comparison of knockdown efficiency in 3T3 (magenta), S12 (blue) and IMCD3 HTR6-GFP (green) cells by TaqMan analysis. The mean and SDs of one (3T3 and S12) or two (IMCD3) triplicate experiments plotting the percentage of target mRNA remaining compared to the NTC are shown. Raw Ct values of each cell line treated with NTC siRNA are also shown on the x-axis for comparison of expression levels (higher Ct values indicate lower mRNA levels).

B) Control SV40-luciferase assay shows no off-target effects of siRNAs on luciferase levels. C3H10T1/2 cells stably transfected with the luciferase reporter gene under control of a constitutive SV40 promoter were transfected for 64 h with the indicated pooled siRNAs and the luciferase levels measured following a further 24 h serum starvation and Hh addition (mimicking the S12 assay conditions). Top screen gene hits are typed in red and controls in blue. The means and SDs of one experiment performed in triplicate plates are shown. The class numbers are labeled in magenta, with “No effect” meaning the genes with no cilia phenotypes due to lack of either knockdown or expression.

C) Hh signaling defects are not driven by changes in cell viability. Hh signaling was measured by the S12 *Gli*-luciferase assay (black) and cell viability was measured using the CellTiter-Glo assay (white) in parallel plates following serum starvation

and Hh stimulation for 24 h. Genes are sorted in increasing order of S12 Hh signaling (lowest on left). Top screen gene hits typed in red and controls in blue. The means and SDs of three independent triplicate S12 experiments and one viability experiment performed in triplicate are shown.

Supplemental Figure 3. 3T3 SEM and IMCD3 SEM phenotype quantitation.

A) Representative SEM images of 3T3 cells. Under these conditions, it is difficult to distinguish primary cilia from microvilli. Scale bar represents 20 μm in left panel (500x magnification) and 2.5 μm in the right panel (4000x magnification image of the boxed region).

B) Histogram of cilia lengths in siRNA-treated IMCD3 HTR6-GFP cells. The lengths of up to 60 cilia per siRNA transfection were measured in Adobe Photoshop® and the results plotted as histograms. In accordance with the immunofluorescence data, depletion of *Ift88*, *Dync2h1*, and the class I genes *Ssa2* and *mC21orf2* greatly reduced the number of ciliated cells and also the mean length of the remaining cilia (from >4 μm to ~1 μm) compared to the NTC. DYNC2H1 is a subunit of the retrograde IFT motor (Huangfu and Anderson, 2005), used as a control for inhibited retrograde transport. By contrast, knockdown of class III genes *Ptpdc1* and *Iqub* increased cilia lengths, in agreement with the immunofluorescence measurements in Figure 5, D and E. Class V gene disruption did not affect the mean cilia length, but in the case of *Ssna1* did slightly decrease the total number cilia in IMCD3 cells (an effect that was not seen in 3T3 cells; Figure 6B).

Supplemental Figure 4. Validation of anti-SSNA1 and anti-NME7 antibodies.

A) *Ptpdc1* knockdown increases the total amount of Gli3 per cilium. The ~200 cilia in the three experiments scored qualitatively for the presence of Gli3 at tips in Figure 5B were measured for fluorescence intensity using Image J (using a circular area of 52). The means and SDs of the three independent experiments are plotted and reveal an average 1.7x increase in Gli3 intensity with *Ptpdc1* knockdown (*, $p \leq 0.05$ vs. NTC).

B) Anti-SSNA1 and anti-NME7 polyclonal antibodies recognize their murine homologs. COS7 cells were transfected with C-terminally GFP tagged *Ssna1* (top) or *Nme7* (bottom) for 21 h, methanol fixed and stained with rabbit anti-SSNA1 (ProteinTech 11797-1-AP at 2.5 μg/mL) or rabbit anti-NME7 (Santa Cruz H278 at 1 μg/mL), respectively, followed by Cy3-conjugated anti-rabbit secondary antibodies (left panels). GFP fluorescence (green, center); merged overlays with DAPI (blue, right). Note that transient overexpression of *Ssna1*-GFP (and untagged *Ssna1*, not shown) results in variably sized inclusion bodies (arrows; a relatively low expressing cell is shown), while NME7-GFP staining is predominantly cytoplasmic. However, both antibodies specifically recognized their respective antigens, co-localizing with GFP fluorescence. Upon stable expression, both proteins were detected at centrosomes (Figure 7A). Scale bar is 20 μm.

C) HTR6-GFP fluorescence intensity in IMCD3 cilia of class V depleted cells. HTR6-GFP fluorescence intensity along the entire cilium was measured using Image J and normalized to the acetylated tubulin signal for each cilium to control for differences in focus or image brightness. The means and SDs of ≥120 cilia are shown (* $p \leq 0.05$ vs. NTC). *Nme7* knockdown decreases the average ciliary intensity of HTR6, while *Ssna1* depletion increases it, consistent with their proposed roles in transport to and from the cilium, respectively.

D) Western blot validation of the anti-NME7 polyclonal antibody. Left panel: 10% SDS-PAGE Western blot of 293 cells alone (lane 1) or transfected with untagged *Nme7* (lane 2), *Nme7*-GFP (lanes 3,5) or GFP-*Nme7* (lanes 4,6) (both LAP tags) using rabbit anti-NME7 (H287, Santa Cruz) in lanes 1-4 or anti-GFP (JL-8, Clontech) in lanes 5,6. Anti-NME7 recognizes bands of the expected sizes for NME7 at ~47kDa (lane 2) and at ~75 kDa for GFP-tagged NME7, also recognized by anti-GFP. Asterisk denotes a non-specific band in 293 cells. Right panel: Lanes 7,8: *Nme7*-GFP stably transfected in 3T3 cells detected with anti-GFP or anti-NME7 respectively. Lanes 9-11: untransfected 3T3, IMCD3 and hTERT-RPE1 (RPE1) cells blotted with anti-NME7; a potential band of the correct size is detected, but also many other nonspecific bands. Lanes 1-6 were

developed with ECL reagent, and lanes 7-11 were with the more sensitive ChemiGlow. Molecular weights (kDa) are indicated on the left. Samples were all run on the same blot, with white gaps indicating cuts.

E) Western blot validation of the anti-SSNA1 polyclonal antibody. Left panel 14% SDS-PAGE Western blot of COS cells alone (lane 1) or transiently transfected with untagged *Ssna1* (lane 2) or *Ssna1*-GFP (LAP tag) (lanes 3,4) and blotted with anti-SSNA1 (ProteinTech 11797-1-AP, lanes 1-3) or anti-GFP (lane 4). Anti-SSNA1 specifically recognizes untagged SSNA1 at ~14 kDa and SSNA1-GFP at ~52 kDa (the LAP S-GFP tag is larger than GFP). Lanes 5,6: *Ssna1*-GFP 3T3 stables blotted with anti-GFP (5) or anti-SSNA1 (6). The blot was developed with ECL reagent, with white gaps indicating the positions of cuts permitting different antibody stainings. Molecular weights (kDa) are indicated on the left.

F) Validation of anti-SSNA1 and anti-NME7 antibodies on endogenous proteins by Western blotting. IMCD3 HTR6-GFP (left panel) or GFP-SMO 3T3 cells (right panel) were transfected with two different pools of NTC siRNAs (NTC and NTCp), or siRNAs to *Ssna1* or *Nme7*, serum starved for 16 h, and lysed in RIPA buffer. 70 µg (IMCD3) or 50 µg (3T3) lysates were run on an 18% Tris-Glycine gel and transferred to 0.2 µm nitrocellulose. The top (36-64 kDa) was immunoblotted with rabbit antibodies to NME7 (H278, Santa Cruz Sc-98518 at 1:400) and the bottom (< 36 kDa) with rabbit antibodies to SSNA1 (ProteinTech 11797-1-AP at 1:1600) and detected with HRP-anti-rabbit and ChemiGlow (overnight exposure). The 36-64 kDa section was then re-probed with anti-tubulin (1A2, 1:10,000) and HRP-anti-mouse to ascertain even loading. (Above 64 kDa was used to stain the GFP cargo and re-probed for transferrin receptor as in Figure 8D). Anti-NME7 was quite dirty and picked up several non-specific bands (asterisks), but did specifically detect a 50 kDa band that was partially depleted upon *Nme7* knockdown. Anti-SSNA1 was much cleaner, but detected a strong nonspecific band at 17 kDa, and a moderately strong band at 25 kDa (asterisks) in addition to SSNA1 itself at 14kDa, which disappeared upon knockdown.

G) SSNA1 depletion does not alter endogenous or transfected SMO levels. GFP-SMO 3T3 stables (left two lanes) or wild-type 3T3 (right four lanes) were

transfected with siNTC (-) or si*Ssna1* (+) and western blotted for endogenous SMO (14A5 mAb, 2µg/ml), tubulin (1A2 mAb, Sigma, 1:10,000) or anti-SSNA1 as in (E). The SMO blot was stripped and reprobed with anti-GFP (1µg/ml JL-8, Clontech).

H) Quantitation of four (NME7) or eight (SSNA1) Western blots such as the ones shown in (F) revealed SSNA1 depletion was 85% or 88% effective in IMCD3 or 3T3 cells, respectively, in agreement with the > 80% mRNA knockdown (Figure S2A). NME7 appeared only to be depleted by 50% in IMCD3 and 25% in 3T3 cells, but is likely due to a nonspecific band at the same position, since the antibody was dirty and the mRNA was depleted in IMCD3 and 3T3 cells by 90% and 80%, respectively (Figure S2A).

Supplemental Figure 5. Hedgehog responsiveness and cilia length depends on cell type.

A-C) 3T3 cells transfected with siRNAs to class I and class II (A), class V (B) or class III (C) genes were treated for 24 h with Hh (+) or just serum starved (-) and immunoblotted with 6F5 anti-Gli3N mAb as in Figure 9. Gli3FL and Gli3R were quantitated relative to tubulin loading control and NTC without Hh as in Figure 9 (average of 3-5 blots per experiment).

D) *Iqub* knockdown does not affect cilia length in S12 cells. IMCD3 HTR6 (top), 3T3 (middle row) or S12 (bottom) were treated with the indicated class III siRNAs and stained for acetylated and gamma tubulin. *Ptpdc1* knockdown increased cilia length in all three cell types, whereas *Iqub* knockdown only had this effect in 3T3 and IMCD3, not S12, cells. Scale bar is 20 µm.

E) IMCD3 and IMCD3 HTR6-GFP cells do not respond to Hh, while S12 cells are ~10x more responsive than 3T3 cells. *Gli1* mRNA levels were measured following 24 h of Hh treatment, normalized to *Rpl19* and plotted as fold increase

over non-Hh treated cells. The means and SDs of 4 triplicate experiments are plotted (except IMCD3 wt, n=1).

Supplemental Table 1.

A) Summary table of cilia phenotypes in the primary screen.

Table of primary screen data in Figure 2. Genes were ranked in each cilia phenotype class from strongest mRNA knockdown (top) to weakest or no mRNA detectable (bottom). 'low taq' (pink) indicates genes with very low to undetectable levels of mRNA (Ct > 34.5, crosses in Figure 2E) as measured by TaqMan analysis with multiple probe sets (see B). Genes chosen for further analysis are highlighted in yellow and the top ones of those following validation in orange. Blue, white and pink shading indicates the severity of the measured cilia phenotypes, as indicated in the key (top).

B) List of siRNA sequences and/or catalog numbers, cloning primer sequences, and q-PCR primer and probe sets. For genes with a low qRT-PCR signal using the first set of primers tested, a second set of primers to different locations within the gene were synthesized (sequence shown) or ordered from Applied Biosystems (catalog number shown).

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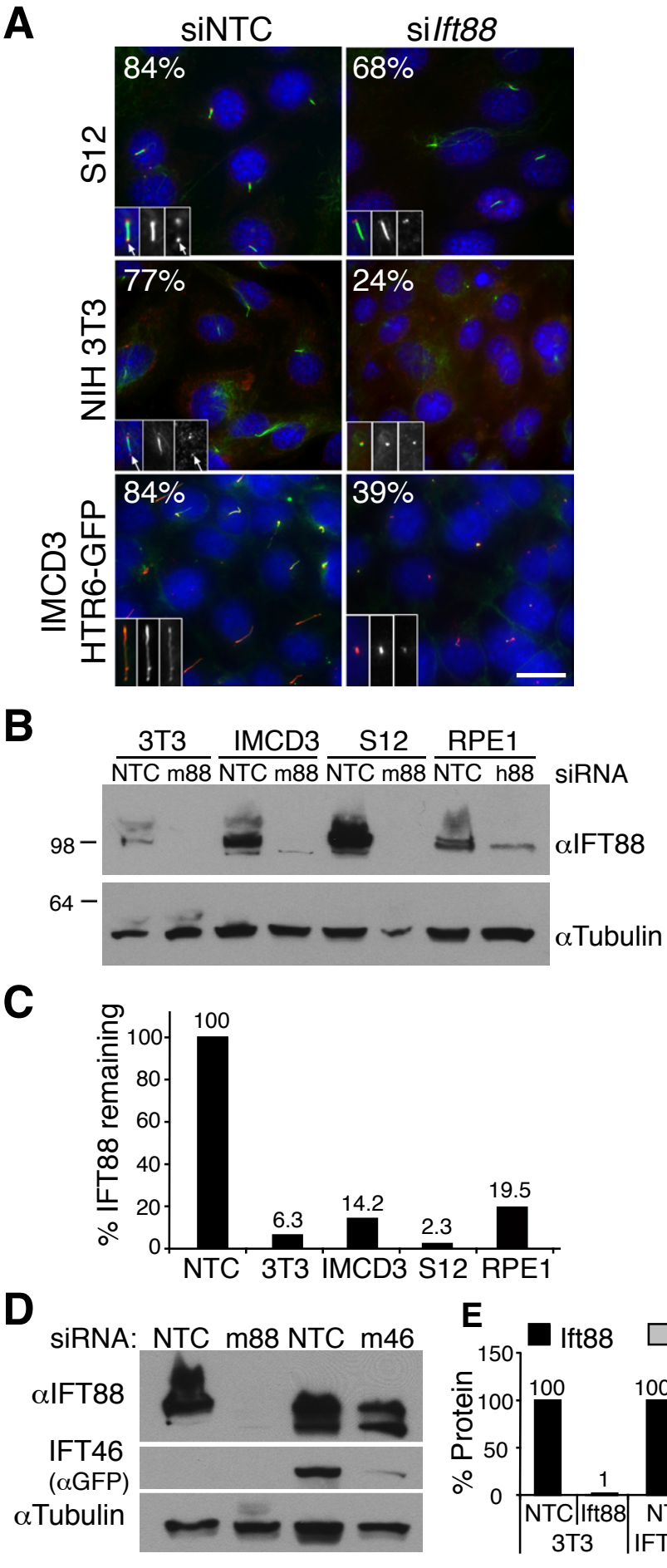

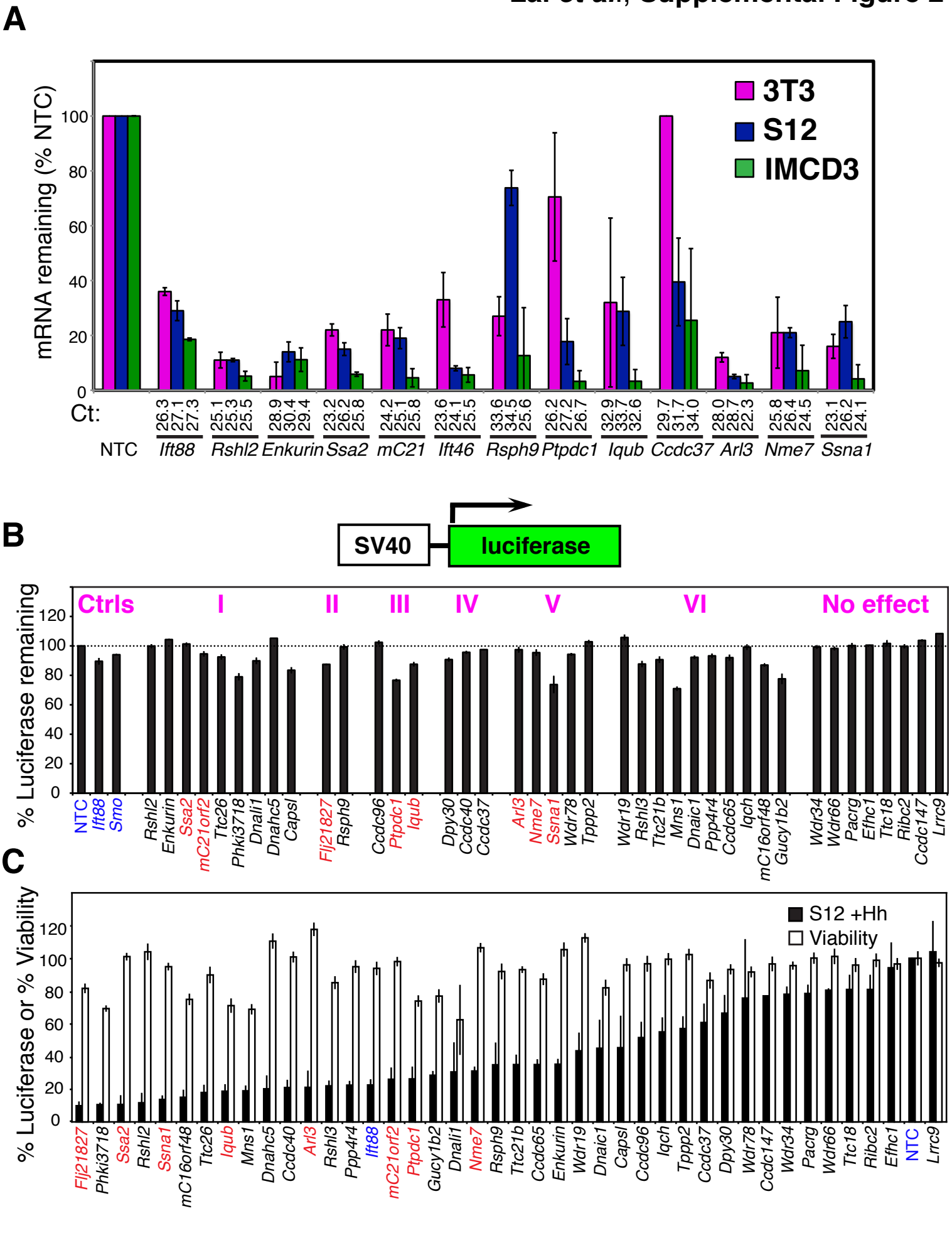
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Rsph9
Ptpdc1
Iqub
Ccdc37
Arl3
Nme7
Ssna1
B
SV40
luciferase
% Luciferase remaining
Ctrls
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III
IV
V
VI
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C
% Luciferase or % Viability
S12 +Hh
Viability

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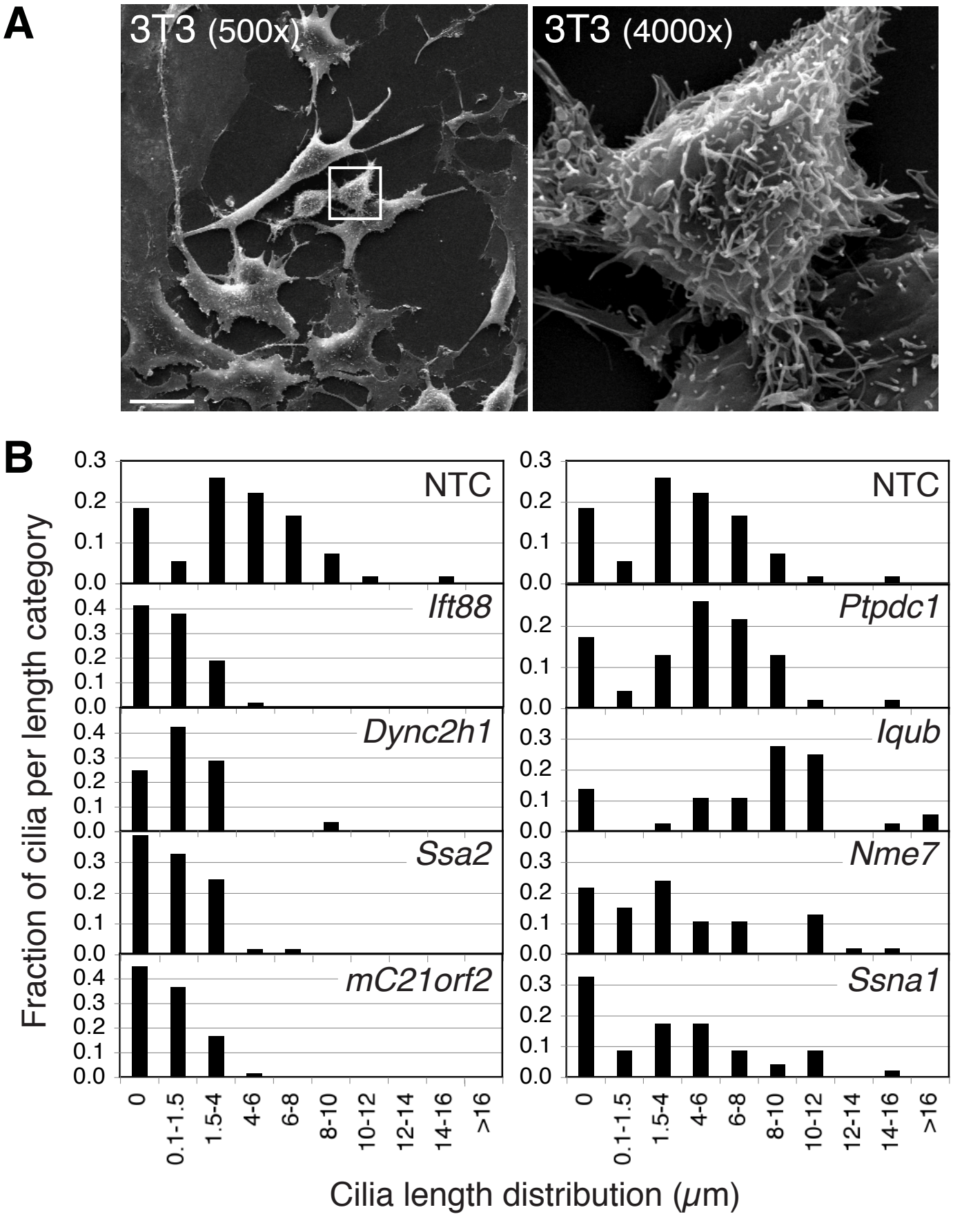

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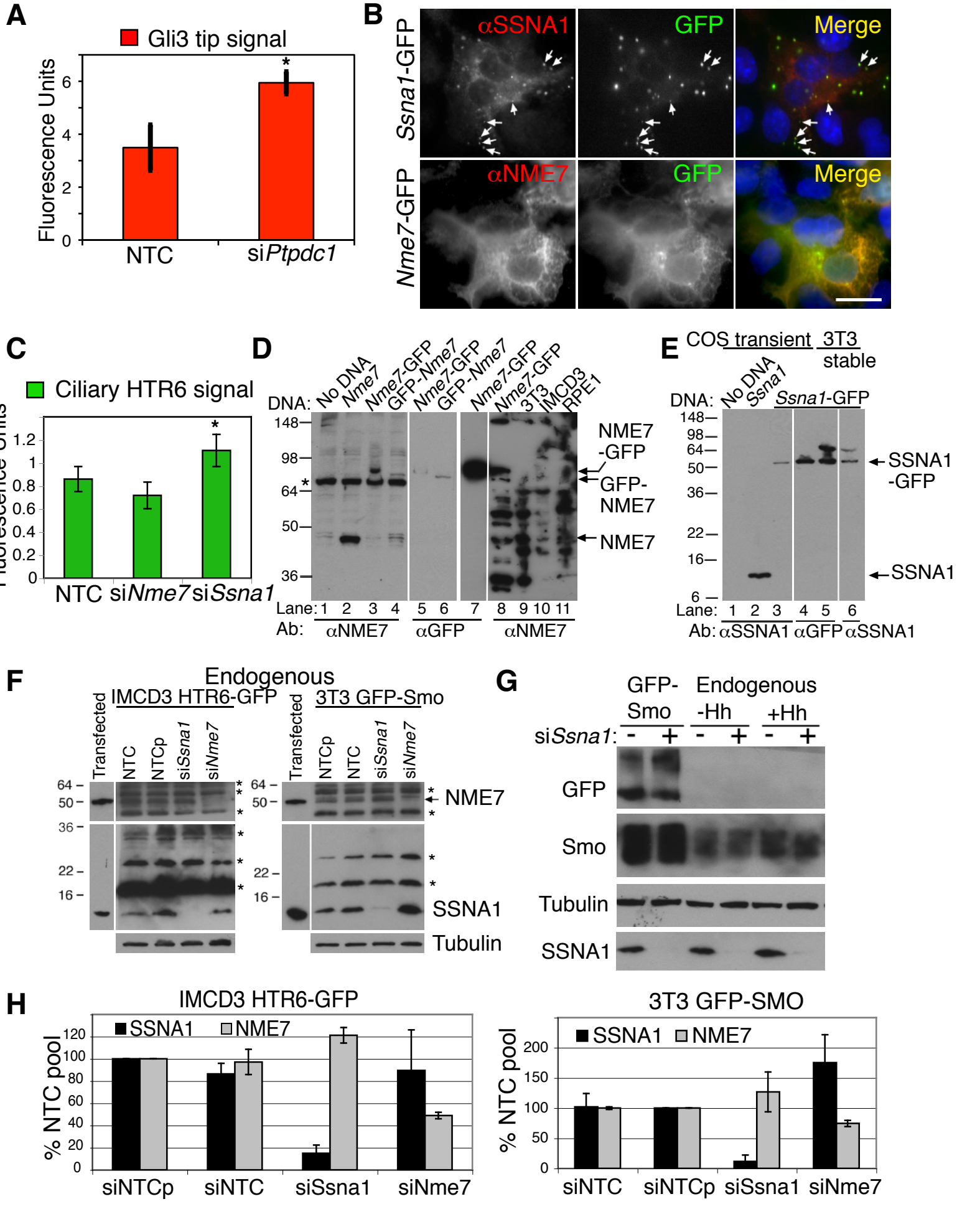

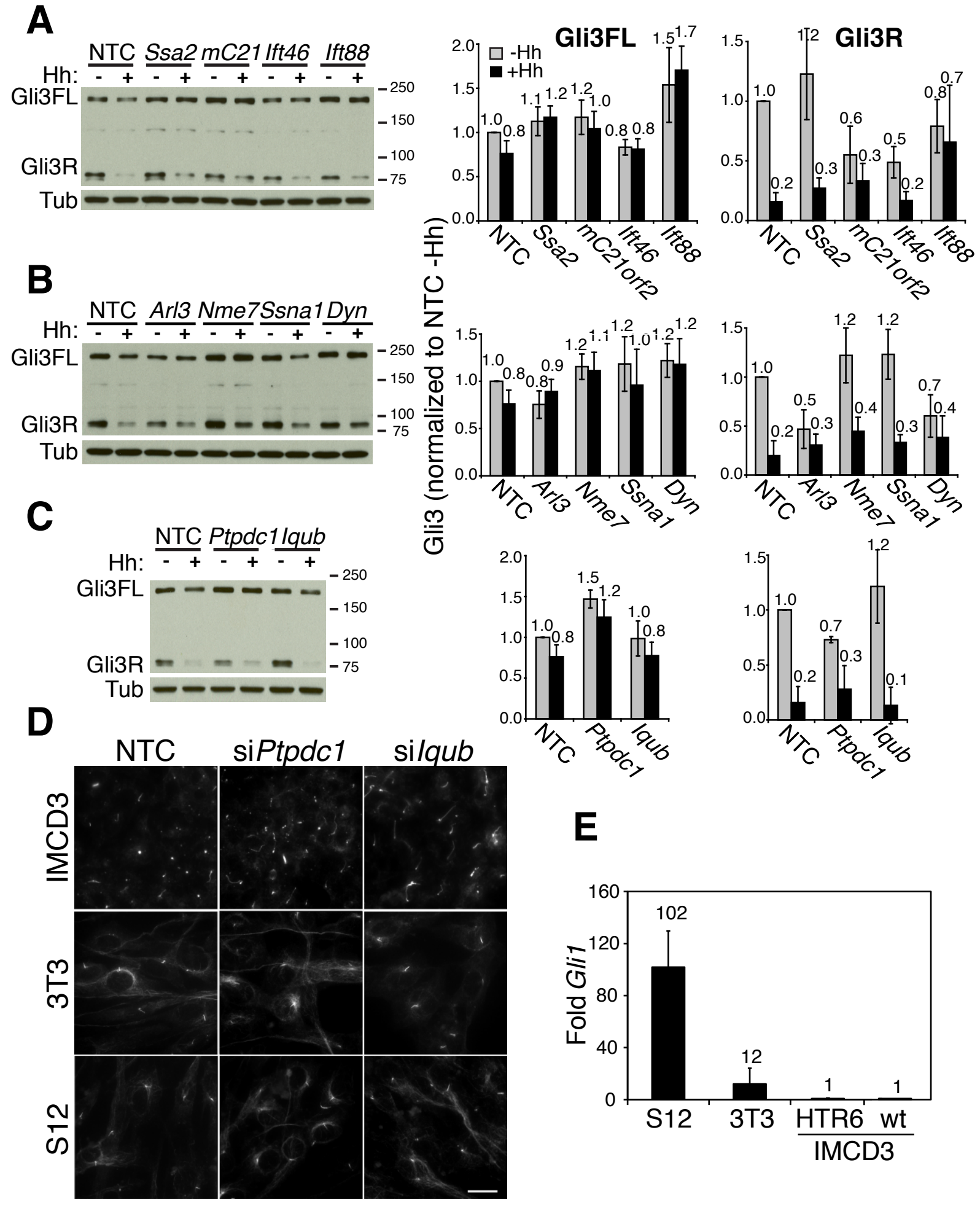
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Gli3R
-Hh
+Hh
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