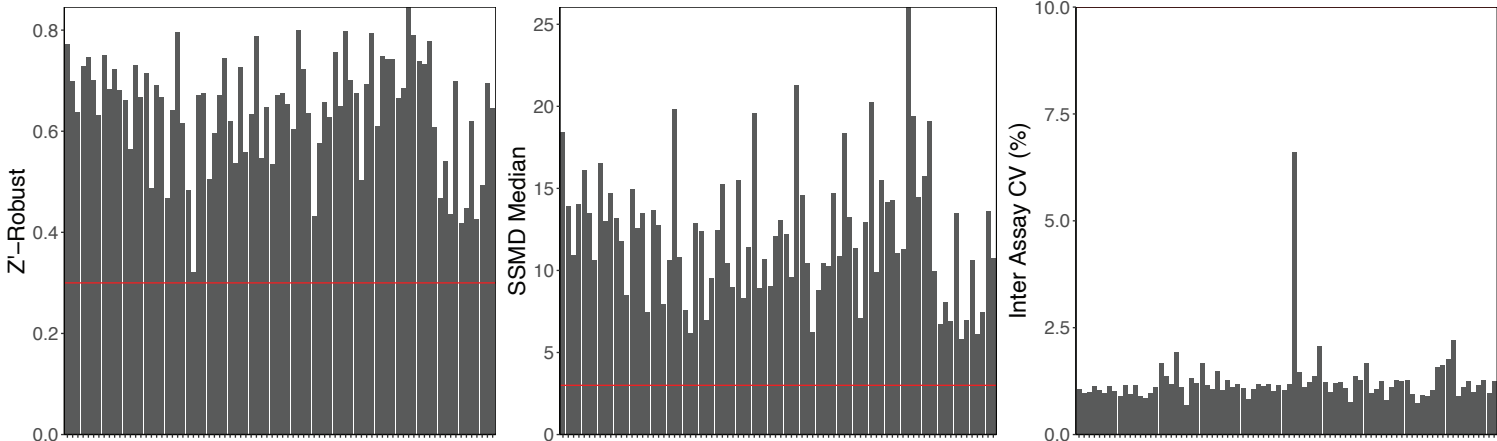
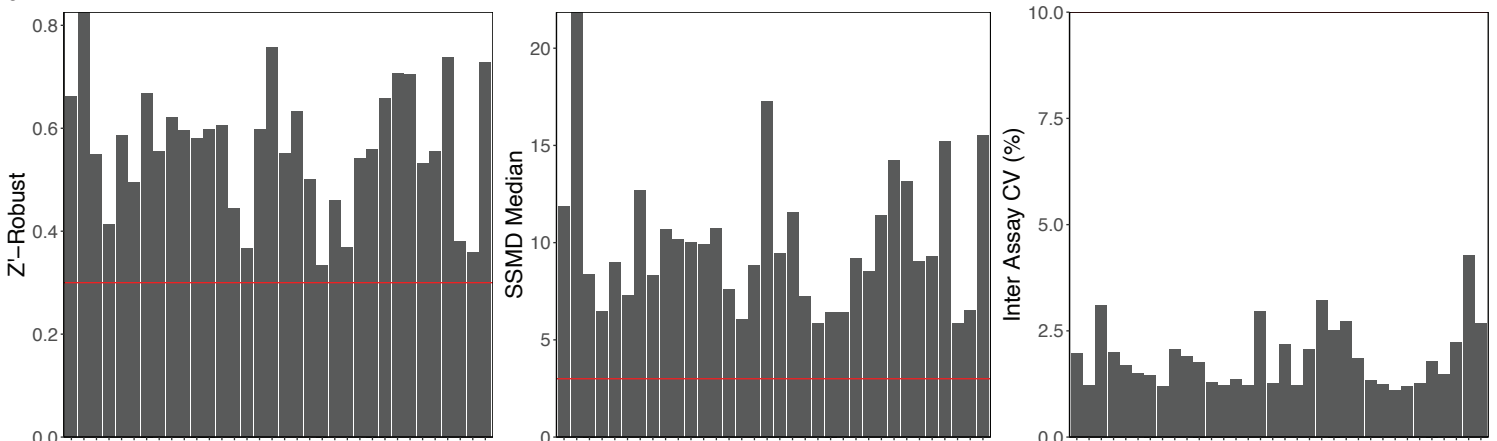


Supplementary Figure 1.

**a**



**b**

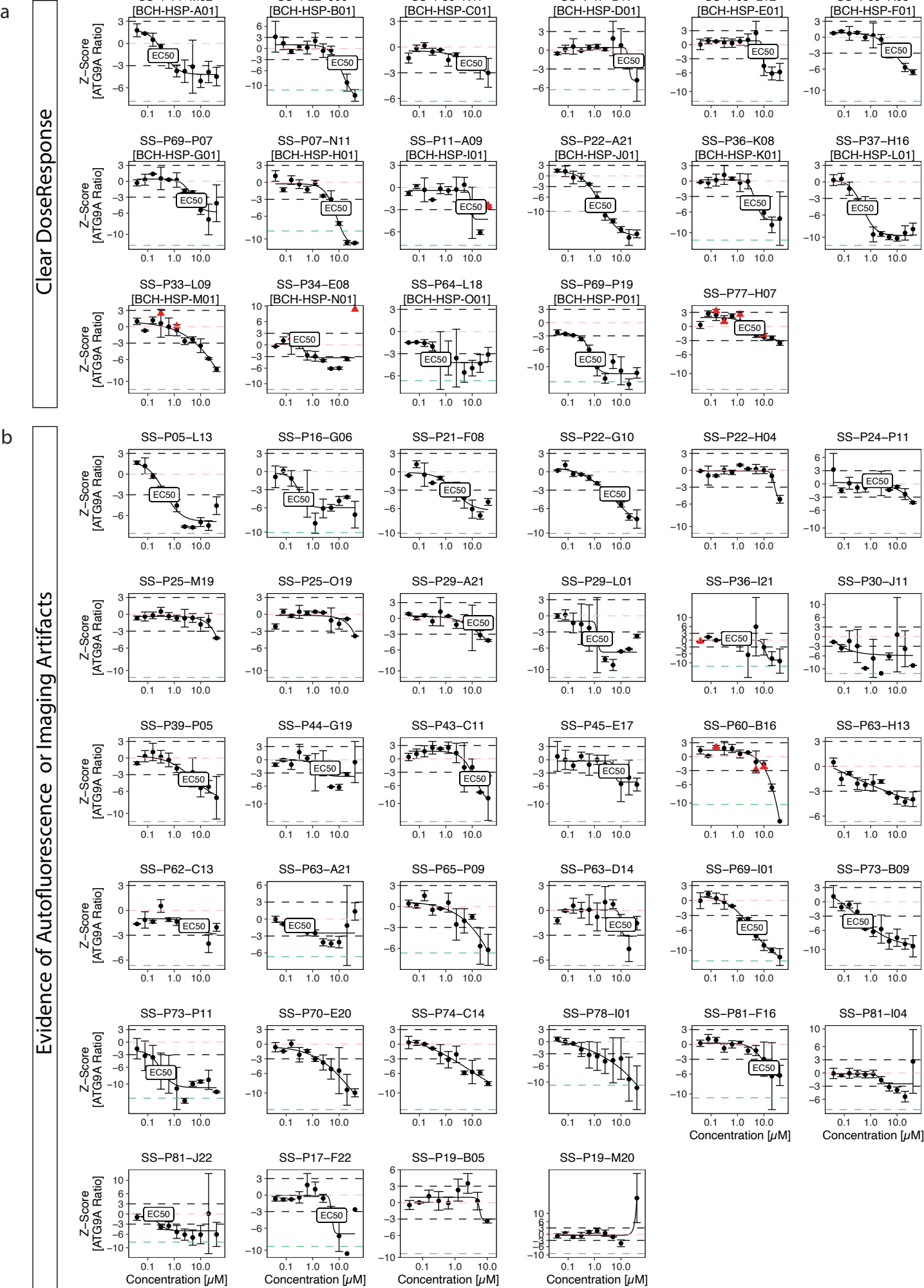


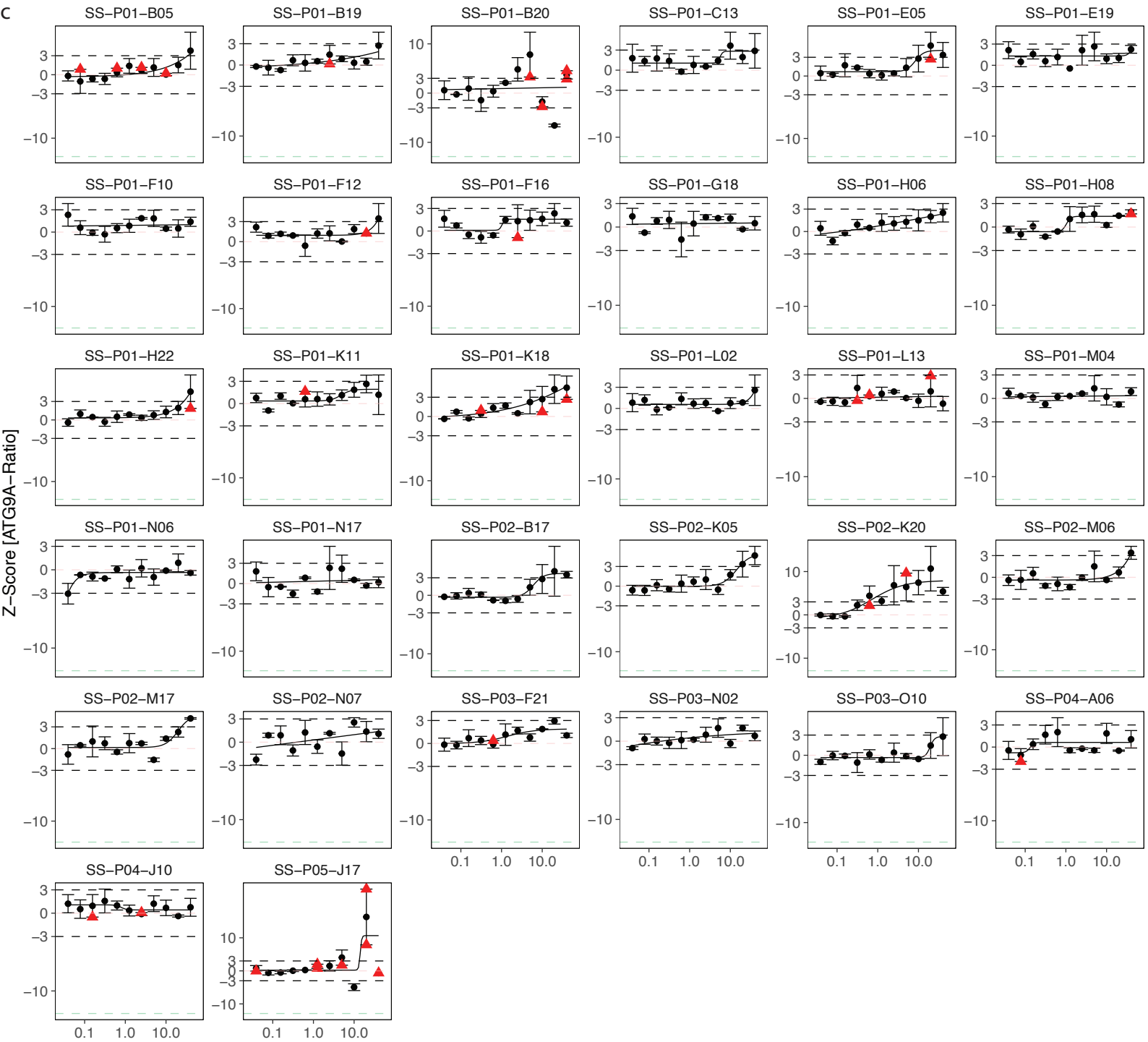
**Supplementary Figure 1. Quality metrics of the ATG9A translocation assay in the primary screen and counter-screen.**

Assay performance was monitored in the (a) primary screen and (b) counter-screen using criteria proposed by Zhang *et al.* and included a  $Z'$  robust  $\geq 0.3$ , a strictly standardized median difference (SSMD)  $\geq 3$  and an inter-assay coefficient of variation  $\leq 10\%$ . All metrics were calculated with respect to the positive and negative controls of the same assay plate to avoid bias by inter-plate variability. Predefined thresholds (red lines) were met by all assay plates.



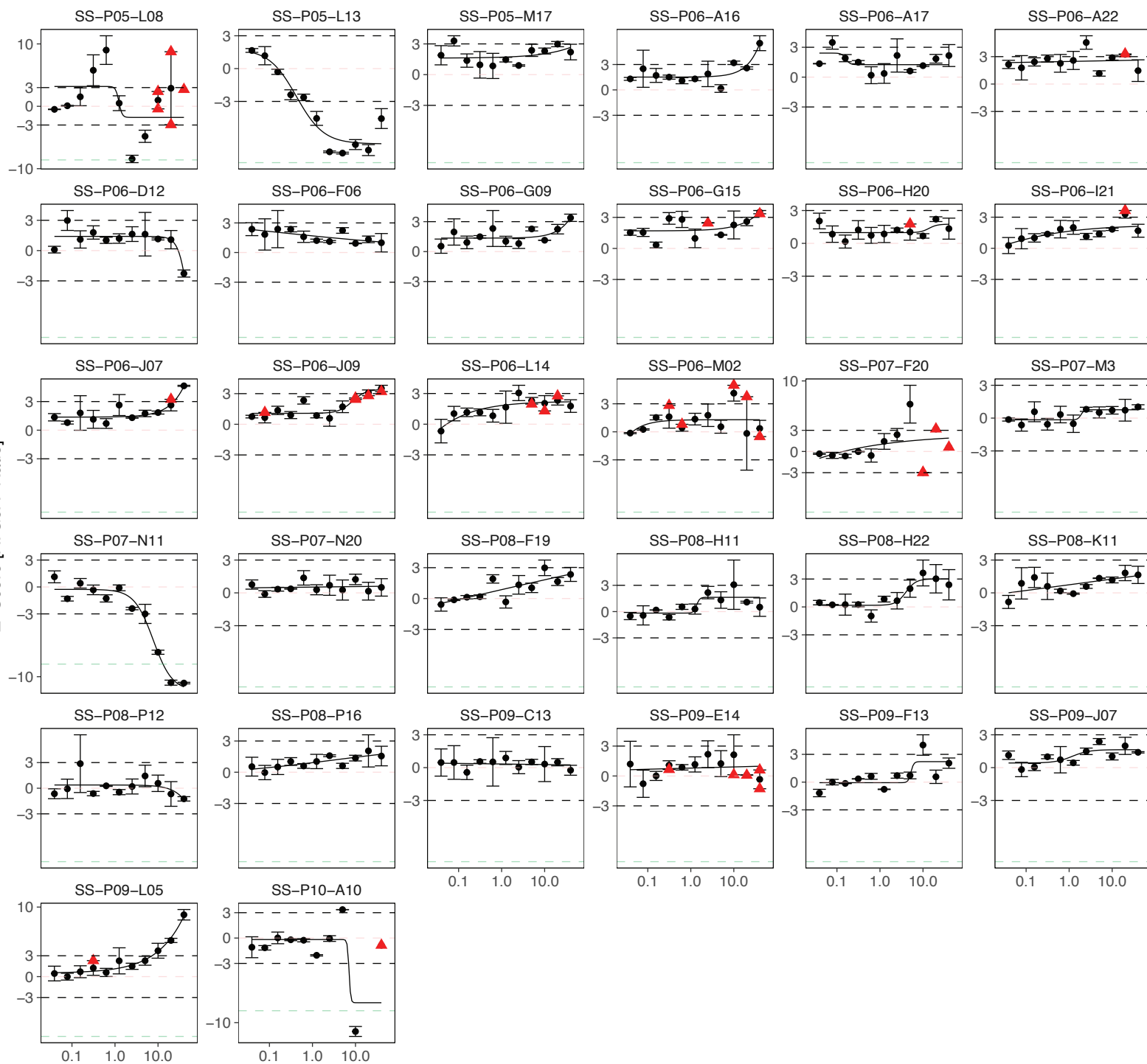
Supplementary Figure 2.





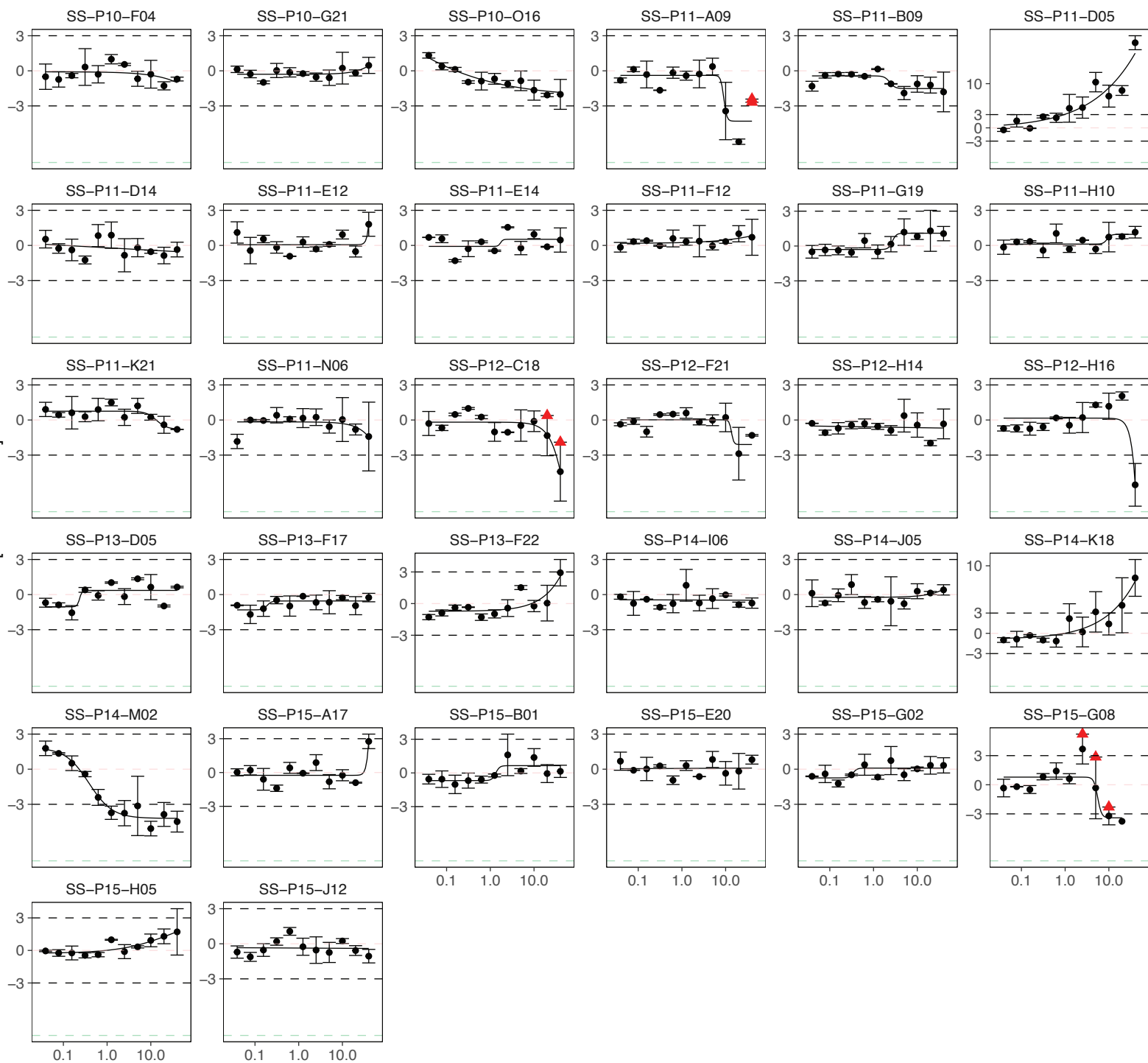
Secondary Screen - Plate 1

Z-Score [ATG9A-Ratio]

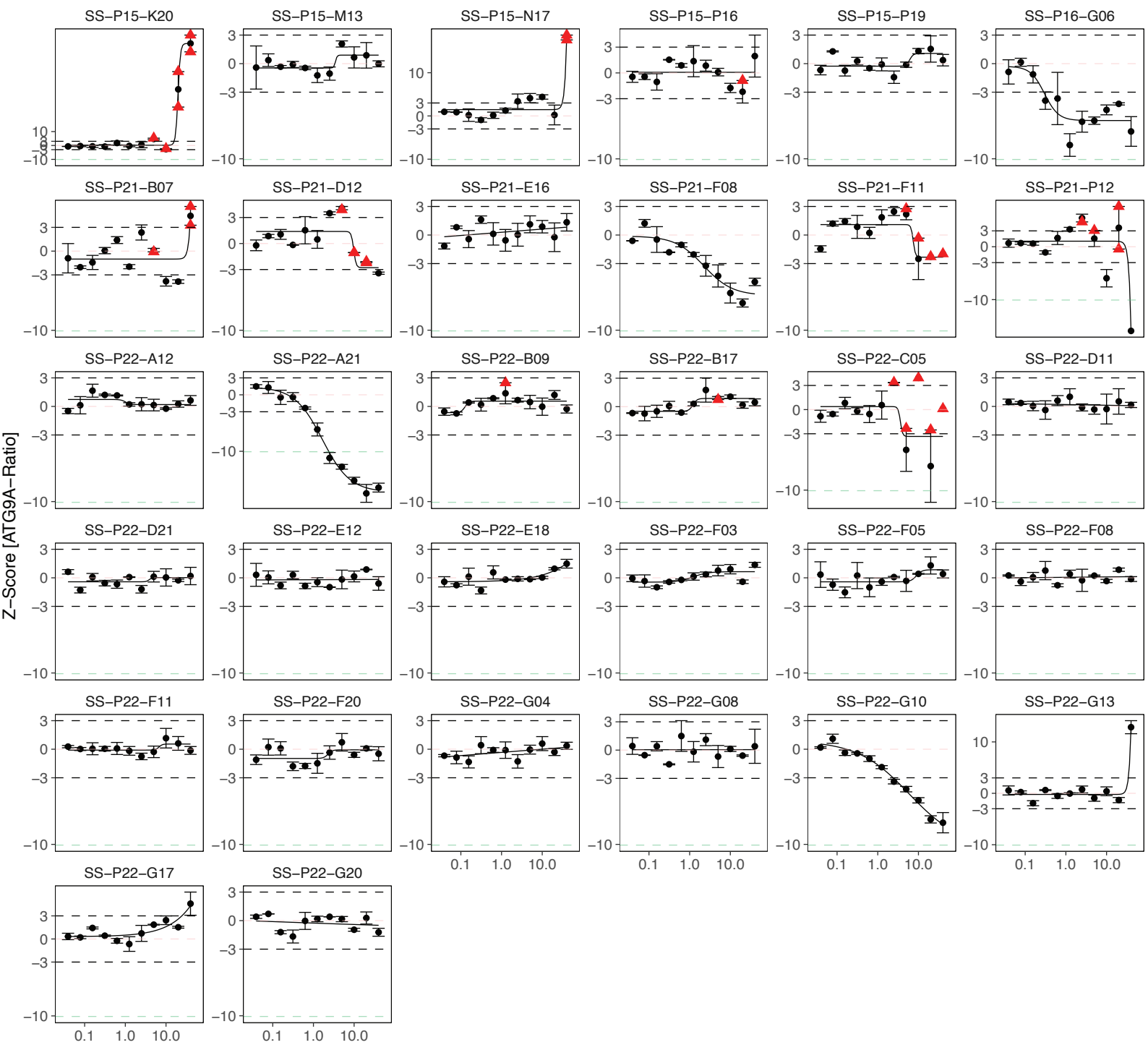


Secondary Screen – Plate 2

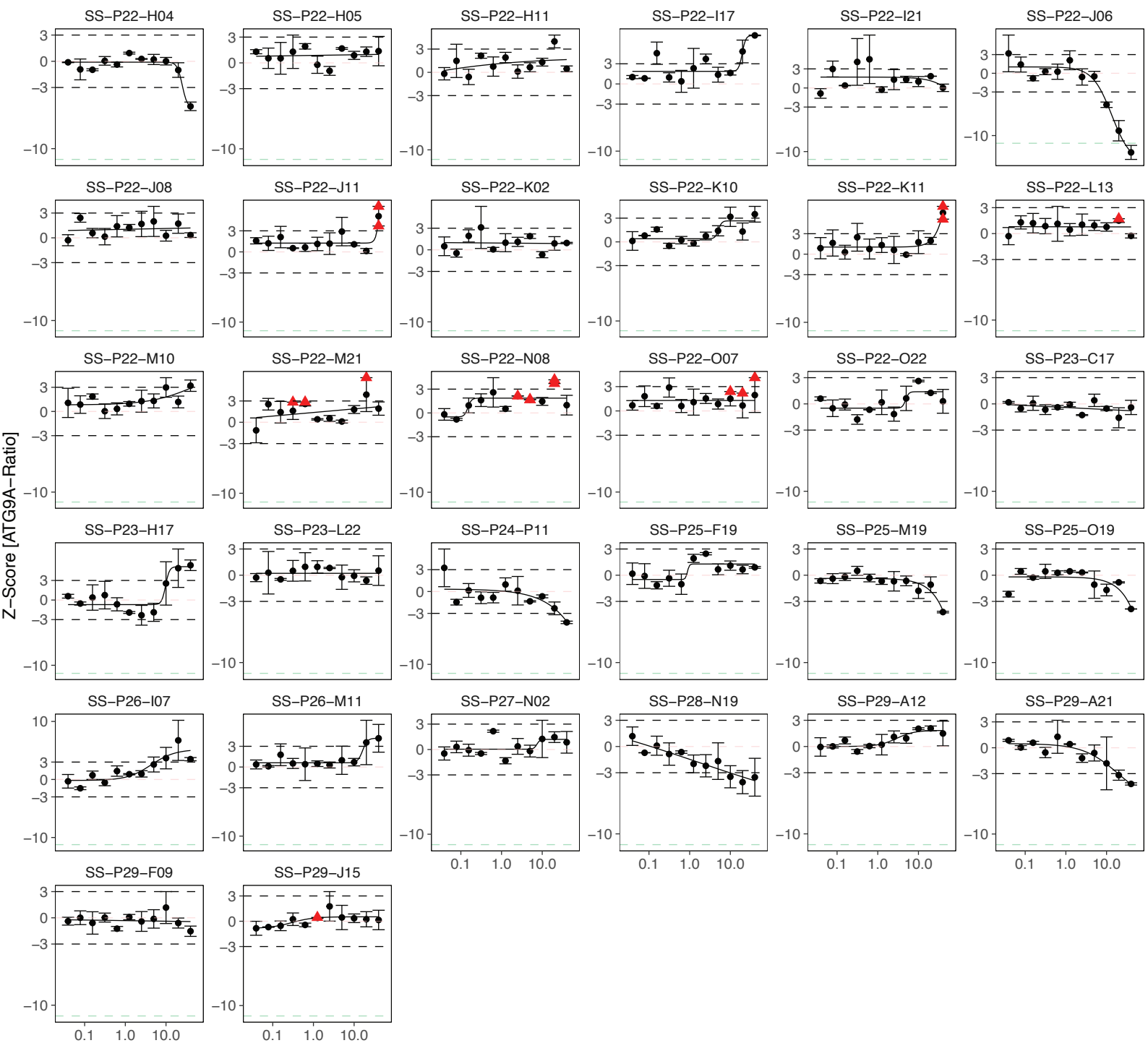
Z-Score [ATG9A-Ratio]



Secondary Screen – Plate 3

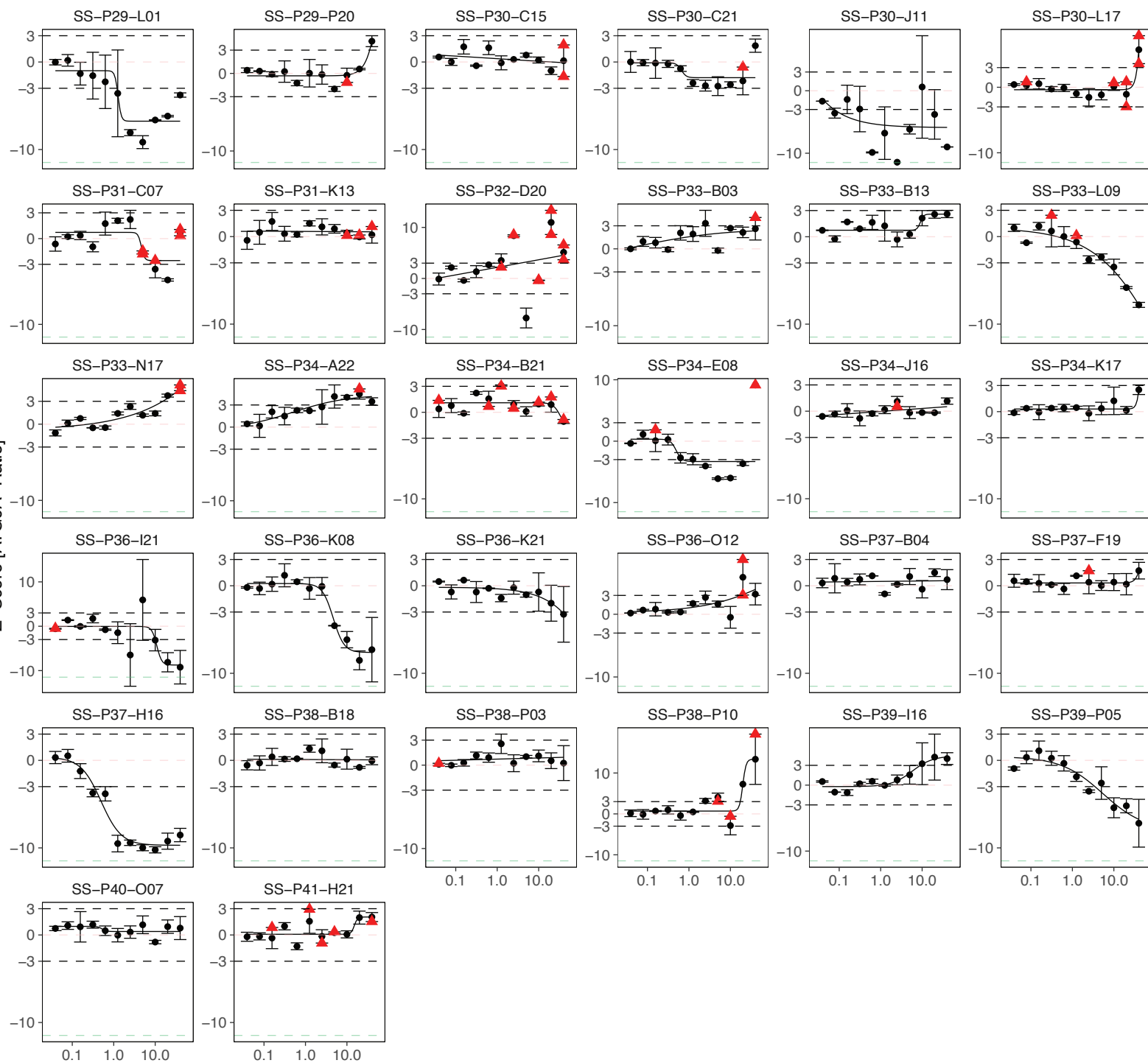


Secondary Screen – Plate 4



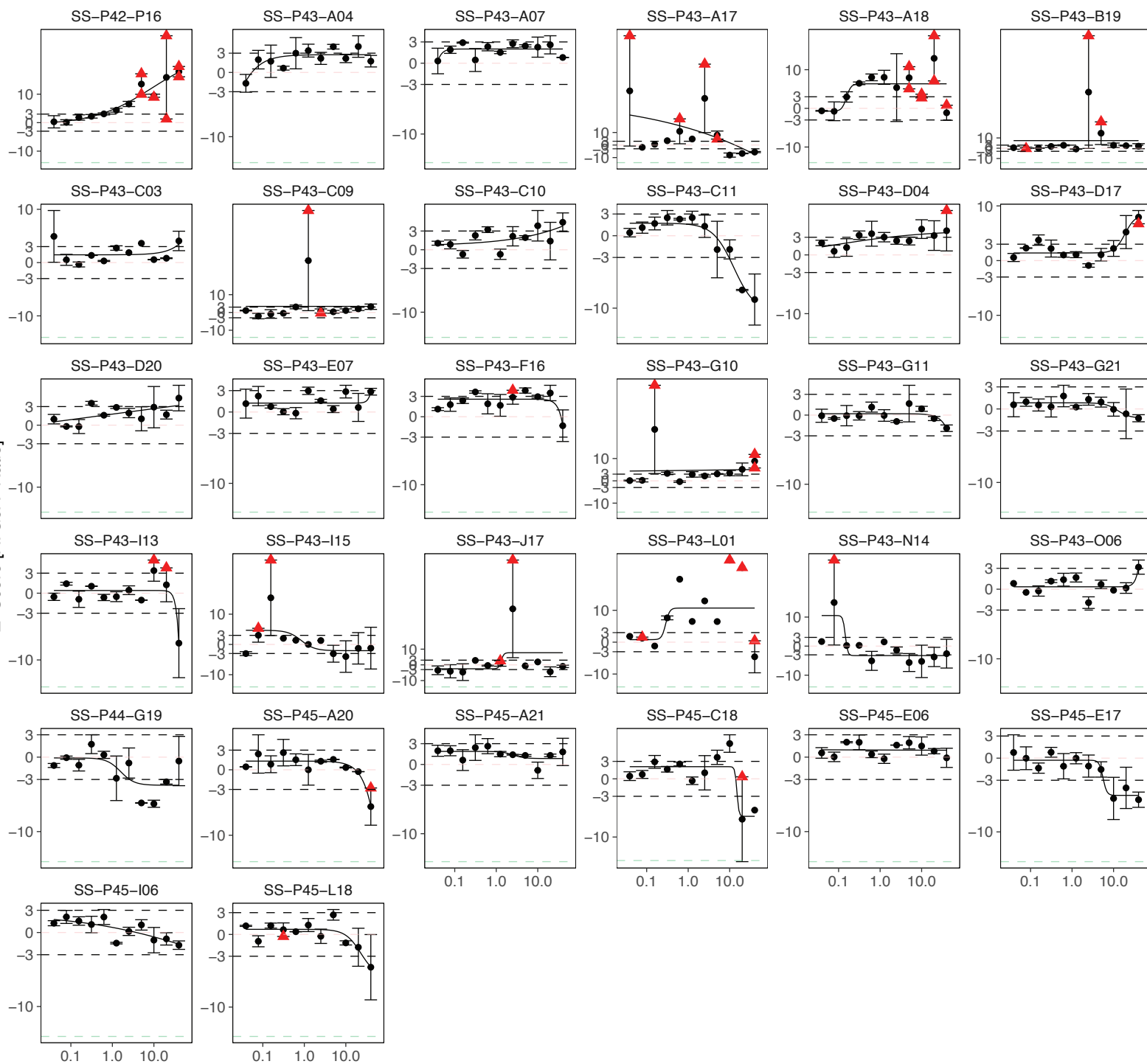
Secondary Screen – Plate 5

Z-Score [ATG9A-Ratio]



Secondary Screen – Plate 6

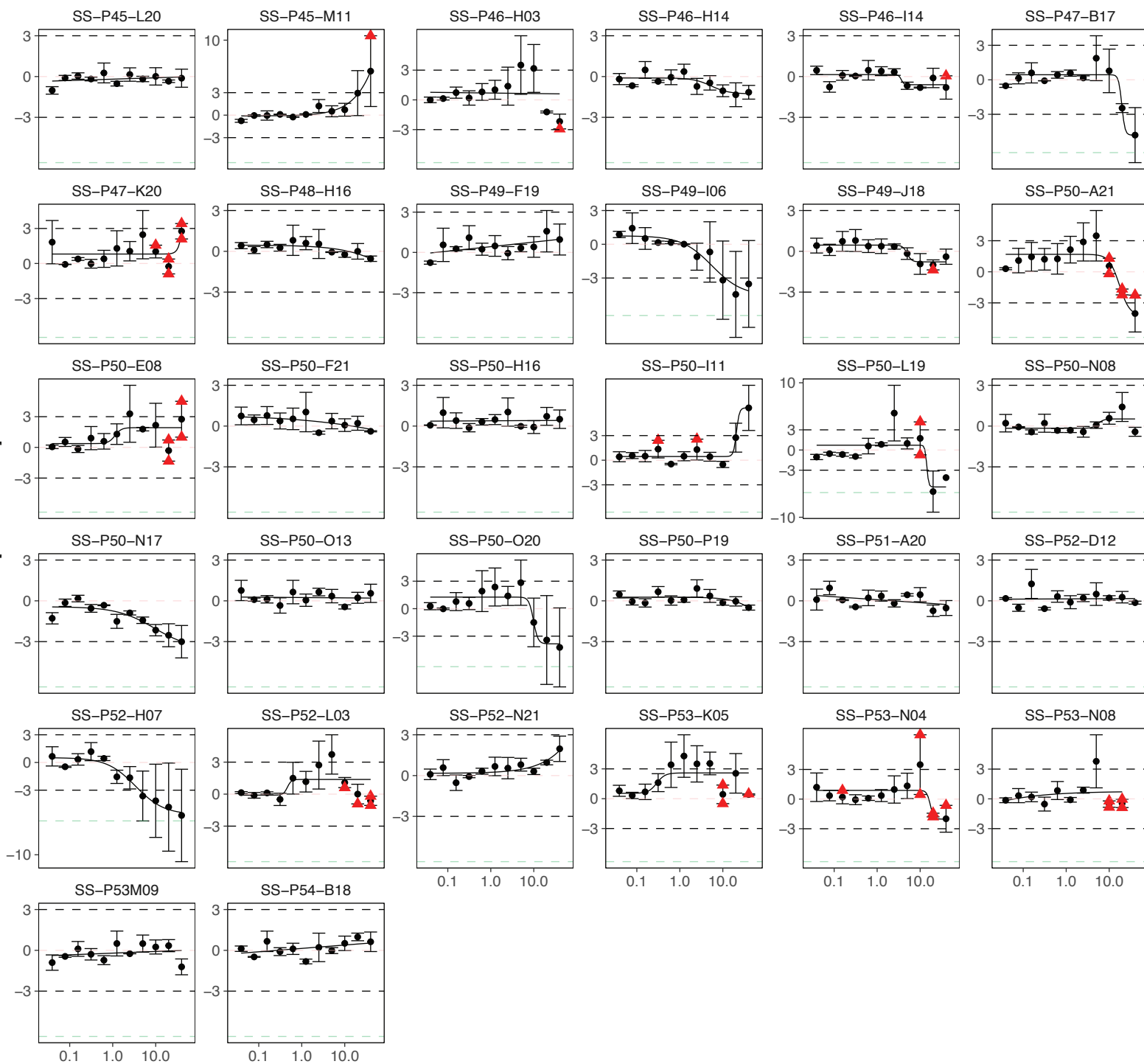
Z-Score [ATG9A-Ratio]



Secondary Screen – Plate 7

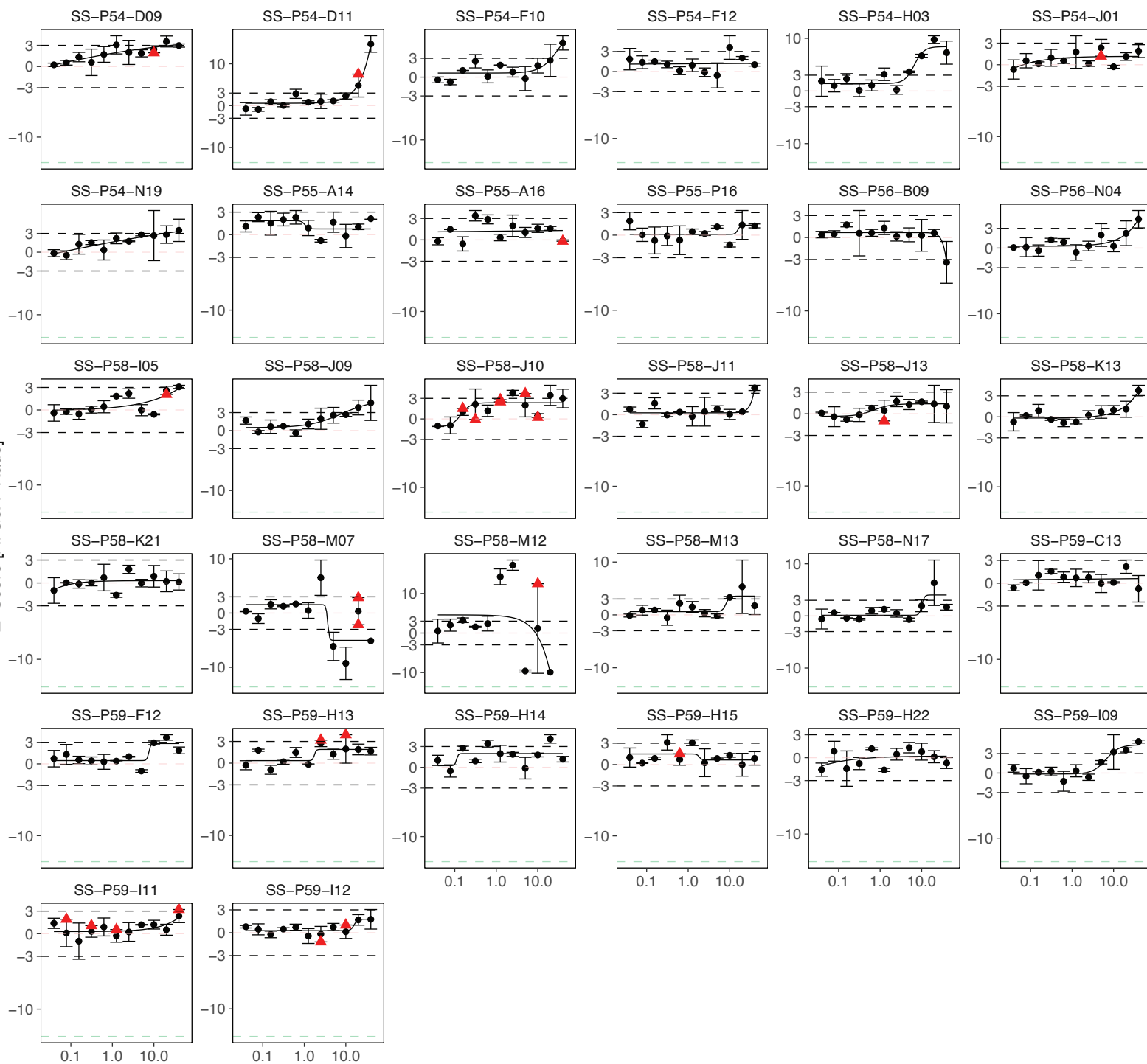


Z-Score [ATG9A-Ratio]

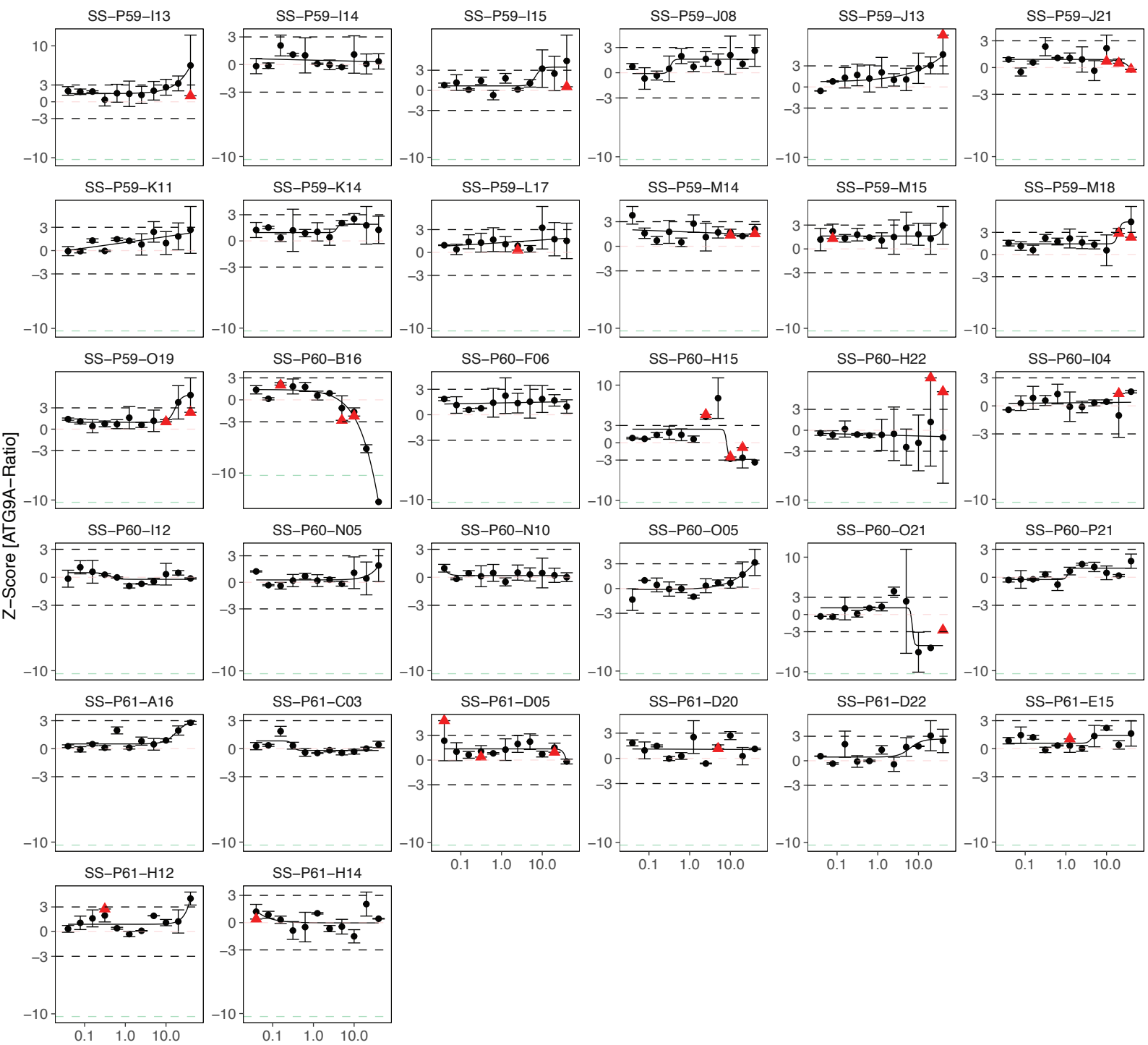


Secondary Screen – Plate 8

Z-Score [ATG9A-Ratio]

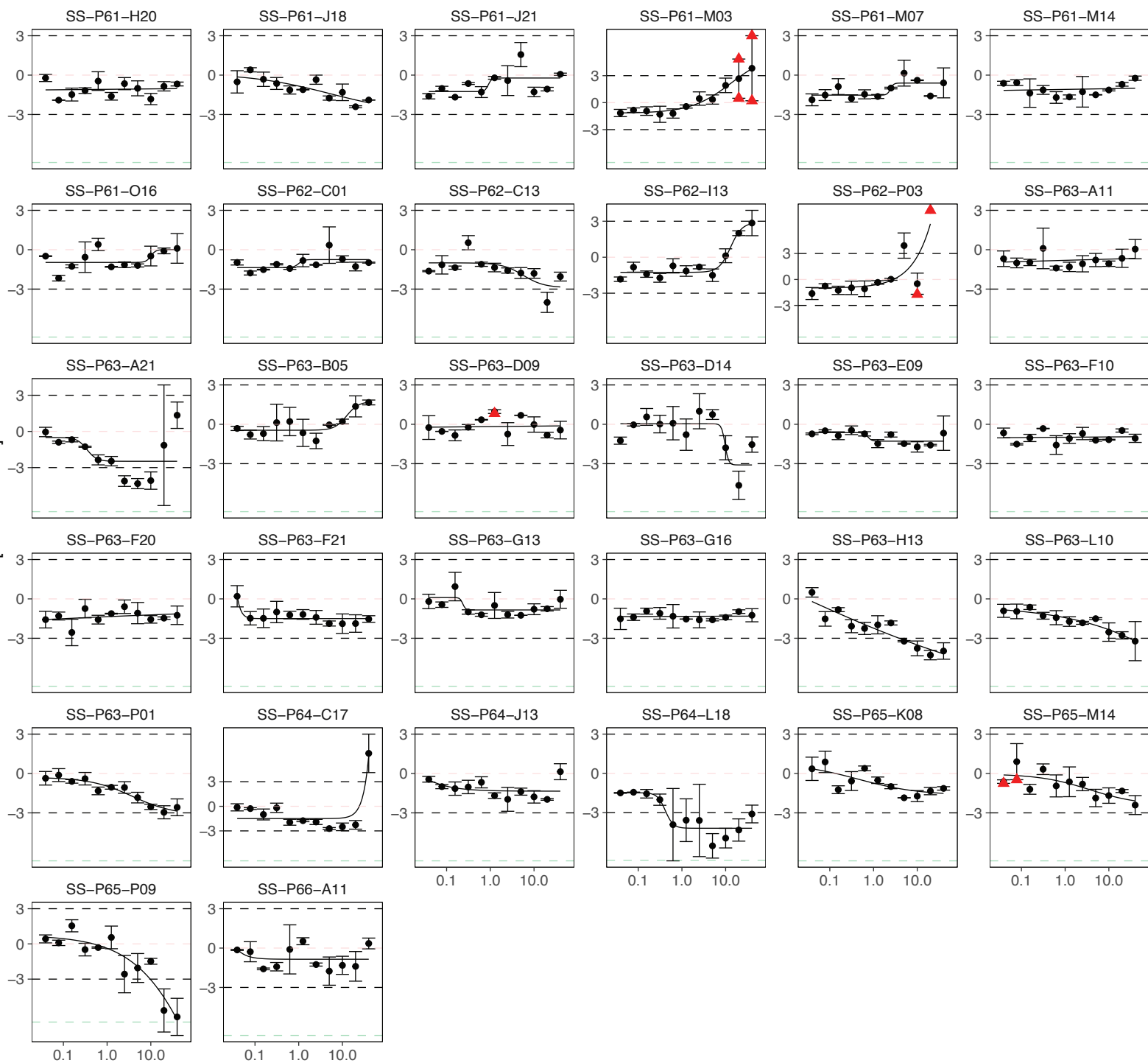


Secondary Screen – Plate 9



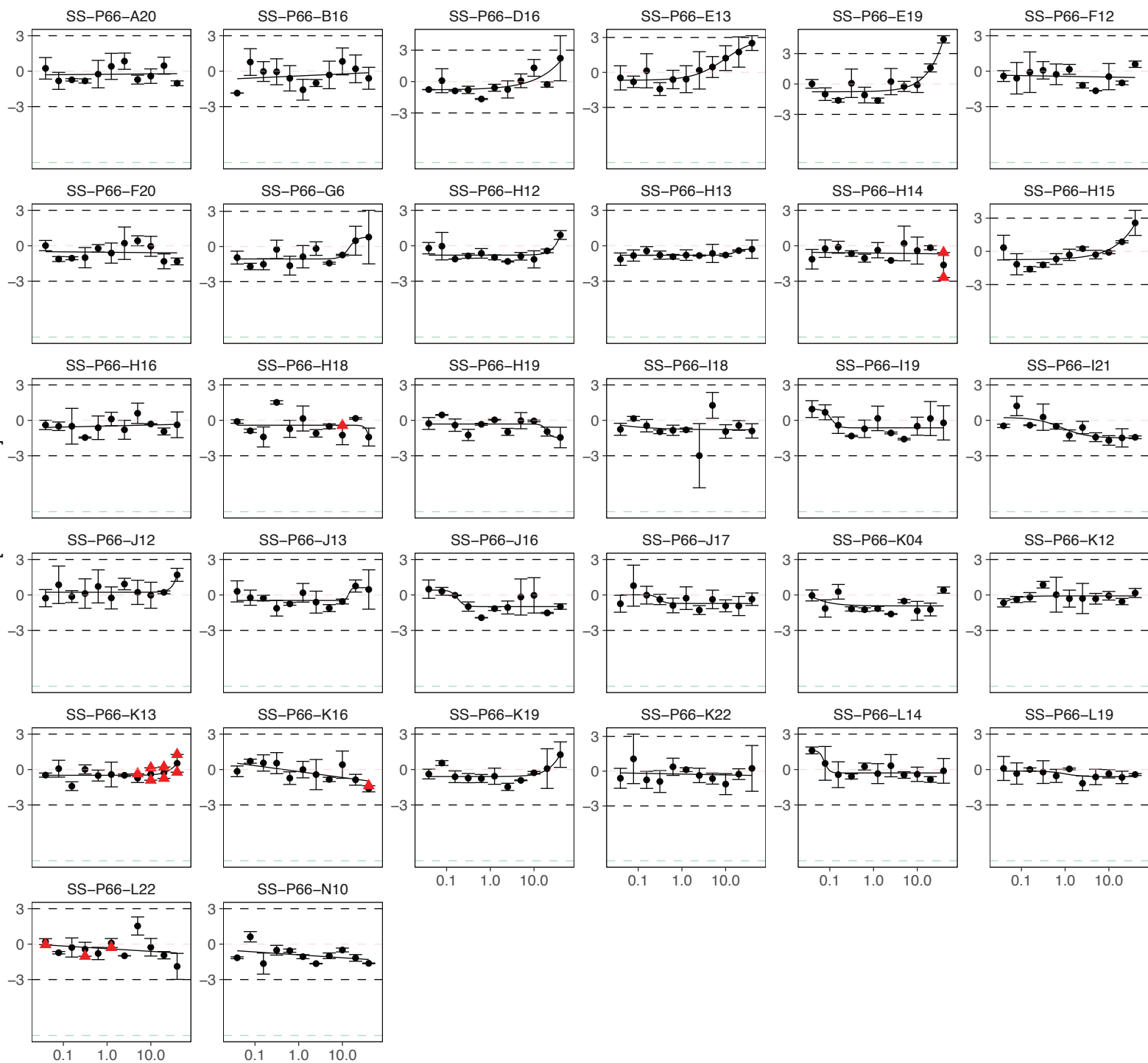
Secondary Screen – Plate 10

Z-Score [ATG9A-Ratio]



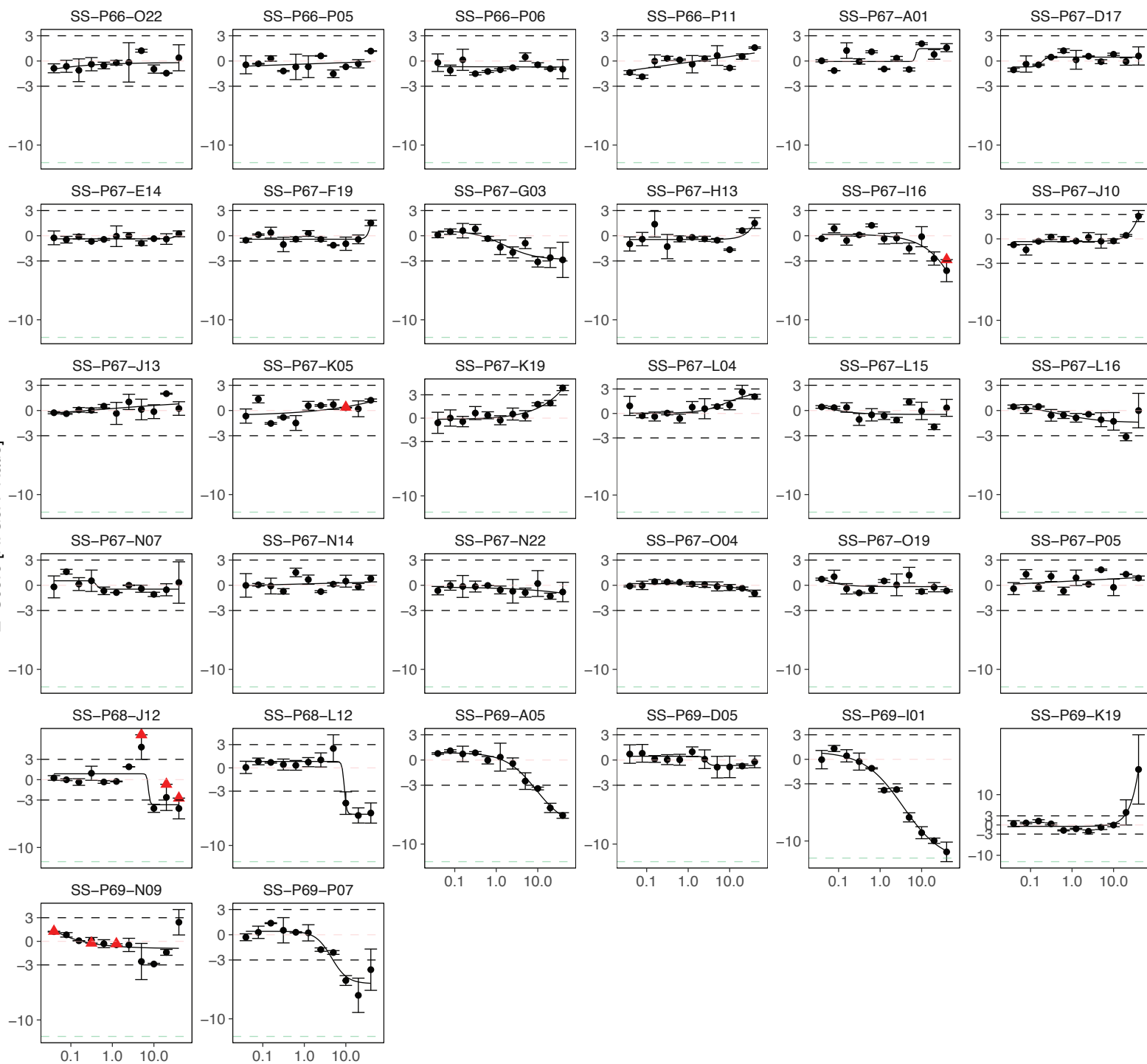
Secondary Screen - Plate 11

Z-Score [ATG9A-Ratio]



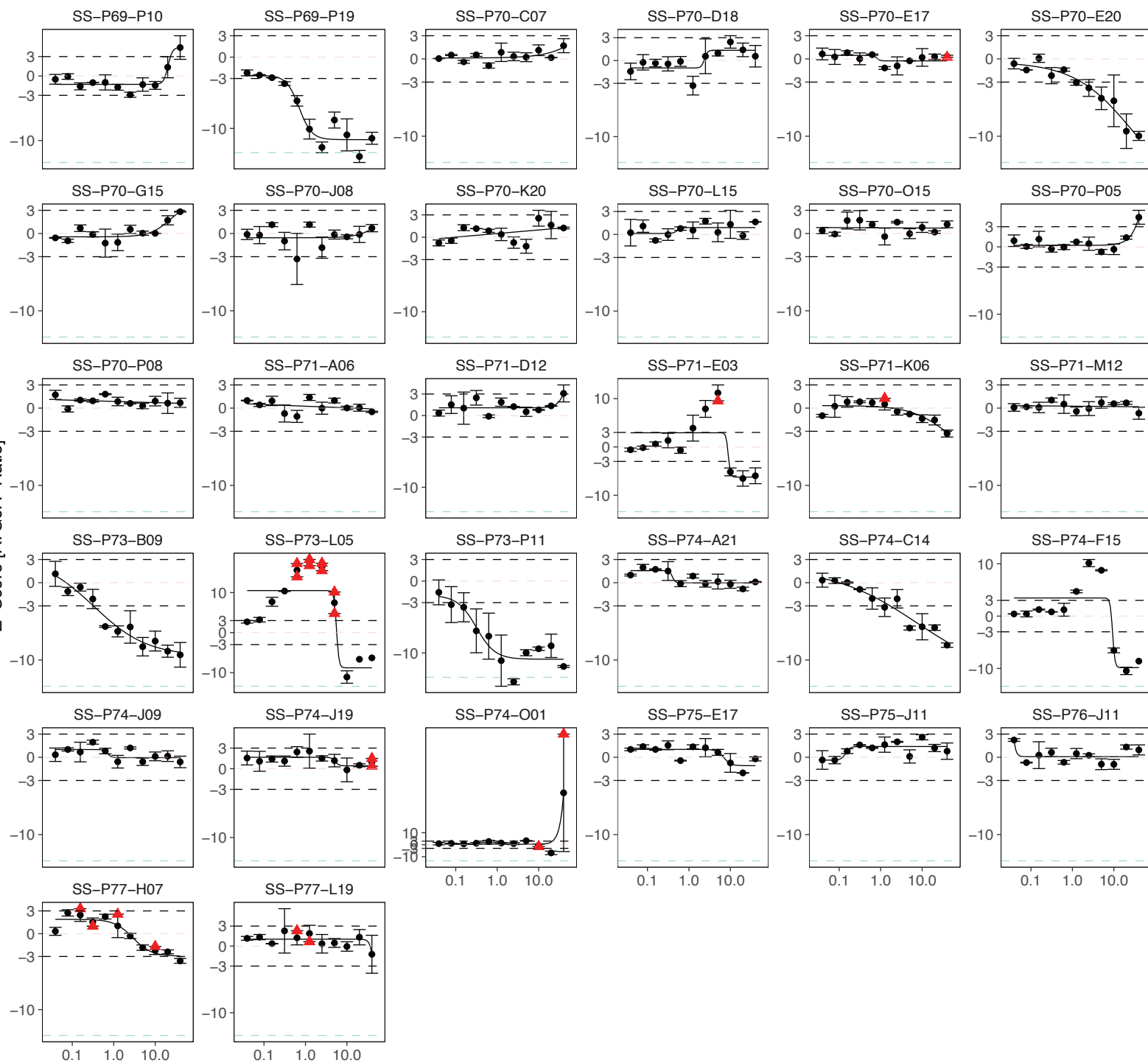
Secondary Screen – Plate 12

Z-Score [ATG9A-Ratio]



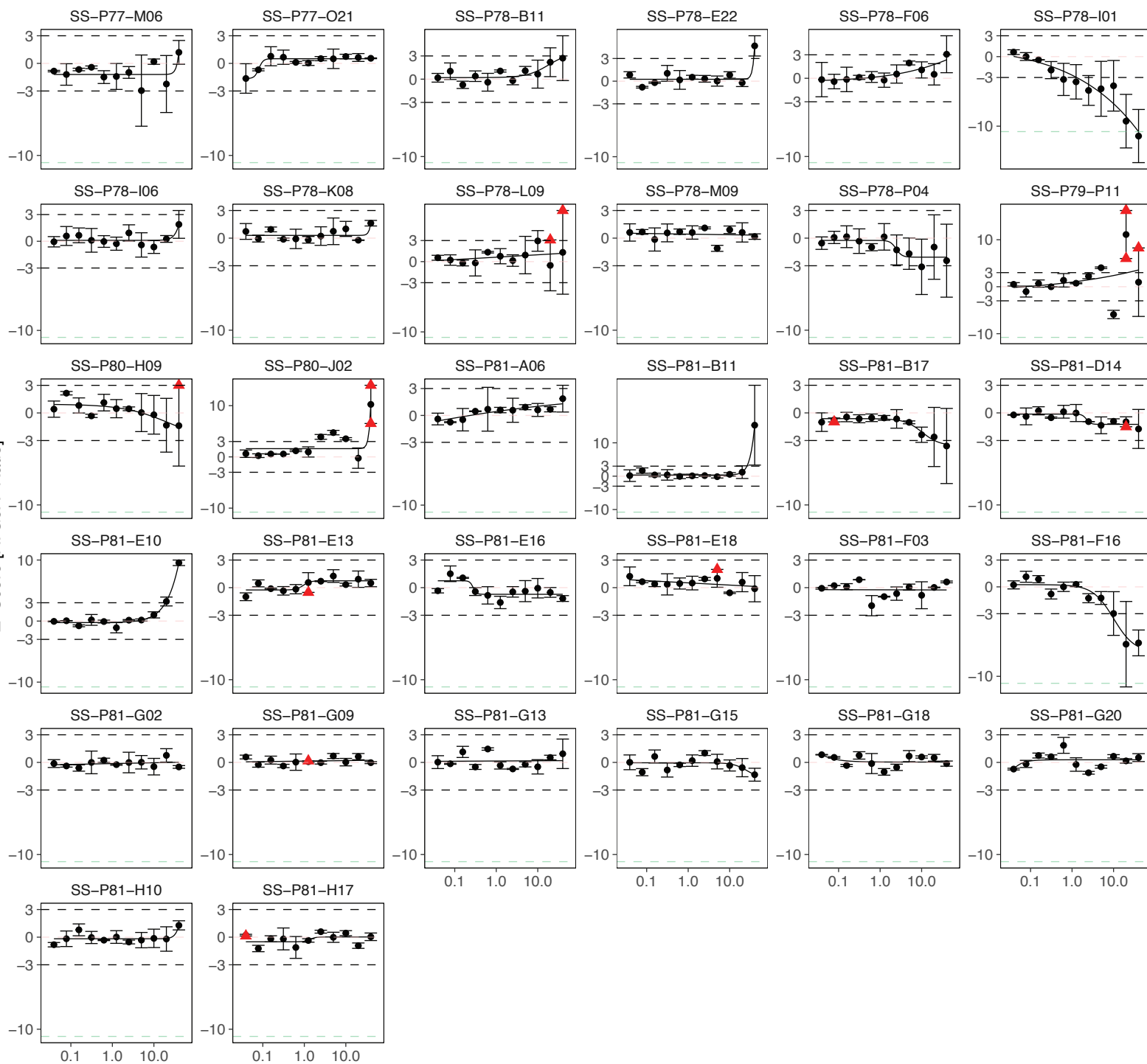
Secondary Screen – Plate 13

Z-Score [ATG9A-Ratio]



Secondary Screen – Plate 14

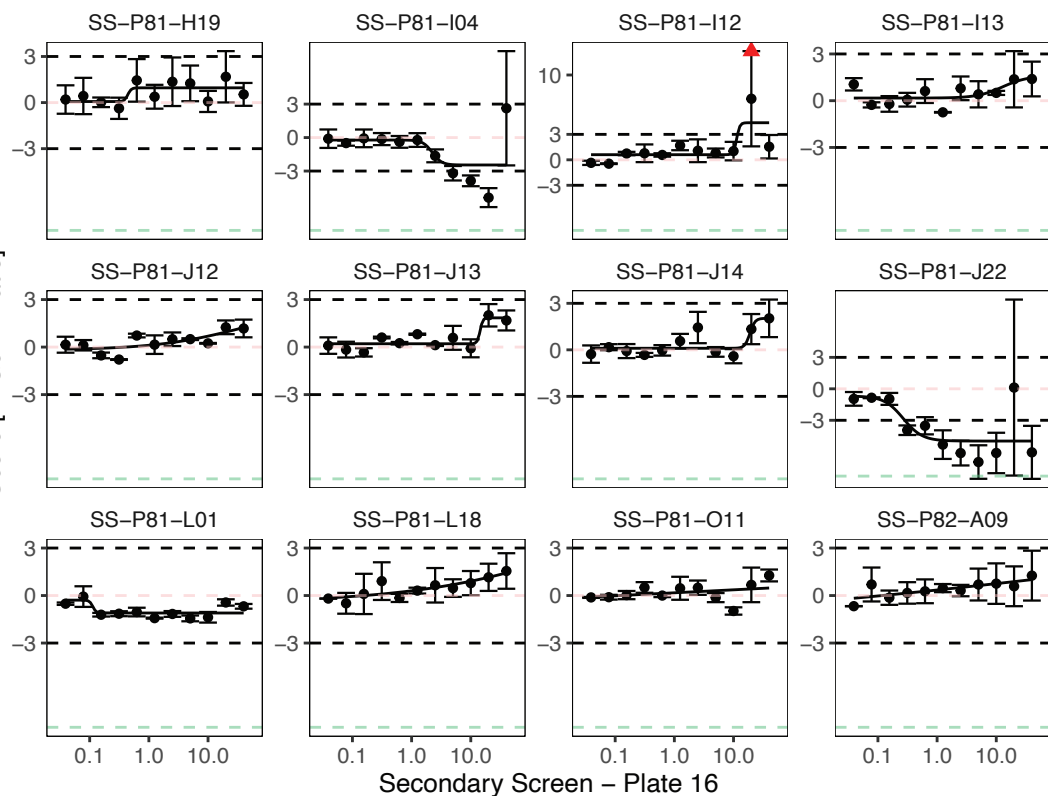
Z-Score [ATG9A-Ratio]



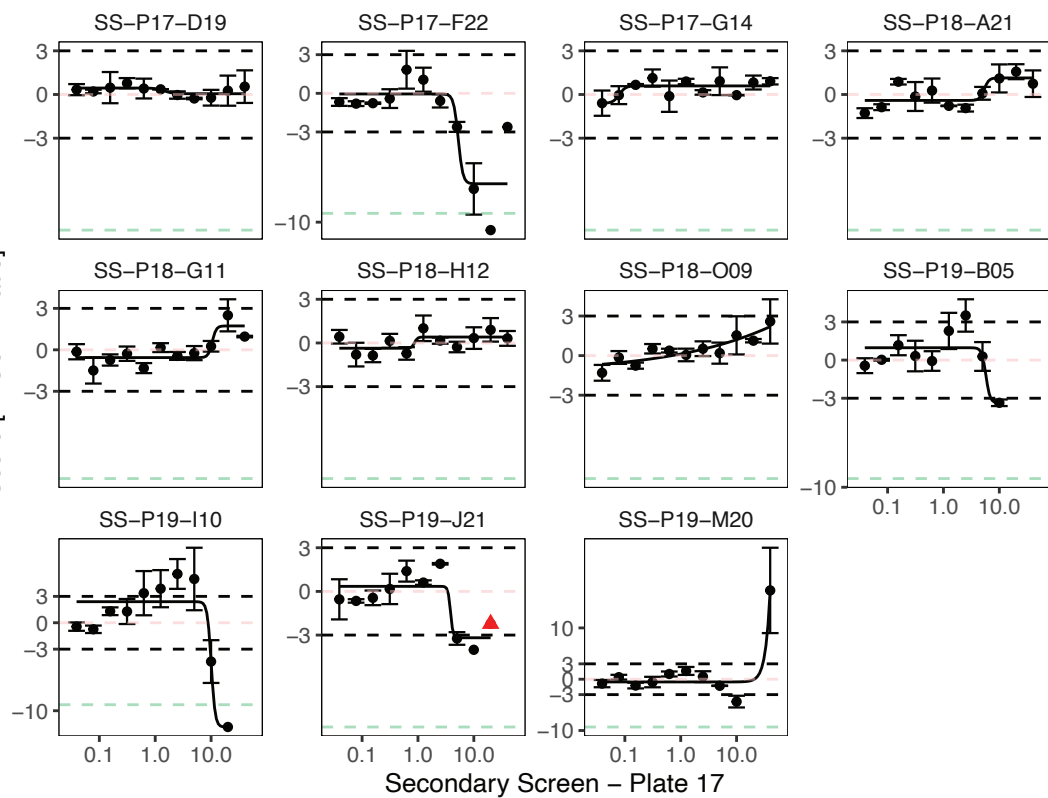
Secondary Screen – Plate 15



Z-Score [ATG9A-Ratio]



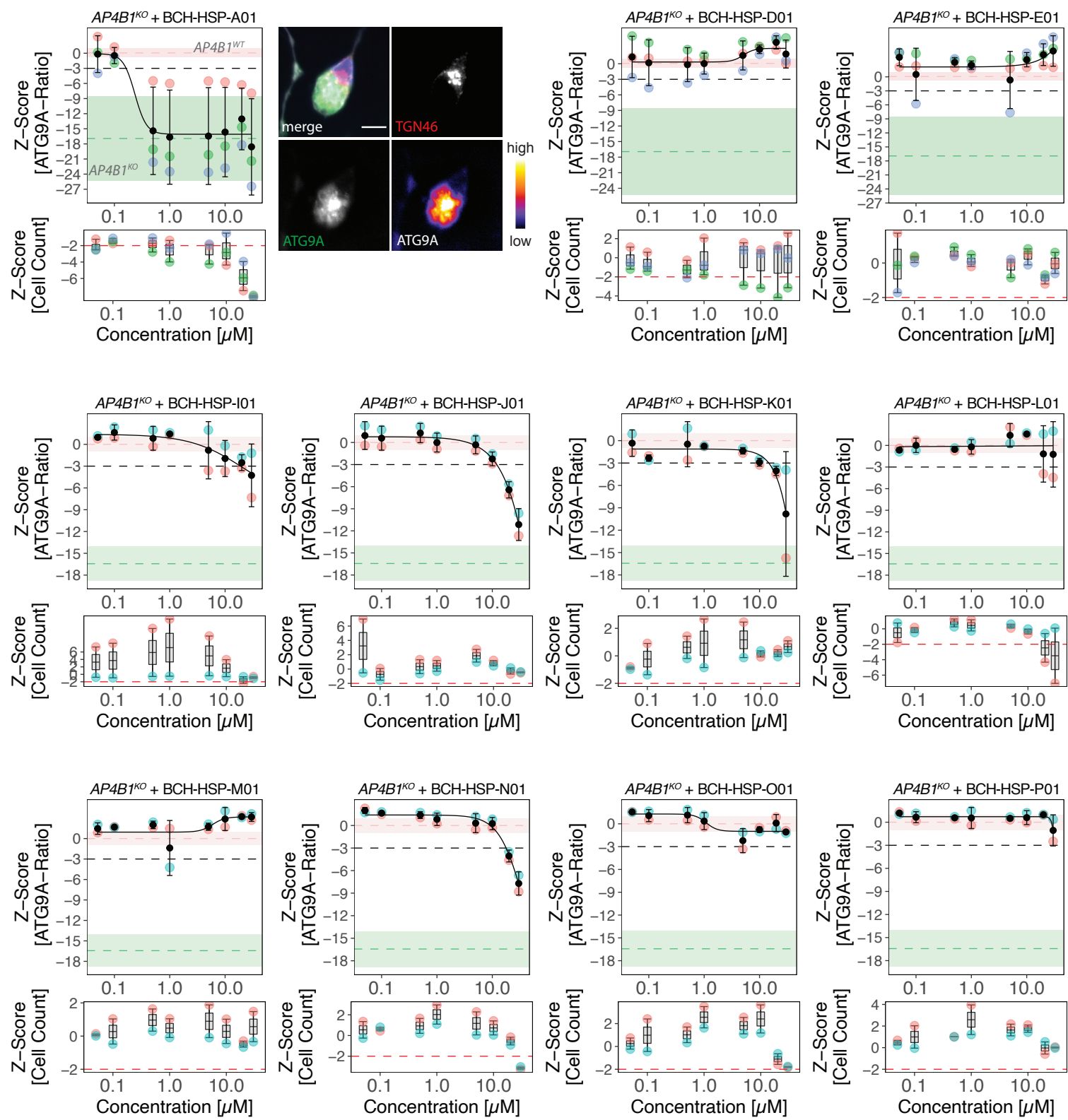
Z-Score [ATG9A-Ratio]



**Supplementary Figure 2. Summary of the counter-screen in AP-4-HSP patient-derived fibroblasts.**

Overview of the counter-screen of the 503 active compounds identified in the primary screen. To assess for dose-dependent effects, compounds were screened in AP-4-HSP patient-derived fibroblasts in 384-well microplates using 11-point titrations ranging from 40 nM to 40  $\mu$ M. All concentrations were screened in duplicates. Black dots and error bars represent mean  $\pm$  1 SD. Active compounds were *a priori* defined as those reducing the ATG9A ratio by at least 3 SD compared to negative controls, in more than one concentration. Toxicity was defined as a reduction of cell count of at least 2 SD compared to the negative control. Green dotted lines represent the mean of the positive controls, while red dotted lines indicate the mean of the negative controls. Red triangles indicate toxic concentrations. EC50 are indicated where possible. a) 17 compounds demonstrated a clear and reproducible dose-response relationship and raised no suspicion for autofluorescence on automated and manual review. b) 34 compounds were active but showed autofluorescence or resulted in imaging artifacts. c) Dose-response curves for all 503 compounds tested in the secondary screen.

Supplementary Figure 3.

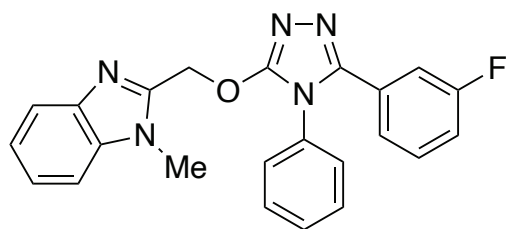


**Supplementary Figure 3. Orthogonal screen identifies 11 compounds that show no activity in *AP4BI*<sup>KO</sup> SH-SY5Y cells.**

Dose-response curves for ATG9A ratios and cell counts in *AB4BI*<sup>KO</sup> cells treated with different compounds. Data points represent per well means from 3 (BCH-HSP-A01, BCH-HSP-D01 and BCH-HSP-E01) or 2 (BCH-HSP-I01, BCH-HSP-J01, BCH-HSP-K01, BCH-HSP-L01, BCH-HSP-M01, BCH-HSP-N01, BCH-HSP-O01, BCH-HSP-P01) different assay plates. Black dots and error bars represent mean  $\pm$  1 SD. Dashed lines show mean Z-scores for positive (green) and negative (salmon) controls. Shaded areas represent  $\pm$  1 SD. Box plots show medians (center), upper and lower quartiles (hinges) and 1.5 x IQR (whiskers). Eleven of 16 compounds were excluded due to either lacking activity (*D-01*, *E-01*, *L-01*, *M-01*, *N-01*, *O-01*, *P-01*), suspicion for artefacts or autofluorescence (*I-01*, *J-01*, *K-01*), or obvious changes in cellular morphology (*A-01*).

Supplementary Figure 4.

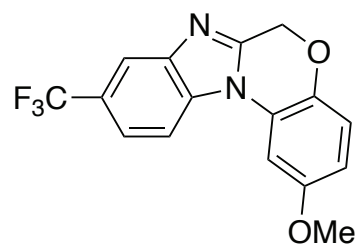
**BCH-HSP-B01**



**1**

Chemical Formula:  $C_{23}H_{18}FN_5O$   
Molecular Weight: 399.43

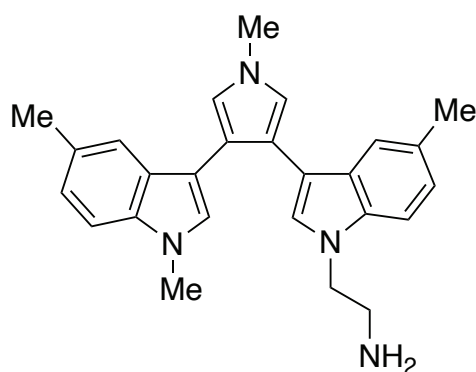
**BCH-HSP-G01**



**2**

Chemical Formula:  $C_{16}H_{11}F_3N_2O_2$   
Molecular Weight: 320.27

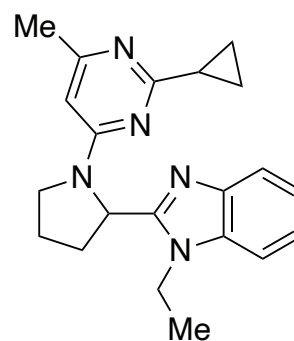
**BCH-HSP-F01**



**3**

Chemical Formula:  $C_{26}H_{28}N_4$   
Molecular Weight: 396.54

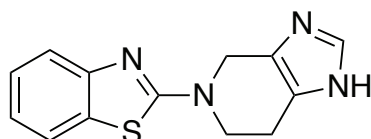
**BCH-HSP-H01**



**4**

Chemical Formula:  $C_{21}H_{25}N_5$   
Molecular Weight: 347.47

**BCH-HSP-C01**



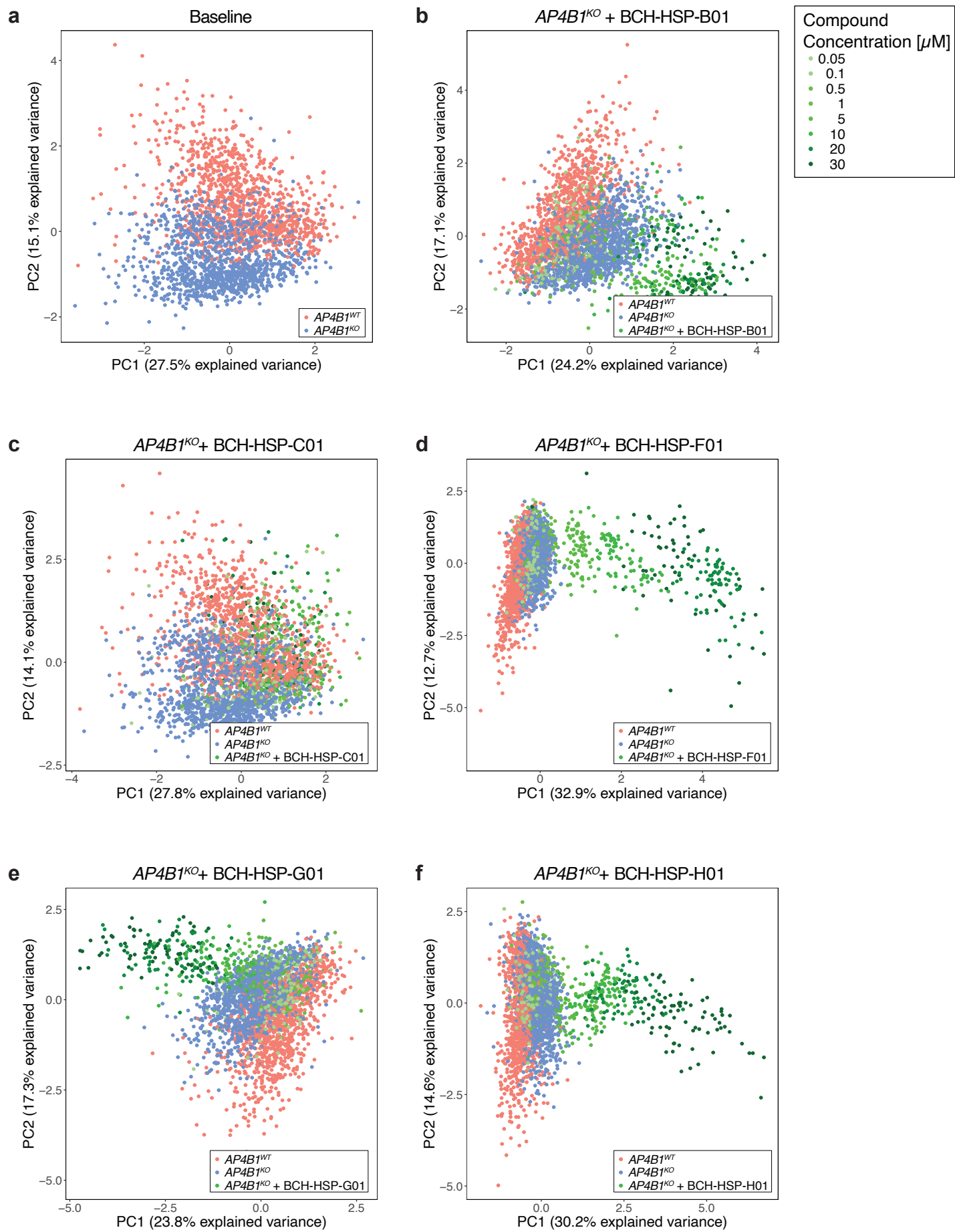
**5**

Chemical Formula:  $C_{13}H_{12}N_4S$   
Molecular Weight: 256.33

**Supplementary Figure 4. Chemical structures of five active compounds.**

Chemical structures of the five active compounds in *AP4B1*<sup>ko</sup> SH-SY5Y cells along with their chemical formula and molecular weight.

Supplementary Figure 5.



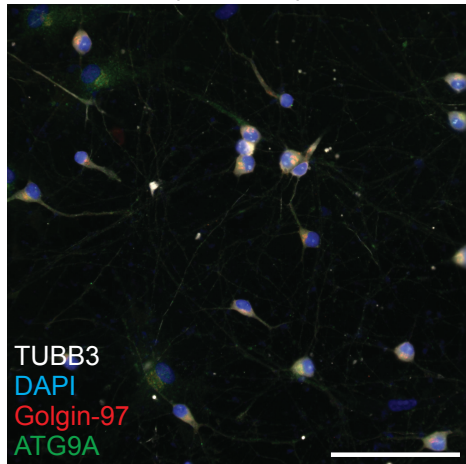
**Supplementary Figure 5. Multiparametric profiling of 5 active compounds in *AP4B1*<sup>KO</sup> SH-SY5Y cells.**

PCA analysis of 85 extracted features of the nucleus, cytoskeleton/global cell morphology, TGN and ATG9A vesicles. (a) Baseline analysis of *AP4B1*<sup>WT</sup> and *AP4B1*<sup>KO</sup> cells. Cell lines clustered closely together and were only separated by the ATG9A signal. (b-f) Spatial clustering of the 5 active compounds in relation to the positive and negative controls. Compound concentrations are depicted by the legend. While BCH-HSP-C01 treated *AP4B1*<sup>KO</sup> cells clustered closely with the controls (c), suggesting no significant off-target effects, all other compounds led to changes in overall cellular morphology in a dose-dependent manner. The most significant changes were seen for BCH-HSP-F01 (d) and BCH-HSP-H01 (f), suggesting off-target effects.

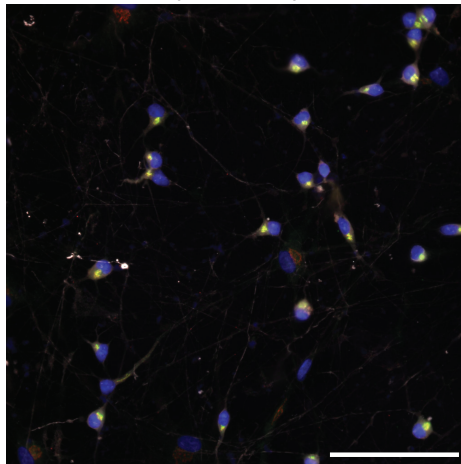


Supplementary Figure 6.

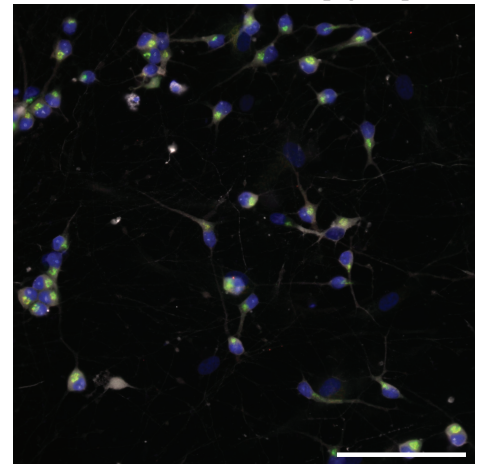
WT/LoF  
(Control)



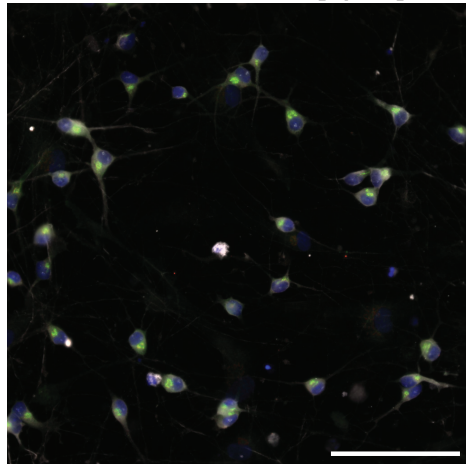
LoF/LoF  
(Patient)



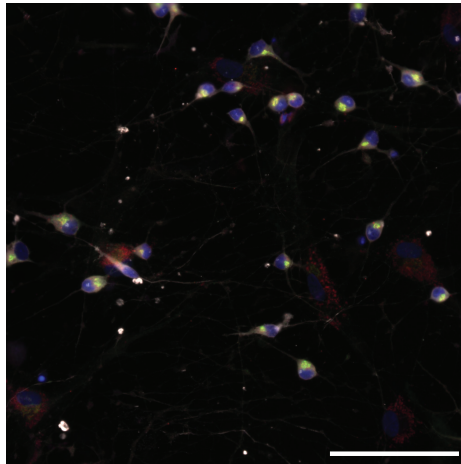
LoF/LoF +  
BCH-HSP-B01 [5 $\mu$ M]



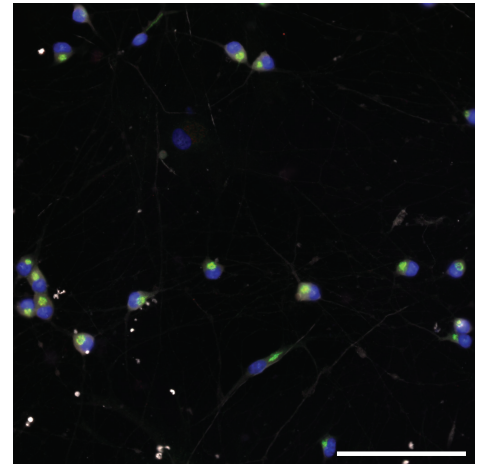
LoF/LoF +  
BCH-HSP-C01 [5 $\mu$ M]



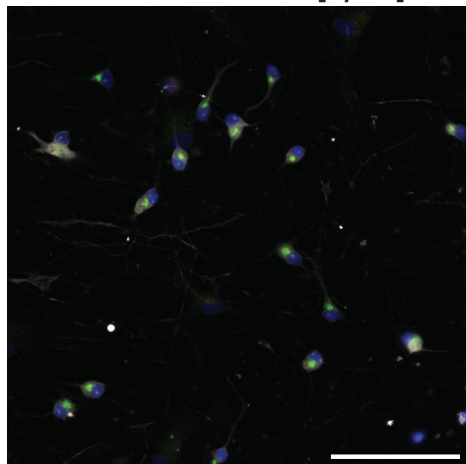
LoF/LoF +  
BCH-HSP-F01 [5 $\mu$ M]



LoF/LoF +  
BCH-HSP-G01 [5 $\mu$ M]



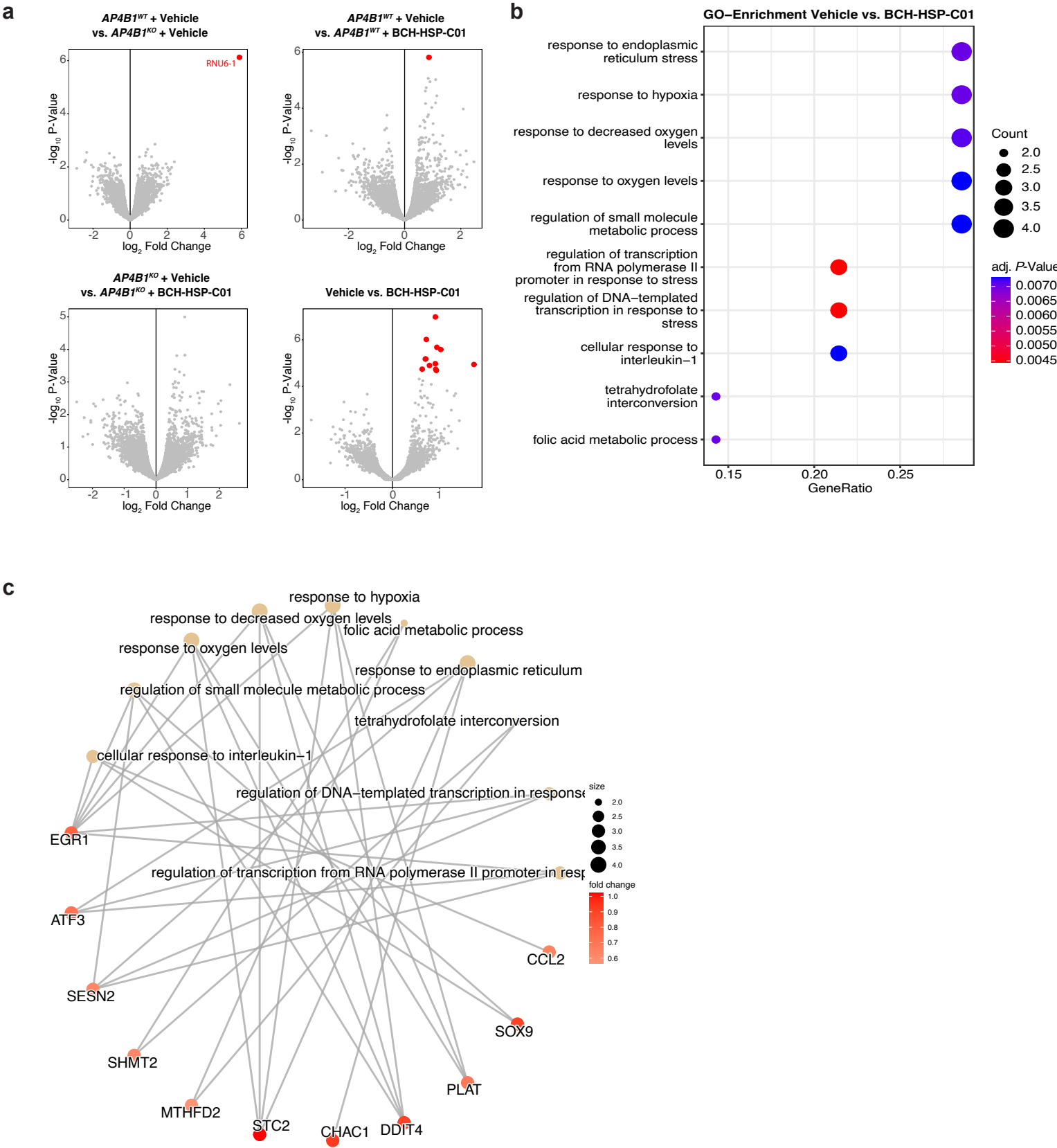
LoF/LoF +  
BCH-HSP-H01 [5 $\mu$ M]



**Supplementary Figure 6. Low magnification images of AP-4-HSP patient-derived hiPSC-neurons treated with 5 active compounds.**

Low magnification images of the testing of 5 active compounds in hiPSC-derived cortical neurons from a patient with *AP4M1*-associated SPG50 compared to a heterozygous control (same-sex parent). The merge shows beta-3 tubulin (grey), DAPI (blue), the Golgi (red) and ATG9A (green). Scale bar: 100  $\mu\text{m}$ .

Supplementary Figure 7.



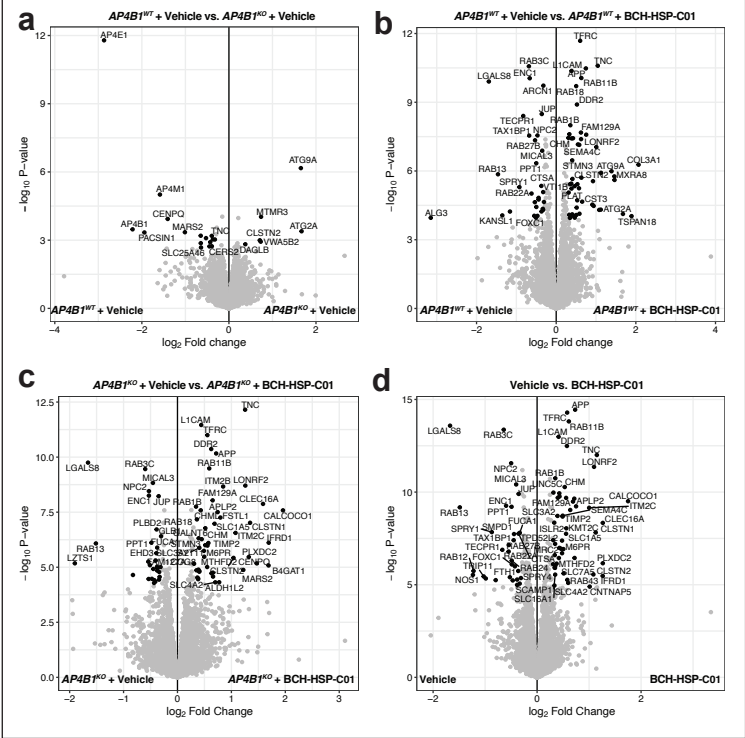
**Supplementary Figure 7. Bulk RNA sequencing in *AP4BI*<sup>KO</sup> SH-SY5Y cells treated with BCH-HSP-C01 shows a small number of differentially expressed genes, mainly involved in ER stress rese.**

(a) Volcano plots depicting the results of bulk RNA Sequencing in different experimental conditions in SH-SY5Y cells (*AP4BI*<sup>WT</sup> vs. *AP4BI*<sup>KO</sup> treated with vehicle, *AP4BI*<sup>WT</sup> treated with vehicle vs. *AP4BI*<sup>WT</sup> treated with BCH-HSP-C01, *AP4BI*<sup>KO</sup> treated with vehicle vs. *AP4BI*<sup>KO</sup> treated with BCH-HSP-C01, *AP4BI*<sup>WT</sup> and *AP4BI*<sup>KO</sup> cells pooled in two groups, vehicle vs. BCH-HSP-C01). Differential expression analysis was done following the TREAT approach developed by McCarthy and Smyth (2009). Red colored dots represent differentially expressed genes with a log<sub>2</sub> fold change > 0.3 and a FDR < 0.05.

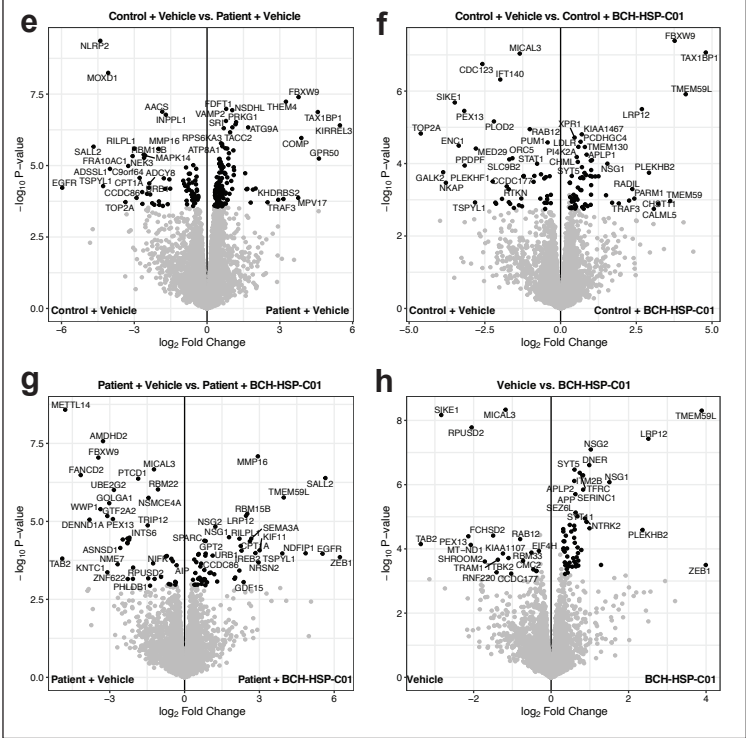
(b) Gene ontology analysis shows enriched pathways of the pooled analysis. Pathways were considered differentially expressed with a FDR < 0.05. (c) Gene-Concept Network showing differentially expressed genes and their pathway membership.

Supplementary Figure 8.

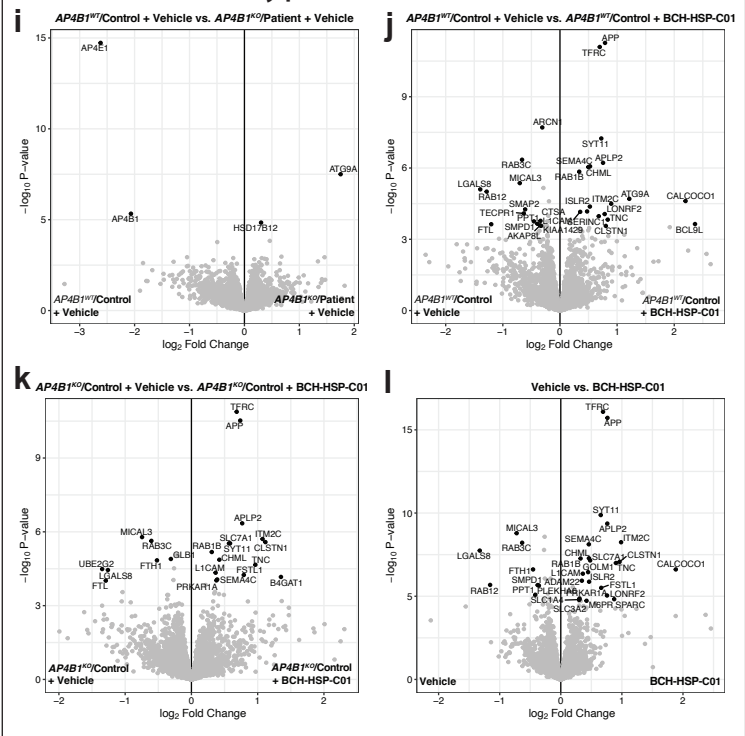
SH-SY5Y Cells



iPSC Neurons

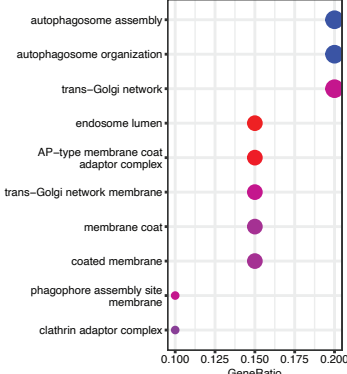


Cell Types Combined



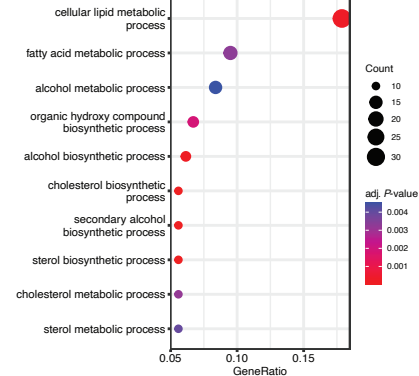
m

GO-Analysis:  
*AP4B1*<sup>WT</sup> + vehicle vs. *AP4B1*<sup>KO</sup> + vehicle



n

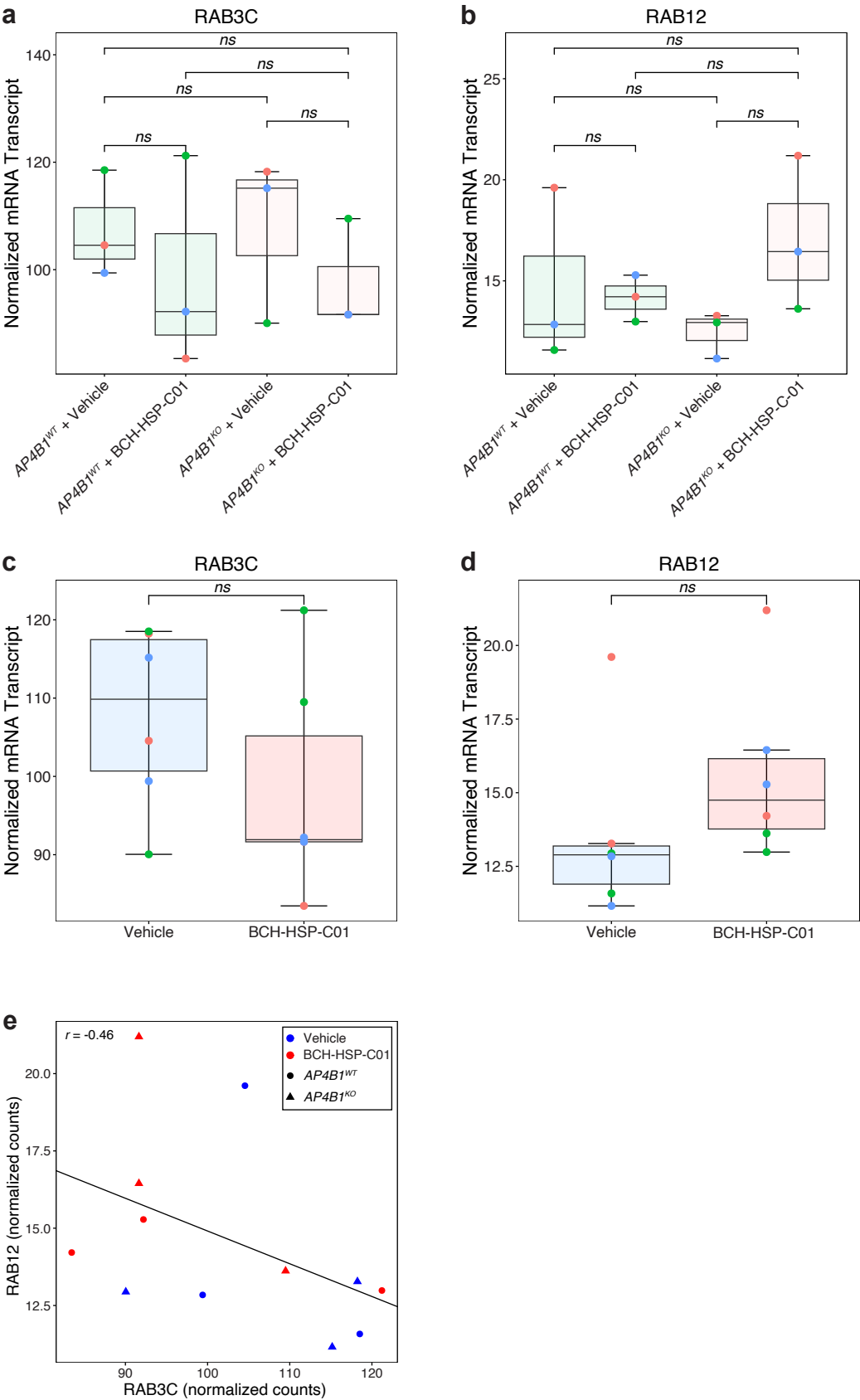
GO-Analysis:  
iPSC neurons Control + vehicle vs. Patient + vehicle



**Supplementary Figure 8. Unbiased quantitative proteomics in *AP4BI*<sup>KO</sup> SH-SY5Y cells and AP-4-HSP patient-derived hiPSC-neurons treated with BCH-HSP-C01.**

(a-d) SH-SY5Y cells: 8141 unique proteins were analyzed. Volcano plots summarize differential protein enrichment for different experimental conditions: (a) *AP4BI*<sup>WT</sup> vs. *AP4BI*<sup>KO</sup> treated with vehicle, (b) *AP4BI*<sup>WT</sup> treated with vehicle vs. *AP4BI*<sup>WT</sup> treated with BCH-HSP-C01, (c) *AP4BI*<sup>KO</sup> treated with vehicle vs. *AP4BI*<sup>KO</sup> treated with BCH-HSP-C01, (d) *AP4BI*<sup>WT</sup> and *AP4BI*<sup>KO</sup> cells pooled treated with vehicle vs. BCH-HSP-C01. Differentially enriched proteins are depicted in black. (e-h) hiPSC-derived neurons: 7386 unique proteins were analyzed. Volcano plots summarize differential protein enrichment for different experimental conditions: (e) controls vs. patient-derived neurons treated with vehicle, (f) controls treated with vehicle vs. controls treated with BCH-HSP-C01, (g) patient-derived neurons treated with vehicle vs. patient-derived neurons treated with BCH-HSP-C01, (h) controls and patient-derived neurons pooled in two groups, treated with vehicle vs. BCH-HSP-C01. Differentially enriched proteins are depicted in black. (i-l) Integrated analysis of SH-SY5Y cells and hiPSC-derived neurons: 5357 unique proteins were analyzed. Volcano plots summarize differential protein enrichment for different experimental conditions: (i) controls vs. AP-4-deficient cells treated with vehicle, (j) controls treated with vehicle vs. controls treated with BCH-HSP-C01, (k) AP-4-deficient cells treated with vehicle vs. AP-4-deficient cells treated with BCH-HSP-C01, (l) controls and AP-4-deficient cells pooled into two groups, vehicle vs. BCH-HSP-C01. Differentially enriched proteins are depicted in black. In all datasets statistical testing for differential protein enrichment was done using protein-wise linear models and empirical Bayes statistics. Proteins were considered as differentially enriched with a FDR < 0.05 and a log<sub>2</sub> fold change > 0.3. (m & n) Gene ontology analysis of *AP4BI*<sup>WT</sup> vs. *AP4BI*<sup>KO</sup> SH-SY5Y cells (m) as well as control vs. patient hiPSC-derived neurons (n) treated with vehicle shows enrichment of various cellular processes. Pathways were considered differentially enriched with a FDR < 0.05.

Supplementary Figure 9.

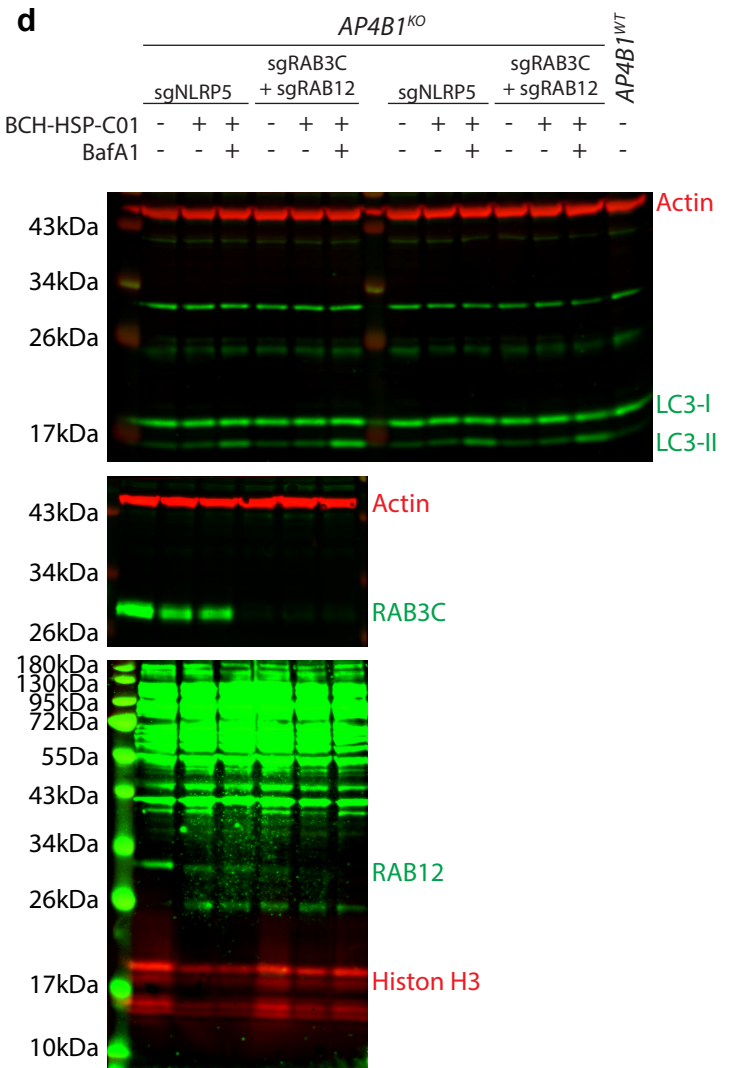
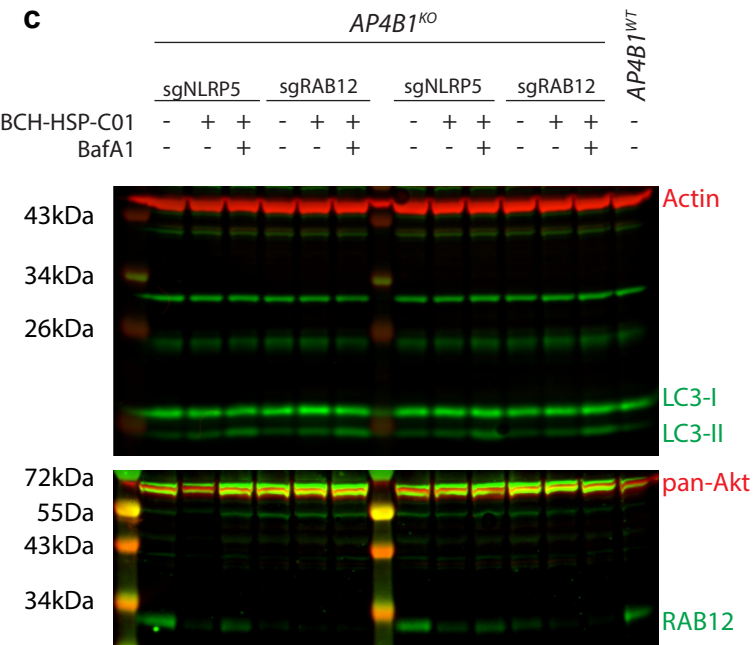
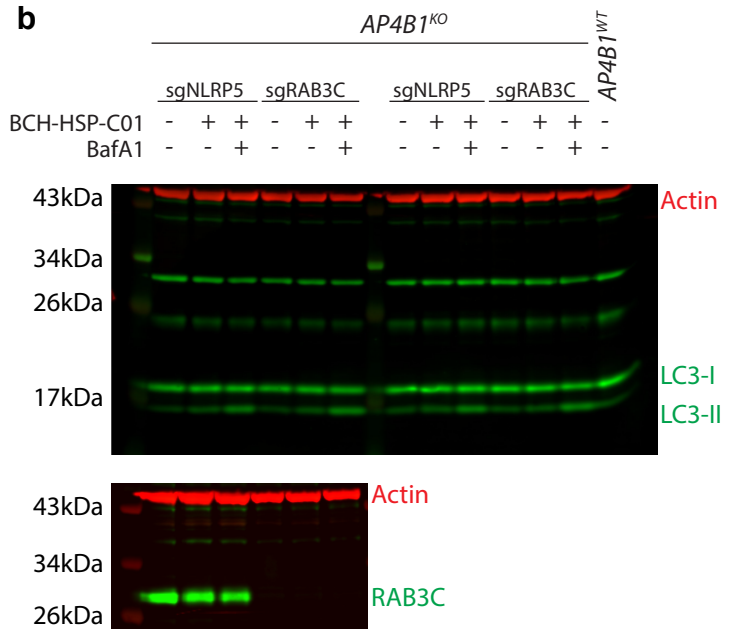
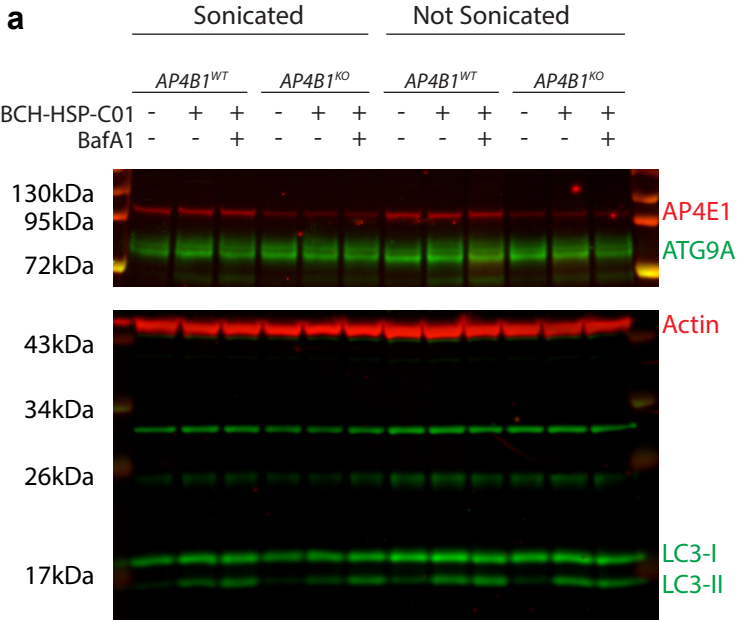


**Supplementary Figure 9. mRNA transcript expression and correlation analysis of *RAB3C* and *RAB12*.**

Normalized mRNA transcript counts for *RAB3C* (a, c) and *RAB12* (b, d) across different experimental conditions in SH-SY5Y cells (*AP4B1*<sup>WT</sup> treated with vehicle, *AP4B1*<sup>WT</sup> treated with BCH-HSP-C01, *AP4B1*<sup>KO</sup> treated with vehicle, *AP4B1*<sup>KO</sup> treated with BCH-HSP-C01) (a, b), as well as *AP4B1*<sup>WT</sup> and *AP4B1*<sup>KO</sup> cells pooled treated with vehicle vs. BCH-HSP-C01 (c, d). No statistically significant differences were detected. Statistical testing was done using pairwise T-tests. *P*-values have been adjusted for multiple testing using the Benjamini-Hochberg procedure. (e) Correlation analysis of *RAB3C* and *RAB12* gene expression in *AP4B1*<sup>WT</sup> (n = 6 samples) and *AP4B1*<sup>KO</sup> (n = 6 samples) SH-SY5Y cells shows a moderate inverse correlation measured by the Pearson correlation coefficient (*r*).



Supplementary Figure 10.



**Supplementary Figure 10. Original Western blots.**

(a) Uncropped, original blots corresponding to Fig. 8c. (b) Uncropped, original blot corresponding to Figure 8j. (c) Uncropped, original blot corresponding to Fig. 8k. (d) Uncropped, original blot corresponding to Fig. 8l.