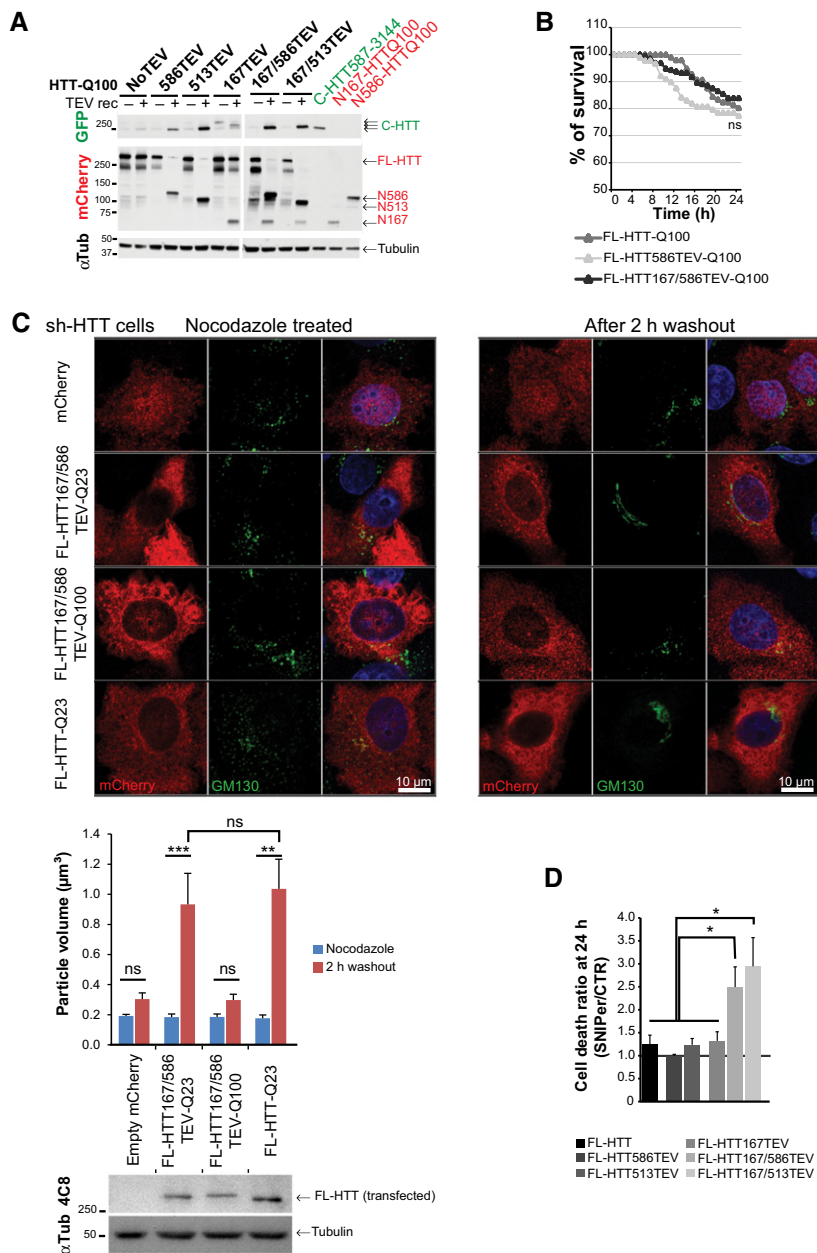


## Expanded View Figures



**Figure EV1. Time- and site-specific control of huntingtin proteolysis.**

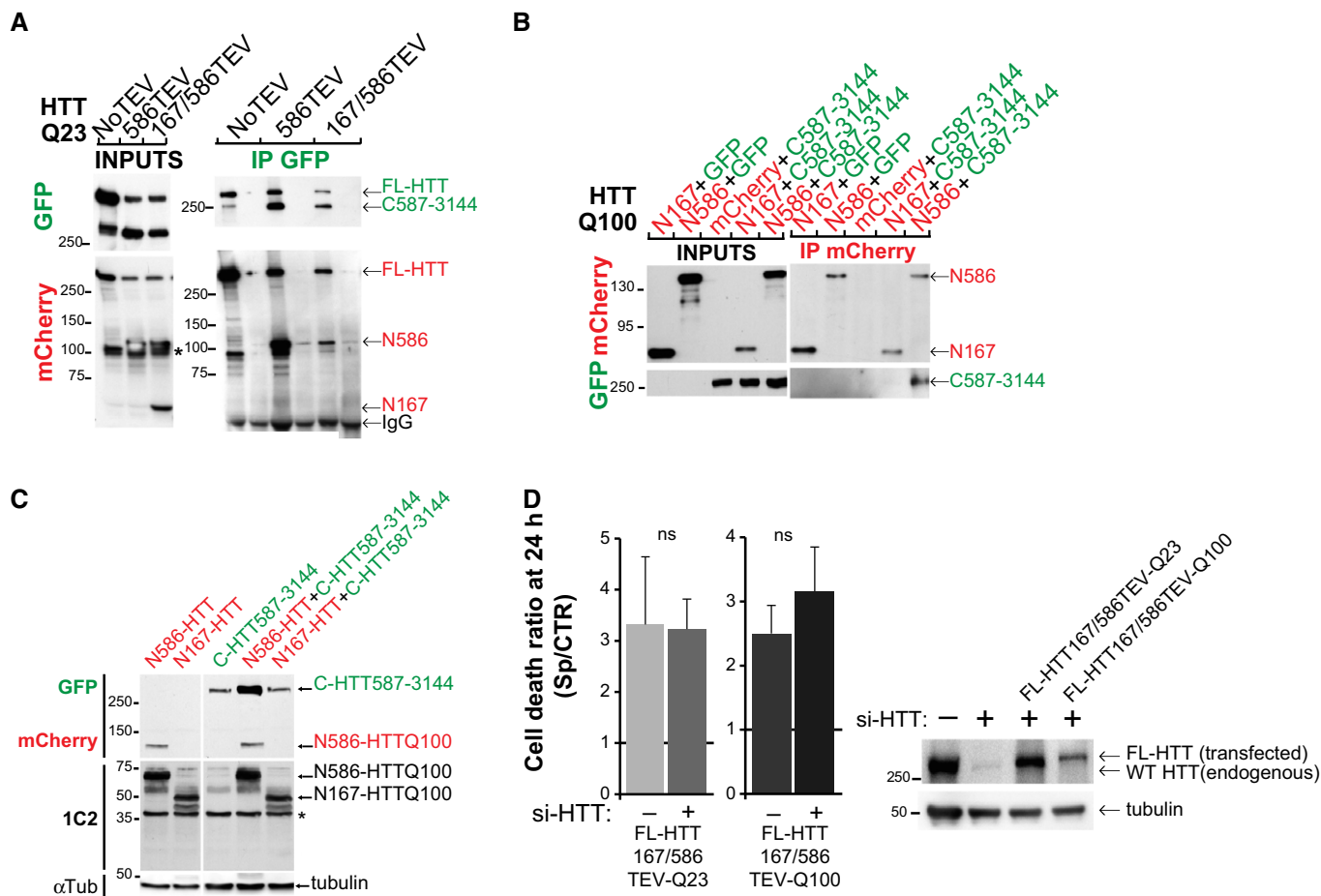
**A** Immunoblotting analyses of HTT N-ter and C-ter fragments generated after cleavage of the indicated constructs expressed in HEK293T cells and using recombinant TEV system. “no TEV” referred to FL-HTT-Q100 constructs, without TEVrcs.

**B** Survival curves of striatal cells expressing FL-HTT constructs containing no TEV, one or two TEVrcs. Toxicity is expressed as the percentage of survival over time.

**C** FL-HTT constructs with TEV sequence insertions at positions 167 and 586 are as functional as FL-HTT on the dynein-dependent Golgi reassembly assay. Upper panels show representative HeLa cells stably silenced for endogenous HTT and that re-express FL-HTT167/586TEV-Q23, HTT167/586TEV-Q100 or FL-HTT-Q23 and treated with nocodazole for 2 h (left panels) and after 2-h washout (right panels). Western blotting shows the expression level of transfected FL-HTT in sh-HTT cell line.

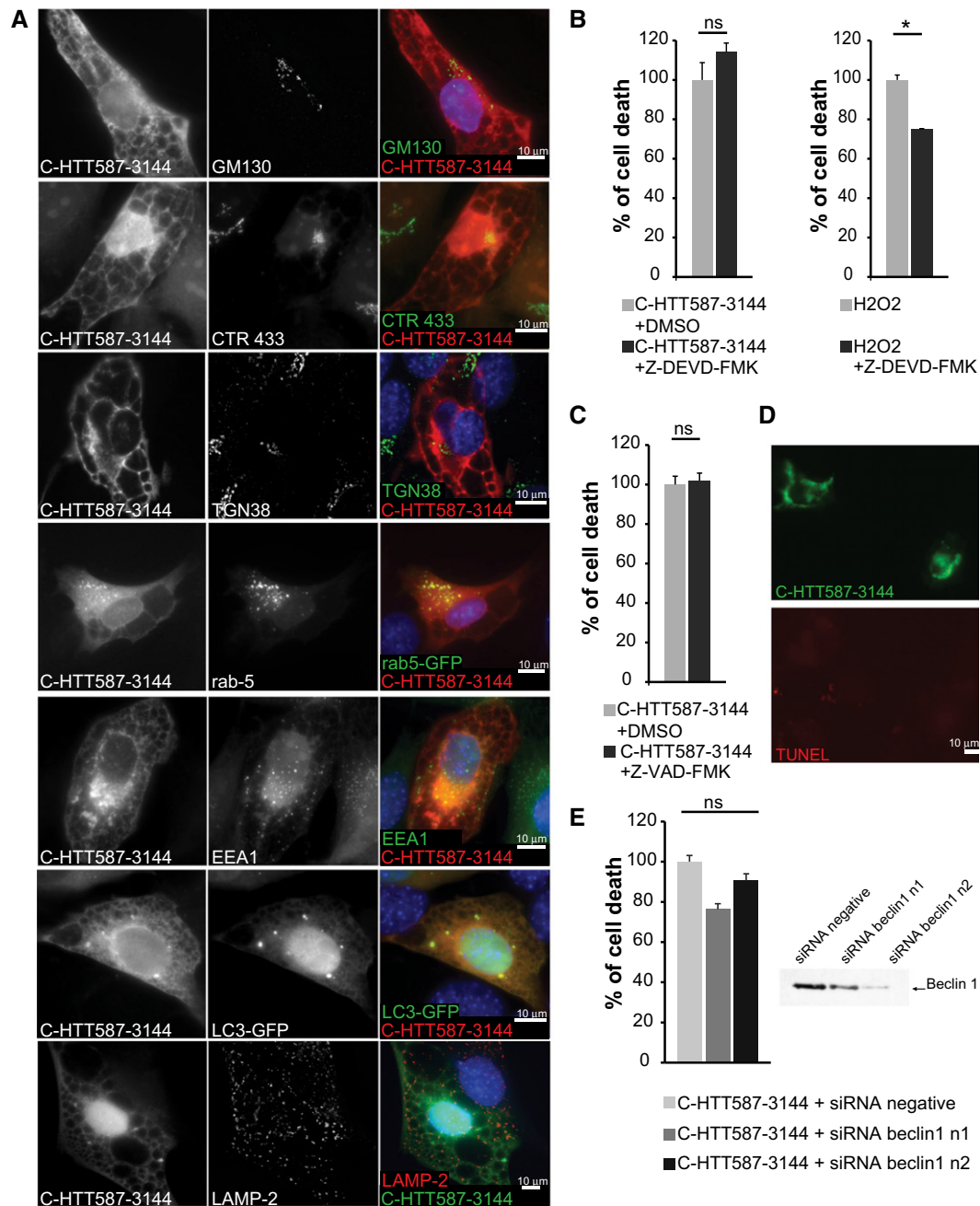
**D** Graph summarising cell death ratio at 24 h of FL-HTT-Q100 analysed in Fig 1D. HTT proteolysis-induced toxicity is shown as a ratio of the percentage of cell death obtained in SNIPer-TEV condition over pcDNA condition.

Data information: The graphs (mean ± SEM) display pooled data from three to four independent experiments (B), from two independent experiments performed in duplicates (C) or from three to four independent experiments (D). In (B), total number of cells and number of independent experiments are as follows: FL-HTT-Q100 + pcDNA: 96 (n = 3) and FL-HTT586TEV-Q100 + pcDNA: 88 (n = 3) and FL-HTT167/586TEV-Q100 + pcDNA: 192 (n = 4). In (C), total number of cells is as follows: nocodazole treated: 6, washout condition: 8. In (D), total number of cells is as in Fig 1D with 140 cells for FL-HTT. Statistics were done by Kaplan–Meier, log-rank test (B), one-way ANOVA with Bonferroni’s multiple comparison tests (C) and ordinary one-way ANOVA with Fisher LSD test (D). ns: non-significant, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.



**Figure EV2. Intramolecular interactions between proteolytic huntingtin fragments.**

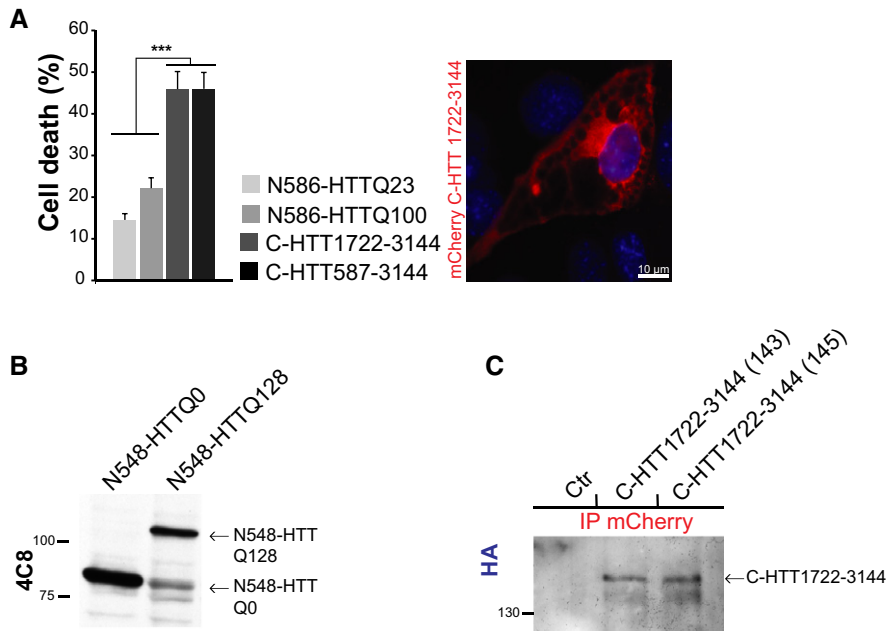
A Immunoprecipitations upon cleavage of FL-HTT-TEV-Q23 by the SNIPer-TEV performed in HEK293T cell. \* indicates a non-specific band.  
 B Immunoprecipitations of HeLa cell extracts expressing mCherry-N-HTTQ100 constructs or mCherry and C-HTT587-3144-GFP or GFP using anti-mCherry antibody.  
 C Immunoblotting analysis of the expression level of N- and C-HTT alone or in combination with striatal cells. \* indicates a non-specific band.  
 D Toxicity in striatal cells transfected with FL-HTT167/586TEV-Q23 and FL-HTT167/586TEV-Q100 and siRNA targeted against endogenous HTT (si-HTT). Graph summarising cell death ratio at 24 h of proteolysis-induced toxicity is shown as a ratio of the percentage of cell death obtained in SNIPer-TEV condition over pcDNA condition. Downregulation of HTT expression was assessed by immunoblotting (right). The bar graphs (mean ± SEM) display pooled data from two to four independent experiments. Total number of cells is as follows: FL-HTT167/586TEV-Q23 without si-HTT: 399 cells; with si-HTT: 230 cells; FL-HTT167/586TEV-Q100 without si-HTT: 430 cells; with si-HTT: 199 cells. Statistics were done by unpaired t-test. ns: non-significant.



**Figure EV3. Released C-terminal HTT fragment elicits endoplasmic reticulum dilation, stress and cell death.**

- A Immunostainings of striatal cells transfected with mCherry-tagged C-HTT587-3144 fragment. Golgi apparatus compartments were immunostained with anti-GM130 (cis-Golgi), anti-CTR433 (medial-Golgi) and anti-TGN38 (trans-Golgi) antibodies. Early endosomes were either stained with anti-EEA1 or labelled with Rab5-GFP-expressing constructs. LC3-GFP-labelled autophagosomes and lysosomes were immunostained with anti-LAMP2.
- B Left: Striatal cells transfected with C-HTT587-3144 were treated with Z-DEVD-FMK, a caspase-3 inhibitor and analysed for cell death. Right: Non-transfected cells were treated for 2 h with 1 mM of H<sub>2</sub>O<sub>2</sub> and Z-DEVD-FMK.
- C Toxicity in striatal cells transfected with C-HTT587-3144 and treated with Z-VAD-FMK.
- D Assessment of DNA fragmentation in C-HTT587-3144-expressing striatal cells using a TUNEL assay.
- E Toxicity in striatal cells transfected with C-HTT587-3144 fragment and siRNA targeted against Beclin-1 (two independent siRNA n1 & n2). Downregulation of Beclin-1 expression was assessed by immunoblotting.

Data information: The bar graphs (mean  $\pm$  SEM) display pooled data from three to five independent experiments in triplicates. For the quantification of cell death after H<sub>2</sub>O<sub>2</sub> treatment (B), one experiment was performed in duplicate and more than 1,000 cells were analysed. Statistics were done by unpaired *t*-test (B, C) or one-way ANOVA with Bonferroni's multiple comparison test. ns: Non-significant, \**P* < 0.05.

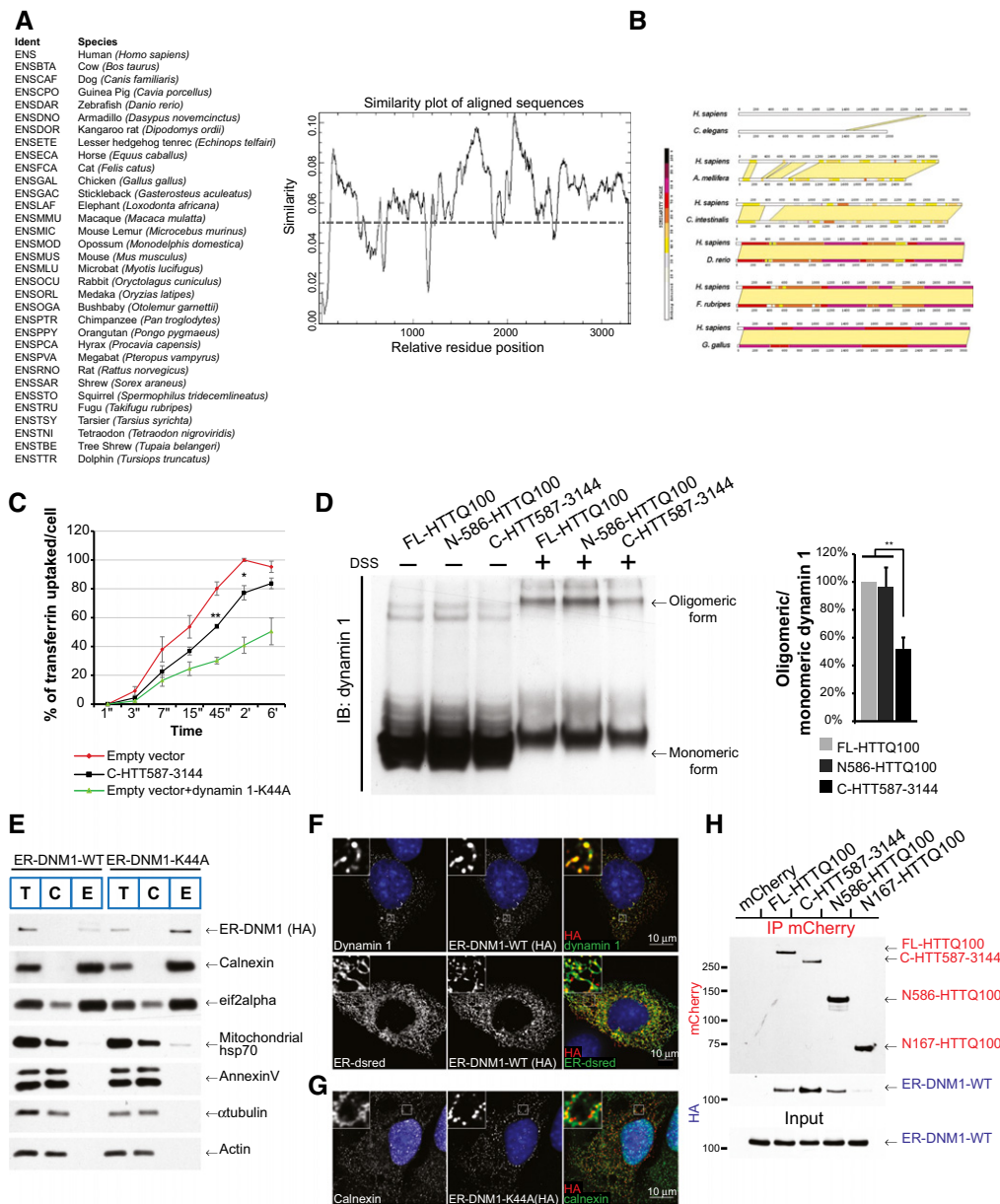


**Figure EV4. C-ter huntingtin causes toxicity in fly.**

**A** Analysis of C-HTT1722–3144 toxicity in striatal cells (left) and representative image of vacuolation in C-HTT1722–3144-expressing cells (right). The graph (mean ± SEM) displays pooled data from three independent experiments in duplicate. Total number of cells is as follows: N586-HTTQ23: 2285, N586-HTTQ100: 1698, C-HTT1722–3144: 483, C-HTT587-3144: 708. Statistics were done by one-way ANOVA with Bonferroni's multiple comparison test, \*\*\* $p < 0.001$ .

**B** Assessment of N548-HTT transgene expression on total brain lysates of adult flies (4 days of age) by immunoblotting.

**C** Detection of C-HTT1722–3144 transgenes in two independent fly strains (143, 145) by immunoprecipitation. Control flies (ctr) are  $W^{1118}$  flies.



**Figure EV5. C-terminal HTT impairs the activity of ER-localised dynamin 1 that induces ER dilation and death.**

- A** Identification of conserved regions in huntingtin. Search of primary sequence of HTT orthologues for highly conserved regions. Analysis of 32 orthologous sequences and generation of a residue conservation plot using the EMBOSS package tool "plocon". Smoothing was applied to the results revealing regions with higher degrees of identity.
- B** Pairwise comparison of human HTT versus *C. elegans*, *A. mellifera*, *C. intestinalis*, *D. rerio*, *F. rubripes* or *G. gallus* Htt. Very N-ter part of HTT (residues 170–400) and regions starting from 1200 to 3144 showed high similarity between the different species.
- C** Assessment of dynamin 1 activity by transferrin uptake in striatal cells. GFP-tagged dynamin 1-K44A construct was used as a positive control for endocytosis inhibition. The curves indicate the percentage of transferrin uptake over time in s (") and min (').
- D** Dynamin 1 oligomeric profile in HTT-expressing cells treated or not with DSS, a non-cleavable cross-linker. HEK293T cells were transfected with the indicated HTT fragments and dynamin 1. Endogenous HTT was downregulated using siRNA which do not target the HTT constructs.
- E** Localisation of engineered ER-targeted dynamin 1 (ER-DNM1-WT or ER-DNM1-K44A) constructs at ER by cellular fractionation in striatal cells. Total (T), cytosolic (C) and ER membrane (E) fractions. ER-DNM1 was detected using anti-HA.
- F, G** Analysis of localisation of engineered ER-targeted dynamin 1 (ER-DNM1-WT or ER-DNM1-K44A) using anti-HA immunostaining in striatal cells. Note the artificial high co-localisation on ER network as shown by immunostaining with ER-dsRed and calnexin.
- H** Striatal cells expressing the indicated mCherry-tagged HTT fragments and ER-DNM1-WT were subjected to immunoprecipitation and analysed by immunoblotting.

Data information: The graphs (mean  $\pm$  SEM) display pooled data from three independent experiments in triplicate. In (C), total number of cells is 10,000 cells per experiment. Statistics were done by unpaired *t*-test (C) or one-way ANOVA with Bonferroni's multiple comparison test (D). \**P* < 0.05, \*\**P* < 0.01.