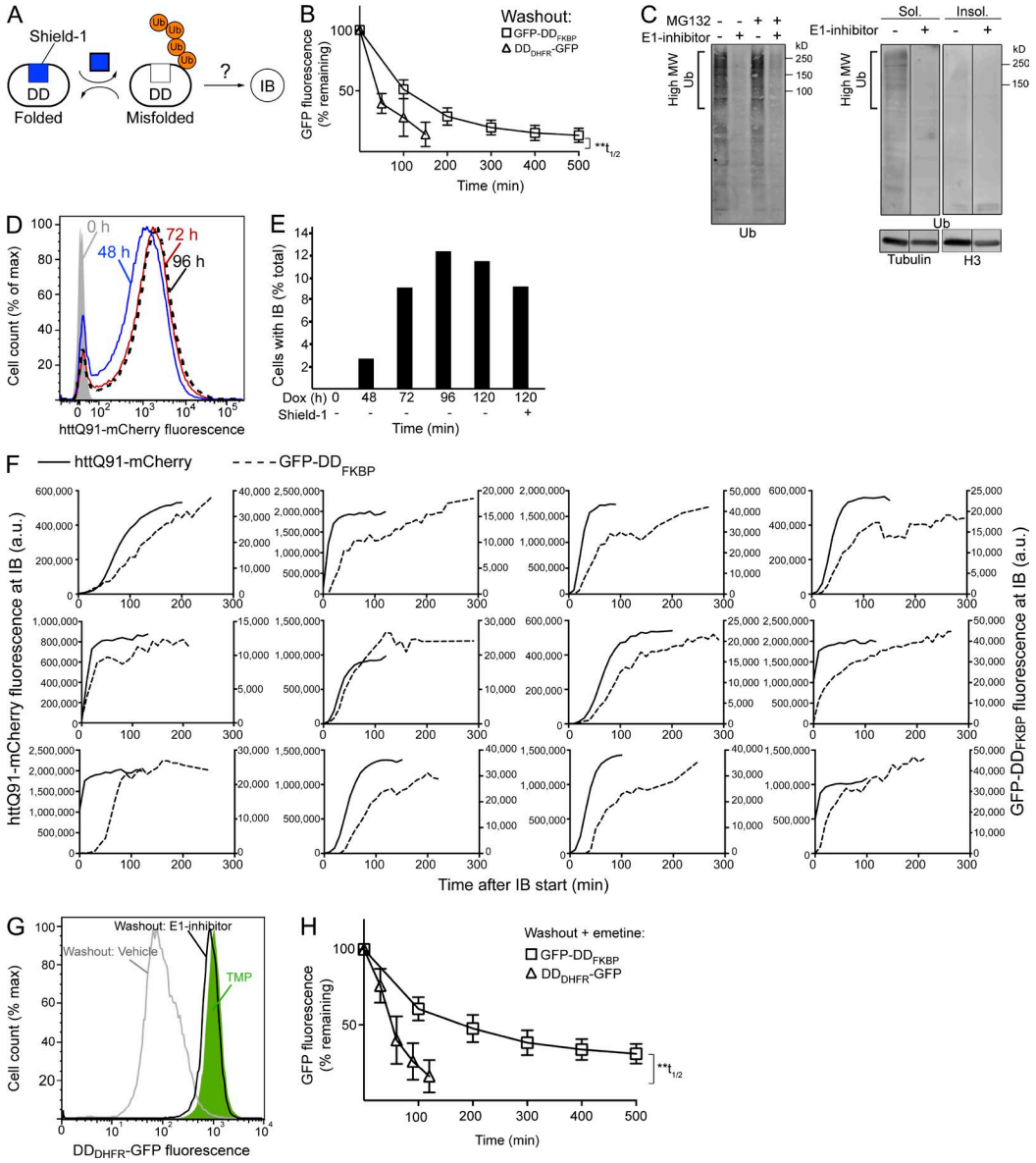

Figure S1. **Additional characterization of reporter cell lines and effects of E1 inhibitor on levels of poly-Ub conjugates.** (A) Strategy used to assess the role of Ub conjugation in recruitment of proteins to htt IBs. The degradation domain (DD) is folded when it is reversibly bound to the synthetic ligand shield-1. DD becomes misfolded and ubiquitylated after removal of shield-1 and thus can be used as a conditionally ubiquitylated reporter for IB recruitment. (B) Decay rate of GFP-DD$_{FKBP}$ and DD$_{DHFR}$-GFP fluorescence after removal of stabilizing ligands. U2-DD^{FK}Q91 and U2-DD^{DH}Q91 cells were treated with 1 µM shield-1 or 10 µM TMP, respectively, for 24 h, and mean GFP fluorescence of cells (n > 15) after washout of ligands was determined by time-lapse microscopy. Data points indicate mean ± SD. (C) E1 inhibitor promotes deubiquitylation of Ub conjugates. (Left) Cells were treated with 10 µM E1 inhibitor, 10 µM MG132, or both for 3 h, and levels of Ub conjugates in whole cell lysates were determined by SDS-PAGE and immunoblotting with anti-Ub antibody. (Right) Cells were treated with 10 µM E1 inhibitor, lysed in 1% Triton X-100, and centrifuged to generate soluble and insoluble fractions. Levels of Ub conjugates were determined by immunoblotting with anti-Ub antibody, and immunoblots with anti-tubulin and anti-histone H3 antibodies were used as loading controls. The bracket indicates high molecular weight (MW) Ub conjugates. (D) Increase in total httQ91-mCherry levels after addition of dox. U2-DD^{FK}Q91 cells were treated with 1 µg/ml dox for 0, 48, 72, or 96 h and analyzed by flow cytometry. (E) Induction of httQ91-mCherry expression results in accumulation of IBs over time. The percentage of U2-DD^{FK}Q91 cells with IBs was determined by PuISA after addition of 1 µg/ml dox over 96 h. (F) Quantification of total httQ91-mCherry and GFP-DD$_{FKBP}$ fluorescence at IBs in single U2-DD^{FK}Q91 cells. t = 0 indicates the frame in which httQ91-mCherry IBs were first observed. a.u., arbitrary units. (G) DD$_{DHFR}$-GFP undergoes Ub-dependent degradation. GFP fluorescence of U2-DD^{DH}Q91 cells was determined by flow cytometry analysis in the presence of 10 µM TMP or after washout of TMP for 6 h in the presence or absence of 10 µM E1 inhibitor. (H) DD$_{DHFR}$-GFP is degraded with a shorter half-life than GFP-DD$_{FKBP}$. U2-DD^{FK}Q91 and U2-DD^{DH}Q91 cells were treated with 1 µM shield-1 or 10 µM TMP, respectively, for 24 h, and mean GFP fluorescence of cells (n > 10) was determined by time-lapse microscopy after inhibition of translation with 25 µM emetine. Data points indicate mean ± SD. Western blots are representative of at least two independent experiments. For flow cytometry data, n > 10,000 cells were analyzed. Statistically relevant differences (α = 0.05): **, P < 0.0001.

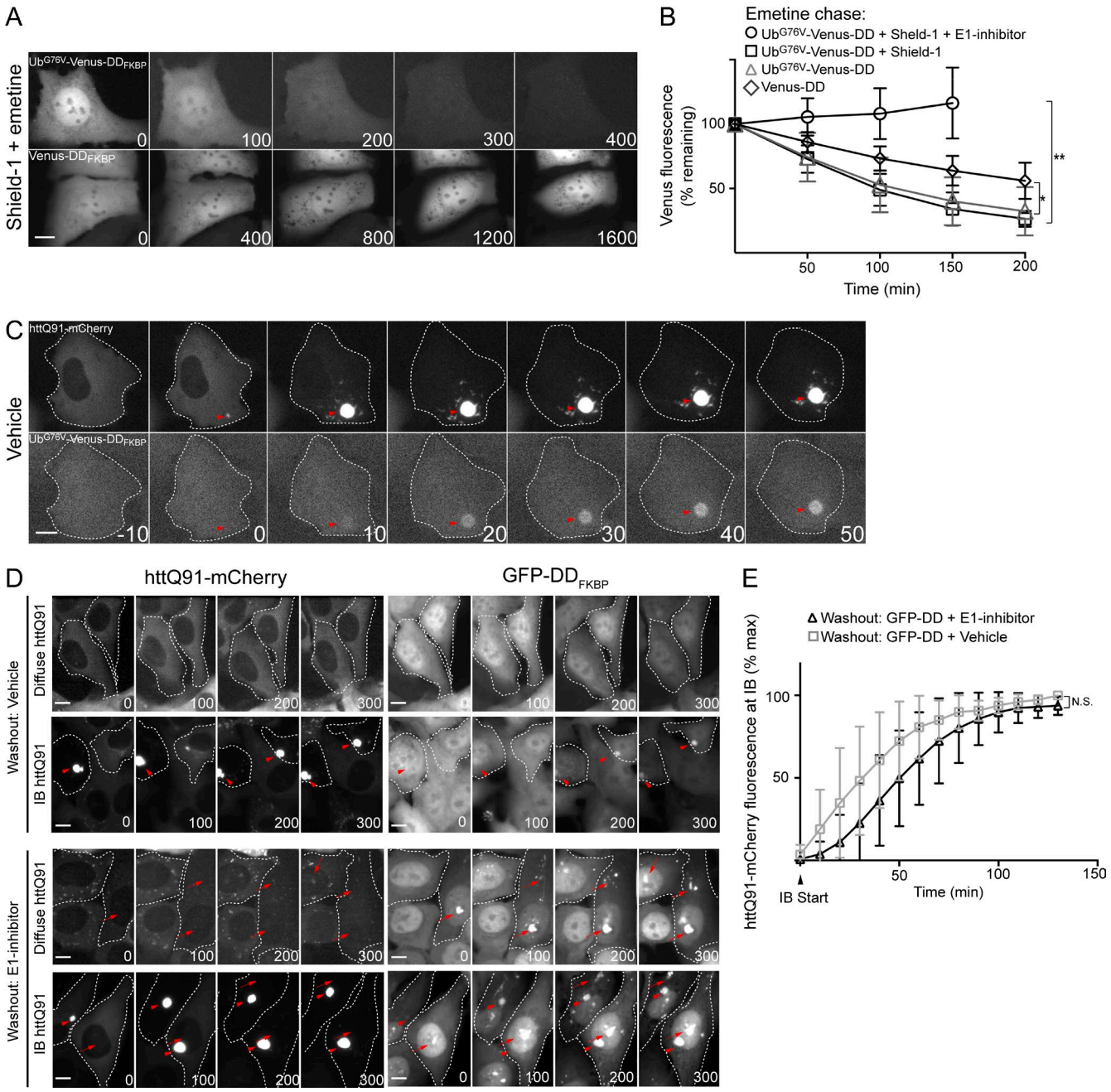

Figure S2. **Half-life of the Ub^{G76V}-Venus-DD reporter and the effect of E1 inhibitor on GFP-DD$_{FKBP}$ localization and recruitment of httQ91-mCherry to IBs.** (A) Folded Ub^{G76V}-Venus-DD$_{FKBP}$ is rapidly turned over. Cells were transiently transfected with Ub^{G76V}-Venus-DD$_{FKBP}$ or Venus-DD$_{FKBP}$ for 48 h in the presence of 1 µM shield-1 and imaged by time-lapse microscopy after addition of 25 µM emetine. (B) Ub^{G76V} fused to Venus-DD$_{FKBP}$ constitutes a dominant, Ub-dependent degradation signal. Cells (n > 15) were transiently transfected with Venus-DD$_{FKBP}$, Ub^{G76V}-Venus-DD$_{FKBP}$, or Ub^{G76V}-Venus-DD$_{FKBP}$ in the presence of 1 µM shield-1 for 48 h and imaged by time-lapse microscopy after addition of 25 µM emetine. A separate population of cells transfected with Ub^{G76V}-Venus-DD$_{FKBP}$ in the presence of shield-1 was treated with 10 µM E1 inhibitor before imaging. (C) Misfolded Ub^{G76V}-Venus-DD$_{FKBP}$ is recruited to IBs. Cells transiently transfected with httQ91-mCherry and Ub^{G76V}-Venus-DD$_{FKBP}$ for 48 h in the absence of shield-1 were imaged by time-lapse microscopy. t = 0 min indicates the frame in which IBs were first observed, and negative values indicate time in minutes preceding this frame. Arrowheads indicate IBs. (D) E1 inhibitor blocks degradation and promotes aggregation of misfolded GFP-DD$_{FKBP}$. U2-DD^{FK}Q91 cells were treated with 1 µg/ml dox and 1 µM shield-1 for 48 h. Cells were subsequently treated with 10 µM E1 inhibitor or vehicle for 3 h and imaged by time-lapse microscopy after shield-1 washout. Arrowheads indicate httQ91-mCherry IBs, and arrows indicate GFP-DD$_{FKBP}$ foci not associated with IBs. (E) E1 inhibitor does not block recruitment of httQ91-mCherry into IBs. httQ91-mCherry fluorescence at IBs was measured in U2-DD^{FK}Q91 cells (n > 10) imaged in Fig. 2 G. IB start indicates the frame in which IBs were first observed. Data points indicate mean ± SD. Time stamps indicate elapsed time in minutes. Microscopy panels are representative of n > 20 observations. Bars, 10 µm. Statistically relevant differences (α = 0.05): *, P < 0.05; **, P < 0.0001.

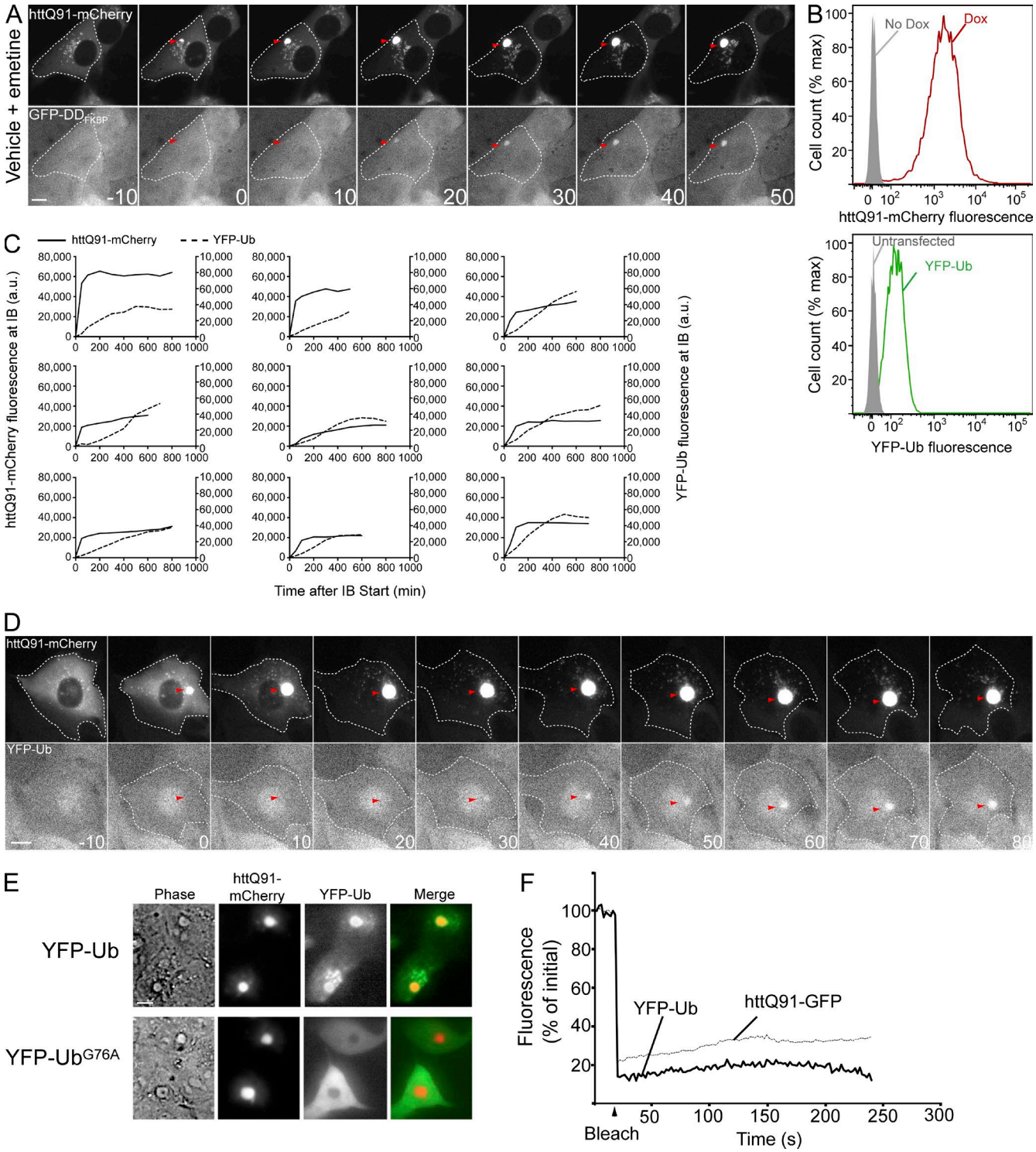

Figure S3. **Additional characterization of U2-Ub-Q91 cells and YFP-Ub FRAP data.** (A) Inhibition of translation does not block recruitment of GFP-DD$_{FKBP}$ to IBs. U2-DD^{FK}Q91 cells were treated with 1 µg/ml dox for 48 h and imaged by time-lapse microscopy after addition of 25 µM emetine. (B) U2-Ub-Q91 cells were treated with 1 µg/ml dox for 72 h and analyzed by flow cytometry. (C) YFP-Ub is recruited to IBs after a delay. U2-Ub-Q91 cells were treated with 1 µg/ml dox for 48 h, and httQ91-mCherry and YFP-Ub fluorescence at IBs was determined in single cells by time-lapse microscopy. t = 0 indicates the frame in which IBs were first observed. a.u., arbitrary units. (D) YFP-Ub is recruited to IBs independently of httQ91-mCherry. U2-Ub-Q91 cells were treated with 1 µg/ml dox for 48 h and imaged by time-lapse microscopy. (E) Conjugation-deficient YFP-Ub is not recruited to IBs. Cells transiently transfected with httQ91-mCherry and YFP-Ub or YFP-Ub^{G76A} were imaged by fluorescence microscopy. (F) YFP-Ub at IBs is immobile. Cells stably expressing YFP-Ub were transiently transfected with httQ91-mCherry. Recovery of YFP fluorescence at mCherry IBs after photobleaching was measured by time-lapse microscopy. In a separate experiment, cells were transiently transfected with httQ91-GFP, and recovery of GFP fluorescence at IBs was measured after photobleaching. Microscopy in A and D is representative of $n > 10$ and $n > 30$ observations, respectively. Bars, 10 µm. Arrowheads indicate IBs. For flow cytometry, $n > 10{,}000$ cells were analyzed. FRAP data are representative of at least two independent experiments.

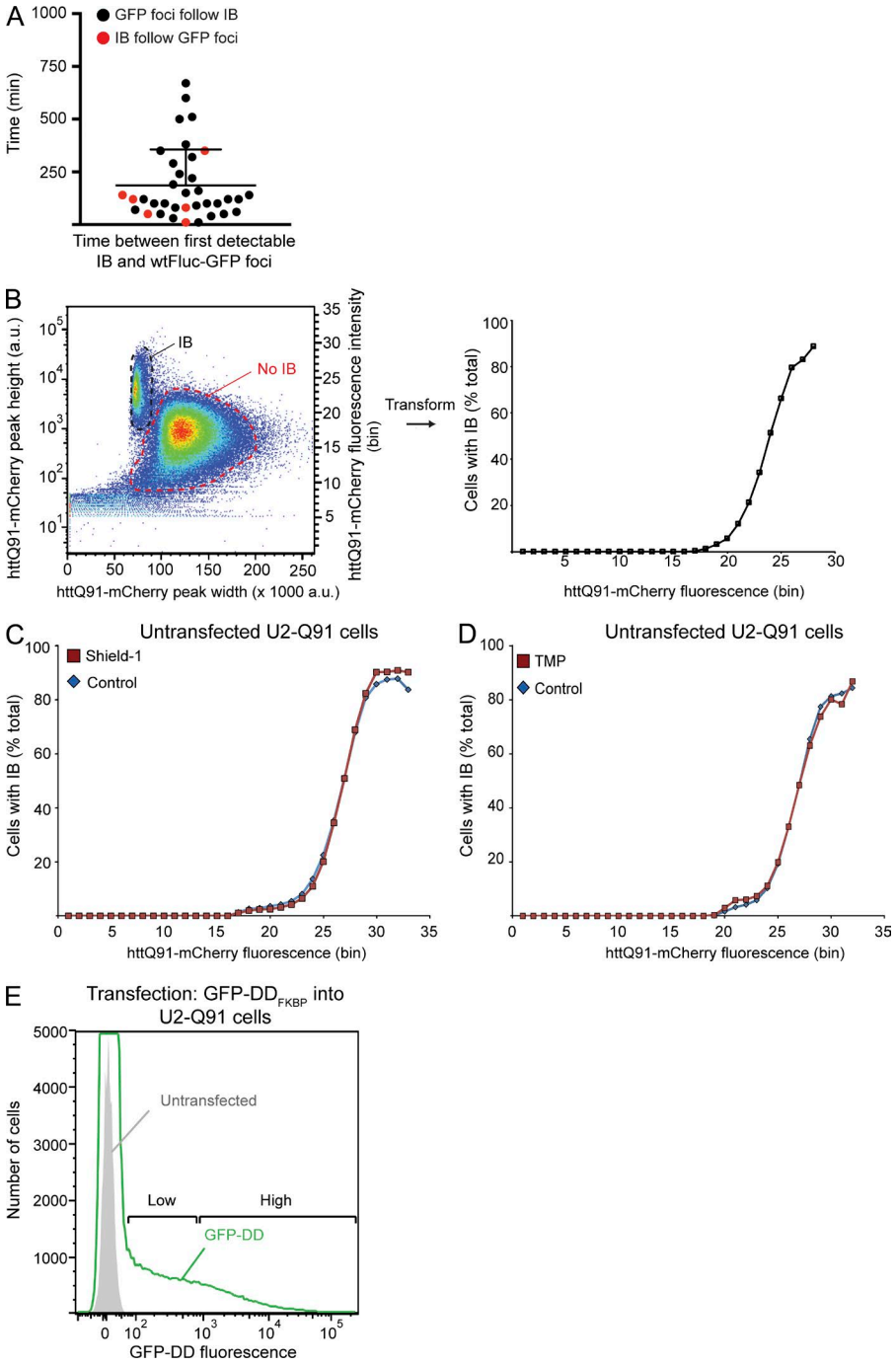

Figure S4. **PulSA method and control experiments for the PulSA experiments.** (A) wtFluc-GFP is recruited into foci before and after IBs form. Cells were transiently transected with wtFluc-GFP and httQ91-mCherry for 48 h, and the time between appearance of detectable httQ91-mCherry IBs and wtFluc-GFP foci was determined by time-lapse microscopy. Red symbols indicate cells in which the emergence of IBs followed the appearance of wtFluc-GFP foci. (B) PulSA flow cytometric analysis of cells expressing httQ91-mCherry. Cells stably expressing tetracycline-inducible httQ91-mCherry (U2-Q91) were treated with 1 µg/ml dox for 72 h and analyzed by flow cytometry. The pulse height of the mCherry fluorescence signal from single cells was plotted against pulse width to generate a gate that demarcates cells with IBs (left). The httQ91-mCherry pulse height (or area) axis was divided into 41 equally sized bins, and the percentage of cells with IBs was plotted as a function of bin number (right). a.u., arbitrary units. (C and D) Addition of TMP or shield-1 does not affect the concentration at which httQ91-mCherry forms IBs. U2-Q91 cells were treated with 1 µg/ml dox and 1 µM shield-1 (C) or 10 µM TMP (D) for 72 h and analyzed by PulSA flow cytometry. (E) Transient expression of low or high levels of GFP reporters in cells. U2-Q91 cells were transiently transfected with GFP-DD$_{FKBP}$ for 72 h and analyzed by flow cytometry. Interval gates indicate populations of cells with low and high levels of GFP fluorescence. Flow cytometry panels are representative experiments of at least two independent repeats.