# Appendix

**RNase H2 degrades toxic RNA:DNA hybrids**
**behind stalled forks to promote replication restart**

Jonathan Heuzé[1*], Samira Kemiha[1*], Antoine Barthe[1], Alba Torán Vilarrubias[1], Elyès Aouadi[1], Umberto Aiello[2,4], Domenico Libri[2,5], Yea-Lih Lin[1], Armelle Lengronne[1], Jérôme Poli[1,3] and Philippe Pasero[1]

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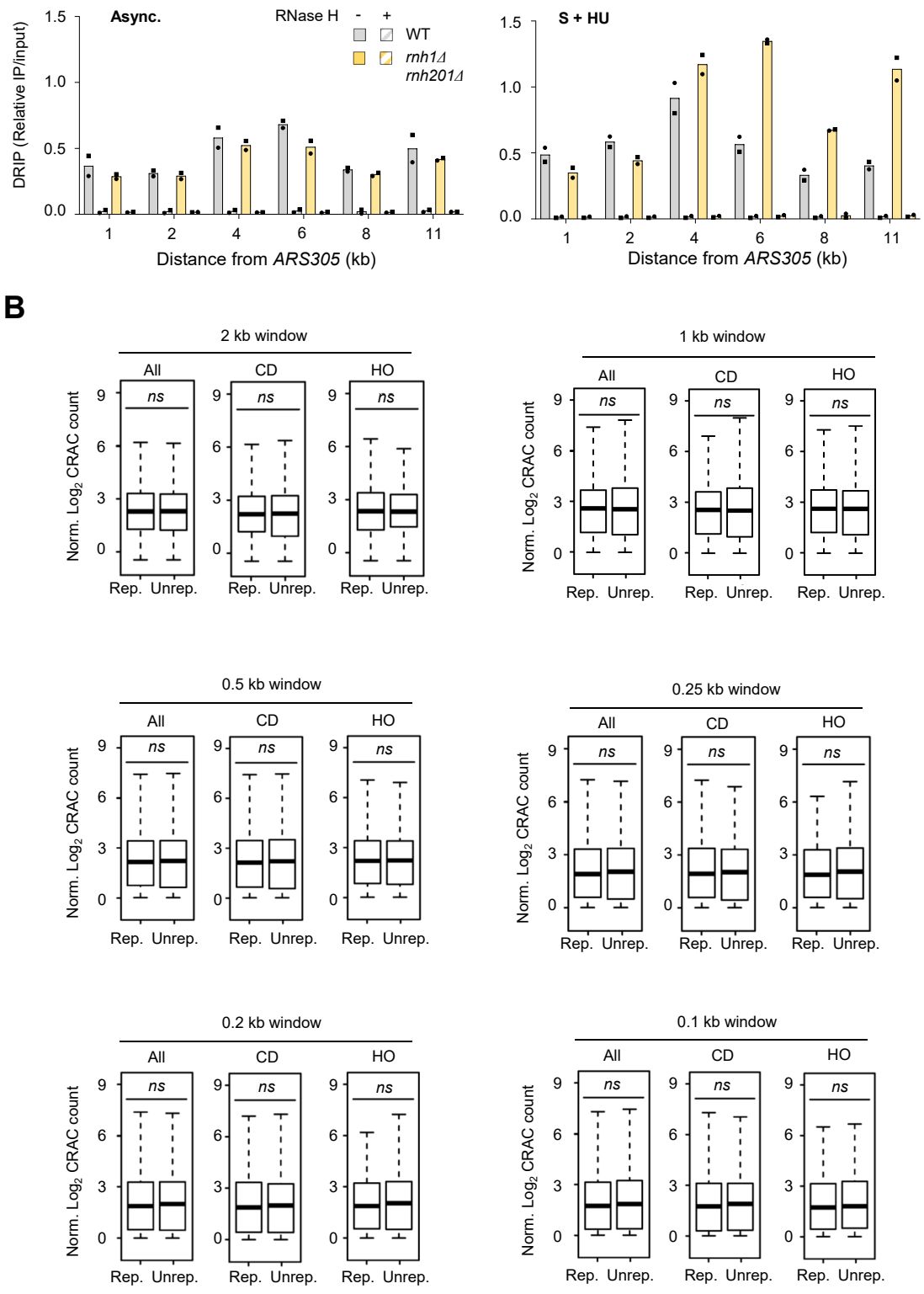
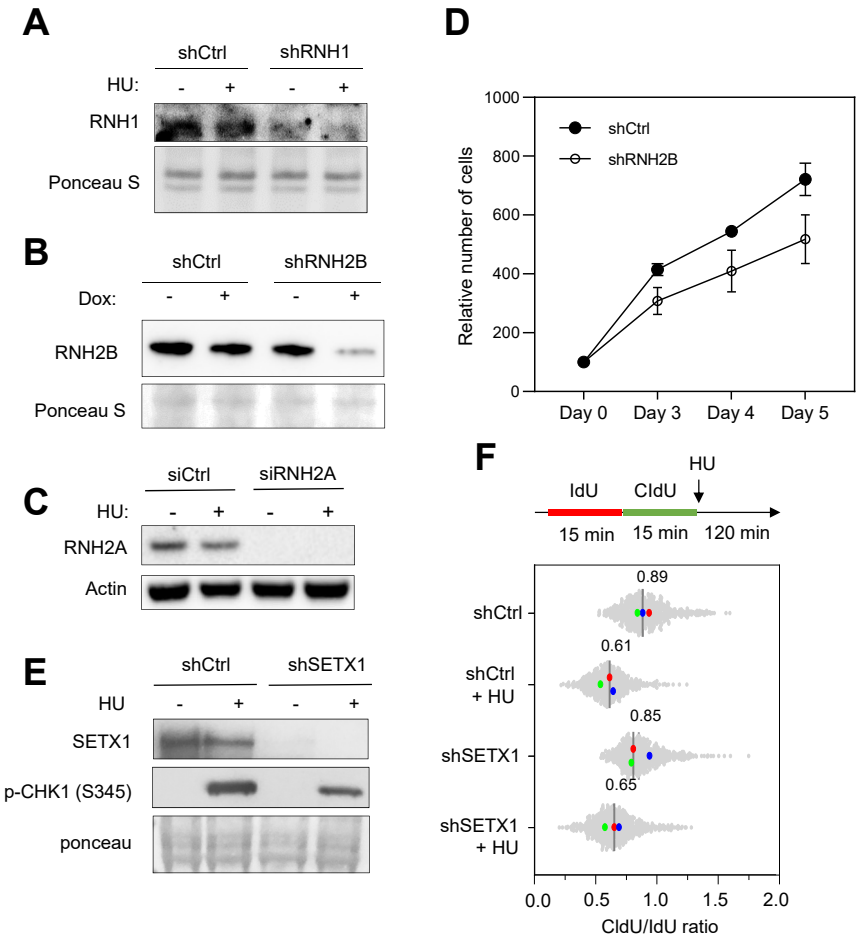
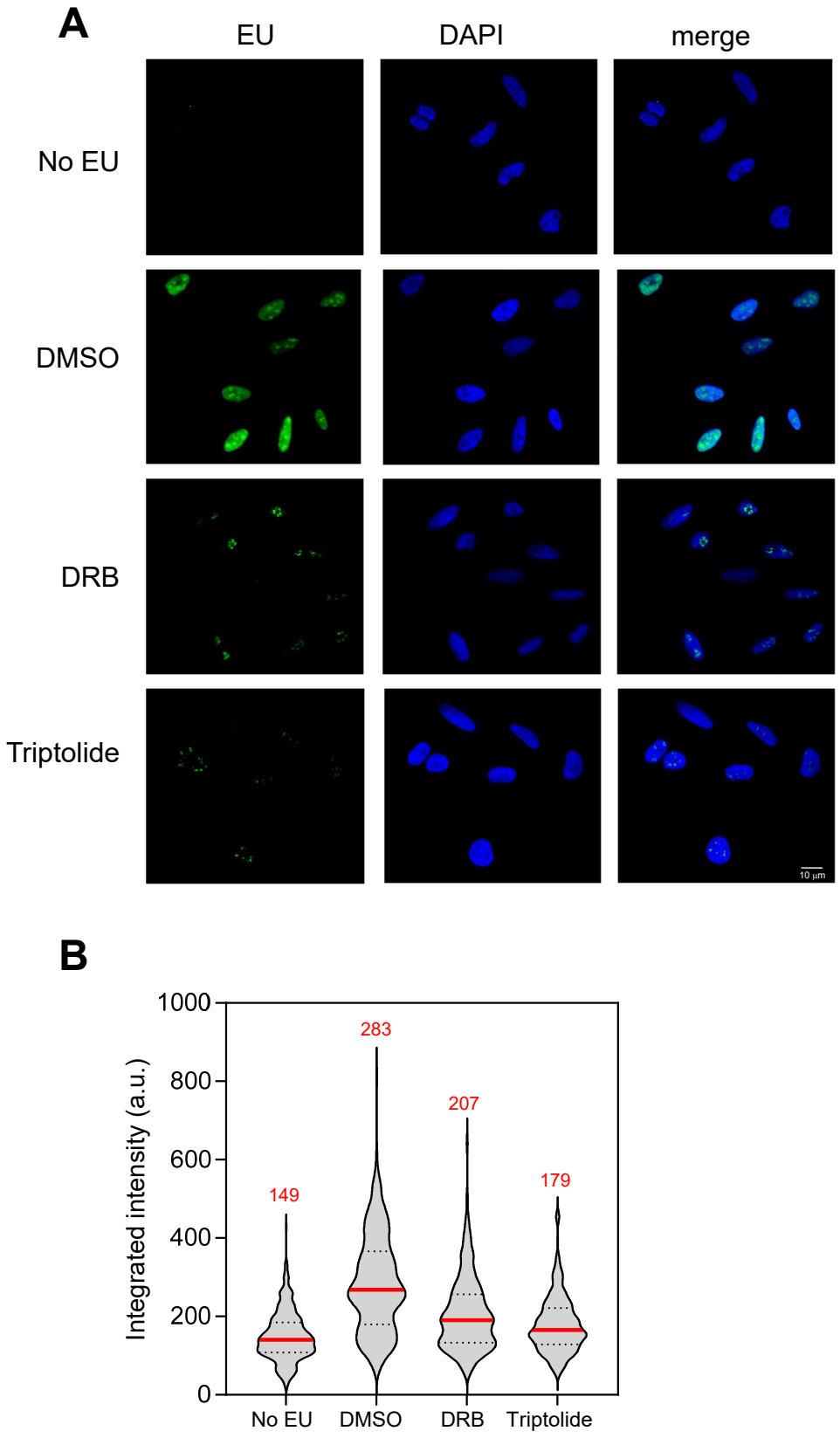



Appendix Figure S1: **Analysis of RNA:DNA hybrids and nascent RNA around HU-arrested forks in budding yeast.**(**A**) DRIP-qPCR analysis of RNA:DNA hybrid enrichment at loci positioned 1 to 11 kb away from *ARS305* in wild type and *rnh1Δ rnh201Δ* cells +/- *in vitro* RNase H treatment. Samples were collected from asynchronous cultures (async.) or from cells arrested in $G_1$ with $\alpha$-factor and released for 90 minutes into S phase in the presence of 200 mM HU (S+HU). Data are expressed as a percentage of input. Individual points are indicated (n=2). (**B**) Rpb1-HTP CRAC analysis of nascent RNA at HU-arrested forks. Boxplot comparisons of the log2 Rpb1-HTP CRAC count computed over several windows (1, 0.5, 0.25, 0.2, 0.1 Kb) and normalized to the corresponding window size on replicated and unreplicated side of HU-arrested forks. Signal was measured for RNAPII transcribing in a co-directional (CD), head-on (HO) or both (all) configuration relative to the fork, as indicated. Untreated conditions from (Aiello *et al.*, 2022). *ns*: $p > 0.05$, t-test.




Appendix Figure S2: **Depletion of RNase H2 induces a proliferation defect in HeLa cells.** (**A**, **B**) Western blot analysis of the levels of RNase H1 and RNase H2B in HeLa cells expressing tetracycline-inducible shRNAs. Cells were treated with 10 μg/ml doxycycline for 72 hours. (**C**) Levels of RNase H2A in HeLa cells transfected for 48 hours with siRNA against RNase H2A (siRNH2A) or a control sequence (siCtrl) with or without exposure to 4 mM HU for 2 hours. Actin was used as a loading control. (**D**) Growth of control (shCtrl) and RNase H2B-depleted (shRNH2B) HeLa cells. Cell proliferation was quantified using WST-1 assay at indicated time points after doxycycline treatment (10 μg/ml). Cell number is expressed relative to the number of control cells at Day 0. (**E-F**) DNA fiber analysis of fork resection in control HeLa cells (shCtrl) and in HeLa cells depleted for SETX (shSETX1) and exposed or not to 4 mM HU. Cells were treated with doxycycline (4 μg/ml) for 72 hours and sequentially labelled for 15 min with IdU and for 15 min with CldU. Then, they were either collected immediately or treated for 2 h with 4 mM hydroxyurea (HU) before DNA fiber analysis. The lengths of the IdU and CldU tracks were plotted as the ratio of CldU to IdU. Mean of 3 independent experiments was indicated.




Appendix Figure S3: **Effect of DRB and triptolide transcription on EU incorporation in HeLa cells.** (**A**) Cells were treated with DMSO as control, 100 µM DRB for 2.5 hours or 1 µM triptolide for 1.5 hours before 100 µM EU labeling for 30 min, as indicated in Fig. 6A. The EU signal was detected using click-it chemistry and fluorescence microscopy. Representative images of EU incorporation are shown. (**B**) EU signal of was quantified using CellProfiler. Median integrated intensity is indicated in red.