A novel role for UPF1 in safeguarding DNA replication in S phase and beyond.

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Abstract

Maintaining DNA replication fidelity is paramount in allowing for the successful duplication of genetic information, that will be passed on to the next generation of daughter cells. Interfering with this process leads to replication stress that can promote mutagenesis, double-stranded DNA breaks and loss of genetic stability. The fundamental process of transcription is a well described promotor of DNA replication stress through changes in chromatin dynamics, DNA structure and direct transcription-replication collisions (TRCS). Here we have identified a novel function for the RNA/DNA helicase UPF1 in preventing harmful consequences between the DNA replicative and transcriptional machineries. Loss of UPF1 resulted in an increase in spontaneous transcription-dependent replication fork stalling and double stranded breaks. UPF1-deficient cells following mild replicative stress show a reduced frequency of replication fork stalling, mitotic delay and the DNA replication salvage pathway of mitotic DNA synthesis (MiDAS). Together these data demonstrate UPF1 as vital for protecting the genome from spontaneous TRCs, most likely through its role in regulating the dissociation of nascent R-loop associated-mRNAs. Loss or down-regulation of UPF1 also represents a potential mechanism whereby cells could display resistance to DNA replication-targeting chemotherapeutics. Targeting UPF1-deficient cancers or UPF1 inhibition with drugs known to synergize with high levels of TRCs such as PARP inhibitors, could be a promising therapeutic approach.

Introduction

Up frameshift 1 (UPF1) was first identified and characterised as a helicase with a central role in mRNA decay pathways, the best characterised of which being nonsense mediated decay (NMD). Together with 6 other proteins (UPF2, UPF3, SMG1, SMG5, SMG6 and SMG7) UPF1 facilitates the selective destruction of newly synthesised mRNAs that possess a nonsense mutation [1]. In human cells, UPF1 also mediates other NMD independent RNA decay pathways including Staufen1-mediated decay and replication-dependent histone mediated decay [2, 3]. More recently, it has become clear that UPF1 plays a wider role in cellular RNA metabolism, regulating the stability and decay of a variety of specific RNAs including histone mRNA, long non-coding RNAs (lncRNAs) [4, 5] and microRNAs [6].

Aside from its canonical role in RNA surveillance, UPF1 has also more recently been implicated in playing a major role in other cellular processes such as DNA replication [7], DNA repair [8] and cell cycle control [9, 10] however the precise mechanism of UPF1 in these pathways is unclear. Azzalin *et al* have shown that cells stably transfected with UPF1 shRNA experience a considerable replication defect, marked by an accumulation in early S phase and reduced BrdU incorporation. They also observed elevated PCNA on replication forks, indicative of replication fork stalling, as well as persistent activation of ATR and an increase in spontaneous levels of γH2AX in UPF1 deficient cells. Together these observations suggest that UPF1 deficient cells experience spontaneous replication fork collapse [7]. UPF1 has also been shown to interact with DNA polymerase delta [7, 11] further supporting a direct role in DNA replication. However, others have argued that the negative effects of UPF1 deficiency on replication could be due to defects in NMD altering gene expression [12]. Indeed UPF1's role in the regulation of mRNAs has been linked to the DNA replication process previously, specifically through the controlled degradation of histone mRNAs. We previously identified this DNA-PK activated S phase checkpoint, which links histone transcription to DNA replication which leads to UPF1 activation [9, 10].

In S phase of the cell cycle, transcription and replication occur simultaneously and as both use DNA as a template, careful coordination is required to avoid collisions between the machineries required for these two essential processes. These collisions are known as transcription-replication conflicts (TRCs) and are a major cause of genomic instability and DNA damage [13]. Recent single molecule studies which give direct visualisation of TRCs revealed post-replicative DNA:RNA hybrids behind replication forks following a TRC which resulted in replication fork slowing and reversal [14]. Replication stress during S-phase can result in a failure to complete DNA replication, leading to the emergence of under-replicated DNA (UR-DNA) regions outside of S-phase. Processing of this UR-DNA

has been suggested to occur through distinct DNA replication processes in G2 [15, 16] and beyond into mitosis through mitotic DNA synthesis (MiDAS) [17-19]. G2 replication (termed G-mids) has also been shown to occur at transcription start sites [16] and MiDAS is known to be caused by TRCs specifically in BRCA2- deficient cells [20].

UPF1 is a highly processive DNA:RNA helicase which has been shown to have the ability to bind a multitude of DNA or RNA structures, including DNA:RNA hybrids possessing single strands of DNA or RNA with equal specificity, to catalyse the resolution of such structures [21].

Here we show an important role for UPF1 in DNA synthesis under normal conditions and replication stress. UPF1 deficiency through either siRNA or CRISPR leads to elevated replication fork stalling and reduced replication speeds in unperturbed conditions. However, under circumstances of replication stress induced by DNA polymerase inhibition, UPF1 deficient cells exhibit far lower levels of stalled replication forks than their wild type counterparts. Our data indicates that instead of acting on replication directly, UPF1 influences DNA replication stalling by regulating transcription. UPF1 is required for effective transcription and UPF1 loss leads to an increased level of transcription/ replication conflicts resulting in stalled and collapsed replication forks leading to a replication defect.

Materials and Methods

Cell Culture and Reagents

HeLa cells (ATCC) were cultured as previously described [22]. RPE UPF1^{WT} and RPA UPF^{KO} were a kind gift of Dr Greg Ngo. Where indicated, cells were treated with Carboplatin (Sigma-Aldrich), aphidicolin (Santa Cruz), RO3306 (Cayman Chemical), at indicated concentrations and with indicated length of period. Antibodies against UPF1 and RNAP II Ser2 were from Cell Signaling, γH2AX was from Novus Bio, and β-Tubulin was from Sigma. Appropriate secondary antibodies conjugated to horseradish peroxidase (DAKO) were used for the western blotting experiments, and Alexa-Fluor 488 and Alexa-Fluor 594 secondary antibodies (Invitrogen) were used for immunofluorescence.

Flow cytometry

Cells were fixed via in 70 % ethanol prior to staining. Following PBS washes to remove ethanol, cells were permeabilised by incubated in Flow Buffer 1 (0.5% BSA, 0.25% Triton-X). Flow Buffer 1 was removed by centrifugation and the cells were incubated in pH3 antibody (EMD Millipore, 3018868) diluted 1:100 in Flow Buffer 1 for 1 hour and 30 minutes at room temperature. Cells were washed 3 times in Flow Buffer 2 (0.25% Triton-X) then resuspended in 100 µL Goat pAb anti-Rabbit IgG FITC secondary antibody, diluted 1:100 in Flow Buffer 1 and incubated for 30 minutes at room

temperature in the dark. The cell pellets were resuspended in 400 µL PI (10 µg/µL stock solution in PBS) containing RNAse A (80µg/ml) and incubated for at least 30 minutes at 4 °C until processing.

The samples were processed on a FACSCalibur (BD Sciences) and analysed using Flowjo.

RNAi and DNA transfection

siRNA transfections were performed using Dharmafect 1 siRNA transfection reagent (Horizon), DNA transfections using Lipofectamine 2000 (Thermo-Fisher Scientific) and siRNA/ DNA contranfections using Dharmafect Duo (Horizon) according to the manufacturer's instructions. Cells were treated 48 hours post-transfection. siControl, siUPF1, siUPF2, siUPF3b, siSTAU1 siGenome SMARTpool siRNA pools were from Horizon Discovery.

Western blotting

Cells were lysed using RIPA buffer and protein concentrations determined using a Bradford Assay. Lysates were separated by SDS-PAGE and transferred to nitocellulose. Blots were blocked in 5 % Milk prior to overnight incubation with specified antibodies at 1:1000 in milk.

Immunofluorescence

HeLa cells (5 x 10^4) were seeded directly onto coverslips fixed in methanol or paraformaldehyde, permeabilised in 0.2% Triton-X100, blocked in 5% BSA and stained with the indicated antibodies. Alexa-Fluor 488 and Alexa-Fluor 594 secondary antibodies (Invitrogen) were used. In the final wash, cells were incubated with DAPI (Life technologies) and mounted to slides using Immumount (Thermo Fisher Scientific). Images were captured using a Nikon ECLIPSE Ti2 confocal microscope.

Live cell Imaging

48 hours post transfection with indicated siRNAs, cells were trypsinised, exposed to ionising radiation (IR) whilst in suspension and reseeded to 24 well plates. Once adhered to the plates (3-4 hours post IR), the cells were loaded into imaging system, or following chemical administration as indicated. Live cell images were captured using ZEISS Cell discoverer 7 microscope every 5 minutes for a duration of 20 hours.

Mitotic EdU incorporation assay

MiDAS assay was performed 48 hours post transfection as described by Garribba et al [23] were captured using Nikon ECLIPSE Ti2 confocal microscope.

Results

UPF1 is required for DNA damage induced mitotic delay

We recently published a high-throughput screen for proteins required for a DNA damage induced delay in mitotic progression (REF). From this screen we identified that UPF1 knockdown resulted in a significant reduction in mitotic population following ionizing radiation.. Here we show that siRNA mediated depletion of UPF1 leads to the loss of a carboplatin-induced mitotic delay (**Figure 1A**). UPF2, which binds to UPF1 and brings about a conformational change required for its activation [24, 25], is also required (**Figure 1A**). Whilst UPF2 interacts with UPF1 in a range of functions, siRNA-mediated depletion of UPF1 recruitment factors UPF3b and STAU1, which specifically recruit UPF1 to target mRNAs for NMD and SMD respectively, had no impact on mitotic progression (**Figure 1B**), Together this indicates that the function of UPF1 impacting mitotic progression could, at least in part be independent of NMD and SMD. Ectopic over-expression of wild-type UPF1 was sufficient to rescue the effects of siRNA mediated depletion of endogenous UPF1, demonstrating the change in phenotype is due to the specific loss of UPF1 (**Figure 1C**). Previous studies have demonstrated a role of ATR but not ATM in facilitating the chromatin recruitment of UPF1 for a seemingly NMD independent function [7]. We found that whilst DNA damage induced mitotic delay does not require active ATM or ATR, small molecule inhibition of ATR is also sufficient, like ionizing radiation or carboplatin, to induce spontaneous mitotic delay, which is dependent on UPF1 (**Figure 1D**).

Due to the known roles of UPF1 in mRNA metabolism and R-loop homeostasis, we tested to see if UPF1-dependent DNA damage induced mitotic delay was affected by the transcription inhibitor α-amanitin or by overexpression of the R-loop resolving enzyme, RNAseH1. We found that both amanitin treatment (**Figure 1E**) and RNAseH1 overexpression (**Figure 1F**) resulted in the loss of UPF1-dependent mitotic delay suggesting that the DNA damage-dependent delay in mitotic progression induced by carboplatin is both transcription and R-loop dependent.

UPF1 depletion results in reduced MiDAS under conditions of replication stress

Whilst it is unclear why cells undergo a delayed mitotic transit in response to DNA damage and replication stress, work from our lab and others has revealed a close link between mitotic duration and the occurrence of mitotic DNA synthesis (MiDAS) in prophase [26-28]. Additionally, UPF1 is known to interact with POLD3 and has been implicated in R-loop metabolism, both of which have been frequently associated with MiDAS. We have previously found that low dose aphidicolin, which is a known inducer of MiDAS, also induces mitotic delay. This delay was found also to be UPF1-

dependent (**Figure 2A**). Using standard conditions to induce MiDAS (**Figure 2B**), we found that siRNA-mediated depletion of UPF1 also leads to a reduction in EdU incorporation in mitotic cells (**Figure 2C-D**), indicating a reduction in mitotic DNA synthesis under these conditions.

MiDAS was first identified in cells exposed to mild replication stress and was proposed as a mechanism by which cells can restart DNA synthesis in mitosis of under replicated regions associated with difficult to replicate loci known as chromosome fragile sites [18, 19, 29]. In all these studies, the CDK1 inhibitor RO3306 was used to arrest cells at the G2/M boundary in order to enhance the mitotic population for analysis. More recently it has been demonstrated that the phenomenon of replication restart in mitosis is brought about through off-target effects of RO3306 on CDK2 [15]. It is suggested that DNA synthesis does in fact not stop at the end of S phase, and under conditions of mild replication stress, continues into G2 and beyond into mitosis. [15]. Furthermore, DNA synthesis detected in G2, in the absence of RO3306, appears to be independent of several canonical MiDAS proteins including MUS81 and POLD3. To determine whether the involvement of UPF1 in MiDAS was due to forced replication restart, brought about by RO3306 treatment, we assessed DNA replication dynamics throughout the cell cycle in asynchronous conditions in paired RPE^{WT} and $RPE^{UPF1\ KO}$ cells (**Figure 3**). Following treatment with aphidicolin, cells were pulse-labelled for 30 minutes with EdU, then harvested and stained for EdU, phospho-histone H3 and DAPI to allow for the determination of each cell cycle phase (**Figure 3B-C)**. As with the mitotic DNA synthesis observed in **Figure 2**, there was a significant reduction in replication stress-induced EdU foci in G2 cells in the absence of UPF1 (**Figure 3D**). Interestingly however, there was also a reduction in overall EdU intensity in S phase cells in the absence of UPF1 which led us to question whether UPF1 may also have a role in unperturbed global DNA replication (**Figure 3F**). Our data also shows that the $RPE^{UPF1\ KO}$ cells have slightly elevated levels of FancD2 foci in S and G2 in untreated conditions indicating that UPF1 loss on its own may lead to replication stress (**Figure 3E,G**). Contradictorily however, under conditions of replication stress, UPF1 depletion led to a reduction in FancD2 foci, indicating that whilst loss of UPF1 promotes replication stress in unperturbed cells, it also could protect against aphidicolin-induced replication stress.

Azzalin et al have suggested that UPF1 is required for S phase progression showing that cells lacking UPF1 accumulate in early S phase [7]. UPF1 heterozygous knockout cells however are viable but do in fact proliferate slower than their wild type counterparts [8] suggesting a possible replication defect. To confirm the reduction in S phase EdU intensity in UPF1-deficient cells observed by microscopy, we studied the cell lines by flow cytometry (**Figure 4A**). Whilst no obvious cell cycle

defects were observed in cells deficient for UPF1 in untreated or mild replicative stress conditions (**Figure 4B-C**), a significant reduction in median BrdU intensity was seen in UPF1-deficient S-phase cells compared to WT counterparts (**Figure 4D**), suggesting a defect in DNA replication. Since we had now observed significant reductions in DNA base incorporation of UPF1-deficient cells by immunofluorescence and flow cytometry we performed DNA fibre analysis to assess the exact effect UPF1-deficiency was having on DNA replication. DNA fibre analysis revealed significant reductions in both replication fork speed (**Figure 4F**) and decreased sister fork symmetry (**Figure 4G**) in siUPF1 cells compared to the siControl in unperturbed conditions. Together this suggests that loss of UPF1 leads to the spontaneous stalling of replication forks. Treatment with low-dose aphidicolin resulted in significantly reduced fork speeds and induced replication fork stalling, as expected in siControl cells, however, consistent with our FancD2 data (**Figure 3G**), aphidicolin treatment in combination with UPF1 depletion, whilst not affecting replication speeds, significantly increased sister fork symmetry (**Figure 4G**).

UPF1 has previously been shown to associate to gene coding DNA regions and correlate with Pol II transcription and RNA levels [30], and plays a role in the release of newly transcribed mRNA molecules from the gene locus [31]. Given our data in **Figure 1E**, that short-term inhibition of transcription rescues UPF1-induced damage-dependent mitotic delay, we tested the effect of the transcription inhibition on UPF1-deficiency-induced phenotypes. Using the CDK9 inhibitor 5,6-dichlorobenzimidazole (DRB), we found that short-term transcription inhibition significantly reduced both aphidicolin-induced and spontaneous γH2AX foci which form in UPF1 deficient cells (**Figure 5B**). DRB treatment also rescued the slowed replication speeds and loss of siter fork symmetry which occurs in UPF1 deficient cells (**Figure 5D-E**), indicating that the effect of UPF1-deficiency on replication fork stalling is dependent on active transcription. We hypothesise that loss of UPF1 causes a transcription defect which leads to increased TRCs through replication machinery colliding with stalled transcription machinery or secondary DNA structures such as R-loops formed *in cis,* resulting in the stalling and collapse of replication forks. In control cells, aphidicolin reduces replication speed resulting in TRCs through the transcription machinery colliding with the stalled replication machinery, however in the absence of UPF1, the slowed transcription machinery compensates for the slowed replication machinery, meaning there are fewer TRCs and replication is able to continue (**Figure 6).**

Discussion

Here we identify a novel role for UPF1 in the maintenance of proper DNA replication. We observed that cells lacking UPF1 exhibit a DNA replication defect even during an unperturbed cell cycle. This defect leads to; reduced replication fork progression, increased replication fork stalling and DNA damage, likely through increased levels of TRCs. Under conditions of low-level replication stress induced by inhibition of the DNA polymerase, cells with reduced UPF1 expression exhibit fewer stalled forks and fewer TRCs than those with higher levels of UPF1 expression. Taken together with the finding that replication fork stalling and DNA damage, induced by the absence of UPF1, is dependent on active transcription we propose that in the absence of UPF1, a transcriptional barrier promotes DNA replication fork stalling through increased levels of TRCs. This pathway could represent a mechanism whereby cancer cells could show resistance to chemotherapeutics that inflict DNA damage by targeting replication.

Replication problems have previously been suggested previously following loss of UPF1. It has previously been reported that shRNA-mediated depletion of UPF1 led to an accumulation of cells in early S-phase, defective replication and spontaneous DSBs in the absence of UPF1 that was dependent on ATR [7]. They proposed that UPF1 was required for S-phase progression and that due to an interaction with DNA polymerase delta (Polδ), UPF1 was directly required for DNA replication or repair. However, whilst the observation of an early S-phase arrest has not replicated by other groups, [8]. It has also been suggested that UPF1 is required to directly maintain leading-strand DNA replication at telomeres in HeLa cells, through an association with telomerase and TPP1 [32]. However no direct quantification of telomeric replication was carried out by this group, these conclusions were drawn on the noted loss of telomeres in the absence of UPF1 and a proposed interaction observed between UPF1 and the regulatory subunit of Polδ [11, 32]. Whilst we show that loss of UPF1 appears to promote DNA replication fork stalling, rather than a direct reduction in DNA synthesis rates, a non-significant but modest reduction in fork progression is still observed even following rescue with DRB, in the UPF1 KO line compared to the WT untreated condition. This could be suggestive of a small replication issue persisting even following rescue of fork stalling with transcriptional inhibition though further work into this would be required.

Taken together our data strongly suggest that UPF1 maintains proper transcription allowing for the coordinate with replication to prevent fork stalling and collapse. Loss of UPF1 not only results in spontaneous replication stress within these cells but also modulates the way in which these cells respond to exogenous replication stress.

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Figure 1: UPF1 and UPF2 are required for promoting a DNA damage-dependent mitotic delay.

Time-lapse live cell microscopy analysis for the mean time taken to complete mitosis. **(A-B)** HeLa cells transfected with the indicated siRNAs and treated with 1μM Carboplatin as shown. **(C)** FLP-IN TReX UPF1^{WT} HeLa cells were treated as indicated with 1μg/ml Doxycycline, transfected with the indicated siRNAs for 48hrs. **(D)** Hela cells transfected with the indicated siRNAs and treated with 1μM ATRi or 1μM ATMi as indicated. **(E)** HeLa cells transfected with the indicated siRNAs and treated with 2μg/ml α-amanitin as indicated. **(G)** HeLa cells transfected with the indicated siRNAs in combination with plasmid encoding RNaseH1^{WT} or RNaseH1^{D210N} as shown. 50 cells for each condition were counted and the data illustrated represents the overall mean of each independent experiment +/- SEM (N=3). One way ANOVA with Dunnett's correction test for multiple comparisons was performed to determine statistical significance. (* denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$ and **** denotes $p \leq 0.0001$). *Ctrl = Control, VC = Vehicle Control, Dox = Doxycycline*

Figure 2: UPF1 is required for Mitotic DNA synthesis following mild replication stress.

(A) Time lapse live cell microscopy analysis for the mean time taken to complete mitosis of HeLa cells transfected with the indicated siRNAs and treated with 0.4μM aphidicolin. **(B)** Schematic depicting experimental outline. Cells were transfected with indicated siRNAs and induced with 1μg/ml doxycycline as described. Cells were treated with 0.4μM Aphidicolin as indicated for 23hrs prior to mitotic shake-off and incubation with 20μM EdU for 25mins prior to fixation. **(C)** Representative immunofluorescence images showing EdU and DAPI of prophase cells. **(D)** Quantification of EdU foci per prometaphase cell. 50 cells for each condition were quantified and the data illustrated represents individual EdU foci from one biological repeat. The error bars represent the mean +/- SD (N=3). One way ANOVA with Dunnett's correction test for multiple comparisons was performed to determine statistical significance (* denotes $p \leq 0.05$, ** denotes $p \leq 0.01$ and *** denotes $p \leq 0.001$). *Dox = Doxycycline, Aph = Aphidicolin*

Figure 3: UPF1 is required for continuous DNA synthesis in G2 following mild replication stress.

(A) Representative immunofluorescence images for RPE^{WT} cells at each stage of the cell cycle untreated and with 0.4μM aphidicolin for 24hrs, stained for EdU, FANCD2, H3PS10 and DAPI. **(B)** Representative X-Y plots for Mean EdU intensity vs Raw DAPI intensity × Raw H3S10 intensity in RPE^{WT} and $RPE^{UPF1\ KO}$ cells treated with 0.4μM aphidicolin as stated. Gating for G1, S and G2 populations demonstrated with red dashed lines. A minimum of 250 individual cells imaged per biological repeat. **(C)** Mean cell cycle phase distribution determined by gating of X-Y plots in **B**. Data points represent mean cell cycle phase distribution +/- SEM (N=3). **(D-E)** Quantification of nuclear EdU and FANCD2 foci per G2 cells respectively. **(F-G)** Quantification of mean nuclear EdU intensity and FANCD2 foci per S-phase cells respectively. **(H-I)** Quantification of mean nuclear EdU and FANCD2 foci per G1 cells respectively. Data illustrated are a representative graph taken from one biological replicate. For EdU foci, intensity and FANCD2 foci in all cell cycle phases a One-way ANOVA with Dunnett's correction test for multiple comparisons was performed on the mean nuclear foci from each biological replicate to determine statistical significance (N=3) (* denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, **** denotes $p \leq 0.0001$).

Figure 4: Loss of UPF1 promotes spontaneous replication fork stalling.

(A) Representative FACS plots for Propidium Iodide staining vs BrdU staining in RPE^{WT} and $RPE^{UPF1\ KO}$ cells treated without and with 0.4μM aphidicolin for 4hrs or 24hrs. Gating examples can be seen on all plots. A negative control of cells not incubated with BrdU was used to define non-S-Phase populations. **(B-C)** Mean cell cycle phase distribution of RPE^{WT} and $RPE^{UPF1\ KO}$ cells respectively. **(D)** Average normalised median EdU intensity of total S-phase population +/- SEM (N=3). One way ANOVA with Dunnett's correction test for multiple comparisons was performed to determine statistical significance (* denotes $p \leq 0.05$). **(E)** Labelling scheme of 100μM CldU and 500μM IdU pulses of HeLa cells and the subsequent DNA fibre structures analysed for **F-G**. **(F)** Quantification of progressing fork speeds. **(G)** Quantification of sister fork symmetry. Data points represent individual DNA fibres analysed from one biological repeat, the black line and error bars represent the mean +/- SD (N=3). A minimum of 200 individual DNA fibres were analysed per condition and a One-way ANOVA with Dunnett's correction test for multiple comparisons was performed on the mean of each biological replicate to determine statistical significance (* denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, **** denotes $p \leq 0.0001$).

Figure 5: Replication fork stalling and DNA damage following UPF1 loss are transcription dependent.

(A) Representative western blot for Phospho-RNAPII CTD (Ser2), UPF1, γ-H2AX (Ser139) and β-actin in RPE^{WT} and $RPE^{UPF1\ KO}$ cells prior to treatment without or with 24hrs 0.4μM aphidicolin and 2hr 100μM DRB. **(B-D)** Quantification of γ-H2AX (Ser139) or phospho-RNAPII CTD (Ser2) in response to treatments indicated, adjusted to β-actin levels to allow for loading variation and expressed as a fold-change of the untreated RPE^{WT} cells (N=3). One-way ANOVA test with Dunnett's correction test for multiple comparisons was carried out on the mean of each biological replicate to determine statistical significance (ns denotes $p > 0.5$ and ** denotes $p \leq 0.01$). **(D)** Quantification of progressing fork speeds. **(E)** Quantification of sister fork symmetry. Data points represent individual DNA fibres analysed, the black line and error bars represent the mean +/- SD (N=3). A minimum of 200 individual replication tracts were analysed per condition for fork speed analysis and 100 individual sister forks for symmetry analysis. For both a One-way ANOVA with Dunnett's correction test for multiple comparisons was performed on the mean of each biological replicate to determine statistical significance (* denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, **** denotes $p \leq 0.0001$).

Figure 6: UPF1 model.

(A) In normal cells, transcription and replication exist in S phase in a careful balance meaning there are no collisions or conflict. **(B)** UPF1 deficiency leads to a transcriptional defect which results in slowing of the transcription machinery causing the replication machinery to collide with it. **(C)** In normal cells, replication-stress inducing agents such as aphidicolin, slow the replication machinery, causing the transcription machinery to collide with it. **(D)** When replication-stress inducing agents are used in UPF1-deficient cells, both transcription and replication are slowed meaning they are once again in balance.

Figure 1

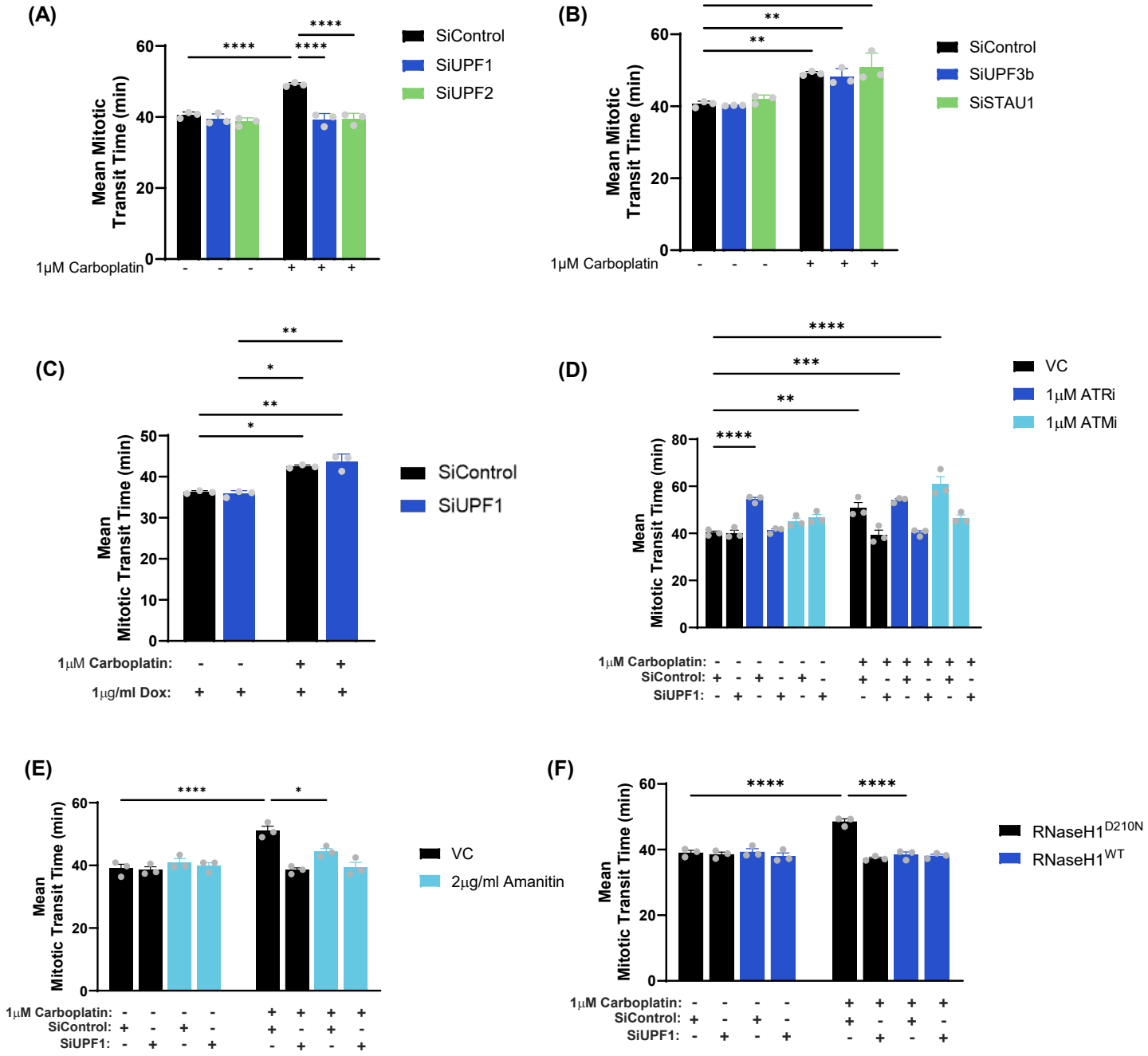

(A)
Mean Mitotic Transit Time (min)
SiControl
SiUPF1
SiUPF2
1μM Carboplatin
(B)
Mean Mitotic Transit Time (min)
SiControl
SiUPF3b
SiSTAU1
1μM Carboplatin
(C)
Mean Mitotic Transit Time (min)
SiControl
SiUPF1
1μM Carboplatin:
1μg/ml Dox:
(D)
Mean Mitotic Transit Time (min)
VC
1μM ATRi
1μM ATMi
1μM Carboplatin:
SiControl:
SiUPF1:
(E)
Mean Mitotic Transit Time (min)
VC
2μg/ml Amanitin
1μM Carboplatin:
SiControl:
SiUPF1:
(F)
Mean Mitotic Transit Time (min)
RNaseH1D210N
RNaseH1WT
1μM Carboplatin:
SiControl:
SiUPF1:

Figure 2

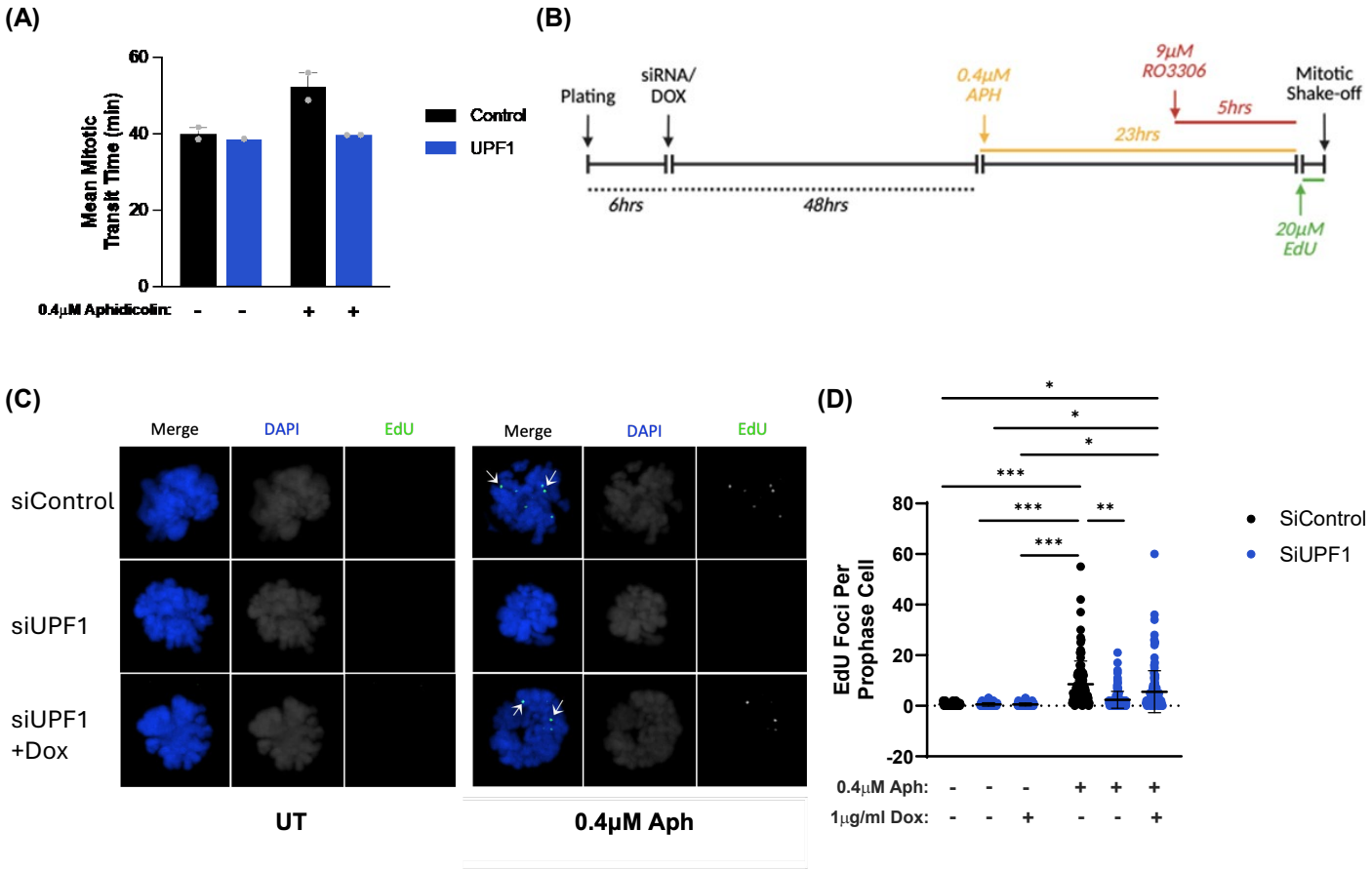

(A)
Mean Mitotic Transit Time (min)
60
40
20
0
Control
UPF1
0.4μM Aphidicolin: - - + +
(B)
Plating
siRNA/ DOX
0.4μM APH
9μM RO3306
5hrs
23hrs
Mitotic Shake-off
6hrs
48hrs
20μM EdU
(C)
Merge
DAPI
EdU
siControl
siUPF1
siUPF1 +Dox
UT
0.4μM Aph
(D)
EdU Foci Per Prophase Cell
80
60
40
20
0
-20
SiControl
SiUPF1
0.4μM Aph: - - - + + +
1μg/ml Dox: - - + - - +

Figure 3

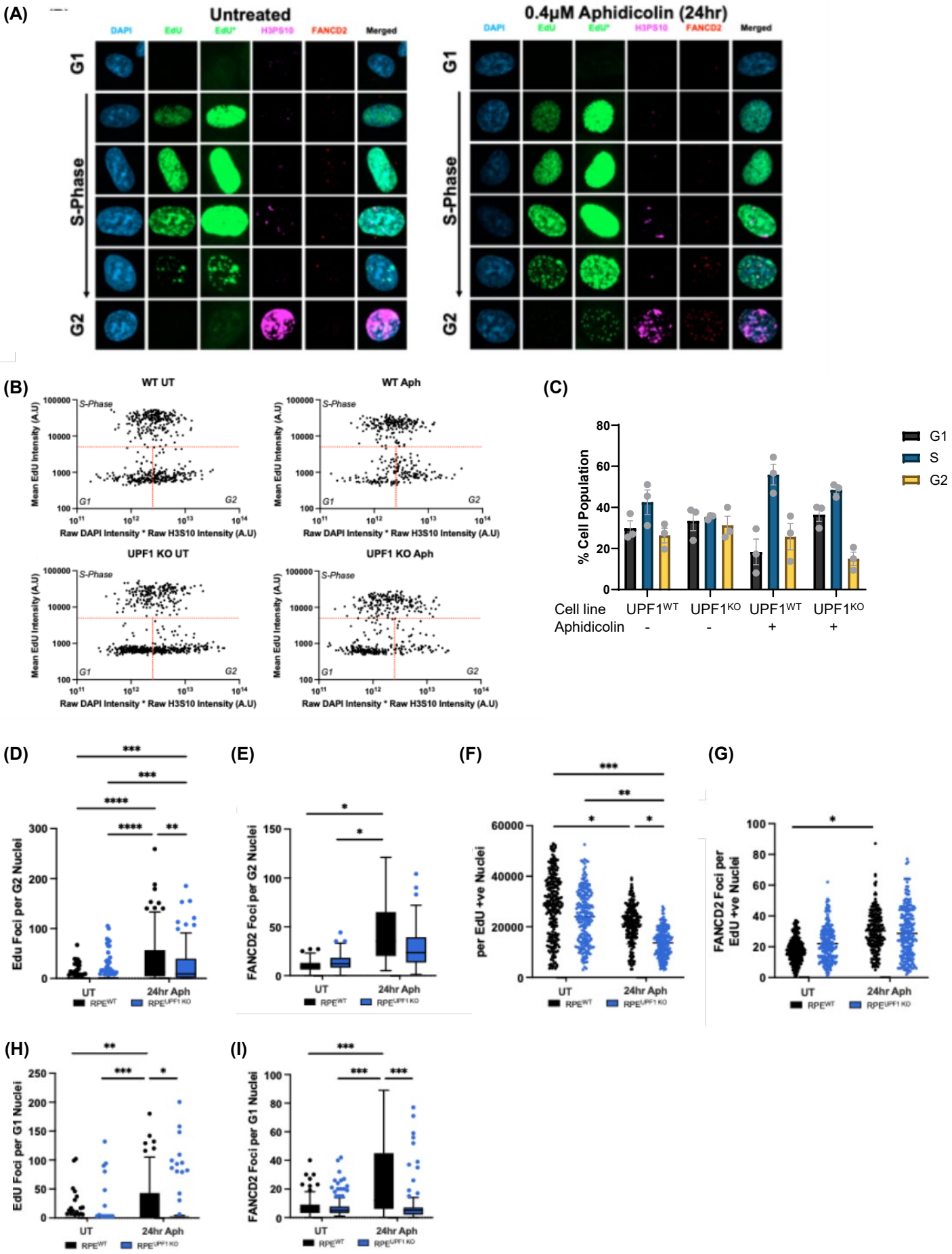

(A)
Untreated
0.4μM Aphidicolin (24hr)
DAPI
EdU
EdU*
H3PS10
FANCD2
Merged
G1
S-Phase
G2
(B)
WT UT
WT Aph
UPF1 KO UT
UPF1 KO Aph
S-Phase
G1
G2
Mean EdU Intensity (A.U)
Raw DAPI Intensity * Raw H3S10 Intensity (A.U)
(C)
% Cell Population
G1
S
G2
Cell line
Aphidicolin
UPF1WT
UPF1KO
(D)
Edu Foci per G2 Nuclei
(E)
FANCD2 Foci per G2 Nuclei
(F)
per EdU +ve Nuclei
(G)
FANCD2 Foci per EdU +ve Nuclei
(H)
EdU Foci per G1 Nuclei
(I)
FANCD2 Foci per G1 Nuclei
UT
24hr Aph
RPEWT
RPEUPF1 KO

Figure 4

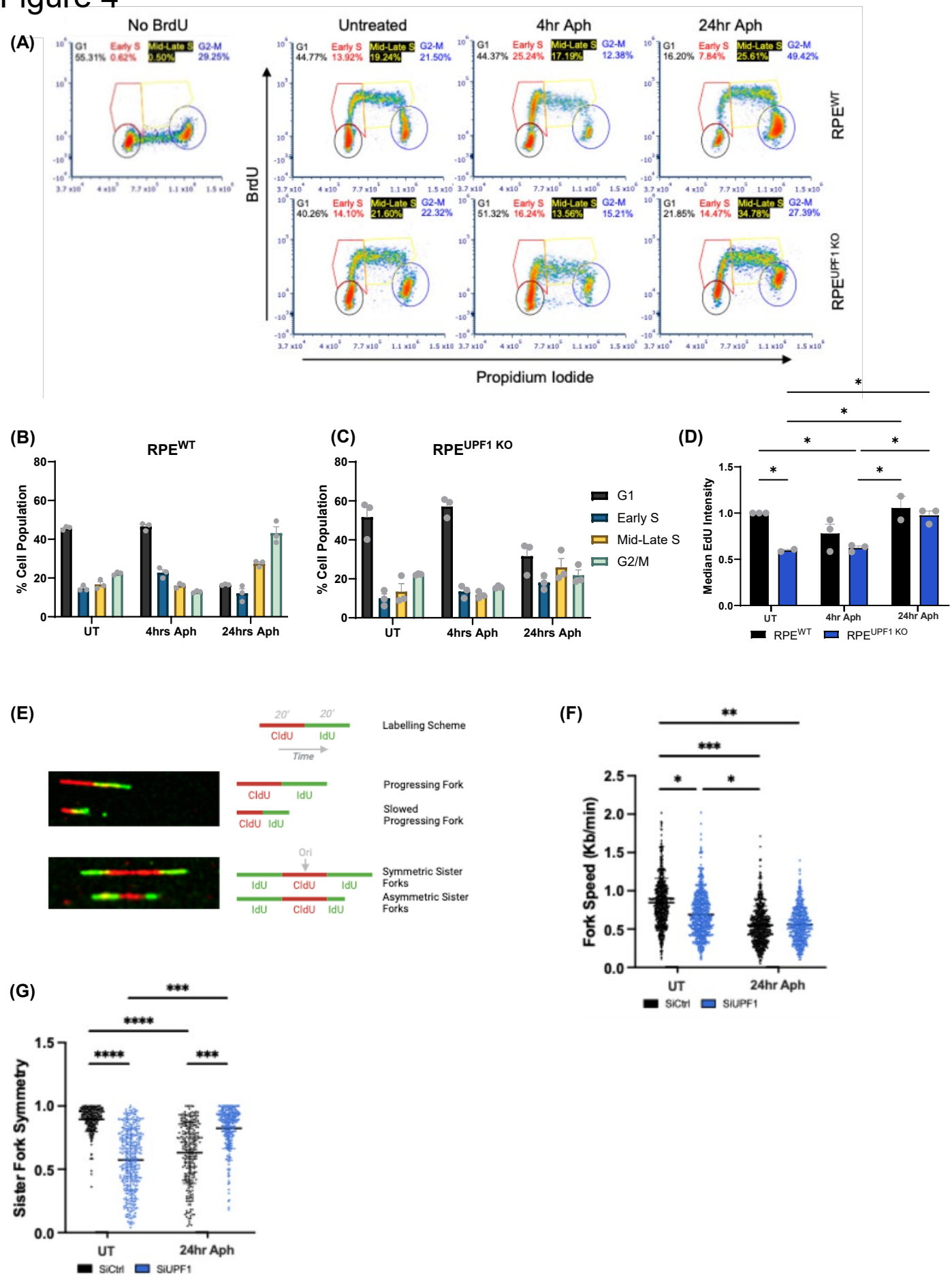

(A)
No BrdU
Untreated
4hr Aph
24hr Aph
G1 55.31% Early S 0.62% Mid-Late S 0.50% G2-M 29.25%
G1 44.77% Early S 13.92% Mid-Late S 19.24% G2-M 21.50%
G1 44.37% Early S 25.24% Mid-Late S 17.19% G2-M 12.38%
G1 16.20% Early S 7.84% Mid-Late S 25.61% G2-M 49.42%
G1 40.26% Early S 14.10% Mid-Late S 21.60% G2-M 22.32%
G1 51.32% Early S 16.24% Mid-Late S 13.56% G2-M 15.21%
G1 21.85% Early S 14.47% Mid-Late S 34.78% G2-M 27.39%
RPE^WT
RPE^UPF1 KO
BrdU
Propidium Iodide
(B)
RPE^WT
% Cell Population
UT
4hrs Aph
24hrs Aph
(C)
RPE^UPF1 KO
% Cell Population
UT
4hrs Aph
24hrs Aph
G1
Early S
Mid-Late S
G2/M
(D)
Median EdU Intensity
UT
4hr Aph
24hr Aph
RPE^WT
RPE^UPF1 KO
(E)
20'
20'
CldU
IdU
Time
Labelling Scheme
Progressing Fork
Slowed Progressing Fork
Ori
Symmetric Sister Forks
Asymmetric Sister Forks
(F)
Fork Speed (Kb/min)
UT
24hr Aph
SiCtrl
SiUPF1
(G)
Sister Fork Symmetry
UT
24hr Aph
SiCtrl
SiUPF1

Figure 5

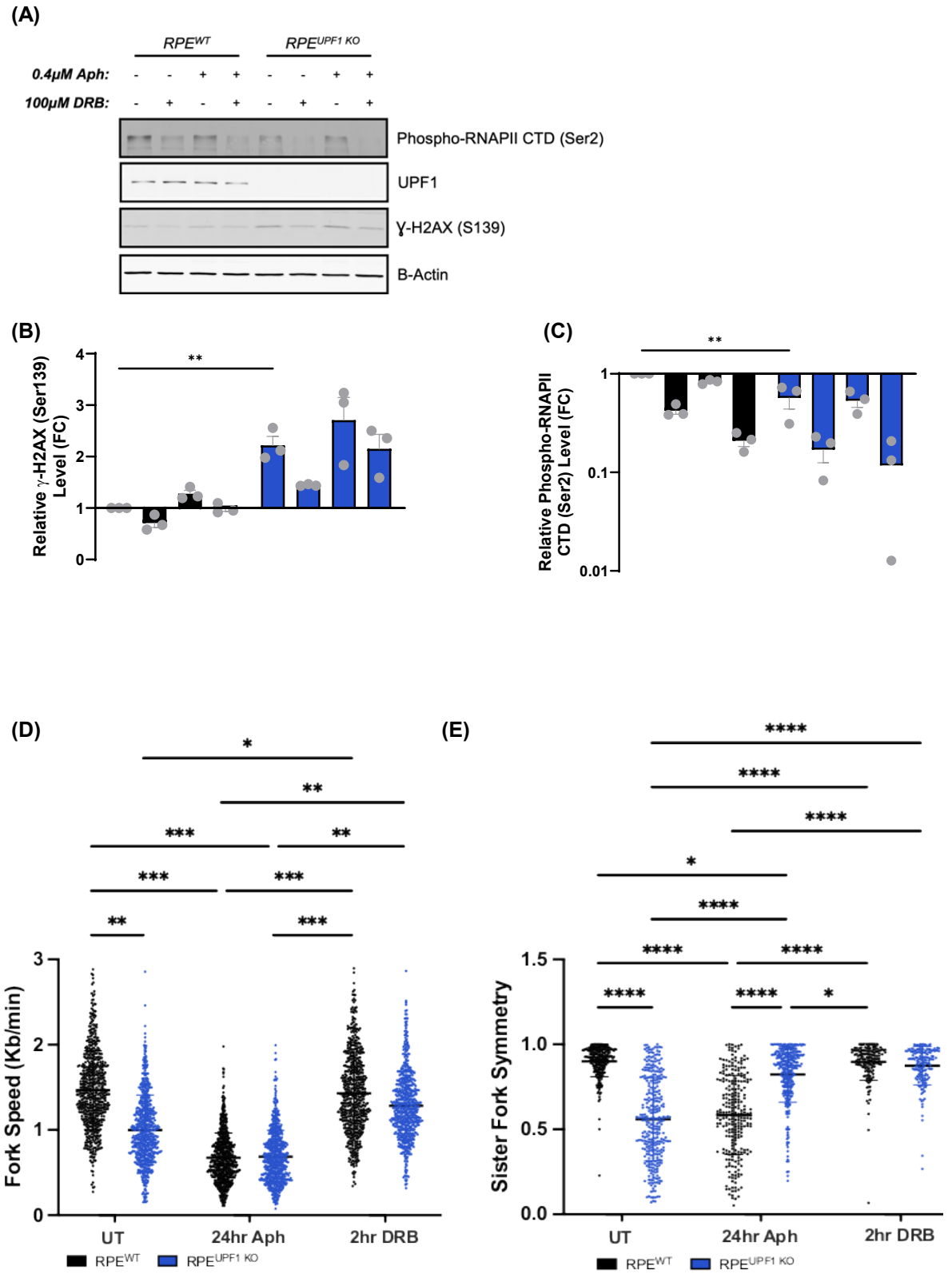
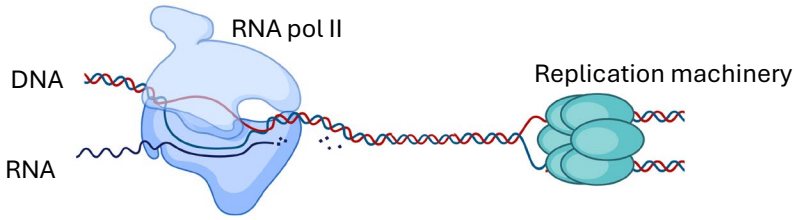
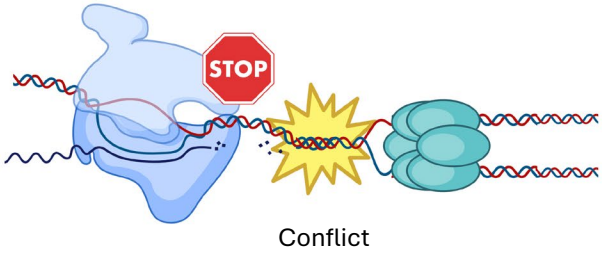

(A)
RPE^WT
RPE^UPF1 KO
0.4μM Aph:
100μM DRB:
Phospho-RNAPII CTD (Ser2)
UPF1
γ-H2AX (S139)
B-Actin
(B)
Relative γ-H2AX (Ser139) Level (FC)
(C)
Relative Phospho-RNAPII CTD (Ser2) Level (FC)
(D)
Fork Speed (Kb/min)
UT
24hr Aph
2hr DRB
RPE^WT
RPE^UPF1 KO
(E)
Sister Fork Symmetry
UT
24hr Aph
2hr DRB
RPE^WT
RPE^UPF1 KO

Figure 6

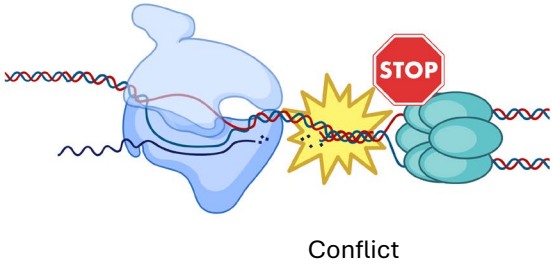
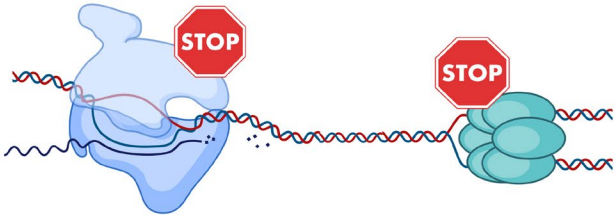
A. Wild type cell



B. UPF1-deficient cell



C. Wild type cell
+ aphidicolin



D. UPF1-deficient cell
+aphidicolin