Pathways of Resistance to Thymineless Death in *Escherichia coli* and the Function of UvrD

Natalie C. Fonville,*,†,1 Zalman Vaksman,‡,1 Jessica DeNapoli,*,† P. J. Hastings,*
and Susan M. Rosenberg*,†,§,2
*Department of Molecular and Human Genetics and, †Interdepartmental Program in Cellular and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030-3411, ‡Department of Microbiology and Molecular Genetics, University of Texas, Houston, Texas 77030, and §Departments of Biochemistry and Molecular Biology, Molecular Virology and Microbiology, and the Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, Texas 77030-3411

ABSTRACT Thymineless death (TLD) is the rapid loss of viability in bacterial, yeast, and human cells starved of thymine. TLD is the mode of action of common anticancer drugs and some antibiotics. TLD in *Escherichia coli* is accompanied by blocked replication and chromosomal DNA loss and recent work identified activities of recombination protein RecA and the SOS DNA-damage response as causes of TLD. Here, we examine the basis of hypersensitivity to thymine deprivation (hyper-TLD) in mutants that lack the UvrD helicase, which opposes RecA action and participates in some DNA repair mechanisms, RecBCD exonuclease, which degrades double-stranded linear DNA and works with RecA in double-strand-break repair and SOS induction, and RuvABC Holliday-junction resolvase. We report that hyper-TLD in Δ*uvrD* cells is partly RecA dependent and cannot be attributed to accumulation of intermediates in mismatch repair or nucleotide-excision repair. These data imply that both its known role in opposing RecA and an additional as-yet-unknown function of UvrD promote TLD resistance. The hyper-TLD of Δ*ruvABC* cells requires RecA but not RecQ or RecJ. The hyper-TLD of *recB* cells requires neither RecA nor RecQ, implying that neither recombination nor SOS induction causes hyper-TLD in *recB* cells, and RecQ is not the sole source of double-strand ends (DSEs) during TLD, as previously proposed; models are suggested. These results define pathways by which cells resist TLD and suggest strategies for combating TLD resistance during chemotherapies.

BACTERIAL, yeast, and human cells deprived of thymine rapidly lose the ability to form colonies, a phenomenon known as thymineless death (TLD) (Barner and Cohen 1954; Ahmad *et al.* 1998). TLD is the mode of action of several common chemotherapeutic drugs including anticancer agents 5-fluorouracil (5-FU), raltitrexed (Tomudex), (Takemura and Jackman 1997) and methotrexate, and the antibiotic trimethoprim (Mcguire 2003). Yet until recently and despite extensive study, how TLD occurs remained elusive. TLD occurs in replicating cells (*e.g.*, Cummings and Kusy 1970), probably because of DNA damage sustained during replication in the absence of thymine. First, sedimentation analysis revealed accumulation of single-strand (ss)-DNA breaks in plasmid (Freifelder 1969) and chromosomal (Nakayama and Hanawalt 1975) DNA following thymine deprivation. Second, pulsed-field-gel electrophoresis and electron microscopy showed aberrant DNA structures containing large (∼1–3 kb) regions of ssDNA (Nakayama *et al.* 1994). Implicating replication-generated DNA damage, recent work from the Khodursky group and our laboratory using DNA microarrays and fluorescent *in situ* hybridization (FISH) revealed that *Escherichia coli* cells undergoing TLD specifically lose origin-proximal DNA sequences early during thymine deprivation (Fonville *et al.* 2010a; Sangurdekar *et al.* 2010), followed by loss of replication-terminus-proximal DNA after extended thymine deprivation (Fonville *et al.* 2010a). Such DNA loss would be expected to contribute to death.

An important window on TLD mechanisms has been afforded by analyses of proteins and pathways that promote TLD in *E. coli*. Homologous recombination (HR) proteins RecF, RecQ, and RecJ are required for TLD (H. Nakayama *et al.* 1982, 1984; K. Nakayama *et al.* 1988). Although this would suggest that HR is a major contributor to the lethality

---

doi: 10.1534/genetics.111.130161
Manuscript received April 29, 2011; accepted for publication June 6, 2011
Available freely online through the author-supported open access option.
Supporting information is available online at http://www.genetics.org/content/suppl/2011/06/24/genetics.111.130161.DC1.

[1] Present address: Virginia Bioinformatics Institute, Virginia Tech, Washington St. 0477, Blacksburg, VA 24061.

[2] Corresponding author: Department of Molecular and Human Genetics, Baylor College of Medicine, 1 Baylor Plaza, Rm. S809 Mail Stop BCM225, Houston, TX 77030-3411. E-mail: smr@bcm.edu

observed under thymine deprivation, confusion surrounded the role for the major recombinase, RecA. Although one group reported that RecA promoted TLD (Inouye 1971), others found no role for RecA (Anderson and Barbour 1973; Nakayama *et al.* 1982). Recent work reexamining the role of RecA has established that, as Inouye reported, RecA is required for a major fraction of TLD (Fonville *et al.* 2010a; Kuong and Kuzminov 2010), compatible with the hypothesis that HR is part of TLD process(es). Both groups found a small, early anti-TLD role of RecA, seen as increased TLD in Δ*recA* cells early during thymine starvation, followed by a large pro-TLD role, seen as far less TLD in Δ*recA* cells later during starvation. The later large pro-TLD role is the one discussed in experiments using Δ*recA* here.

RecA functions not only in HR but also in induction of the bacterial response to DNA damage: the SOS response (Ennis *et al.* 1985). Whereas Morganroth and Hanawalt (2006) tested and rejected the hypothesis that the SOS response promotes TLD, recent work from three groups has reversed this conclusion. First, Sangurdekar *et al.* (2010) found that SOS-controlled genes are upregulated upon thymine deprivation. Second, we (Fonville *et al.* 2010a) and Kuong and Kuzminov (2010) found that the SOS response is required for one of a few operative TLD pathways in that TLD is blocked by mutations that block SOS-response induction. Whereas Kuong and Kuzminov (2010) suggested that SOS might promote TLD via upregulation of RecA, resulting in increased HR (Kuong and Kuzminov 2010), we showed that SOS-induced levels of RecA did not substitute for a functional SOS response in promoting TLD. Thus, SOS-promoted upregulation of another gene(s) promotes TLD, and we found that the SOS function responsible is SulA, an inhibitor of cell division (Fonville *et al.* 2010a). Ultimately, we showed that the main function of RecA and an important role of RecF in the mechanisms of TLD is induction of the SOS DNA-damage response and SulA, leading to permanently arrested cell division and thus inability to form colonies during thymine starvation (Fonville *et al.* 2010a).

The SOS-mediated transcriptional upregulation of SulA underlies one pathway of TLD, which results in at least one log of killing under thymine starvation (Fonville *et al.* 2010a). Alternatively and simultaneously, we found that RecQ and RecJ promote TLD via a separate pathway independent of RecA/SOS/SulA, causing an additional log of killing (Fonville *et al.* 2010a). Although these two pathways (RecA/SOS/SulA pathway and RecA-independent, RecQ/RecJ pathway) contribute to TLD, removing both of them did not abolish TLD completely (Fonville *et al.* 2010a). An additional RecA- and RecQ-independent pathway(s) not yet identified also contributed about one more log of TLD. Thus, at least three pathways underlie TLD: a RecA-SOS-SulA-dependent pathway, a RecA-independent/RecQ/RecJ-dependent pathway, and a third pathway requiring neither RecA/SOS/SulA nor RecQ/RecJ.

Although the recent work of multiple groups has begun to illuminate the pathways of and DNA intermediates that accompany TLD, important enigmas remain, particularly concerning DNA-repair proteins that participate in pathways by which cells *resist* TLD. Mutants lacking these proteins show *faster* loss of colony-forming ability during thymine starvation, referred to here as hyper-TLD. For example, RecBCD double-strand exonuclease functions with RecA both in repair of DNA double-strand breaks and double-strand ends (DSBs/DSEs) by HR and in induction of the SOS response by DSB-inducing agents (Mcpartland *et al.* 1980; Clark and Sandler 1994), and yet, although both HR and SOS induction promote TLD (Fonville *et al.* 2010a; Kuong and Kuzminov 2010), cells lacking RecBCD are TLD hypersensitive (Nakayama *et al.* 1982), not resistant as *recA* cells are. RecBCD creates single-strand DNA onto which it then loads RecA at the start of both HR and SOS induction (Arnold and Kowalczykowski 2000). Perhaps more understandably, RuvABC, a Holliday-junction resolvase (Friedberg *et al.* 2005) that removes the intermolecular recombination intermediates (IRIs) that RecA creates, promotes TLD resistance in that cells that lack RuvABC are TLD hypersensitive (Fonville *et al.* 2010a). The hyper-TLD in *ruv* cells is RecA dependent, implying that RecA-generated IRIs, left unresolved in the absence of Ruv, kill cells. Such "death-by-recombination," in which unresolved IRIs kill cells by preventing chromosome segregation, was also observed in mutants that promote extra accumulation of HR intermediates (Magner *et al.* 2007; Fonville *et al.* 2010b). Finally, UvrD, a helicase with multiple functions *in vivo*, one of which is to remove RecA from ssDNA (Veaute *et al.* 2005), also promotes TLD resistance in that *uvrD* null mutants are TLD hypersensitive (Siegal 1973). Understanding how cells become TLD hypersensitive and defining the pathways and mechanisms of action of the proteins that allow cells to resist TLD is likely to be important to maximizing TLD-inducing chemotherapies and combating resistance. In this study we define pathways by which UvrD, RuvABC, and RecBCD allow cells to resist TLD.

Materials and Methods

Strains used in this study are given in Table 1. P1 transductions were as described (Miller 1972). TLD experiments were as described (Fonville *et al.* 2010a). Cells were grown to stationary phase in M9 minimal medium with 50 μg/ml thymine, 0.1% glucose, and 0.5% casamino acids (thy$^+$ growth medium) then diluted 1:20 into fresh thy$^+$ growth medium, and incubated at 37° for ~1 hr 10 min (1 hr 40 min for strains containing a Δ*recB* allele) to allow them to exit stationary phase and enter early log phase. We found the timing of incubation of cells prior to resuspension in TLD medium to be critical for seeing consistent levels of TLD. One milliliter of cells was washed twice with M9 with 0.1% glucose and 0.5% casamino acids but lacking thymine (TLD medium), then resuspended in 2 ml of TLD medium at $\sim 5 \times 10^6$ cells/ml and incubated at 37° for 5 hr with aliquots taken and dilutions plated at the indicated times. Colony-forming units (CFU) were scored on a Microbiology International ProtoCOL colony counter after 24 hr at 37°. Longer incubations verified that all CFU were apparent at 24 hr.

For microscopy, cultures were started as for TLD assays with stationary cultures diluted 1:20 into 5 ml of fresh thy$^+$ growth medium and incubated at 37° for ~1 hr until cells had entered early log phase (OD450 ≈ 0.3). DAPI, 2 μg/ml, was added to the cells 10 min prior to washing. One milliliter of cells was washed twice with TLD medium and then resuspended in 0.1 (*recB*) or 0.5 (parental) ml of TLD medium with 1 μg/ml of DAPI and 1 μg/ml of propidium iodide (PI). Ten microliters of cells was spotted onto a TLD-medium plate (TLD medium solidified with 1.3% agar) and allowed to dry. Agar squares, 1 cm^2, containing the spots were cut from the agar and inverted onto a microscope slide. Moist Kim Wipes were placed next to the agar plugs to maintain humidity during the incubation. Microscopy was performed using an Olympus 81× inverted fluorescence microscope with a Hamamatsu HD camera. Cells were maintained at 34° to 36° using a custom-built Precision Weather Station and imaged every 10 min for brightfield and DAPI with PI imaging (TRITC filter) every 30 min. The microscope was set to autofocus every 30 min. Slidebook software was used to program the microscope and to process the images.

Error bars represent 1 SEM of ≥ 3 independent experiments. Statistical analyses were performed using SigmaStat (SYSTAT) and/or SPSS (PASW Statistics) software. For TLD assays significance was determined as $P < 0.05$ using two-way repeated measure ANOVA to analyze the curve data and Tukey post-hoc analysis.

Results

Two aspects of the TLD protocol were controlled to allow comparisons between strains of different growth rates. First, TLD was reported to be growth-phase dependent: inefficient in stationary phase but efficient in log-phase cells (Kuong and Kuzminov 2010). To allow sufficient time for *recB* strains, which grow slowly, to exit stationary phase, these were incubated in growth medium for an additional 30 min prior to thymine starvation (*Materials and Methods*). However, second, we found that cells taken straight from stationary phase and diluted to an OD of 0.3 (the density at which early log-phase cells were subjected to TLD) showed similar or greater sensitivity to TLD than cells in early log phase (supporting information, Figure S1). These data indicate a cell-density effect on TLD and highlight the need to examine cells at constant, low density to see maximal TLD. Thus, all strains were resuspended in TLD medium at ~5 × 10^6 cells/ml at 0 min (*Materials and Methods*).

Removal of DNA-repair intermediates is not how UvrD promotes TLD resistance

We wished to understand the basis of the hypersensitivity of Δ*uvrD* mutants to TLD (Siegal 1973). UvrD is a DNA helicase that resolves/removes intermediates in mismatch repair (MMR) and nucleotide excision repair (NER) (Friedberg *et al.* 2005) and also opposes HR by stripping RecA off ssDNA (Veaute *et al.* 2005). MMR-defective Δ*mutS* cells are not affected in TLD (Figure 1A; Kuong and Kuzminov 2010), implying that the absence of MMR ability *per se* cannot explain the TLD hypersensitivity of Δ*uvrD* mutants. However, MutS acts early in MMR, by binding DNA mismatches, such that cells that lack MutS do not initiate any MMR reaction. By contrast, UvrD acts after MutS and MutL have bound a DNA mismatch and MutH endonuclease has cleaved DNA near the mismatch, to unwind the DNA, removing the MMR intermediate of protein-bound nicked DNA (Li 2008). Therefore, unlike Δ*mutS* cells, Δ*uvrD* single-mutant cells will accumulate DNA mismatches bound by MutS and MutL with single-strand nicks nearby. In Figure 1A, we show that Δ*uvrD* Δ*mutS* double mutants, which do not begin MMR, are as hypersensitive to TLD as Δ*uvrD* single mutants, which begin but fail to complete MMR. We conclude that neither lack of MMR nor accumulation of MMR intermediates is the primary reason for the hyper-TLD of Δ*uvrD* cells.

UvrD also unwinds NER intermediates (Sancar 1996). NER-defective Δ*uvrC* cells have a TLD sensitivity similar to their parental strain (Figure 1B; Morganroth and Hanawalt 2006) indicating that loss of NER *per se* does not cause TLD. As with MMR, UvrD works late in NER to unwind the damaged, nicked DNA created by UvrA, UvrB, and UvrC (Sancar 1996). Therefore, UvrABC-initiated NER intermediates will persist in the absence of UvrD and might underlie the hyper TLD sensitivity of Δ*uvrD* mutants. We find that both Δ*uvrA* Δ*uvrD* and Δ*uvrC* Δ*uvrD* double mutants, which do not begin NER, are as sensitive as Δ*uvrD* single mutants, which begin but fail to complete NER (Figure 1B). Therefore, we conclude that, as with MMR, accumulated NER intermediates are not the main cause of hyper-TLD of Δ*uvrD* cells.

Part of how UvrD resists TLD is by opposing RecA and RecF but not SOS

UvrD helicase removes RecA from ssDNA, opposing HR (Flores *et al.* 2005; Veaute *et al.* 2005). If the TLD hypersensitivity of Δ*uvrD* mutants resulted exclusively from the greater abundance of RecA-DNA filaments (Veaute *et al.* 2005), then we would expect Δ*uvrD* Δ*recA* double mutants to have TLD resistance similar to that of Δ*recA* cells. In Figure 1C we show that removing RecA from Δ*uvrD* cells (using a Δ*uvrD* Δ*recA* double mutant) alleviates some but not all of the hypersensitivity of Δ*uvrD* cells to TLD. This appears to differ from recent results that show a complete rescue of the *uvrD* sensitivity by Δ*recA* (Kuong and Kuzminov 2010). However, whereas we used a null allele (deletion) of *uvrD*, the *uvrD* allele used by Kuong and Kuzminov encodes a truncated 230-amino-acid UvrD protein, which may still contain ATP binding and other activity. Thus, their somewhat different result might reflect altered function or partial activities of the mutant UvrD protein rather than UvrD removal. The data in Figure 1C imply that the increased TLD in strains lacking UvrD results from two separate causes: part from the

Table 1 E.*coli* K-12 strains and plasmids used in this study

Plasmid/strain	Relevant genotype	Source/reference
pCP20	FLP recombinase vector	Datsenko and Wanner (2000)
AB1157	F^- *thi-1 hisG4* Δ(*gpt-proA*)62 *argE3 thr-1 leuB6 araC14 lacY1 galK2 xylA5 mtl-1 rpsL31 tsx-33 glnV44 rfbC1 mgl-51 rpoS396 kdgK51*	CGSC1157[a] (Bachmann 1972)
AB2497	AB1157 *thyA12 deoB6*	CGSG2497[a] (Howard-Flanders *et al.* 1966)
BW26355	BW25113 Δ*recA635*::FRTKanFRT	CGSC7651[a] (Datsenko and Wanner 2000)
DM49	*lexA3*	CGSC6368[a]
GY8322	AB1157 *sfiA11* Δ(*srlR-recA*)*306*::Tn*10* [mini-F K5353 $recA^+$]	S. Sommers (Gif sur Yvette); ENZ280 (Dri *et al.* 1991) carrying the K5353 mini-F plasmid (Dutreix *et al.* 1989)
JW2703	Δ*mutS*::FRTKanFRT	Baba *et al.* (2006)
JW2860	Δ*recJ*::FRTKanFRT	Baba *et al.* (2006)
MG1655	Sequenced wild-type *E. coli* K-12 F^- λ^-	Blattner *et al.* (1997)
RTC0013	MG1655 Δ*recB*::Kan	Cirz *et al.* (2005)
SMR85	*recA801 srlC300*::Tn*10*	Lab Collection
SMR6201	R594 Δ*recQ1801*::FRT*cat*FRT	Lopez *et al.* (2005)
SMR8097	FC40 Δ*recF1804*::FRTKanFRT	Pennington and Rosenberg (2007)
SMR8547	MG1655 Δ*uvrA402*::Gm	Lab collection
SMR8548	MG1655 Δ*uvrC403*::Gm	Lab collection (Slack *et al.* 2006)
SMR9811	Δ*uvrD404*::FRT*cat*FRT *metE163*::Tn*10*	Magner *et al.* (2007)
SMR9812	Δ*uvrD404*::FRT*cat*FRT Δ*recQ1906*::FRT *metE163*::Tn*10*	Magner *et al.* (2007)
SMR10253	MG1655 Δ*mutS*::FRTKanFRT	MG1655 × P1(JW2703)
SMR10399	AB1157 Δ*ruvABC*::*cat zea-3*::Tn*10*	Fonville *et al.* (2010a)
SMR10433	AB2497 Δ*recA635*::FRTKanFRT	Fonville *et al.* (2010a)
SMR10445	AB2497 Δ*mutS*::FRTKanFRT	AB2497 × P1(SMR10253)
SMR10660	AB2497 Δ*ruvABC*::*cat zea3*::Tn*10*	Fonville *et al.* (2010a)
SMR10665	AB2497 Δ*recB*::Kan	AB2497 × P1(RTC0013)
SMR10669	AB2497 *lexA3 malB*::Tn*9*	Fonville *et al.* (2010a)
SMR10670	AB2497 Δ(*srlR-recA*)*306*::Tn*10*	Fonville *et al.* (2010a)
SMR10671	AB2497 Δ(*srlR-recA*)*306*::Tn*10* Δ*recB*::Kan	SMR10665 × P1(GY8322)
SMR10672	AB2497 Δ*topB*::FRTKanFRT	AB2497 × P1(JW1752)
SMR10681	AB2497 Δ*recQ1906*::FRT	Fonville *et al.* (2010a)
SMR10691	AB2497 Δ*recF1804*::FRTKanFRT	Fonville *et al.* (2010a)
SMR10692	AB2497 *lexA3* Δ*recF1804*::FRTKanFRT	Fonville *et al.* (2010a)
SMR10913	AB2497 Δ*recQ1906*::FRT Δ*recA635*::FRTKanFRT	Fonville *et al.* (2010a)
SMR11118	AB2497 Δ*ruvABC*::*cat zea3*::Tn*10* Δ*recA635*::FRTKanFRT	Fonville *et al.* (2010a)
SMR11193	AB2497 Δ*uvrD404*::FRT*cat*FRT	AB2497 × P1(SMR9811)
SMR11194	AB2497 Δ*uvrD404*::FRT	SMR11193 × pCP20
SMR11196	AB2497 Δ*recQ1906*::FRT Δ*ruvABC*::*cat*	SMR10681 × P1(SMR10399)
SMR11197	AB2497 Δ*uvrD404*::FRT*cat*FRT Δ*recQ1906*::FRT *metE163*::Tn*10*	AB2497 × P1(SMR9812)
SMR11199	AB2497 Δ*uvrD404*::FRT*cat*FRT Δ*recA635*::FRTKanFRT	SMR11193 × P1(BW26355)
SMR11206	AB2497 Δ*uvrD404*::FRT Δ*mutS*::FRTKanFRT	SMR11194 × P1(SMR10253)
SMR11207	AB2497 Δ*uvrC403*::Gm	AB2497 × P1(SMR8548)
SMR11214	AB2497 Δ*recQ1906*::FRT Δ*recB*::Kan	SMR10681 × P1(RTC0013)
SMR11233	AB2497 Δ*uvrD404*::FRT*cat*FRT Δ*recQ1906*::FRT *metE163*::Tn*10* Δ*recA635*::FRTKanFRT	SMR11197 × P1(BW26355)
SMR11235	AB2497 Δ*recF1804*::FRTKanFRT Δ*uvrD404*::FRT*cat*FRT	SMR10691 × P1(SMR9811)
SMR11310	AB2497 *recA801 srlC300*::Tn*10*	SMR10278 × P1(SMR85)
SMR11312	AB2497 Δ*recF1804*::FRTKanFRT *recA801 srlC300*::Tn*10*	SMR10691 × P1(SMR85)
SMR11314	AB2497 *lexA3*	SMR10669 × P1(DM49)
SMR11317	AB2497 *lexA3* Δ*uvrD404*::FRT*cat*FRT	SMR11314 × P1(SMR9811)
SMR12992	AB2497 *lexA3* Δ*recF1804*::FRTKanFRT *recA801 srlC300*::Tn*10*	SMR10692 × P1(SMR85)
SMR12994	AB2497 *lexA3 recA801 srlC300*::Tn*10*	SMR10669 × P1(SMR85)
SMR12996	AB2497 Δ*uvrC403*::Gm Δ*uvrD404*::FRT*cat*FRT	SMR11207 × P1(SMR9811)
SMR12998	AB2497 Δ*recF1804*::FRT	SMR10691 × pCP20
SMR12999	AB2497 Δ*recF1804*::FRT Δ*uvrD404*::FRT	SMR11235 × pCP20
SMR13000	AB2497 Δ*recF1804*::FRT Δ*recA635*::FRTKanFRT	SMR12998 × P1(BW26355)
SMR13001	AB2497 Δ*recF1804*::FRT Δ*uvrD404*::FRT Δ*recA635*::FRTKanFRT	SMR12999 × P1(BW26355)
SMR13003	AB2497 Δ*recQ1906*::FRTΔ*ruvABC*::*cat* Δ*recA635*::FRTKanFRT	SMR11196 × P1(BW26355)
SMR13005	AB2497 Δ*uvrA402*::Gm	AB2497 × P1(SMR8547)
SMR13007	AB2497 Δ*uvrA402*::Gm Δ*uvrD404*::FRT	SMR11194 × P1(SMR8547)
SMR14220	AB2497 Δ*recJ*::FRTKanFRT	AB2497 × P1(JW2860)

(continued)

Table 1 Continued

Plasmid/strain	Relevant genotype	Source/reference
SMR14228	AB2497 Δ*recJ*::FRTKanFRT Δ*ruvABC*::*cat*	SMR14220 × P1(SMR10399)
SMR14238	AB2497 Δ*recJ*::FRT	SMR14220 × pCP20
SMR14239	AB2497 Δ*recJ*::FRT Δ*uvrD404*::FRT*cat*FRT	SMR14238 × P1(SMR9811)
SMR14241	AB2497 Δ*recJ*::FRT Δ*uvrD404*::FRT*cat*FRT Δ*recQ1906*::FRT *metE163*::Tn*10*	SMR14238 × P1(SMR9812)
SMR14242	AB2497 Δ*recJ*::FRT Δ*recF1804*::FRTKanFRT	SMR14238 × P1(SMR8097)
SMR14244	AB2497 Δ*recJ*::FRT Δ*recA635*::FRTKanFRT	SMR14238 × P1(BW26355)
SMR14249	AB2497 Δ*recJ*::FRT Δ*uvrD404*::FRT*cat*FRT Δ*recF1804*::FRTKanFRT	SMR14239 × P1(SMR8097)
SMR14251	AB2497 Δ*recJ*::FRT Δ*uvrD404*::FRT*cat*FRT Δ*recA635*::FRTKanFRT	SMR14239 × P1(BW26355)
SMR14253	AB2497 Δ*recJ*::FRT Δ*recQ1801*::FRT*cat*FRT	SMR14238 × P1(SMR6201)
SMR14490	AB2497 Δ*recJ*::FRT Δ*recQ1801*::FRT*cat*FRT Δ*recA635*::FRTKanFRT	SMR14253 × P1(BW26355)

[a] CGSC, The *E. coli* Genetic Stock Center (Yale University).

increased persistence of RecA on DNA when UvrD is absent and part independent of the enhancement of a RecA-dependent TLD pathway.

The RecA-dependent component of the hyper-TLD in Δ*uvrD* cells could, in principle, be caused by increased HR or an increased SOS response. Cells that lack UvrD show increased spontaneous SOS induction (SaiSree *et al.* 2000), and SOS induction causes TLD via expression of SulA (Fonville *et al.* 2010a). However, we find that blocking induction of the SOS response using an uncleavable LexA repressor protein, encoded by *lexA3*(Ind$^-$), did not alleviate the hypersensitivity of Δ*uvrD* cells (Figure 1D). The data imply that hyperrecombination not hyper-SOS induction is likely to underlie the RecA-dependent component of the hyper-TLD of Δ*uvrD* cells.

RecF helps RecA load onto ssDNA (Friedberg *et al.* 2005). If RecF loaded the RecA that promotes the hyper-TLD of Δ*uvrD* cells, then TLD should be partially blocked in the absence of *recF* as was seen in the absence of *recA*. Indeed, Figure 2A shows that Δ*recF* partially ameliorates the TLD hypersensitivity of Δ*uvrD* cells. This again differs from what was seen using a truncated UvrD protein, with which TLD was relieved to a level similar to or greater than that in UvrD$^+$ cells lacking *recF* (Kuong and Kuzminov 2010). These data suggest that part of the sensitivity of Δ*uvrD* cells to thymine deprivation might be due to the lack of opposition to RecF-promoted loading of RecA onto ssDNA. To test whether RecF and RecA act via the same (epistatic) or separate (additive) pathways in the absence of *uvrD*, we compared a Δ*uvrD* Δ*recA* Δ*recF* triple mutant with Δ*uvrD* Δ*recF* and Δ*uvrD* Δ*recA* cells. We find that although the Δ*uvrD* Δ*recA* Δ*recF* triple mutant is as resistant to TLD as Δ*uvrD* Δ*recF* cells ($P = 0.2$ at $t \geq 180$ min; Figure 2B), as expected, the Δ*uvrD* Δ*recA* Δ*recF* triple mutant is significantly more

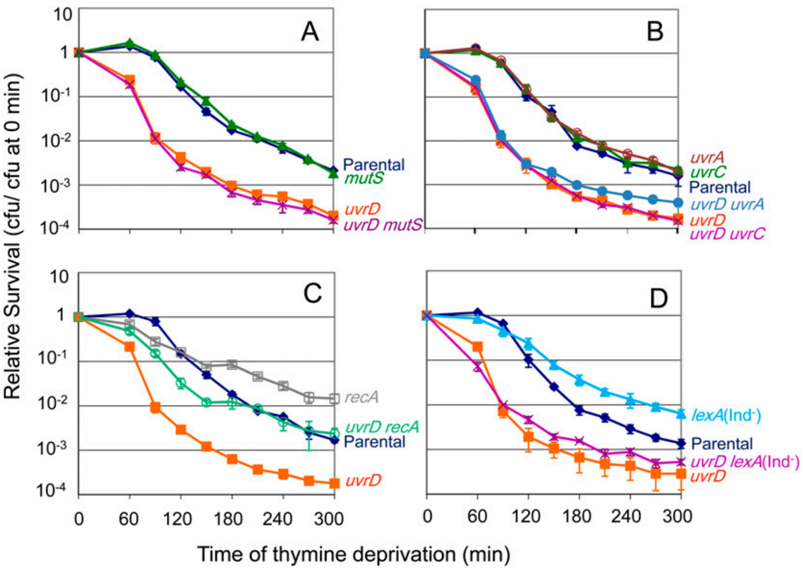

Figure 1 RecA contributes SOS independently to the hyper-TLD of Δ*uvrD* cells, but neither NER nor MMR intermediates do. (A) Mismatch-repair intermediates are not the main cause of hyper-TLD of Δ*uvrD* cells. First, mismatch-repair-defective Δ*mutS* (SMR10445; solid green triangle) cells are not significantly different from the parental strain (AB2497; solid blue diamond). Second, a Δ*uvrD* Δ*mutS* double mutant (SMR11206; purple X) showed the same hypersensitivity to TLD as Δ*uvrD* (SMR11194; solid orange square) alone, indicating that the accumulation of MMR intermediates created by MutS did not cause most hyper-TLD of Δ*uvrD* cells. (B) NER intermediates are not the main cause of the hyper-TLD of Δ*uvrD* cells. NER-defective Δ*uvrA* (SMR13005; open red circle) and Δ*uvrC* (SMR11207; solid green triangle) cells are not significantly different from the parental strain (AB2497; solid blue diamond). Neither a Δ*uvrD* Δ*uvrA* double mutant (SMR13007; solid blue circle), nor a Δ*uvrD* Δ*uvrC* double mutant (SMR12996; purple X) showed less hypersensitivity to TLD than Δ*uvrD* cells (SMR11194; solid orange square) indicating that UvrABC-generated NER intermediates are not the main cause of the Δ*uvrD* hyper-TLD. (C) RecA is partially required for the hypersensitivity to TLD of Δ*uvrD* cells. A Δ*uvrD* Δ*recA* double mutant (SMR11199; open green circle) was not as resistant to TLD as the Δ*recA* single mutant (SMR10433; open gray square; $P < 0.05$), but was significantly more resistant than the Δ*uvrD* single mutant (SMR11193; solid orange square; $P < 0.05$). Parental: AB2497; solid blue diamond. (D) The SOS response is not required for the hyper-TLD of Δ*uvrD* cells. The SOS-blocking *lexA*(Ind$^-$) allele did not relieve the hypersensitivity of Δ*uvrD* cells. Strains used from top to bottom: *lexA*(Ind$^-$) (SMR11314; solid blue triangle), parental (AB2497; solid blue diamond), Δ*uvrD* *lexA*(Ind$^-$) (SMR11317; purple X), Δ*uvrD* (SMR11193; solid orange square). Means ±SEM of three independent experiments.

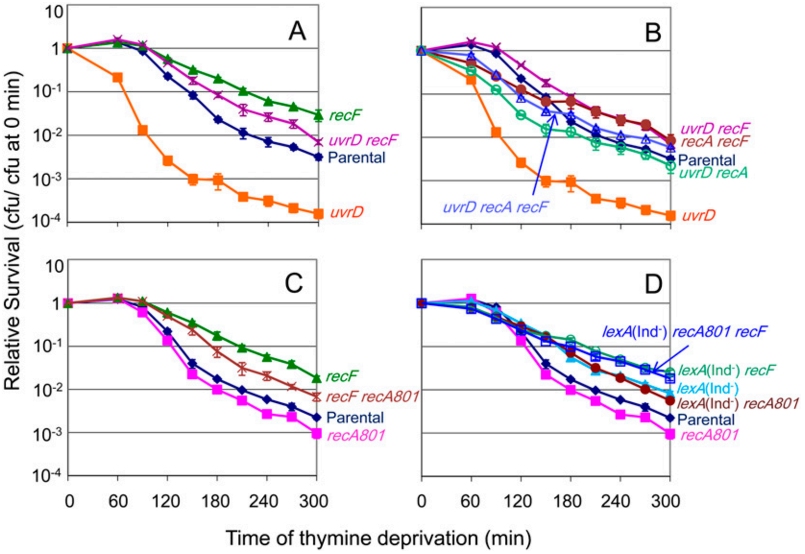

Figure 2 UvrD action resists two RecF-dependent TLD pathways: one RecA dependent and one RecA independent. (A) RecF is partially required for much but not all of the hypersensitivity of Δ*uvrD* cells to TLD. A Δ*uvrD* Δ*recF* double mutant (SMR11235; purple X) was more resistant to TLD than Δ*uvrD* (SMR11193; solid orange square; $P < 0.05$) alone, but not as resistant as Δ*recF* (SMR10691; solid green triangle; $P < 0.05$). Parental (AB2497; solid blue diamond). (B) RecF and RecA function in independent death pathways in the absence of *uvrD*. The Δ*uvrD* Δ*recF* (SMR12999; purple X) double mutant is more resistant than the Δ*uvrD* Δ*recA* (SMR11202; open green circle; $P < 0.05$) mutant indicating that, in Δ*uvrD* cells, RecF plays a role in TLD in addition to loading RecA. The Δ*uvrD* Δ*recA* Δ*recF* triple mutant (SMR13001; open blue triangle) is as resistant to TLD as the Δ*uvrD* Δ*recF* double mutant ($P = 0.17$) but is more resistant than Δ*uvrD* Δ*recA* cells ($P < 0.05$). Δ*uvrD* (SMR11193; solid orange square), Δ*recA* Δ*recF* (SMR13000; solid brown circle), parental (AB2497; solid blue diamond). Data in A and B are from experiments run in parallel, thus can be compared directly. (C) *recA801* partially compensates for RecF in TLD, indicating that part of the RecF role in TLD is loading of RecA. Partial suppression of the Δ*recF* phenotype (SMR10691; solid green triangle) by *recA801* in the Δ*recF recA801* double mutant (SMR11312; brown X; $P < 0.05$ compared with Δ*recF*). Therefore, much of the RecF role in TLD is loading RecA onto ssDNA. *recA801* (SMR11310; solid pink square), parental (AB2497; solid blue diamond). The data do not exclude the additional RecA-independent role for RecF in TLD shown in B. (D) The RecA-dependent component of RecF-mediated TLD is *via* SOS activation. *lexA*(Ind⁻) *recA801* (SMR12994; solid brown circle) cells are as resistant to TLD as *lexA*(Ind⁻) cells (epistatic, same pathway). Thus, the sole role of *recA801* in TLD is promoting an SOS response. As expected *lexA*(Ind⁻) *recA801* Δ*recF* (SMR12992; open blue square) cells are more resistant to TLD than the *lexA*(Ind⁻) *recA801* double mutant ($P < 0.05$, additive effects), reiterating that RecF functions in a pathway in addition to that of loading RecA and promoting SOS. *lexA*(Ind⁻) Δ*recF* (SMR10692; open green circle). Parental (AB2497; solid blue diamond), *recA801* (SMR11310; solid pink square), *lexA*(Ind⁻) (SMR10669; solid blue triangle). Data in C and D are from experiments run in parallel, thus can be compared directly. Means ±SEM of three independent experiments.

resistant than Δ*uvrD* Δ*recA* cells ($P = 0.005$ at $t \geq 180$ min). These data indicate that RecF promotes TLD via both the RecA-dependent pathway, possibly by loading RecA, and by an additional RecA-independent pathway and mechanism. Because RecF appears not to play the early anti-TLD role that RecA does (Figure 2A and Figure 2B, no early drop in survival of the Δ*recF* strains), it seems likely that RecBCD loads RecA in its early anti-TLD role but that RecF loads RecA in its late pro-TLD role. This implies that the early anti-TLD role of RecA is in DSB repair whereas the late pro-TLD role pertains to ssDNA gaps, the RecBCD and RecF substrates, respectively.

RecF promotes TLD by RecA-dependent and RecA-independent pathways

In hyper-TLD of Δ*uvrD* cells (above), and TLD in UvrD⁺ cells (Fonville *et al.* 2010a), RecF and RecA acted in the same pathway, which, in UvrD⁺ cells, is a pathway of death by induction of the SOS response and SulA (Fonville *et al.* 2010a). To support the conclusion that part of how RecF promotes TLD in UvrD⁺ cells is by loading of RecA, and to probe whether SOS induction or HR is the TLD-promoting outcome, we tested whether an allele of *recA* (*recA801*) that encodes a RecF-independent RecA protein makes RecF unnecessary. *recA801* encodes a RecA protein from a class of mutant RecA's that compete against single-strand-binding protein (SSB) for binding of ssDNA better than wild-type RecA does (Volkert and Hartke 1987; Madiraju *et al.* 1992). *recA801* compensates for the loss of *recF* in recombinational DNA repair, but only partially compensates for loss of *recF* in SOS induction *in vivo* (Volkert and Hartke 1987). Figure 2C shows that *recA801* partially suppresses the TLD resistance of Δ*recF*, that is, makes Δ*recF* cells more TLD sensitive (Δ*recF recA801 vs.* Δ*recF*, $P < 0.01$). The partial suppression of the *recF* TLD resistance by *recA801* might reflect the inability of *recA801* to compensate fully for the loss of *recF* in induction of the SOS response (Volkert and Hartke 1987). We used the SOS-response-blocking *lexA* (Ind⁻) mutation to remove induction of the SOS response as a complicating factor and found that *recA801* could no longer compensate for the loss of *recF* in the absence of the SOS response (Figure 2D). This, and the additivity of the effect of blocking SOS with removal of RecF [the *lexA*(Ind⁻) Δ*recF* double mutant is more resistant than the *lexA*(Ind⁻) mutant; Figure 2D; $P < 0.05$], indicates that although part of the RecF role in promoting TLD is loading RecA onto ssDNA and promoting an SOS response as previously determined (Fonville *et al.* 2010a), RecF plays an additional RecA/SOS-independent role in promoting TLD, both in the presence and absence of *uvrD*. One possibility for a RecA-loading/SOS-induction-independent role of RecF in promoting TLD might be RecF-promoted stabilization of stalled replication forks, discussed further below.

UvrD resists RecQ-dependent and RecQ-independent TLD pathways

RecQ is required for an SOS-independent pathway of TLD in *E. coli* (Fonville *et al.* 2010a). This RecQ-dependent TLD pathway might have promoted part of the hyper-TLD in the absence of *uvrD*. Indeed, removing *recQ* partially rescues the hyper-TLD of Δ*uvrD* cells (Figure 3A). This indicates that one of the TLD pathways that UvrD resists is RecQ promoted. However, in Δ*uvrD* cells RecQ might promote TLD either through a RecA-dependent pathway of hyperaccumulation of toxic intermolecular HR intermediates as described previously in cells lacking UvrD (Magner *et al.* 2007; Fonville *et al.* 2010b) or through a RecA-independent TLD pathway as it does when UvrD is present (Fonville *et al.* 2010a). We find that the triple Δ*uvrD* Δ*recA* Δ*recQ* mutant is not significantly more resistant to TLD than Δ*uvrD* Δ*recA* or the Δ*uvrD* Δ*recQ* cells (Figure 3B; $P = 0.99$ for both comparisons at $t \geq 180$ min). That is, *recA* and *recQ* are epistatic. This indicates that the role of RecQ in promoting hyper-TLD in Δ*uvrD* cells occurs through the same pathway as RecA and supports a death-by-recombination pathway similar to those described previously (Magner *et al.* 2007; Fonville *et al.* 2010b). Additionally, we conclude that part of the hyper-TLD observed in Δ*uvrD* cells occurs via a RecA- and RecQ-independent pathway because triply mutant Δ*uvrD* Δ*recA* Δ*recQ* cells were significantly more sensitive to TLD than Δ*recA* Δ*recQ* double-mutant cells ($P \leq 0.001$ at $t \geq 180$ min; Figure 3B).

RecJ functions in the RecQ- RecF- RecA-dependent pathway of hyper-TLD in ΔuvrD cells

RecJ is required for the RecQ-dependent pathway of TLD (Fonville *et al.* 2010a). We show that RecJ is also required for hyper-TLD in Δ*uvrD* cells (Figure 4A), in which Δ*recJ* confers a greater relief of hyper-TLD of Δ*uvrD* cells (Δ*recJ* Δ*uvrD*; Figure 4A) than does Δ*recQ* (Δ*recQ* Δ*uvrD*; Figure 4A, $P < 0.01$). RecJ might promote the same or a different pathway of hyper-TLD in Δ*uvrD* cells as RecQ and RecA. First, Δ*recJ* Δ*recQ* Δ*uvrD* cells were as sensitive to TLD as Δ*recJ* Δ*uvrD* cells, indicating that RecQ and RecJ do not have additive effects (are epistatic; Figure 4A), but Δ*recJ* confers greater resistance than Δ*recQ*. We conclude that RecJ and RecQ function in the same pathway of hyper-TLD in Δ*uvrD* cells, and the greater RecJ effect indicates a possible additional RecQ-independent role of RecJ. Second, Δ*recJ* Δ*recA* Δ*uvrD* cells were as sensitive to TLD as Δ*recA* Δ*uvrD* cells, indicating that RecA and RecJ do not have additive effects (Figure 4C). We conclude that RecJ acts in the RecA-dependent pathway of hyper-TLD in Δ*uvrD* cells. Third, Δ*recJ* Δ*recF* Δ*uvrD* cells were as sensitive to TLD as Δ*recJ* Δ*uvrD* cells, not additively so (Figure 4D). This implies that RecJ and RecF act in the same pathway of hyper-TLD in Δ*uvrD* cells. These data show that unlike the separate RecJ/Q- *vs.* RecA/F-dependent pathways of TLD in UvrD$^+$ cells (Fonville *et al.* 2010a), hyper-TLD in Δ*uvrD* cells is promoted by RecJ, RecQ, RecF, and RecA acting primarily in a single pathway.

RecJ functions in both the RecQ and RecA-dependent TLD pathways in UvrD$^+$ cells

Whereas, RecA, RecF, RecQ, and RecJ act in one linear pathway of hyper-TLD in Δ*uvrD* cells (Figures 3B and Figure 4, A, C, and D), RecQ and RecJ were shown previously to act in one pathway of TLD in UvrD$^+$ cells while RecA and RecF acted in a second SOS-response-dependent pathway that is independent of RecQ (Fonville *et al.* 2010a). Whether RecJ might also function in the RecA/F-dependent (RecQ-independent) TLD pathway in UvrD$^+$ cells had not been tested. Figure 4C shows that Δ*recJ* cells are slightly more resistant than Δ*recA* cells ($P = 0.03$) and that Δ*recA* Δ*recJ* cells are similar in resistance to *recA* suggesting action in the same pathway. The slightly greater resistance of Δ*recJ* than Δ*recA* and Δ*recA* Δ*recJ* cells might be because all Δ*recA* cells suffer an early reduction in survival (dip in curves prior to 180 min) that then lowers the point at which the second, resistant phase of Δ*recA* curves begins (Fonville *et al.* 2010a; Kuong and Kuzminov 2010), or because RecJ acts partly in a pathway separate from RecA. Either way, these results support the action of RecJ at least partially in the RecA-dependent (RecQ-independent) pathway of TLD in UvrD$^+$ cells. Also supporting this interpretation, Δ*recJ*

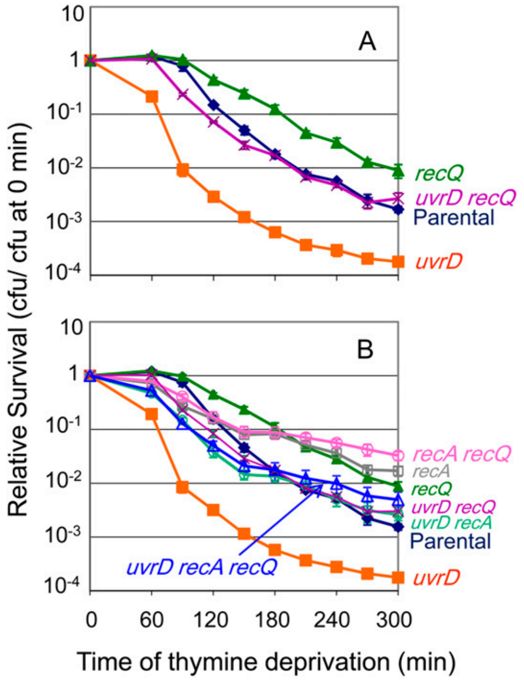

Figure 3 (A) UvrD action resists TLD by a RecQ-dependent and a RecQ-independent pathway. RecQ is partially required for the hypersensitivity of Δ*uvrD* cells to TLD. The Δ*uvrD* Δ*recQ* (SMR11197; purple X) double mutant is more resistant to TLD than Δ*uvrD* (SMR11193; solid orange square; $P < 0.05$), but not as resistant as Δ*recQ* cells (SMR10681; solid green triangle; $P < 0.05$). Parental (AB2497; solid blue triangle). (B) The RecQ-dependent pathway that UvrD resists is also RecA dependent, in that RecA and RecQ promote TLD hypersensitivity of Δ*uvrD* cells *via* the same (epistatic) pathway. The triple Δ*uvrD* Δ*recA* Δ*recQ* mutant was not significantly more resistant to TLD than either the Δ*uvrD* Δ*recA* or Δ*uvrD* Δ*recQ* double mutant, but was not as resistant to TLD as the Δ*recA* Δ*recQ* double mutant ($P < 0.05$). Strains from top to bottom: Δ*recA* Δ*recQ* (SMR10913; open pink circle), Δ*recA* (SMR10433; open gray square), Δ*recQ* (SMR10681; solid green triangle), Δ*uvrD* Δ*recA* Δ*recQ* (SMR11233; open blue triangle), Δ*uvrD* Δ*recQ* (SMR11197; purple X), Δ*uvrD* Δ*recA* (SMR11199; open green circle), parental (AB2497; solid blue triangle); Δ*uvrD* (SMR11193; solid orange square). Means ±SEM of three independent experiments.

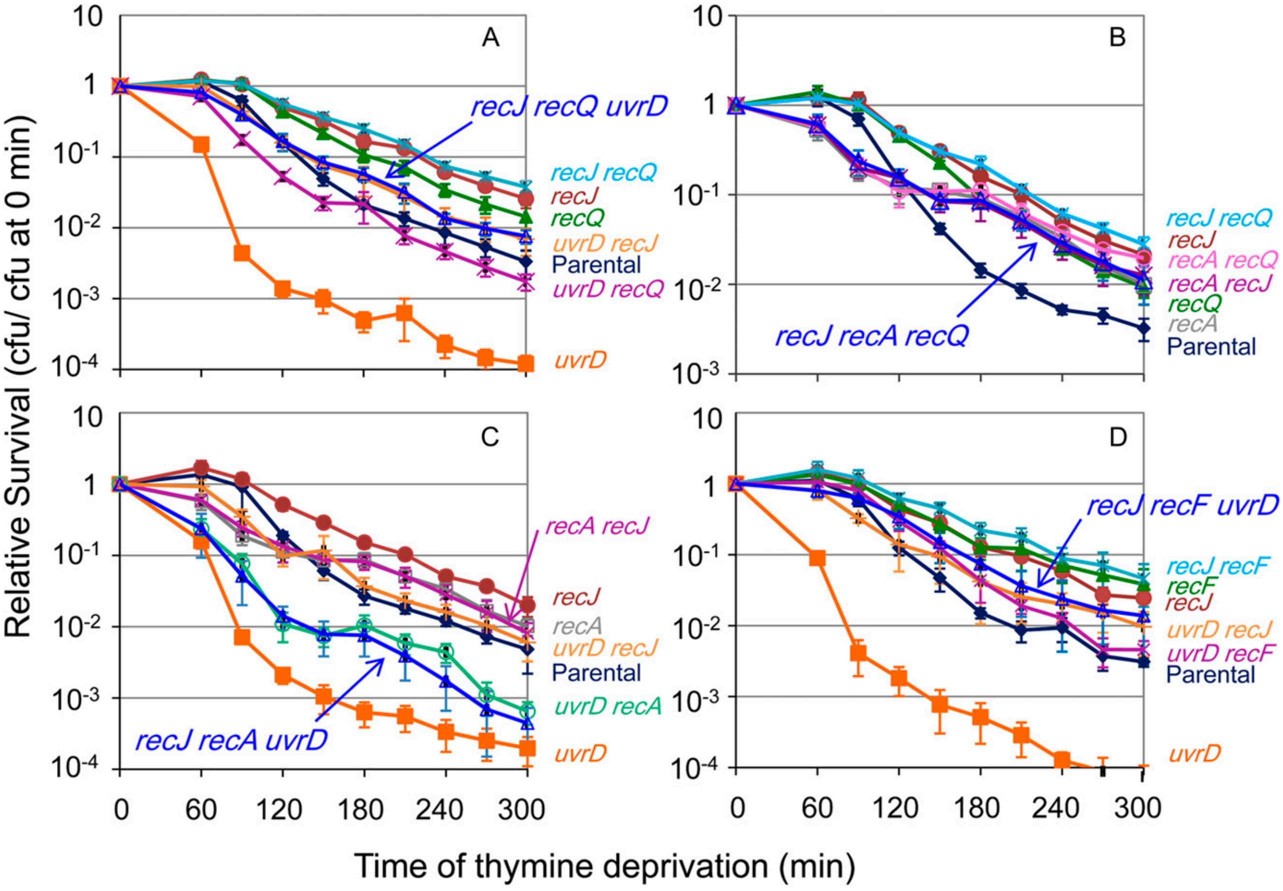

Figure 4 (A) RecJ is partially required for the hyper-TLD of Δ*uvrD* cells, and loss of RecJ relieves the hyper-TLD to a greater level than loss of RecQ. The loss of both RecJ and RecQ relieves the hyper-TLD of Δ*uvrD* cells to the same degree of RecJ alone. Strains from top to bottom: Δ*recJ* Δ*recQ* (SMR14253; blue asterisk), Δ*recJ* (SMR14238; solid brown circle), Δ*recQ* (SMR10681; solid green triangle), Δ*uvrD* Δ*recJ* (SMR14239; orange +), Δ*recJ* Δ*recQ* Δ*uvrD* (SMR14241; open blue triangle), parental (AB2497; solid blue diamond), Δ*uvrD* Δ*recQ* (SMR11197; purple X), Δ*uvrD* (SMR11193; solid orange square). (B) Δ*recA* Δ*recJ* cells were not significantly more resistant to TLD than Δ*recA* cells and removing RecQ did not have an additional effect in that Δ*recJ* Δ*recA* Δ*recQ* cells showed no additional TLD resistance above that in Δ*recA* Δ*recJ* cells. Strains from top to bottom: Δ*recJ* Δ*recQ* (SMR14253; blue asterisk), Δ*recJ* (SMR14238; solid brown circle), Δ*recA* Δ*recQ* (SMR10913; open pink circle), Δ*recA* Δ*recJ* (SMR14244; purple X), Δ*recJ* Δ*recA* Δ*recQ* (SMR14490; open blue triangle), Δ*recQ* (SMR10681; solid green triangle), Δ*recA* (SMR10670; open gray square), parental (AB2497; solid blue diamond). (C) Δ*recJ* Δ*recA* Δ*uvrD* cells are as sensitive to TLD as Δ*recA* Δ*uvrD* cells. Strains from top to bottom: Δ*recJ* (SMR14238; solid brown circle), Δ*recA* (SMR10670; open gray square), Δ*recA* Δ*recJ* (SMR14244; purple X), Δ*uvrD* Δ*recJ* (SMR14239; orange +), parental (AB2497; solid blue diamond), Δ*uvrD* Δ*recA* (SMR11199; open green circle), Δ*recJ* Δ*recA* Δ*uvrD* (SMR14251; open blue triangle), Δ*uvrD* (SMR11193; solid orange square). (D) Δ*recJ* Δ*recF* Δ*uvrD* cells are as resistant to TLD as Δ*uvrD* Δ*recJ* cells, but are more resistant than Δ*uvrD* Δ*recF* cells. Strains from top to bottom: Δ*recJ* Δ*recF* (SMR14242; blue asterisk), Δ*recF* (SMR12998; solid green triangle), Δ*recJ* (SMR14238; solid brown circle), Δ*recJ* Δ*recF* Δ*uvrD* (SMR14249; open blue triangle), Δ*uvrD* Δ*recJ* (SMR14239; orange +), Δ*uvrD* Δ*recF* (SMR12999; purple X), parental (AB2497; solid blue diamond), Δ*uvrD* (SMR11193; solid orange square). Means ±SEM of three independent experiments.

cells are more resistant than Δ*recQ* ($P = 0.01$) with the Δ*recJ* Δ*recQ* double mutants resembling Δ*recJ* (Figure 4B; Fonville *et al.* 2010a). Additionally, we find that RecJ and RecF function in a single TLD pathway in that Δ*recJ* Δ*recF* cells were not significantly more resistant to TLD than Δ*recF* cells (Figure 4D; $P = 0.86$ at $t \geq 180$ min), having an epistatic interaction diagnostic of a single pathway, not an additive interaction diagnostic of separate pathways.

The hyper-TLD pathway opposed by RuvABC is RecQ- and RecJ-independent

Δ*ruvABC* cells are hypersensitive to TLD and this hypersensitivity requires RecA (Fonville *et al.* 2010a). We suggested that death-by-recombination, in which unresolved IRIs block chromosome segregation (Magner *et al.* 2007), might cause the hyper-TLD of Δ*ruvABC* cells (Fonville *et al.* 2010a) as it does death of *ruv*-defective Δ*uvrD* cells (Magner *et al.* 2007) and appears to cause part of hyper-TLD in Δ*uvrD* cells (Figure 1D). RecQ and RecJ promote death-by-recombination in *ruv uvrD* (Magner *et al.* 2007) or *recG uvrD* cells (Fonville *et al.* 2010b). However, we find that neither Δ*recQ* (Figure 5A) nor Δ*recJ* (Figure 5B) relieves the hyper-TLD of Δ*ruvABC* cells. Also Δ*ruvABC* Δ*recQ* Δ*recA* cells are as resistant to TLD as Δ*recA* Δ*ruvABC* cells (Figure 5A). These results indicate that although the hyper-TLD in cells lacking *ruvABC* is RecA dependent and probably occurs via death-

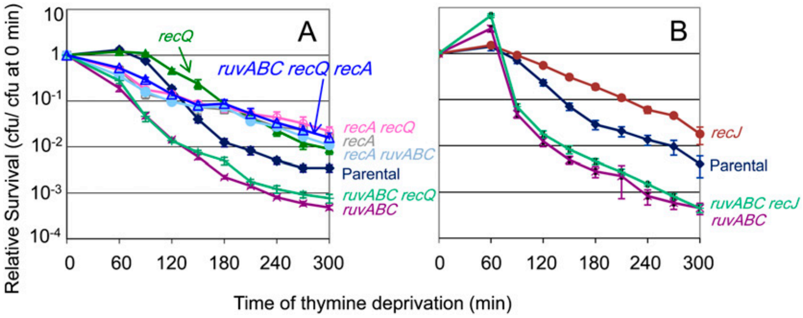

Figure 5 The RecA-dependent TLD pathway resisted by RuvABC is not RecQ/J-dependent. (A) Δ*ruvABC* Δ*recQ* cells (SMR11196; green +) are as hypersensitive to TLD as Δ*ruvABC* cells (SMR10660; purple X); also, triply mutant Δ*ruvABC* Δ*recQ* Δ*recA* cells (SMR13003; open blue triangle) are as resistant to TLD as Δ*recA* Δ*ruvABC* cells (SMR11118; solid blue circle). Δ*recA* Δ*recQ* (SMR10913; open pink circle), Δ*recQ* (SMR10681; solid green triangle), Δ*recA* (SMR10433; open gray square), parental (AB2497; solid blue diamond). (B) Δ*ruvABC* Δ*recJ* cells (SMR14228; green +) are as hypersensitive to TLD as Δ*ruvABC* cells (SMR10660; purple X). Δ*recJ* (SMR14220; solid brown circle), parental (AB2497; solid blue diamond). Means ±SEM of three independent experiments.

by-recombination (Fonville *et al.* 2010a), neither RecQ nor RecJ plays a role in it, a surprising result discussed below.

The main role of RecBCD in TLD-resistance is not protection from SOS, recombination, or RecQ-generated DNA ends

Cells lacking RecB, which processes DSEs into substrates for recombination, are hypersensitive to TLD (Nakayama *et al.* 1982). Previously we suggested that RecQ might promote TLD by creating DSEs generated by overlap of tracts of single-strand degradation by RecQ helicase with RecJ exonuclease at nearby sites (Fonville *et al.* 2010a). RecB would then degrade these DSEs, possibly removing extra *ori*-proximal DNA segments from uncompleted chromosome replication and releasing nucleotides that might extend survival. If processing RecQ-generated DSEs were the *sole* role of RecBCD in TLD resistance, then RecBCD would confer no TLD resistance in cells lacking RecQ. To test whether DSE formation by RecQ is responsible for creating the DNA substrate that then must be processed by RecBCD for resistance to TLD, we examined the sensitivity of a Δ*recB* Δ*recQ* double mutant and found that it was only slightly, though significantly, more resistant to TLD than Δ*recB* (Figure 6A; $P < 0.05$). This suggests that if RecQ creates some, it does not create most, of the DSEs/DSBs the processing of which by RecBCD allows survival of thymine starvation. That is, RecBCD (*i.e.*, DSE processing) is still required for surviving thymine starvation, even in Δ*recQ* cells.

Additionally, cells that lack RecB fail to repair DSEs. In the absence of RecB, RecJ single-strand-dependent exonuclease appears to be able to prepare DSEs for RecA filament formation and, in cooperation with a DNA helicase, allows induction of an SOS response (Vlasic *et al.* 2008) and some recombination (Ivancic-Bace *et al.* 2005). To test whether activation of SOS causes most of the sensitivity of Δ*recB* cells to TLD, we asked whether Δ*recB* hyper-TLD is RecA dependent. We found that Δ*recA* Δ*recB* cells showed only slightly but significantly more resistance to thymine deprivation than Δ*recB* cells (Figure 6B; $P < 0.05$). Therefore, neither RecA-promoted recombination nor SOS induction is the main cause of hyper-TLD in Δ*recB* mutants. The data imply that RecBC-mediated DNA degradation improves survival during thymine starvation, even in the absence of RecA. The slight increase in TLD resistance of both Δ*recB* Δ*recQ* cells (Figure 6A) and Δ*recA* Δ*recB* cells (Figure 6B) over Δ*recB* cells is likely to result from the additivity of the RecQ- and RecA-promoted pathways operative in wild-type cells with the more robust alternative hyper-TLD pathway that dominates in Δ*recB* cells.

The hypothesis that RecBCD double-strand exonuclease activity promotes recovery from TLD was supported further by visualizing nucleoids (bacterial chromosomes) of cells undergoing TLD. Upon thymine depletion, parental cells cease cell division, as seen previously (Fonville *et al.* 2010a), and the DNA appears diffuse within the cell, *i.e.*, mostly not visible with DAPI, although a subset of cells possess a single compact centrally localized nucleoid (Figure 6C). By contrast, the DNA in Δ*recB* cells appeared fragmented as many small DAPI foci dispersed throughout the cells (Figure 6C). Such foci were not seen in Δ*recB* cells before thymine deprivation. RecBCD exonuclease and DSE-repair activities may help maintain the nucleoid, allowing recovery upon plating in the presence of thymine.

Discussion

This study examined the pathways by which UvrD, RuvABC, and RecBCD protect cells from TLD. We sought to identify proteins in the pathways that produce the DNA substrates that kill cells more rapidly in the absence of these DNA-repair proteins.

The anti-TLD role of UvrD

In cells lacking *uvrD*, we found that the hyper-TLD does not result primarily from either incomplete NER or MMR intermediates (Figure 1, A and B) and partially requires RecA, RecF, RecQ, and RecJ (Figures 1C, 2A, and 3A) acting in a single pathway (Figure 4, A, C, and D), but does not require induction of the SOS response (Figure 1D). These data suggest that the UvrD anti-recombination role (Veaute *et al.* 2005) could account for protection against the RecA-dependent component of hyper-TLD seen in Δ*uvrD* cells. This could occur by a death-by-recombination model, such as in Figure 7B, in which unresolved IRIs kill cells by blocking chromosome segregation. Because RecF loads RecA onto ssDNA in single-strand gaps (whereas RecBCD loads RecA onto ssDNA at

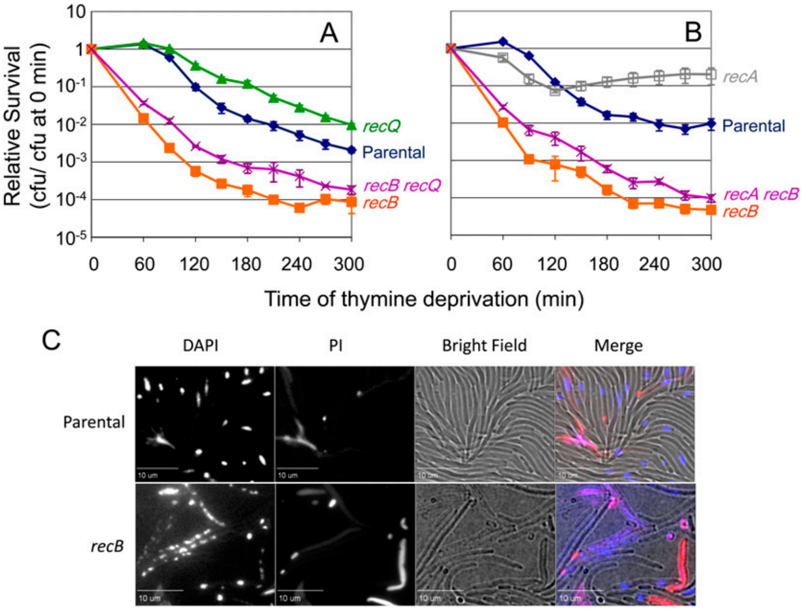

Figure 6 RecBCD resists a RecA-, SOS-, and RecQ-independent TLD pathway. (A) Much of the RecB role in TLD survival is independent of RecQ. Δ*recB* Δ*recQ* (SMR11214; purple X) cells are slightly less sensitive to TLD than Δ*recB* cells (SMR10665; solid orange square; $P < 0.05$). Δ*recQ* (SMR10681; solid green triangle), parental (AB2497; solid blue diamond). (B) Neither SOS nor homologous recombination creates the problem that $RecB^+$ action resists in that RecB is required for TLD survival in the absence of RecA. Δ*recA* Δ*recB* cells (SMR10671; purple X) are slightly less sensitive to TLD than Δ*recB* cells (SMR10665; solid orange square; $P < 0.05$). Δ*recA* (SMR10670; open gray square), parental (AB2497; solid blue diamond). Means ±SEM of three independent experiments. (C) Representative pictures of parental (AB2497) and Δ*recB* cells (SMR10665) after 12 hr of TLD. Note that although many cells or the Rec^+ parent are visible in brightfield, few display compact DAPI-stained nucleoids, whereas nearly all of the *recB* cells visible in brightfield show strings of small fragmented-looking nucleoids, which were not visible before thymine deprivation. In the merged image, DAPI (DNA stain) is blue and propidium iodide (PI, stain for dead cells) is red.

double-strand ends) (Holthausen *et al.* 2010), the RecF dependence of this death route implies that ssDNA gaps are the DNA substrate at which most of the UvrD anti-RecA anti-TLD activity is focused. We have drawn these gaps at stalled replication forks in Figure 7, A and B.

Although we found this pathway to account for only part of the hyper-TLD of $UvrD^-$ cells, others reported that hyper-TLD in a different *uvrD* mutant was totally RecA dependent (Kuong and Kuzminov 2010). Whereas we used a complete deletion (null) allele of *uvrD*, they used an allele that encodes a truncated 230 amino acid UvrD protein, which may still contain ATP binding and other activity. This, or their different growth temperature (28° as opposed to our 37°), or their slightly different minimal medium from ours may account for the different results. Whereas both labs observed the major RecA-dependent hyper-TLD pathway, we also observed an additional RecA-independent hyper-TLD pathway operative in *uvrD*-null cells.

The ΔruvABC and other death-by-recombination pathways require RecQ only when UvrD is absent

We provide evidence here that hyperaccumulation of IRIs, which occurs in Δ*uvrD* cells (Veaute *et al.* 2005; Magner *et al.* 2007; Fonville *et al.* 2010b), contributes to the hyper-TLD of Δ*uvrD* cells. That is, Δ*uvrD* cells appear to die a death-by-recombination (illustrated Figure 7B), implied by the RecQ/RecJ/RecF/RecA dependence but SOS independence of their hyper-TLD (Figures 1–4). It is therefore surprising that RecQ was not required for the hyper-TLD of Δ*ruvABC* cells (Figure 5A), which lack IRI-resolution capacity and die a RecA-dependent hyper-TLD most probably also via death-by-recombination (Fonville *et al.* 2010a). This was surprising because death-by-recombination in IRI-resolution-defective cells was shown previously to be RecQ dependent (Magner *et al.* 2007); however, in the latter study, those cells lacked UvrD. The lack of a role for RecQ in hyper-TLD by probable death-by-recombination in $RuvABC^-$ $UvrD^+$ cells (Figure 5A), despite the requirement for RecQ in death-by-recombination in cells lacking UvrD (Magner *et al.* 2007; Fonville *et al.* 2010b), including the Δ*uvrD* hyper-TLD studied here (Figure 2), can be explained by two nonmutually exclusive hypotheses.

First, the role for RecQ in death-by-recombination might be to promote the net accumulation of a specific IRI-precursor DNA substrate that is normally opposed by UvrD, and so is a minor contributor to IRI formation in $UvrD^+$ cells but a major contributor in $UvrD^-$ cells. Figure 7B shows a possible example of this. In it, in the absence of UvrD, RecQ promotes unwinding of the lagging strand of a stalled replication fork, allowing the ssDNA gap created to invade the leading-strand duplex and form an IRI, which must then be resolved. Perhaps UvrD excels at removing RecA from this particular gapped ssDNA-RecA intermediate created by RecQ/J, but is not as robust at removing RecA from RecQ/J-independent IRI precursors, such as DSEs (Figure 7D), which are processed by RecBCD and not RecQ/J. If so, DSEs might be a major source of IRIs resolved by RuvABC in $UvrD^+$ cells and so the major cause of death-by-recombination in $UvrD^+$ cells lacking RuvABC (Figure 7D). By this view, in the absence of UvrD, both RecQ/J and RecA-promoted death-by-recombination will contribute to TLD; however, in $UvrD^+$ $RuvABC^-$ cells, RecA-promoted death-by-recombination causes hyper-TLD without help from RecQ/J (as observed; Figure 5) because UvrD opposed the RecQ/J-generated IRI precursors (Figure 7A), leaving other IRI precursors (*e.g.*, DSEs; Figure 7D) to predominate instead.

Note that the general hypothesis here does not demand that the UvrD-resistant IRI precursor *is* a DSE; that is just one possibility. An alternative possibility is that UvrD strips RecA specifically from ssDNA gaps only at forks, or at fork-

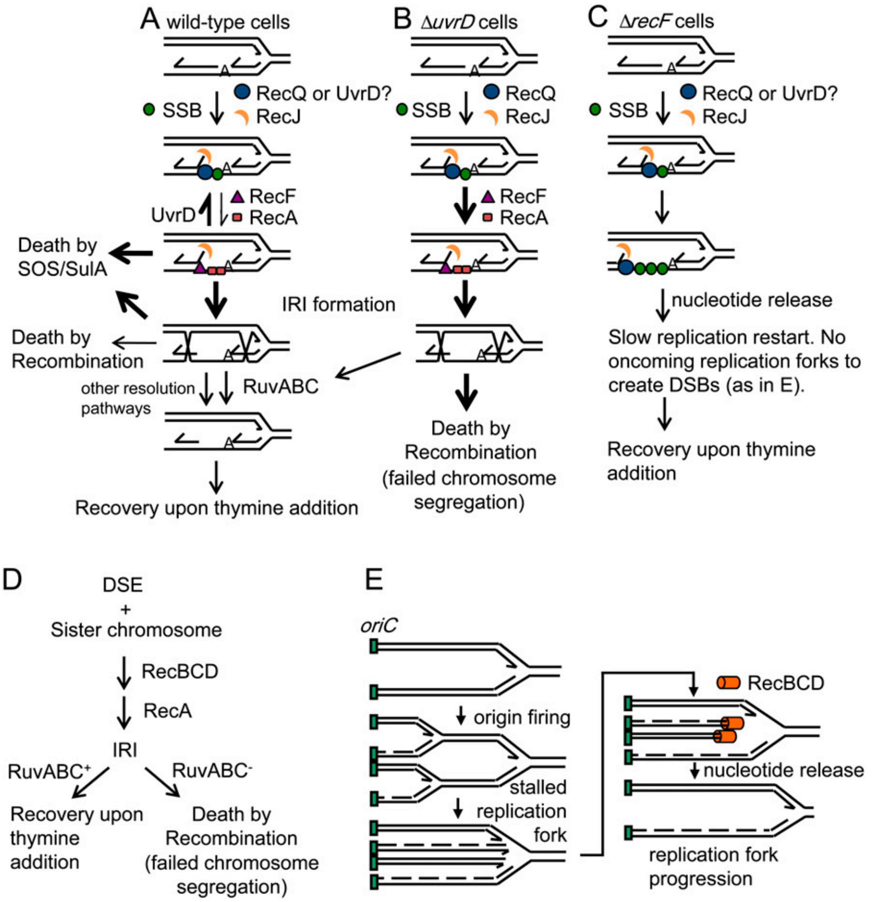

Figure 7 Models for UvrD-, RuvABC-, and RecB-promoted resistance to TLD. (A) In the presence of UvrD, its action to oppose RecA filament formation and a possible additional role in opposing the action of RecQ might maintain the majority of stalled replication forks in a manner that would allow them to recover and for replication to proceed when cells are returned to thymine, thus allowing colonies to form. UvrD could do this partly by directly or indirectly inhibiting RecQ action, in that hyper-TLD in Δ*uvrD* cells is partly RecQ dependent (Figure 3) and by removing RecA from ssDNA thus preventing death-by-recombination. The fraction of replication forks that do become entangled by IRIs require RuvABC for their resolution prior to replication fork restart. (B) In the absence of *uvrD*, RecQ and RecA are unopposed and the majority of replication forks might then be converted into IRIs, in that few can recover upon re-introduction of thymine and this death is RecA and RecQ dependent (Figures 1 and 3). Death-by-recombination might result if cell division is attempted before all the IRIs are resolved by RuvABC, resulting in tearing of the chromosomes. There is also a RecQ and RecA-independent component to TLD in Δ*uvrD* cells *via* an unknown mechanism that is not depicted. (C) Cells lacking *recF* are resistant to TLD largely due to failure to load RecA onto regions of ssDNA, thus to failure to initiate IRI formation and induce the SOS response (Fonville *et al.* 2010a). In addition, unwinding and degradation of nascent DNA at stalled replication forks in the absence of RecF might release nucleotides that could be used to prolong survival while leaving ssDNA protected by SSB, potentially explaining the RecA-independent component of RecF-dependent TLD. (D) The RecA dependence but RecQ/J independence of hyper-TLD in RuvABC$^-$ (UvrABC$^+$) cells could be explained by a death-by-recombination model in which most of the IRIs formed in UvrABC$^+$ cells and resolved by RuvABC are instigated by DSEs, such that lethal IRIs form without RecQ/J involvement. (E) Model: RecBCD resists TLD by degradation of DNA ends, releasing nucleotides that forestall TLD. This hypothesis can account for RecQ-/RecA-independent production of DSEs during TLD. DSEs might form when an oncoming replication fork collides with a stalled replication fork. The beneficial role of RecB during TLD could be degradation of these DSEs, releasing nucleotides that could be used to advance the stalled fork and, at the same time, RecBCD could prevent nonproductive recombination. Lines represent strands of DNA, arrows represent 3′-DNA ends, dashed lines represent the lagging strands of the oncoming replication forks. Green boxes represent the origin of replication. IRI, interchromosomal recombination intermediate.

lagging strands, and that other ssDNA gaps that form RecQ/J independently predominate in UvrD$^+$ cells and so cause death-by-recombination in UvrD$^+$ RuvABC$^-$ cells.

Second, the possibility that UvrD and RecQ share specific DNA substrates was suggested for *E. coli* (Lestini and Michel 2008) and observed in *Deinococcus radiodurans* (Bentchikou *et al.* 2010). Perhaps RecQ is not needed for the RecA/F/J- and SOS-dependent TLD pathway in UvrD$^+$ cells (Fonville *et al.* 2010a) because UvrD can substitute for RecQ, for example, in unwinding DNA for RecJ single-strand exonuclease activity (Figure 7A). UvrD substitution for RecQ could explain why in UvrD$^+$ cells RecA, RecF (Fonville *et al.* 2010a), and RecJ (Figure 4B) work together in a TLD pathway that does not require RecQ (Fonville *et al.* 2010a), and by contrast why RecQ *is* required for RecA/F/J-dependent death-by-recombination of either *ruv* or *recG* cells that lack UvrD (Magner *et al.* 2007; Fonville *et al.* 2010b), and RecA/F/J-dependent death-by-recombinational hyper-TLD of UvrD$^-$ cells (Figures 3 and 4). This hypothesis might seem not to explain why RecA (Fonville *et al.* 2010a) but *neither* RecQ *nor RecJ* is required for the hyper-TLD death-by-recombination pathway in RuvABC$^-$ cells that possess UvrD. If UvrD had simply substituted for RecQ, RecJ might still have been expected to be required per the model in Figure 7A, as it is in RecQ-dependent death by recombination (Magner *et al.* 2007; Fonville *et al.* 2010b) and RecQ-dependent TLD in UvrD$^+$ cells (Fonville *et al.* 2010a). However, perhaps UvrD, but not RecQ, might work with a different 5′ exonuclease that then substitutes for RecJ. Thus, either hypothesis might explain why RecQ is required for apparent death-by-recombination pathways only in the absence of UvrD.

A RecF-promoted TLD pathway independent of RecA

We found that there is a RecF-dependent but RecA-independent pathway of both the hyper-TLD in UvrD$^-$ cells (Figure 4D) and TLD in UvrD$^+$ cells (Fonville *et al.* 2010a). Although one role of RecF could be creation of double-strand

DNA ends by nonconservative (nonreciprocal) recombination (Takahashi *et al.* 1992), this process was RecA-dependent, and so cannot readily account for the RecA-independent role of RecF in TLD described here. Alternatively, RecF was suggested to stabilize stalled replication forks, protecting the nascent lagging strand from degradation and allowing efficient replication restart (Courcelle *et al.* 1997). In the absence of RecF, restart of stalled replication forks is slowed, and initiation of new rounds of replication from the origin is delayed (Rudolph *et al.* 2008). The RecF promotion of initiations from the *ori* after fork stalling might be its RecA-independent TLD-promoting role (Figure 7C), both because the new forks allow more fork-stalling TLD opportunities and because the new forks may create lethal DSEs if they hit stalled forks (Figure 7E). Additionally, the generation of large ssDNA regions in the absence of RecF might oppose TLD by releasing nucleotides including thymidine (Figure 7C).

Roles of RecJ in TLD

Surprisingly, although we confirmed our previous finding that RecJ and RecQ work in together in a pathway to promote TLD (Fonville *et al.* 2010a), we discovered that RecJ also participates in the RecA/F/SOS-dependent TLD pathway (Figure 4). These data imply that RecJ functions both with RecA/F (*e.g.*, Figure 7A) as well as with RecQ independently of RecA to promote TLD (*e.g.*, Figure 8). We suggested previously that RecJ and RecQ might promote TLD RecA independently by degrading nascent strands from stalled forks back to the *ori*, creating large ssDNA regions that could form secondary structures that lead to DSBs and promote death (Fonville *et al.* 2010a). These might explain the *ori*-specific DNA loss early in TLD observed both by FISH (Fonville *et al.* 2010a) and DNA microarrays (Sangurdekar *et al.* 2010).

How RecBCD opposes TLD

Whether hypothetical RecQ/J-promoted DSEs (Figure 8) cause DNA breakage during TLD (Fonville *et al.* 2010a) is not known. However, our data indicate that if processing such DSEs is how RecBCD avoids TLD, then RecQ is not the sole creator of those DSEs, because hyper-TLD in *recB* null cells is RecQ independent (Figure 6A). However, given the possible redundancy of UvrD and RecQ helicase activities discussed above, it could be that such DSBs are a major substrate for RecBCD during TLD, but are generated by UvrD when RecQ is absent (Figure 8). We also show that how RecBCD opposes TLD is not via its roles in RecA-promoted recombination or SOS induction because RecB$^+$ protects even RecA$^-$ cells from TLD (Figure 6B). One possible model is that the primary role of RecBCD in TLD resistance is degradation of DSEs releasing nucleotides that would otherwise be trapped in DNA and that the released nucleotides forestall eventual TLD. The DSEs could result from new replication forks colliding with stalled forks (Figure 7E), RecQ/UvrD and RecJ action on nascent lagging strands from stalled forks (Figure 8), or replication-fork regression, which occurs at stalled forks creating "chicken-foot" structures with an exposed DSE (Seigneur *et al.* 1998; not illustrated). All of these models predict the *ori*-specific DNA loss seen early in TLD in RecBCD$^+$ cells (Fonville *et al.* 2010a; Sangurdekar *et al.* 2010).

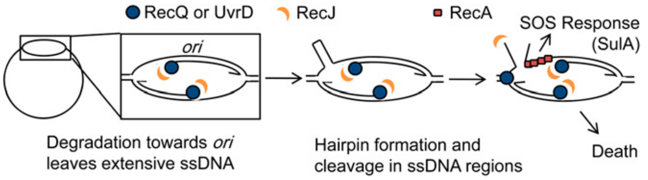

Figure 8 Model of RecQ/J- or UvrD/RecJ-mediated DNA fragmentation, a possible mechanism for the RecA-independent contribution of RecQ or UvrD to TLD. The RecQ- or UvrD-mediated unwinding of nascent DNA at stalled replication forks toward the *ori* may lead to RecA-independent DNA destruction. This might be used to restore arrested replication bubbles to the duplex state if the unwinding and RecJ-mediated degradation continues to the opposite stalled fork; however. it also creates extensive regions of ssDNA. Breakage of the DNA, shown here to occur if a hairpin forms and is cleaved by a hairpin endonuclease (but possible with other secondary structures), opens the whole chromosome up to degradation by RecQ or UvrD and RecJ, or RecBCD. Arrows represent 3′-DNA ends.

Alternatively, in cells that lack RecBCD, chicken feet formed at stalled forks persist and so are subject to endonucleolytic cleavage by RuvABC double-strand endonuclease leading to chromosome breakage (Seigneur *et al.* 1998). Such chicken-foot cleavage might cause hyper-TLD in *recB* null cells (not illustrated). This would fit with chromosome breakage seen in Δ*recBCD* cells during TLD (Kuong and Kuzminov 2010) and with the fragmented DAPI-stained nucleoids in Δ*recB* cells (Figure 6C). Other models are also possible (*e.g.*, Kuong and Kuzminov 2010).

Cancer and chemotherapies

Our findings bear on chemotherapeutic strategies. In addition to mutations in DNA replication proteins, which are associated with TLD resistance in human carcinomas (Yamao *et al.* 1993), human counterparts of the *E. coli* DNA repair and damage-response proteins could affect sensitivity importantly. Humans have several RecA homologs including RAD51, the DSB-repair function of which is disrupted in *BRCA*-defective cells (Moynahan and Jasin 2010), which underlie several cancers (Thacker 2005; Somyajit *et al.* 2010). Human BRCA2 (Jensen *et al.* 2010; Liu *et al.* 2010) and RAD50 (Koroleva *et al.* 2007) are functional analogs of *E. coli* RecF. Humans have five RecQ homologs, defects in three of which underlie cancer predisposition syndromes and any of which may be mutated in sporadic cancers (Monnat 2010). Mutations in these genes, others in their pathways, and genes of the DNA-damage response are probable or known in many cancers and might be predicted to confer TLD resistance. Screening cancers for mutations in these genes could help customize more effective chemotherapies, allowing avoidance of TLD-inducing drugs in mutant tumors that are likely to be resistant.

GEN1 and SLX1/SLX4 are human analogs of RuvABC (Ip *et al.* 2008; Fekairi *et al.* 2009) and any of several human DNA helicases may function like *E. coli* UvrD, and so might promote TLD resistance. One might also argue, given the role of the BRCA complex in DSB repair, that the BRCA complex might act more like RecBCD, promoting TLD resistance, than like RecF, promoting TLD, a point that should be tested. Inhibitors designed to target these proteins' functions might provide powerful adjuncts to TLD-inducing chemotherapies by sensitizing cells to TLD.

Identification of the proteins that cause and protect cells from TLD in bacteria, and their counterparts in humans, is likely to allow customized and improved chemotherapies.

Acknowledgments

We thank Janet Gibson for initiating construction and Ryan Frisch for PCR analysis of *recJ* mutants. N.C.F. and Z.V. thank Drs. Lei Li and Heidi Kaplan for their support during the manuscript revision and the Department of Microbiology and Molecular Genetics at the University of Texas for the use of the microscope. This work was supported by National Institutes of Health grants R01-GM64022 (P.J.H.) and R01-CA85777 (S.M.R.).

Literature Cited

Ahmad, S. I., S. H. Kirk, and A. Eisenstark, 1998 Thymine metabolism and thymineless death in prokaryotes and eukaryotes. Annu. Rev. Microbiol. 52: 591–625.

Anderson, J. A., and S. D. Barbour, 1973 Effect of thymine starvation on deoxyribonucleic acid repair systems of *Escherichia coli* K-12. J. Bacteriol. 113: 114–121.

Arnold, D. A., and S. C. Kowalczykowski, 2000 Facilitated loading of RecA protein is essential to recombination by RecBCD enzyme. J. Biol. Chem. 275: 12261–12265.

Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura *et al.*, 2006 Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2:** 2006.0008.

Bachmann, B. J., 1972 Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. 36: 525–557.

Barner, H. D., and S. S. Cohen, 1954 The induction of thymine synthesis by T2 infection of a thymine requiring mutant of Escherichia coli. J. Bacteriol. 68: 80–88.

Bentchikou, E., P. Servant, G. Coste, and S. Sommer, 2010 A major role of the RecFOR pathway in DNA double-strand-break repair through ESDSA in *Deinococcus radiodurans*. PLoS Genet. 6: e1000774.

Blattner, F. R., G. Plunkett 3rd, C. A. Bloch, N. T. Perna, V. Burland *et al.*, 1997 The complete genome sequence of *Escherichia coli* K-12. Science 277: 1453–1474.

Cirz, R. T., J. K. Chin, D. R. Andes, V. de Crecy-Lagard, W. A. Craig *et al.*, 2005 Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biol. 3: e176.

Clark, A. J., and S. J. Sandler, 1994 Homologous genetic recombination: the pieces begin to fall into place. Crit. Rev. Microbiol. 20: 125–142.

Courcelle, J., C. Carswell-Crumpton, and P. C. Hanawalt, 1997 *recF* and *recR* are required for the resumption of replication at DNA replication forks in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 94: 3714–3719.

Cummings, D. J., and A. R. Kusy, 1970 Thymineless death in *Escherichia coli*: deoxyribonucleic acid replication and the immune state. J. Bacteriol. 102: 106–117.

Datsenko, K. A., and B. L. Wanner, 2000 One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97: 6640–6645.

Dri, A. M., J. Rouviere-Yaniv, and P. L. Moreau, 1991 Inhibition of cell division in hupA hupB mutant bacteria lacking HU protein. J. Bacteriol. 173: 2852–2863.

Dutreix, M., P. L. Moreau, A. Bailone, F. Galibert, J. R. Battista *et al.*, 1989 New *recA* mutations that dissociate the various RecA protein activities in *Escherichia coli* provide evidence for an additional role for RecA protein in UV mutagenesis. J. Bacteriol. 171: 2415–2423.

Ennis, D. G., B. Fisher, S. Edmiston, and D. W. Mount, 1985 Dual role for *Escherichia coli* RecA protein in SOS mutagenesis. Proc. Natl. Acad. Sci. USA 82: 3325–3329.

Fekairi, S., S. Scaglione, C. Chahwan, E. R. Taylor, A. Tissier *et al.*, 2009 Human SLX4 is a Holliday junction resolvase subunit that binds multiple DNA repair/recombination endonucleases. Cell 138: 78–89.

Flores, M. J., N. Sanchez, and B. Michel, 2005 A fork-clearing role for UvrD. Mol. Microbiol. 57: 1664–1675.

Fonville, N. C., D. Bates, P. J. Hastings, P. C. Hanawalt, and S. M. Rosenberg, 2010a Role of RecA and the SOS Response in Thymineless Death in *Escherichia coli*. PLoS Genet. 6: e1000865.

Fonville, N. C., M. D. Blankschien, D. B. Magner, and S. M. Rosenberg, 2010b RecQ-dependent death-by-recombination in cells lacking RecG and UvrD. DNA Repair 9: 403–413.

Freifelder, D., 1969 Single-strand breaks in bacterial DNA associated with thymine starvation. J. Mol. Biol. 45: 1–7.

Friedberg, E., G. Walker, W. Siede, R. Wood, R. Schultz *et al.*, 2005 *DNA Repair and Mutagenesis*, Ed. 2. ASM Press, Washington, DC.

Holthausen, J. T., C. Wyman, and R. Kanaar, 2010 Regulation of DNA strand exchange in homologous recombination. DNA Repair 9: 1264–1272.

Howard-Flanders, P., R. P. Boyce, and L. Theriot, 1966 Three loci in *Escherichia coli* K-12 that control the excision of pyrimidine dimers and certain other mutagen products from DNA. Genetics 53: 1119–1136.

Inouye, M., 1971 Pleiotropic effect of the *recA* gene of *Escherichia coli*: uncoupling of cell division from deoxyribonucleic acid replication. J. Bacteriol. 106: 539–542.

Ip, S. C., U. Rass, M. G. Blanco, H. R. Flynn, J. M. Skehel *et al.*, 2008 Identification of Holliday junction resolvases from humans and yeast. Nature 456: 357–361.

Ivancic-Bace, I., E. Salaj-Smic, and K. Brcic-Kostic, 2005 Effects of *recJ*, *recQ*, and *recFOR* mutations on recombination in nuclease-deficient *recB recD* double mutants of *Escherichia coli*. J. Bacteriol. 187: 1350–1356.

Jensen, R. B., A. Carreira, and S. C. Kowalczykowski, 2010 Purified human BRCA2 stimulates RAD51-mediated recombination. Nature 467: 678–683.

Koroleva, O., N. Makharashvili, C. T. Courcelle, J. Courcelle, and S. Korolev, 2007 Structural conservation of RecF and Rad50: implications for DNA recognition and RecF function. EMBO J. 26: 867–877.

Kuong, K. J., and A. Kuzminov, 2010 Stalled replication fork repair and misrepair during thymineless death in *Escherichia coli*. Genes Cells 15: 619–634.

Lestini, R., and B. Michel, 2008 UvrD and UvrD252 counteract RecQ, RecJ, and RecFOR in a rep mutant of *Escherichia coli*. J. Bacteriol. 190: 5995–6001.

Li, G. M., 2008 Mechanisms and functions of DNA mismatch repair. Cell Res. 18: 85–98.

Liu, J., T. Doty, B. Gibson, and W. D. Heyer, 2010 Human BRCA2 protein promotes RAD51 filament formation on RPA-covered single-stranded DNA. Nat. Struct. Mol. Biol. 17: 1260–1262.

Lopez, C. R., S. Yang, R. W. Deibler, S. A. Ray, J. M. Pennington *et al.*, 2005 A role for topoisomerase III in a recombination pathway alternative to RuvABC. Mol. Microbiol. 58: 80–101.

Madiraju, M. V., P. E. Lavery, S. C. Kowalczykowski, and A. J. Clark, 1992 Enzymatic properties of the RecA803 protein, a partial suppressor of recF mutations. Biochemistry 31: 10529–10535.

Magner, D. B., M. D. Blankschien, J. A. Lee, J. M. Pennington, J. R. Lupski *et al.*, 2007 RecQ promotes toxic recombination in cells lacking recombination intermediate-removal proteins. Mol. Cell 26: 273–286.

McGuire, J. J., 2003 Anticancer antifolates: current status and future directions. Curr. Pharm. Des. 9: 2593–2613.

McPartland, A., L. Green, and H. Echols, 1980 Control of *recA* gene RNA in *E. coli*: regulatory and signal genes. Cell 20: 731–737.

Miller, J., 1972 *Generalized Transduction: Use of P1 in Strain Construction*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Monnat, R. J., Jr., 2010 Human RECQ helicases: roles in DNA metabolism, mutagenesis and cancer biology. Semin. Cancer Biol. 20: 329–339.

Morganroth, P. A., and P. C. Hanawalt, 2006 Role of DNA replication and repair in thymineless death in *Escherichia coli*. J. Bacteriol. 188: 5286–5288.

Moynahan, M. E., and M. Jasin, 2010 Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. Nat. Rev. Mol. Cell Biol. 11: 196–207.

Nakayama, H., and P. Hanawalt, 1975 Sedimentation analysis of deoxyribonucleic acid from thymine-starved *Escherichia coli*. J. Bacteriol. 121: 537–547.

Nakayama, H., K. Nakayama, R. Nakayama, and Y. Nakayama 1982 Recombination-deficient mutations and thymineless death in *Escherichia coli* K12: reciprocal effects of *recBC* and *recF* and indifference of *recA* mutations. Can. J. Microbiol. 28: 425–430.

Nakayama, H., K. Nakayama, R. Nakayama, N. Irino, Y. Nakayama *et al.*, 1984 Isolation and genetic characterization of a thymineless death-resistant mutant of *Escherichia coli* K12: identification of a new mutation (*recQ1*) that blocks the RecF recombination pathway. Mol. Gen. Genet. 195: 474–480.

Nakayama, K., S. Shiota, and H. Nakayama, 1988 Thymineless death in *Escherichia coli* mutants deficient in the RecF recombination pathway. Can. J. Microbiol. 34: 905–907.

Nakayama, K., K. Kusano, N. Irino, and H. Nakayama, 1994 Thymine starvation-induced structural changes in *Escherichia coli* DNA: detection by pulsed field gel electrophoresis and evidence for involvement of homologous recombination. J. Mol. Biol. 243: 611–620.

Pennington, J. M., and S. M. Rosenberg, 2007 Spontaneous DNA breakage in single living *Escherichia coli* cells. Nat. Genet. 39: 797–802.

Rudolph, C. J., A. L. Upton, and R. G. Lloyd, 2008 Maintaining replication fork integrity in UV-irradiated *Escherichia coli* cells. DNA Repair 7: 1589–1602.

SaiSree, L., M. Reddy, and J. Gowrishankar, 2000 *lon* incompatibility associated with mutations causing SOS induction: null *uvrD* alleles induce an SOS response in *Escherichia coli*. J. Bacteriol. **182:** 3151–3157.

Sancar, A., 1996 DNA excision repair. Annu. Rev. Biochem. 65: 43–81.

Sangurdekar, D. P., B. L. Hamann, D. Smirnov, F. Srienc, P. C. Hanawalt *et al.*, 2010 Thymineless death is associated with loss of essential genetic information from the replication origin. Mol. Microbiol.

Seigneur, M., V. Bidnenko, S. D. Ehrlich, and B. Michel, 1998 RuvAB acts at arrested replication forks. Cell 95: 419–430.

Siegal, E., 1973 Ultraviolet-sensitive mutator strain of *Escherichia coli* K-12. J. Bacteriol. 113: 145–160.

Slack, A., P. C. Thornton, D. B. Magner, S. M. Rosenberg, and P. J. Hastings, 2006 On the mechanism of gene amplification induced under stress in *Escherichia coli*. PLoS Genet. 2: e48.

Somyajit, K., S. Subramanya, and G. Nagaraju, 2010 RAD51C: a novel cancer susceptibility gene is linked to Fanconi anemia and breast cancer. Carcinogenesis 31: 2031–2038.

Takahashi, N. K., K. Yamamoto, Y. Kitamura, S. Q. Luo, H. Yoshikura *et al.*, 1992 Nonconservative recombination in Escherichia coli. Proc. Natl. Acad. Sci. USA 89: 5912–5916.

Takemura, Y., and A. L. Jackman, 1997 Folate-based thymidylate synthase inhibitors in cancer chemotherapy. Anticancer Drugs 8: 3–16.

Thacker, J., 2005 The RAD51 gene family, genetic instability and cancer. Cancer Lett. 219: 125–135.

Veaute, X., S. Delmas, M. Selva, J. Jeusset, E. Le Cam *et al.*, 2005 UvrD helicase, unlike Rep helicase, dismantles RecA nucleoprotein filaments in *Escherichia coli*. EMBO J. 24: 180–189.

Vlasic, I., I. Ivancic-Bace, M. Imesek, B. Mihaljevic, and K. Brcic-Kostic, 2008 RecJ nuclease is required for SOS induction after introduction of a double-strand break in a RecA loading deficient recB mutant of *Escherichia coli*. Biochimie 90: 1347–1355.

Volkert, M. R., and M. A. Hartke, 1987 Effects of the *Escherichia coli recF* suppressor mutation, *recA801*, on *recF*-dependent DNA-repair associated phenomena. Mutat. Res. 184: 181–186.

Yamao, F., Y. Nagai, S. Kaneda, S. Yoshida, and T. Seno, 1993 Conditional resistance to thymineless death predominantly selects DNA synthesis-deficient mutants of mammalian cells. Mutat. Res. 289: 83–89.

Communicating editor: D. Leach

GENETICS

Supporting Information
http://www.genetics.org/content/suppl/2011/06/24/genetics.111.130161.DC1

Pathways of Resistance to Thymineless Death in *Escherichia coli* and the Function of UvrD

Natalie C. Fonville, Zalman Vaksman, Jessica DeNapoli, P. J. Hastings, and Susan M. Rosenberg

DOI: 10.1534/genetics.111.130161

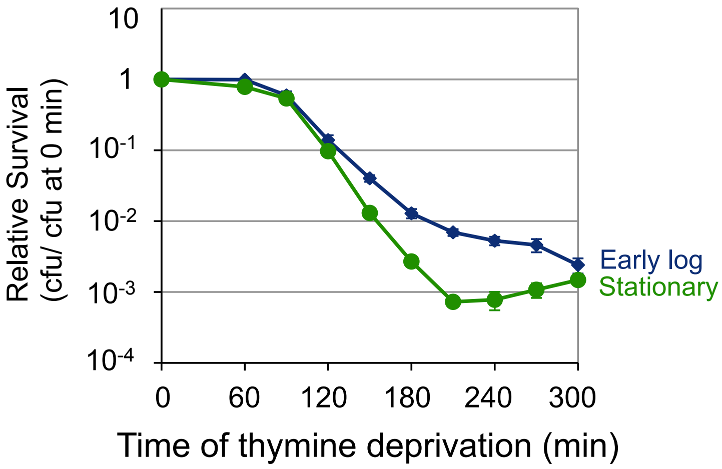

Figure S1 Stationary-phase cells undergo TLD when placed at low density. Stationary-phase AB2497 cells were diluted (1:20) and allowed to exit stationary phase for 1 hour (early-log) or diluted 1:10 and immediately assayed for viability (stationary phase.) The dilution was adjusted so that the stationary and early-log cells were at a similar density at 0 min for the viability assay. Mean ± SEM of 3 independent cultures.