Folate stress induces SLX1- and RAD51-dependent mitotic DNA synthesis at the fragile X locus in human cells

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Folate deprivation drives the instability of a group of rare fragile sites (RFSs) characterized by CGG trinucleotide repeat (TNR) sequences. Pathological expansion of the TNR within the *FRAXA* locus perturbs DNA replication and is the major causative factor for fragile X syndrome, a sex-linked disorder associated with cognitive impairment. Although folate-sensitive RFSs share many features with common fragile sites (CFSs; which are found in all individuals), they are induced by different stresses and share no sequence similarity. It is known that a pathway (termed MiDAS) is employed to complete the replication of CFSs in early mitosis. This process requires RAD52 and is implicated in generating translocations and copy number changes at CFSs in cancers. However, it is unclear whether RFSs also utilize MiDAS and to what extent the fragility of CFSs and RFSs arises by shared or distinct mechanisms. Here, we demonstrate that MiDAS does occur at *FRAXA* following folate deprivation but proceeds via a pathway that shows some mechanistic differences from that at CFSs, being dependent on RAD51, SLX1, and POLD3. A failure to complete MiDAS at *FRAXA* leads to severe locus instability and missegregation in mitosis. We propose that break-induced DNA replication is required for the replication of *FRAXA* under folate stress and define a cellular function for human SLX1. These findings provide insights into how folate deprivation drives instability in the human genome.

MiDAS | structure-specific endonucleases | chromosome fragile sites | homologous recombination | break-induced DNA replication (BIR)

Folate is a B type vitamin that functions as a carrier for one-carbon units, which are essential for DNA and RNA synthesis. Humans cannot synthesize folate and, therefore, rely on dietary sources of this nutrient. In human populations in which folic acid supplementation is absent, folate deficiency is observed frequently (1–4). Because of the requirement for folate in the synthesis of nucleotides, folate deficiency can destabilize the human genome through influencing the fidelity of DNA replication. In particular, it is established that a subgroup of so-called rare fragile sites (RFSs), which are found in less than 5% of the human population, are highly sensitive to folate deprivation. These folate-sensitive RFSs generally encompass CGG trinucleotide repeat sequences, which are prone to expand in length via a mechanism that remains to be fully elucidated. Most intriguingly, when these CGG repeats expand beyond a certain length, the locus exhibits fragility in metaphase when cells are challenged with "folate stress" conditions, such as when cells are deprived of folate or exposed to the thymidylate synthase inhibitor, fluorodeoxyuridine (FdU) (5). It is well-established that, when the copy number of the TNR sequences expands beyond a critical size, the development of specific neurological diseases such as fragile X syndrome (FXS) can be triggered (6–9). The genomic locus associated with FXS, *FRAXA*, contains unstable CGG repeats located at the 5′-untranslated region (5′-UTR) of the *FMR1* gene. In the general population, the *FRAXA*-associated TNR consists of less than 55 CGG repeats, while the mutant form of this locus has over 200 repeats. Abnormal expansion of this TNR can lead to hypermethylation and epigenetic silencing of *FMR1*, which leads to the development of FXS (10). To our knowledge, 24 folate-sensitive RFSs have been identified (5) but only 6 of them (including *FRAXA*) have been characterized for their precise genomic location (11).

RFSs appear on metaphase chromosomes as gaps or breaks in otherwise fully condensed chromatin (called RFS "expression"). Much of our understanding of the underlying cause of chromosome fragile site expression has been derived from studies of another class of fragile sites that exist in all individuals, known as common fragile sites (CFSs). It is generally considered that CFS

Significance

Folate deficiency is associated with multiple disorders in humans. Through the analysis of the fragile X syndrome locus (*FRAXA*) in immortalized human lymphocytes or fibroblasts, we demonstrate that *FRAXA* undergoes DNA synthesis in mitosis (MiDAS). We demonstrate that this process occurs via break-induced DNA replication and requires the SLX1/SLX4 endonuclease complex, the RAD51 recombinase and POLD3, a subunit of polymerase delta. We also demonstrate that other loci undergo MiDAS upon folate stress. This study reveals a function of human SLX1 in the maintenance of *FRAXA* stability and provides evidence that, in addition to *FRAXA*, MiDAS occurs at other loci following folate deprivation. These findings provide insight into the diverse and detrimental consequences of folate deficiency in human cells.

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expression results from a localized failure to undergo adequate compaction of the locus during early mitosis due to incomplete DNA replication during interphase (12). CFSs are recognized as being a key driver of genome instability in cancer cells (13, 14). In cultured cells, the fragility of CFSs can be strongly enhanced if the cells experience DNA replication stress, such as traversing S phase in the presence of a low dose of the DNA polymerase inhibitor, aphidicolin (APH). In human cancers, however, this stress is widespread and intrinsic, because it is driven by the action of activated oncogenes. In response to replication stress, human cells activate a signaling pathway that leads to the localization of DNA repair proteins, including the SLX4-associated, structure-specific endonuclease (SSE), MUS81-EME1 to CFS loci (15–17). This, in turn, facilitates the completion of DNA replication at CFSs only after cells have entered early mitosis, using a form of unscheduled DNA synthesis known as "MiDAS" (mitotic DNA synthesis) (18, 19). Mechanistically, MiDAS likely represents a form of break-induced DNA replication (BIR), which is a subpathway of homologous recombination repair acting at sites of broken/collapsed DNA replication forks (20). BIR has largely been characterized in budding yeast, where it has been shown to be a conservative form of DNA synthesis dependent on the Rad51 recombinase and Pol32 (21, 22). At CFSs, MiDAS is apparently triggered during the onset of chromatin condensation in the mitotic prophase and is facilitated not only by MUS81-EME1, but also by POLD3, the human homolog of yeast Pol32 (18, 19). Curiously, however, MiDAS at CFSs does not require RAD51, but instead utilizes RAD52, in a pathway that might be analogous to a poorly characterized, Rad52-dependent, BIR subpathway defined in yeast (21, 22). Moreover, a very similar MiDAS process also occurs at telomeres following replication stress (23, 24). If the MiDAS pathway is not activated or the levels of replication stress are excessive, cells display chromosomal abnormalities later in mitosis, such as the formation of ultrafine anaphase DNA bridges (UFBs) and lagging chromatin in anaphase/telophase (18, 25).

We analyzed previously the segregation of mutant *FRAXA* loci in mitosis when cells are cultured under "folate stress" conditions (26). That study indicated that folate stress promotes mitotic abnormalities similar to those observed at CFSs, including an increased frequency of chromatin bridges and UFBs. However, one striking difference from CFSs is that the UFBs associated with *FRAXA* are almost exclusively RPA-coated (and therefore composed of single-stranded DNA), while those arising from CFSs under APH conditions are PICH-coated double-stranded DNA UFBs (27). This indicates that homologous recombination could play a role in processing *FRAXA* under folate stress conditions, since RPA-coated UFBs have been suggested to represent unresolved HR intermediates (28). Moreover, cells expressing mutant *FRAXA* show a strikingly high frequency of missegregation. Around 50% of the *FRAXA* loci form lagging DNA associated with a UFB, which represents a much higher percentage of missegregation than is seen at any CFS locus studied thus far (29). Based on these considerations, we postulated that folate stress might have a different (and more detrimental) effect on mutant *FRAXA* than is seen at CFSs exposed to APH-induced replication stress. In this article, we report that MiDAS also occurs at fragile *FRAXA* loci during folate stress, but that the pathway utilized differs in some respects from that characterized previously at CFSs. Our data demonstrate that completion of DNA replication at *FRAXA* in early mitosis requires the SLX1 endonuclease and likely proceeds via a form of RAD51-dependent and POLD3-dependent BIR.

Results

MiDAS Occurs at *FRAXA* under Folate Stress Conditions. To analyze the effect of folate stress on the stability of a RFS in human cells, we used three lymphocyte cell lines. These were GM06865 with a normal *FRAXA* allele; GM06891 with a moderately expanded, premutation allele; and GM09237 with a severely expanded, full mutation allele. In addition, analyses requiring small interfering RNA (siRNA)-mediated protein depletion were performed on an hTERT-immortalized fibroblast cell line (GM05848) that has a full mutation *FRAXA* allele (*SI Appendix*, Fig. S1).

We first analyzed whether folate stress can induce MiDAS at the *FRAXA* locus or elsewhere in the human genome. For this, we treated GM06865, GM06891, and GM09237 cells with 0.5 μM FdU for 17 h, arrested them at the G_2/M phase boundary with the CDK1 inhibitor, RO3306, and then released the cells into mitosis in the presence of the thymidine analog, EdU, to mark sites of new DNA synthesis. EdU detection was then performed on metaphase chromosome spreads (18, 30) (Fig. 1*A*). We observed that a small number of EdU foci (typically up to three) could be detected in all cell lines in the absence of folate stress, but that exposure to FdU induced a significantly higher number of EdU foci (Fig. 1 *B* and *C*). This suggested that several loci in the human genome can be affected by folate stress. Interestingly, the vast majority (73–94% on average depending on the cell line) of the EdU foci detected were located on only one sister-chromatid (Fig. 1 *B* and *D*), which is a much higher percentage than that observed at CFSs in cells treated with APH (19). These data indicate that the majority of folate stress-induced MiDAS events occur via a conservative form of DNA synthesis, which has been proposed previously to be mediated via BIR (18, 19). Of most relevance to the current study, when combining EdU detection with fluorescence in situ hybridization (FISH) using a probe targeting *FRAXA* (Fig. 1*A*), we confirmed that MiDAS occurs at *FRAXA* in the GM09237 cell line that has a full mutation *FRAXA* allele (Fig. 1 *E* and *F*). Moreover, MiDAS was highly associated with *FRAXA* fragility, as all of the EdU foci detected were localized at loci that were visibly fragile (Fig. 1*G*). Nevertheless, not all fragile *FRAXA* loci displayed EdU incorporation (~35%; Fig. 1*F*), a frequency that is comparable to that observed at the CFS, *FRA16D,* following APH treatment in GM05848 mutant *FRAXA* cell line (*SI Appendix*, Fig. S2). Importantly, we failed to detect EdU incorporation at a measurable frequency at *FRAXA* in GM06891 cells that have a premutation *FRAXA* allele (118 CGG repeats) (Fig. 1*F*).

It was shown previously that folate stress can induce the expression of some CFSs, although at a very low frequency (31, 32). To address whether folate stress might induce MiDAS at CFSs in GM09237 cells, we examined whether EdU incorporation could occur at either of two widely studied CFSs, *FRA16D* and *FRA3B* (14). We observed that folate stress did not induce EdU incorporation at *FRA16D* or *FRA3B* (Fig. 1*H*). These data are consistent with our previous finding that folate stress does not induce the fragility at CFSs (26). Taken together, these results indicate that, in response to folate stress, MiDAS can occur at the fully expanded *FRAXA* locus and at a small number of other loci in human genome that remain to be identified.

SLX1/4 Plays a Role in Fragility and MiDAS at *FRAXA*. Next, we sought to understand why there was a high incidence of EdU foci on only one sister-chromatid at *FRAXA*. Because this pattern was strongly suggestive of a conservative, BIR-like form of DNA synthesis, we first analyzed known BIR/MiDAS factors for their role in inducing fragility at *FRAXA* under folate stress conditions. First, we analyzed the MUS81-EME1 SSE, which is known to affect both fragility and the frequency of MiDAS at CFSs. We depleted MUS81 using a validated short hairpin RNA (17) and then treated cells with FdU before harvesting metaphase chromosomes for analysis of fragility (*SI Appendix*, Fig. S3 *A* and *B*). We observed that MUS81 depletion did not affect the frequency of fragility at *FRAXA* (*SI Appendix*, Fig. S3 *C* and *D*). Given the apparent lack of a role for the MUS81-EME1 endonuclease at the locus, we considered the possibility that fragility at *FRAXA* might be mediated by a pathway utilizing SSEs other than MUS81. We therefore analyzed whether SLX4, the scaffold protein that is known to bind to and regulate the activity of three

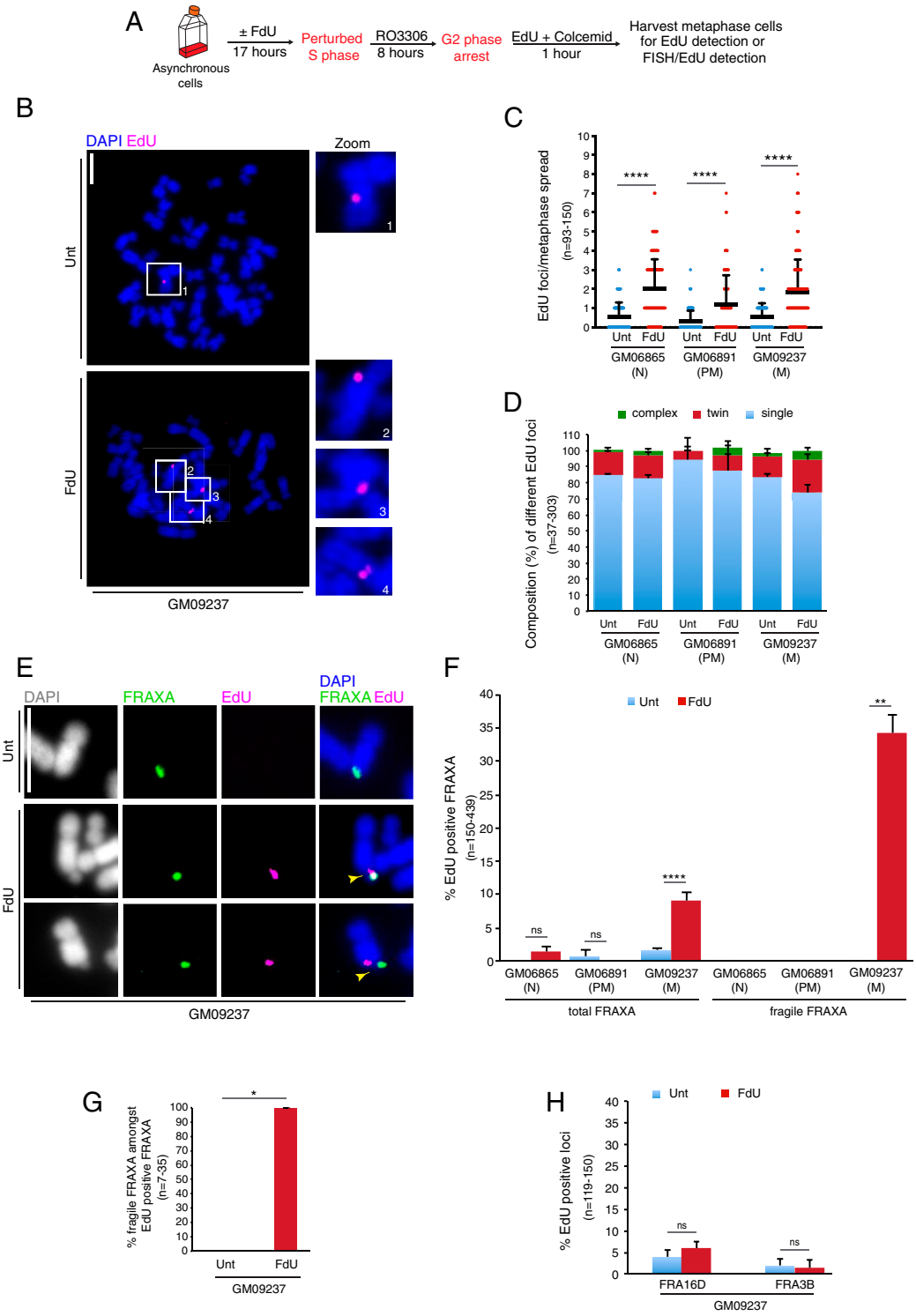

Fig. 1. MiDAS occurs at *FRAXA* in response to folate stress. (*A*) Experimental workflow for the analysis of EdU incorporation in metaphase chromosomes following FdU treatment in GM06865, GM06891, and GM09237 cells. The location of *FRAXA* was validated using a FISH probe. (*B*) Representative images of EdU foci (magenta) on metaphase chromosomes. Zoomed images (*Right*) are arranged according to the numbers in the boxed areas. (*C*) Quantification of EdU foci in metaphase chromosomes. (*D*) Quantification of EdU foci located on either a single sister-chromatid (single) or both sister-chromatids (twin) in cell lines containing a normal *FRAXA* allele (N), a premutation *FRAXA* allele (PM), or a full mutation *FRAXA* allele (M). In some cases, the pattern of EdU staining was more difficult to assign as it produced a complex pattern involving one/both sister-chromatids (complex). There were no statistically significant differences among the two patterns of EdU foci in the three cell lines analyzed. (*E*) Representative images of EdU foci (magenta) located at *FRAXA* (green) on metaphase chromosomes. Yellow arrowheads indicate examples of fragile *FRAXA* loci. (*F*) Quantification of EdU foci colocalized with *FRAXA*. (*G*) Quantification of EdU foci that are located at fragile *FRAXA*. (*H*) Quantification of EdU foci colocalized with *FRA16D* and *FRA3B*. M, full mutation *FRAXA* allele; N, normal *FRAXA* allele; PM, premutation *FRAXA* allele; Unt, untreated. (Scale bar, 5 μm.) Error bars represent SDs from at least three independent experiments. ns, not significant; $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$.

SSEs (MUS81-EME1, XPF-ERCC1, and SLX1; ref. 33), might play a role at *FRAXA*. In these experiments, we used the GM05848 fibroblast cell line to increase the efficiency of siRNA-mediated protein depletion (*SI Appendix*, Fig. S1). We observed that SLX4 depletion strongly reduced the incidence of fragility at *FRAXA* (Fig. 2 *A–E*). We therefore investigated whether SLX4-associated SSEs other than MUS81 might be required for *FRAXA* fragility. We observed that depletion of SLX1, but not XPF, strongly reduced the frequency of fragility at *FRAXA* following FdU treatment (Fig. 2 *B–E*). It is known that SLX1 needs to be bound to SLX4 to be active as a nuclease (34) and, therefore, to exclude the possibility that SLX1 depletion led to a destabilization of the SLX4 protein, as has been reported by others (35), we quantified the level of SLX4 in each experiment. We observed that depletion of SLX1 did not significantly affect the level of SLX4 in the GM05848 cell line (Fig. 2*C*). Collectively, these data suggest that, although very similar in cytological appearance, the mechanism by which fragility arises at *FRAXA* and at CFSs is mechanistically different.

At CFSs, it has been proposed that fragility is a consequence of a lack of chromatin compaction at loci still undergoing DNA synthesis in mitosis (18). Hence, we assessed whether MiDAS at *FRAXA* was affected by the depletion of SLX1 and SLX4. We observed that depletion of either SLX1 or SLX4 led to a dramatic reduction in the frequency of MiDAS at *FRAXA* (Fig. 2 *F–L*). Consistent with the data on fragility discussed above, we confirmed that MUS81 is not required for MiDAS at this locus (*SI Appendix*, Fig. S3 *E–H*). We conclude, therefore, that the appearance of fragility and MiDAS at the *FRAXA* locus requires the SLX1/SLX4 endonuclease.

Next, we sought to confirm that SLX1 and SLX4 were actively recruited to *FRAXA* following FdU treatment in mitosis using a protocol combining immunofluorescence and FISH (Fig. 3*A*). For this, we used two cell lines with the full mutation *FRAXA* allele (GM05848 and GM09237) (*SI Appendix*, Fig. S1). We observed that, following FdU treatment, both SLX1 and SLX4 were recruited to the *FRAXA* locus (Fig. 3 *B*, *C*, *E*, and *F*). In the GM05848 cells, a significant proportion of the fragile *FRAXA* loci were marked by SLX1 (>35%) or SLX4 (>45%) (Fig. 3 *D* and *G*). In addition, SLX4 was recruited to a higher percentage (70%) of fragile *FRAXA* loci in GM09237 cells, which might reflect the fact that the number of CGG repeats is higher in this cell line than in the GM05848 cells (Fig. 3*G*). We also observed that the recruitment of SLX4 to *FRAXA* was not affected by depletion of SLX1 (*SI Appendix*, Fig. S4), which further demonstrates that the SLX4 protein is not significantly destabilized upon SLX1 depletion in our system.

MiDAS at *FRAXA* Is Promoted by RAD51. Previous studies have indicated that CFS-associated MiDAS requires RAD52 and replicative polymerases, but not RAD51 (18, 19). Rather, depletion of RAD51 leads to an increase in the frequency of MiDAS at CFSs, suggesting that RAD51 plays a role in preventing replication fork perturbation within CFSs when cells experience replication stress (19). To investigate whether RAD52 and replicative polymerases might play a role in MiDAS at *FRAXA* following folate stress, we first sought to confirm that these factors were required for CFS MiDAS under APH stress in the mutant *FRAXA* cell line, GM05848. For this, we examined the commonly observed CFS locus, *FRA16D* (*SI Appendix*, Fig. S2), in cells treated with either a RAD52 or a RAD51 inhibitor in late G_2 phase/early mitosis, or a high dose of APH in mitosis to inhibit replicative DNA polymerases (36) (*SI Appendix*, Fig. S2*A*). Consistent with previous findings, we observed that MiDAS at *FRA16D* was strongly reduced in cells exposed to high-dose APH or the RAD52 inhibitor, but not by RAD51 inhibition (*SI Appendix*, Fig. S2 *B* and *D*). We then analyzed the effect of FdU-induced folate stress in this cell line (Fig. 4*A*). We observed that the frequency of both fragility and MiDAS at *FRAXA* was markedly reduced by high-dose APH in mitosis (Fig. 4 *B* and *C*), as is seen at CFSs. This result was further confirmed by depletion of POLD3, a noncatalytic subunit of DNA polymerase delta (37), which led to a significantly reduced frequency of fragility at *FRAXA* and a dramatic decrease in the frequency of MiDAS (*SI Appendix*, Fig. S5 *A–D*). However, there was a striking difference in the requirement for homologous recombination factors at *FRAXA* compared to *FRA16D*, in that both fragility and MiDAS at *FRAXA* required RAD51, but not RAD52 (Fig. 4 *B* and *C*). We confirmed these findings obtained using chemical inhibitors by conducting siRNA-mediated depletion of either RAD52 or RAD51 (*SI Appendix*, Fig. S5 *E–J*). Consistent with these results, we confirmed that RAD51 is recruited to a significant fraction of all *FRAXA* loci following FdU treatment, and to ∼40% of the visibly fragile *FRAXA* loci (Fig. 4 *D–G*). Taken together, these data indicate that MiDAS at *FRAXA* occurs via a BIR-like process dependent on POLD3 and RAD51 (and not RAD52 as at CFSs).

RAD51 and SLX1/4 Are Recruited Independently of *FRAXA* in Mitosis. Next, we set to determine whether RAD51 and SLX1/4 are recruited independently or not to *FRAXA* in mitosis under folate stress conditions. For this, we depleted SLX1 or SLX4, treated the cells with FdU, and then harvested metaphase cells for immunofluorescence (IF)-FISH analysis (*SI Appendix*, Fig. S6). We observed that neither SLX1 nor SLX4 was required for RAD51 loading onto *FRAXA* (*SI Appendix*, Fig. S6 *A–F*). We then addressed whether RAD51 might be required for SLX1/4 loading onto *FRAXA*, but again this was not the case (*SI Appendix*, Fig. S6 *G–I*). These data indicate that the mitotic recruitment of RAD51 and SLX1/4 to *FRAXA* occurs via independent mechanisms.

MiDAS Inhibition Exacerbates *FRAXA* Mitotic Missegregation. We also analyzed whether inhibition of MiDAS might affect the segregation of *FRAXA* following folate stress. For this, we depleted either SLX1 or inhibited RAD51 in the late G_2 phase/early mitosis, before harvesting mitotic cells for anaphase/telophase analysis by FISH (Fig. 5 *A–C* and *F*). We observed that impairment of either SLX1 or RAD51 function led to a significant increase in *FRAXA* missegregation (Fig. 5 *D*, *E*, and *G*). These data suggest that MiDAS plays a critical role in preventing *FRAXA* missegregation in mitosis.

Discussion

MiDAS is proposed to be a salvage pathway that is employed by cells to ensure that genomic regions that remain underreplicated at the end of the S/G_2 phases are duplicated in mitosis (18, 38). Previous studies have indicated that several fragile loci (including CFSs and telomeres) are "hotspots" for MiDAS, particularly in cancer cells experiencing replication stress due to oncogene activation (18, 19, 23, 24). In this study, we addressed whether MiDAS was utilized by noncancer cells to rescue the replication of a fully expanded *FRAXA* locus under folate stress conditions. Our data revealed that MiDAS does occur in these noncancer cells even when they are not exposed to FdU or lack an abnormally expanded CGG repeat sequence at *FRAXA*. Nevertheless, the frequency of MiDAS is strongly elevated when these cells are treated with FdU, and MiDAS is also clearly evident at the *FRAXA* locus with a full mutation allele. These observations are reminiscent of the MiDAS that occurs at CFSs, which is also evident in noncancer cells, albeit at a very low frequency, and indicate that MiDAS is a universal rescue pathway used by cells experiencing different forms of replication stress.

A second similarity to the MiDAS occurring at CFSs is that most of the FdU-induced DNA synthesis detected is confined to only one of the two sister-chromatids, reflecting the fact that MiDAS frequently occurs via a conservative form of DNA synthesis. However, we found no evidence for a role RAD52 at

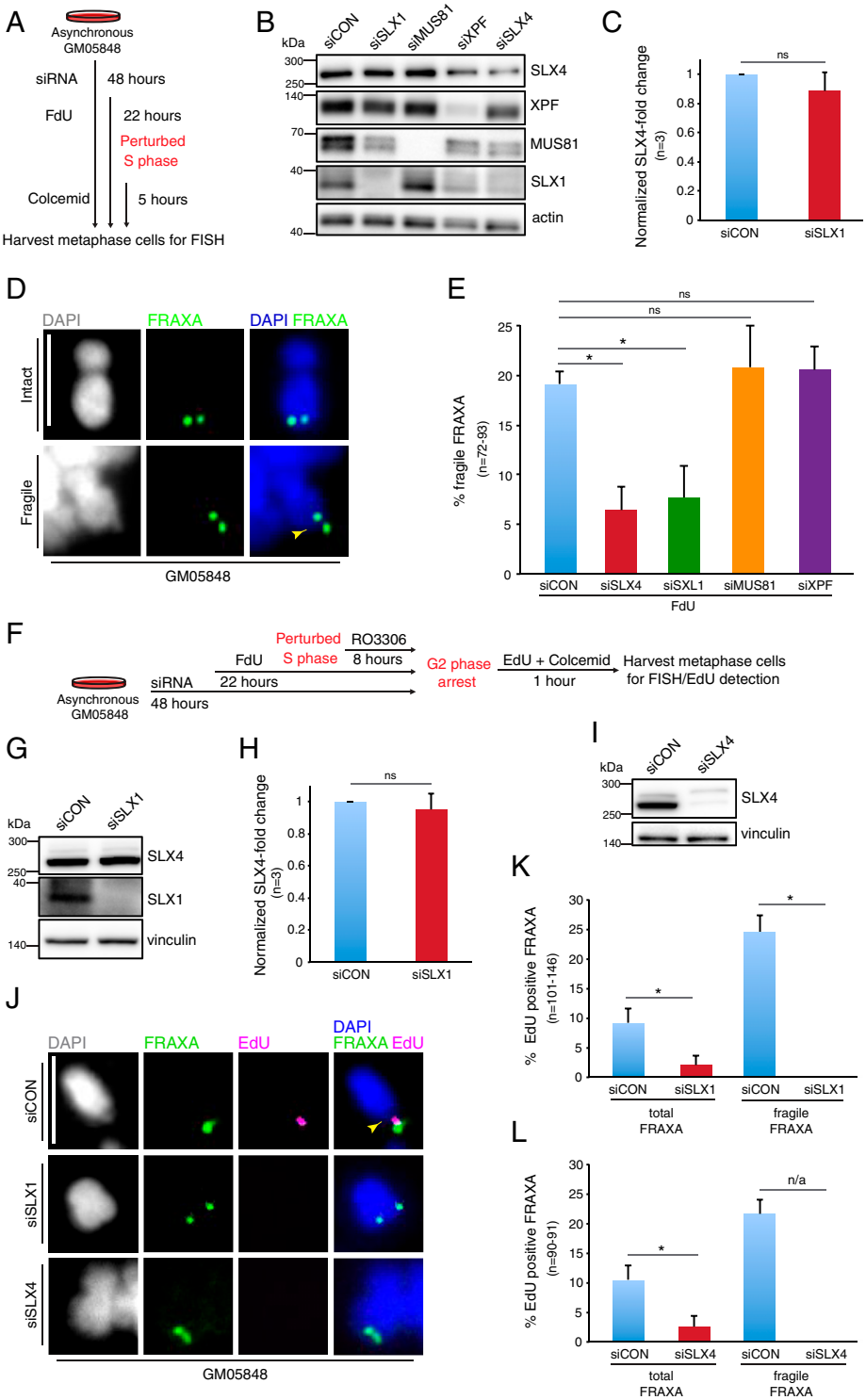

Fig. 2. SLX1 and SLX4 promote *FRAXA* fragility and are required for MiDAS at *FRAXA* in response to folate stress. (*A*) Experimental workflow for the analysis of fragility at *FRAXA* on metaphase chromosomes following siRNA silencing of genes of interest in GM05848 cells. The location of *FRAXA* was validated using a FISH probe. (*B*) Western blot analysis of the SLX4, XPF, MUS81, and SLX1 proteins following siRNA-mediated depletion. Actin was used as a loading control. (*C*) The relative expression of SLX4 following SLX1 depletion. Data show the average of three independent analyses. (*D*) Representative images of the *FRAXA* locus (green) on metaphase chromosomes in GM05848 cells. The yellow arrowhead indicates an example of a fragile *FRAXA* locus. (*E*) Quantification of fragility at *FRAXA* following the depletion of proteins of interest. (*F*) Experimental workflow for the analysis of EdU incorporation on metaphase chromosomes following siRNA silencing of either SLX1 or SLX4 in GM05848 cells. The location of *FRAXA* was validated using a FISH probe. (*G*) Western blot analysis of SLX1 and SLX4 proteins following siRNA silencing. Vinculin was used as a loading control. (*H*) The relative expression of SLX4 following SLX1 depletion in three independent experiments. (*I*) Western blot analysis of SLX4 protein following siRNA silencing. (*J*) Representative images of EdU (magenta) colocalized with *FRAXA* (green) in GM05848 cells. The yellow arrowhead indicates an example of a fragile *FRAXA* locus. (*K*) Quantification of EdU foci colocalized with *FRAXA* following SLX1 depletion. (*L*) Quantification of EdU foci colocalized with *FRAXA* following SLX4 depletion. siCON, control siRNA. (Scale bar, 5 μm.) Error bars represent SDs from at least three independent experiments. n/a, statistical analysis was not possible due to insufficient number of fragile *FRAXA* in "siSLX4" sample; ns, not significant; $*P < 0.05$.

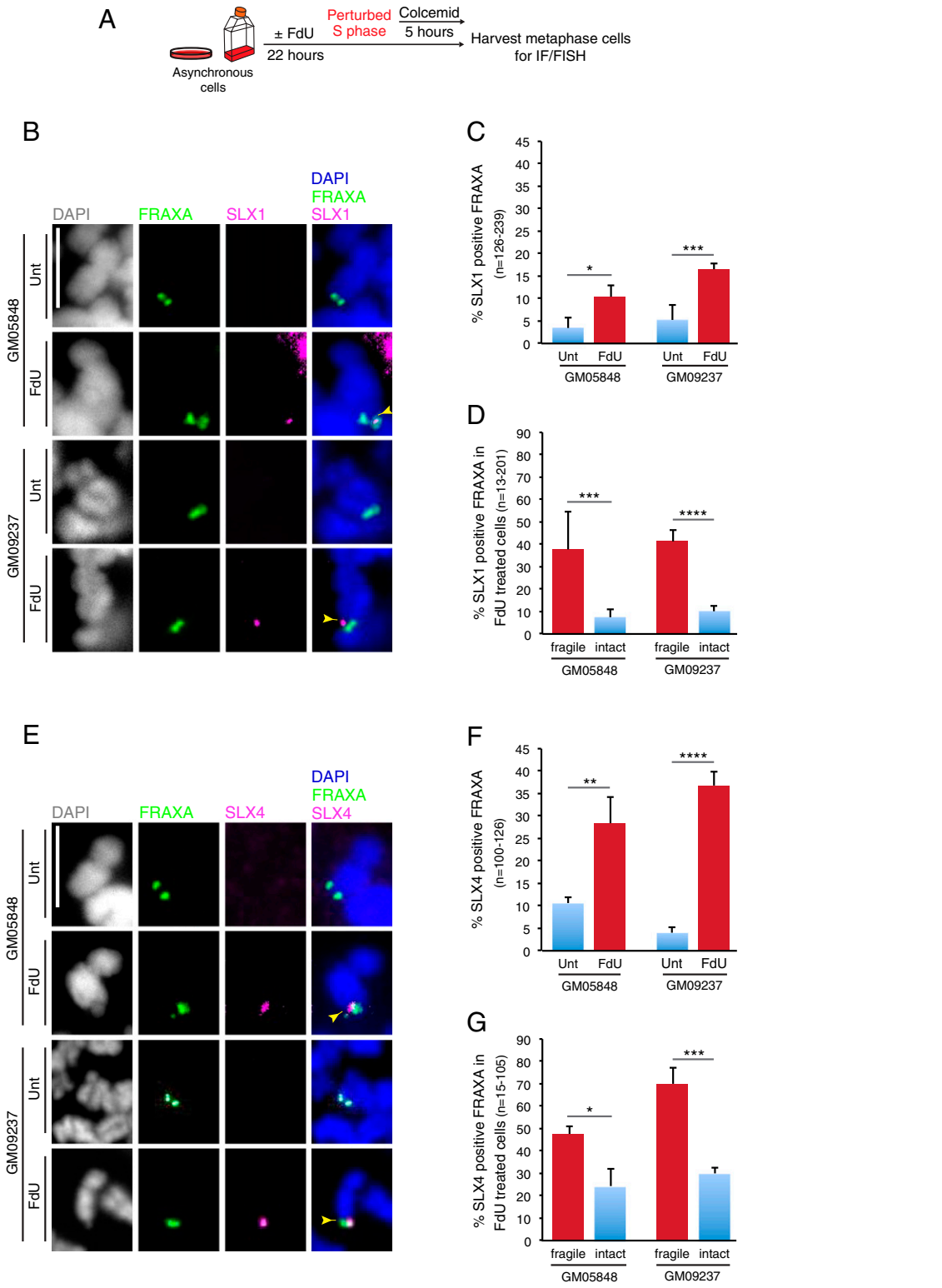

Fig. 3. SLX1 and SLX4 are recruited to *FRAXA* in mitosis following FdU treatment. (*A*) Experimental workflow for the detection of SLX1 or SLX4 on metaphase chromosomes following FdU treatment in GM05848 or GM09237 cells. (*B*) Representative images of the colocalization of SLX1 (magenta) with *FRAXA* (green) on metaphase chromosomes. Quantification of SLX1 colocalized with *FRAXA* (*C*) and the fraction of intact or fragile *FRAXA* loci that are SLX1-positive in FdU-treated cells (*D*). (*E*) Representative images of the colocalization of SLX4 (magenta) with *FRAXA* (green) on metaphase chromosomes. Quantification of SLX4 colocalized with *FRAXA* (*F*) and the fraction of intact or fragile *FRAXA* loci that are SLX4-positive in FdU-treated cells (*G*). Unt, untreated. Yellow arrowheads indicate examples of fragile *FRAXA* loci. (Scale bar, 5 μm.) Error bars represent SDs from at least three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$; $****P < 0.001$.

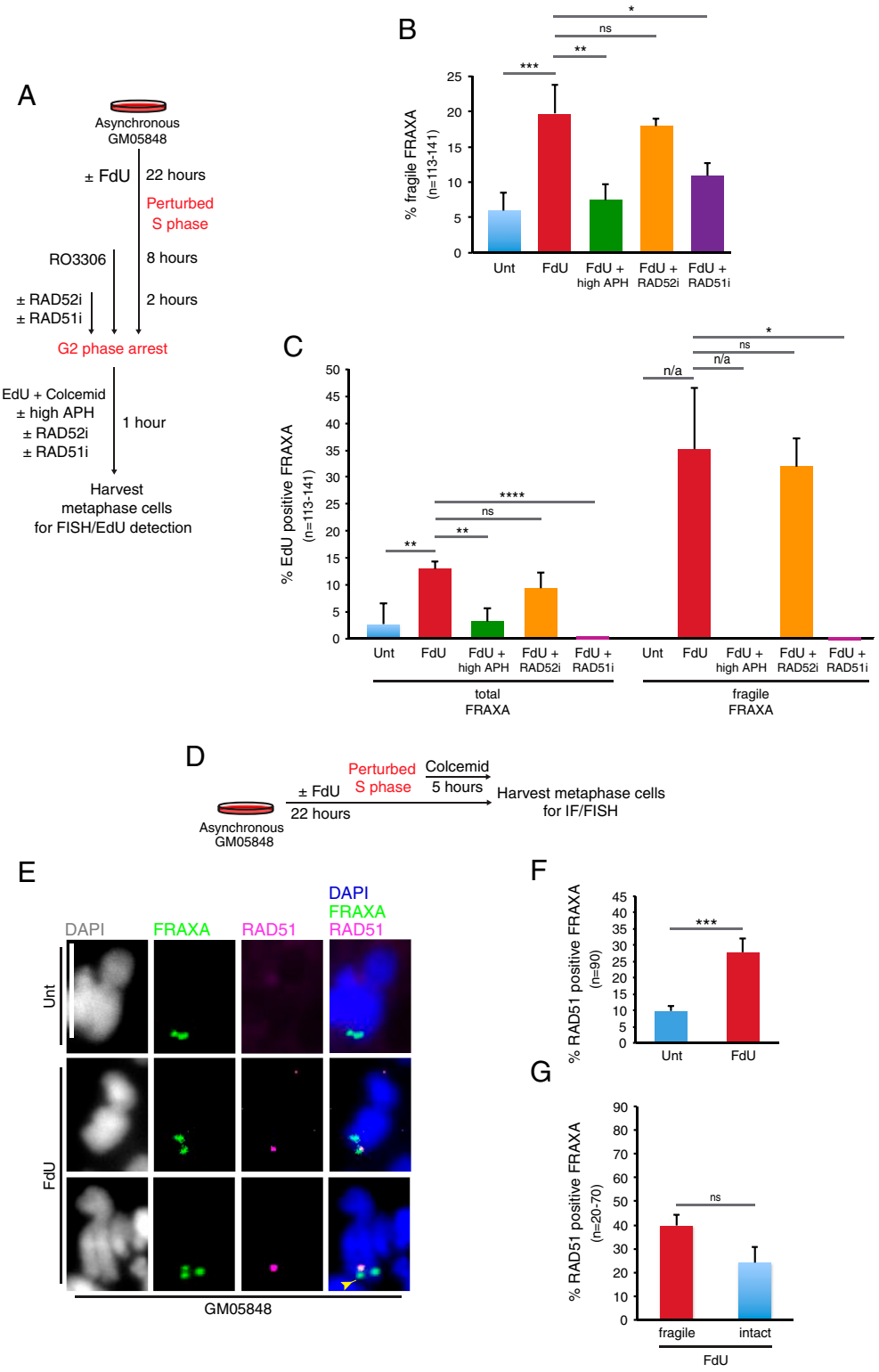

Fig. 4. RAD51 promotes MiDAS at *FRAXA* and localizes to the locus following folate stress. (*A*) Experimental workflow for the analysis of EdU incorporation at *FRAXA* on metaphase chromosomes in GM05848 cells. Various compounds, as indicated, were added prior to the harvesting of metaphase chromosomes. The location of *FRAXA* was validated using a FISH probe. (*B*) Quantification of the frequency of fragility at *FRAXA* under the indicated conditions. (*C*) Quantification of the colocalization of EdU foci with *FRAXA* under the indicated conditions. (*D*) Experimental workflow for the detection of RAD51 on metaphase chromosomes following FdU treatment in GM05848 cells. (*E*) Representative images of RAD51 (magenta) colocalization with *FRAXA* (green) on metaphase chromosomes. Quantification of the colocalization of RAD51 with *FRAXA* (*F*), and the fraction of intact or fragile *FRAXA* loci that are RAD51-positive in FdU-treated cells (*G*). Unt, untreated. (Scale bar, 5 μm.) Error bars represent SDs from at least three independent experiments. n/a, statistical analysis was not possible due to insufficient number of fragile *FRAXA* in "Unt" and "FdU + high APH" samples; ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.005$; $****P < 0.001$.

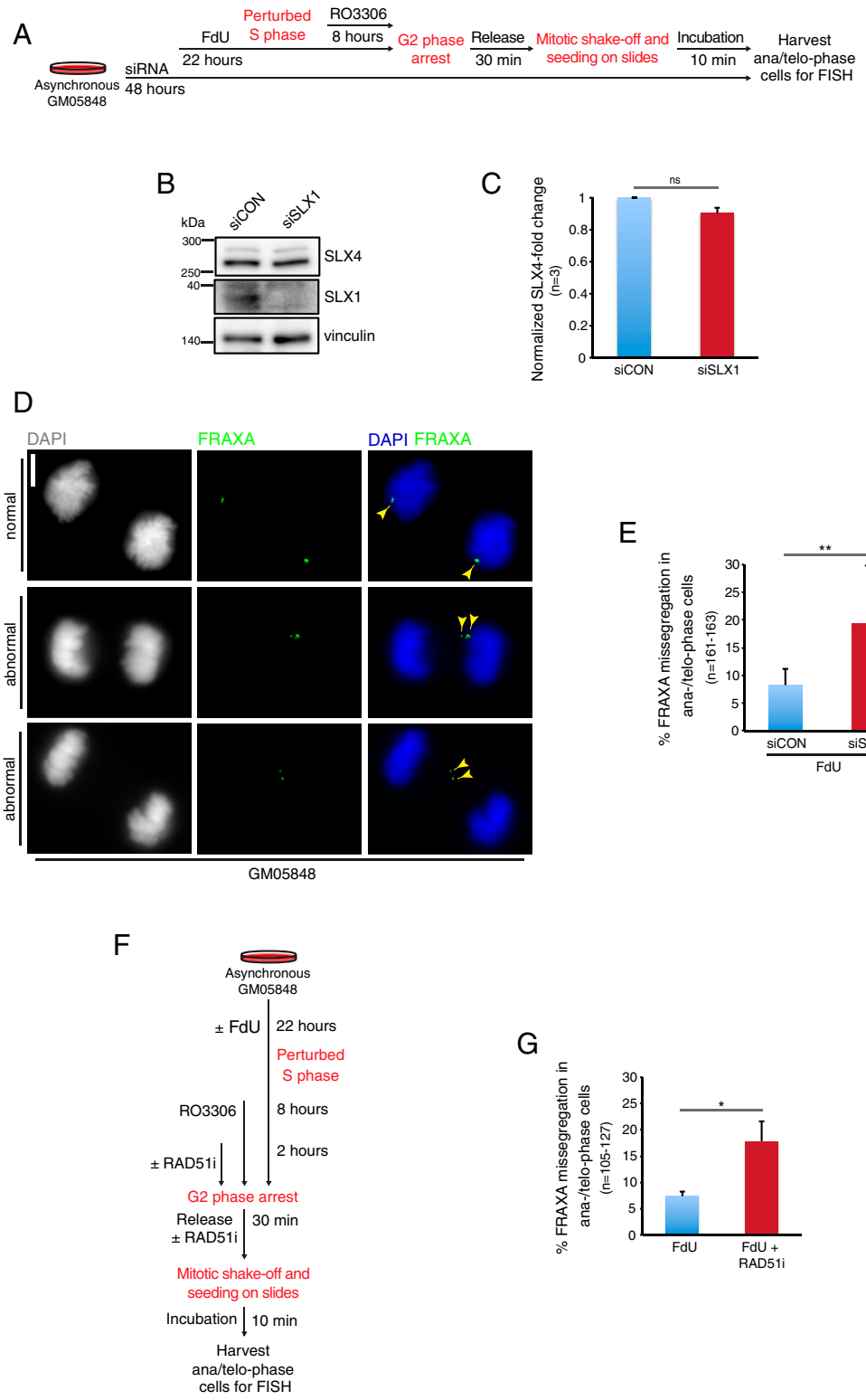

Fig. 5. SLX1 depletion or RAD51 inhibition exacerbates mitotic missegregation of *FRAXA* following folate stress. (*A*) Experimental workflow for the analysis of *FRAXA* mitotic segregation in anaphase/telophase cells following siRNA silencing of SLX1 in GM05848 cells. The location of *FRAXA* was validated using a FISH probe. (*B*) Western blot analysis of SLX4 and SLX1 proteins following siRNA silencing. Vinculin was used as a loading control. (*C*) The relative expression of SLX4 following SLX1 depletion in three replicates. (*D*) Representative images of normal or abnormal segregation of *FRAXA* (green). Yellow arrowheads indicate the location of *FRAXA*. (*E*) Quantification of the frequency of *FRAXA* missegregation in anaphase/telophase cells. (*F*) Experimental workflow for the analysis of *FRAXA* mitotic segregation in anaphase/telophase cells following RAD51 inhibition in GM05848 cells. The location of *FRAXA* was validated using a FISH probe. (*G*) Quantification of the frequency of *FRAXA* missegregation in anaphase/telophase cells. siCON, control siRNA. (Scale bar, 5 μm.) Error bars represent SDs from at least three independent experiments. ns, not significant; $*P < 0.05$; $**P < 0.01$.

FRAXA. Instead, we have shown that the RAD51 recombinase promotes *FRAXA*-associated MiDAS. In addition, it was intriguing to see that MUS81-EME1 is also not required for FdU-induced fragility or MiDAS at *FRAXA* (Fig. 2 and *SI Appendix*, Fig. S3), although another SSE complex, SLX1/4, is required. Hence, MiDAS at CFSs and RFSs apparently utilizes different subpathways of BIR.

Despite these differences, it would appear that the proteins required to promote MiDAS at both CFSs and *FRAXA* in each case contribute to the fragility of these loci. This begs two questions: (*i*) what is the connection between fragility and MiDAS? (*ii*) why do some CFS or *FRAXA* loci not show fragility after replication perturbation? One plausible explanation for the connection between MiDAS and fragility at CFSs or *FRAXA* is that fragility is an indication of delayed chromosome condensation resulting from MiDAS being ongoing during the time period when condensation normally occurs. For the second question, we propose that those cases where there is lack of fragility represent loci that completed replication in interphase and, therefore, have no requirement for MiDAS. Future research is certainly warranted to test these hypotheses.

Taken together, our data suggest the following model: upon exposure to folate stress in S phase, DNA replication forks stall within the expanded CGG repeats at *FRAXA* and other loci. These stalled replication forks are recognized and cleaved by the SLX1/4 nuclease. The cleavage of the leading strand template would generate a free 3′ DNA tail that could be used by RAD51 to create a D-loop, which would subsequently initiate BIR-based DNA synthesis in early mitosis promoted by POLD3 (*SI Appendix*, Fig. S7). It remains to be clarified how SLX1/4 and RAD51 are recruited to *FRAXA* in early mitosis, although we provide evidence that they are recruited independently. This is intriguing, as the recruitment of RAD51 is normally triggered by a double-strand break (DSB) (39). It is possible that, when SLX1 is depleted under the circumstances we have analyzed, an alternative SSE is able to initiate cleavage at *FRAXA*, thus allowing the recruitment of RAD51. However, judging by the fact that there is a reduction in the frequency of fragility and MiDAS when SLX1 is depleted (although RAD51 can still be recruited to *FRAXA*), we propose that MiDAS requires the concerted action of both SLX1 and RAD51. Further investigation is required to clarify the exact role of RAD51 in MiDAS at *FRAXA*. In particular, it will be important to determine whether RAD51 requires SLX1-mediated DNA cleavage to mediate BIR at *FRAXA*.

If MiDAS is successful, it can facilitate the completion of replication at *FRAXA*, allowing the faithful segregation of the locus into daughter cells in anaphase. However, in those cases where MiDAS does not occur and replication at *FRAXA* fails to be completed, the *FRAXA* locus would not display overt fragility, but would be missegregated in anaphase. Subsequently, this can lead to *FRAXA* exclusion into micronuclei in the daughter cells, or trigger the abortion of cytokinesis and the formation of a tetraploid progeny, as described previously (26). It remains unclear why some of the fragile *FRAXA* loci seemingly fail to activate MiDAS at all. There are two possible explanations for this. First, that MiDAS cannot be adequately initiated or completed because key components of the pathway fail to be recruited effectively in prophase at some loci. Second, that this reflects a failure to detect MiDAS at *FRAXA* loci where the tract of incorporated EdU is very short.

It is known that the three SLX4-associated SSEs (MUS81-EME1, XPF-ERCC1, and SLX1) play an important role in cleaving a variety of branched DNA structures including stalled replication forks, and three- and four-way recombination intermediates (e.g., Holliday junctions; HJs). While XPF-ERCC1 and MUS81-EME1 function as heterodimers and have quite specific requirements for substrate binding, SLX1 is thought to be a more promiscuous nuclease able to cleave various DNA structures, including nicked and intact HJs, DNA flaps, and replication forks (16, 34). Indeed, it has been proposed previously that SLX1 acts as a "first-responder" that can nick branched DNA structures and convert them into a suitable substrate for processing by other enzymes, including other SLX4-associated SSEs (33). Based on the findings of our study, it is probable that SLX1/SLX4 is able to cut a nonconventional stalled replication fork structure at *FRAXA*, which cannot be recognized by MUS81-EME1. We propose that this atypical replication fork structure might arise because of a DNA secondary structure that forms within the long CGG repeats (40, 41). Therefore, the differences in the specific enzymatic requirements for MiDAS to be promoted at *FRAXA* and CFSs might be explained by the distinct structural features of the template DNA at these loci. CFSs are known to be associated in many cases with loci harboring long and transcriptionally active genes, which could lead to conflicts between replication and transcription machineries (42). In contrast, the mutant *FRAXA* locus is epigenetically silenced (10) and, therefore, it is highly unlikely to experience replication-transcription conflicts.

The findings reported here further our understanding of the repair mechanisms that human cells employ to counteract different forms of replication stress. We propose that the pathway identified here might be one of the mechanisms by which repeat expansion or contraction occurs at *FRAXA*. Consistent with this, a recent study using a selectable cassette carrying the HyTK gene under the control of a CGG repeat-containing *FMR1* promoter in mouse cells showed that CGG repeat instability is reduced upon depletion of proteins involved in BIR, including POLD3 and RAD51 (43). Furthermore, it is clear that, in addition to *FRAXA*, folate stress induces MiDAS at several undefined loci in the human genome. This, in turn, might be one of the underlying causes of the wide range of pathological conditions associated with folate deficiency, including anemia, fetal neural tube defects, and cancer (44–48). Indeed, it is very unlikely that RFSs (which are present only in 5% of the population) are responsible for the range of health problems associated with folate deprivation. In the future, it will be important to identify these additional genomic loci that are sensitive to folate stress.

Materials and Methods

The full details of cell lines, cell culture, cell synchronization, and treatment are described in *SI Appendix*. The procedures or methods for FISH, FISH combined with EdU detection or IF, flow cytometry, RNA interference, Western blot analysis, and statistical analysis are also described in *SI Appendix*.

Data and Materials Availability. All additional data and information are included in *SI Appendix* as supplementary figures, additional legends, and references.

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