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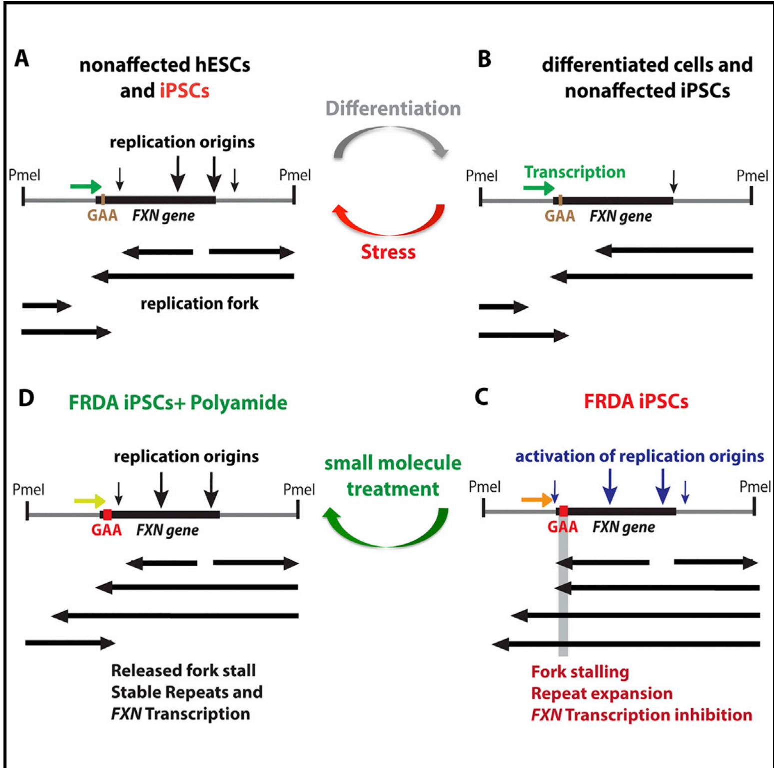
Stalled DNA Replication Forks at the Endogenous GAA Repeats Drive Repeat Expansion in Friedreich's Ataxia Cells

Graphical Abstract



Highlights

- DNA replication is altered at the endogenous *FXN* locus in FRDA iPSCs
- Developmentally regulated origins are activated in the *FXN* gene in FRDA iPSCs
- Replication forks stall at the expanded GAA repeats in FRDA cells
- GAA-specific polyamides rescue replication fork stalling in FRDA iPSCs

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In Brief

Gerhardt et al. demonstrate that stable secondary structures, formed at the expanded GAA repeats in Friedreich's ataxia patient cells, stall DNA replication. In addition, using a single DNA molecule approach to visualize the *Frataxin* locus, they show that aberrant activation of origins downstream of the GAA repeats alters replication fork direction.

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Stalled DNA Replication Forks at the Endogenous GAA Repeats Drive Repeat Expansion in Friedreich's Ataxia Cells

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SUMMARY

Friedreich's ataxia (FRDA) is caused by the expansion of GAA repeats located in the Frataxin (FXN) gene. The GAA repeats continue to expand in FRDA patients, aggravating symptoms and contributing to disease progression. The mechanism leading to repeat expansion and decreased FXN transcription remains unclear. Using single-molecule analysis of replicated DNA, we detected that expanded GAA repeats present a substantial obstacle for the replication machinery at the FXN locus in FRDA cells. Furthermore, aberrant origin activation and lack of a proper stress response to rescue the stalled forks in FRDA cells cause an increase in 3′-5′ progressing forks, which could enhance repeat expansion and hinder FXN transcription by head-on collision with RNA polymerases. Treatment of FRDA cells with GAA-specific polyamides rescues DNA replication fork stalling and alleviates expansion of the GAA repeats, implicating DNA triplexes as a replication impediment and suggesting that fork stalling might be a therapeutic target for FRDA.

INTRODUCTION

Friedreich's ataxia (FRDA) is the most commonly inherited ataxia in the Caucasian population. FRDA is an autosomal recessive disease with an estimated prevalence of 1:29,000 and a carrier frequency of about ~1:100 (Delatycki et al., 2000). FRDA is caused by a GAA repeat expansion in the first intron of the *Frataxin* (*FXN*) gene. In affected individuals, the GAA repeat increases from a normal range of 6–34 repeats to a pathogenic range of 66–1,700 repeats (Campuzano et al., 1996). GAA repeat expansion leads to decreased transcription of the *FXN* gene and reduced expression of FXN, an iron-binding protein responsible for biogenesis of iron-sulfur clusters (reviewed by Schmucker and Puccio, 2010). Frataxin deficiency causes mitochondrial iron overload, which severely impacts cardiomyocytes and neurons (Koeppen, 2011). Progressive damage to the nervous system leads to symptoms such as gait disturbance, vision and hearing impairment, and speech difficulties. There is currently no cure for this fatal disease. Expanded GAA repeats are inherited from both parents and expand further post-zygotically in FRDA patients (De Biase et al., 2007; De Michele et al., 1998). Recently, progressive GAA repeat expansions were discovered in human induced pluripotent stem cells (iPSCs) derived from FRDA fibroblasts (Ku et al., 2010), allowing for analyses of the expansion mechanism in the natural context of the *FXN* locus.

The mechanism for GAA repeat expansion in FRDA patients remains ambiguous. GAA repeats are able to form unusual non-B DNA structures, such as triplexes, intramolecular H-DNA, as well as intermolecular sticky DNA (Wells, 2008), which potentially could block the replication and transcription machineries in patient cells. Expanded GAA repeats also form R-loops in vitro and in vivo (Groh et al., 2014; Li et al., 2016). It is shown that GAA repeats are able to impede transcription elongation (Krasilnikova et al., 2007; Ohshima et al., 1998) and the DNA replication fork (Krasilnikova and Mirkin, 2004) in vitro or in plasmid-based model systems in vivo. Also, R-loops formed at the expanded GAA tracts could block elongation of RNA polymerase II (Pol II) as well as the replication machinery. However, until now it was not possible to determine whether the GAA repeats in FRDA cells stall the replication machinery and could block *FXN* transcription in vivo at the endogenous locus. A stalled replication fork could lead to fork reversal, double-strand break formation, and DNA polymerase slippage, resulting in GAA repeat expansions (Mirkin, 2007).

Besides replication fork stalling, studies in model systems have shown that GAA repeat instability could depend on the orientation of replication fork movement through the repeats (Rindler et al., 2006). Several models have been proposed where activation or deactivation of replication origins changes their position and distance relative to the repeat tract (origin switch and origin shift model), which influences the replication fork direction through the repeats. In addition, altered replication fork progression (fork shift model) could play an important role in the mechanism leading to repeat expansion or contraction (Mirkin, 2007). However, it still has to be revealed which of these models applies

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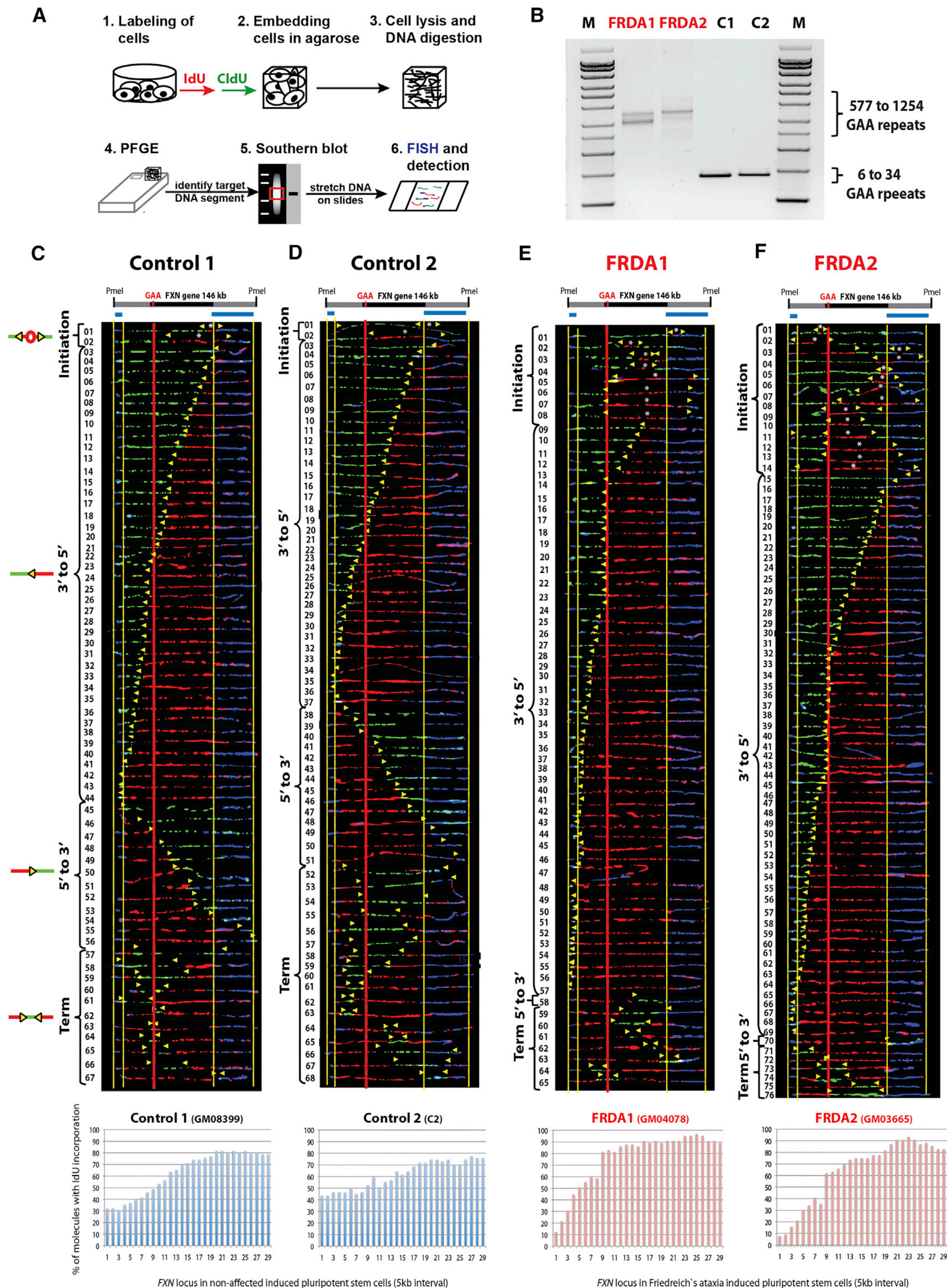

Figure 1. The DNA Replication Program Is Altered at the Endogenous *FXN* Locus in FRDA iPSCs

(A) Schematic of the steps of SMARD that enable visualization of single-replicating DNA molecules (adapted from Gerhardt et al., 2014a). First, cells are pulsed with IdU (red) and CldU (green) and then embedded in agarose and lysed. After digestion with the *PmeI* enzyme, DNA molecules are separated by pulsed-field gel electrophoresis (PFGE). Next the DNA segment containing the *FXN* locus is identified, excised from the gel, and stretched on silanized glass slides. The labeled DNA molecules are detected by immunostaining (red and green), and two FISH probes surrounding the repeats are used to orient the *FXN* locus.

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to GAA repeat expansion and whether replication forks stall at the *FXN* locus in FRDA cells.

We determined that errors in the DNA replication program can affect GAA repeat expansion in FRDA iPSCs at the endogenous *FXN* locus. Using single-molecule analysis of replicated DNA (SMARD), we observed a significant stalling of replication forks at the expanded GAA repeats, along with aberrant replication origin activation and altered replication fork direction through the repeats at the *FXN* locus in FRDA iPSCs and fibroblasts. The magnitude of replication fork stalling described herein has not been previously reported in human cells. Treatment of FRDA iPSCs with a GAA-specific polyamide, which prevents GAA triplex formation in vitro and stabilizes GAA repeats in vivo (Du et al., 2012), released fork stalling. This result implicates replication fork stalling at GAA triplexes in the repeat expansion mechanism in FRDA patient cells.

RESULTS

The DNA Replication Program Is Altered at the Endogenous FXN Locus in FRDA iPSCs

In vitro models predict that repeat expansion is caused by DNA polymerase slippage at stalled replication forks (Mirkin, 2007). Using a plasmid-based model system, it has been shown that the replication fork stalls at expanded GAA repeats (Chandok et al., 2012; Krasilnikova and Mirkin, 2004) and that altered replication fork progression results in repeat instability (Cleary et al., 2002; Pelletier et al., 2003; Rindler et al., 2006; Shishkin et al., 2009). It is still unknown, however, how the replication fork proceeds through the endogenous *FXN* locus in the native chromatin environment and whether the replication machinery stalls at the GAA repeats in FRDA cells. To determine the replication program at the endogenous *FXN* locus, we employed SMARD (Figure 1A) (Gerhardt et al., 2014a; Norio and Schildkraut, 2001). We analyzed the replication profile of a 146-kb genomic DNA segment containing the *FXN* gene and flanking sequence recognized by two fluorescence in situ hybridization (FISH) probes (detecting a short and a long DNA fragment at either end of the segment) in two unaffected control and two FRDA iPSC lines (FRDA1: GM04078: ~577 and 743 GAA repeats and FRDA2: GM03665 containing ~874 and 1,254 GAA repeats at passage 6 after reprogramming; Figure 1B). FRDA iPSCs exhibit decreased *FXN* transcript levels and expanding GAA repeats (Ku et al., 2010) (Figures S1A and S1B). Flow cytometry analyses showed that the FRDA iPSC and control iPSC lines have similar cell-cycle profiles (Figures S1C and S1D).

First, we asked whether the GAA repeats were replicated differently in FRDA iPSCs compared to unaffected control iPSCs (Figures 1C–1F). In unaffected iPSCs (controls 1 and 2), the replication fork moved from both directions through the GAA repeats (Figures 1C and 1D). We detected DNA molecules with replication forks progressing from either the 5′-3′ or 3′-5′ direction or both directions. Very few replication origins (2.5% of all molecules) were seen at the *FXN* locus in unaffected iPSCs. In contrast, we detected a significantly altered replication program in FRDA iPSCs, with replication forks progressing predominantly in the 3′-5′ direction through the *FXN* locus and GAA repeats (Figures 1E and 1F). In addition, we detected increased replication initiation at the *FXN* locus. Replication origins were detected in 12.3% of molecules in FRDA1 iPSCs and in 18.3% of molecules in FRDA2 iPSCs. Replication origins were activated mostly downstream of the repeats in the *FXN* gene, which increases the number of replication forks progressing in the 3′-5′ direction through the repeats. In FRDA fibroblasts, from which FRDA1 iPSCs were derived, we observed a similar replication profile as in FRDA iPSCs (Figures S2A and S2B), suggesting that altered replication fork progression through the repeats could lead to repeat expansion in FRDA patient cells.

In iPSCs harboring one expanded and one short GAA tract, progressive expansions occur exclusively within the long GAA repeats, identifying the length threshold as a key determinant of the expansions (Ku et al., 2010). Additionally, repeat expansion takes place solely at the disease locus, indicating that specific *cis* elements are crucial for the mechanism. Therefore, the length of the repeat tract together with activation or inactivation of local replication origins, which alter fork progression, are the primary determinants of the repeat expansion (Gerhardt et al., 2014b). These *cis* elements accompanied by the activities of *trans* factors, such as mismatch repair protein Msh2, facilitate GAA repeat expansions (Du et al., 2012; Ku et al., 2010). To separate the effect of *cis* elements from potential cell-specific differences in *trans* factors, we determined the replication profile in FRDA iPSCs containing one expanded allele and one allele with a short, stable GAA repeat tract (FRDA P iPSCs; Figures S2A and S2C). As anticipated, we observed a mixed replication profile in FRDA P iPSCs, with replication forks progressing from both directions through the repeats, similar to unaffected iPSCs, and an increase in replication origins (16% of molecules) at the *FXN* gene, similar to FRDA iPSCs (Figure S2C). These results show that two different replication programs, presenting in SMARD analyses as a mixed profile, are governed by the length of the GAA tract and co-exist in cells heterozygous for the GAA repeat expansion.

Replication Forks Stall at the GAA Repeats in FRDA iPSCs

Replication forks stall in both directions at the GAA repeats when replicated as episomal DNA in human cells (Follonier and Lopes, 2014; Krasilnikova and Mirkin, 2004). To determine whether stalling occurs at the endogenous *FXN* locus, we divided the *FXN* segment into 5-kb intervals, and we counted the replication forks that proceed through each 5-kb interval. Our results

(B) The repeat lengths in two unaffected (C1 and 2) and two FRDA (FRDA1 and 2) iPSC lines were determined by PCR.
(C–F) Top (also shown in Figures 3 and 4): map of the *PmeI* segment containing the *FXN* gene and GAA repeats. The positions of the FISH probes are marked in blue and the GAA repeats are indicated by a red vertical bar. Middle: photomicrographs of labeled DNA molecules from control 1 (C), control 2 (D), FRDA1 (E), and FRDA2 (F) iPSCs are ordered according to replication fork (yellow arrows) progression in the 5′-3′ and 3′-5′ directions, replication initiation, and termination. White asterisks mark the replication initiation sites (also shown in Figures 3 and 4). Bottom: the percentage of molecules with IdU incorporation (first pulse) is calculated from the DNA molecules shown above. Results of cell cycle, *FXN* mRNA quantitation, and repeat expansion analyses in FRDA iPSCs are shown in Figure S1.

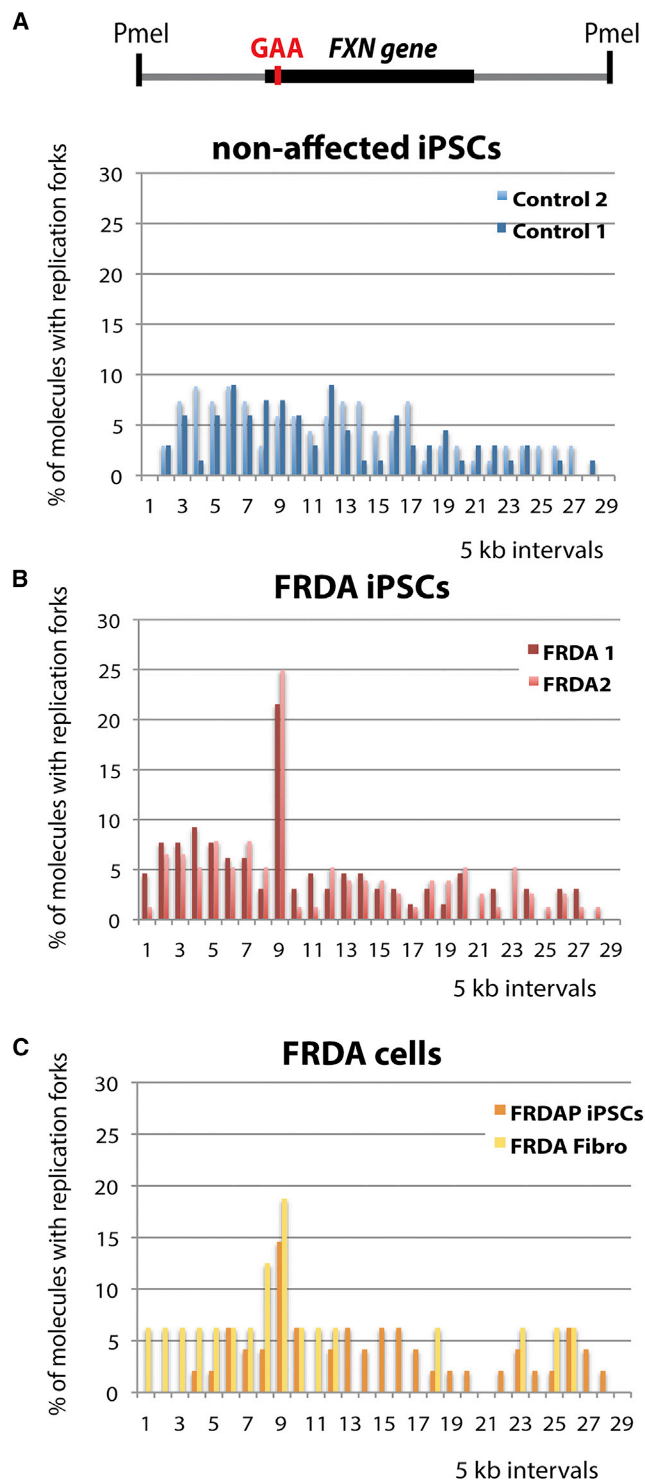


Figure 2. Replication Forks Stall at the GAA Repeats in FRDA Cells
(A–C) The percentage of molecules with replication forks for each 5-kb DNA interval was calculated at the *FXN* locus in unaffected iPSCs (controls 1 and 2) (A), FRDA iPSCs (FRDA1 and 2) (B), and FRDA fibroblasts and FRDA iPSCs carrying one expanded and one short GAA allele (FRDA P) (C). SMARD profiles are shown in Figure S2.

show no significant replication fork stalling at the GAA repeats in two control iPSC lines (Figure 2A). In contrast, in FRDA iPSCs, replication fork stalling was detected at the expanded GAA repeats (Figure 2B). FRDA2 iPSCs with longer GAA repeats showed slightly more stalled replication forks (25% of molecules) than FRDA1 iPSCs with shorter repeats (21.5%). While we observed replication fork stalling in both directions (Figures 1E and 1F), a major block of the replication machinery occurred at the repeats in the 3′-5′ direction. We also detected replication fork stalling in FRDA P iPSCs (14.6% of molecules) and FRDA fibroblasts (18.7% of molecules, Figure 2C). These results show that the replication machinery encounters obstacles at the endogenous *FXN* locus or that it collides with the Pol II transcription complex.

Replication Origins Are Developmentally Regulated at the FXN Locus

Next, we determined whether the replication origins are activated in response to the stalled fork or if activation of origins and increased number of replication forks progressing in the 3′-5′ direction cause fork stalling. As the replication origins in FRDA iPSCs seem to be activated in a false direction to rescue stalled replication forks, we were interested to establish whether these replication origins are dormant rescue origins or more likely developmentally regulated origins.

To this end, we examined the replication program at the *FXN* locus in unaffected human embryonic stem cells (hESCs) by SMARD. Because it has been shown that the position and number of replication initiation sites can change during cell fate determination (Gray et al., 2007; Norio et al., 2005), we decided to analyze the DNA replication at the *FXN* locus before and after hESC differentiation. We observed ∼20% of molecules with replication initiation sites at the *FXN* locus in two hESC lines (hESC1 [H9] and hESC2 [H14]) (Figures 3A and 3B; Figure S3A). The replication origins were located downstream of the repeats at the *FXN* gene (Figures 3A and 3B). In addition, the replication forks progressed in both directions through the *FXN* locus at normal speeds without fork stalling in each of these lines (Figures S3B and S3C). After differentiation of hESCs to neurospheres, we detected only a single initiation event and replication forks progressing from both directions through the repeats (Figure 3C). The replication profile in these differentiated cells more closely resembles the replication profile of the *FXN* locus in control iPSCs rather than that of the undifferentiated hESCs (Figures 1C, 1D, and 3). These results show that replication origins in the *FXN* gene do not fire after cell differentiation and that reprogramming of the replication profile at the *FXN* locus in unaffected iPSCs was incomplete. In summary, the presence of active replication origins at the *FXN* locus in undifferentiated hESCs, but not differentiated cells, confirms that replication origins at the *FXN* locus are developmentally regulated.

Replication Origins Are Activated Upstream and Downstream of the Repeats in Unaffected Cells but Not FRDA iPSCs

Because origin placement influences the direction of replication fork movement through the GAA repeats, we next investigated

whether and where replication origins are activated at the *FXN* gene after induction of replicative stress by inhibition of the DNA polymerase and replication machinery (Anglana et al., 2003; Doksani et al., 2009). It has been shown that, in response to replicative stress including fork stalling, dormant origins are activated to rescue the stalled replication fork. After treatment of control iPSCs with aphidicolin, an inducer of replicative stress (Figure 3D), we observed a slower replication fork progression probably caused by replication fork pausing (Figures S3C and S3D). As expected, to counteract disrupted replication fork progression, we detected activation of dormant replication origins in 25% of DNA molecules (Figures 3D and 3E). These replication origins were activated in control iPSCs in the *FXN* gene downstream of the repeat region at locations similar to those found in FRDA iPSCs (Figures 1E and 1F). However, in contrast to FRDA iPSCs, in control cells the replication forks progressed from both directions through the repeats after induction of replicative stress and replication fork pausing. These results indicate that, under replicative stress, replication origins are activated not only downstream of the repeats in the *FXN* gene but also upstream of the *FXN* gene in cells lacking the expanded GAA repeat tract.

In plasmid-based experiments, an increase in instability of particular expansions was detected when TTC repeats were located on the lagging-strand template (3′-5′ direction at the endogenous locus) (Rindler et al., 2006). To determine an accurate direction of the replication fork, we calculated the percentage of molecules with replication forks progressing through the GAA repeats in either the 5′-3′ or 3′-5′ direction in control hESCs, control iPSCs, FRDA iPSCs, and FRDA fibroblasts (Figure 3F). Quantification of directionality through the repeat region showed that, in control cell lines, the replication fork moved from both directions, with a slight bias toward progression in the 3′-5′ direction. In control iPSCs treated with aphidicolin, more replication forks progressed in the 5′-3′ direction. However, a significant alteration in the replication fork direction was only detected in FRDA cell lines, with most of the replication forks moving through the GAA repeats in a 3′-5′ direction (Figure 3F). These results suggest that, in FRDA cells, the stalled replication fork is not rescued by activation of dormant origins upstream of the repeats and, instead, activation of origins downstream of the repeats leads to preferential progression of DNA polymerase in the 3′-5′ direction. This altered replication fork progression and stalling in the 3′-5′ direction could promote repeat expansion and lead to collision with the transcription machinery at the endogenous *FXN* locus.

GAA-Specific Polyamide Treatment Releases Replication Fork Stalling in FRDA iPSCs

A released replication block should alleviate GAA repeat expansion as well as minimize collision between transcription and replication machineries. We wanted to know if treatment of FRDA iPSCs with a GAA-specific polyamide (FA1) (Figure 4B), which is known to inhibit triplex formation in vitro (Burnett et al., 2006; Du et al., 2012), would release the stalled replication fork at the GAA repeats at the endogenous *FXN* locus in vivo. It was shown that this beta-alanine-linked polyamide prevents GAA repeat expansion in FRDA iPSCs (Du et al., 2012) and increases *FXN* transcription in FRDA cells (Burnett et al., 2006). However, it is not clear yet how this compound exactly leads to stabilization of the GAA repeats in FRDA iPSCs. It has been suggested that GAA-specific polyamides could prevent the formation of DNA triplexes in vivo at the endogenous *FXN* locus and, thus, avoid polymerase stalling (Burnett et al., 2006).

To test whether a GAA-specific polyamide can prevent the replication fork stall in vivo at the *FXN* locus, we treated FRDA iPSCs for 48 hr with compound FA1 and analyzed the replication program in these cells by SMARD. Although we observed a similar replication profile in both treated and non-treated cells (Figures 4A and 4B), in FRDA iPSCs treated with FA1 we found no significant replication fork stalling at the GAA repeats in contrast to non-treated FRDA iPSCs (Figures 4C and 4D). Thus, these results show that the GAA-specific FA1 polyamide indeed released the replication fork stalling in FRDA iPSCs (Figure 4D). In addition, replication initiation was unchanged upon FA1 treatment of FRDA iPSCs (Figures 4A and 4B; Figure S3E). In summary, our results show that culturing FRDA iPSCs with a GAA-specific polyamide, which alleviates formation of secondary GAA repeat structures, released the observed replication impediment in FRDA iPSCs. The release of replication fork stalling by FA1 could explain the observed stabilization of the expanded GAA repeats in FRDA iPSCs (Du et al., 2012).

DISCUSSION

Several inherited repeat expansion disorders like FRDA, fragile X syndrome (FXS), myotonic dystrophy (DM1), Huntington's disease, and spinocerebellar ataxia type 7 (SCA7) are caused by expansions of trinucleotide repeat sequences (Iyer et al., 2015). We examined the DNA replication program in FRDA patient cells to determine if defects in DNA replication processes cause GAA repeat expansions. We found that replication fork progression at the *FXN* gene is altered in FRDA cells to a much greater extent than the replication impediment we previously detected at the *FMR1* locus in unaffected and FXS hESCs using SMARD (Gerhardt et al., 2014a, 2014b). The major stall detected in FRDA cells suggests that the replication fork indeed encounters a block imposed by stable non-canonical DNA structures (e.g., triplexes) formed by expanded GAA repeats at the endogenous *FXN* locus. The replication fork also could encounter paused transcription machinery in FRDA cells and R-loop structures. Our observation of a released replication fork block after treatment with a DNA-binding GAA-specific polyamide suggests that stable GAA DNA triplex conformations are responsible for replication fork stalling in FRDA cells.

We also found that the directionality of the replication fork was altered in FRDA cells. In unaffected cells, which have short GAA repeats that lack the propensity for non-canonical DNA structure or R-loop formation, replication forks progressed from both directions through the endogenous *FXN* locus, which could lead to a balance between contraction and expansion events that stabilize the short GAA repeats. In contrast, in FRDA cells harboring expanded GAA repeats, we observed fork progression predominantly in the 3′-5′ direction, which, in conjunction with a higher probability for secondary structure formation, could promote repeat expansion. In plasmid-based

A hESCs1

B hESCs2

C diff. cells

D iPSCs + Aphidicolin

PmeI GAA FXN gene 146 kb PmeI

Initiation

3′ to 5′

5′ to 3′

Term

hESCs 2 (H14)

hESCs 1 (H9)

diff. cells

iPSCs + Stress

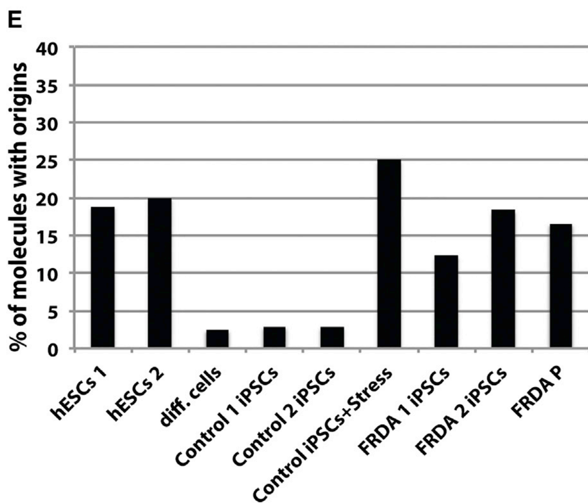
% of molecules with IdU incorporation

FXN locus in non-affected hESCs (5kb interval)

FXN locus in non-affected cells (5kb interval)

FXN locus in non-affected iPSCs (5kb interval)

E



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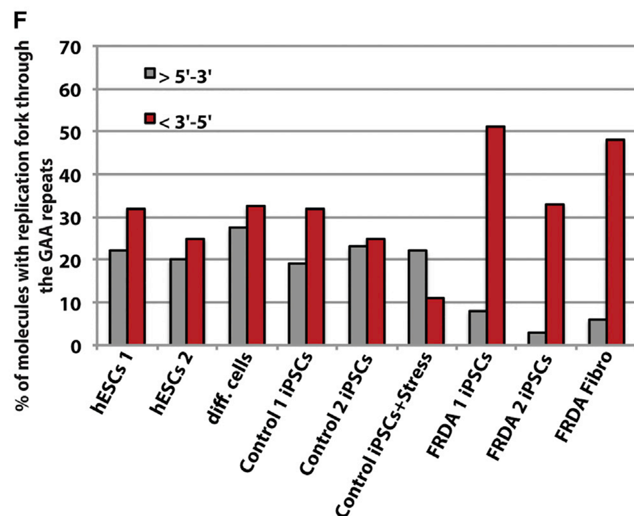

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model systems, replication fork progression from 5′-3′ through GAA repeats led to repeat contraction and expansion. However, replication forks progressing from the 3′-5′ direction (TTC sequence as the template for lagging-strand synthesis) mostly resulted in repeat expansion (Rindler et al., 2006). We postulate that in FRDA iPSCs the replication fork direction is switched due to the activation of proximal replication origins downstream of the repeats. Replication forks progressing from the 5′-3′ direction (upstream of the repeats) rarely reach the expanded GAA repeat region in FRDA cells, as these replication forks originate from replication origins located farther away from the repeat tract than the newly activated replication origins in FRDA cells. Our results confirm findings from plasmid-based experiments that a switch in the replication fork direction occurs at the endogenous *FXN* locus (Figure S4), which could facilitate the observed GAA repeat expansion in FRDA iPSCs.

The FRDA fibroblasts exhibit a similar replication program to FRDA iPSCs, with replication forks predominantly progressing in a 3′-5′ direction through the GAA repeats. However, in contrast to FRDA iPSCs, the expanded repeats are relatively stable in FRDA fibroblasts (Figure S2A). Stability of the GAA repeats could be explained by lack of additional *trans* factors necessary for repeat instability, such as Msh2, which have been shown to bind in close proximity to the repeats and facilitate repeat expansion in FRDA iPSCs (Ku et al., 2010). Human pluripotent stem cells express increased levels of Msh2, and its downregulation leads to a significant alleviation of expansion rates (Du et al., 2012; Gerhardt, 2015; Ku et al., 2010). However, increased Msh2 level is not sufficient to cause repeat instability, as in FRDA P iPSCs where the *FXN* allele of normal repeat length does not expand while the allele with the long GAA tract does. Thus, co-existence of both the appropriate *cis* environment (structure-forming expanded repeats and replication origins) and *trans* factors (high expression levels of MMR proteins) is necessary for expansions to occur.

Our results suggest that the altered replication fork direction is caused by a failure to activate the proper dormant origins to rescue the stalled forks at the *FXN* locus in FRDA iPSCs. It is not known which factors or elements lead to origin activation or deactivation in human cells (Méchali, 2010). Under replicative stress and fork-stalling conditions induced by aphidicolin treatment, replication forks progress bidirectionally in unaffected cells, indicating that replication initiation events take place both upstream and downstream of the repeats. In FRDA cells, however, the majority of replication forks progress only in the 3′-5′ direction, originating from initiation sites downstream of the GAA repeats. A similar phenomenon of unidirectional fork movement through the CGG repeats at the *FMR1* locus was observed in FXS hESCs (Gerhardt et al., 2014a, 2014b). However, inactivation of a replication origin upstream of the expanded CGG repeats by a point mutation was required to elicit the alteration in replication fork direction in FXS hESCs. Replication initiation was unchanged upon FA1 treatment of FRDA iPSCs (Figures 4A and 4B; Figure S3E). These results suggest that replication origin activation in FRDA iPSCs might not be due to replication fork stalling, but rather an independent event causing replication fork progression in a 3′-5′ direction that drives repeat expansion in FRDA cells. However, further experiments are needed to test this theory.

Besides DNA sequence, changes in chromatin modifications could impact origin activation (Dorn and Cook, 2011; Rivera et al., 2014). At the *FXN* locus, origins are active in unaffected hESCs with unperturbed *FXN* transcription and a euchromatic environment as well as in FRDA cells having the *FXN* locus in a heterochromatic state and decreased *FXN* transcription. These results show that epigenetic modifications associated with neither transcription initiation nor active transcription itself are likely to account for differences in origin firing between FRDA and control cells. Besides epigenetic marks and local chromatin structure, chromatin looping, nuclear position, and binding of specific *trans* factors could influence origin de/activation (Marks et al., 2016). Although preferential localization of expanded GAA-FXN loci to the nuclear lamina was discovered recently (Silva et al., 2015), such analyses have not been conducted in pluripotent cells.

It remains to be determined whether the replication machinery runs into co-transcriptional R-loops or if the transcription complex collides with the stalled replication machinery at the unresolved DNA structures stabilized by DNA-binding proteins (Du et al., 2012; Ku et al., 2010). Recently it was shown that prevention of R-loop formation increases *FXN* transcription in FRDA fibroblasts (Li et al., 2016). The contributions of triplexes and R-loops to expansion and transcription may vary between cell types depending on the availability of *trans* factors. This could explain the different levels of *FXN* gene transcription in FRDA cells with similar levels of fork stalling. It also has been proposed that R-loops at GAA repeats may stimulate formation of DNA triplexes, as RNA-DNA hybrids render one of the DNA strands single stranded and capable of interaction with double-stranded DNA (dsDNA) to form a stable triple helical conformation (Grabczyk and Usdin, 2000). Head-on collision between transcription and replication machineries has been recurrently linked to genetic instability and chromosomal rearrangements; therefore, it is likely that repeat expansions are stimulated by the temporary

Figure 3. Replication Origins Are Developmentally Regulated and Are Activated Upstream and Downstream of the Repeats in Unaffected iPSCs after Induction of Replicative Stress

(A–D) Photomicrographs of labeled DNA molecules from unaffected hESCs1 (H9) (A), hESCs2 (H14) (B), differentiated H9 cells (C), and unaffected iPSCs treated with aphidicolin (D) are ordered according to replication fork (yellow arrows) progression in the 5′-3′ and 3′–5′ directions, replication initiation, and termination. Bottom: the percentage of molecules with IdU incorporation (first pulse) is calculated from the DNA molecules shown above.

(E) Graph shows the percentage of DNA molecules with replication initiation sites in unaffected hESCs, differentiated cells, unaffected iPSCs with or without aphidicolin, and FRDA iPSCs.

(F) The percentage of DNA molecules with replication forks progressing through the GAA repeats at the *FXN* locus either in the 3′-5′ or 5′-3′ direction in unaffected hESCs, differentiated cells, control iPSCs (with and without aphidicolin treatment), and FRDA iPSCs and fibroblasts. Results of hESC pluripotency assessment as well as replication fork quantitation and speed calculations with and without aphidicolin treatment are given in Figure S3.

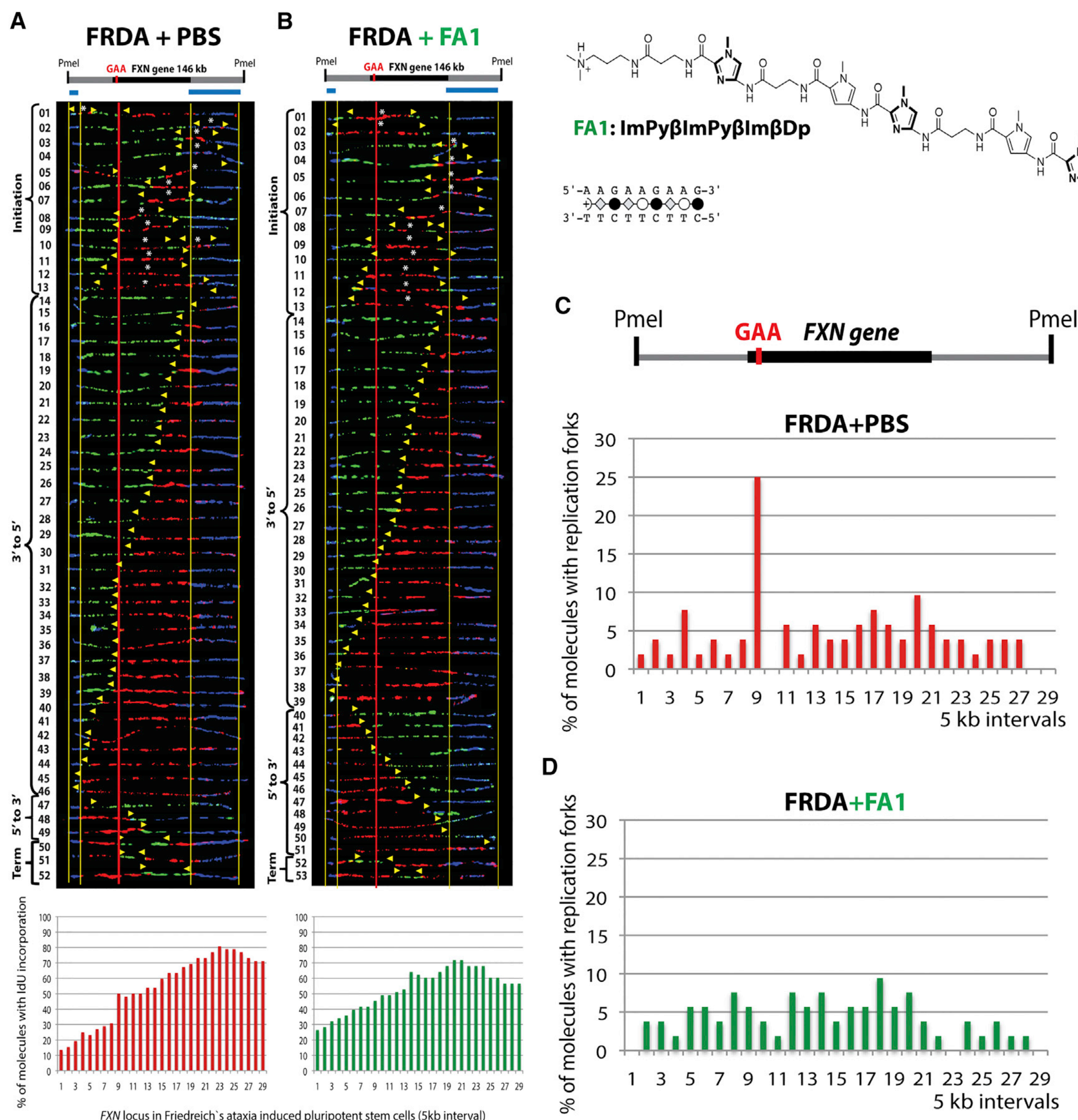

Figure 4. Stalled Replication Forks at the GAA Repeats in FRDA iPSCs Are Released by Polyamide Treatment

(A and B) Photomicrographs of labeled DNA molecules from FRDA iPSCs incubated with PBS (A) or polyamide FA1 (B). Bottom: the percentage of molecules with IdU incorporation (first pulse) is calculated from the DNA molecules shown above. (B, right) Polyamide structure of FA1: β-alanine (β)-linked Pyrrole (Py)-Imidazole (Im)-dimethylaminopropylamine (Dp) and a model depicting the linear FA1 polyamide binding in a C-terminal to N-terminal orientation with respect to 5′- to -3′ sequence of the GAA repeat target in the minor groove are shown.

(C and D) The percentage of molecules with replication forks for each 5-kb DNA interval was counted at the *FXN* locus in FRDA iPSCs incubated with PBS (C) or polyamide FA1 (D). A model depicting the basis for altered replication fork movement at the *FXN* locus in FRDA cells is shown in Figure S4.

conflict between replication and transcription at the expanded GAAs.

In summary, our data demonstrate that the replication machinery stalls at the endogenous expanded GAA repeats in the *FXN* gene while progressing in the 3′-5′ direction. Replication fork stalling may result in DNA polymerase slippage and stimulate the progressive repeat expansions seen in FRDA iPSCs. Moreover, our results show that the GAA-specific polyamide FA1 releases replication fork stalling at the GAA repeats in FRDA iPSCs, suggesting that expanded repeats may indeed form triplexes at the endogenous *FXN* locus that impede the replication complexes. Thus, compounds capable of alleviating replication fork stalling can be of therapeutic significance in the treatment of FRDA.

EXPERIMENTAL PROCEDURES

Cell Culture

Control 1 and FRDA 1 and FRDA 2 fibroblasts were obtained from Coriell Cell repositories (lines GM08399, GM04078, and GM03665, respectively). Control 2 and FRDA P fibroblasts (lines C2 and 4491, respectively) were derived from

skin biopsies conducted at Children's Hospital of Philadelphia (CHOP) according to approved institutional review board (IRB) protocols, as described in Li et al. (2015a). Detailed culturing, differentiation, and reprogramming protocols can be found in the Supplemental Experimental Procedures.

Flow Cytometry

Antibodies against SSEA-3, SSEA-4, OCT4, SOX2, PAX6, PLZF, and TRA-1-60 were used to assess iPSC and hESC pluripotency. Antibody information and detailed protocols are provided in the Supplemental Experimental Procedures.

Determining Number of GAA Repeats and FXN mRNA Expression

PCR analyses of the GAA repeat number were conducted using two sets of primers FXN_short (forward and reverse; 498 bp of the GAA repeats flanking sequences) and FXN_long (forward and reverse; 1,370 bp of the GAA repeats flanking sequences), exactly as described in Li et al. (2015b). All qRT-PCR analyses were conducted using the Power SYBR Green RNA-to-C_T 1-Step Kit (7500 Fast Real Time–PCR System, Applied Biosystems), according to Li et al. (2015a).

SMARD

The complete SMARD protocol is described in the Supplemental Experimental Procedures.

Statistical Analyses

Statistical analyses were conducted using GraphPad Prism 6. Statistical significance was determined by performing Student's t test and $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.075.

AUTHOR CONTRIBUTIONS

J.G. and M.N. conceived and designed the study. J.G., A.D.B., and J.S.B. performed the experiments and analyzed the results. P.B.D. and J.W.P. generated and provided the polyamide. J.G., J.S.B., and M.N. wrote the manuscript. A.D.B. and Z.R. edited the manuscript.

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