PostDoc Journal
Vol. 4, No. 5, May 2016

Journal of Postdoctoral Research
www.postdocjournal.com

Fanconi anemia pathway regulates convergent transcription-induced cell death at trinucleotide repeats in human cells

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Abstract Almost 20 incurable neurodegenerative disorders are caused by trinucleotide repeat (TNR) expansion beyond a certain threshold, with disease time of onset and severity positively correlating with repeat length. Typically, long TNRs display a bias toward further expansion and repeats continue to expand not only during germline transmissions from parents to offspring, but also remain highly unstable in somatic tissues of patients. Hence, understanding TNR instability mechanisms sheds light on underlying disease pathology. Recently, we showed that activated ATR (ataxia-telangiectasia mutated [ATM] and Rad3 related) is the major signal for convergent-transcription-induced cell death at CAG repeats and is regulated by the mismatch repair (MMR) pathway. Additionally, components of other DNA repair pathways such as transcription-coupled nucleotide excision repair (TC-NER) and R-loop resolution by RNaseH (ribonuclease H) reduce cell death. Because activated ATR signals the Fanconi anemia (FA) pathway for repair of interstrand DNA crosslink, we asked whether the FA pathway also modulates convergent-transcription-induced cell death at expanded CAG repeats. We show here that siRNA knockdown of FA components—FANCI, FANCJ, FANCM, FANCA, and FANCD2—decreases cell death, suggesting that FA proteins, like MMR proteins, are activators of cell death during convergent transcription.

Keywords: Fanconi anemia, convergent transcription, trinucleotide repeat instability, neurodegenerative diseases, cell death

Introduction

Trinucleotide repeats (TNRs) are hypermutable microsatellite sequences, capable of gaining or losing repeat units at a high frequency, a phenomenon referred to as repeat instability [1]. TNRs are distributed throughout genes, in exons, introns, and 5' and 3'-UTRs [2]. Normally, variations in TNR tract length—repeat instability—acts to fine tune gene expression with attendant evolutionary benefits [3, 4]. However, their continued expansion beyond a certain threshold results in neurodegenerative disorders [5]. Repeat instability occurs in both the germline and somatic tissues of affected individuals, where their continued instability in different tissues exacerbates disease symptoms [6-10]. Disease-associated TNRs tend to form intramolecular secondary structures during DNA metabolic processes, and these structures then become the substrates for toxic DNA damage responses. Therefore, understanding the underlying molecular mechanisms that drive instability at expanded repeats will shed light on disease pathogenesis.

In the past, studies in model organisms have suggested that a range of DNA metabolic processes such as replication, transcription, DNA repair, genome wide demethylation, and rereplication modulate TNR instability [7, 11-17]. Additionally, we showed that transcription in one direction across long CAG repeats and transcription in both directions (convergent

transcription) induce repeat instability in human cells, and that DNA repair pathways play a vital modulatory role [12, 18]. More importantly, convergent transcription across expanded repeats was also found to cause a synergistic increase in cell death, which we have termed DNA toxicity to emphasize the role of the repeat DNA sequences in cell death. Convergent transcription also induces DNA toxicity across other expanded TNRs, including GAA, CGG, and CCTG, signifying the commonality of repeat-dependent, convergent-transcription-induced cell death [19]. Because convergent transcription is common in the human genome, including many TNR disease genes [20-24], DNA toxicity may contribute to neuronal cell death in neurodegenerative patients.

Previously, we showed that during convergent transcription at long CAG repeats, ATR (ataxia-telangiectasia mutated [ATM] and Rad3 related) DNA damage response is activated and is the key mediator of cell death [18]. Additionally, we found that transcription-coupled nucleotide excision repair (TC-NER) and R-loop resolution enzymes lower DNA toxicity of convergent transcription, whereas MMR components increase cell death by activating ATR DNA damage response during convergent transcription [25-28]. Because activated ATR is an important trigger for the Fanconi anemia (FA) pathway to repair interstrand DNA crosslinks [29], we asked whether FA components could also modulate convergent-transcription-induced cell death at CAG repeats.

FA pathway comprises 19 distinct functional complementation groups that collaborate to repair interstrand DNA crosslinks [30]. Mutations in FA genes result in a chromosomal instability disorder, which in patients is characterized by developmental deformities, bone marrow failure, and cancer predisposition [31]. In response to crosslinking agents such as diepoxybutane, mitomycin C, and cisplatin, the FA core complex (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM and FANCT) forms at the damage site [30]. This complex enhances ATR activation by localizing ATRIP (ATR interacting protein) to the damage site [32]. Activated ATR phosphorylates FANCI protein, which is the major switch for the FA pathway [33]. Phosphorylated FANCI enables monoubiquitination of FANCD2, which in turn maintains FANCI monoubiquitination [34]. Together, the FANCI-D2 complex enables incision and recruitment of downstream recombinational and nucleolytic proteins (FANCD1, FANCJ, FANCN, FANCO, FANCP, FANCQ, FANCR and FANCS), along with a translesion synthesis enzyme to complete the repair [35-37].

In this study, we show that knockdown of FA components—FANCI, FANCJ, FANCM, FANCA, and FANCD2—during convergent transcription across CAG repeats suppresses cell death. This novel result suggests that the FA pathway normally is involved in enhancing convergent-transcription-induced cell death at CAG repeat tracts.

Materials and Methods

Cell Lines and Culture. DIT7 cells used in this study were derived from HT1080 via the intermediate RS11 cell line, as described previously [18]**.** Briefly, DIT7 cells carry a single integrated copy of an *HPRT* (hypoxanthine-guanine phosphoribosyltransferase) minigene that carries a CAG_{95} tract in its intron and is flanked by the promoters pTRE-CMV^{mini} and pNERB-X1, which drive sense and antisense transcription, respectively. The doxycycline-inducible pTRE-CMV^{mini} promoter contains a downstream binding site for the rtTA protein, which is a fusion construct of reverse tetracycline repressor and the HSV VP16 transcription activation domain. The pNERB-X1 promoter is inducible by RSL1 (Rheoswitch ligand 1, NEB) (Figure 1). DIT7-R103 containing 15 repeat units was derived from DIT7 by spontaneous contraction of its 95 repeat units. The cells were grown at 37°C with 5% CO_2 in DMEM/F-12 medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone, Thermo Scientific) and 1% MEM nonessential amino

acids (Gibco). 0.25% trypsin-EDTA (Gibco) was used for passaging.

Experimental Outline and siRNA Treatments

We tested the effects of the FA pathway on cell death induced by convergent transcription through CAG repeats by using siRNA-mediated knockdown of selected FA genes. In the experimental protocol, convergent transcription was initiated at day 0 by addition of the inducers doxycycline and RSL1 to the medium at a concentration of 2 μg/mL and 500 nM, respectively. Seventy-two hours prior to that start point (day –3), 100,000 DIT7 or DIT7-R103 cells were plated in each well of 6-well plates. Twenty-four hours after plating (day –2), siRNAs were added to a final concentration of 200 nM using Oligofectamine (Invitrogen), per the manufacturer's protocol. For each gene knockdown, a target-specific siRNA and a control vimentin siRNA (which has no effect on cells) were mixed and added to a final concentration of 100 nM each. The control knockdown consisted of vimentin siRNA at a final concentration of 200 nM. The siRNA sequences (Dharmacon Thermo Scientific) used in this study are listed in Table 1. A second round of siRNA treatment was administered on day 0 at the same time convergent transcription was induced by addition of inducers. From day 1 through day 4, additional doxycycline was added at a concentration of 1 μg/mL because the half-life of doxycycline is 24 hours. No additional RSL1 was added after day 0. Knockdown efficiencies were evaluated by isolating RNA on day 1 and measuring percentage knockdown by real time RT-PCR, as described previously [38]. At least 70% reduction of gene expression was achieved by targeted siRNA treatment.

Table 1: Sequences of siRNAs used for gene knockdown

Gene	siRNA Sequence
Vimentin	GAAUGGUACAAAUCCAAGU
FANCI	CTGGCTAATCACCAAGCTTAA
FANCJ	GUACAGUACCCCACCUUAU
FANCA	GGAAGATATCCTGGCTGGCACTCTT
FANCM	AAGCTCATAAAGCTCTCGGAA
FANCD2	AATAGACGACAACTTATCCATCACC

Dead Cell Measurements

Dead cell percentages were calculated by dividing the number of cells that did not adhere to the plate (that is, the floating dead cells), by the total number of cells (the sum of adherent and nonadherent cells). Nonadherent cells were determined by Coulter counting of cells in the medium from gently rinsed plates; adherent cells were counted after trypsinization of cells that were attached to the plates. Adherent cells are defined as viable cells, and nonadherent cells are the dead cells [18]. Previously, we confirmed the viability of adherent cells by using propidium iodide (a dye retained in dead cells only), which was incorporated in <4% of adherent cells, indicating the >96% of the adherent cells were viable [18]. The very small percentage of dead cells present in the adherent population was ignored in the final calculations. Both floating and adherent cells were counted on day 5.

Statistics

In all experiments, Student's *t* test was used to determine the statistical significance of the differences between the FA gene knockdowns and the control vimentin knockdowns, using means and standard deviations in the calculations.

Results and Discussion

In this study we used two human cell lines—one carrying an HPRT minigene with 95 CAG units in its intron (DIT7), and the other, with 15 CAG units (DIT7-R103)—to test the effects of convergent transcription on CAG repeat tracts. The modified HPRT minegene has a doxycycline-inducible cytomegalovirus (CMV) promoter pTRE-CMV^{mini} that controls sense transcription from 5' side and a RSL1-inducible pNEBRX1 promoter controls antisense transcription from 3' side (Figure 1). Transcription induction at both the sense and antisense promoters causes a synergistic increase in cell death at diverse TNRs, triggered by an activated ATR DNA damage response [18, 19, 39]. DNA repair pathways modulate this DNA toxicity effect, with an additional role of the MMR pathway in regulating ATR activation during convergent transcription [13, 28].

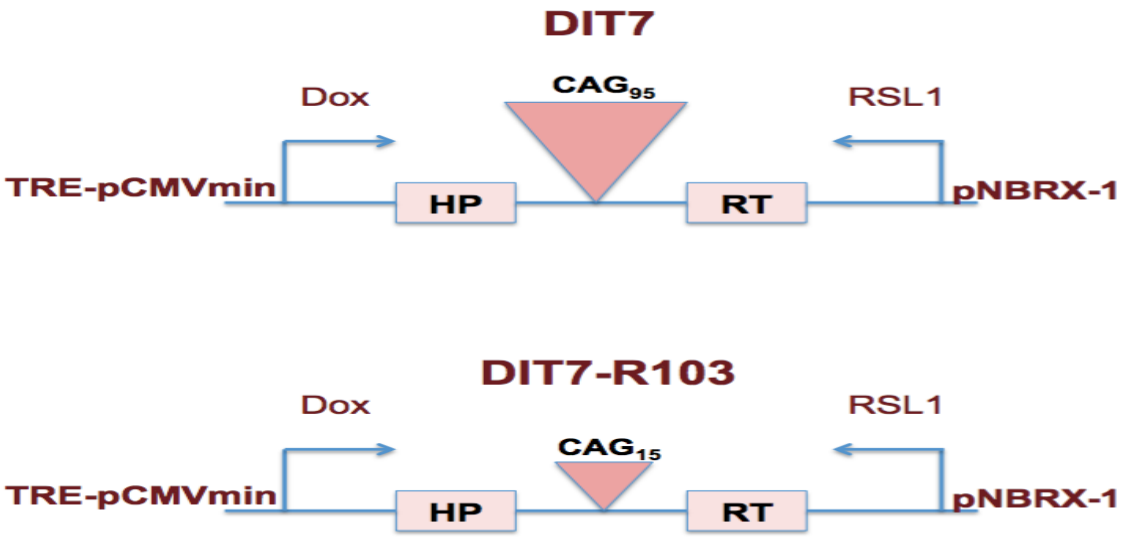

Figure 1. Schematics of the *HPRT* minigenes in the DIT7 and DIT7-R103 cell lines. The *HPRT* minigene in DIT7 cells and DIT7-R103 cells carry 95 and 15 units of CAG repeat tract respectively within the 2.1 kb long intron. The promoters pTRE-CMV^{mini} in the sense direction and pNEBR-X1 in the antisense direction are regulated by doxycycline and RSL1, respectively.

Typically, ATR activation entails the presence of RPA coated ssDNA or unligated nicks, along with activating factors such as ATRIP, the 9-1-1 complex (Rad9-Hus1-Rad1), and TopBP1 (topoisomerase II binding protein 1). Once activated, ATR phosphorylates downstream proteins—Chk1 (checkpoint kinase 1), Nbs1 (nibrin), SMC1 (structural maintenance of proteins 1), and p53 (tumor protein p53), thus enabling repair, cell cycle arrest, and apoptosis [40-43]. In addition, activated ATR is also the major switch activating FANCI by phosphorylation in instances of interstrand crosslink DNA damage [33]. Phosphorylated FANCI triggers FANCD2 monoubiquitination and together, as the FANCI-D2 complex, initiate the Fanconi anemia repair pathway.

Because activated ATR could potentially activate the FA cascade at CAG repeats, we sought to establish the role of FA pathway during convergent-transcription-induced cell death at CAG repeats, using siRNA-mediated knockdowns of key FA components. Initially, we tested the effects of knockdowns of FANCI and FANCD2, the two components of the FANCI-D2 complex, which instigates downstream repair, following assembly of the FA core complex. We found that FANCI knockdown and FANCD2 knockdown each significantly reduced the level

of cell death induced by convergent transcription in DIT7 cells and in DIT7-R103 cells (Figure 2 and 3; Table 2). These results indicate that the FANCI-D2 complex normally acts to induce cell death during convergent transcription across CAG repeats. It seems likely that convergent transcription activates ATR kinase, which then phosphorylate FANCI, activating the FA pathway; however, this hypothesis must be tested in future studies.

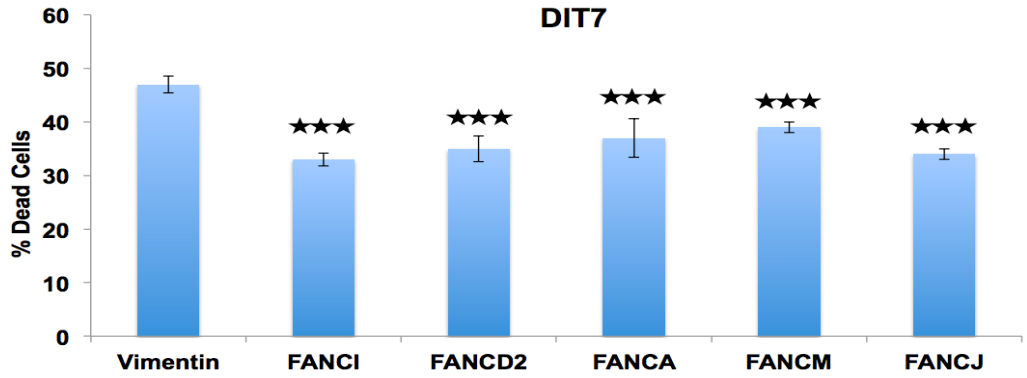

Figure 2. The effect of knockdown of FA pathway genes in DIT7 cells on cell death induced by convergent transcription. The graph shows the percentage of dead DIT7 cells (95 CAG repeat units) generated by convergent transcription following siRNA-mediated knockdowns. The frequencies of dead cells in each case were: vimentin (47%), FANCI (33%), FANCD2 (35%), FANCA (37%), FANCM (39%), and FANCJ (34%). The siRNAs sequences are shown in Table 1. Data are the average of 2 independent siRNA knockdown experiments each with 3 replicates. Error bars represent standard deviations. *P* values are indicated: ★★★P<0.001.

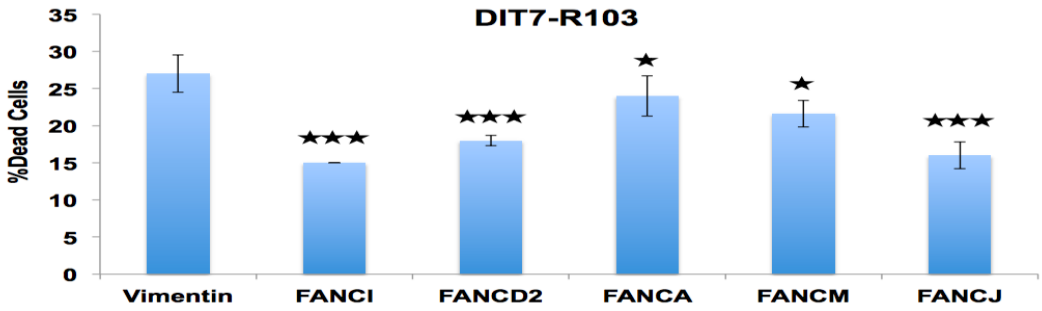

Figure 3. The effect of knockdown of FA pathway genes in DIT7-R103 cells on cell death induced by convergent transcription. The graph shows the percentage of dead DIT7-R103 cells (15 CAG repeat units) generated by convergent transcription following siRNA-mediated knockdowns. The frequencies of dead cells in each case were: vimentin (27%), FANCI (15%), FANCD2 (18%), FANCA (24%), FANCM (22%), and FANCJ (16%). The siRNAs sequences are shown in Table 1. Data are the average of 2 independent siRNA knockdown experiments each with 3 replicates. Error bars represent standard deviations. *P* values are indicated: ★P<0.05, ★★★P<0.001.

Table 2: Decrease in percentage of dead cells after siRNA treatment

siRNA Treatment	Decrease in Cell Death%[a]	
	DIT7	DIT7-R103
Vimentin	0	0
FANCI	30	44
FANCJ	28	42
FANCM	16	20
FANCA	21	12
FANCD2	25	37

Next, we tested the knockdown of two critical FA core components—FANCA and FANCM—to determine whether the FA core complex could also modulate convergent-transcription-induced cell death. It is known that FANCA of the FA core complex enhances binding and localization of ATRIP, which together with RPA (replication protein A) allows activation of ATR-TopBP1 [32]. Thus, if FANCA were to potentiate the ATR pathway, then its knockdown should result in a reduction in cell death from convergent transcription. Similarly, FANCM has a direct role in accumulating RPA and TopBP1 following DNA damage and thus has a role in ATR activation [44, 45]. Moreover, FANCM, in complex with FAAP24 (FA core complex associated protein 24) can signal ATR activation independent of the FA core complex [45, 46]. In this study, we found that knockdown of either FANCA or FANCM in DIT7 and DIT7-R103 cells reduced convergent-transcription-induced cell death (Figure 2 and 3; table 2). These results suggest that the FA core complex normally acts to induce cell death during convergent transcription across CAG repeats.

Finally, we tested knockdown of one of the downstream FA proteins involved in interstrand cross link repair, FANCJ, which is a helicase involved in opening the helix so that other proteins can repair the damage [47]. FANCJ knockdown in both DIT7 and DIT7-R103 cells causes a reduction in cell death from convergent transcription induced cell death (Figure 2 and Figure 3; Table 2), suggesting that the helicase activity of FANCJ may normally act to increase DNA toxicity at CAG repeats during convergent transcription. Whether other proteins in the FA pathway are also involved in enhancing cell death at CAG repeats remains to be tested.

Conclusion

In this study, we have shown that the FA pathway proteins, including the FANCI-D2 complex (FANCI and FANCD2), the FA core complex (FANCA and FANCM), and the downstream repair protein (FANCJ), contribute to convergent-transcription-induced cell death at CAG repeats. This novel result suggests that the FA DNA repair pathway is involved in processing aberrant CAG repeat structures that arise during convergent transcription. Mechanistically, we speculate that MMR-mediated ATR DNA damage response triggers the FA pathway via FANCI, but ATR itself may also influence the FA core proteins—FANCA and FANCM. Once the FA pathway is activated via the FANCI-D2 complex, the downstream proteins such FANCJ may aid in processing the CAG repeats and enhance convergent transcription induced cell death.

Acknowledgements

We thank members of Wilson lab for helpful discussions. This project was supported by a grant from the NIH (GM38219) to J.H.W.

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