FANCM-family branchpoint translocases remove co-transcriptional R-loops

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Abstract

Co-transcriptional R-loops arise from physiological or aberrant stalling of RNA polymerase, leading to formation of stable DNA:RNA hybrids. Unresolved R-loops can promote genome instability. Here, we show that the Fanconi anemia- and breast cancer-associated FANCM protein can directly unwind DNA-RNA hybrids from co-transcriptional R-loops *in vitro*. FANCM processively unwinds both short and long R-loops, irrespective of sequence, topology or coating by replication protein A. R-loops can also be unwound in the same assay by the yeast and bacterial orthologs of FANCM, Mph1 and RecG, indicating an evolutionary conserved function. Consistent with this biochemical activity of FANCM, we show that FANCM deficient cells are sensitive to drugs that stabilize R-loop formation. **Our work reveals a mechanistic basis for R-loop metabolism that is critical for genome stability.**

1   Introduction

2   R-loops form when RNA anneals within duplex DNA, and displaces a corresponding single-

3   strand DNA (ssDNA) patch. R-loops can arise directly, from transcription of difficult to transcribe

4   regions, or by enzyme driven integration of RNA, such as R-loops created by the Cas9 protein

5   during CRISPR (Ginno *et al*, 2012, Szczelkun *et al*, 2014). Persistent R-loops can be a threat to

6   genome stability because the displaced ssDNA within an R-loop is prone to cleavage by

7   nucleases (creating DNA breaks)(Arudchandran *et al*, 2004), recombination with distant DNA

8   sequences (creating chromosome rearrangements)(Huertas & Aguilera, 2003) and atypical

9   modification by ssDNA viral defence proteins such as APOBEC enzymes (creating base

10   substitutions)(Sollier & Cimprich, 2015).

11   Several studies have shown DNA replication-dependent genome instability is partially

12   prevented when the rate of transcription is reduced, indicating that R-loops cause DNA damage

13   predominantly during S phase (Hamperl *et al*, 2017, Helmrich *et al*, 2011, Schwab *et al*, 2015).

14   In particular, the Fanconi anemia (FA) DNA repair pathway is activated by R-loop accumulation,

15   culminating in formation of monoubiquitinated FANCD2 at R-loop rich regions (Garcia-Rubio *et*

16   *al*, 2015, Madireddy *et al*, 2016, Schwab *et al*, 2015). As FANCD2 monoubiquitination is

17   normally activated by direct barriers to the replication machinery (such as DNA double strand

18   breaks or interstrand crosslinks) (Deans & West, 2011), R-loops most likely also activate the FA

19   pathway by blocking DNA replication. In support of this hypothesis, accumulation of

20   monoubiquitinated FANCD2 during normal replication (but in the absence of exogenous DNA

21   damage) is suppressed by over-expression of RNAseH1 (Madireddy *et al*, 2016, Schwab *et al*,

2

22   2015). This nuclease specifically removes DNA:RNA hybrids in the nucleus (Nakamura *et al*,

23   1991).

24   FANCM is a component of the FA pathway that is essential for activation of FANCD2

25   ubiquitination (Coulthard *et al*, 2013), but also has additional FA pathway-independent

26   functions. These include direct remodeling of DNA replication fork structures, recruitment of

27   DNA repair complexes, activation of the ATR checkpoint pathway and suppression of meiotic

28   crossovers (Collis *et al*, 2008, Crismani *et al*, 2012, Deans & West, 2009, Gari *et al*, 2008a). We

29   recently reported elevated formation of R-loops in FANCM-defective cells (Schwab *et al*, 2015).

30   This was not because of increased transcription, but because of failure to properly remove R-

31   loops (Schwab *et al*, 2015). Similarly, R-loops accumulate at telomeres after FANCM depletion,

32   particularly in cells that utilize the ALT pathway of telomere maintenance(Pan *et al*, 2017). As

33   such, FANCM may also prevent accumulation of DNA:RNA hybrids by the TERRA long non-

34   coding RNA, which is essential for maintenance of ALT (Arora *et al*, 2014).

35   FANCM is a functional ortholog of the yeast Mph1 and Fml1 branchpoint translocase proteins

36   (Whitby, 2010). Like these enzymes, FANCM contains an N-terminal SF2 helicase domain whose

37   ATPase activity is activated only by branched DNA molecules (such as those found at stalled

38   DNA replication or transcription bubbles)(Coulthard *et al*, 2013), and can translocate replication

39   forks and Holliday junction DNA structures. Here we show for the first time that FANCM, and its

40   yeast homolog Mph1, are also efficient in R-loop processing. Like for the bacterial RecG protein

41   (Hong *et al*, 1995, Vincent *et al*, 1996), this function depends upon ATP hydrolysis and involves

42   branch migration, to directly remove RNA trapped in co-transcriptionally formed R-loops.

43   FANCM can unwind R-loops of different size and sequence, including a highly stable R-loop

44   formed by transcription across a telomeric repeat. Importantly, we also show that FANCM

45   deficient cells, but not those lacking another FA group member, are highly sensitive to small

46   molecule drugs that promote R-loop formation.

47 **Results**

48 ***In vitro* unwinding of co-transcriptional R-loops by the FANCM-FAAP24 complex**

49 We previously showed that FANCM can unwind DNA:RNA hybrids within a homologous duplex

50 (Schwab *et al*, 2015), a property it shares with other human enzymes such as BLM and

51 replicative helicases (Chang *et al*, 2017, Shin & Kelman, 2006). Because this type of structure

52 may not accurately represent the formation of a co-transcriptional R-loop, we set about

53 establishing a more sophisticated substrate that contains the high GC skew, closed triplex

54 structure and longer length of native R-loops (Ginno *et al*, 2013). To do this we used a pUC19

55 plasmid containing the mouse immunoglobulin class switch recombination sequence (sμ

56 region) in between a T7 promotor and terminator (Supplemental Figure 1a). Using T7

57 polymerase and ^{32}P-UTP we generated co-transcriptional R-loops using techniques previously

58 described by Roy et al (Roy *et al*, 2008), where the formation of R-loops can be measured by

59 both a change in plasmid mobility, and retention of the radiolabeled nascent RNA (Figure 1a).

60 Treatment with RnaseH but not Rnase A led to loss of the R-loop structure on the gel, and loss

61 of signal on autoradiographs, confirming the presence of RNA-DNA hybrids within the plasmids

62 (Figure 1b lane4). RNase H degraded the RNA molecule down to nucleotide sized fragments,

63 however addition of FANCM (in heterodimer with its stabilization partner FAAP24) led to

64 release of the RNA transcript without degradation (Figure 1c). This process was ATP dependent:

65 the R-loop remained intact when wild-type FANCM:FAAP24 was added in the absence of ATP

66 (Figure 1c, lane 3) or when the ATPase dead FANCM mutant complex FANCM[K117R]-FAAP24 was

67 used (Figure 1c). We also tested whether other members of the FA pathway could act on R-

68   loops, given that *in vivo* studies suggest the entire FA pathway (Supplemental Figure 2a) may be

69   required for R-loop regulation (Garcia-Rubio *et al*, 2015, Schwab *et al*, 2015). No component of

70   the FA core complex of proteins, or the FANCI:FANCD2 heterodimer had any direct effect on co-

71   transcriptional R-loops in our assay. Under these experimental conditions only the FANCM-

72   FAAP24 complex can unwind an R-loop structure. (Supplemental Figure 2b).

73   **FANCM-FAAP24 unwinding of co-transcriptional R-loops is processive and not blocked by RPA**

74   **binding**

75   *In vivo*, ssDNA displaced within an R-loop is most likely bound by ssDNA binding proteins such

76   as Replication Protein A (RPA), to protect it from attack by DNA modifying enzymes (Nguyen *et*

77   *al*, 2017). To determine whether FANCM-FAAP24 could still unwind R-loops in which the

78   displaced strand is coated in RPA filament, we incubated plasmid R-loops with molar excess of

79   RPA for 15 minutes prior to adding FANCM-FAAP24. As expected, a uniform shift in

80   electrophoretic mobility was observed when the displaced DNA strand in the R-loop became

81   bound by RPA (Figure 2a). After addition of FANCM-FAAP24, 70% of the RPA bound R-loops

82   were unwound within 5 minutes, whereas 92% of uncoated R-loops were unwound. By 10

83   minutes, almost all of the uncoated R-loops and approximately 85% of RPA bound R-loops were

84   unbound (Figure 2b-c). This observation was not due to addition of extra protein to the reaction

85   because high concentrations of Bovine Serum Albumin (BSA) did not cause an effect

86   (Supplemental Figure 3). Together, these data suggest FANCM-FAAP24 acts in a processive

87   manner on both naked and RPA-coated R-loops, and that RPA has a slight inhibitory effect on

88   the unwinding ability of FANCM.

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90   FANCM-FAAP24 can unwind R-loops of different topology

91   Topological changes in DNA occur during the formation of R-loops, because the bound RNA

92   creates underwound or overwound (supercoiled) regions. Topological effects may both

93   promote R-loop formation during transcription (El Hage *et al*, 2010, Powell *et al*, 2013) or

94   prevent R-loop removal, including by FANCM or other enzymes. To test this, we first assessed

95   whether our purified plasmid R-loops were stable upon induction of topological change. As

96   linearization removes all covalent topology from DNA, we treated our plasmid R-loops with

97   restriction enzymes *Eco*RI and *Hind*III. We found that linearization had no effect on R-loop

98   stability (Figure 3a). Second, R-loops were also stable in a un-super-coiled covalently closed

99   circular (ccc) plasmid generated by *E.coli* Topo1 or human Topoisomerase IIIα:RMI1:RMI2

100   complex, with which FANCM associates in cells (Deans & West, 2009) (Figure 3a). Together,

101   these data show that once formed, transcriptional R-loop structures are stable regardless of

102   changes in DNA topology. These observations and others (Wilson-Sali & Hsieh, 2002) support

103   the idea that type I topoisomerase role in transcription is in prevention of R-loop formation, but

104   not the direct removal of RNA trapped within an R-loop.

105   We next tested the ability of FANCM-FAAP24 to unwind the topologically distinct R-loop forms.

106   In time course assays, FANCM showed no preference in activity towards either supercoiled,

107   linear or ccc R-loops (Figure 3b-c) unwinding all at essentially equal rates. This suggests that

108   DNA topology does not affect R-loop unwinding activity of FANCM against a native R-loop

109   structure.

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FANCM-FAAP24 R-loop unwinding activity is sequence and length independent

112 R-loops accumulate in different regions of the genome including highly transcribed genes, GC-

113 skewed promoters and telomeric repeats (Arora *et al*, 2014, Ginno *et al*, 2012, Powell *et al*,

114 2013). To test whether R-loop processing by FANCM showed any sequence preference, we

115 tested several sequences that were previously demonstrated to be strongly R-loop prone

116 (Ginno *et al*, 2012). These sequences include the human *APOE* or *SNRPN* and mouse *Airn*

117 genomic loci, which we cloned into our *in vitro* R-loops test plasmid (Supplementary Figure 1).

118 These regions were assessed for their percentage of GC skew using *genskew.csb.univie.ac.at*

119 (Figure 4a). All were unwound rapidly and efficiently by FANCM:FAAP24 (Figure 4a). FANCM

120 could also remove R-loops formed by transcription through the telomeric repeat sequence,

121 otherwise known as TERRA transcripts (Figure 4b). These R-loops have previously been shown

122 to have a strong G-quadraplex forming ability (Martadinata & Phan, 2013), suggesting that

123 FANCM's R-loop processing capability extends to R-loops that contain G-quadraplexes either

124 within the displaced strand or between the RNA and the displaced strand (Arora *et al*, 2014),

125 and supports a role for FANCM in the TERRA-dependent maintenance of telomeres by the ALT

126 pathway of telomere maintenance (Lee *et al*, 2014, Pan *et al*, 2017).

127 As R-loops of greater than 600bp can be detected in a cellular context (Ginno *et al*, 2012) we

128 examined whether FANCM-FAAP24 could processively unwind R-loops containing different

129 lengths of RNA. To do this we generated a plasmid containing ~1148bp of repeats of the

130 immunoglobulin class switch sμ sequence (Supplementary Figure 1) downstream of a T7

131   promoter. We compared the size of RNA trapped within these R-loops to those found in the

132   standard R-loop (consisting of a single sμ repeat of 143 bp) by subjecting the purified R-loops to

133   Urea-PAGE. Denaturation revealed multiple RNA species of different lengths within the

134   population of R-loops (Fig.4c). One explanation for this observation is that RNase A could cleave

135   unpaired single strand regions within a longer R-loop, hence making the actual hybrid pieces

136   appear shorter. Alternatively, T7 RNA polymerase could be stalling stochastically at different

137   points along the template. Importantly though, the RNA fragments between the standard and

138   long R-loops gave different average sizes of ~150 and ~300 nucleotides respectively (Figure 4c).

139   We could therefore compare the rate of unwinding on R-loops of similar sequence but different

140   length. Using the defined conditions of this assay FANCM-FAAP24 can unwind 100% of short

141   plasmid R-loops within 5 minutes, with ~55% unwinding occurring within the first minute

142   (Figure 4d). In contrast FANCM-FAAP24 takes 10 minutes to achieve ~85% unwinding of longer

143   plasmid R-loops, a similar rate of unwinding when corrected for length (Figure 4d).

144   Collectively, these data further support the concept that FANCM:FAAP24 traverses long tracks

145   of DNA without dissociation (i.e. processivity), to remove RNA molecules trapped within

146   extended R-loop sequences.

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148   **R-loop displacement is a conserved feature of FANCM family proteins**

149   FANCM protein is a 230kDa protein, with an N-terminal translocase domain, a C-terminal ERCC4

150   structure specific DNA binding domain, and multiple protein:protein interaction domains that

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151   recruit additional DNA repair factors (Blackford *et al*, 2013, Coulthard *et al*, 2013, Deans &

152   West, 2009) (Figure 5a). Only the N-terminal translocase domain is conserved in homologs from

153   lower eukaryotes, such as Mph1 from *Saccharomyces cerevisiae*, and Fml1 from *S. pombe*

154   (Whitby, 2010). RecG proteins are thought to be the closest relatives of FANCM/Mph1/Fml1 in

155   bacteria (Gari *et al*, 2008a, Sun *et al*, 2008). To test whether R-loop unwinding is a conserved

156   feature of these "FANCM family" members we purified recombinant *S.cerevesae* Mph1

157   *S.pombe* Fml1 or *Thermatoga maritima* RecG and tested their activity against our co-

158   transcriptional R-loops (Figure 5b). RecG has previously been demonstrated to unwind

159   synthetic linear R-loops (Vincent *et al*, 1996) but Mph1 and Fml1 have never been tested.

160   Further data, collected using a FANCM fragment (aa 1-800), revealed the N-terminal conserved

161   translocase domain of FANCM is sufficient for *in vitro* R-loop removal (Figure 5c). All of these

162   enzymes displaced the co-transcriptional R-loops with similar activity, indicating a conserved

163   function of FANCM-family proteins.

164   FANCM protein (and Mph1 and RecG (McGlynn *et al*, 1997, Prakash *et al*, 2009)) can also

165   unwind D-loops, which share structural similarities with R-loops (see Discussion). D-loop

166   formation is an intermediate in DNA repair by homologous recombination which requires

167   RAD51 or RecA recombinase. In addition to FANCM-family members, the RecQ helicase BLM

168   (mutated in Bloom's Syndrome) can unwind D-loops (Bachrati *et al*, 2006). We therefore tested

169   BLM for its ability to unwind co-transcriptional R-loops. We carried out the assay with

170   increasing amounts of BLM (Figure 5d). While BLM protein could unwind a very small fraction of

171   the plasmid R-loops at high molar ratios of enzyme to plasmid (highest concentration 40nM, to

172   1nM substrate), it did this very slowly compared to FANCM:FAAP24, which rapidly unwound R-

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173   loops at stoichiometric and sub-stoichiometric concentrations (1nM for Figure 5d). In contrast

174   to its much lower activity towards R-loop substrates, BLM was as efficient as FANCM in assays

175   using a plasmid D-loop substrate (Supplemental Figure 4). These data suggest that BLM is

176   capable of unwinding R-loop structures, but unlike for FANCM, they are not its preferred

177   catalytic substrate.

178   **FANCM KO cells but not FANCL KO cells are sensitive to agents that induce R-loop**

179   **stabilization**

180   We recently demonstrated that FANCM knockout cells, or those expressing a translocation

181   deficient FANCM^{K117R} mutant protein, accumulate excessive R-loops under normal cell culture

182   growth conditions (Schwab *et al*, 2015). Several chemicals have also been shown to increase R-

183   loop prevalence, each by a different mechanism. These include inhibitors of the spliceosome

184   (that promote R-loops through retention of intronic sequences), topoisomerase 1 inhibitors

185   such as topotecan (that promote R-loops by stalling transcription), and reactive aldehydes

186   (unknown mechanism) (Powell *et al*, 2013, Schwab *et al*, 2015, Wan *et al*, 2015). We used

187   isogenic FANCM KO, FANCL KO or parental HCT116 cells (previously characterized by Wang et al

188   (Wang *et al*, 2013)) and tested their sensitivity to increasing concentrations of these R-loop

189   promoting compounds. We found that FANCM deficient cells are particularly sensitive to

190   topotecan and the spliceosome inhibitor pladienolide B, while FANCL-deficient cells are not

191   (Figure 6a-b). Both FANCM and FANCL deficient cells are exquisitely sensitive to acetyl

192   aldehyde, which also generates DNA interstrand crosslink damage in addition to R-loop

193   accumulation.

194   Importantly, sensitivity to both topotecan and pladienolide B correlated with an inflection point

195   in total cellular R-loop levels in response to these drugs. This was measured by slot blot of

196   genomic DNA probed with anti-DNA:RNA hybrid monoclonal antibody S9.6 (Figure 6c-d). R-loop

197   levels and LD50 dosage were highly related, and suggests that cellular viability in response to

198   these compounds correlates with a threshold R-loop level. This maximum tolerated level is

199   reached with lower doses of either topotecan or pladienolide B in FANCM-deficient cells. This

200   result indicates that R-loop metabolism requires FANCM activity independent of the other FA

201   ICL repair proteins, to catalytically unwind R-loops formed under both physiological and drug-

202   induced conditions.

Discussion

203

204   Even though FANCM contains a SF2 helicase domain, exhaustive investigations have never

205   uncovered a direct helicase activity of the protein against any DNA substrate (Coulthard *et al*,

206   2013, Gari *et al*, 2008a, Gari *et al*, 2008b, Meetei *et al*, 2005, Mosedale *et al*, 2005, Whitby,

207   2010, Xue *et al*, 2008). Instead, FANCM is thought to act on junctions in DNA as a branch point

208   translocase (Gari *et al*, 2008a, Gari *et al*, 2008b). In this manner, it utilizes its ATP-dependent

209   motor to reanneal DNA and further displace annealed strands ahead of the junction, without

210   directly acting to unwind DNA like a helicase. This has been proposed for two different DNA

211   structures: (i) stalled replication forks, whereby the annealing of nascent DNA strands by

212   FANCM catalyzes replication fork reversal and the formation of a chicken foot structure and (ii)

213   during recombination and D-loop formation, FANCM can catalyze displacement of the invading

214   structure (Figure 7). Both of these activities have also been described for yeast Mph1 and

215   bacterial RecG (McGlynn *et al*, 1997, Muller & West, 1994, Prakash *et al*, 2009). We propose a

216   similar function for FANCM, Mph1 and RecG at R-loops at stalled transcription complexes. The

217   branchpoint of all three structures, and the catalytic mechanism required for their

218   translocation, is identical. In the case of stalled replication forks, FANCM creates the substrate

219   necessary for resumption of replication (Gari *et al*, 2008a). But for D-loops and R-loops, FANCM

220   is acting to suppress illegitimate recombination and/or barriers to DNA replication and

221   transcription.

222   R-loop branch migration does not appear to be a general property of all translocases and

223   helicases. For example, another SF2 helicase, BLM, can displace RNA from the R-loop, but only

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224 weakly when used at high concentration; similar to observations made for the bacterial enzyme

225 RuvAB (Vincent *et al*, 1996). Like RuvAB, BLM is able to act to directly unwind DNA:RNA

226 heteroduplexes constructed from synthetic oligonucleotides (Chang *et al*, 2017, Popuri *et al*,

227 2008), but these contained heterologous base-pairing, or extensive ssDNA regions that are not

228 present in "native" co-transcriptional R-loops. The weak activity of BLM at high enzyme to

229 substrate ratios (40:1, Figure 5d) on co-transcriptional R-loops, is probably the result of

230 "accidental" helicase activity with the R-loop as the protein moves along double stranded DNA

231 (Cheok *et al*, 2005), rather than the direct branch migration mechanism proposed for FANCM,

232 Fml1, Mph1 and RecG. Future work should also compare the activity of FANCM to that of other

233 proposed R-loop metabolizing enzymes such as Senataxin (Yuce & West, 2013), Aquarius

234 (Sollier *et al*, 2014) and DHX9 (Chakraborty & Grosse, 2011). It should be noted that none of

235 these enzymes in purified form have been directly tested for activity on co-transcriptional R-

236 loops.

237 But what is the nature of R-loops that are acted upon by FANCM? R-loops can form at multiple

238 different loci. Indeed, mathematical modeling suggests that every transcribed region is able to

239 form an R-loop, but that increased observation at some loci comes from the fact that particular

240 sequences are more prone to their formation, such as G-rich sequences (Belotserkovskii *et al*,

241 2017). Another consequence paradoxically, is that within a cell population, highly transcribed

242 genes are more likely to be discovered in an R-loop-bound, arrested state. In this study, we

243 have shown that FANCM can displace RNA from all co-transcriptional R-loops tested, including

244 those that are long and those with very high G-content. FANCM therefore has the potential to

245 act upon any R-loop in the genome. This might include R-loops required to initiate class switch

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246   recombination at the immunoglobulin heavy chain locus in activated B cells, which regulates

247   immune function and occurs during G1 phase of the cell cycle (Schrader *et al*, 2007). Some FA

248   mouse models show minor class switching defects (Nguyen *et al*, 2014), although *Fancm-*

249   deficient mice have not been tested in this respect (Singh *et al*, 2009). We favor the hypothesis

250   that FANCM's R-loop regulation activity concerns their removal ahead of, or encountered by,

251   the replication fork. This is because overwhelming evidence points to a role for the Fanconi

252   anemia pathway in DNA damage during S phase (Deans & West, 2011) and the fact that FANCM

253   protein is enriched in chromatin at sites of ongoing replication (Blackford *et al*, 2013, Castella *et*

254   *al*, 2015). But DNA:RNA hybrids were also recently shown to form by transcription from dsDNA

255   breaks during resection, when RNA polymerases become loaded onto broken DNA ends (Ohle

256   *et al*, 2016). FANCM may play some role in displacing such hybrids in prevention of over-

257   resection, another phenotype of FANCM-deficient cells (Blackford *et al*, 2013).

258   Finally, the maintenance of telomeres is also regulated by R-loops in some cancers (Azzalin *et*

259   *al*, 2007). TERRA transcripts are produced by transcription of the C-rich telomeric DNA strand

260   and are essential for the ALT mechanism of telomere maintenance by recombination. FANCM is

261   necessary for ALT (Pan *et al*, 2017), and it is possible that this is because it acts on TERRA

262   transcripts to permit telomere replication or somehow regulate telomere recombination. Our *in*

263   *vitro* experiments demonstrate that FANCM displaces the G-quadraplex stabilized TERRA

264   transcripts as efficiently as it acts on promoter- or switch-region- based R-loops.

265   While several physiological R-loops may be targeted by FANCM activity, it is also clear that

266   chemically induced (or pathological) R-loops accumulate more rapidly, and for longer periods,

267 in FANCM-deficient cells (Figure 6). In particular, R-loop promoting inhibitors of topoisomerase

268 1 or splicing promote R-loop accumulation at lower concentrations in FANCM-knockout cells,

269 and this correlates with increased cell death induced by these drugs. Camptothecin sensitivity

270 was also observed in cells from *Fancm*-deficient mice (Bakker *et al*, 2009), Mph1 deficient yeast

271 (Scheller *et al*, 2000) and RecG deficient bacteria (Sutherland & Tse-Dinh, 2010). Such sensitivity

272 is not a common property of homologous recombination deficiency, but is seen for a subset of

273 HR proteins that also play a role in R-loop metabolism, such as BRCA1 and BRCA2 (Bhatia *et al*,

274 2014, Hill *et al*, 2014). Recent evidence suggests that transcription replication collisions

275 promoted by splicing inhibitors or topoisomerase poisons could provide a major mechanism for

276 the therapeutic action of these drugs (Sollier & Cimprich, 2015). As such, FANCM-deficiency or

277 FANCM-overexpression could modulate the tumour and normal cellular response to these

278 drugs in clinical use.

279 In conclusion, our biochemical reconstitution of co-transcriptional R-loop formation has

280 established branch point translocation as a major mechanism of R-loop release. Given the

281 emerging role of R-loops in cancer (Sollier & Cimprich, 2015, Stork *et al*, 2016), it is possible that

282 a defect in FANCM mediated R-loop metabolism is directly responsible for the tumour prone

283 phenotype of *FANCM*-associated Fanconi anaemia (homozygous mutations) and familial breast

284 cancer (heterozygous carriers). The highly processive *in vitro* activity of FANCM, and strong R-

285 loop phenotype after treatment with various chemotherapy drugs is consistent with an

286 important role for processing of R-loops by FANCM in human disease.

287

Methods

Co-transcriptional R-loop plasmid design and construction

Co-transcriptional R-loop sequences for the human APOE or SNRPN promoter sequences or murine µ-switch repeat or AIRN promoter sequences were synthesized with a 5'-flanking T7 promotor and 3'-T7-terminator sequence and cloned into the EcoRI and HindIII sites of pUC19 (followed by sequence verification) by General Biosystems (Sequences are provided in Supplemental Figure 3). To generate the longer R-loop plasmid (pUC19 SR long), successive rounds of restriction cloning was undertaken to concatermerize the µ-switch repeat through repetitive subcloning of AvrII/HindIII digested fragments into SpeI/HindIII digested pUC19-SR. Plasmids were transformed into NEB 10-beta cells (NEB), plated and midi prepped (Qiagen). DNA concentration was established by nanodrop. R-loop forming plasmids have been deposited at Addgene. pcDNA6-Telo and TeloR contain a 0.8 kb fragment of human telomeric repeat cloned downstream of a T7 promoter (Arora *et al*, 2014).

R-loop generation and purification

2µg of plasmid DNA was incubated in a final reaction volume of 200µl containing 1x T7 polymerase reaction buffer (NEB), 25 units of T7 polymerase (NEB), 2.25mM of each nucleotide CTP, GTP, ATP and 825nM UTP-α-^{32}P 3000 Ci/mmol (Perkin Elmer) for 1 hour at 37°C. The reaction was stopped by heat denaturation at 65°C for 20mins. 100µl of 1.05M NaCl and 0.03M MgCl$_2$ buffer was added to each reaction plus 2.5µg of Rnase A (EpiCentre) for 1hr at 37°C. R-loops where then purified by 2x phenol/chloroform using phase lock tubes (Quanta Bio), precipitated in a final concentration of 0.3M Na Acetate and 70% ethanol at -20°C overnight. Next day the samples were centrifuged at 13,000 *x g* in table top centrifuge for 30min.

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310   Supernatant was removed and samples were washed with 70% ethanol and centrifuged for a

311   further 10min. Supernatant was removed and pellets were left to air dry. R-loops were

312   resuspended in 10mM Tris pH8, then ran through 2x S-400 columns (GE Healthcare) to remove

313   unincorporated nucleotides, quantified using nanodrop and stored at 4°C.

314   **Protein Purification**

315   The following were purified as previously described: FLAG-FANCM-FAAP24 and FLAG-

316   FANCM$_{K117R}$-FAAP24 (Coulthard *et al*, 2013), RPA (Henricksen *et al*, 1994), RecG (Singleton *et al*,

317   2001) (a kind gift of Steve West, Francis Crick Institute), Flag-BLM, Flag-Mph1 and Flag-

318   Topoisomerase IIIα-RMI1-RMI2 expression vectors were cloned into pFL or pUCDM baculovirus

319   vectors and subsequently integrated into the Multibac Bacmid (Berger *et al*, 2004). For these

320   proteins, 1L High 5 *Trichoplusia ni* cells (1 x 10^6/ml, Invitrogen) were infected with virus

321   (MOI=2.5). Cells were harvested 72 hours after infection at 500 x *g*, 4°C and pellets washed with

322   1xPBS. Cells were lysed on ice in 0.5M NaCl, 0.02M TEA pH 7.5, 1mM DTT, 10% glycerol plus

323   mammalian protease inhibitors (Sigma P8340-5ml) and sonicated on ice 5x 10 sec bursts.

324   Lysates was clarified by centrifugation at 35,000G for 40 minutes at 4°C. Clarified supernatant

325   was then incubated with equilibrated Flag M2 resin (Sigma) for 1hr on a roller at 4°C. Flag resin

326   was then subjected to 4x batch washes with lysis buffer (without mammalian protease

327   inhibitors) with 5 minutes on a roller at 4°c between each spin. The resin was then placed into

328   gravity flow column for a final wash and protein was eluted with 100μg/ml Flag peptide. Flag-

329   Mph1 and Flag-FANCM-FAAP24 complexes were subjected to further purification by ssDNA

330   affinity resin (Sigma): Flag elutions containing FANCM were pooled and diluted to have a final

331   concentration of 100mM NaCl, 20mM TEA pH7.5, 10% glycerol, 1mM DTT (buffer B) and added

332   to 400µl ssDNA resin overnight on a roller at 4°C. The resin was then placed down gravity flow

333   column and washed with 10CV of buffer B. FANCM-FAAP24 complexes were eluted with buffer

334   B containing 0.5M NaCl. Proteins were quantified using BSA titrations on SDS-PAGE gels. All

335   proteins were flash frozen in their final buffers and stored at -80°C. Topoisomerase I from *E.coli*

336   and all restriction enzymes were purchased from New England Biolabs.

337   **R-loop unwinding assays**

338   R-loop unwinding reactions (10µl) contained 1nM of R-loop, 1mM ATP, 2µl of protein (protein

339   concentrations stated in main text) in R-loop buffer (6.6mM Tris pH7.5, 3% glycerol, 0.1mM

340   EDTA, 1mM DTT, 0.5mM MgCl$_2$) and incubated at 37°C for time as shown in figures. Reactions

341   were stopped by adding 2µl of stop buffer (10mg.ml^{-1} proteinase K (NEB), 1% SDS) and

342   incubated for 15min at 37°C. 2µl of 50% glycerol was added to samples prior to loading onto 1%

343   or 0.8% agarose TAE gels, run at 100V in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA)

344   for 60-90 mins. Gels were then crushed between precut biodyene B membranes (Pall) for

345   1hour, exposed overnight to a GE phosphor-screen and imaged on a Typhoo scanner (GE

346   Biosciences). To visualize DNA, agarose gels were post stained with Sybr gold (Thermofisher) 1

347   in 10,000 in TAE.

348   Quantification of R-loop unwinding was performed using Image J and Prism software.

349   **Cell based assays**

350   HCT116- or FANCM-/- and FANCL-/- derivatives were provided by Lei Li (University of Texas MD

351   Anderson). Cell lines were authenticated by G-banding (St Vincent's Cytogenetics) and

352   maintained in DMEM + 10% fetal bovine serum at 37'C, 5% CO$_2$ in a humidified chamber. For

353   drug sensitivity assays, cells were plated in 96-well plates at 1,500 cells/well, then treated 24hrs

354   later with various concentrations of topotecan (aka Hycamptin®, GSK) or pladienolamide B

355   (Calbiochem). After 72hr, survival was measured using sulforhodamine B assay read at 550nm

356   on a EnSpire Plate reader (Perkin Elmer).

357   To measure total cellular R-loop levels, HCT116 cells were treated with drug or vehicle for 4hr.

358   Total genomic DNA was extracted using Isolate II kit (Bioline). 1µg of genomic DNA was slot

359   blotted, using a BioRad Microfiltration apparatus, onto Biodyne B Nylon membrane (Thermo

360   Fisher), which was then air-dried and blocked in Odyssey blocking buffer (LiCor). The membrane

361   was then probed with 0.5µg/mL S9.6 anti-DNA:RNA monoclonal antibody (produced and

362   purified in house from S9.6 hybridoma (ATCC)) and 10ng/ml anti-ssDNA (F7-26, Millipore).

363   Atto800-anti-mouse (LiCor) and Cy5-conjugated anti-IgM antibody (Millipore) were used to

364   visualize the level of DNA:RNA hybrids and total DNA detected by the primary antibodies, and

365   visualized and quantified using Odyssey LiCor dual color imaging system and accompanying

366   software.

367

Author contributions

369   Conceptualization, C.H., J.J.O, and A.J.D.; Methodology, C.H., J.J.O., S.v.T., V.J.M., and A.J.D.;

370   Investigation, all authors.; Writing - Original Draft, C.H. and A.J.D..; Writing - Review & Editing

371   C.H., J.J.O, E.D. and A.J.D.; Funding Acquisition, A.J.D.; and Supervision, C.H., and A.J.D.

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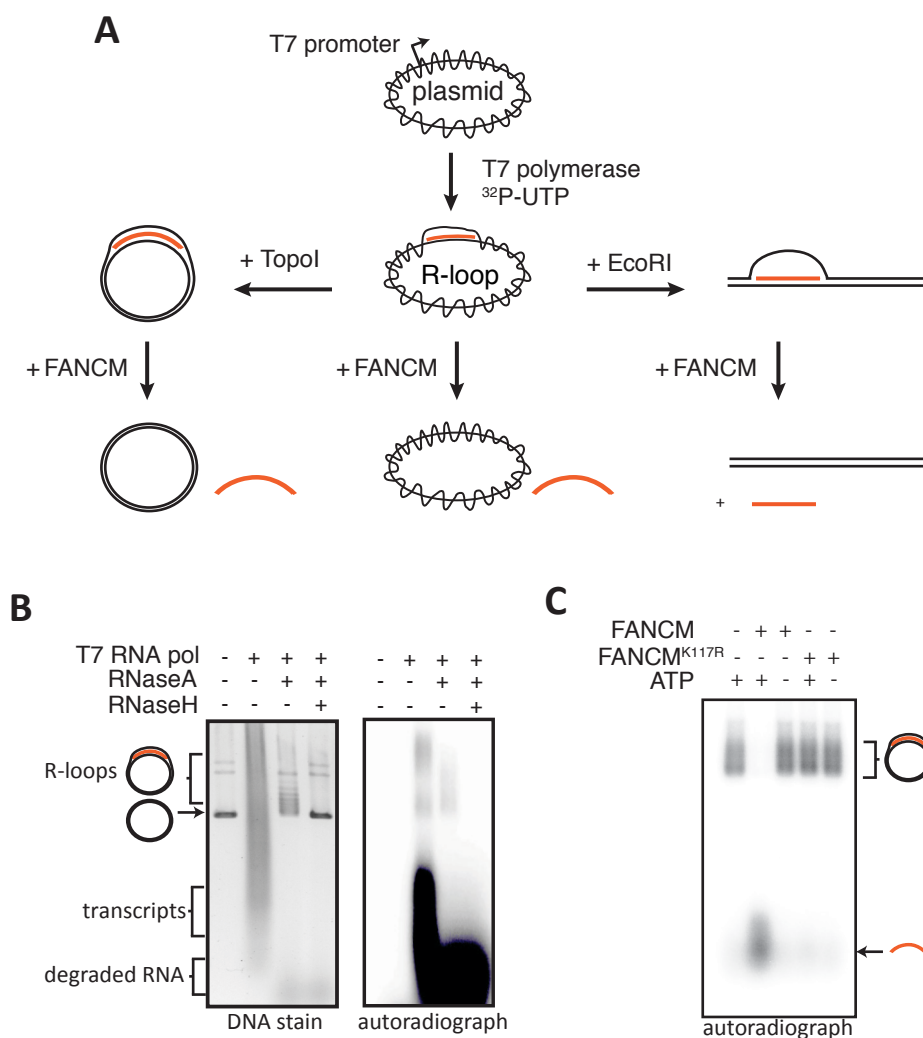549 Cell Biol* 33: 406-17
550

Figure 1: Generation of co-transcriptional R-loops in vitro and their processing by FANCM-FAAP24. A) Schematic of method used to generate and unwind R-loops of different topological states. DNA is coloured black and RNA orange. B) Plasmid based R-loops observed by gel electrophoresis. Sybr gold stain of plasmid DNA molecules reveals the topological changes to the plasmid DNA upon R-loop formation. Right panel is an autoradiograph identifying the 32P-UTP incorporation into R-loop, transcripts or post-RNAse treatment. C) Autoradiograph showing FANCM-FAAP24 (1nM) unwinding purified R-loops (1nM) in an ATP dependent manner. FANCM^{K117R} is a translocase activity deficient mutant.
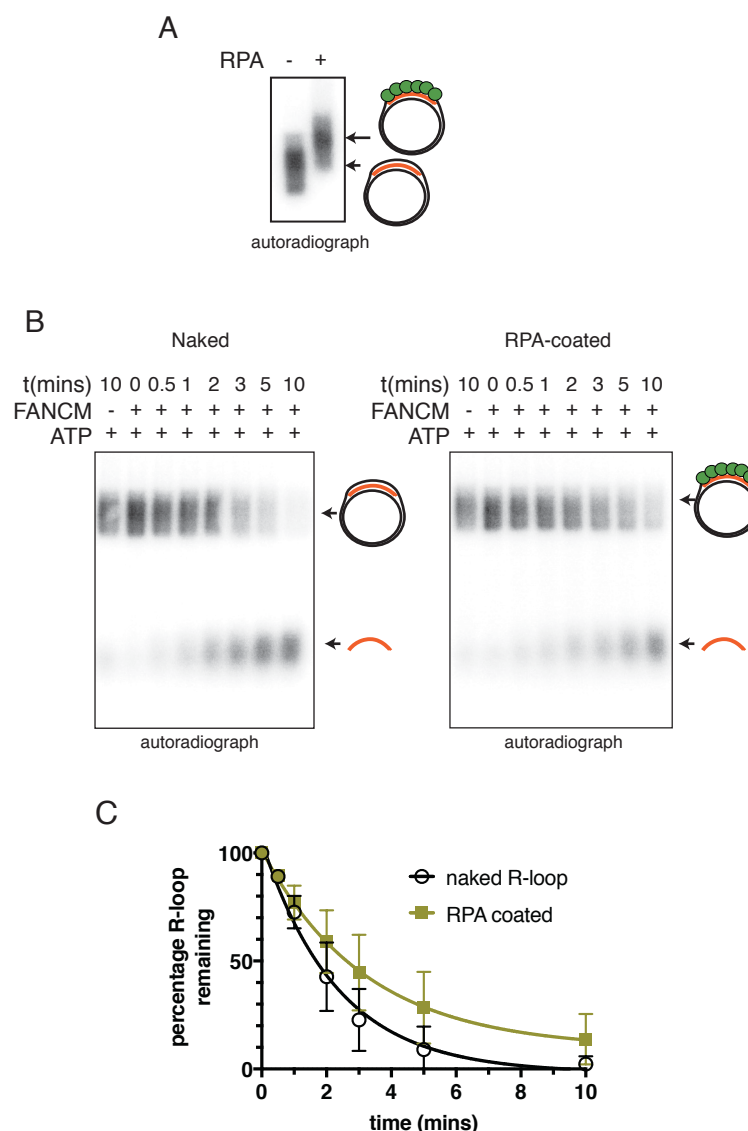
Figure 2: FANCM-FAAP24 acts on protein coated R-loops.
A) Autoradiograph of an EMSA showing RPA bound to plasmid based R-loops. B) Representative Autoradiographs of time course assays of FANCM (0.25nM) activity on naked R-loops (1nM, left panel) versus RPA coated R-loops (1nM right panel) C) Quantification of unwinding activity (± stderr) from n>3 experiments.

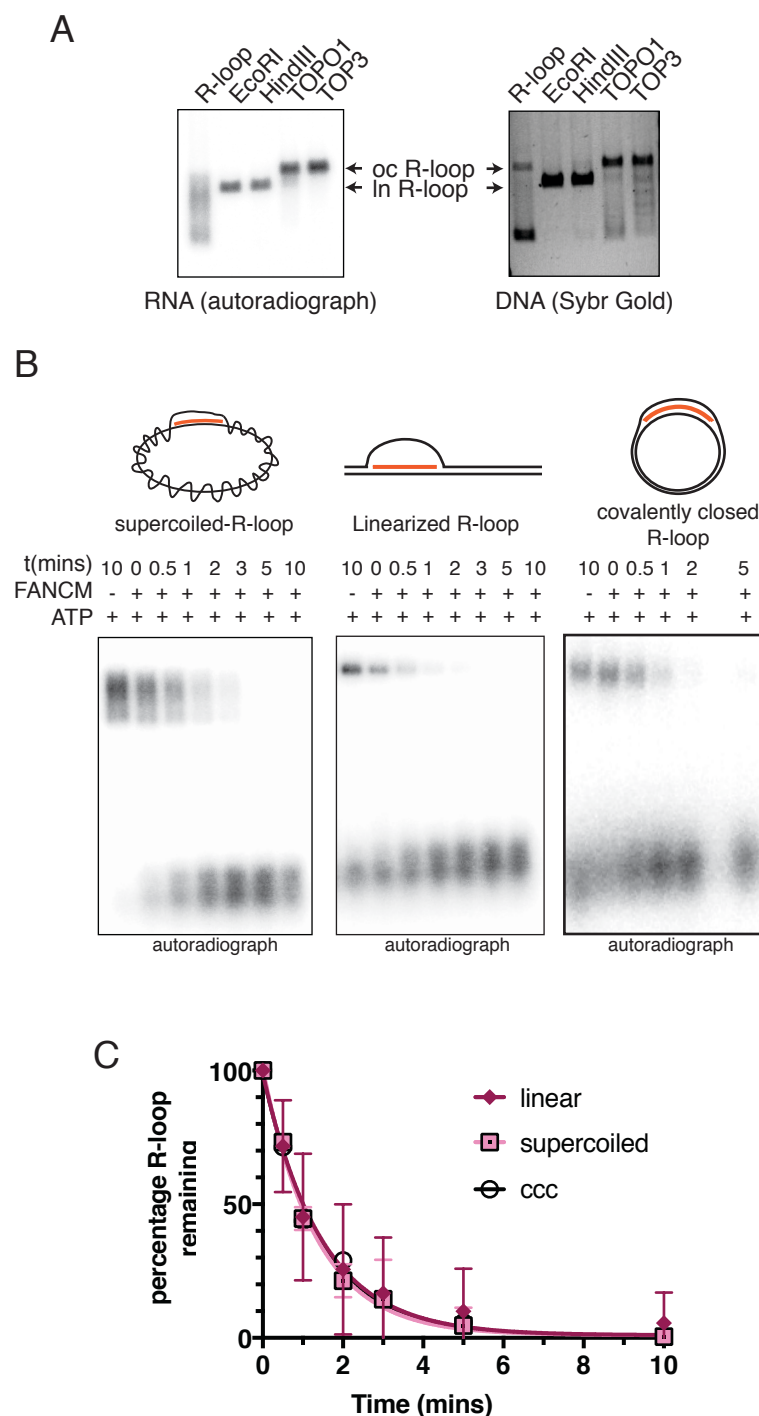**Figure 3: FANCM unwinds R-loops with different DNA topologies**

A) Characterization of topological isoforms of plasmid based R-loops after generation of linear form by restriction enzyme digestion by EcoRI or HindIII, or covalently closed circle form (ccc) by topoisomerases 1 or III treatment. Left panel is an autoradiograph depicting the RNA molecules. The right panel is sybr gold stain of the DNA molecules. B) Autoradiographs of time course assays of plasmid (left panel) versus linear (middle panel) based R-loops. The graph is the average (n=3) % of R-loop unwound (y-axis) of each time point (x-axis) with standard error bars. Final concentrations of R-loops and FANCM for these assays was 1nM and 0.25nM respectively.
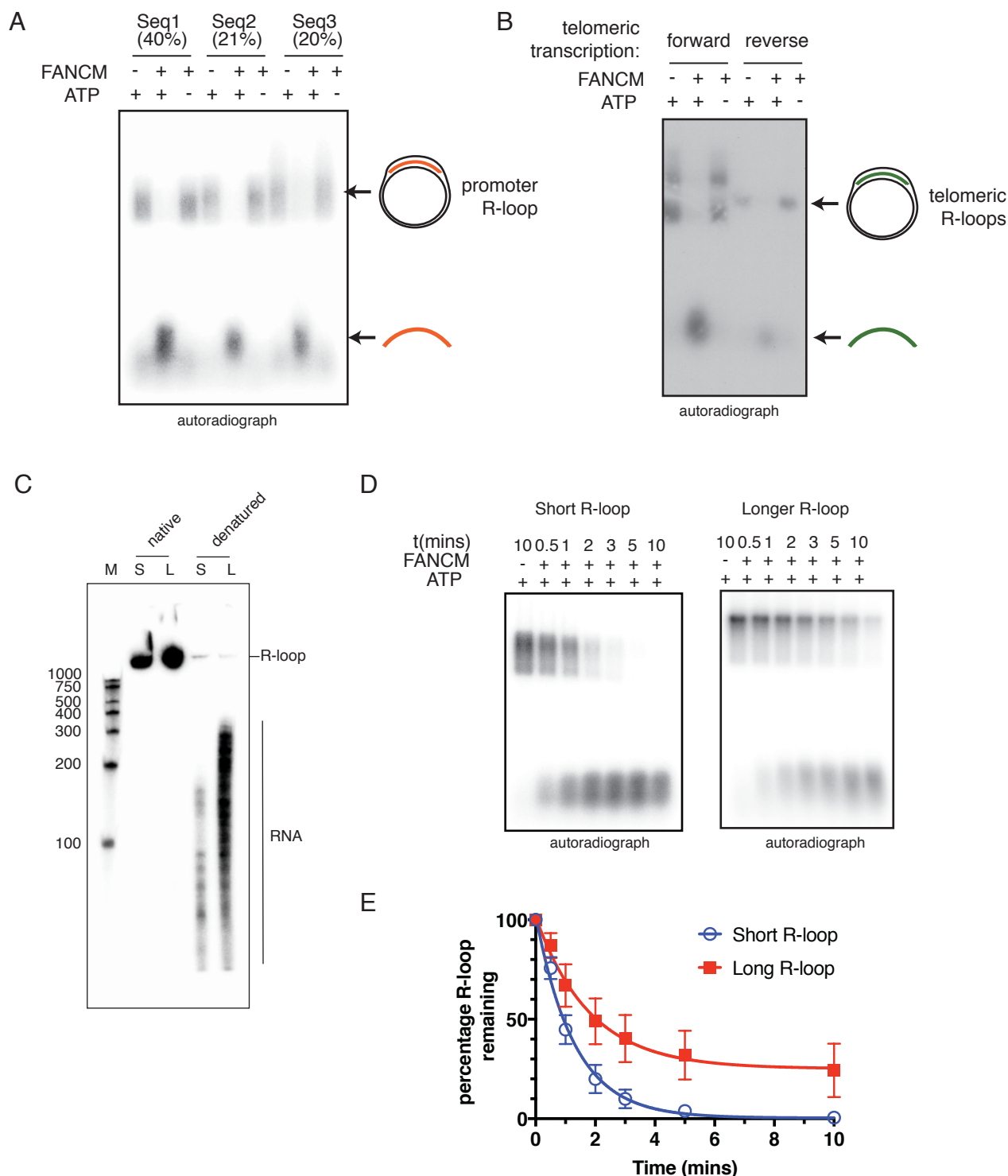
Figure 4: FANCM-FAAP24 can process R-loops irrespective of sequence, length or genomic origin. A) R-loop processing assay with three independently derived R-loop forming sequences. The GC skew % of each sequence is indicated in brackets above the autoradiograph. B) R-loops can be formed by *in vitro* transcription in a forward or reverse direction through a 1.4kb telomeric repeat sequence. FANCM:FAAP24 was tested against both products. C) R-loops of similar sequence but different length were generated (see materials and methods) and shown to produce trapped RNA species of different sizes on a PAGE gel. Short (S) and long (L) plasmid based R-loops are shown in native or denatured states. D-E) Substrates from C were incubated in a representative timecourse with FANCM:FAAP24 and R-loop remaining plotted from an average of 3 experiments (±stderr).

Figure 5 Conserved action of FANCM-like proteins in R-loop metabolism.
A) Domain organization of FANCM orthologs, and BLM showing conserved domains. For FANCM, interaction sites are shown for: MHF=MHF1/2 complex, FAcc=Fanconi anemia core complex, TRR=Top3A-RMI1-RMI2 complex. B) MPH1 and RecG (1nM) unwind plasmid based R-loops in an ATP dependent manner, in a 10 minute reaction. C) FANCM (1nM) 1-800 retains the ability to unwind R-loops *in vitro*. D) Autoradiograph showing BLM unwinding plasmid based R-loops is ATP dependent but less efficient then FANCM. The concentrations of BLM used was 1, 10, 20, 40, 40nM (lane2-6) or 1nM for FANCM-FAAP24 (lane7-8) .
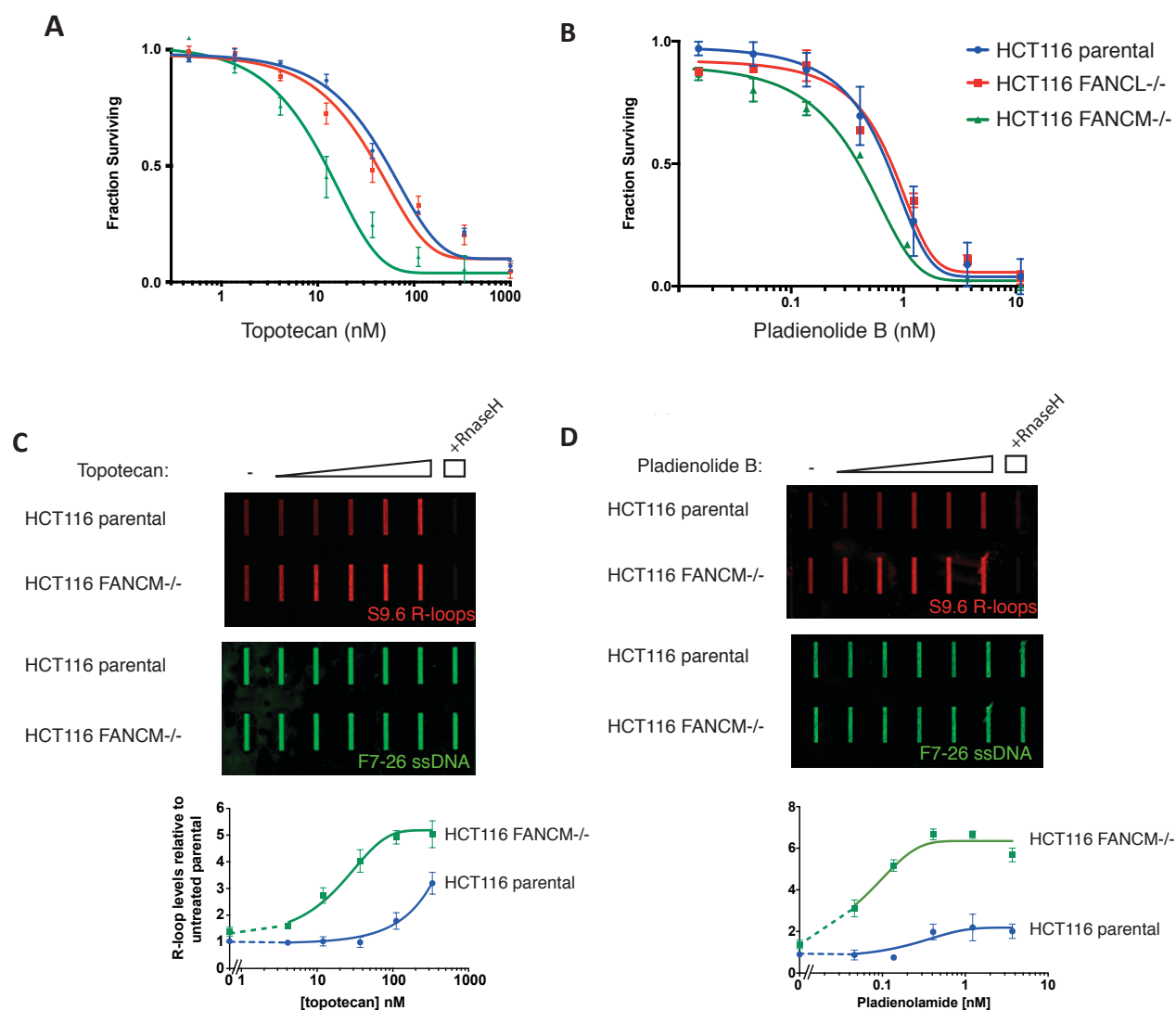
Figure 6 FANCM deficient cells are sensitive to R-loop stabilizing compounds
A-B) Dose response curves of parental and FANCM-/- HCT116 cell lines exposed to topotecan (left) or pladienolide B (right) for 72hours and stained with sulforhodamine B. C-D) Total R-loop levels measured by slot blotting of HCT116 genomic DNA extracted after increasing dose of topotecan or pladienolide B. Blots were probed with S9.6 anti-DNA:RNA hybrid or F7-26 anti-DNA, far-red secondary antibodies and detected by LiCor Odyssey imaging. RNaseH treated genomic DNA was used as a control for S9.6 DNA:RNA hybrid specificity. Quantification of S9.6 verses F7-26 slot blot signal from 3 experiments +/- sterr is shown in graphed form.
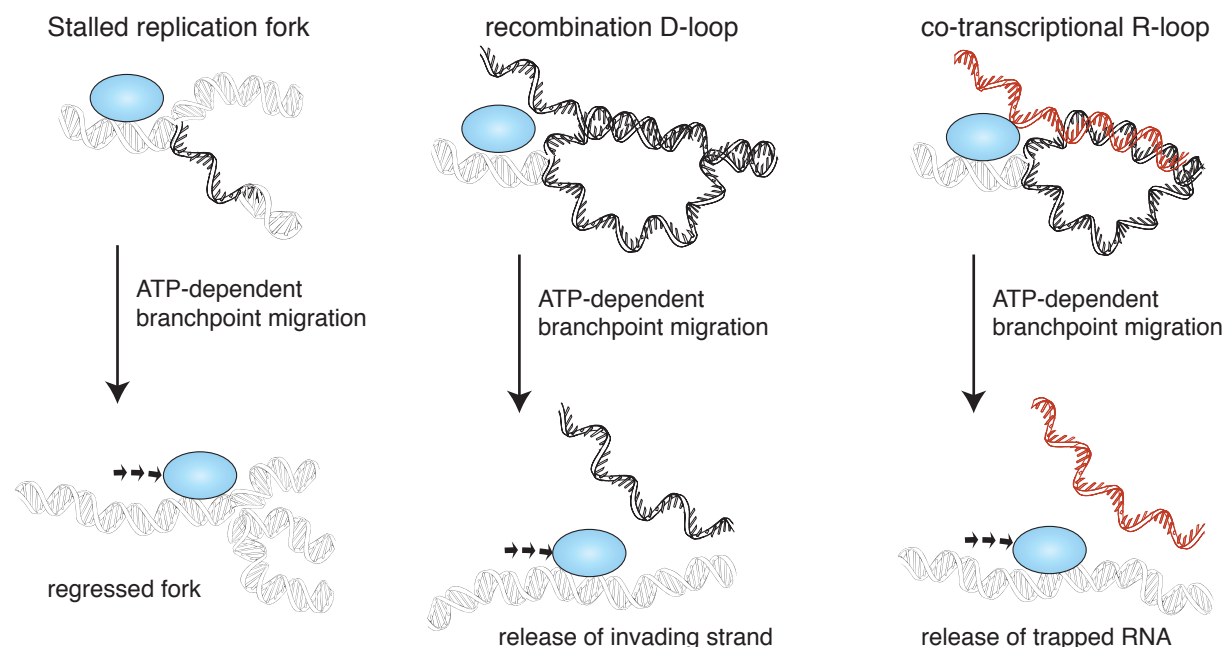
Figure 7 Model for FANCM-family-mediated maintenance of genome stability by a common mechanism of branchpoint translocation:

Branchpoint translocation is indicated for 3 different DNA structures associated with genome instability. FANCM/Mph1/RecG or other family member (blue sphere in cartoon) binds specifically to junction structure in DNA. This engages the motor activity of the enzyme, to continuously push the junction. For a stalled replication fork (left) this leads to formation of a regressed fork, as the nascent DNA strands anneal. For a D-loop or R-loop, branchpoint migration leads to displacement of a ssDNA or RNA molecule respectively.