Towards development of an *in vitro* repair assay of the ALS-associated *C9orf72* repeat

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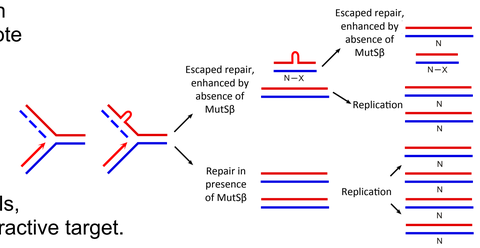
Highlights

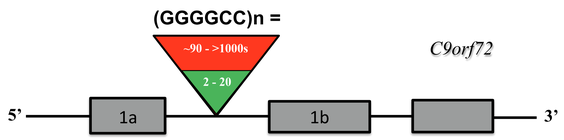
- DNA metabolism, specifically repair, is thought to promote ongoing disease-associated repeat expansion.
- Processing of the *C9orf72* repeat during DNA repair remains uncharacterized. This *in vitro* assay aims to assess the effect of:
  - nick location on directing repair.
  - size of the repeat slip-out on repair efficiency.
  - DNA metabolic proteins on repair outcome.
- DNA plasmid substrates with n = 6, 25, or 42 (G_4C_2•G_2C_4) repeats were constructed to contain nickase recognition sites.
- Preliminary repair assay results indicate an ability to process the slipped heteroduplex (SI-DNA) structures to multiple forms.

Background

- Previous work has demonstrated that expanded repeat sequences undergo DNA metabolism differently than non-repetitive sequences.
- (CAG•CTG) repeat-containing DNA sequences have shown that:
  - nick location relative to the repeat sequence effects efficiency of repair → result in either an expansion or contraction product.
  - repeat slip-out size changes the ability for DNA repair to occur, with larger slip-outs being poorly repaired.
  - DNA metabolic proteins can affect repair outcome, promoting disease-associated expansions in some cases.
- The mismatch repair protein MutSβ, which can bind slip-outs for repair, has been shown to promote error-prone repair (see image at right).
- Absence of MutSβ induces contractions in mouse models, making it an attractive target.

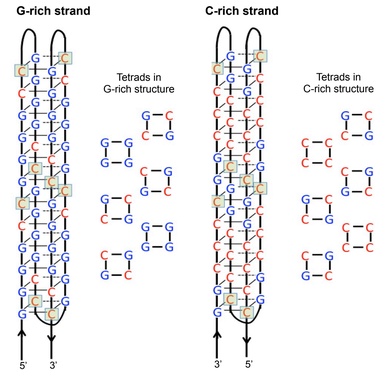


C9orf72 repeat expansion



The expanded *C9orf72* repeat is the leading genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).
The repeat exists over a polymorphic range of lengths, and when expanded, is prone to further instability (expansion or contraction). Unaffected individuals usually carry between 2 - 20 of these repeats, while clinically affected individuals generally present with >90 repeats.

G-rich & C-rich quadruplex structure

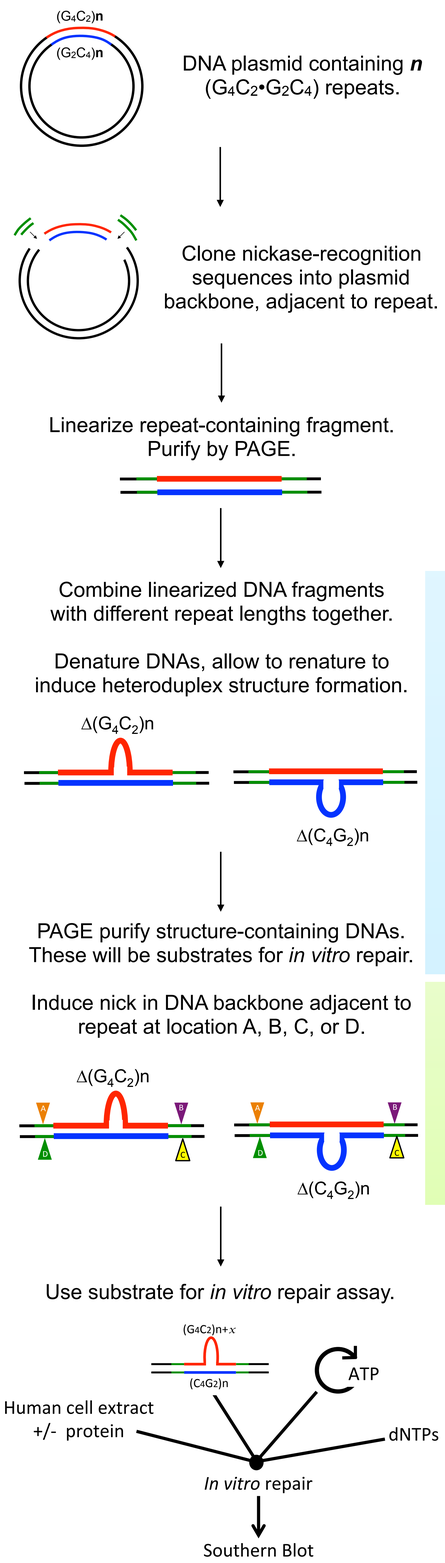


Recent work in our lab demonstrated that the expanded *C9orf72* repeat forms quadruplex structures in both the G-rich and C-rich strands (Zamiri, Mirceta *et al.*, 2015).

The effects of binding by DNA metabolic proteins to these structures on DNA repair is unknown.

The *in vitro* repair assay will provide an avenue for assessment of the effects of proteins on these structures and the DNA products resulting from metabolism.

In vitro repair assay methodology and development



Structure induction and polyacrylamide gel electrophoresis (PAGE) purification of slipped-intermediate DNAs (SI-DNA)

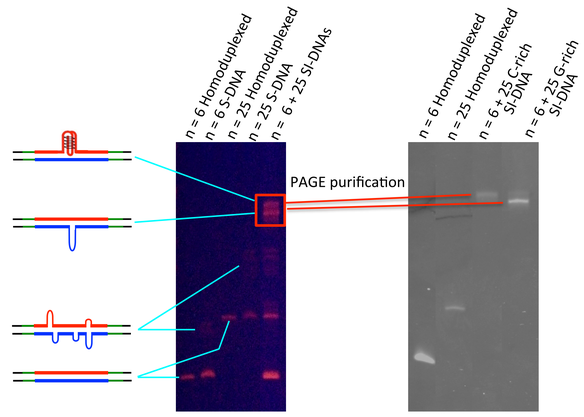


***Figure 1.* Purified linear DNA fragments containing 6 or 25 repeats were alkaline denatured and slowly renatured allowing formation of slipped-DNAs (S-DNAs) and SI-DNAs.** S-DNAs result from slip-out formation in both strands containing the same number of repeats. SI-DNAs result from renaturation of two strands, each containing a different number of repeats: $(G_4C_2)_6$ + $(G_2C_4)_{25}$ or $(G_4C_2)_{25}$ + $(G_2C_4)_6$ (upper and lower bands, respectively).

Nicking Endonuclease (NEase) assay development

The nick in the DNA backbone directs repair machinery.

Thus, efficiency of nick-induction in purified, linear substrates must be determined prior to use of the substrate in the *in vitro* repair assay.

The NEase assay will be used to quantify [nicked]:[non-nicked] linear substrate present in a sample.

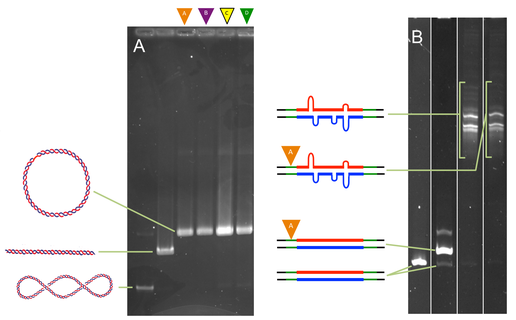
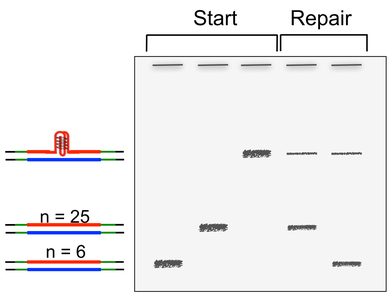


***Figure 2.* Nicking efficiency is tested prior to linearization and post-structure-induction.** *(A)* Circular plasmid constructs are tested. Supercoiled and linear controls are run alongside nicked plasmids to compare migration of the forms of DNA in agarose. Efficient nicking at all sites is observed. *(B)* The same DNA substrates, now linearized +/- structures, are assessed on a denaturing PAG for changes in migration following nicking. Homoduplex nicked DNA is resolved.

Expected outcome of *in vitro* repair assay

Previous use of the *in vitro* repair assay with (CAG•CTG) substrates provides a reference for expected outcomes, as visualized by Southern Blot:

- Homoduplexed DNAs of known repeat lengths are run alongside non-repaired heteroduplexed (SI-DNA) substrate(s).
- *In vitro* repair products are run alongside the controls, and evaluated by densitometric analysis.
- Level of repair is determined by the amount of product that co-migrates with the homoduplexed DNA versus the SI-DNA.
- Strand bias for repair is determined by the repeat length of the product.



(G_4C_2•G_2C_4) SI-DNAs are processed following *in vitro* repair assay

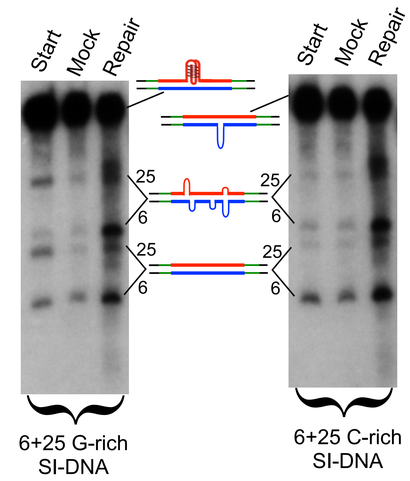


***Figure 3.* Preliminary results show changes in DNA structure composition following *in vitro* repair.**
SI-DNAs containing Δ(G_4C_2•G_2C_4) = 19 underwent *in vitro* repair with HeLa cell extract.

In lane 1, "Start", it is seen that PAG-pure SI-DNAs contain some amount of homoduplexed and S-DNA contaminant of both lengths of the constituent DNAs (6 and 25 repeats).

Each repair reaction is paired with a control of "Mock" repair reaction, in which no cell extract is added. The relative percentage of each structure-containing DNA is unchanged in the control.

Following *in vitro* repair of the SI-DNAs (nicked 5' of the G-rich strand at site A), an increase in the formation of homoduplexed and S-DNA species is observed, with a correlating decrease in the amount of SI-DNA material.

Future Directions

Establishment of the *in vitro* repair assay for *C9orf72* requires:

- optimization of the NEase assay to clearly visualize nicked vs. non-nicked linear fragments containing slipped-DNA structures.
- exploration of alternative avenues for the NEase assay (mung bean nuclease, snake venom phosphodiesterase, PARP binding).
- calibration of the *in vitro* repair products using short, synthetically-synthesized and highly-pure repeat-containing linear substrates

The *C9orf72 in vitro* repair assay holds great potential for exploration of the effects of DNA metabolic proteins on G-rich sequences. In particular, this assay provides a methodical and reproducible approach for exploration of how highly stable quadruplex structures formed by this sequence affect DNA metabolism. The results of this will inform understanding of secondary and tertiary structure on DNA repair, and may uncover potential therapeutic targets.

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Acknowledgements and Funding

Many thanks to Dr. Laura Ranum for the gift of repeat-containing DNA plasmids. Thanks to Dr. Yuh-Hwa Wang for her skilled imaging of purified DNA by electron microscopy. Finally, thanks to the whole Pearson Lab for ongoing discussions surrounding this project.