Localization of a FUS oncogene ortholog: Cabeza

Elaine Rose and Dr. Douglas A. Gray, Faculty of Medicine, University of Ottawa

Introduction

FUS (Fused in Sarcoma) is an intrinsically disordered human protein. Translocation of the FUS gene has been shown in human solid tumors of the mesenchymal lineage, as well as its mutation in neurodegenerative diseases. FUS is an unusual protein in that it lacks a stable secondary and tertiary structure. Cabeza is a fruit fly (*Drosophila melanogaster*) ortholog of FUS that is also disordered and shows similar overall function (by mutational analysis). Their specific sequences are divergent, but both proteins have a comparable organization and richness for certain amino acids. FUS and Cabeza have previously been compared structurally, however, little is known about their functional resemblances. Through analysis using a Bayesian search algorithm, it is believed that certain segments of these two proteins may be functionally interchangeable. The functional segments of FUS have not yet been researched in depth, but comparing the protein to one of its orthologs may help in determining these unknowns. Expressing Cabeza in a fluorescent protein fusion will form a platform for its detection in living cells. Time-lapse imaging will be performed to determine the localization and trafficking of Cabeza at the subcellular level.

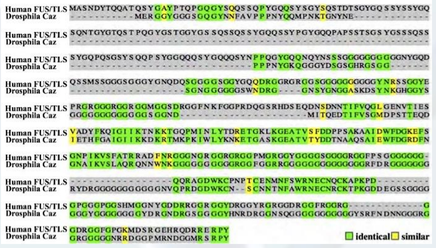

Figure 1. Amino acid sequences of Human FUS protein and *Drosophila m.* Cabeza protein.

Methodology

Cabeza was individually fused to both green and red fluorescent proteins using:

- PCR (Polymerase Chain Reaction) was used to amplify the desired gene, Cabeza.

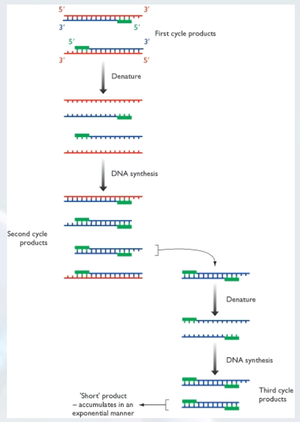

Figure 2. Stages of Polymerase Chain Reaction. Region to be copied (Cabeza) is shown in red and newly synthesized DNA is shown in blue. Heat denaturation occurred at 95°C, primer annealing at 70°C, and primer extension at 72°C.

- Plasmid transformation for molecular cloning and transfection. DNA was annealed to a plasmid vector using restriction enzymes. Molecular cloning allowed many copies of the plasmid DNA through transfection in E. Coli.
- The plasmid vectors allowed for fusion of a fluorescent protein and also contained an ampicillin resistance gene.
- Fidelity of the previous PCR reaction was confirmed by direct sequencing of the resulting plasmid.

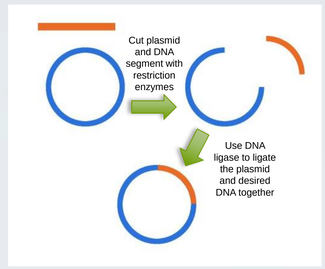

Figure 3. Steps in Plasmid transformation. Plasmid vector used had a green or red fluorescent protein gene adjacent to the insertion site. The restriction enzymes used were AsiSI and MluI.

- More copies of expression vectors containing Cabeza were then transfected into HEK (Human Embryonic Kidney) 293T cells along with plasmids containing wild type FUS, mutant FUS, and the gene for Huntingtin protein (associated with Huntington's disease), individually. Live-cell imaging and time-lapse microscopy were employed to study translocation of the proteins.
- The variants of mammalian FUS used are FUSY (wild-type) and FUSF (a mutant isoform that is prone to mislocalization).
- These names indicate whether the last residue of the protein is either tyrosine (Y) or phenylalanine (F).

Results

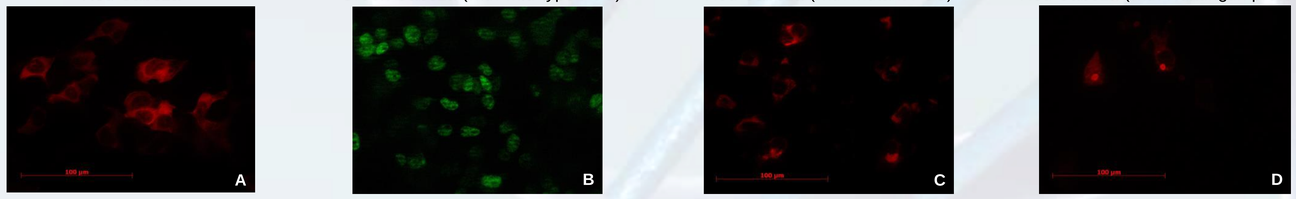

Figure 4. From each 293T cell culture transfection, the microscopy image channels specifically for each Cabeza fluorescent protein fusion are shown. Trafficking and behaviour of the protein can be compared through these images. **A.** Cells contain only mK-Cabeza in the presence of no other transfected proteins. **B.** Cells contain mG-Cabeza and wild-type mK-FUSY. **C.** Cells contain mK-Cabeza and mutant mG-FUSF. **D.** Cells contain mK-Cabeza and Q103-YFP (Huntingtin protein-Yellow fluorescent protein fusion).

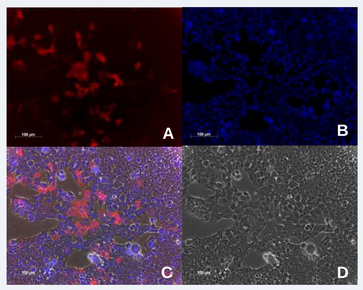

Figure 5. Cells transfected with only mK-Cabeza. **A.** Red channel mK-Cabeza fluorescence. **B.** Cell stain fluorescence. **C.** Merge image. **D.** Gray scale

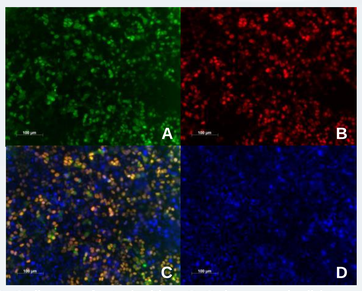

Figure 6. Cells transfected with mG-Cabeza and wild-type mK-FUSY. **A.** Green channel mG-Cabeza fluorescence. **B.** Red channel mK-FUSY. **C.** Merge image. **D.** Cell stain fluorescence.

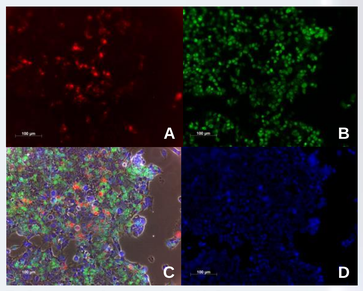

Figure 7. Cells transfected with mK-Cabeza and mutant mG-FUSF. **A.** Red channel mK-Cabeza fluorescence. **B.** Green channel mG-FUSF. **C.** Merge image. **D.** Cell stain fluorescence.

All images labeled 'Cell stain fluorescence' display fluorescence from the blue stain, DAPI (4',6-diamidino-2-phenylindole). This acts as a DNA-binding reagent that stains cell nuclei.

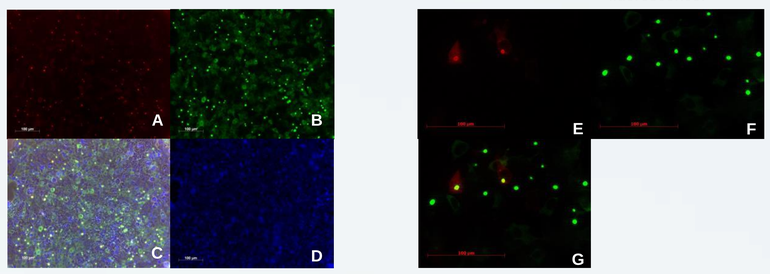

Figure 8. Cells transfected with mK-Cabeza and Huntington Q103-YFP. **A, E.** Red channel mK-Cabeza fluorescence. **B,F.** Green channel Q103-YFP fluorescence. **C.** Merge image. **D.** Cell stain fluorescence. **G.** Merge image of red and green channel fluorescence.

Each protein individually in 293T cells is expected to be localized as follows: Cabeza is found mainly in the cytoplasm, wild-type FUS is found in the nucleus, and mutant FUS is found in the cytoplasm. Huntingtin (Q103) protein forms large protein aggregates that also form in cell nuclei.

These results demonstrate that:

- Cabeza, by itself, is localized in the cytoplasm.
- In the presence of wild-type (normal) human FUS, Cabeza becomes localized in the nucleus along with the FUSY protein.
- When mutant FUS is transfected with Cabeza, the FUSF protein is mainly seen in the nucleus (as opposed to its usual cytoplasm localization) and Cabeza forms small aggregates in the cytoplasm of the cell.
- While Huntingtin protein is transfected in the same cells as Cabeza, its usual nuclear aggregates form. The aggregates, however, do not only contain Huntingtin protein, but also Cabeza. While some Cabeza still remains cytoplasmic, the inclusions contain a large amount of the protein.

Conclusion

- Cabeza can be expressed as a fluorescent protein fusion in mammalian cells.
- Furthermore, other proteins, especially those orthologous to Cabeza, demonstrate a significant influence on trafficking and localization of Cabeza *in vivo*.
- From these results, it can be said that Cabeza is affected strongly by its human ortholog, FUS (both wild-type and mutant versions), through changes in its subcellular localization.
- Cabeza may also be linked to aggregation since it is attracted to inclusions formed by Huntingtin protein.

Therefore, the difference in sequence between Cabeza and FUS apparently leads to mislocalization of Cabeza in mammalian cells. Despite this divergence, the tendency for Cabeza to aggregate (for example, in the presence of Huntingtin protein) is maintained.

Many more experiments may be conducted to determine the cause of these proteins' impact on Cabeza's behaviour and subcellular localization.
In the future, it will be possible to map the sequences responsible for these properties by swapping domains between Cabeza and FUS.

Acknowledgements and Contact Information

- I would like to acknowledge and thank my faculty sponsor, Dr. Douglas A. Gray.
- This research was made possible by the Undergraduate Research Opportunity Program at the University of Ottawa and the Ottawa Hospital Research Institute.

- Inquiries can be directed to: erose013@uottawa.ca

References

Brown, T.A. Genomes. 2nd edition. Oxford: Wiley-Liss; 2002. Chapter 4, Studying DNA. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK21129/>.

Gray, D.A. and Woulfe, J. (2013) Structural disorder and the loss of RNA homeostasis in aging and neurodegenerative disease. Front. Genet. **4**:149. doi: 10.3389/fgene.2013.00149

Wang, J. and Brent, J., et al. (2011). The ALS-associated proteins FUS and TDP-43 function together to affect Drosophila locomotion and life span. J Clin Invest. **121**(10):4118–4126. doi: 10.1172/JCI57883.