Corso di Dottorato di Ricerca in Scienze della Vita e dell'Ambiente – Ciclo XXXIX

Structural and Functional Analysis of Human DEAD-box Helicase 3X responsible for DDX3X Syndrome

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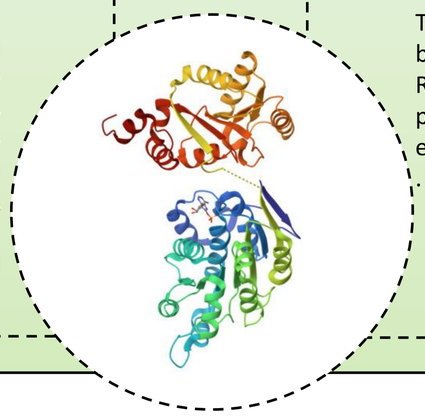
INTRODUCTION

DDX3X is a human RNA-binding DEAD-box helicase, acting as RNA-unwinding enzymes, chaperones for RNA folding, and regulators of protein-RNA complex assembly/disassembly on every stage of RNA metabolism. It comprises conserved domains 1 and 2 with nine signature motifs for DNA and RNA binding, as well as ATP hydrolysis [1]. DDX3X follows a catalytic cycle as a dimer with three stages: an apo state, a pre-unwinding state with RNA binding, and a post-unwinding state where each monomer releases an RNA strand after separation. DDX3X has a crucial role in cell growth control, mRNA transport, and translation. Its influence on RNA metabolism impacts various biological processes, and altered functionality can lead to diseases, including DDX3X syndrome, a rare intellectual disability (ID) linked to mutations in the X-linked *ddx3x* gene, that approximately corresponds to 2% of intellectual disability in female [2].



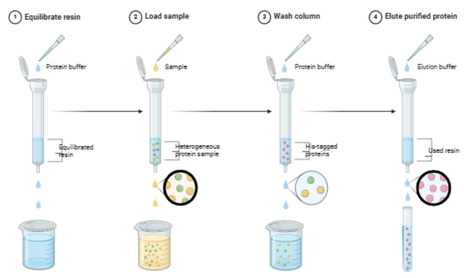
AIM

The aim of the project is to investigate, through computational simulations and fluorescence-based enzymatic activity assays, the impact of some of the most frequent DDX3X mutations (i.e., R351Q, R362C, and R376C) on the enzyme's catalytic cycle. These genetic variants are of particular research interest, as they are associated with severe neurodevelopmental disorders in early childhood.

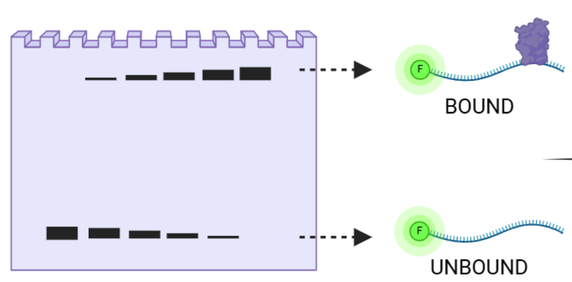
METHODS

MOLECULAR DYNAMICS SIMULATIONS

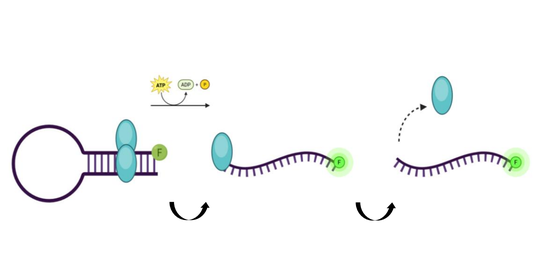
PROTEIN PURIFICATION



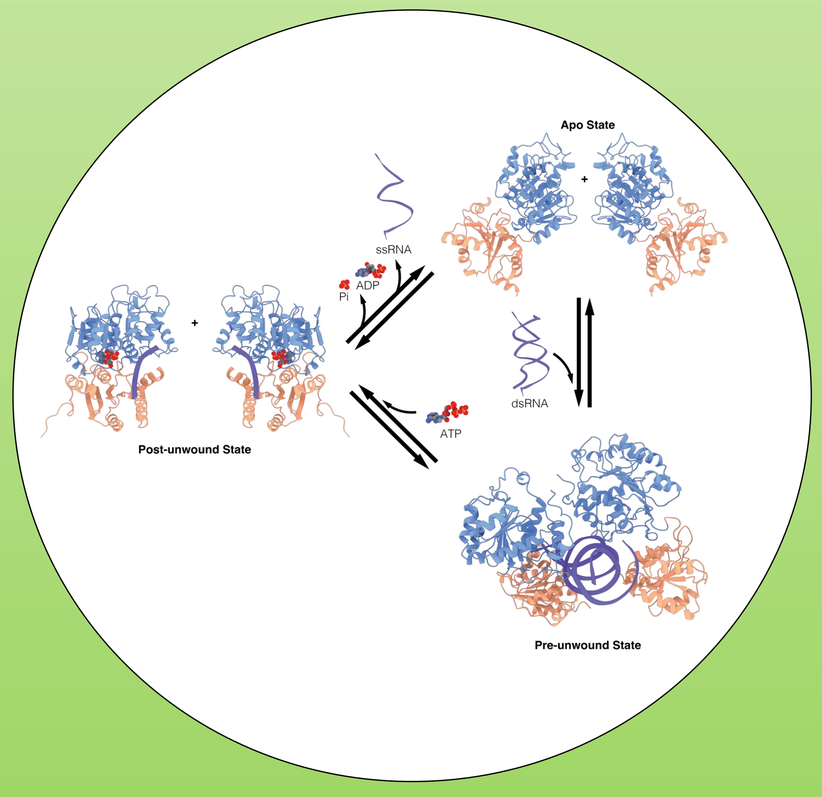
EMSA ASSAY



FLUORESCENCE-BASED HELICASE ASSAY

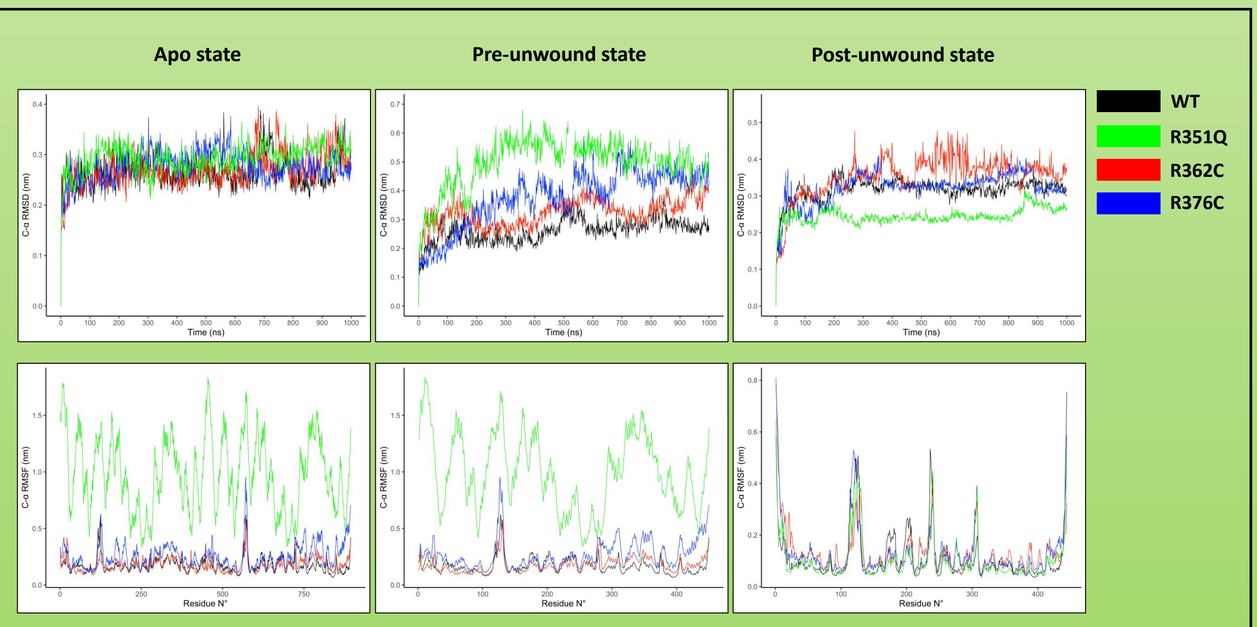


RESULTS



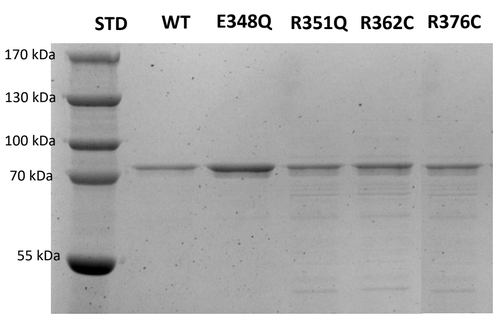
Ribbon three-dimensional (3D) structural representation of the three catalytic states of WT DDX3X. The Apo, Pre-unwound, and Post-unwound states of WT DDX3X were depicted using Chimera X software [PDB ID: 2I4I; PDB ID: 6O5F].

COMPUTATIONAL SIMULATIONS



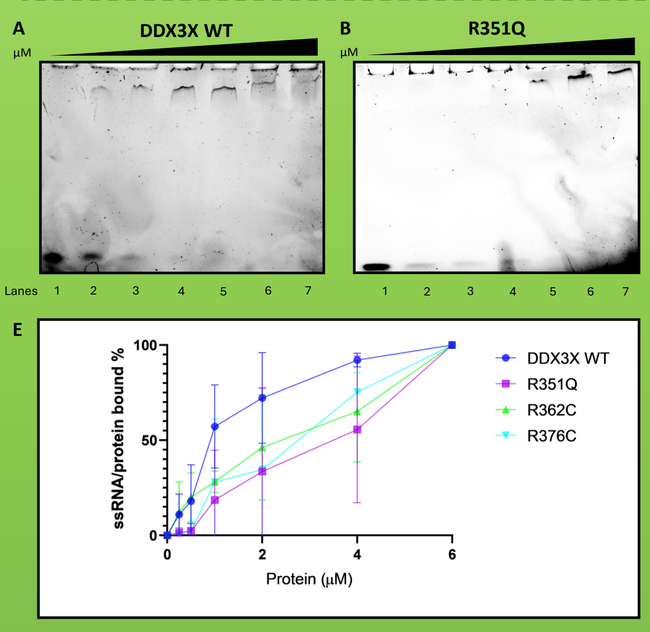
To investigate the effect of the R351Q, R362C and R376C DDX3X mutants, we conducted a series of molecular dynamics simulations [5] for the enzyme's three catalytic states. Our analysis of Root Mean Square Deviation (RMSD) revealed a pronounced destabilization of the mutants in both pre- and post-unwound states, indicating that the mutations indeed affect the enzyme-RNA complex (upper side of panel). Furthermore, the ATP-binding domain of the DDX3X mutants in the pre-unwound state exhibited higher Root Mean Square Fluctuation (RMSF), which may be associated with a reduced RNA unwinding capacity in these mutant enzymes. In particular, the R351Q mutant appears to be the most destabilized during the first two phases of the catalytic cycle (lower side of panel).

PURIFICATION



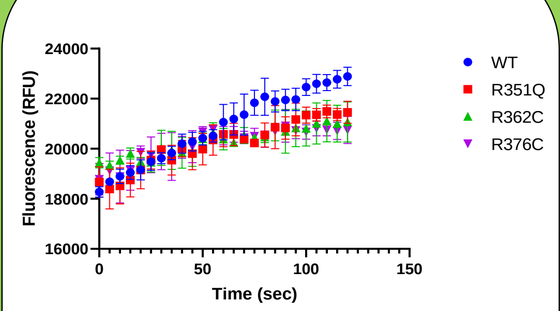
Purification of DDX3X and mutants. Proteins expression in *E.coli* BL21 cells. Bands (79 kDa) of DDX3Xwt, E348Q, R351Q, R362C, R376C.

EMSA ASSAY



Electrophoretic Mobility Shift Assay (EMSA). (A) 50 nM of fluorescent ssRNA substrate was incubated with DDX3X WT and **(B)(C)(D)** mutants at the indicated concentrations. **(E)** Quantitative analysis of binding portion between DDX3Xwt and ssRNA, comparing to mutants. The bands on the gel were quantified by densitometry using the ImageLab tool, while the graphs and statistical analyses were generated using Prism 8 software.

FLUORESCENCE-BASED HELICASE ASSAY



DDX3X WT and Mutants Fluorescence-based Helicase Assay. Activity assay to evaluate enzymatic helicase efficiency,, measured as an increase in fluorescence intensity over time.

CONCLUSIONS

In conclusion, This study presents structural and functional analyses of DDX3X mutations identified in patients with DDX3X syndrome. Computational simulations revealed that these mutations alter the enzyme's dynamics, particularly in RNA-bound states, as shown by increased RMSD values. These findings suggest that the most destabilized phases of the catalytic cycle involve RNA binding, potentially reflecting changes in RNA–protein affinity and, consequently, affecting helicase activity. In particular, the ATP-binding domain of DDX3X mutants in the pre-unwound state exhibited elevated RMSF values, which may be linked to reduced RNA unwinding efficiency. These results support the hypothesis of an impaired catalytic cycle in mutant proteins. To experimentally validate the molecular dynamics data, we established an efficient purification protocol for both wild-type and mutant DDX3X proteins and developed two fluorescence-based assays to monitor helicase activity and RNA-binding capacity [3][4]. Overall, our analyses indicate that these mutations can impair DDX3X function, although the precise step(s) of the catalytic cycle affected remain to be clarified. Notably, the R351Q variant shows the most pronounced alteration in RNA-binding affinity, a result consistently supported by both computational and experimental approaches.

REFERENCES

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