Development of Assays to Support Identification and Characterization of Modulators of DEAH-Box Helicase DHX9

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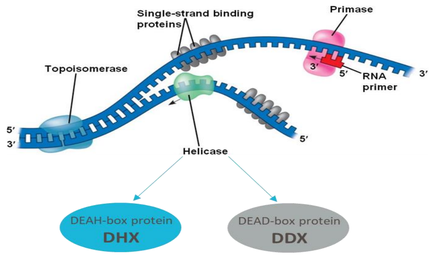
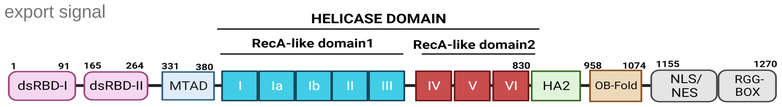
DHX9: DEAH-Box RNA Helicase

DHX9, also known as Nuclear DNA Helicase (NDH II) and RNA Helicase A (RHA), is an NTP-dependent helicase enzyme. DHX9 is a member of DEAH-box helicase family with regulatory roles in transcription and translation, RNA processing and transport, microRNA processing and maintenance of genomic stability[1]. DHX9 localizes to both the the nucleus and the cytoplasm and is ubiquitously expressed in all tissue types.

NTPase activity is stimulated by RNA in both DEAD-box and DEAH-box helicases but DEAD-box proteins use only ATP, whereas DEAH box proteins are more promiscuous in their NTP usage[2].



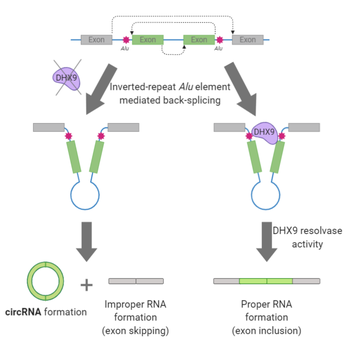
The domain structure of DHX9 is shown below: dsRBD, double-stranded RNA binding domain; MTAD, minimal transactivation domain; HA2, helicase-associated domain 2; OB-fold, oligonucleotide/oligosaccharide binding fold; NLS, nuclear localization signal; NES, nuclear export signal



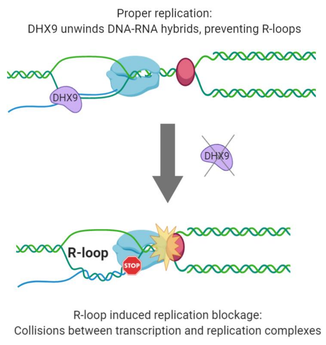
DHX9 As A Therapeutic Target For Oncology

In humans and primates, retrotransposons called Alu elements are copied via reverse transcription into RNA and integrate into genomic DNA at new loci. Inverted repeat Alu elements can cause circRNA during pre-mRNA back-splicing, leading to genomic instability. DHX9 has been shown to specifically bind to Alu elements and resolve circRNA[3]. DHX9 has also been shown to unwind R-loops and other secondary RNA/DNA structures during transcription and replication. R-loop formation results in transcriptional pausing and can lead to DNA damage by a number of different mechanisms.[4] DHX9 is overexpressed in many different cancers, and its involvement in the DNA damage response and genomic instability makes it an attractive target for oncology.

Alu element-induced circRNA formation



R-loop prevention of DNA replication

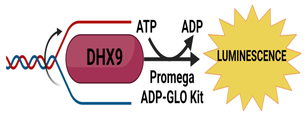


References

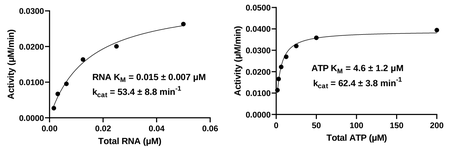
[1]Lee et al., Oncotarget (2016)
[2]Dominissni et al., Nature (2012)
[3]Atkas et al., Nature (2017)
[4]"Valuable Waste" Science & Tech. Research News (2017)
[5]Vu et al, Nature Medicine (2017)
[6]LHanson et al. Methods Enzymology (2012)
[7]Schutz et al, J. Mol. Biol (2010)
[8]Prabu et al, Mol. Cell (2015)

ATPase Assay Developed As Primary Biochemical Assay

DHX9 ATPase assay was developed with 31-mer double-stranded RNA substrate[5]



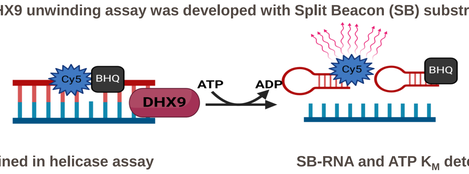
RNA and ATP K_M determined



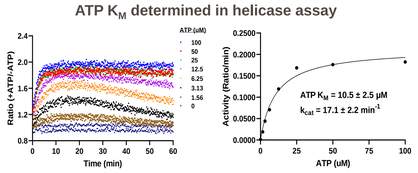
- DHX9 can unwind double stranded DNA or RNA in an ATP-dependent manner
- Promega's ADP-Glo kinase assay kit was used to monitor ADP formation during unwinding
- Several literature sourced substrates were tested for unwinding dependent ATPase activity
- A 31-mer double stranded RNA with a single nucleotide, 5' overhang provided optimum activation of ATPase activity
- The selected substrate was utilized to develop primary biochemical assay under balanced conditions and validated with a nucleotide analog

Helicase Assay Developed As Secondary Assay

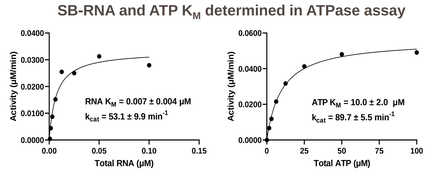
DHX9 unwinding assay was developed with Split Beacon (SB) substrate[6]



ATP K_M determined in helicase assay



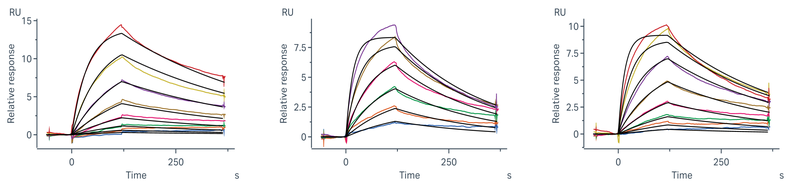
SB-RNA and ATP K_M determined in ATPase assay



- DHX9 helicase activity can be monitored with a fluorescently-quenched double-stranded Split Beacon (SB) RNA substrate[5]
- SB RNA and ATP were evaluated in the ATPase and helicase assays to corroborate balanced assay conditions across both assays
- The ATP K_M in the ATPase assay is within 2-fold of the ATP K_M in the helicase assay
- The SB-RNA K_M was not generated in helicase assay due to technical challenges, but SB-RNA K_M in ATPase assay is within 2-fold of 31-mer double-stranded RNA K_M

SPR Assay Established For Orthogonal Validation

DHX9 SPR assay confirms binding of ATP and other nucleotide analogs

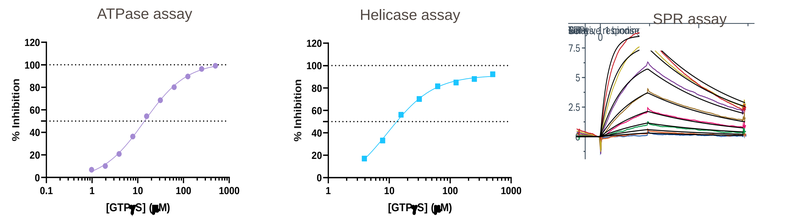


ATP K_D = 1.20 ± 0.07 µM | ATP K_D = 0.62 ± 0.39 µM | ATP K_D = 0.98 ± 0.31 µM

- Surface plasmon resonance (SPR) is a label-free technology enabling measurement of small molecule affinity to target proteins
- An SPR assay was developed as an orthogonal biophysical assay to further evaluate chemical matter
- SPR confirmed binding of ATP, ADP and GTP with affinities in the low micromolar range
- Kinetic analysis show slow association and dissociation for all tested nucleotide analogs

Assay Suite Confirms GTPγS As A DHX9 Inhibitor

Non-hydrolysable nucleotide validated as a tool compound

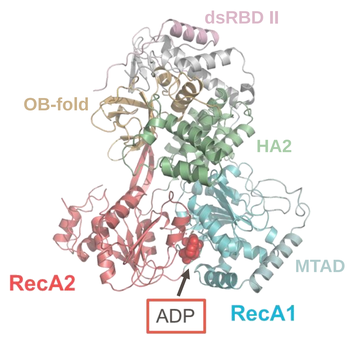


Nucleotide analog	ATPase assay (IC_{50}, µM)	Helicase assay (IC_{50}, µM)	SPR assay (K_D, µM)
GTPγS	13.9 ± 1.4	9.6 ± 3.9	0.74 ± 0.22

- Several non-hydrolysable nucleotide analogs were evaluated in both biochemical assays and SPR, including ATPαS, ATPγS, GTPαS and GTPγS
- GTPγS binding correlated well across the biochemical and biophysical assays and was selected as a tool inhibitor

First Mammalian DHX9 Crystal Structure Solved

Structure of cat DHX9 bound to ADP



- 2.7 Å structure of cat DHX9 solved by X-ray crystallography; ribbon colors indicate structural domains
- ADP (sphere representation) and a coordinated magnesium ion were found at the expected nucleotide binding site between RecA1 and RecA2 domains
- High structural similarity with human DHX9 RecA1 domain (PDB ID: 3LLM[7]; RMSD: 0.38 Å; sequence identity: 98.3 %)
- Comparison of DHX9-ADP structure to Drosophila MLE (DHX9 ortholog) bound to RNA (PDB ID: 5AOR[8]; sequence identity = 51.9%) shows overall architecture conservation with expected conformational differences due to lack of RNA substrate

Conclusions

- Robust primary and secondary biochemical assays were developed to enable hit-finding efforts for DHX9
- SPR assay established to confirm specific and reversible compound binding to DHX9
- First mammalian DHX9 structure was solved
- Together these assays and structure enablement provide a toolkit for screening, validation, characterization and optimization of small molecule modulators of DHX9

Acknowledgements

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