PROJECT PROPOSAL:

Decoding AGO1's Role in Paraspeckle Assembly: Direct vs. NEAT1-Mediated Interactions with Paraspeckle Proteins

Background: Paraspeckles are membrane-less nuclear substructures that regulate gene expression, primarily by sequestering RNA and associated proteins (reviewed in Fox et al., 2018). Their assembly is driven by the lncRNA NEAT1, which recruits key RNA-binding proteins to form functional nuclear condensates through liquid-liquid phase separation (reviewed in Taiana et al., 2020). Recent work by Shuaib et al. (2025) has identified AGO1 as a component of paraspeckles, where it interacts with NEAT1 and paraspeckle proteins (PSPs) to regulate nuclear architecture. Furthermore, AGO1 depletion has been shown to weaken NEAT1-PSP associations and disrupt paraspeckle integrity (Shuaib et al., 2025). These findings suggest that AGO1 may directly interact with PSPs to stabilize their association with NEAT1. However, AlphaFold2 predictions revealed minimal potential interaction sites between AGO1 and key PSPs such as HNRNPK, SFPQ, or NONO (Figure 1A). This raises the possibility that AGO1's interaction with these proteins is indirectly mediated by NEAT1, which likely acts as a central scaffold to colocalize these components. Based on this, we aim to test the **hypothesis** that AGO1's interaction with PSPs is indirectly mediated by NEAT1.

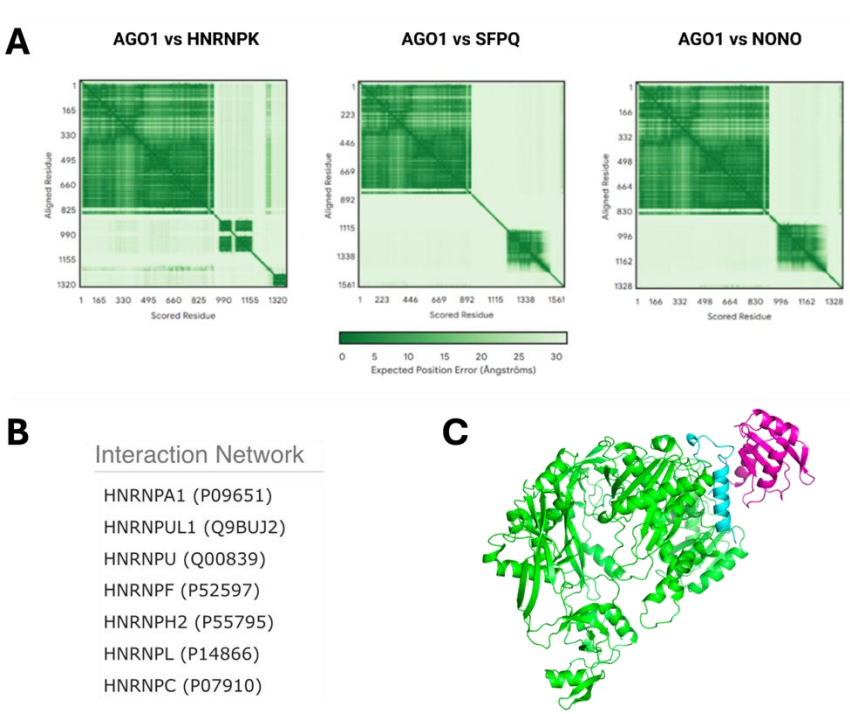


Figure 1. Computational and experimental analyses of AGO1 interactions with paraspeckle-associated proteins.

A) AlphaFold2 Predicted Aligned Error (PAE) heatmaps show the predicted structural alignment confidence between AGO1 and three paraspeckle proteins: HNRNPK (left), SFPQ (middle), and NONO (right). The x-axis and y-axis represent the aligned residues of AGO1 and the respective paraspeckle protein. The color gradient indicates the expected position error in Angstroms (Å), with darker green denoting higher confidence (lower error) and lighter green indicating lower confidence (higher error). Minimal strong interaction signals suggest weak or indirect binding between AGO1 and these PSPs; **B)** Overlapping AGO1 interactors from the IntAct database, highlighting HNRNP family members (e.g., HNRNPU, HNRNPUL1) that align with experimental data from Shuaib et al. (2025). **C)** AlphaFold3-predicted ternary complex of AGO1 (green) with the C-terminus of HNRNPU (blue) and residues 70–168 of HNRNPUL1 (purple).

Supporting evidence for our Hypothesis: Multiple lines of evidence support the hypothesis that NEAT1 acts as a scaffold to mediate AGO1-PSP interactions. First, AlphaFold2 predictions reveal minimal direct interaction interfaces between AGO1 and core paraspeckle proteins (HNRNPK, SFPQ, NONO), suggesting weak or RNA-bridged binding (Figure 1A). Second, while the IntAct database and experimental studies (Shuaib et al., 2025) identify HNRNP family members (e.g., HNRNPU, HNRNPUL1) as AGO1 interactors (Figure 1B), AlphaFold3 predicts these complexes adopt conformations resembling

AGO1-RNA binding (Figure 1C) – a likely artifact of overfitting, given the absence of RNA in the simulation. Third, experimental AGO1 proteomics studies have confirmed interactions between AGO1 and HNRNP family members, but these interactions were disrupted by RNAse treatment (Höck et al., 2007), indicating that they are RNA-dependent.

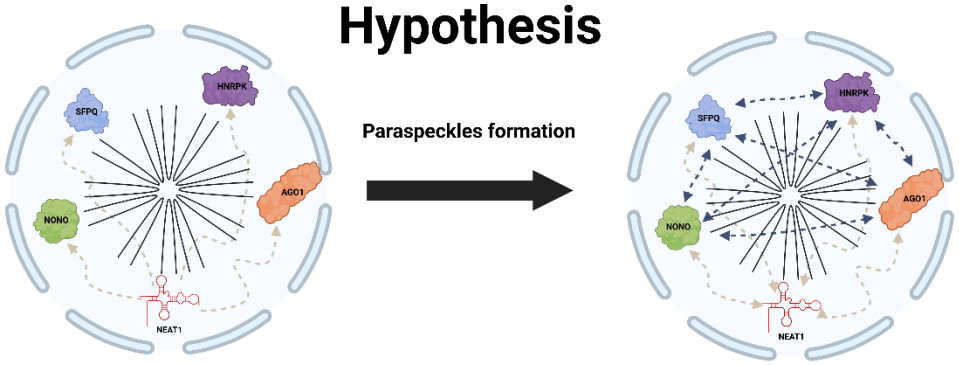
Experimental design: To investigate NEAT1's role in scaffolding AGO1-PSP interactions we will use chromatin-enriched fractions and employ titrated CRISPRi to achieve partial NEAT1 knockdown (50-70% reduction confirmed by qPCR), preserving paraspeckle architecture while enabling analysis of dosage-dependent effects on complex assembly. Three complementary approaches will be applied: First, **quantitative expansion microscopy** will map AGO1-PSP spatial relationships with 100-nm resolution. We will analyze minimum inter-protein distances in ≥50 paraspeckles per condition using automated particle detection and nearest-neighbor algorithms. This approach will reveal whether NEAT1 reduction alters the nanoscale organization of AGO1 within paraspeckle hubs. Second, we will employ time-controlled **TurboID-AGO1 labeling** (10-min biotin pulse) followed by streptavidin pull-down and quantitative Western blotting for core PSPs (SFPQ, NONO, HNRNPU). Signal intensities will be normalized to total biotinylated protein (streptavidin-HRP) and compared between: (i) non-targeting controls, (ii) NEAT1 knockdown, and (iii) rescue with ASO-resistant NEAT1_2. Parallel RNase A treatment will distinguish RNA-dependent interactions. Third, to probe the role of structured RNA in AGO1-PSP interactions, we will perform **RNase III-sensitive co-IPs** targeting NEAT1's dsRNA domains to specifically probe dsRNA-bridged AGO1-PSP complexes. Input and bound fractions will be analyzed by Western blotting for quantification of binding ratios. Critical controls include: (1) cytoplasmic fraction analysis to exclude nonspecific associations, (2) monitoring of NEAT1-independent complexes (AGO1-DICER1), and (3) dose-response validation of knockdown effects. The integrated data will provide: (a) spatial mapping of interaction distances, (b) quantitative binding affinities under controlled NEAT1 reduction, and (c) mechanistic insight into RNA-mediated scaffolding - collectively testing whether AGO1-PSP coordination requires stoichiometric NEAT1 levels in chromatin-associated paraspeckles.

Expected outcome: If our hypothesis is correct, we anticipate three consistent findings: partial NEAT1 knockdown will increase AGO1-PSP distances in chromatin fractions (quantified by expansion microscopy), TurboID-AGO1 proximity labeling will show reduced PSP biotinylation in NEAT1-depleted cells, and RNase-sensitive co-IPs will confirm RNA-dependent AGO1-PSP binding. These results would demonstrate that NEAT1 scaffolds AGO1 within paraspeckles via RNA tethering. Conversely, if AGO1-PSP interactions persist despite NEAT1 reduction or resist RNase treatment, this would refute our Hypothesis, suggesting direct AGO1-PSP binding.

Significance of findings: This study will resolve a key mechanistic question in nuclear organization by determining whether NEAT1 scaffolds AGO1-PSP interactions within paraspeckles. A positive finding would establish a novel localization mechanism for AGO1, functionally separating its nuclear roles from canonical cytoplasmic RNAi pathways. Given NEAT1's involvement in cancer and neurodegenerative diseases (reviewed in Gu et al., 2022 and An et al., 2018), and AGO1's essential cellular functions (reviewed in Müller et al., 2020), these results could open new avenues for targeting nuclear-specific AGO1 activity while preserving its RNAi machinery – a potential breakthrough for selective therapeutic intervention.

Summarizing figure:

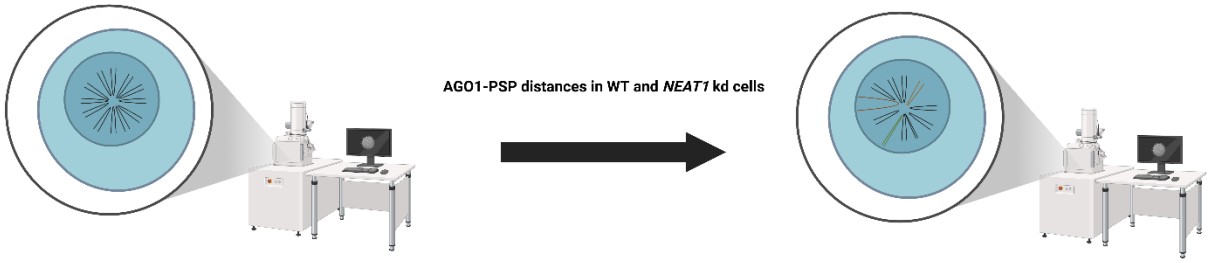
Hypothesis



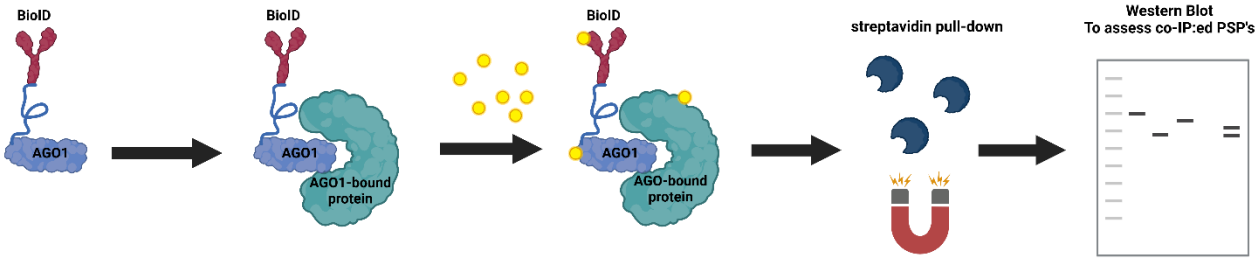
Is AGO1's interaction with PSPs indirectly mediated by NEAT1 ?

Methods

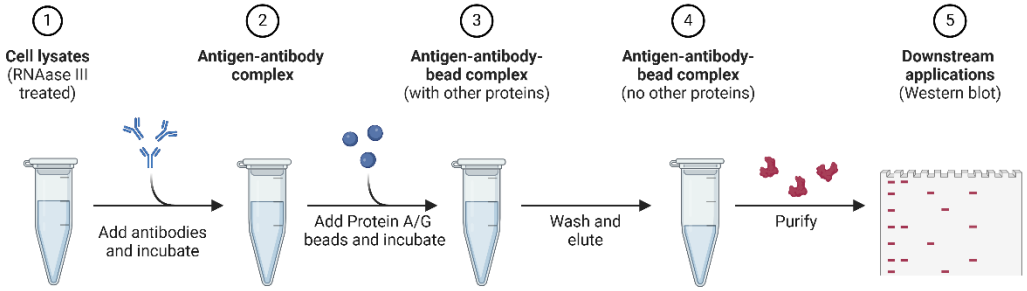
1. Quantitative Expansion Microscopy



2. TurboID-AGO1 labeling



3. RNase III-sensitive Co-Immunoprecipitation (RNA-dependency Test)



References

1. An, H., Williams, N. G., & Shelkovnikova, T. A. (2018). NEAT1 and paraspeckles in neurodegenerative diseases: A missing lnc found?. *Non-coding RNA research*, *3*(4), 243-252.
2. Fox, A. H., Nakagawa, S., Hirose, T., & Bond, C. S. (2018). Paraspeckles: where long noncoding RNA meets phase separation. *Trends in biochemical sciences*, *43*(2), 124-135.
3. Gu, J., Zhang, B., An, R., Qian, W., Han, L., Duan, W., ... & Ma, Q. (2022). Molecular interactions of the long noncoding RNA NEAT1 in cancer. *Cancers*, *14*(16), 4009.
4. Höck, J., Weinmann, L., Ender, C., Rüdel, S., Kremmer, E., Raabe, M., ... & Meister, G. (2007). Proteomic and functional analysis of Argonaute-containing mRNA–protein complexes in human cells. *EMBO reports*, *8*(11), 1052-1060.
5. Müller, M., Fazi, F., & Ciaudo, C. (2020). Argonaute proteins: from structure to function in development and pathological cell fate determination. *Frontiers in Cell and Developmental Biology*, *7*, 360.
6. Shuaib, M., Mannen, T., Yamazaki, T., Adroub, S. A., Ghosheh, Y., Albawardi, W., ... & Orlando, V. (2025). AGO1 interacts with NEAT1 lncRNA and impacts nuclear compartments. *bioRxiv*, 2025-01.
7. Taiana, E., Ronchetti, D., Todoerti, K., Nobili, L., Tassone, P., Amodio, N., & Neri, A. (2020). LncRNA NEAT1 in paraspeckles: a structural scaffold for cellular DNA damage response systems? *Non-coding RNA*, *6*(3), 26.