What's behind the dot?

- Wholistic Content Analysis of the Paraspeckle Complex -

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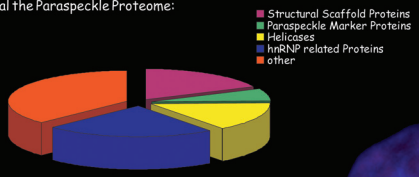
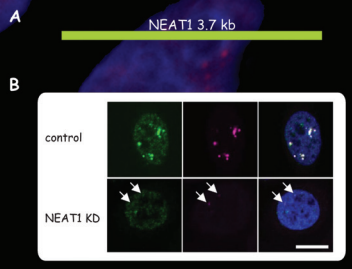
1 NONO-IP Mass Spectrometry

NONO is a abundant nuclear RNA binding protein that is enriched in Paraspeckles. We chose Immuno-Precipitation followed by Mass Spectrometry to reveal the Paraspeckle Proteome:



We were able to obtain an initial paraspeckluar proteome containing 79 candidate proteins. The pie diagram above shows the relative distribution of these proteins in five different functional classes.
Because of the wide nuclear distribution of NONO we cannot be confident that all found proteins are genuine.

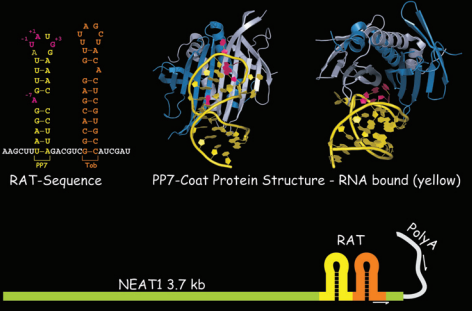
2 NEAT1 is the essential structural scaffold

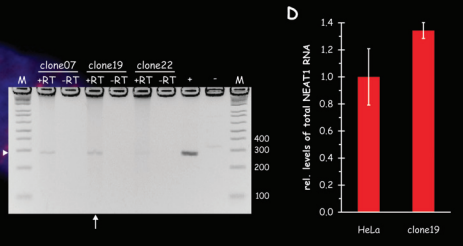


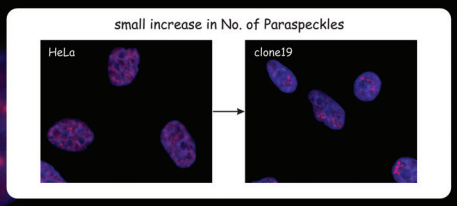
The long non-coding RNA (lncRNA) NEAT1 (3.7 kb) is located specifically in the Paraspeckle complex and essentially builds its structural scaffold. **A**: Green bar: Schematic view of NEAT1 (3.7 kb). **B**: Sasaki et al. [2] show disappearing Paraspeckles (green) after NEAT1 knock down (pink). Arrows indicate remnant Paraspeckles, white bar: 10 µm.

3 RNA-Affinity Purification and Stable Cell Line

Using Hogg et al. [4] RNA-Affinity-Tag (RAT), we will be able to affinity purify the Paraspeckle complex with a much higher order of specificity:

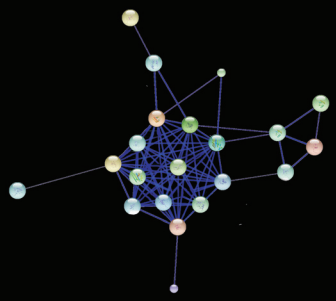






RNA-Affinity-Tag strategy. **A**: Sequence of the RNA-Affinity-Tag (RAT) developed by Hogg et al. [4]. The crystal structure of the bacteriophage Pseudomonas aeruginosa coat protein (PP7-CP) shows the mode of binding to its target RNA [5]. Essential nt for binding are highlighted in pink. **B**: Schematic view of the used NEAT1-RAT construct. Arrows indicate position of oligos used for construct validation. **C**: End point-PCR of cDNA derived from different stable integrated HeLa clones. Arrowhead indicated expected amplicon of 279 bp. To ensure that the PCR product is result of transcribed RNA we used -RT (Reverse Transcriptase) reactions as controls. + = pos. control, - = neg. control. **D**: qPCR showing NEAT1 total levels. In the tested clone #19 are about 40% more NEAT1 RNA present due to the over expressed NEAT1-RAT construct. **E**: Accodring to Clemson et al. [6] overexpression o NEAT1 should result in more Paraspeckles. Compared to HeLa one can see slightly more Paraspeckles in the stable clone#19. Blue: DAPI, Red: NONO immunostain with TRITC.

Outlook



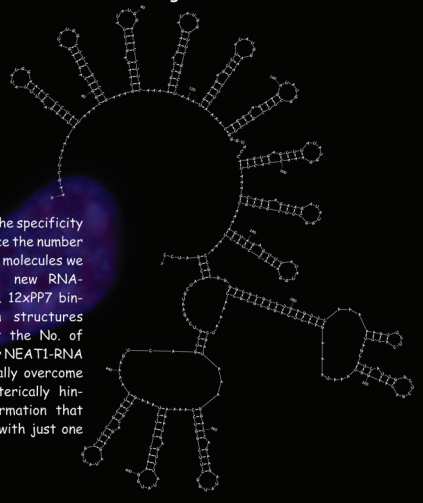
The initial obtained protein list resulting from NONO-IPs will be used in a Gateway-Cloning based medium throughput interaction pipeline to unwind both protein-protein and protein-RNA interaction within the paraspeckle complex. The RNA based Affinity purification strategy will help to increase the quality of the candidate list.
Hand in hand the Proteome, RNome and Interactome will lead us to understand the functional role of the not yet fully elucidated nuclear complex.

Introduction

Paraspeckles are nuclear substructures in which the nuclear proteins PSPC1, NONO and SFPQ are enriched [1]. The Paraspeckle formation is depending on the presence of a long non-coding RNA (lncRNA) NEAT1 which two isoforms reach from 3.7 kb up to 23 kb in human [2]. Currently the most detailed described function of the Paraspeckle complex is the retention of inverted repeat (IR) containing RNAs that thereby trapped in the nucleus and prevented from being translated [3]. Therefore Paraspeckles seem to play a role in gene regulation on a so far yet poorly understood level.

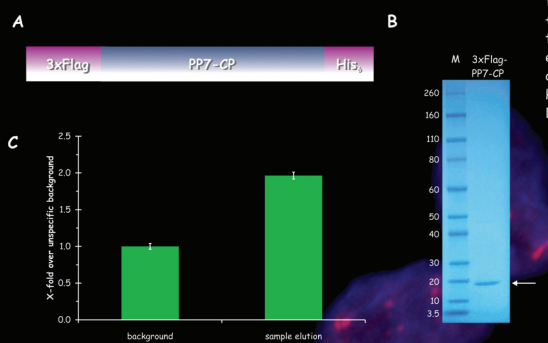
Aim Our aim is to unwind the whole and unbiased protein and RNA content of the Paraspeckle complex. Downstream protein-protein and Protein-RNA interaction analysis will reveal the complete Interactome of the Paraspeckle thereby providing further implications on their function and signaling pathways.

5 Improvement of RNA-Tag



To increase the specificity and to enhance the number of PP7 bound molecules we designed a new RNA-Affinity Tag. 12xPP7 binding hairpin structures should boost the No. of molecules per NEAT1-RNA and additionally overcome hidden or sterically hindered conformation that might occur with just one repeat.

4 Paraspeckle Purification



A: Schematic view of the 3xFlag tagged PP7-CP including the C-term. His$_6$-Tag. **B**: Result of Ni^{2+}-NTA Affinity purification after heterolog expression in E. coli. Marker in kDa, arrow indicates target protein of the correct molecular weight (17.1 kDa). Protein of this quality was used for Paraspeckle purification. **C**: Affinity purification of Paraspeckle was performed using nuclear extracts of clone#19 cells. Those were incubated either with (+) or without (-) 3xFlag-PP7-CP and immuno-precipitated afterwards with Flag-Agarose beads. IP-qPCR using RAT specific oligos revealed a 2x increased level of NEAT1-RAT over background (-).

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References

[1] Fox et al., Curr. Biol., 2002, 12(**1**):13-25.
[2] Sasaki et al., PNAS, 2009, 106(**8**):2525-2530.
[3] Prasanth et al., Cell, 2005, 123(**2**):249-263.
[4] Hogg et al., RNA, 2007, 13(**6**):868-880.
[5] Chao et al., NSMB, 2008, 15(**1**):103-105.
[6] Clemson et al., Mol. Cell, 2009, 33(**6**):717-726.