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The nuclear ATPase/adenylate kinase hCINAP is recruited to perinucleolar caps generated upon RNA pol.II inhibition

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ABSTRACT

hCINAP is an atypical nucleoplasmic enzyme, combining structural features of adenylate kinases and ATPases, which exhibits dual enzymatic activity. It interacts with the Cajal Body marker coilin and its level of expression and enzymatic activity influence Cajal Body numbers. Here we show that upon specific transcriptional inhibition of RNA pol.II, hCINAP segregates in perinuclear caps identified as Dark Nucleolar Caps (DNCs). These are distinct from perinucleolar caps where coilin and fibrillarin (both Cajal Body components) accumulate. In DNCs, hCINAP co-localizes with Paraspeckle Protein (PSP1) and also co-segregates with PSP1, and not coilin, in nuclear and nucleolar foci upon UV irradiation.

Structured summary:
MINT-8048545: *hCINAP* (uniprotkb:Q9Y3D8) and *PSP1* (uniprotkb:Q8WXF1) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

Human Coilin Interacting Nuclear ATPase Protein (hCINAP) is a nuclear factor, originally identified as a protein interacting with the Cajal Body marker protein, p80 coilin [1]. hCINAP exhibits several unusual or unique properties. First, the hCINAP mRNA is an alternatively spliced transcript from the TAF9 locus, which also encodes the basal transcription factor $TAFIID_{32}$, although the two proteins have no identity in their sequence due to differential usage of ATG starting codons and reading frames in the translation of the alternative transcripts [1]. Second, crystallographic analysis shows that while hCINAP has a structure typical for an adenylate kinase (AK), it also contains features characteristic of ATPase/GTPase proteins. Furthermore, it displays dual enzymatic activity of both an atypical AK, with unusually broad substrate specificity, and of an ATPase, an activity not reported for any other human adenylate kinase [2,3]. Intriguingly, His79, a B-motif amino acid residue, is crucial for the regulation of hCINAP's dual enzyme selectivity, in response to intracellular substrate concentration [3].

At steady state hCINAP has a diffuse nucleoplasmic localization, excluding nucleoli, and although it does not concentrate in Cajal Bodies (CBs), its levels of expression and also its enzymatic activity influence CB organization. Specifically, overexpression of hCINAP decreases the average number of CBs per nucleus [1], while depletion of hCINAP causes defects in CB formation and redistribution of CB components [4]. Expression of an hCINAP mutant with His79 changed to Gly deregulates CB number, both increasing the average number of CBs per nucleus and also dramatically altering the frequency distribution of CBs, with numbers ranging from 0 to 30 per cell rather than 1–7 as seen when wild-type hCINAP is exogenously expressed [3]. Cajal Bodies are conserved nuclear organelles that serve as macromolecular assembly platforms (scaffolds), facilitating the maturation of splicing snRNPs and snoRNPs and other small nuclear RNPs involved in nuclear metabolic processes [5–8].

These findings have highlighted the putative importance of hCINAP in nucleotide homeostasis in the mammalian nucleus and in the assembly and/or stability of CBs. The challenge remains however to elucidate what is the mechanism through which hCINAP can impact on nuclear organization and eventually what is the biological function that is associated with hCINAP's AK and ATPase activity in the nucleus, where movement or assembly of chromatin and nuclear organelles are ATP-dependent processes [9,10]. In this study we present new findings on hCINAP's role in nuclear dynamics and organization.

Abbreviations: DRB, 5,6-dichloro-β-D-ribofuranosylbenzimidazole; GFP, green fluorescent protein; hCINAP, human coilin-interacting protein; PSP1, Paraspeckle Protein 1; RT-PCR, reverse transcription-polymerase chain reaction

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2. Materials and methods

2.1. Cell culture and drug treatments

HeLa cells were cultured in GMax-DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin at 37 °C with 5% CO_2.

DRB (5,6-dichloro-β-D-ribofuranosylbenzimidazole) was added at a final concentration of 25 or 50 μg/ml and Actinomycin D at 0.04 μg/ml or 1 μg/ml. Cells were incubated for 3 h before sampling.

2.2. UV-C irradiation

Semiconfluent cells were washed with PBS and the medium was collected and kept at 37 °C. The cells were irradiated in a UV Stratalinker 2400 oven at 254 nm with 30 J/m^2. The saved medium was added back and cells were incubated for 6 h, prior to microscopic examination.

2.3. Generation of HeLa$^{GFP\text{-}hCINAP}$ stable cell lines

For the establishment of HeLa$^{GFP\text{-}hCINAP}$, 5 μg of EGFP-hCINAP plasmid [circular (C) or linear (L)] was transfected into a 6 cm dish of HeLa cells using Lipofectamine 2000 (Invitrogen). After 18 h, cells were split at different dilutions (1:10–1:500) and medium containing 400 μg/ml G418 was added to select for cells that had stably incorporated the plasmid into their genomic DNA. After 14 days, visible colonies were picked, subcloned and expanded for biochemical and microscopic analyses.

2.4. Antibodies

Primary antibodies were: rabbit anti-hCINAP [1:1000 for Western blot (WB) and 1:800 for immunofluorescence (IF)] [1], mouse monoclonal anti-coilin 5P10 (1:50, IF) [11], rabbit anti-PSP1_48 (1:250, IF) [12], mouse monoclonal anti-fibrillarin AFB01 (1:200, IF, tebu-bio), mouse anti-GFP (1:1000, WB, Roche), mouse monoclonal anti-dynein (1:600, WB, Santa Cruz) and mouse monoclonal anti-α tubulin T5168 (1:6000, IF, Sigma). Secondary antibodies were: TRITC-conjugated goat anti rabbit IgGs (1:500, Jackson ImmunoResearch Laboratories), goat Cy5 anti mouse IgG (1:100, Jackson ImmunoResearch Laboratories), donkey Alexa Fluor 568 anti-rabbit IgG (1:600, Molecular Probes), donkey Alexa Fluor 555 anti-mouse IgG (1:1500, Molecular Probes), goat Alexa Fluor 350 anti-mouse IgG (H+L) (1:100, Molecular Probes), sheep HRP anti mouse IgG (1:6000, Amersham Pharmacia Biotech) and donkey HRP anti rabbit IgG (1:30 000, Santa Cruz). Nuclei were stained with Hoechst 33342 (0.5 μg/ml, Invitrogen).

2.5. Immunofluorescence microscopy

Immunofluorescence labeling was performed as previously described [3]. Deconvoluted fluorescent images were acquired with a Deltavision Restoration Microscope (Applied Precision) and a Micromax KAF1400 (Kodak) camera, and conventional fluorescent images with a C. Zeiss Axiovert 200M inverted fluorescence microscope equipped with an AxioCam MRm camera, using a ×63 1.3 or ×100 oil Plan-Apochromat objective lenses.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Poly A$^+$ RNA from HeLa cultures was purified with the RNeasy Mini Kit (Qiagen) and cDNA was reverse transcribed from 2 μg of RNA using the Protoscript Kit (New England Biolabs). For the detection of endogenous hCINAP (excluding GFP-hCINAP) by semi-quantitative PCR, the upstream primer CINAP5UTR (gtagagcaaagggcacgtgagcgag) and the downstream CINAPRV (ccggaattcttaagtagctagccttataag) were used (product size 620 bp). For the detection of GFP-hCINAP (excluding endogenous hCINAP) the upstream primer GFPUP (ctcgtgaccaccctgacctac) was used in conjunction with primer CINAPRV (product size 1055 bp). The amplification of house keeping gene Pumilio 1 (PUM1) with primers PUM1UP (tgggaacaagagggcatctg) and PUM1RV (tgaggtgtgcaccatgaac) (product size 186 bp), was used as an internal reference reaction to normalize reaction conditions across samples. A mock RT reaction (2 μg of RNA, no reverse transcriptase) was used as negative control.

2.7. Cell cycle analysis by flow cytometry and analysis of mitotic progression by microscopy

To analyze cell cycle progression, cells in exponential growth were fixed with 70% ethanol for 2 h, stained for DNA content with propidium iodide, scored for their fluorescence on a FACSVantage SE and analyzed for their cell-cycle distribution using ModFit (Verity Software House).

The determination of the percentage of mitotic cells (mitotic index) and their assignment to mitotic subphases (n = 6000 cells) was performed by visual analysis using fluorescence microscopy of cells immunofluorescently labeled for α-tubulin and counterstained with Hoechst 33342. Statistical significance was assigned by two-way ANOVA analysis with Bonferroni post-test (GraphPad *Prism*).

3. Results and discussion

As a starting point, we constructed a HeLa cell line stably expressing GFP-hCINAP, following integration into the genome. Two stable clones, designated 33C and 62L, were pursued after purification with limiting dilution and expansion. Both stable clones displayed correct nuclear localization of GFP-hCINAP (Fig. 1A1, A2, B1, and B2, respectively), identical to GFP-hCINAP localization observed after transient transfection (Fig. 1C1 and C2) and also identical to endogenous hCINAP (Fig. 1D1 and D2). The level of expression of GFP-hCINAP was markedly greater in clone 33C compared with 62L, as assessed by fluorescence microscopy (note that exposure time in Fig. 1A1 is seven times lower than in B1) and confirmed (a) by RT-PCR, using oligonucleotide primers that would specifically amplify the GFP-hCINAP transcript (Fig. 1E, top panel) and (b) by Western immunoblotting, using anti-GFP tag antibodies (Fig. 1F, middle left panel) and anti-hCINAP antibodies (Fig. 1F, middle right panel). Expression levels of endogenous hCINAP transcript and endogenous protein in both clones were comparable to wild-type HeLa cells (Fig. 1E, middle panel; Fig. 1F, bottom right panel) while expression of GFP-hCINAP protein in clone 33C was much higher than that of endogenous hCINAP protein (note that the exposure time of right top immunoblot panel in Fig. 1F is half of that in the right bottom panel). We further characterized clones 33C and 62L, by evaluating their cell cycle profiles (Suppl. Fig. S1 and Suppl. Table 1), quantifying their mitotic indexes and mitotic phase distribution (Suppl. Fig. S2), and measuring the number of nuclei per cell (data not shown). There were minor differences in both clones as compared with wild-type cells, most notably a small increase in the percentage of aneuploid cells (Suppl. Fig. S1 and Suppl. Table 1) and small fluctuations in the distribution of cell cycle or mitotic subphases (Suppl. Fig. S2). However, both stable clones maintained a robust proliferation rate, normal morphology, stable expression and correct localization of hCINAP. Stable clone 33C was utilized for subsequent experiments.

We subjected clone 33C cells to transcriptional arrest, using Actinomycin D at a concentration that causes inhibition of both