Mechanism and regulation of dUTPase nucleocytoplasmic transport with an outlook on cell cycle dependent nuclear proteome reconstruction

Theses of the PhD dissertation

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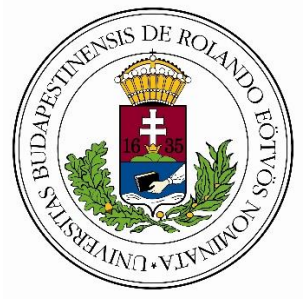

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1. Introduction

The representatives of the dUTPase enzyme family play an important role in genome integrity maintenance by being an essential component of the *de novo* thymidylate biosynthesis. dUTPases catalyze the pyrophosphorolysis of dUTP into dUMP and inorganic pyrophosphate, thereby lowering the cellular dUTP level and preventing the incorporation of uracil moieties into DNA. Lack or inhibition of dUTPase within the cell leads to major perturbations of the dUTP/dTTP levels and increases the uracil content of DNA [1]. Since *de novo* thymidylate biosynthesis plays a key role in dNTP synthesis, it is often the target of different anti-cancer treatments (fluoropyrimidine, antifolates, etc.) positioning dUTPase inhibition in a potential medical aspect. It has been also shown that depletion of dUTPase significantly sensitized human cells to fluoropyrimidine treatment [2]. Both in humans and *Drosophila melanogaster* two dUTPase isoforms are present. One of these is nuclear, while the other is either mitochondrial (human) or cytoplasmic (*D. melanogaster*). The subcellular localization of nuclear dUTPase isoform is consistent with the finding that *de novo* thymidylate biosynthesis could only effectively prevent genomic uracil incorporation if it takes place in the nucleus. Thus key components of the pathway are actively transported into the nucleus after sumoylation during the onset of S-phase [3]. Since dUTPases have molecular weights (~55 kDa) that do not permit their passive nuclear diffusion, we wished to clarify how they are transported and what regulates these processes. The nucleocytoplasmic transport of macromolecules above 40 kDa depends on karyopherins, a group of proteins specifically recognizing short linear motifs in cargo proteins. Peptide segments involved in these processes are termed nuclear localization signals and nuclear export signals (NLSs and NESs, respectively) [4]. Nucleocytoplasmic trafficking is under multi-level regulatory control to orchestrate cellular events. For instance, phosphorylation adjacent to NLSs can modulate (either inhibit or enhance) nucleocytoplasmic trafficking in several cases [5]. Literature shows that import characteristics of a cargo is mainly regulated through the affinity and the concentration of cargos and importins. Therefore in many cases phosphorylation directly influences cargo:importin binding affinity [6]. The nuclear isoform of the human dUTPase was previously reported to be phosphorylated in the vicinity of its NLS, however using hypophosphorylation mimicking alanine substitution in the cognate position showed no alteration in localization, activity or subunit association [7]. Hypophosphorylation mimicking mutants on their own cannot provide a full understanding of the potential effects, hence it would be also feasible to check the effects of a hyperphosphorlyation mimicking glutamic acid mutation. dUTPases have a typical homotrimeric structural arrangement which means that the nuclear isoforms possess three

cognate NLSs. Before starting the present study it was unknown whether this feature is essential for the protein to achieve its proper localization pattern.

2. Experimental aims

The two isoforms of vertebrate dUTPases strictly localize to compartments where DNA synthesis occurs, namely the nucleus and the mitochondria. We wished to gain a better understanding how dUTPase is imported into the nuclear compartment, whether the transport process is under regulation and if so, what is the mechanism. We wished to check if other mechanisms might also limit dUTPase presence in the nucleus.

2.1 *D. melanogaster* dUTPase proved to be an *in vivo* calpain substrate [8]. Based on this we wished to check if the human dUTPase is also a calpain substrate or not. If so, this might have an effect on dUTPase distribution. Calpain driven partial proteolysis might limit dUTPase presence leading to altered dNTP homeostasis.

2.2 The nuclear isoform of the human dUTPase was reported to be phosphorylated in actively dividing cells, putatively by Cdk1, in the vicinity of its NLS (on the S11 residue) [9].
2.2.1 We wished to check the effects of this phosphorylation on intracellular localization with a glutamic acid substitution (S11E) combined with an isosteric control, a S11Q mutation.
2.2.2 Based on NLS composition, dUTPase is expected to utilize the classical nuclear import pathway relying on importin-α. We wished to confirm this interaction through a variety of biophysical methods and check the effects of phosphorylation on this putative interaction.
2.2.3 A systematical overview of Cdk1 regulated nuclear transport processes was previously done in yeast, describing several novel proteins having a cell-cycle dependent localization pattern driven by Cdk1 [10]. Combining *in silico* screening methods along with high-throughput experimental validation we wished to investigate the putative relevant regulatory phenomenon in the context of the human proteome.

2.3 One of the conserved features of dUTPases is that they are homotrimeric enzymes. This means that the human nuclear isoform has three NLSs. We were interested in how NLS copy number might influence the transport processes of the enzyme. We wished to do this by comparing the nuclear targeting along with importin-α binding capabilities of dUTPase variants that contain one or three NLSs. The unique feature of *Drosophila virilis* dUTPases provided us with a good model system for this since it is built up by a single polypeptide chain (with one

NLS) but structurally resembling the arrangement of homotrimeric dUTPases. These results might shed light on how NLS copy number regulates nuclear transport processes overall.

3. Applied methods

To answer the question aimed in the **2.1** point of the Experimental aims section, m-calpain and the nuclear isoform of the human dUTPase was prepared by recombinant DNA technology, produced and purified from *E.coli*. Cleavage was tested in an *in vitro* digestion assay. Calpain cleavage site was identified by mass-spectrometry. Calpains were activated in HeLa cells with ionomycin and cell extracts were probed for dUTPase over a time series with western blots.

To achieve aim **2.2.1** we have tagged dUTPase with the DsRed fluorescent protein and generated the S11E, S11Q mutants. Cellular localization was verified using confocal microscopy. Timing of the phosphorylation was determined with immunocytochemistry using a dUTPase phospho-specific antibody. Dynamics of localization during cell cycle were followed with video microscopy.

To investigate the questions of aim **2.2.2**, complex formation among purified dUTPase and importin-α was analyzed by native gel electrophoresis, analytical gel filtration, isothermal titration microcalorimetry and circular dichroism measurements. Structure of the dUTPase NLS:importin-α complex was determined by X-ray crystallography.

Aim **2.2.3** was addressed by screening the human proteome for NLS containing proteins (using NucImport) that harbor a Cdk1 site in the vicinity of their NLSs (using Predikin) in the P0 or P-1 position. Gene Ontology was used to characterize the nature of the findings. Several of the hits were experimentally validated by an NLS reporter assay.

To investigate the questions of aim **2.3** we have validated the presence of the single unique dUTPase isoform of *D. virilis* on mRNA (with 5'rapid amplification of cDNA ends) and on protein level (with western blots). Localization properties of the enzyme compared to *D. melanogaster* isoforms were addressed by fusing them to EGFP or AU1 tag. Thus the effects of NLS copy number was evaluated by confocal microscopy. Localization was checked in both insect and mammalian cell lines. Localization of the endogenous dUTPase pool in both *Drosophila* species were checked in tissues and in cell lines with immunocytochemistry. Complex formation among the different NLS number bearing dUTPases and importin-α was characterized by native gel electrophoresis, analytical gel filtration, and isothermal titration microcalorimetry.

4. Scientific results and conclusions

Theses of aim 2.1 (cf. Bozoky Z., Rona G., et al., 2011 *PlosOne*)

- The nuclear isoform of the human dUTPase is partly digested by m-calpain *in vitro.*
- Calpain cleavages sites are located on the N-terminal end of dUTPase. Cleavage occurs between the following positions: ^{4}SE^{5}; ^{7}TP^{8}; ^{31}LS^{32} as identified by mass-spectrometry.
- Calpain cleavage is calcium dependent and the substrate analogue α,β-imido-dUTP does not protect dUTPase from cleavage.
- Calpain cleavage does not have an effect on the catalytical activity of dUTPase, which is identical to the intact form.
- Cleavage at the ^{31}LS^{32} results in the loss of the NLS of dUTPase possibly having an impact on the proper localization of the enzyme.
- Ionomycin induced HeLa cell activation results in the degradation of the dUTPase pool after 24 hours of treatment.

Theses of aim 2.2.1: (cf. Rona G., et al., 2013 *Acta D.*; Rona G., et al., 2014 *Cell Cycle*)

- The glutamic acid (Glu) point mutant of the human dUTPase (S11E) mimicking a constitutively phosphorylated serine residue is cytoplasmic. The WT protein as well as the S11Q mutant are predominantly nuclear. Latter is the isosteric control for the Glu substitution but it is not charged and represents a non-phosphorylatable mutation. This localization is consistent in a variety of cell lines (MCF-7, COS7, HeLa, NIH-3T3 and 293T) with diverse genetic backgrounds differing in both origin and modes of transformation.
- The CRM1 inhibitor, leptomycin B, does not affect the localization pattern of the S11E mutant arguing for the fact that the cytoplasmic enrichment of the mutant is not caused by accelerated nuclear export processes.
- Phosphorylation of the S11 position takes place during the G2/M-phase in the cell cycle. Staining of the dUTPase phospho-NLS specific antibody coincides with the phospho-S10-Histon 3 M-phase marker.
- Video microscopy shows that WT dUTPase gets re-imported to the nucleus following division after a delay (~200 minutes) in 293T cells. In contrast, the re-import of the S11Q mutant starts directly after division. Once the import starts both constructs show similar import kinetics. The cognate phosphorylation event on the S11 position of the WT DsRed-tagged exogenous dUTPase was validated by western blot.

Theses of aim 2.2.2: (cf. Rona G., et al., 2013 *Acta D.*)

- Analytical gel filtration showed that in the mixture of WT dUTPase and importin-α, a molecular species with higher molecular mass than either components applied separately is present. This argues for a complex formed between the two proteins. The chromatogram profile was different when the S11E mutant dUTPase was used, still having a peak eluting earlier than any of the components alone, but later than the peak associated with the WT dUTPase:importin-α complex. This behavior might be explained by assuming that the complex formed between S11E dUTPase and importin-α is characterized with faster dynamic equilibrium, i.e. it is less stable as compared to the WT dUTPase:importin-α complex.
- Native-PAGE results show that WT and S11Q mutant dUTPase forms a complex with importin-α, showing up at a new position on the gel, whereas the band of uncomplexed importin-α disappears. No complex formation could be detected in case of the S11E mutant dUTPase.
- Importin-α is stabilized against heat induced unfolding due to the interaction with dUTPase and also renders the thermal unfolding transition more cooperative. Heat-induced unfolding was followed by circular dichroism spectroscopy at 210 nm. Importin-α stabilization could be observed with the WT (shift from 35.7 °C to 43.6 °C) and the S11Q mutant dUTPase (35.7 °C to 44.4 °C) but not for the S11E mutant.
- Isothermal titration microcalorimetry was used to specify the K_d values of the dUTPase:importin-α complexes and to also shed light on the stoichiometry of the complex. A change of one order of magnitude in the dissociation constant was observed for the S11E mutant compared to the WT and S11Q dUTPase (K_d was 0.79 μM, 0.76 μM and 9.62 μM for WT, S11Q and S11E dUTPases, respectively). The determined stoichiometry supports a model where each NLS of the trimeric dUTPase binds an importin-α (as the molar binding ratio is ~ 1.0). Thus all NLSs could be bound to importin-α at ideal conditions.
- The crystal structures of importin-α with the WT NLS and the S11E mutant NLS showed the structural background of the interaction. The differently arranged accommodation of the S11E peptide within the NLS binding site results in the loss of several interactions between the NLS peptide and the importin-α surface leading to the weaker binding properties of the phosphorylation mimicking NLS.

Theses of aim 2.2.3: (cf. Rona G., et al., 2014 *Cell Cycle*)

- Computational analysis of the human proteome to identify putative Cdk1-dependent phosphorylation sites in the vicinity of NLSs resulted in 72 hits (50 hits for the P0 position and 22 for P-1).
- According to Gene Ontology (GO) annotations, we found proteins involved in DNA damage recognition and repair, gene expression, epigenetics, RNA-editing, cell cycle regulation and several transcription factors.
- 13 of the hits (7 of the P-1 position and 6 of the P0 position) were validated experimentally by using our NLS-reporter construct. The NLSs of the protein hits were fused to an inert cargo (β-galactosidase-DsRed reporter). The resulting localization data show that in each case, replacement of the appropriate Ser or Thr residues (in P-1 or P0 position), predicted to be phosphorylated by Cdk1, by Glu in the NLSs always leads to significantly weaker nuclear accumulation or even to complete nuclear exclusion, compared to the WT.
- Swi6 NLS "glutamic acid" scanning shows that NLS function is inhibited by a P0 or P-1 position phosphorylation but is enhanced by a P-2 position phosphorylation. This is in agreement with the information obtained from known importin-α peptide based inhibitors.
- Phosphorylation sites of known Cdk1 targets: UNG2 (S14), UBA1 (S4) and p53 (S315) are all located in the P-2 position and could facilitate the nuclear re-entry of these proteins after division.
- We hypothesize that Cdk1 has an important role in regulating nuclear proteome re-establishment after cell division through inhibitory phosphorylation of P-1 and P0 positions in NLSs or through enhancing nuclear transport by P-2 position phosphorylation.

Theses of aim 2.3: (cf. Rona G., et al., 2014 *Febs J.*)

- We have validated the presence of a single endogenous dUTPase isoform in *D. virilis* (called ABC) on both mRNA (based on 5'RACE results) and protein level (based on western blots). The gene encoding dUTPase is present in a repetition of three copies in a one open reading frame which results in a single polypeptide chain. The three domains are not sequentially identical having small differences among them, resulting in a structure we call a "pseudo-heterotrimer" having only one NLS.
- An artificial dUTPase construct (expected to form a homotrimer, termed "AAA") having three NLS signals was generated. Localization of EGFP/AU1 tagged AAA and ABC constructs were compared to each other and to previously established *D. melanogaster* dUTPases constructs. AAA construct is strictly nuclear while the ABC construct could also

be found in the cytoplasm. These results were independent of the tag used (EGFP or AU1) and showed the same pattern in both insect and in a variety of mammalian cell lines (293T, HeLa, COS7).

- Endogenous dUTPases both for *D. melanogaster* and *D. virilis* (in cell lines and tissues) could be found in the nucleus and also in the cytoplasm. In case of *D. melanogaster* this is achieved through two distinct isoforms while *D. virilis* achieves this localization pattern with its single dUTPase isoform.
- Analytical gel filtration and native gel electrophoresis showed complex formation among AAA:importin-α and ABC: importin-α. During gel filtration a molecular species with higher molecular mass arises when AAA or ABC dUTPases and importin-α are mixed, suggesting complex formation. The chromatography profile of the two putative complexes markedly differ proposing alterations in the complex composition. The AAA dUTPase:importin-α complex has a slightly earlier elution peak, indication of having a higher molecular mass. In addition, the chromatography profile for the AAA dUTPase:importin-α complex is highly asymmetric, hinting the possibility of formation of multiple species with different molecular masses.
- Isothermal titration microcalorimetry provided quantitative information about the complex formation. The molecular binding ratio is the same for the two construct, showing that each NLS could be occupied by importin-α under ideal conditions. The interaction between importin-α and the NLS deleted constructs (ΔNLS-AAA and ΔNLS-ABC) were fully abolished. We suggest that the ABC pseudo-heterotrimeric dUTPase binds one importin-α molecule with its single NLS whereas the AAA homotrimeric dUTPase binds three importin-α molecules with the three NLSs.
- Taking into account the similar K_d values, the difference in the cellular localization pattern between the two construct thus most likely come from the fact that the AAA construct, having three NLSs, can compete more efficiently for importin-α in the intracellular environment. We, therefore show that NLS copy number in oligomers may contribute to the regulation of nucleocytoplasmic trafficking along with the affinity of the NLSs.

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6. PEER-REVIEWED PUBLICATIONS RELATED TO THE DOCTORAL THESIS

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*equal contribution

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8. PUBLICATIONS IN PRESS AT PEER-REVIEWED JOURNALS

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9. COMMENT ARTICLE AT PEER-REVIEWED JOURNALS

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