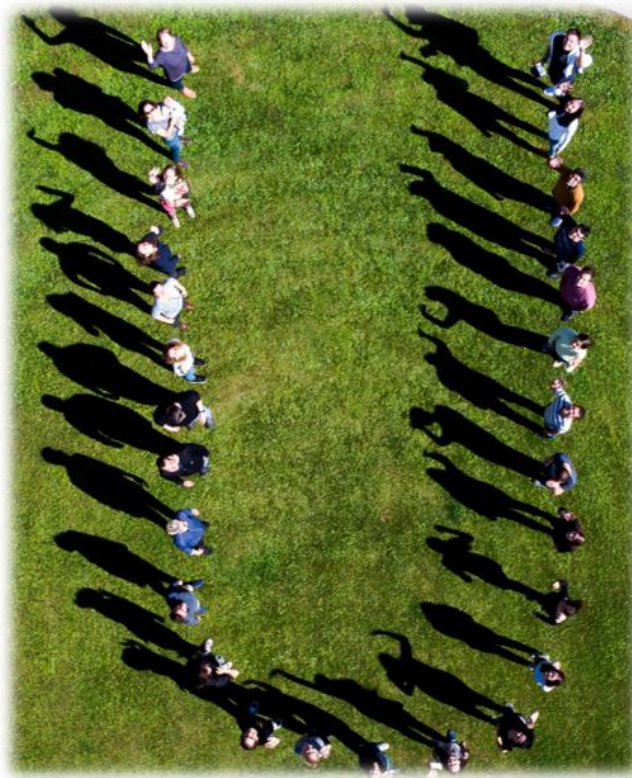


ANNUAL MEETING 2024

Genome Metabolism Research Group

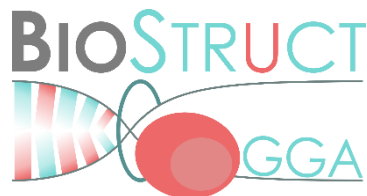
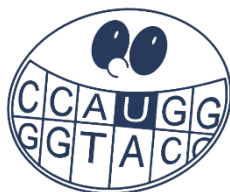
Institute of Molecular Life Sciences

HUN-REN Research Centre for Natural Sciences



Motto: “Research is seeing what everybody else has seen
and thinking what nobody else has thought.”

Albert Szent-Györgyi



Budapest

19-20 November 2024

STATEMENT OF MISSION

Similarly to the previous years, we decided to come together for these 2 days in order to synthesize our recent work and to tell other lab members what we achieved during the last year. This occasion provides an excellent opportunity to acquire a deeper understanding of the issues we are working on and to put them into a wider scientific context. Discussions after each presentation are expected to initiate fresh ideas and novel routes of successful project realization. The intensive 2-days training is also intended to enhance teamwork and knowledge transfer between lab members.

PRACTICAL INFORMATION

Presentations will cover the work performed during the last year in the lab.

Chairs are kindly requested to observe the time allowed for presentations (10-15 min) and lead the discussion sessions (10-15 min).

Everybody is fully expected to take active part in the discussions.

Venue: HUN-REN TTK Research Centre for Natural Sciences, Budapest 1117, Magyar tudósok krt 2.

PARTICIPANTS

Angéla Békési

Eszter Oláh

Gergely Döbrössy

Viktória Perey-Simon

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Beáta G. Vértessy

Júlia Cs. Nagy-Lászlóffy

Fanni Vigh

Nikolett Nagy

PROGRAMME

Tuesday, 19th November 2024

9:00 Morning coffee

Session 1: RT-qPCR analysis of reference genes and dUTPase

Chair: Beáta G. Vértessy

9:30 **Máté Tóth:** Analysis of reference genes with stable mRNA expression during cell cycle

9:50 **Otília Tóth:** Identification of reference genes and expression of dUTPase during the cell cycle in human cancer cell lines

10:10 **Nikolett Nagy:** Characterization of dUTPase expression in mouse postnatal development and adult neurogenesis

10:30 Coffee break

Session 2: Moonlighting function of dUTPase in mitosis

Chair: Judit Tóth

11:00 **Bianka Kozma:** Identification and role of dUTPase protein-binding partners in a mouse embryo model

11:20 **Eszter Oláh:** Isoforms and Modified Variants of Human dUTPase Enzyme and Experimental Design of Silencing dUTPase Using shRNAi in HCT116 Cells

11:40 **Otília Tóth, Eszter Oláh, Nikolett Nagy:** Investigation of the role of dUTPase in mitosis

12:20 **Fanni Vigh:** Clathrin is required for the function of the mitotic spindle

12:50-14:00 Lunch break (Delirest)

Session 3: Characterization of NDST3 heparan sulfate biosynthesis enzyme

Chair: Angéla Békési

14:00 **Réka Iván:** Recombinant production and biochemical analysis of the N-sulfotransferase domain of the heparan sulfate biosynthesis enzyme NDST3

14:20 **Kende V. Kárpáti:** Recombinant production and preliminary characterization of the NDST3 deacetylase domain of NDST3 heparan sulfate biosynthesis enzyme

14:40 **Júlia Cs. Nagy-Lászlóffy:** Recombinant production and preliminary analysis of the full luminal region of the NDST3 heparan sulfate biosynthesis enzyme

15:10 **Gergely N. Nagy:** Understanding the sulfation specificity of heparan sulfate glycosaminoglycans: NDST3 characterization project overview

Wednesday, 20th November 2024

9:00 Morning coffee

Session 4: Effects of thymidylate synthase inhibitors and the role of genomic uracilation patterns in embryonic development

Chair: Rita V. Hirmondó

9:30 **Angéla Békési:** Genomic uracilation and transcriptional regulation upon thymidylate synthase inhibition in HCT116 cells

10:00 **Eszter Holub:** Factors of genome editing and RNA regulation upon thymidylate synthase inhibitory drug treatments

10:30 **Milda Szajkó:** Characterization of genomic uracil patterns: sensor development and functional analysis in an embryonic development model

11:00 Coffee break

Session 5: New insights into mycobacterial stress response and DNA repair

Chair: Gergely N. Nagy

11:30 **Dániel Molnár:** New insights into the mycobacterial stress response and adaptation

12:00 **Rita V. Hirmondó:** Unveiling DNA repair pathways and cell structure in mycobacteria using fluorescence reporter constructs

12:30 **Gergely Döbrössy:** Developing a CRISPR-based method to investigate pathway choice for DNA double-strand break repair in mycobacteria

13:00-14:00 Lunch break (Delirest)

Session 6: The importance of enzymes in embryonal development, in biocatalysis and degradation of toxic agents

Chair: Nikolett Nagy

14:00 **Viktória Perey-Simon:** Role and effect of uracil metabolism on zebrafish embryonic development

14:30 **András Telek:** The RnKIREd story: biocatalytic reductive aminations and cell-free enzyme engineering

15:00 **Zoé Tóth:** Identification and characterisation of novel enzymes presumably involved in hydrocarbon biodegradation

15:30 **Nikolett Emődi:** Enzyme-based protection against mycotoxins: The search for novel zearlenone hydrolases

16:00 **Beáta G. Vértessy:** Closing remarks

Analysis of reference genes with stable mRNA expression during cell cycle

M. Tóth¹, N. Nagy^{2,3}, O. Tóth^{1,2}, B. G. Vértessy^{1,2}

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Reverse transcription-coupled quantitative PCR is one of the most widely used molecular biology methods today. It is widely used in research and medical diagnostic procedures (Kubista et al. 2006). mRNA expression requires the use of appropriate reference genes, but finding these is particularly challenging (Kozera and Rapacz 2013). Our research group has already successfully identified reference genes that are well applicable across different human cell lines (Rácz et al. 2021). In my work, I investigated the mRNA expression of previously used reference genes (*RPL30*, *SNW1*, *PUM*, *ACTB*, *TBP*, *HNRNPL*, *CNOT4*, *PPIA*, *GAPDH*, *UBC*, *PCBP1*, *IPO8*) in two human cancer cell lines (U937, MOLT4) during the cell cycle using RT-qPCR technique. To obtain information about the expression patterns specific to a particular cell cycle phase, I had to synchronize the cells and then analyze the samples at different time points. The success of the synchronization was verified by FACS. The data obtained from RT-qPCR measurements were evaluated using three different independent methods: the comparative deltaCt method, BestKeeper and NormFinder softwares. My aim was to identify reference genes whose expression remains stable throughout the cell cycle, thus aiding the study of cell cycle-dependent processes, which has a significant role in cancer research. Based on my results, I was able to identify suitable reference genes for both cell lines, *CNOT4*, *TBP*, *GAPDH* genes in the MOLT4 cell line and *SNW1*, *TBP* and *RPL30* genes in the U937 cell line. Summarizing the results, I also successfully identified reference genes with stable expression in both cell lines, which are *SNW1* and *GAPDH* genes. The results obtained are of significant help in designing cell cycle-dependent gene expression assays. My research also highlights the critical importance of checking the stability of reference genes for all cell lines.

Identification of reference genes and expression of dUTPase during the cell cycle in human cancer cell lines

O. Tóth^{1,2}, G. A. Rácz², E. Oláh^{2,3}, M. Tóth¹, E. Szabó², G. Várady², B. G. Vértessy^{1,2}, N. Nagy^{2,3}

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Cell cycle-dependent gene expression analysis is particularly important as numerous genes show tightly regulated expression patterns at different phases of the cell cycle. For cancer cells, analysis of cell-cycle related events is of paramount significance since tumorigenesis is characteristically coupled to cell-cycle perturbations. RT-qPCR is a highly sensitive technique to investigate cell cycle-dependent transcriptional regulation. However, for reliable evaluation of qPCR data reference genes with stable mRNA expression are required. Although several studies investigating cell cycle-dependent gene expression employ frequently used reference genes, moreover, such potential genes for cell cycle analysis have not been described in the literature. Therefore, we aimed to identify reference genes characterized with stable expression throughout the cell cycle in MOLT4 and U937 human cancer cell lines using RT-qPCR. Here we present a highly robust method used both for identification of stable reference genes and for determination of cell cycle-dependent gene expression of dUTPase. Enzyme dUTPase has a crucial role maintaining genomic integrity to prevent uracil misincorporation into the DNA [1]. Recently, two novel isoforms, DUT-3 and DUT-4 have been characterized in human cell lines [2], however, localization and cell cycle-dependent expression of these isoforms are not yet characterized. Therefore we investigated the mRNA expression of dUTPase isoforms throughout the cell cycle using RT-qPCR, and the localization of novel DUT-3 and DUT-4 isoforms using immunostaining. Here we show, that the previously identified *SNW1* and *CNOT4* genes [3] are suitable for cell cycle-dependent gene expression analysis, therefore we used these reference genes for normalization to examine the expression of dUTPase isoforms during the cell cycle. According to our results, DUT-N and DUT-M isoforms are shown to be S-phase dependent, while cytoplasmic DUT-3 and nuclear DUT-4 are found to be constitutive and G2-phase dependent, respectively.

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3. G. A. Rácz, N. Nagy, G. Várady, J. Tóvári, Á. Apáti, B. G. Vértessy, *Sci Rep.*, (2021)

Characterization of dUTPase expression in mouse postnatal development and adult neurogenesis

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The enzyme dUTPase has an essential role in maintaining genomic integrity. In mouse, nuclear (nDut) and mitochondrial (mDut) isoforms of the enzyme have been described [1]. We investigated the isoform-specific mRNA expression levels in different murine organs during development using RT-qPCR. In this study, we analyzed brain, gonad and liver samples derived from 14.5-day embryos. Furthermore, heart, kidney, lung, spleen and thymus samples were also investigated in postnatal 2-, 4-, 10-week- and 13-month-old mice to represent the milestones during development using FVB/N mouse strain. With the analysis of these organs, we could cover the three germ layers: brain (ectoderm); heart, kidney, ovary and testis (mesoderm); thymus, lung and liver (endoderm). We demonstrate organ-, sex- and developmental stage-specific differences in the mRNA expression levels of both isoforms.

The nuclear isoform of dUTPase is more abundant than the mitochondrial isoform based on their mRNA expression. In addition, the changes observed during development and among the organs show larger differences in case of the nuclear isoform. We found that expression of nDut in the lymphoid organs, i.e., the thymus and spleen, shows uniformly high level throughout development. Regarding the reproductive organs, differences can be observed between the sexes at the 4-week-old stage and after, notably the expression of nDut in the adult testis is threefold higher than in the adult ovary. In the liver the expression of nDut is 20-fold higher in the E14.5 samples than in the 4- and 10-week-old groups. In some 13-month-old tissues we observed a slight increase in the expression of *Dut* isoforms, notably in the brain and gonad samples. Various studies have reported an age-related decline in DNA repair processes, importantly in the BER pathway in mouse brain and germ cells [2,3,4]. During aging the activity and expression of DNA glycosylases, particularly uracil-DNA-glycosylases was shown to decrease. Accordingly, we propose that dUTPase can act as a compensatory enzyme by preventing dUTP nucleotide misincorporation, hence attempt to maintain genomic integrity during aging.

We found high mRNA expression level of the nuclear isoform in the embryo brain, and the expression level remained relatively high in the adult brain as well. This was surprising, since dUTPase is known to play an important role in proliferating cells, and mass production of neural cells is completed by adulthood. However, the importance of appropriate dTTP biosynthesis was described in neuronal development, since in human and in zebrafish erroneous DTYMK led to severe postnatal neurodegenerative disease [5,6]. Thus, we investigated the pattern of the dUTPase protein expression specifically in the adult brain with immunostaining and found that dUTPase is present in the germinative zones, the subventricular and the subgranular zones, where neurogenesis occurs and in the rostral migratory stream where neuroblasts migrate to the olfactory bulb (Figure 1).

We showed that dUTPase is expressed in the intensely proliferating intermediate progenitor cells, presumably from the start of the mitotic activity, and is present during mitosis. Postmitotic neuroblasts

also keep on expressing the enzyme, which partially dislocates to their cytoplasm. The elevated dUTPase protein expression in these regions may account for the measured high mRNA level, as estimates suggest that several thousand neural cells are generated in different compartments of the young adult mouse brain [7].

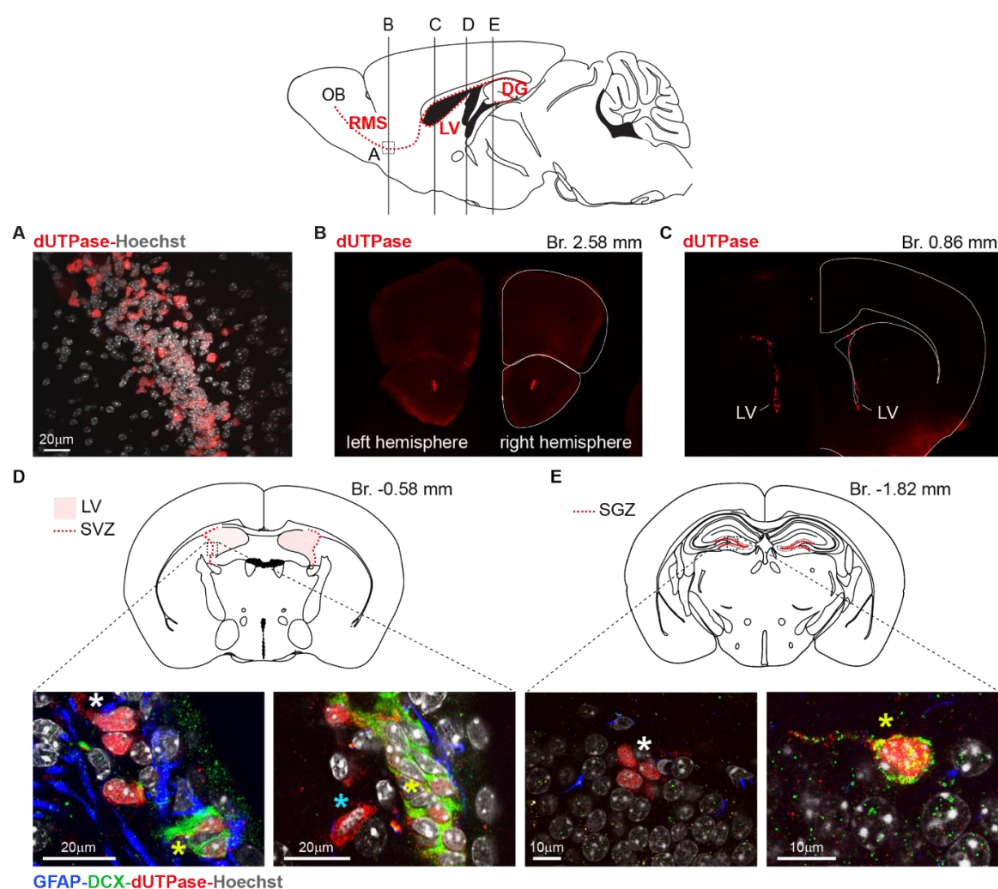


Figure 1. Protein expression of dUTPase in adult mouse brain.

Next to our extensive isoforms-specific gene expression analysis in the different mouse organs during development, we showed that dUTPase protein expression in the adult mouse brain is mainly observed in the progenitor cells in the germinative zones and the rostral migratory stream [8]. Our results suggest that dUTPase expression is critical during cell differentiation and development and indicate that accurate dTTP biosynthesis can be vital, especially in neurogenesis.

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Identification and role of dUTPase protein-binding partners in a mouse embryo model

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DNA stability is critical for maintaining genomic integrity and normal cell function. For this purpose, several mechanisms are activated in cells, of which the dUTPase enzyme plays an important role in preventive repair. The enzyme catalyses the hydrolysis of dUTP, thereby preventing the misincorporation of uracil in DNA instead of thymine, and provides cells with the dUMP precursor required for *de novo* biosynthesis of the dTTP nucleotide.

Nevertheless, our group has previously shown that, in addition to its long-known function, the enzyme may also be involved in critical processes of cell division and embryonic development. My research aimed to study these processes in detail during early mouse embryonic development. To this end, I investigated the localisation of the dUTPase enzyme in mouse embryos at 3.5, 4.5 and 6.5 days of blastocyst stage using immunofluorescence staining. My results showed that the cytoplasmic and nuclear distribution of dUTPase varied with embryonic development stage, which could be explained by the different mitotic activity of different embryonic cell types.

In addition, a definite colocalization between the dUTPase enzyme and tubulin was detected in dividing cells of early-stage blastocysts at the mitotic and central spindle and in the trophectoderm of late-stage embryos at the microtubule network. Based on my results, it is likely that dUTPase interacts with the constantly evolving microtubule network, which justified the need for further experiments. To this end, I have identified α - and β -tubulin as well as several subunits of the TRiC complex as potential interacting partners of the human dUTPase enzyme using a co-immunoprecipitation coupled LC-MS/MS procedure.

My results indicate that dUTPase enzyme may exceed its previously known preventive role and interact with microtubules at stages of cell division and differentiation where cells undergo active cytoskeletal rearrangement. A better understanding of the role of dUTPase enzyme in living organisms and its successful inhibition could make it a promising therapeutic target for cancer treatment.

Isoforms and Modified Variants of Human dUTPase Enzyme and Experimental Design of Silencing dUTPase Using shRNAi in HCT116 Cells

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The enzyme dUTPase is an evolutionarily conserved protein found in organisms ranging from viruses and bacteria to humans, where it plays a critical role in establishing and maintaining genomic integrity. The well-characterized primary function of dUTPase is the hydrolysis of dUTP, yielding inorganic pyrophosphate and dUMP, which serves as the precursor of dTMP and substrate of thymidylate synthase (TS) [1]. The protein typically assembles from three identical subunits, each contributing to the overall rigidity and catalytic activity of the enzyme to form a homotrimeric structure with three active sites located at subunit interfaces. Characteristic core domain, conserved sequence motifs, and key residues responsible for the trimeric structure, substrate binding and hydrolytic function have previously been identified and investigated in detail [2]. To date, four different isoforms of dUTPase containing all five conserved motifs have been described in human cells. Besides the well-known nuclear and mitochondrial isoforms, two novel variants, DUT-3 and DUT-4 were identified recently [3]. Encoded by the *DUT* gene, isoforms are generated by alternative splicing under the regulation of three different alternative promoters [4]. Sequence of the mRNA products at the 3' end is shared between all isoforms, as well as the corresponding C-terminus at the protein level. The previously discovered DUT-N and DUT-M variants differ only in their first exon, as this region contains the nuclear and mitochondrial localization signals, respectively [4, 5]. DUT-3 was thought to be localized in the cytoplasm as it lacks any localization signals, while the fourth form, DUT-4, closely resembles DUT-N, the only difference being a few amino acid residues at the N-terminal region of the protein; upstream locus of its first exon however suggests alternate promoter usage and regulation [3]. Phosphorylation of dUTPase adjacent to its localization signal by Cdk1 kinase in G2 phase results in dynamic cell cycle-dependent distribution of the protein and has been shown to regulate stability and subcellular localization of dUTPase [5]. Although the phosphorylation-dependent cytoplasmic accumulation of dUTPase has been described, the purpose of this modification is yet to be clarified [6, 7].

Previous results and recent observations suggest that dUTPase may have an undocumented role in cellular processes besides its canonical function that needs to be investigated in detail. CRISPR/Cas9-mediated knockout of dUTPase was shown to be lethal at the preimplantation (blastocyst) stage in *dut*^{-/-} mouse embryos, a critical period when the meiotic to mitotic transition occurs, indicating an important role of dUTPase in mitosis. Moreover, several proteins known to be important in mitotic processes have been identified as potential interacting partners of dUTPase using co-IP-MS in mitotic HCT116 cells, further supporting our hypothesis. Therefore, our aim was to thoroughly investigate the subcellular localization patterns of all isoforms via the transient transfection of HCT116 cells overexpressing recombinant DUT-N, DUT-3 and DUT-4 fused to eGFP at their C-termini. Furthermore, two phosphorylation-state mimicking (S11E and S11Q), and two catalytically inactive (D102N and A98F), eGFP-fused mutant variants were generated via site-directed mutagenesis to assess the effect of phosphorylation and the relevance of enzyme activity in the hitherto unknown role of dUTPase in mitosis.

Cytoplasmic localization of the DUT-3 isoform and nuclear localization of DUT-4 was recently confirmed by immunofluorescent staining (ICC) as well as through the transient transfection of HCT116 cells with overexpressed recombinant constructs of dUTPase, highlighting the significance of its

presence in the cytoplasm. Characterization of the spatial and temporal distribution of different isoforms, the enzymatically inactive, and the phosphorylation-state mimicking variants throughout the various phases of eukaryotic cell division can provide invaluable insights regarding the role of dUTPase in cellular processes.

Downregulating dUTPase levels using siRNAi was previously shown to perturb other enzymes of thymidylate metabolism and to affect cell proliferation in cancer cell lines [8, 9]. To examine the effect of silencing dUTPase in mitosis, we designed 4 shRNAi constructs targeting the 3'-UTR region of the protein for the effective post-transcriptional degradation of the mRNA products. Experimental design, proposed analytical methods and validation of inducible dUTPase knockdown will be discussed in detail. Together, the study of inactive and modified forms, alongside shRNA silencing, provides a comprehensive picture for exploring the hitherto unknown function and regulatory mechanisms of dUTPase, with potential implications for targeting nucleotide metabolism in diseases characterized by genomic instability.

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Investigation of the role of dUTPase in mitosis

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The well-known role of dUTPase in the hydrolysis of dUTP to dUMP is extensively described in the literature. While maintaining low cellular dUTP/dTTP ratio is critical, other function of dUTPase has not been reported so far. Recently, two novel isoforms of dUTPase have been characterized in human cells, giving four isoforms of the enzyme, i.e. a nuclear, a mitochondrial and presumably a cytoplasmic and a secondary nuclear isoform [1]. Previously, it was shown that dUTPase is phosphorylated in mitosis at the Ser11 residue adjacent to the nuclear localization signal and the phosphorylated form is excluded from the nucleus as interaction with importin- α is abolished [2,3]. Importantly, it was reported that knockout of the *Dut* gene in mouse lead to early embryonic lethality, since embryos with *Dut*^{-/-} genotype died after the blastocyst stage [4]. Investigation of dUTPase mRNA expression in different mouse organs during development highlighted that dUTPase expression is the highest in tissues characterized with extensive cell proliferation, i.e. thymus, spleen and reproductive organs [5,6]. Overexpression of dUTPase is also described in cancer cells, moreover the enzyme is a potential chemotherapeutic target as part of the thymidylate metabolism pathway [7]. Although the elevated expression of dUTPase in proliferating cells is explained by the importance of dUTP hydrolysis, no targeted investigation was conducted in mitotic cells.

Therefore, we analysed the localization of dUTPase with immunofluorescent staining in different samples. We characterized the localization of dUTPase (DUT) and phosphorylated dUTPase (P-DUT) during mitosis in HCT116, HEK293 and MEF cells. Moreover, we examined the localization in iHPSC B7 and XCL1 neural progenitor cells (NPC) and mouse blastocysts. We found that dUTPase localizes to the mitotic spindle in metaphase, and it is detected in the centrosomes as well. Interestingly, in trophectoderm cells of mouse blastocysts and human NPCs dUTPase shows colocalization with the microtubule protofilament network.

Furthermore, we performed coIP-MS on mitotic cells analysing the cytoplasm and found several interacting partners of dUTPase which have important roles in mitosis (α -, and β -tubulin, TPT1, myosin, profilin, cofilin). Moreover, six subunits of the chaperon responsible for tubulin folding (TRiC) was also identified. Importantly, interaction of the TRiC complex with murine cytomegalovirus M72 dUTPase homolog was described in the literature [8]. Previously, using Y2H technique two proteins important for microtubule organizing centers (MTOC), i.e. centrosomes and Golgi, cenexin (ODF2) and myomegalin (PDE4DIP) were identified as interacting partners.

Localization pattern and potential interactions with several proteins involved in MTOC formation and microtubule binding propose a particularly important role of dUTPase in mitosis. We suggest that the unique structure of homotrimer dUTPase may be responsible for interactions essential for mitosis. Interestingly, several proteins important for mitotic processes are characterized with trimeric structure (clathrin, MCC, AMPK). During mitosis, the cell undergoes dramatic changes in shape and the different cellular components are subjected to mechanical forces. The symmetry of trimer proteins ensures structural stability, allows simultaneous binding to multiple partners and coordinates the highly dynamic microtubule network.

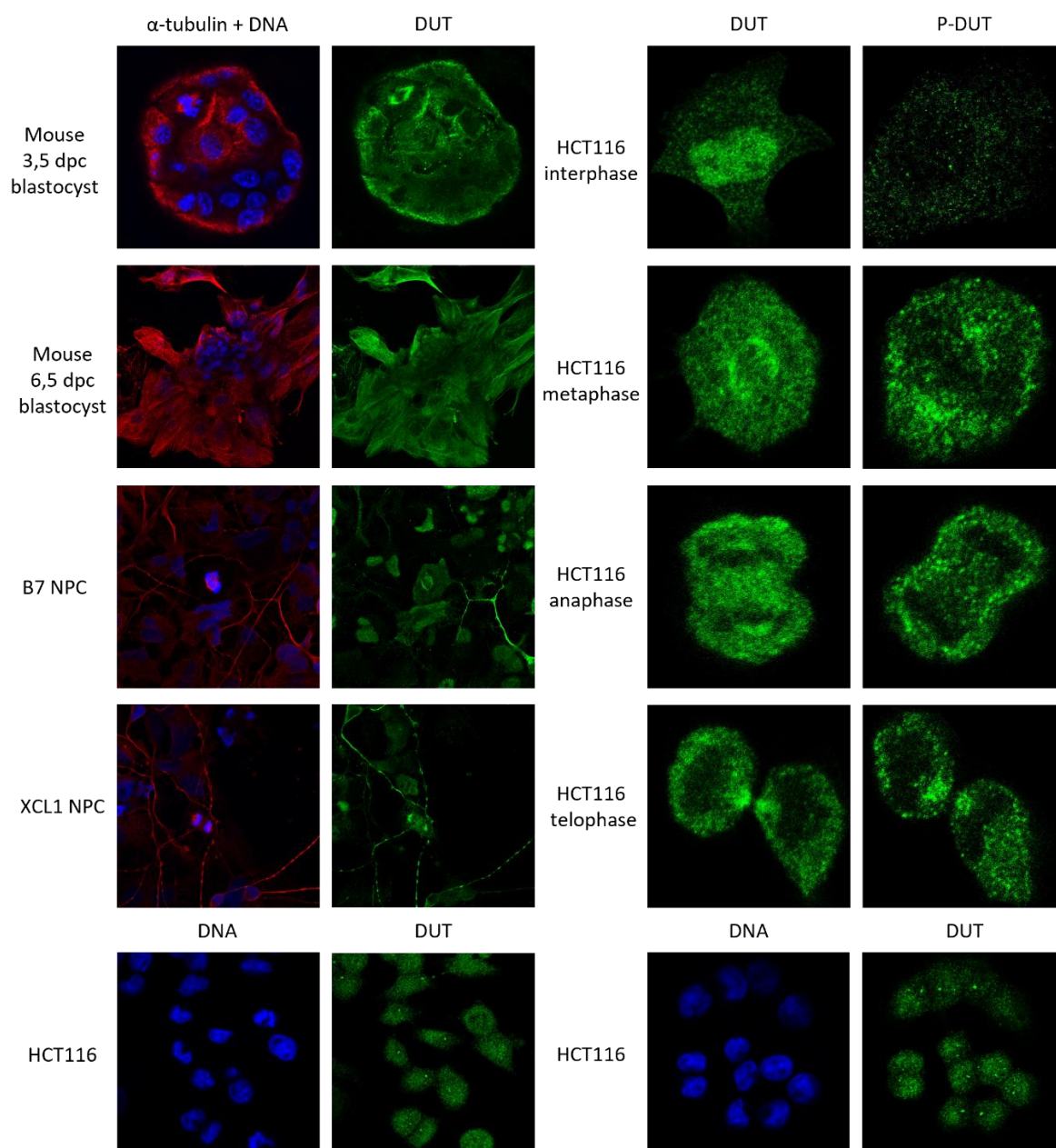


Figure 1. Immunofluorescent staining of dUTPase.

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Clathrin is required for the function of the mitotic spindle

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This is an introductory presentation about a Nature study titled above [1]. The importance of clathrin is known in cells during interphase regarding the transportation of vesicles. In this study it is introduced that it has a second function during mitosis, which is the stabilization of kinetochore fibres of the spindle apparatus. Using various GFP constructs of clathrin, it is shown that the N-terminal of the protein's heavy chain binds to the spindle. Using RNA silencing, it is presented that the depletion of clathrin heavy chain prolongs mitosis, while causing misalignment of chromosomes to the metaphase plate, without attaching spindle fibres. Through Mad2 GFP construct, it was observed that the prolonged mitosis is caused by persistent activation of the spindle checkpoint. Rescue experiments were done with clathrin triskelia in cells depleted of endogenous clathrin.

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Recombinant production and biochemical analysis of the N-sulfotransferase domain of the heparan sulfate biosynthesis enzyme NDST3

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Heparan sulfates (HS) are linear cell-surface proteoglycan-linked polysaccharides that play important roles in a variety of biological processes, including cell-to-cell communication, cell-surface signaling, connective tissue matrix stability, and host-pathogen interactions. The molecular interactions of HS chains are critically influenced by their negative charge pattern, which is dictated by their huge variety of sulfate modifications. Following the polymerization of the HS chain, the initial sulfate modifications are provided by the bifunctional N-deacetylase-N-sulfotransferase (NDST) enzymes that perform N-deacetylation and re-sulfation of glucosamine units. (1) However, these modifications are not uniform along the HS chain, and therefore the four human NDST isoenzymes (NDST1-4) create divergent glucosamine patterns. Since NDST enzymatic modifications serve as templates for enzymes catalyzing further HS sulfation and epimerization, the function of NDST isoenzymes fundamentally determines the structure and interactions of the mature HS. (2) The expression of NDST3 is confined to neuronal tissues, and it has important roles in embryonic development and neuropathologies. (3) Peculiarly, NDST3 has a lower sulfotransferase activity compared to other NDST isoenzymes, but the underlying molecular basis for this is currently not resolved. (4)

The aim of my research is to characterize the unique sulfotransferase specificity of NDST3. To this end, I developed a methodological pipeline based on literature and protein engineering considerations for recombinant production of thermostable and enzymatically active NDST sulfotransferase protein constructs in an E coli expression system. For successful protein production, I assessed the effects of protein domain boundaries used, compared fusion protein constructs to assist protein production and purification, and optimized the protein production process. I used this methodology to synthesize the sulfotransferase domains of NDST3 and NDST1 and verified that NDST3 has a moderate sulfotransferase activity compared to NDST1 using a fluorimetry-based coupled enzyme assay. Overall, the optimization of protein production and enzyme activity methods has provided the basis for an experimental system that allows the comparative characterization of the sulfotransferase specificity of NDST1-4 enzymes, which shed light on the different NDST enzymatic functions and contributions to HS biosynthesis.

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Recombinant production and preliminary characterization of the NDST3 deacetylase domain of NDST3 heparan sulfate biosynthesis enzyme

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Heparan sulfate proteoglycans (HSPGs) are involved in several crucial biological roles, such as cell signaling, adhesion and migration, anticoagulation and pathogen binding. The biosynthesis and maturation of HS is a complex process involving numerous membrane-bound enzymes located in the endoplasmic reticulum and the Golgi apparatus [1]. The first key modification is performed by NDST enzymes, of which four isoenzymes (NDST1-4) are known in mammals. NDST enzymes consist of three domains, including the non-catalytic carbohydrate-binding domain (CBM), the N-deacetylase domain (NDase), and the N-sulfotransferase domain (NST). NDSTs catalyze the bifunctional N-deacetylation and N-sulfation processes on N-acetyl-D-glucosamine units within the HS polymer chain, forming the starting points for diverse sulfation patterns that enable a variety of functional roles [2]. The NDST1-4 isoenzymes exhibit different activities relative to each other and are found in distinct tissues and organs. The distribution of NDST3 and NDST4 isoenzymes is the most limited, with their expression being significant only in the brain and the nervous system. Among the four isoenzymes, NDST3 has shown moderate activity in N-deacetylation compared to the other isoenzymes, while its sulfotransferase domain was the least active [3,4].

The aim of my research is to investigate the deacetylase function of NDST3. To this end, I performed cloning of CBM+NDase domain constructs of NDST3 and tested their recombinant expression in *Escherichia coli*. I tested several E coli strains as expression hosts and found that protein expression in the cytosol was only effective when a chaperonin was also co-expressed either as a fusion protein partner (His-Trigger Factor-CBM-NDase cloned in pCold vector) or as a separate protein, in ArcticExpress strain. The His-Trigger Factor-CBM-NDase was purified using nickel affinity chromatography and its was analyzed subsequently by heparin affinity chromatography. Nevertheless, its further characterization was limited due to its low stability at room temperature. Accordingly, we designed new CBM-NDase constructs engineered to improve thermal stability whose production and characterization is underway.

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Recombinant production and preliminary analysis of the full luminal region of the NDST3 heparan sulfate biosynthesis enzyme

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Heparan sulfate (HS) glycosaminoglycans are key linear polysaccharide components of the extracellular matrix with a diverse pattern of sulfation modifications that governs their interactions in a large variety of biological roles, including adhesion and cell surface signalling [1]. The initial sulfate modifications on HS chains are provided by the bifunctional heparan sulfate N-deacetylase N-sulfotransferase enzymes (NDST1-4) which provide glucosamine deacetylation and sulfation modifications that influence further sulfations during HS biosynthesis [2]. NDST1-4 enzymes are displaying different deacetylase and sulfotransferase efficiencies, while NDST1 and 2 are efficient bifunctional catalysts, NDST3 was described as a reasonable deacetylase but poor sulfotransferase [3].

My research aimed to achieve recombinant production of the full luminal form of NDST3, which could allow in-depth characterization of its bifunctional enzyme activities and its contribution to HS biosynthesis. Accordingly, I designed two chimera NDST3 constructs with different N-termini and fused them to the C-terminus of a Trigger Factor chaperonin. I managed to produce these remarkably large protein constructs (>140 kDa) in an *E. coli* expression system. To assess the substrate binding capacity of NDST3, I tested its heparin interactions as the latter has been described as a high-affinity feedback inhibitor of NDST1 and 2 [4]. The heparin affinity chromatography assays indicated potent NDST3-heparin interactions which confirmed the glycosaminoglycan binding capacity of the recombinant NDST3 constructs. Unfortunately, subsequent biochemical characterizations were hindered by the limited thermal stability of the protein constructs, therefore, I assessed the possibility for NDST3 production in a mammalian expression system relying on HEK293T cells. Using molecular cloning I have created several protein expression constructs for secretory expression that include both the full luminal and sulfotransferase regions of NDST3. Initial protein expression tests confirmed NDST3 sulfotransferase construct production in HEK cells although their secretion requires further optimization. Collectively I have reached important milestones in production and initial characterization of NDST3, which could guide subsequent studies in recombinant production of a functional NDST3 and characterization of its enzymatic specificity.

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Understanding the sulfation specificity of heparan sulfate glycosaminoglycans: NDST3 characterization project overview

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Heparan sulfate (HS) glycosaminoglycans (GAGs) are major components of the extracellular matrix (EM) with diverse biological functions, including cell signalling and communication, connective tissue integrity, and host-pathogen interactions ¹. Heparin, a form of HS, is an essential anticoagulant medicine. HS biosynthesis enzymes work together in ways yet poorly understood to assemble and decorate GAG polysaccharide chains leading to great structural and functional diversity ². The bifunctional N-deacetylase/N-sulfotransferase (NDST) enzymes catalyze the first steps toward the formation of HS N-sulfated domains. Preliminary results from our collaborator Rebecca Miller indicate that the variability of the HS sulfation patterns is grounded in the various specificities of the NDST isoenzymes. However, the mechanism of how these specificities are brought about remains elusive. Our project intends to fill this gap in knowledge with a multidisciplinary approach to study the modification mechanism of HS GAG polysaccharide substrates by NDST3. Our project has three overarching objectives: 1) to address the functional role and cooperation of NDST3 enzyme domains; 2) to decipher how the molecular structure defines the specificity of NDST3 and 3) to dissect how NDST3-induced HS modifications modulate the overall HS epitope diversity. With Réka, Julia, and Kende, we set off to produce protein reagents encompassing the separate deacetylase and sulfotransferase regions as well as the full bifunctional NDST3, using *E. coli* or HEK293T cells as expression hosts. I my talk I will summarize our advancements and discuss emerging opportunities for research line progress in 2025.

I will also report on the progress of continued collaboration with my postdoctoral research group in Oxford. I have been investigating the extracellular interactions of semaphorin-plexin cell guidance ligand-receptor complexes, which have important roles in neuronal development and plasticity, immunology, and cancer ³. Within, signaling of class 5 semaphorins (Sema) to Plexin A (Plxn) receptors are reported to guide the development of the retina ⁴ and hippocampus ⁵, and their *de novo* variants are associated with various neuropathies ⁶. During my postdoctoral research, I unveiled the structural basis of Sema5A interactions with glycosaminoglycans ⁷ while also conducting structural studies to dissect the molecular basis of Sema5A-PlxnA2 extracellular interactions. Since 2024, this work has been carried forward by a recently joined postdoc, Richard Karlsson. In a coordinated effort, a Sema5A three-domain structure has been solved, providing insights into the ectodomain geometry of Sema5s that is structurally unrelated to other semaphorins. Further progress has been made in demonstrating the *in vitro* specificity of Sema5A towards different PlxnA 1/2/4 members.

I have also been co-supervising the progress of Emily Rowland, D. Phil student in Oxford, who has submitted her D. Phil thesis with the Viva exam being held on the 15th of November. Her research focuses on the neuropilin receptor specificity of class 3 type of semaphorins while investigating extracellular Sema3G-Nrp2-PlxnA4/PlxnD1 interactions. Her advances in completing a comprehensive *in vitro* binding analysis, cryo-EM structural studies, and writing up research in the thesis would hopefully result in manuscript writing and submission in 2025.

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Genomic uracilation and transcriptional regulation upon thymidylate synthase inhibition in HCT116 cells

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The colon cancer cell lines HCT116, with altered DNA repair capacities (both MMR proficient and deficient, as well as UNG inhibited versions), have been used as a model system to study genomic uracil incorporation following thymidylate synthase (TS) inhibitory drug treatments [1]. Despite targeting the same gene, the two TS inhibitors – the antifolate RTX and the nucleotide analogue 5FdUR – exhibit different effects in terms of cytotoxicity, genomic patterns of uracil incorporation, and induced mutagenicity (manuscript under publication). Additionally, we have whole transcriptome sequencing data available for this model, and one of our objectives is to dissect the relationship between uracilation and transcription regulation.

Last year, we refined our analysis of RNA-seq data for both long and short RNAs and identified differently affected pathways, such as the p53 pathway, resulting from the two drug treatments, which were also influenced by the cellular repair capacity. Meanwhile, the differential manifestation of induced APOBEC activities within the genome in response to the two drug treatments raised questions about alterations in genome accessibility, particularly regarding ssDNA structures as potential APOBEC substrates. We further developed the U-DNA-Seq approach, which preserves the ssDNA component of genomic DNA isolates that may have been lost or underrepresented in previous methods. To achieve this, we compared several DNA isolation techniques using specific Qubit assays and enzymes for the selective digestion of either ssDNA, dsDNA, or RNA-DNA hybrids. The MN kit and the Epicentre precipitation-based method were found to better preserve ssDNA components than the previously used Zymo kit. The genomic uracil profiles generated by ssU-DNA-Seq closely resembled the corresponding original profiles, with only minor differences. However, preprocessing required a special approach to remove the 3' tails synthesized in a template-free manner along with the adapter sequences. Without this rigorous data trimming, the base-substitution profiles calculated from the input data using the variant calling (GATK mutect2 tool) were markedly altered, differing significantly from any known spectra. If this is indeed due to the increased vulnerability of ssDNA, which escapes detection upon conventional NGS library preparation, our results may provide important insights regarding the detection of DNA damage/mutations in NGS data in general.

We also made efforts to improve the comparative analysis of U-DNA profiles, within collaborative projects too, and we collected relevant functional genomic data to perform new GIGGLE searches and genome segmentation analyses.

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Factors of genome editing and RNA regulation upon thymidylate synthase inhibitory drug treatments

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One of the most desired enzyme target in chemotherapy is thymidylate synthase (TS)[1] as it is the only enzyme which is responsible for the formation of the DNA precursor dTMP from dUMP and 5,10-methylenetetrahydrofolate [2]. Upon its inhibition dTMP cannot be efficiently formed and the cellular dUTP:dTTP ratio gets elevated, leading to increased incorporation of uracil moieties into the DNA during replication [3]. The genomic uracils are targeted by the base excision repair (BER) pathway which initiating enzymes are the uracil-DNA-glycosylases (UDGs)[4]. Upon persisting high dUTP:dTTP ratio, uracils are incorporated again during the repair synthesis, leading to hyperactive futile repair cycles and finally cell death [5]. Interestingly, upon inhibition of the main UDG, treatment with TS inhibitors still result in cell death[6], suggesting either that the incorporated uracils might interfere with vital cellular functions, and/or that the TS inhibitory drugs might have some additional mechanism of action.

We study two selective TS inhibitors, the base analogue 5-fluoro-2'-deoxyuridine (5FdUR), which covalently binds to the active site [7], and the antifolate raltitrexed (RTX), which competitively inhibits the enzyme [8]. We investigate the effects of these drugs in mismatch repair deficient or proficient colorectal cancer cell line HCT116 expressing the protein inhibitor of the main UDG (UGI). We have already identified differences between the two drug treatments. In case of high-dose 5FdUR treatment decreased cytotoxic effect was measured compared to the lower concentration of 5FdUR or any concentration of RTX. Elevated C-to-T transition frequency and APOBEC3-like mutational signature was detected only upon high-dose 5FdUR treatment, suggesting activation of the APOBEC3 (A3) DNA-cytidine deaminases. Gene expression measurements revealed elevated mRNA level of A3B in case of both drug treatments, and smaller but high-dose 5FdUR selective increase of A3C, A3D and A3H (under publication). To better understand the dynamics of their activation during the course of the treatments, gene expression of the A3s are measured after 12 h, 24 h and 36 h (ongoing project).

The protein level of A3B after 48 h treatment was found to be elevated in immunocytochemistry (ICC) and showed similar pattern upon both drug treatments. However, no change was detected in the protein level of A3B on western blot (WB), which might suggest an alternative splice variant which might be recognized in ICC with the polyclonal antibody but not on WB with a monoclonal antibody.

The protein level of A3C and A3D showed non-significant increase on WB, while A3H has not been measured yet (no available antibody yet). Additionally, a possible difference in the localisation of the A3s could also explain the high-dose 5FdUR specific genomic effect. A3D showed strong colocalization with nuclear speckles (marker SC-35) with ICC staining but had multiple bands on WB so to be sure in the microscopy results the specificity of the primary antibody is being checked with IP-MS (ongoing).

We attempted to optimise the ICC staining of A3C with no success so far, so new antibody is being ordered soon.

Another factor of the high-dose 5FdUR specific cytosine deaminations might be a difference in the availability of the DNA, since A3s are mainly active on single stranded substrates. To check whether the A3 action is also dependent on the DNA structure, ssDNA content is being checked in time during the two drug treatments, for which RPA staining has been optimised.

To determine the contribution of the different A3s to the detected single-base-substitutions as well as to decide whether there is a causative relation between the C-to-T mutations and the decreased cytotoxic effect upon high-dose 5FdUR treatment, CRISPR knockout constructs are planned (ongoing project, no results yet). Three Single-cell colonies were founded, karyotyped, checked for cell-viability and A3B A3C and A3D proteins were characterised upon the drug treatments. Interestingly, one of the clones did not show decreased cytotoxicity in case of 5FdUR treatment but seemingly had similar A3B, A3C and A3D levels as the other clones, which display the expected viability effect. Unfortunately, there have been no successful CRISPR knockout yet.

Additionally, the drug target TS is also able to modulate RNA processing. It was already presented that it regulates the translation of its own mRNA [9], which is believed to be one of the mechanisms of resistance occurring upon TS inhibitory chemotherapy [10]. It was also shown that TS can bind to p53 mRNA [11], an important tumorsuppressor transcription factor. Since RNA-protein interactions are primarily RNA structure mediated, it can easily happen that TS can bind to yet unidentified RNAs [12][13]. We hypothesize that upon the drug treatments (competitive vs. covalent inhibitors) the RNA interactome of TS might be differently influenced which can lead to different cellular responses. To characterize the TS-RNA interactome upon TS inhibitory treatments we optimised the TS-coupled-RNA-immunoprecipitation (TS-RIP) protocol and samples have been prepared for sequencing (ongoing project).

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Characterization of genomic uracil patterns: sensor development and functional analysis in an embryonic development model

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The presence of uracil in the nucleotides that compose DNA is highly restricted. Cells typically interpret it as an error and remove it through a base excision repair mechanism initiated by uracil-DNA glycosylases [1]. Uracil can appear in cells as a result of cytosine deamination or through thymine-replacing incorporation. The prevention of the latter is ensured by the extremely low dUTP/dTTP ratio, a disruption of which has long been used as a cancer therapy strategy. However, uracil in DNA may also have biological functions, such as playing roles in immune responses [2] or in programmed cell death during *Drosophila* metamorphosis [3].

Recent findings from our research group suggest that in early zebrafish embryonic stage, both the dUTP/dTTP ratio and the level of uracil in DNA are elevated. Its functional significance is supported by the fact that disturbing this state by microinjecting an active dUTP-degrading enzyme (dUTPase) leads to severe developmental defects. Using uracil-DNA immunoprecipitation coupled with sequencing (U-DNA-Seq, [4]) developed in the group, the genomic uracil pattern was mapped in 2.5-hour zebrafish embryos. My research aims to explore the developmental role of the observed elevated genomic uracil level. For this, I performed in silico analyses, comparing the identified uracilation patterns with functional genomic data collected from databases of zebrafish embryos, utilizing the GIGGLE Search package and the Segway genome segmentation learning algorithm. The uracil-enriched regions showed significant overlap with certain satellite elements, including the BRSATI associated with centromeres [5], and correlated with multiple transcriptional regulatory proteins and accessible DNA regions. Reliable sensors are key for effective uracil-DNA investigation. While we currently use uracil-DNA glycosylase-based sensors in the lab, their sensitivity and stability are not always optimal, especially for samples with low uracil content isolated from complex matrices. Therefore, my goal is to further develop U-DNA sensors that combine previously stable constructs, preserving their N-terminal, with a C-terminal AviTag. This allows for enzymatic biotinylation, which, through specific biotin-avidin interaction, enhances the sensitivity and applicability of the sensors.

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New insights into the mycobacterial stress response and adaptation

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Mycobacteria, the causative agents of tuberculosis (TB) are among the most ancient human pathogens. Despite many efforts TB is still a global health concern. In the fight against TB, it is particularly important to expand our knowledge of the stress tolerance, and adaptability of mycobacteria. To unravel the effects of antibiotic pressure on genome stability, we determined the genetic variability, phenotypic tolerance, DNA repair system activation, and dNTP pool upon treatment with current antibiotics using *Mycobacterium smegmatis*.

Whole-genome sequencing revealed no significant increase in mutation rates after prolonged exposure to first-line antibiotics. However, the phenotypic fluctuation assay indicated rapid adaptation to antibiotics mediated by non-genetic factors.

In this talk I will show the conclusion of a very long and stressful research story with its promising new directions.

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Unveiling DNA repair pathways and cell structure in mycobacteria using fluorescence reporter constructs

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Tuberculosis (TB) continues to rank among the top global health threats, and posing a particular challenge with rising cases of multidrug-resistant TB (MDR-TB). As an intracellular pathogen, *Mycobacteria* has evolved specific and efficient DNA repair mechanisms, especially those associated with homologous recombination (HR), to preserve genome stability under these stressful conditions. Utilizing advanced genome sequencing and CRISPR-based tools allows us to examine these repair mechanisms in remarkable detail, uncovering new functions of critical enzymes and proteins essential for mycobacterial survival. This study presents a novel *in vivo* system for analyzing DNA repair using fluorescence-based reporter constructs, along with research into the structure and diversity of mycobacterial cells.

To reveal possible moonlighting function of the dUTPase enzyme and investigating homolog recombination, we established an *in vivo* system comprising specific CRISPR cleavage on a fluorescent reporter. By analyzing fluorescence changes (signal intensity, wavelength) we can deduce the mode of DNA repair and its effectiveness in different mutant strains.

Advancements in genome sequencing technologies have further enhanced our understanding to uncover genetic diversity, track evolutionary adaptations, identify resistance mutations, and understand the complex interactions within bacterial populations. Our sequencing analyses indicate significant genomic heterogeneity within mycobacterial colonies derived from single cells without mutation rate increase. To address this, we designed a fluorescent reporter-based system to assess the clonal diversity within mycobacterial populations. In addition to this experimental work, we plan to analyze publicly available whole-genome sequencing (WGS) data across multiple species.

Beyond genetic repair mechanisms, the complex cell wall structure of mycobacteria, composed of mycolic acid and arabinogalactan layers, requires specialized cell division processes. In many rod-shaped bacteria, MreB family proteins, homologous to eukaryotic actin, form helical structures to regulate growth and morphology. We aim to investigate the role of MSMEG_4349 as a potential actin homolog in mycobacteria, which may contribute to cell shape regulation and structural integrity.

Developing a CRISPR-based method to investigate pathway choice for DNA double-strand break repair in mycobacteria

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Understanding the mechanisms of DNA double-strand break (DSB) repair is crucial for developing new insights into mycobacterial survival strategies, particularly in the context of antibiotic resistance in *Mycobacterium tuberculosis*. The choice of pathway to repair DSBs, primarily homologous recombination (HR) or non-homologous end joining (NHEJ), is critical for maintaining genome stability under stress conditions faced by intracellular pathogens. In comparison to Shuman's earlier method, which achieved DSB induction using a restriction enzyme [1], we are developing a new CRISPR-based method to enable precise in vivo interrogation of DSB repair pathways in mycobacteria.

This study introduces an innovative system combining CRISPR-mediated DSB induction with a fluorescence-based reporter construct to monitor repair pathway activity. Given the toxicity of the commonly used CRISPR-Cas9 system in *Mycobacterium smegmatis* [2], we employ the Cpf1 (Cas12a) nuclease in our approach. By analyzing changes in fluorescence signals, we can assess the repair pathway preference and efficacy across various mycobacterial strains and mutants. This approach allows us to characterize the roles of key enzymes, including potential novel activities such as those of dUTPase, within the repair pathways, and to explore any "moonlighting" functions they may have in pathway choice.

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Role and effect of uracil metabolism on zebrafish embryonic development

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Uracil base is a building block of RNA. Its presence in DNA in most cases is mutagenic, which needs to be repaired. Uracil can enter DNA in two ways. On the one hand through the oxidative deamination of cytosine basis, on the other hand DNA polymerases can't distinguish between dUTP and dTTP, so if the dTTP:dUTP ratio is inappropriate in the cells, dUTP will be incorporated into the DNA^[1]. dUTPase and Uracil-DNA glycosylase (UNG) are the main repair enzymes which are responsible for keeping the genomic uracil level low. dUTPase catalyses the hydrolysis of dUTP to dUMP thus reducing the cellular dUTP:dTTP level, at the same time providing a dUMP substrate for de novo thymidylate biosynthesis^[2].

Our experiments investigate the early stages of embryonic development. As a model organism, we used zebrafish, which is nowadays very popular among researchers because it is easy to keep, requires little cost, develops quite rapidly and shows good survivability against various post-fertilization procedures^[3]. In previous results we found high uracil-DNA and dUTP levels before the maternal to zygotic transition (MZT). During MZT, the zygotic genome begins transcription, maternal mRNAs are actively cleared, and developmental control is transferred to the nucleus^[4]. To find out what is the main reasons for these high levels we measured the mRNA expression levels of genes involved in the uracil metabolism. In addition we have started to measure the dUTPase levels in the embryos of different ages, but these measurements need more optimisation. We have also performed microinjection experiments to figure out what happens when we reduce the dUTP levels, by microinjecting active and inactive form of the dUTPase enzyme into fertilised oocytes. We were also curious to see if there is any pattern in the uracilation of the DNA, to examine that we made U-DNA sequencing using immune precipitation sample preparation.

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The *Rn*KIRED story: biocatalytic reductive aminations and cell-free enzyme engineering

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Chiral secondary amine dicarboxylic acid derivatives are key building blocks of several peptidomimetic ACE2 inhibitors. Reductive amination of α -ketoacids with α -amino esters can yield orthogonally functionalized derivatives facilitating further chemical modifications. Biocatalysis offers a sustainable and selective way to implement this synthetic strategy. In our work we have selected a ketimine reductase enzyme (*Rn*KIRED) for this transformation from a pool of 3 separate enzyme classes (opine dehydrogenases[1], imino acid reductases[2], and imine reductases[3]). We have extended the substrate scope of this enzyme among α -ketoacids and α -amino esters and demonstrated the synthetic applicability of our approach in 16 preparative scale reactions[4]. In addition, we have discovered a unique substrate controlled stereoselective behaviour of *Rn*KIRED. Coupling small amino esters to large ketoacids yields S configuration, while coupling large amino esters with small ketoacids results in R configuration on the newly formed stereocenter (**Figure 1**).

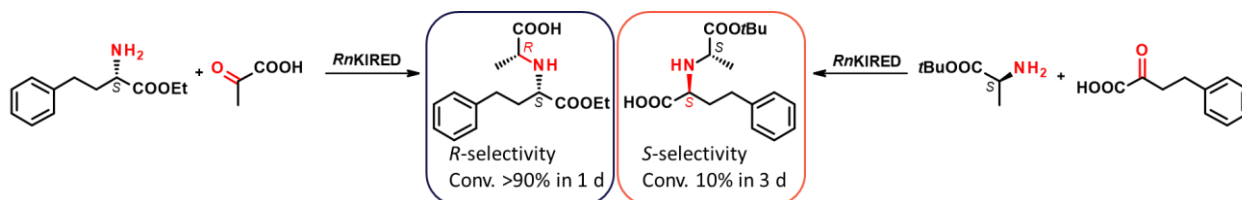


Figure 1. Reductive aminations catalyzed by *Rn*KIRED resulting in different stereoconfigurations.

While this phenomenon enables educated planning of synthetic routes for chemists it also results in challenges concerning the application of *Rn*KIRED in specific reaction settings. Thus, we explored the molecular background for the unique stereospecificity both to understand the underlying mechanisms and to be able to engineer the enzyme for further applications.

When searching for an improved biocatalyst, the quick access to variants is crucial. In an organic chemistry lab cell-free protein synthesis (CFPS) allows the production of enzymes from an appropriate DNA template without the need of manipulating bacteria. This method can be used for miniaturized parallel synthesis and purification to enable fast and reliable activity screenings[5]. To apply this technique in enzyme engineering, we have designed a 60-member variant library of *Rn*KIRED using computational tools[6]. To quickly access the DNA encoding for each variant we have developed an innovative PCR-based method that yields linear templates compatible with CFPS. The combined workflow can provide activity information from an enzyme variant library within 2 days (**Figure 2**). From the 60-member library we have identified single-point mutants that improve the activity and/or modify the stereoselectivity of *Rn*KIRED towards the target molecule. Next, we have combined the mutations according to the FRISM principle[7], to obtain double mutant variants that exhibit further improvements in activity and stereoselectivity.

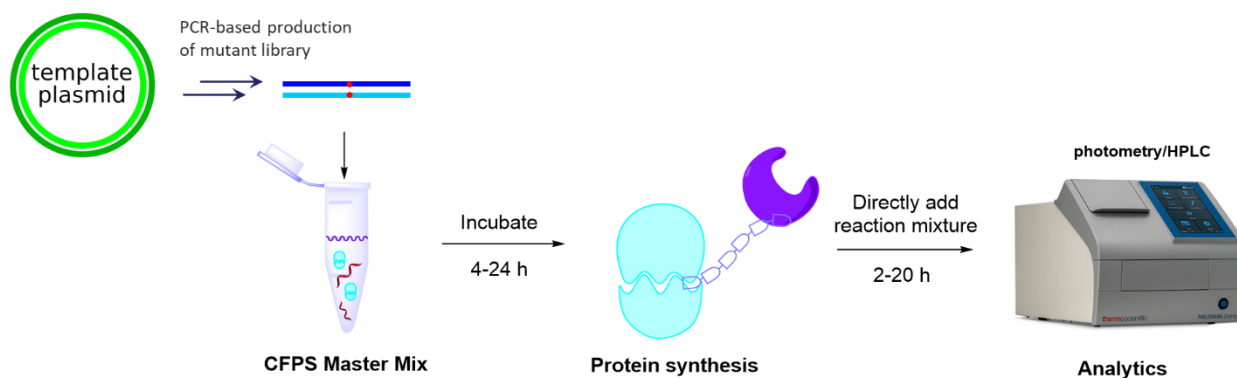


Figure 2. The general workflow of cell-free enzyme engineering.

The presented workflow is completely cell-free thus can be performed even in a chemistry lab without huge investments in specialized laboratories. This bridges a significant gap between the use of natural enzymes and the access to improved variants for industrial applications[8].

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Identification and characterisation of novel enzymes presumably involved in hydrocarbon biodegradation

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The environmental contamination of hydrocarbons used as energy sources is an increasing problem as the hydrocarbon compounds can accumulate in soils and waters, which may endanger human health and biodiversity [1]. Thus, the remediation of these polluted areas is of key importance. Therefore, the identification of novel enzymes contributing to the degradation of these compounds may be a good approach. Metagenomic databases provide a useful platform to identify enzymes from microorganisms adapted to extreme conditions such as hydrocarbon contamination.

Therefore, we searched for novel enzymes with potential monooxygenase activity using the previously developed MetaHMM methodology [2]. From the metagenomic hits we selected five gene sequences, which are identified from metagenome present in tar-contaminated soil sample collected in Israel. The enzymes encoded by the selected gene sequences are probably members of Cytochrome P450 enzyme superfamily, which are heme-containing monooxygenases with a wide range of substrate selectivity [3].

In order to characterise these enzymes, we performed BLAST searches to identify potential substrates and the enzyme subfamilies. To further analyse their function, we carried out protein expression experiments and predicted the three-dimensional structures using AlphaFold. We concluded that the selected enzymes may adapt similar structures as compared to the Cytochrome P450 enzymes sharing high level of sequence similarity with resolved structures.

To test the activity of these enzymes and to further predict substrate selectivity we plan to perform cytochrome reduction assays and molecular docking simulations, respectively.

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Enzyme-based protection against mycotoxins: The search for novel zearalenone hydrolases

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Mycotoxins are secondary metabolites produced primarily by fungi and molds. Contamination of grains reduces feed quality, causing significant losses to the agricultural industry. Mycotoxins produced by *Fusarium* sp., such as zearalenone (ZEN), lead to adverse health issues in humans and livestock upon acute and chronic dietary exposure. Zearalenone's estrogen-like structure disrupts hormone homeostasis and causes reproductive health problems. [1]

Mycotoxins can enter the human food chain indirectly via residues found in milk, meat, eggs, and their derivatives. Therefore, it is crucial to address mycotoxin contamination at the animal feed stage to prevent further risks down the food chain. [2] Currently discarding the grains or the use of adsorbents are the most commonly used strategies to deal with the mycotoxin contamination. Enzymatic degradation of zearalenone enables cereals initially contaminated with mycotoxins to be safely used for animal feed and protects the animals from any negative effects of zearalenone in the feed. [3]

Lactone hydrolases have attracted considerable attention because they can degrade ZEN to a non-toxic form by hydrolyzing the lactone bond in the molecule. [4] Our research focuses on finding new zearalenone lactonases with very low K_M values and high k_{cat} values, so that they can efficiently inactivate the low environmental concentrations of ZEN.

Currently, we are validating existing literature results to establish a baseline of known enzyme sequences. In the next phase of the research, we will focus on discovering novel enzymes, based on the proteins' sequence, and structure. Additionally, we aim to enhance the stability and activity of these lactonases through protein engineering, to develop them as effective feed additives for livestock.

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