Aim 1: How NDK-1/NM23 is able to influence cytoskeleton rearrangement to promote engulfment and clearance of apoptotic cells?

Nm23 gene family members encode nucleoside diphosphate kinases (NDPKs), which catalyze synthesis of nucleoside triphosphates including GTP from corresponding nucleoside diphosphates at the cost of ATP. Fifteen years ago a highly specific genetic interaction was observed between awd (abnormal wing disc), the Drosophila ortholog of Nm23 and Dynamin/shibire, which is a large GTPase functioning in a later stage of endocytosis in eukaryotic cells. AWD was identified as an essential component of endocytosis: a factor required for Dynamin-dependent synaptic vesicle recycling during neurotransmitter internalization in the nervous system (Krishnan et al., 2001).
Recently it was found that Dynamin superfamily proteins and class I human NDPKs function in one complex at membranes (plasma membrane and mitochondrial inner membrane) (Boissan et al., 2014). NDPKs locally fuel atypical GTPase dynamins with GTP allowing these motor proteins to work with high thermodynamic efficiency during membrane remodeling processes.
In previous studies we investigated the role of NDK-1, the Caenorhabditis elegans homolog of group I NDPKs in apoptosis. We first linked an NDPK to apoptotic engulfment and cell corps removal (Fancsalszky et al., 2014). We also found a genetic interaction between ndk-1 and dyn-1, the worm dynamin ortholog, as we generated ndk-1(-);dyn-1(-) double mutants, which were not viable. A phenotypic similarity was found between dying ndk-1(lf) embryos and dyn-1(lf) mutant embryos: late embryonic lethality with persistent cell corpses.

In the current project we further characterized the role of NDK-1/NDPK and DYN-1/Dynamin during apoptotic engulfment and corps clearance in the worm in collaboration with Dr. Zheng Zhou (Baylor College of Medicine, Houston, USA) and Dr. Mathieu Boissan (INSERM, France).
To examine whether the two proteins form a complex in C. elegans, co-immunoprecipitation was performed. Precipitation of worms transgenic for NDK-1::GFP with a monoclonal anti-GFP antibody followed by Western blot detection using a highly specific anti-DYN-1 antibody showed that DYN-1/Dynamin and NDK-1/NDPK function in the same complex. Furthermore, the Zhou lab performed an IP-Mass Spec on wild-type worm extracts with the same anti-DYN-1 antibody and pulled down NDK-1.
In the second year of the project, in order to analyze co-localization of NDK-1 and DYN-1 in vivo, we also performed duolink proximity ligation assay (PLA). PLA experiments were successfully conducted and resulted in 9-13 positive spots/fixed embryo and also verified that NDK-1 and DYN-1 function in the same complex in cells, which engulf apoptotic corpses in nematode embryos.
In order to further characterize the co-localization of NDK-1 and DYN-1 dynamics over the process of apoptotic cell-corps removal during embryogenesis we generated transgenic lines carrying DYN-1::GFP and NDK-1::mCherry constructs. Time-lapse microscopy conducted on the above mentioned, double transgenic embryos showed the expected co-localization of the two transgenes in engulfing cells. This technique also allows us to visualize the phagosomal localization of DYN-1::GFP and NDK-1::mCherry over the engulfment process: both fusion proteins were visible on phagosomal surfaces at the early stage of phagosome maturation (during the first 15 minutes of apoptotic clearance).

Aim 2: We were interested to see whether the positive effect of NDPKs exerted on apoptotic cell removal is evolutionarily conserved

As genetic pathways of apoptosis and apoptotic cell elimination are highly conserved between worms and mammals, we expected that the positive effect of NDK-1 exerted on apoptotic engulfment is evolutionarily conserved. To investigate this issue, we first intended to overexpress NDK-1 and its human counterparts NM23-H1 and H2 (recently also called NME1 and NME2, respectively) in

specialized engulfing cells to examine how elevated NDPK level modifies their engulfment capacity (collaboration with Tamás I. Orbán, Research Center for Natural Sciences, HAS and with Éva Rajnavölgyi, University of Debrecen, Institute of Immunology). The constructs overexpressing the NDPK homologs were nicely introduced by transposon-mediated gene delivery into HEK293 cells (that we used as a positive control to check our materials and tools). We successfully transfected U937 monocytes by the same constructs, but were unable to stabilize transgenic lines as the cells died after transfection. Thus, phagocytosis assays in response to NM23-H1/2 overexpression could not be carried out. Next, we decided to approach the problem in the opposite way: with the help of Zsuzsa Szondy (University of Debrecen) we monitored the phagocytic capacity of mouse bone marrow derived macrophages (BMDMs) after silencing the mouse homologue NM23-M1.
Macrophages were treated by Nm23-M1-specific or non-targeting siRNAs. Western blots showed that Nm23-M1–specific silencing resulted in 55% decrease of the NM23-M1 protein level. Next, an in vitro apoptotic cell phagocytosis assay was performed, where apoptotic thymocytes were incubated with BMDMs and the phagocytic capacity of mouse macrophages was examined after Nm23-M1 silencing. We found that decreased NM23-M1 level in specific siRNAi-treated macrophages caused a 40% loss in phagocytic activity.

Next we focused on the interaction of the human counterparts NME1/Dyn2 during internalisation of phagocytic particles in primary human macrophages derived from blood monocytes in a novel collaboration (Dr. Florence Niedergang and Dr. Mathieu Boissan, INSERM, Paris). We first analyzed the recruitment of endogenous NME1 and Dynamin2 at sites of phagosome formation, using non-opsonized zymosan as target particles and specific antibodies against NME1 and Dynamin2. We found that both NME1 and Dynamin2 are enriched in F-actin positive phagocytic cups.
To determine more precisely if NME1 plays a role in phagosome formation, we depleted the protein by RNA interference. Depletion is never complete in macrophages but there was a clear reduction in NME1 expression in macrophages treated with a specific sequence, as compared with the control. We then performed phagocytosis of IgG-opsonized particles on siRNA-treated cells. The results revealed a significant impairment of phagocytosis in NME1 depleted cells. Next, recruitment of both endogenous NME1 and Dynamin2 was analyzed at sites of phagocytosis in cells treated with siRNA against NME1 and a control. We found a decrease in the recruitment of NME1, but also, more importantly, of Dynamin2, at sites of phagocytosis.
These results show that NME1 controls the recruitment of Dynamin2 and is important for an efficient receptor-mediated phagocytosis in human macrophages.
Thus our experiments in three different models show that the positive effect exerted on apoptotic clearance by NDK-1 is evolutionarily conserved, as mouse and human NDPK homologues, NM23-M1 and NME1 (NM23-H1), respectively, are essential factors promoting phagocytosis. Our study provides evidence that the Dynamin/NDPK cooperation is a global mechanism in phagosome formation.

The presence of uncleared apoptotic cells have been associated with different diseases that involve inflammation, autoimmunity and cancer. It is of relevance to human health that the inflammation that arises as a result of insufficient removal of apoptotic cells, damages organs and might also favour tumour growth and progression. Thus, in the light of our findings, we can propose a novel explanation for the metastasis inhibitor activity of NME1: as this group I NDPK promotes phagocytosis of apoptotic cells through fuelling Dynamin GTPases by GTP, it contributes to avoid inflammation, which would emerge in the presence of cell debris and induce tumour progression.
Our efforts resulted in a publication in *FASEB J*: https://www.ncbi.nlm.nih.gov/pubmed/31242766

In the frame of this project we also examined the dosage dependent effect of NDK-1 exerted on cell migration in C. elegans focusing on the distal tip cells (DTCs, leader cells of gonadal migration). We found that in the absence of NDK-1 the migration of DTCs is incomplete. A half dosage of NDPK - as present in ndk-1 (+/ −) heterozygotes - results in extra turns and overshoots of migrating gonad arms. Conversely, an elevated NDPK level also leads to incomplete gonadal migration owing to a premature stop of DTCs in the third phase of migration, where NDK-1 acts. We found that NDK-1 exerts a dosage-dependent effect on the migration of DTCs. Our results are consistent with AWD's function in Drosophila. The combined data suggest that NDPK enzymes control the availability of surface receptors to regulate cell-sensing cues during cell migration. The dosage of NDPKs may be a coupling factor in cell migration by modulating the efficiency of receptor recycling. We published these data as a part of a review in *Laboratory Investigations*, where we summarized mechanisms, which are linked to the anti-metastatic activity of NM23: https://pubmed.ncbi.nlm.nih.gov/28920944/

In addition we summarized the functions of NM23-H1 and its homologs in major processes linked to metastasis in *Pathology Oncology Research (POR):* https://pubmed.ncbi.nlm.nih.gov/31993913/

Both PhD students involved in this grant, Tamás Szeniczey and Zsolt Farkas defended their thesis and completed their PhD studies during the timeframe of the project (defense of Tamás Szeniczey: summer 2019; defense of Zsolt Farkas under my supevision: spring 2020).

Aim 3: By using co-immunoprecipitation we intended to determine interaction partners of NDK-1/NDPK

By identifying evolutionarily conserved novel interaction partners of NDK-1, we potentially have the opportunity to detect novel NM23 interactors. For these experiments we basically performed co-IP analyses coupled to mass spectrometry using a transgenic strain carrying a rescuing translational NDK-1::GFP construct. Mass spectrometry (MS) was performed by the Proteomics Service of Biological Research Center, Szeged (Dr. Aladár Pettkó-Szandtner and Prof. Katalin Medzihradszky).

So far three IP-MS experiments were conducted and resulted glutamate dehydrogenase (GDH-1) as one of the most promising candidates. Glutamate dehydrogenase converts glutamate to α-ketoglutarate and ammonia, while during this reaction $NAD(P)^+$ is reduced to NAD(P)H. A product of the above process, α-ketoglutarate will be used in the TCA cycle resulting in ATP production. In addition, glutamate dehydrogenase plays an important role in ammonia metabolism or lipid biosynthesis. It is important to note that glutamate dehydrogenase is negatively regulated by GTP, which further suggests a link between GDH-1 and NDPK functions.

Currently we are testing the GDH-1/NDK-1 interaction by generating ndk-1(-);GDH-1(RNAi) double mutants to see their phenotype.

Aim 4.: Identification of factors, which are involved in upstream regulation of NDK-1 (using the worm as model system)

We set up a system in the nematode, which is suitable for screening suppressors or activators of NDK-1: we mutagenized nematode strains transgenic for NDK-1::GFP by EMS, and subsequently screened for specimens with altered GFP expression. Animals showing enhanced GFP expression after mutagenesis carry mutations in suppressors of NDK-1, and worms with reduced transgene expression contain potential mutations in NDK-1's activators. So far two such EMS mutagenesis screens were performed and two candidate lines with reduced NDK-1::GFP expression were isolated. Currently these

lines are under whole genome sequencing and subsequent bioinformatics analysis in the lab of Dr. Attila Patócs (HAS-SE Momentum Hereditary Endocrine Tumour Syndromes Research Group, Hungarian Academy of Sciences and Semmelweis University, Budapest).

Aim 5.: To gain insight into the function of extracellular NM23.

In some tumor types such as AML and neuroblastoma, NM23 is released into the serum, moreover in AML and neuroblastoma patients with poor prognosis NM23-H1 serum level is elevated. We asked the following questions: what is the case in solid tumors? Can NM23 also be detected in the serum of patients suffering in colorectal carcinoma (CRC)? Does serum NM23-H1 level correlate with the appearance of metastases (e. g. can it be used as a biomarker)? In frame of our collaboration with Dr. Zoltan Prohaszka (Semmelweis University, 3rd Department of Medicine, Budapest), we possess a complete series of serum samples derived from CRC patients at different stages of tumor progression (229 CRC serum samples: in 50 patients out of 229 metastasis is verified; and 121 control samples).To answer the above questions we started to establish a sensitive sandwich ELISA system to measure serum NM23-H1 levels.

While we were setting up a sandwich ELISA system from commercially available antibodies, the American company AVIVA put its novel NME1-specific (NM23-H1-specific) ELISA kit on the market. We tested this kit using our positive and negative controls (as a positive control we used sera from AML patients) and found that this system was highly specific and sensitive. We conclude that NM23-H1 level in sera of our CRC collection should be measured using this novel kit. However, as the budget of the current project is limited, and originally we did not plan with the costs of such expensive kits, measurement of NM23-H1 level in our CRC collection will be performed in frame of another grant.