Nuclear transport of nicotinamide phosphoribosyltransferase is cell cycle dependent in mammalian cells and its inhibition slows cell growth

Petr Svoboda1,2, Edita Krizova1, Sarka Sestakova1, Kamila Vapenkova1, Zdenek Knejzlik1, Silvie Rimpelova1, Diana Rayova1, Nikol Volfova1, Ivana Krizova3, Michaela Rumlova3, David Sykora4, Rene Kizek5, Martin Haluzik6,7, Vaclav Zidek2, Jarmila Zidkova1, Vojtech Skop1,*#

From the 1Department of Biochemistry and Microbiology, University of Chemistry and Technology Prague, Prague 6, 166 28, Czech Republic; 2Institute of Physiology, Czech Academy of Sciences, Prague 4, 142 20, Czech Republic; 3Department of Biotechnology, University of Chemistry and Technology Prague, Prague 6, 166 28, Czech Republic; 4Department of Analytical Chemistry, University of Chemistry and Technology Prague, Prague 6, 166 28, Czech Republic; 5Department of Human Pharmacology and Toxicology, University of Veterinary and Pharmaceutical Sciences Brno, Brno, 612 42, Czech Republic; 6Centre for Experimental Medicine and 7Diabetes Centre, Institute for Clinical and Experimental Medicine, Prague 4, 140 21, Czech Republic; 8Institute of Medical Biochemistry and Laboratory Diagnostics, Charles University in Prague and General University Hospital, Prague 2, 128 08, Czech Republic.

Running title: NAMPT nuclear transport

*Present address: Diabetes, Endocrinology, and Obesity Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD 20892, USA.

†To whom correspondence should be addressed: Vojtech Skop: Department of Biochemistry and Microbiology, University of Chemistry and Technology Prague, 166 28 Prague 6, Czech Republic Email: skopv@vscht.cz; Tel: (00420)220445154.

Keywords
Nicotinamide phosphoribosyltransferase (NAMPT), NAD, Nuclear localization, GFP fusion, Cancer, Epigenetics, Sirtuin, Visfatin, Pre-B cell colony enhancing factor (PBEF).

ABSTRACT
Nicotinamide phosphoribosyltransferase (NAMPT) is located in both the nucleus and cytoplasm and has multiple biological functions including catalyzing the rate-limiting step in NAD synthesis. Moreover, up-regulated NAMPT expression has been observed in many cancers. However, the determinants and regulation of NAMPT’s nuclear transport are not known. Here, we constructed a GFP-NAMPT fusion protein to study NAMPT’s subcellular trafficking. We observed that in unsynchronized 3T3-L1 preadipocytes, 25% of cells have higher GFP-NAMPT fluorescence in the cytoplasm and 62% in the nucleus. In HepG2 hepatocytes, 6% of cells had higher GFP-NAMPT fluorescence in the cytoplasm and 84% in the nucleus. In both 3T3-L1 and HepG2 cells, GFP-NAMPT was excluded from the nucleus immediately after mitosis and migrated back into it as the cell cycle progressed. In HepG2 cells, endogenous, untagged NAMPT displayed similar changes with the cell cycle, and in non-mitotic cells, GFP-NAMPT accumulated in the nucleus. Similarly, genotoxic, oxidative, or dicarbonyl stress also caused nuclear NAMPT localization. These interventions also increased poly(ADP-ribosyl) polymerases (PARP) and sirtuins (SIRT) activity, suggesting an increased cellular demand for NAD. We identified a nuclear localization signal in NAMPT and amino acid substitution in this sequence (424RSKK to ASGA), which does not affect its enzymatic activity, blocked nuclear NAMPT transport, slowed cell growth, and increased histone H3 acetylation. These results suggest that NAMPT is transported...
into the nucleus where it presumably increases NAD synthesis, required for cell proliferation. We conclude that specific inhibition of NAMPT transport into the nucleus might be a potential avenue for managing cancer.

Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the rate limiting step in NAD biosynthesis, the reaction between nicotinamide and 5-phosphoribosyl-1-pyrophosphate to form nicotinamide mononucleotide (NMN) (1). NAD is a required cofactor in numerous oxidoreductase reactions (2) and in non-redox processes such as DNA repair and replication, transcription, cell division, and cell death. Two main classes of NAD-consuming enzymes are the poly(ADP-ribosyl) polymerases (PARP) and sirtuins (SIRT). Non-redox, NAD-consuming enzymes are found in the nucleus, mitochondria, and cytosol (3). NAMPT has also been identified as a secreted factor with an extracellular function affecting immune system. Alternate names of the NAMPT are visfatin (4), and pre-B cell colony enhancing factor (PBEF) (5). The fact that NAMPT is both an intracellular enzyme and a secreted hormone, is intriguing and would suggest complex trafficking of this protein.

Since NAD is required throughout the cell, NAMPT might be expected to be distributed in multiple cellular compartments. In dividing Swiss 3T3 and PC-12 cells, NAMPT was predominantly cytosolic, but its localization changed to nuclear when the cell division was stopped (6). Translocation of NAMPT from cytosol to nucleus as a result of cell division arrest has also been observed in colorectal carcinoma cells HCT-116 (7). NAMPT was located in the nucleus in pre-pubertal chicken testis cells (8). In non-stimulated human vascular endothelial cells, NAMPT was mainly nuclear; it localized to the cytoplasm after activation by IL-1β (9). These results suggest that the transport of NAMPT between cytosol and nucleus is a regulated process. However, NAMPT’s transport mechanism, relationship to cell cycle, and role in the nucleus are not known.

To characterize NAMPT transport and its regulation, we studied endogenous NAMPT and GFP-NAMPT during the cell cycle, after inhibition of cell proliferation, and under stress conditions. We identified a required nuclear localization signal. Our data suggest that inhibition of NAMPT nuclear transport has potential as a therapeutic target in cancer treatment.

Results

NAMPT is located both in the nucleus and cytosol

We studied the cellular localization of NAMPT in three cell types: i) human hepatoma HepG2 cells, ii) mouse 3T3-L1 preadipocytes, and iii) differentiated 3T3-L1 adipocytes. Fusion proteins with either EGFP or HA at the N-terminus of NAMPT were constructed. The GFP-NAMPT was transiently transfected and its localization was analyzed by fluorescence microscopy (Figure 1A). HA-tagged NAMPT showed similar localization as GFP-NAMPT (Figure 1B). Fluorescence was detected in both, the nucleus and the cytoplasm with a large intercellular variability in nuclear to cytoplasmic proportion. To quantify NAMPT localization, we counted the number of cells with NAMPT located predominantly in the nucleus or cytoplasm according to average fluorescence. In HepG2 hepatocytes, NAMPT was nuclear in 84% and cytoplasmic in 6% of the cells, without clear preferential localization in the rest. In 3T3-L1 preadipocytes, NAMPT was nuclear in 62% of cells and cytoplasmic in 25%. In contrast, in non-dividing 3T3-L1 adipocytes, NAMPT localization was nuclear in >99% of cells. These results demonstrate that NAMPT localizes to both the nucleus and cytoplasm in multiple cell types and suggest that the GFP does not affect NAMPT distribution.

NAMPT localization changes during cell cycle

To further explore NAMPT localization, we produced stably-transfected HepG2 cell lines expressing GFP-NAMPT and monitored its localization using live cell imaging microscopy (Movie S1). In cells undergoing mitosis during the initial 24 h, GFP-NAMPT location was scored according to average fluorescence until the next mitosis or up to the end of monitoring, whichever occurred first (Figure 2A). Prior to mitosis, NAMPT was always predominantly nuclear, while newly formed nuclei were free of NAMPT and for the subsequent 6.9 ± 1.7 h the average...
fluorescence was higher in cytoplasm. This period was followed by $5.3 \pm 2.6$ h of dual localization, and then NAMPT was observed to be predominantly nuclear for $7.6 \pm 3.6$ h, until the next mitosis. Live-cell microscopy was also performed in 3T3-L1 preadipocytes stably expressing GFP-NAMPT (Movie S2). Two patterns of NAMPT localization were observed (Figure 2B). In 60% of cells, localization changes were similar to those in HepG2 (4.9 ± 1.5 h cytoplasmic, 3.3 ± 1.5 h dual, and 8.2 ± 3.2 h nuclear). The other 40% of cells skipped the nuclear localization phase. After mitosis, the NAMPT in these cells was cytoplasmic (6.7 ± 3.2 h) and then dual (8.9 ± 3.5 h) before the proceeding into the next mitosis. The intensity of non-mitotic nuclear GFP-NAMPT fluorescence appeared to be stable or increasing in both cell lines.

DNA content was measured by flow cytometry to determine cell cycle phase (Figure 2C). In the unsynchronized HepG2 cells carrying GFP-NAMPT, 39.5 ± 2.6% of cells were in G1/G0, 40.0 ± 2.5% in S, and 20.5 ± 1.3% in G2/M phase. In unsynchronized GFP-NAMPT 3T3-L1 cells, 46.8 ± 1.6% were in G1/G0, 26.7 ±1.9% in S, and 26.5 ± 2.2% in G2/M phase. In these unsynchronized cultures, the percentage of cells in a phase reflects the relative duration of the phase.

Localization of endogenous NAMPT was determined in unsynchronized cultures of HepG2 cells. These cells were pre-treated with FUCCI cell cycle sensor kit to determine cell cycle phase (Figure 2D). Most of cells (79 ± 16%) in G1/G0 had higher NAMPT level in cytosol. 71 ± 18% of cells in early S phase and all cells in late S, G2 and M had NAMPT predominantly in nuclei. This result was similar to previous experiment utilizing GFP-NAMPT; therefore, overexpression of GFP-NAMPT did not significantly affect cell cycle associated changes in NAMPT localization.

Taken together, these results demonstrate that NAMPT changes its cellular localization in a cell cycle-dependent manner.

Cell cycle arrest and stress conditions influence NAMPT localization

We next analyzed the effect of cell cycle inhibitors on NAMPT localization (Figure 3A,B). Aphidicolin inhibits DNA replication and arrests at S phase (10), RO-3306 inhibits cyclin dependent kinase 1 and arrests cells at G2/M phase (11), and culture at confluency and differentiation which both arrests cells at G1/G0. Cell cycle inhibition was confirmed by flow cytometry (Table S1). All four manipulations increased nuclear NAMPT. NAMPT was predominantly nuclear in 22% of control cells, vs 86% of aphidicolin treated cells, 78% of RO-3306 treated cells, 67% of confluent cells, and 100% of differentiated cells. All of the cell cycle inhibitors increased SIRT activity (Figure 3C). Confluency reduced cellular NAD levels and NAMPT and NMNAT1 mRNA expression (Figure 3 D, Table S2,S3,S4,S6).

To test the hypothesis that DNA damage and stress conditions affect nuclear transport of NAMPT, we monitored NAMPT localization after DNA damage by UV light or BrdU (12), or induction of oxidative (H$_2$O$_2$) or dicarbonyl (methylglyoxal, MG) stress (Figure 3E). Each treatment increased number of cells with predominantly nuclear NAMPT: UV light, BrdU, H$_2$O$_2$ and MG to 70%, 67%, 38% and 72%, respectively compared to 22% in controls. Increased PARP activity was also observed in all four conditions (Figure 3F). UV illumination and H$_2$O$_2$ (the more aggressive DNA damaging agents) reduced NAD level and mRNA expression of NAMPT and NMNAT1 (Figure 3G, Table S2,S3,S5). In agreement with these results is NAMPT localization after transient plasmid transfection (Figure 1), where NAMPT was nuclear at 62% of cells. Transfection is another example of stress condition associated with activation of nuclear processes.

We further tested effect of SIRT6 and PARP inhibition on NAMPT localization. Trichostatin A (TSA) is an inhibitor of SIRT6 and class I and class II histone deacetylases (13). TSA at 0.75 nmol·l$^{-1}$, which did not affect cell viability, increased the number of cells with cytoplasmic NAMPT localization (Figure 3H) and increased histone H$_3$ acetylation (Figure 3I). High doses of TSA 75 nmol·l$^{-1}$, which caused ~ 50% reduction in cell viability, had the opposite effect, and substantially increased number of cells with predominantly nuclear NAMPT to 66% (Data not shown). Inhibitor of PARP 3-aminobenzamide (3AB) reduced PARP activity but did not affect
NAMPT localization (Figure 3H,J). NAMPT enzymatic activity inhibitor FK866 increased histone H3 acetylation, but had no effect on NAMPT localization or PARP activity.

Overall, these results show that NAMPT is transported into nucleus under situations with increased requirement for nuclear NAD.

**Nuclear localization signal of NAMPT**

Sequence analysis of NAMPT suggested two possible nuclear localization signals, 35SYFECREKTENSKVRKVKYEE and 423KRSKKGR (Figure 4A). Clustered amino acid substitution (RKVK to GAVA or RSKK to ASGA) were introduced into GFP-NAMPT, producing GFP-NAMPTGAVA and GFP-NAMPTASGA. After transient transfection of HepG2 cells, GFP-NAMPTGAVA remained mostly nuclear (nuclear in 74 ± 11% of cells and cytoplasmic in 9 ± 4% of cells), not significantly different from GFP-NAMPTWT. In contrast, localization of GFP-NAMPTASGA was almost completely cytoplasmic (nuclear in 12 ± 7% of cells and cytoplasmic in 77 ± 12% of cells) (Figure 4B). Fluorescence intensities of GFP-NAMPTGAVA and GFP-NAMPTASGA were similar to GFP-NAMPTWT, suggesting that the introduced substitutions did not affect protein expression or stability. Region 424RSKK is located on the surface of NAMPT and it is separated from its active site (Figure 4C). These results demonstrate that 423KRSKKGR region in NAMPT contains a required nuclear localization signal.

**Inhibition of NAMPT nuclear transport slows HepG2 cell growth.**

To explore the functional role of NAMPT nuclear localization, we generated stable HepG2 cell lines overexpressing NAMPTWT, NAMPTASGA, and NAMPT carrying amino acid substitutions in the active site (219D to A, 311R to A and 313D to A) producing enzymatically inactive NAMPT (NAMPTKO) (14,15). The three independent clones expressing NAMPTWT, NAMPTASGA, and NAMPTKO had similar levels of NAMPT protein expression approximately 5 times that of the endogenous enzyme (Figure 5A). Overexpression of NAMPTWT or NAMPTASGA similarly increased the activity of NAD salvage pathway ~6-fold, while NAMPTKO overexpression had no effect on this activity (Figure 5B). These data suggest that the RSKK to ASGA substitution does not affect NAMPT enzymatic activity and verified that NAMPTKO is enzymatically inactive. Exogenous NAMPT mRNA levels had high variability and were slightly higher in NAMPTKO (Figure 5C). Overexpression of NAMPTWT or NAMPTASGA produced approximately 2-fold higher cellular NAD concentrations compared to control or NAMPTKO cells (Figure 5D). Expression of exogenous NAMPT had no effect on the mRNA levels of endogenous NAMPT or NMNAT1 (Figure 5E,F). Taken together, these results demonstrate that the NAMPTWT, NAMPTASGA, and NAMPTKO are comparably overexpressed.

Like transiently expressed NAMPT, the stably expressed NAMPTWT was mostly nuclear (nuclear in 82 ± 10% of cells and cytoplasmic in 7 ± 5% of cells), while the NAMPTASGA mutant localization was almost entirely cytoplasmic (nuclear in 5 ± 3% of cells and cytoplasmic in 86 ± 9% of cells) (Figure 5G).

Overexpression of NAMPTWT accelerated growth, with a 30% decrease in generation time. In contrast, overexpression of NAMPTASGA as well as NAMPTKO did not affect growth (Figure 5H). Accelerated growth in cells overexpressing NAMPTWT was associated with increased proportion of cells in G1/G0 (Table S7). Overexpression of either NAMPTWT or NAMPTASGA but not NAMPTKO made cells less sensitive to FK866, a NAMPT enzymatic activity inhibitor. Importantly, NAMPTWT relative to NAMPTASGA overexpression made cells significantly more resistant, when ~10 times higher concentration of FK866 was required to achieve same effect on cell viability (Figure 5I). Overexpression NAMPTWT reduced histone H3 acetylation by 64%, while in NAMPTASGA cells the reduction was only 38% and no reduction was observed in NAMPTKO (Figure 5J). NAMPTWT, but not NAMPTASGA cells had slightly higher SIRT activity (Table S7). PARP activity was increased by 46% in NAMPTWT overexpressing cells and did not change in NAMPTASGA or NAMPTKO cells, suggesting that nuclear localization and enzymatic activity are required for these effects (Figure 5H,I).

**Discussion**
Cell cycle dependent NAMPT nuclear transport

NAMPT is is localized in the nucleus in non-dividing adipocytes and it is present in both cytoplasm and nucleus in dividing cells. This suggests that NAMPT is transported between nucleus and cytoplasm in a cell-cycle dependent manner. Supporting literature includes the observation that NAMPT translocates from cytosol to nucleus after cell division arrest (6,7). We monitored NAMPT localization continuously in cells which were naturally progressing through the cell cycle. NAMPT was lowest in newly formed nuclei right after mitosis and increased thereafter. This suggests that mitosis regulates nuclear NAMPT levels. Furthermore, NAMPT was present in the nucleus in early S phase suggesting a possible role of this enzyme in DNA replication.

The requirement of NAD for nuclear processes is a likely reason for NAMPT nuclear localization. Nicotinamide is product of all non-redox NAD consuming reactions (3) and in mammalian cells, most of the NAD is synthetized from nicotinamide by a salvage pathway of NAMPT and nicotinamide mononucleotide adenylyl transferase (NMNAT)(16). There is one NAMPT isoform and three NMNAT isoforms: nuclear - NMNAT 1, Golgi surface - NMNAT 2, and mitochondrial - NMNAT 3 (17). Compartmentalization of enzymes of a single metabolic pathway is a general feature of living organisms, which increases the rate and efficiency of synthesis (18). Rapid turnover of NAD occurs in the nucleus (19). Due to nuclear co-localization of NAMPT with NMNAT1, it should be possible to quickly restore NAD from nicotinamide. NMN (product of NAMPT), NAD and nicotinamide can pass through the nuclear pore by passive diffusion (19,20), but the export of nicotinamide and further import of NMN or NAD would significantly prolong the synthesis.

Two main classes of nuclear enzymes utilizing NAD are poly(ADP-ribosyl) polymerases and NAD dependent deacetylases, also known as sirtuins (SIRT). These enzymes regulate DNA repair, chromatin structure, transcription, replication, telomere length, cell division and circadian rhythm (21-27) and are dependent on nuclear NAD availability (16). SIRT are the important histone deacetylases and key factors for chromatin condensation and heterochromatin stabilization (26). Chromosomes are maximally condensed during mitotic segregation, then de-condense and are mostly open during replication (28). To reflect these changes, SIRT should have maximal activity prior to mitosis and lower activity in post-mitotic phase. Our results showing changes of NAMPT localization during naturally progressing cell cycle are in agreement with these cell cycle dependent changes in chromatin structure. NAMPT nuclear content mimics different requirements of NAD owing to SIRT-induced changes in chromatin structure during the cell cycle. Hence, these changes in chromatin structure are possible reasons for different NAMPT localization depending on the cell cycle.

Effect of cell cycle inhibition and stress on NAMPT localization

One of the aims of this study was to find conditions that increase NAMPT nuclear content. Cell cycle inhibition at G1/G0 (confluency and differentiation), S (aphidicolin) and G2/M (RO-3306) increased NAMPT nuclear localization. This suggests that NAMPT is transported into nucleus in all phases of cell cycle and/or that cell cycle arrest activates transport of NAMPT into nucleus. Concurrently with increased NAMPT nuclear localization, we found increased SIRT activity after all types of cell cycle inhibition. This indicates that increased SIRT mediated NAD degradation should be at least partly covered by increased NAD synthesis within nucleus. Trichostatin A (TSA), an inhibitor of SIRT6 and class I and class II histone deacetylases slightly increased cytoplasmic NAMPT. SIRT6 is located in the nucleus and is crucial for maintaining genomic integrity (13). Increased cytoplasmic NAMPT after TSA treatment suggests a possible relationship between SIRT6 activity, histone deacetylation and NAMPT nuclear transport.

We hypothesized that NAMPT is transported into nucleus to provide nuclear NAD, which led us to test NAMPT localization under conditions with extreme demand on nuclear NAD. The most effective NAD consumers are PARP activated by DNA damage (29). Activation of PARP by various stress conditions caused increased NAMPT nuclear localization. UV illumination and H2O2 – the more aggressive DNA damaging agents, also reduced cellular
NAMPT nuclear transport

NAD level. Inhibition of PARP had no effect on NAMPT transport or NAD level, however PARP activity is low without activation by DNA damage (30), therefore PARP inhibition under basal conditions was not expected to not modify nuclear NAD level. NAMPT inhibition also did not change NAMPT localization, suggesting that NAMPT transport is not affected by its total activity or by total cellular NAD content.

In total, we tested several conditions associated with increased nuclear NAD degradation and all of these caused increased nuclear localization of NAMPT. Although it is not possible to exclude other reasons for NAMPT nuclear localization (such as regulation of activity by clustering with enzymes and transcription factors) and mechanisms regulating NAMPT nuclear transport are unknown, we believe that the main reason for NAMPT nuclear localization is NAD synthesis and that NAMPT nuclear transport is one of mechanisms regulating the nuclear NAD level.

**Structure of NAMPT NLS**
The identified NAMPT NLS ($^{423}$KRKKKGR$^{429}$) fits a classical monopartite class 2 NLS (31), suggesting that NAMPT is transported into the nucleus by a mechanism using importins α and β and the small GTPase Ran (32). The regulated, rate limiting step in this type of transport is importin α recognition of the NLS (32). Humans have seven isoforms of importin α, which differ in cargo specificity. Since cargo specificity is not primarily determined by the NLS sequence, once cannot predict which importin α isoform is responsible for NAMPT nuclear transport (33,34).

**Effect of NAMPT nuclear transport inhibition on cell growth**
Recently it has been shown that NAMPT is necessary for prevention of cellular senescence (35). It is not known if NAMPT nuclear localization is required for the cell cycle progression. HepG2 cells overexpressing NAMPTASGA grew slowly and were more prone to pharmacological inhibition of NAMPT compared to cells overexpressing NAMPTWT. These effects occurred despite the same cellular concentration of NAD. Although nuclear NAD levels were not measured, it is possible that they were selectively reduced. Possible mechanisms of cell death or cell cycle arrest caused by reduction of nuclear NAD have been reported (36). Reduced NAD and SIRT activity decreases deacetylation and activates proteins like tumor suppressor protein p53 or FOXO1. These proteins regulate cell cycle arrest and apoptosis through downstream targets (36,37) (Pan 2018). In agreement with this mechanism, we found lower histone H3 acetylation in NAMPTWT and NAMPTASGA, but not in NAMPTKO cells, suggesting higher SIRT-mediated deacetylation due to higher levels of SIRT substrate (NAD) in these cells. Importantly, NAMPTASGA cells had histone H3 acetylation ~2 times higher compared to NAMPTWT, which indicates that nuclear NAD regeneration provides substrate to SIRT more effectively then NAD synthesis in cytoplasm. NAMPTWT cells had also higher PARP activity, which was probably caused by higher nuclear NAD availability, because this effect was not seen in NAMPTASGA or NAMPTKO cells.

Our data demonstrate that all effects of NAMPTWT overexpression were completely abolished in NAMPTKO cells and were either abolished or substantially reduced in NAMPTASGA cells. Thus, NAMPT enzymatic activity and nuclear localization is required for the effect of NAMPT overexpression on cell growth, histone deacetylation and PARP activity, suggesting that NAMPT overexpression affects cell growth by providing NAD for nuclear processes.

**Possible utilization of NAMPT nuclear transport inhibition in cancer treatment**
Overexpression of NAMPT has been observed in many cancers (38,39) and was associated with increased tumor growth, poor prognosis, metastases, and resistance to therapy (40-43). The faster growth of HepG2 cells overexpressing NAMPTWT suggests that NAMPT may directly contribute to the aggressiveness of these tumors. Tumor cells are more sensitive to NAD depletion, because of increased NAD demand for redox (glycolysis and oxidative phosphorylation) (44,45) and non-redox (PARP and SIRT) reactions (23,46). Thus, NAMPT inhibitors have been tested as anti-cancer agents (47,48). NAMPT inhibition was shown to cause a massive reduction of NAD, ATP depletion, and
cell death (49-51). However, a threshold level of NAD depletion must be achieved for the cytotoxic effect, otherwise cells survive and regenerate the NAD pool (52). Dose-limiting toxicities did not permit the use of sufficiently high doses of NAMPT inhibitors (38) and no tumor response was reported in phase I clinical trials (48). NAMPT inhibitor toxicities included thrombocytopenia, lymphopenia, (53,54) gastrointestinal side effects (48), and retinal toxicity (54,55). For this reason, other approaches such as targeted delivery of NAMPT inhibitor into cancer cells or newly developed inhibitors are currently being tested (56,57).

NAMPT activity is essential for mammalian cells to survive (58). Here we show that nuclear transport of NAMPT is a regulated process, with inhibition slowing cell growth without significantly changing total cellular NAD levels. Based on our results and on the information that cancer cells have higher NAD demands for nuclear processes (23,46), and that nuclear NAD content is a key regulator of cell cycle and apoptosis (36,37), we propose inhibition of NAMPT nuclear transport as a new approach for cancer treatment. This would target nuclear processes requiring NAD, sparing metabolic processes in cytosol and mitochondria and is expected to be less toxic than inhibition of NAMPT enzymatic activity. Our data suggest possible efficacy of this approach, although there are some limitations in our study. We were not able to measure nuclear NAD content and all our tested cells had preserved production of endogenous NAMPT. Because this is the first study suggesting possible utilization of NAMPT nuclear transport inhibition in cancer treatment, further research for verification or exclusion of this approach will be necessary. Inhibition of NAMPT nuclear transport might be achieved by blocking the interaction between NAMPT and specific isoform of importin α. It can be expected that this approach should be the most effective in the treatment of tumors, primarily those overexpressing NAMPT. Furthermore, its efficacy could be increased by combination therapy with drugs increasing the requirement of NAD in the nucleus, such as DNA damaging agents.

Experimental procedures

Plasmid vectors

NAMPT cDNA was obtained from 3T3-L1 preadipocytes. The total RNA was extracted using RNeasy Mini Kit (Qiagen, USA). Total RNA was used for reverse transcription (AMV First Strand cDNA Synthesis Kit; NEB, USA) with reverse NAMPT primer followed by PCR amplification (primer sequences are shown in Table S8). Insertion of cDNA into pCMV-HA (Clontech, USA) vector was carried out using restriction endonucleases that produce cohesive ends. The transfer of NAMPT cDNA (or other DNA fragment) into another plasmid was performed either using direct restriction endonuclease digestion or by PCR amplification with primers containing restriction endonuclease sites, followed by the ligation of the received fragment into another plasmid. The structures of all plasmid vectors and their application are described in figure S1. Specific point mutations were obtained by PCR mutagenesis using Phusion Hot start II DNA polymerase (Thermo Scientific, USA) and pEGFP-C1-NAMPT as a template. Primer sequences for PCR mutagenesis are shown in Table S8. The PCR product was purified by MinElute Reaction Cleanup (Qiagen, USA) and the template was digested by DpnI (NEB, USA). Competent cells of E. coli XL1-Blue were used for the amplification of prepared plasmids. The correct structures of all plasmids were verified by restriction digestion followed by electrophoresis and by sequencing (GATC Biotech, Germany).

Cell lines

3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, USA) supplemented with 10% FBS (Biochrom AG, Germany) and 4 mmol\(\cdot\)l\(^{-1}\) L-glutamine (HyClone, USA). These cells were differentiated into adipocytes using 0.4 µmol\(\cdot\)l\(^{-1}\) dexamethasone (Sigma, USA), 0.5 mmol\(\cdot\)l\(^{-1}\) 3-isobutyl-1-methylxanthine (Sigma, USA) and 1.7 µmol\(\cdot\)l\(^{-1}\) insulin (Sigma, USA) as described previously (59). HepG2 hepatocytes were maintained in Minimum Essential Medium (MEM; HyClone, USA) supplemented with 10% FBS, 2 mmol\(\cdot\)l\(^{-1}\) L-glutamine and non-essential amino acids (Sigma, USA). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO\(_2\), 95% air.
3T3-L1 cells were transfected by electroporation using Amaxa Nucleofector Technology (Lonza, Switzerland). HepG2 cells were transfected by lipofection using TransIT-LT1 Transfection Reagent (Mirus Bio, USA). Stable cell lines were prepared by transfection of cells with the plasmid mixture - pcGlobin2-SB100X containing Sleeping Beauty SB100X transposase and one of pT2HB-CAGGS plasmids. 24 h after transfection, the culture medium was changed for the selection medium containing G418 antibiotics (Sigma, USA) in the concentration of 1200 mg·l⁻¹ (HepG2) and 750 mg·l⁻¹ (3T3-L1). Selection was carried out for 2 weeks and was followed by cloning of the cells (a single cell was seeded into one vessel of 96-well plate containing a conditioned medium). The clones producing a required protein were selected 3 weeks after cloning. The verification of properties was carried out by fluorescence microscopy (GFP and GFP-NAMPT producing cells) and by western blotting (GFP-NAMPT, HA-NAMPT and NAMPT overexpressing cells, Figure S2A,B). The cells producing GFP as an inert protein were used as a control due to the eventual effect of transfection and selection on studied parameters.

3·10⁴ cells were seeded into one vessel of a 12-well plate to determine the generation time. The generation time was calculated from the number of viable cells at days 1, 2 and 4 after seeding. The number of cells was determined by hemocytometer after trypsinization and complete resuspension.

The effect of NAMPT inhibition on cell viability was determined by 72 h cultivation of cells with various concentration of FK866 (Sigma, USA). Then, the cell viability was determined using Cell Proliferation Reagent WST-1 (Roche, Switzerland).

Pharmacological treatment, Cell cycle inhibition and stress conditions

24 hours before the start of an experiment, 3T3-L1 preadipocytes with stable GFP-NAMPT production were seeded into a 12-well plate at a density of 2.5·10⁴ cells per well for the purpose of fluorescence microscopy, into a 6-well plate at of 6·10⁴ cells per well for flow cytometry and into a 10-cm dish at a density of 4·10⁵ cells per well for NAD(P), mRNA expression, PARP and SIRT activity determination. At time 0 h, cells were treated with either one of the cell cycle inhibitors: aphidicolin at 6 µmol·l⁻¹ (Sigma, USA), RO-3306 at 10 µmol·l⁻¹ (Sigma, USA), one of the stress inducers: 5-bromo-2'-deoxyuridine at 1 mmol·l⁻¹ (BrdU; Sigma, USA); hydrogen peroxide at 500 µmol·l⁻¹ (Lach-Ner, Czech Republic) and methylglyoxal at 400 µmol·l⁻¹ (MG; Sigma, USA), PARP inhibitor: 3-aminobenzamide at 10 mmol·l⁻¹ (3AB; Sigma, USA), SIRT6 inhibitor: trichostatin A at 0.75 nmol·l⁻¹ or 75 nmol·l⁻¹ (TSA; Sigma, USA), or NAMPT enzymatic activity inhibitor FK866 at 1 mmol·l⁻¹ (Sigma, USA). Genotoxic stress was further induced by UV light for 20 s (wavelength: 253.7 nm). Other ways to induce the cell cycle inhibition were 2-day cultivation of fully confluent 3T3-L1 preadipocytes or their differentiation into adipocytes. The localization of GFP-NAMPT by fluorescence microscopy, SIRT activity, NAD(P) concentration and mRNA expression were analyzed 24 h after the beginning of the experiment. PARP are activated very quickly by damaged DNA, therefore shorter time after initiation of stress conditions was used to obtain this parameter. The PARP activity was analyzed after 120 min incubation with BrdU, 10 min incubation with H₂O₂, 20 min incubation with MG, and 60 min after illumination of cells with UV light.

 Determination of the cell cycle using flow cytometry

Cells were trypsinized, washed with PBS and centrifuged at 250 x g for 5 min. The cell pellet was resuspended in 0.5 ml of PBS and fixed with 4.5 ml 80% ice cold ethanol for 30 min on ice. Fixed cells were then centrifuged at 1500 x g for 5 min, washed with PBS and centrifuged again for 5 min at 1500 x g. Fixed cells were treated with 0.5 ml of RNaseA (1.5 g·l⁻¹) and stained with 0.5 ml of propidium iodide (0.1 g·l⁻¹) for 90 min at 37 °C. Stained cells were analyzed by flow cytometer FACS Aria III (BD) with a 488 nm laser as an excitation source and the emission was separated by a band pass filter at 575/26 nm. The obtained data were analyzed with BD FACSDiva software.
**Determination of NAD metabolism associated parameters**

Activity of NAD salvage enzymes was determined by the previously described method (60). Briefly, cells were trypsinized, washed with PBS and lysed by osmotic shock. Reaction mixture contains the cell lysate in reaction buffer (50 mmol·l⁻¹ Tris-HCl, pH 8.8, 20 mmol·l⁻¹ MgCl₂, protease inhibitors), 20 U alcohol dehydrogenase from yeast (Sigma, USA), 5 mmol·l⁻¹ 5-phospho-α-D-ribose-1-diphosphate (Sigma, USA), 4 mmol·l⁻¹ ATP (Sigma, USA), 50 mmol·l⁻¹ nicotinamide (Sigma, USA), 1% (v/v) ethanol (Lach-Ner, Czech Republic). The reaction mixture was incubated in 37 °C for 16 h and the fluorescence of NADH was measured every 30 min (excitation wavelength of 355 nm and emission wavelength of 460 nm). SIRT activity was analyzed using SirTuin Activity Assay Kit (BioVision, USA). Cell lysates containing 40 µg of protein were used for analysis. Histone H3 acetylation was determined by Histone H3 Acetylation Assay Kit (ab115102, Abcam, USA) and 2 µg of histone proteins were used for analysis. PARP activity was analyzed using HT Universal Colorimetric PARP Assay Kit (Trevigen, USA). Cell lysates containing 40 µg of protein were used for analysis. Manufacturer provided activated DNA was not used for measuring PARP activity in 3T3-L1 cells subjected to genotoxic, oxidative and dicarbonyl stress, where damaged DNA from the samples serves as PARP activator. Cellular content of NAD(P) was measured by high performance liquid chromatography (HPLC) with mass spectrometry (MS) detection. Method is described in detail in supplement 1.

**mRNA quantification**

Total RNA was isolated using RNeasy Mini Kit (Qiagen, USA). Possible DNA contamination was eliminated by DNase treatment (Qiagen, USA). The standard amount of 1 µg of total RNA was used for reverse transcription using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with random primers. 5 µl of cDNA solution was used for quantitative PCR using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) with concentration of primers at 1 µmol·l⁻¹. Primer sequences are described in Table S8. PCR reaction was run on CFX96 Touch™ (Bio-Rad, USA).

**Fluorescence microscopy and immunolocalization**

Fluorescent microscopy (including live cell imaging) was performed by inverted microscopy on an Olympus IX-81 with integrated CO₂ incubator (Olympus, Japan). The brightness and contrast of figures were adjusted using ImageJ 1.49v software (NIH, USA). The immunolocalization of HA-NAMPT was carried out 24 h after cell transfection with pCMV-HA-NAMPT plasmid. Cells were fixed with 4% formaldehyde (P-LAB, Czech Republic) for 15 min and permeabilized using 0.1% Triton X-100 (Pierce, USA) for 20 min. The permeabilized cells were incubated with 1% BSA (Sigma, USA) for 15 min and subsequently with FITC-labeled mouse antibody directed against HA (1:300, H7411, Sigma, USA). After washing, the cells were incubated with 1 µg·ml⁻¹ DAPI (Sigma, USA) for 5 min and observed by fluorescence microscopy. The same protocol of cell fixation and permeabilization was used for the immunolocalization of non-labeled NAMPT. The permeabilized cells were incubated with a primary rabbit antibody directed against NAMPT (1:200, RB-08-0003, RayBiotech, USA) for 2 h. and with Alexa Fluor 488-labeled secondary donkey antibody directed against rabbit IgG (1:600, 111-545-003, Jackson ImmunoResearch, USA) for 1 h. After washing, the cells were incubated with 0.5 µg·ml⁻¹ DAPI (Sigma, USA) for 5 min and observed by fluorescence microscopy. Specificity of antibodies used to NAMPT immunolocalization was validated by immunofluorescence microscopy and western blot (Figure S3).

Fluorescence microscopy filters for excitation and emission, respectively were: 492 nm and 530 nm for EGFP, Alexa Fluor 488 and green fluorescent protein of FUCCI, 350 nm and 457 nm for DAPI, 620 nm and 700 nm for Alexa Fluor 647, and 572 nm and 620 nm for red fluorescent protein of FUCCI. Microscopy images acquired using various filters were merged using ImageJ 1.49v software (NIH, USA), while DAPI staining or bright field were used to verify position of the nucleus. To quantify NAMPT localization, the proportion of cells...
containing NAMPT predominantly in nucleus or in cytoplasm was scored. Two independent investigators in each image counted number of cells which had i) higher average fluorescence in the nucleus (nuclear localization), ii) higher average fluorescence in the cytoplasm (cytoplasmic localization), and iii) average fluorescence in the cytoplasm and the nucleus was similar; it was not possible to distinguish whether fluorescence is higher in nucleus or cytoplasm (dual localization). The proportion of cells with nuclear or cytoplasmic localization was expressed as a percentage of the total number of cells in field of view.

**Colocalization of NAMPT with cell cycle markers**

Cell cycle phase in HepG2 cells with natural expression of NAMPT was determined by transduction Premo™ FUCCI Cell Cycle Sensor (Thermo Scientific, USA), followed by incubation for 48 h. Immunolocalization of NAMPT was performed by similar procedure described in Fluorescence microscopy and immunolocalization. The only difference was in the secondary antibody used for this experiment, which was used Alexa Fluor 647-labeled secondary donkey antibody directed against rabbit IgG (1:600, 111-605-003, Jackson ImmunoResearch, USA) for 1 h.

**Western blot**

Western blot was performed and evaluated as described previously (61). Each lysate was resolved by SDS-PAGE (40 µg protein per lane, 10% gel) (Mini-PROTEAN Tetra Cell; Bio-Rad, USA). The proteins were transferred onto a nitrocellulose membrane by electro-transfer (Mini Trans-Blot Cell; Bio-Rad, USA). The membrane was saturated with 3% BSA for 2 h and incubated with a mixture of primary rabbit antibodies directed against NAMPT (1:5000, RB-08-0003, RayBiotech, USA) and GAPDH (1:50000, G9545, Sigma, USA) for 16 h at 4 °C. Antibody binding was then revealed with a peroxidase-labeled secondary mouse antibody directed against rabbit IgG (1:5000 (W401B, Promega, USA) for 2 h. The chemiluminescent substrate West Pico (Pierce, USA) and camera Alliance 4.7 UVItec camera (UVItec, UK) were used for visualization.

**Bioinformatics**

The nuclear localization signal was identified using several types of software: PSORT (http://psort.hgc.jp/form2.html), NoD (http://www.compbio.dundee.ac.uk/www-nod/), NLS Mapper (http://nls-mapper.iba.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), and NLStradamus (http://www.moseslab.csb.utoronto.ca/NLStradamus/).

**Statistics**

Data are expressed as mean ± SD. Student’s t-test was applied to determine differences between cell samples, with a statistical significance defined as P < 0.05. Data normality was verified by the Shapiro-Wilk W-test
**Acknowledgments:** We wish to thank Marc L Reitman (National Institutes of Health, Bethesda, USA) for his advices and great help in editing the text to the NIH Fellows Editorial Board for text editing. Plasmids pT2HB-CAGGS and pcGlobin2-SB100X with transposase Sleeping Beauty SB100X were a kind gift from Lajos Mátés, (Biological Research Centre, Hungarian Academy of Sciences, Szeged). We wish to thank Monika Cahova (Institute for Clinical and Experimental Medicine, Prague, Czech Republic) for providing Amaxa nucleofector, and to Lucie Peterkova (University of Chemistry and Technology Prague, Prague, Czech Republic) for Sf9 cell cultivation.

**Conflicts of interest:** The authors declare that there is no conflict of interest associated with this manuscript.
References

1. Revollo, J. R., Grimm, A. A., and Imai, S. (2004) The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. J Biol Chem 279, 50754-50763
2. Berger, F., Ramírez-Hernández, M. H., and Ziegler, M. (2004) The new life of a centenarian: signalling functions of NAD(P). Trends Biochem Sci 29, 111-118
3. Cantó, C., Menzies, K. J., and Auwerx, J. (2015) NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus. Cell Metab 22, 31-53
4. Mohammadi, M., Mianabadi, F., and Mehrad-Majd, H. (2018) Circulating visfatin levels and cancers risk: A systematic review and meta-analysis. J Cell Physiol
5. Moschen, A. R., Geiger, S., Gerner, R., and Tilg, H. (2010) Pre-B cell colony enhancing factor/NAMPT/visfatin and its role in inflammation-related bone disease. Mutat Res 690, 95-101
6. Kitani, T., Okuno, S., and Fujisawa, H. (2003) Growth phase-dependent changes in the subcellular localization of pre-B-cell colony-enhancing factor. Febs Letters 544, 74-78
7. Buldak, R. J., Skonieczna, M., Buldak, L., Matysiak, N., Mielanczyk, L., Wyrobiec, G., Kukla, M., Michalski, M., and Zwirska-Korczala, K. (2014) Changes in subcellular localization of visfatin in human colorectal HCT-116 carcinoma cell line after cytochalasin-B treatment. European Journal of Histochemistry 58, 239-246
8. Ocon-Grove, O. M., Krzysik-Walker, S. M., Maddineni, S. R., Hendricks, G. L., and Ramachandran, R. (2010) NAMPT (visfatin) in the chicken testis: influence of sexual maturation on cellular localization, plasma levels and gene and protein expression. Reproduction 139, 217-226
9. Romacho, T., Villalobos, L. A., Cercas, E., Carraro, R., Sanchez-Ferrer, C. F., and Peiro, C. (2013) Visfatin as a Novel Mediator Released by Inflamed Human Endothelial Cells. Plos One 8
10. Baranovskiy, A. G., Babayeva, N. D., Suwa, Y., Gu, J., Pajov, Y. I., and Tahirov, T. H. (2014) Structural basis for inhibition of DNA replication by aphidicolin. Nucleic Acids Res 42, 14013-14021
11. Vassilev, L. T., Tovar, C., Chen, S., Knezevic, D., Zhao, X., Sun, H., Heimbrook, D. C., and Chen, L. (2006) Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. Proc Natl Acad Sci U S A 103, 10660-10665
12. Kaufman, E. R. (1985) Reversion analysis of mutations induced by 5-bromodeoxyuridine mutagenesis in mammalian cells. Mol Cell Biol 5, 3092-3096
13. Wood, M., Rymarchyk, S., Zheng, S., and Cen, Y. (2018) Trichostatin A inhibits deacetylation of histone H3 and p53 by SIRT6. Arch Biochem Biophys 638, 8-17
14. Khan, J. A., Tao, X., and Tong, L. (2006) Molecular basis for the inhibition of human NMPRTase, a novel target for anticancer agents. Nat Struct Mol Biol 13, 582-588
15. Rongvaux, A., Galli, M., Denanglaire, S., Van Gool, F., Dreze, P. L., Szpirer, C., Bureau, F., Andris, F., and Leo, O. (2008) Nicotinamide phosphoribosyl transferase/pre-B cell colony-enhancing factor/visfatin is required for lymphocyte development and cellular resistance to genotoxic stress. J Immunol 181, 4685-4695
16. Di Stefano, M., and Conforti, L. (2013) Diversification of NAD biological role : the importance of location. Fews Letters 280, 4711-4728
17. Berger, F., Lau, C., Dahlmann, M., and Ziegler, M. (2005) Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenyllytransferase isoforms. Journal of Biological Chemistry 280, 36334-36341
18. Zecchin, A., Stapor, P. C., Goveia, J., and Carmeliet, P. (2015) Metabolic pathway compartmentalization: an underappreciated opportunity? Curr Opin Biotechnol 34, 73-81
19. Cambronne, X. A., Stewart, M. L., Kim, D., Jones-Brunette, A. M., Morgan, R. K., Farrens, D. L., Cohen, M. S., and Goodman, R. H. (2016) Biosensor reveals multiple sources for mitochondrial NAD⁺. *Science* **352**, 1474-1477

20. Timney, B. L., Raveh, B., Mironaska, R., Trivedi, J. M., Kim, S. J., Russel, D., Wente, S. R., Sali, A., and Rout, M. P. (2016) Simple rules for passive diffusion through the nuclear pore complex. *J Cell Biol* **215**, 57-76

21. Boehler, C., Gauthier, L. R., Mortusewicz, O., Biard, D. S., Saliou, J. M., Bresson, A., Sanglier-Cianferani, S., Smith, S., Schreiber, V., Boussin, F., and Dantzer, F. (2011) Poly(ADP-ribose) polymerase 3 (PARP3), a newcomer in cellular response to DNA damage and mitotic progression. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 2783-2788

22. Chang, H. C., and Guarente, L. (2013) SIRT1 mediates central circadian control in the SCN by a mechanism that decays with aging. *Cell* **153**, 1448-1460

23. Kozako, T., Suzuki, T., Yoshimitsu, M., Arima, N., Honda, S., and Soeda, S. (2014) Anticancer Agents Targeted to Sirtuins. *Molecules* **19**, 20295-20313

24. Messner, S., and Hottiger, M. O. (2011) Histone ADP-ribosylation in DNA repair, replication and transcription. *Trends in Cell Biology* **21**, 534-542

25. Schreiber, V., Dantzer, F., Ame, J. C., and de Murcia, G. (2006) Poly(ADP-ribose): novel functions for an old molecule. *Nature Reviews Molecular Cell Biology* **7**, 517-528

26. Vaquero, A. (2009) The conserved role of sirtuins in chromatin regulation. *International Journal of Developmental Biology* **53**, 303-322

27. Wang, R. H., Lahusen, T. J., Chen, Q., Xu, X. L., Jenkins, L. M. M., Leo, E., Fu, H. Q., Aladjem, M., Pommier, Y., Appella, E., and Deng, C. X. (2014) SIRT1 Deacetylates TopBP1 and Modulates Intra-S-Phase Checkpoint and DNA Replication Origin Firing. *International Journal of Biological Sciences* **10**, 1193-1202

28. Williamson, W. D., and Pinto, I. (2012) Histones and genome integrity. *Frontiers in Bioscience-Landmark* **17**, 984-995

29. Opitz, C. A., and Heiland, I. (2015) Dynamics of NAD-metabolism: everything but constant. *Biochem Soc Trans* **43**, 1127-1132

30. Vodenicharov, M. D., Ghodgaonkar, M. M., Halappanavar, S. S., Shah, R. G., and Shah, G. M. (2005) Mechanism of early biphasic activation of poly(ADP-ribose) polymerase-1 in response to ultraviolet B radiation. *J Cell Sci* **118**, 589-599

31. Kosugi, S., Hasebe, M., Matsumura, N., Takashima, H., Miyamoto-Sato, E., Tomita, M., and Yanagawa, H. (2009) Six Classes of Nuclear Localization Signals Specific to Different Binding Grooves of Importin alpha. *Journal of Biological Chemistry* **284**, 478-485

32. Christie, M., Chang, C. W., Róna, G., Smith, K. M., Stewart, A. G., Takeda, A. A., Fontes, M. R., Stewart, M., Vértessy, B. G., Forwood, J. K., and Kobe, B. (2016) Structural Biology and Regulation of Protein Import into the Nucleus. *J Mol Biol* **428**, 2060-2090

33. Pumroy, R. A., and Cingolani, G. (2015) Diversification of importin-alpha isoforms in cellular trafficking and disease states. *Biochemical Journal* **466**, 13-28

34. Melen, K., Fagerlund, R., Franje, J., Kohler, M., Kinnunen, L., and Julkunen, I. (2003) Importin alpha nuclear localization signal binding sites for STAT1, STAT2, and influenza a virus nucleoprotein. *Journal of Biological Chemistry* **278**, 28193-28200

35. Khaidizar, F. D., Nakahata, Y., Kume, A., Sumizawa, K., Kohno, K., Matsu, T., and Bessho, Y. (2017) Nicotinamide phosphoribosyltransferase delays cellular senescence by upregulating SIRT1 activity and antioxidant gene expression in mouse cells. *Genes Cells* **22**, 982-992

36. Alaei, M., Khaghani, S., Behroozfar, K., Hesari, Z., Ghorbanhosseini, S. S., and Nourbakhsh, M. (2017) Inhibition of Nicotinamide Phosphoribosyltransferase Induces Apoptosis in Estrogen Receptor-Positive MCF-7 Breast Cancer Cells. *J Breast Cancer* **20**, 20-26
37. Thakur, B. K., Dittrich, T., Chandra, P., Becker, A., Kuehnau, W., Klusmann, J. H., Reinhardt, D., and Welte, K. (2013) Involvement of p53 in the cytotoxic activity of the NAMPT inhibitor FK866 in myeloid leukemic cells. Int J Cancer 132, 766-774
38. Olesen, U. H., Thougaard, A. V., Jensen, P. B., and Sehested, M. (2010) A Preclinical Study on the Rescue of Normal Tissue by Nicotinic Acid in High-Dose Treatment with AP0866, a Specific Nicotinamide Phosphoribosyltransferase Inhibitor. Molecular Cancer Therapeutics 9, 1609-1617
39. Shackelford, R., Hirsh, S., Henry, K., Abdel-Mageed, A., Kandil, E., and Coppola, D. (2013) Nicotinamide Phosphoribosyltransferase and SIRT3 Expression Are Increased in Well-differentiated Thyroid Carcinomas. Anticancer Research 33, 3047-3052
40. Yan, X., Zhao, J., and Zhang, R. (2017) Visfatin mediates doxorubicin resistance in human colorectal cancer cells via up regulation of multidrug resistance 1 (MDR1). Cancer Chemother Pharmacol 80, 395-403
41. Cao, Z., Liang, N., Yang, H., and Li, S. (2017) Visfatin mediates doxorubicin resistance in human non-small-cell lung cancer via Akt-mediated up-regulation of ABCC1. Cell Prolif 50
42. Ke, H. L., Lin, H. H., Li, W. M., Li, C. C., Chang, L. L., Lee, Y. C., Huang, C. N., and Wu, W. J. (2015) High visfatin expression predicts poor prognosis of upper tract urothelial carcinoma patients. Am J Cancer Res 5, 2447-2454
43. Reddy, P. S., Umesh, S., Thota, B., Tandon, A., Pandey, P., Hegde, A. S., Balasubramaniam, A., Chandramouli, B. A., Santosh, V., Rao, M. R. S., Kondaiah, P., and Somasundaram, K. (2008) PBEF1/NAmPRTase/Visfatin - A potential malignant astrocytoma/glioblastoma serum marker with prognostic value. Cancer Biology & Therapy 7, 663-668
44. Chiarugi, A., Dolle, C., Felici, R., and Ziegler, M. (2012) The NAD metabolome - a key determinant of cancer cell biology. Nature Reviews Cancer 12, 741-752
45. Kroemer, G., and Pouyssegur, J. (2008) Tumor cell metabolism: Cancer's Achilles' heel. Cancer Cell 13, 472-482
46. Ossovskaya, V., Koo, I. C., Kaldjian, E. P., Alvares, C., and Sherman, B. M. (2010) Upregulation of Poly (ADP-Ribose) Polymerase-1 (PARP1) in Triple-Negative Breast Cancer and Other Primary Human Tumor Types. Genes Cancer 1, 812-821
47. Khan, J. A., Forouhar, F., Tao, X., and Tong, L. (2007) Nicotinamide adenine dinucleotide metabolism as an attractive target for drug discovery. Expert Opin Ther Targets 11, 695-705
48. von Heideman, A., Berglund, A., Larsson, R., and Nygren, P. (2010) Safety and efficacy of NAD depleting cancer drugs: results of a phase I clinical trial of CHS 828 and overview of published data. Cancer Chemotherapy and Pharmacology 65, 1165-1172
49. Del Nagro, C., Xiao, Y., Rangell, L., Reichelt, M., and O’Brien, T. (2014) Depletion of the Central Metabolite NAD Leads to Oncosis-mediated Cell Death. Journal of Biological Chemistry 289, 35182-35192
50. Hasmann, M., and Schemainda, I. (2003) FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. Cancer Res 63, 7436-7442
51. Tan, B., Young, D. A., Lu, Z. H., Wang, T., Meier, T. I., Shepard, R. L., Roth, K., Zhai, Y., Huss, K., Kuo, M. S., Gillig, J., Parthasarathy, S., Burkholder, T. P., Smith, M. C., Geeganage, S., and Zhao, G. S. (2013) Pharmacological Inhibition of Nicotinamide Phosphoribosyltransferase (NAMPT), an Enzyme Essential for NAD(+) Biosynthesis, in Human Cancer Cells METABOLIC BASIS AND POTENTIAL CLINICAL IMPLICATIONS. Journal of Biological Chemistry 288, 3500-3511
52. Roulston, A., and Shore, G. C. (2016) New strategies to maximize therapeutic opportunities for NAMPT inhibitors in oncology. Mol Cell Oncol 3, e1052180
53. Tarrant, J. M., Dhawan, P., Singh, J., Zabka, T. S., Clarke, E., DosSantos, G., Dragovich, P. S., Sampath, D., Lin, T., McCray, B., La, N., Nguyen, T., Kauss, A., Dambach, D., Misner, D. L., Diaz, D.,
and Uppal, H. (2015) Preclinical models of nicotinamide phosphoribosyltransferase inhibitor-mediated hematotoxicity and mitigation by co-treatment with nicotinic acid. *Toxicology Mechanisms and Methods* **25**, 201-211

54. Holen, K., Saltz, L. B., Hollywood, E., Burk, K., and Hanauske, A. R. (2008) The pharmacokinetics, toxicities, and biologic effects of FK866, a nicotinamide adenine dinucleotide biosynthesis inhibitor. *Investigational New Drugs* **26**, 45-51

55. Zabka, T. S., Singh, J., Dhawan, P., Liederer, B. M., Oeh, J., Kauss, M. A., Xiao, Y., Zak, M., Lin, T., McCray, B., La, N., Nguyen, T., Beyer, J., Farman, C., Uppal, H., Dragovich, P. S., O'Brien, T., Sampath, D., and Misner, D. L. (2015) Retinal Toxicity, in vivo and in vitro, Associated with Inhibition of Nicotinamide Phosphoribosyltransferase. *Toxicological Sciences* **144**, 163-172

56. Neumann, C. S., Olivas, K. C., Anderson, M. E., Cochran, J. H., Jin, S., Li, F., Loftus, L. V., Meyer, D. W., Neale, J., Nix, J. C., Pittman, P. G., Simmons, J. K., Ulrich, M. L., Waight, A. B., Wong, A., Zaval, M. C., Zeng, W., Lyon, R. P., and Senter, P. D. (2018) Targeted Delivery of Cytotoxic NAMPT Inhibitors Using Antibody-Drug Conjugates. *Mol Cancer Ther* **17**, 2633-2642

57. Liederer, B. M., Cheong, J., Chou, K. J., Dragovich, P. S., Le, H., Liang, X., Ly, J., Mukadam, S., Oeh, J., Sampath, D., Wang, L., and Wong, S. (2018) Preclinical Assessment of the ADME, Efficacy and Drug-Drug Interaction Potential of a Novel NAMPT Inhibitor. *Xenobiotica*, 1-56

58. Revollo, J. R., Körner, A., Mills, K. F., Satoh, A., Wang, T., Garten, A., Dasgupta, B., Sasaki, Y., Wolberger, C., Townsend, R. R., Milbrandt, J., Kiess, W., and Imai, S. (2007) Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* **6**, 363-375

59. Skop, V., Cahova, M., Dankova, H., Papackova, Z., Palenickova, E., Svoboda, P., Zidkova, J., and Kazdova, L. (2014) Autophagy inhibition in early but not in later stages prevents 3T3-L1 differentiation: Effect on mitochondrial remodeling. *Differentiation* **87**, 220-229

60. Skop, V., Kontrova, K., Zidek, V., Pravenec, M., Kazdova, L., Mikulik, K., Sajdok, J., and Zidkova, J. (2010) Autocrine Effects of Visfatin on Hepatocyte Sensitivity to Insulin Action. *Physiological Research* **59**, 615-618

61. Svoboda, P., Krizova, E., Cenkova, K., Vapenkova, K., Zidkova, J., Zidek, V., and Skop, V. (2017) Visfatin is actively secreted in vitro from U-937 macrophages, but only passively released from 3T3-L1 adipocytes and HepG2 hepatocytes. *Physiol Res* **66**, 709-714

62. Wang, T., Zhang, X., Bheda, P., Revollo, J. R., Imai, S., and Wolberger, C. (2006) Structure of Nampt/PBEF/visfatin, a mammalian NAD+ biosynthetic enzyme. *Nat Struct Mol Biol* **13**, 661-662
FOOTNOTES
This work was supported by grants TA02010013 from the Technological Agency of the Czech Republic, 14-36804G from the Czech Science Foundation, MH CZ - DRO ("Institute for Clinical and Experimental Medicine - IKEM, IN 00023001"), and by financial support from specific university research (MSMT No 20-SVV/2017). Acquisition of the SpectraMax® i3 Multi-Mode Detection Platform (Molecular Devices) used within the publication was financed by the projects OPPC CZ.2.16/3.1.00/24503 and NPU I LO1601.
Figure - 1: Localization of NAMPT in HepG2 hepatocytes, 3T3-L1 preadipocytes and 3T3-L1 adipocytes.

A: Cells were transfected with plasmid pEGFP-C1-NAMPT and localization of GFP-NAMPT was monitored 24 h post transfection using fluorescence microscopy. B: Cells were transfected with plasmid pCMV-HA-NAMPT and localization of HA-NAMPT was studied by immunostaining using FITC-labeled antibody directed against HA, followed by fluorescence microscopy. Specific nuclear staining was achieved by staining with DAPI.

Quantitative evaluation: proportion of cells containing NAMPT predominantly in nucleus (N) or in cytoplasm (C) was calculated from a random field of view (0.16 mm²) of 5 independent cultures (in 3T3-L1 preadipocytes each view field contained 22 ± 7 transfected cells; in 3T3-L1 adipocytes each view field contained 16 ± 4 transfected adipocytes; in HepG2 hepatocytes each view field contained 20 ± 5 transfected cells). The remaining cells had dual localization (equal cytoplasmic and nuclear) of NAMPT. Data are expressed as mean ± SD. Bar = 10 µm.
Figure - 2: Changes in NAMPT localization during cell cycle.
Localization of GFP-NAMPT was monitored in HepG2 (A) and 3T3-L1 (B) cells using live-cell fluorescence microscopy for 42 and 24 h respectively. Localization of NAMPT was scored (beginning from mitosis) according to NAMPT localization - predominantly cytoplasmic (blue), dual localization (equal cytoplasmic and nuclear) (red) and predominantly nuclear (green). An example of cells with specific NAMPT localization is shown above the bar (these cells are marked with red arrow if the image contains more cells with different NAMPT localization). Scoring was carried out from a random field of view (0.64 mm²) of 5 independent cultures. To avoid the cells with impaired cell cycle, only the cells that have undergone first mitosis during initial 8 h (for 3T3-L1) or 24 h (for HepG2) were scored. The entire cell cycle (two mitoses during the monitored time) was observed in 35% of the 80 analyzed HepG2 cells and in 80% of the 120 analyzed 3T3-L1 preadipocytes. C: Proportion of cells in G1/G0, S, G2/M phase of cell cycle was determined in unsynchronized cultures of HepG2 and 3T3-L1 cells expressing GFP-NAMPT by flow cytometry. D: Co-staining of endogenous NAMPT with cell cycle markers in HepG2 cells. Unsynchronized culture was transduced with Premo™ FUCCI Cell Cycle Sensor. This sensor stains cells in G1, G0 by red, in early S by yellow and in late S, G2, M by green. Endogenous NAMPT was immunolocalized 48 h after transduction together with DAPI nucleus staining. Quantitative evaluation: proportion of cells containing NAMPT predominantly in nucleus (N) or in cytoplasm (C) was calculated from 13 random fields of view, each contained 7 ± 3 FUCCI stained cells. Data are expressed as mean ± SD. Bar (A, B) = 20 µm; bar (D) = 10 µm
Figure - 3: Effect of cell cycle inhibitors and stress conditions on the NAMPT intracellular localization.
A: Control figure and plot for B, E and H. Representative figure and quantitative evaluation of non-treated 3T3-L1 preadipocytes expressing GFP-NAMPT.
Inhibition of cell cycle in 3T3-L1 preadipocytes was accomplished by cultivation with aphidicolin, RO-3306 for 24 h, by cultivation at confluency for 2 days and by differentiation into adipocytes. Within these...
cells the following were analyzed: Localization of GFP-NAMPT using fluorescence microscopy (B); SIRT activity in cell lysates of GFP expressing cells (C); and concentration of NAD in cell lysates of GFP expressing cells (D).

Stress conditions in 3T3-L1 preadipocytes were acquired by illumination with UV light and by cultivation with BrdU (genotoxic stress), H₂O₂ (oxidative stress), methylglyoxal (MG, dicarbonyl stress). 24 h after stress induction in these cells the following were analyzed: Localization of GFP-NAMPT using fluorescence microscopy (E); PARP activity in cell lysates of GFP expressing cells (F); and concentration of NAD in cell lysates of GFP expressing cells (G).

Inhibition of NAMPT, SIRT6 and PARP activity in 3T3-L1 preadipocytes was acquired by cultivation with FK866, trichostatin A (TSA) and 3-aminobenzamide (3AB) respectively. 24 h after activity inhibition in these cells the following were analyzed: Localization of GFP-NAMPT using fluorescence microscopy (H); Histone H3 acetylation (I); PARP activity (J); and concentration of NAD in cell lysates of GFP expressing cells (K).

Quantitative evaluation: proportion of cells containing NAMPT predominantly in nucleus (N) or in cytoplasm (C) based on average fluorescence was calculated from a random field of view (0.64 mm²) of 3-6 independent cultures and related to total number of cells. Total number of cells: control – 134 ± 17; aphidicolin – 34 ± 13; RO-3306 – 46 ± 8; 2 days’ of confluency – 570 ± 30; differentiation – 1224 ± 110; UV – 32 ± 13; BrdU – 37 ± 4; H₂O₂ – 87 ± 22; MG – 100 ± 12; FK866 – 64 ± 10; TSA – 64 ± 10; 3AB – 44 ± 7 Data are expressed as mean ± SD, *P<0.05 compared to control cells. Bar (left A, B, E, H) = 50 µm; bar (right A) = 10 µm.

**Figure - 4: Verification of nuclear localization signal of NAMPT.**

A: Amino acid sequences showing clustered amino acid substitutions 50RKVK to GAVA (NAMPTGAVA) and 425RSKK to ASGA (NAMPTASGA). Colored lines under sequences match the regions found by NLS searching programs Psort (blue), NoD (green), NLStradmus (purple), NLS Mapper (orange) as hypothetical nuclear localization signals.

B: Localization of GFP-NAMPTGAVA and GFP-NAMPTASGA in HepG2 cells 24 h after transient transfection by plasmid pEGFP-C1-NAMPT carrying defined mutations. Figure shows representative fluorescence microscopy images and quantitative evaluation of proportion of cells containing NAMPT predominantly in nucleus (N) or in cytoplasm (C).
C: Location of $^{42}_{4}RSKK$ region and active side in structure of NAMPT. Structure 2H3B was obtained from RCSB PDB and visualized by VMD (NIH, USA). $^{42}_{4}RSKK$ region is labeled by red and amino acids participating in the reaction (62) are colored yellow. Data are expressed as mean ± SD, Bar = 25 µm.
Figure 5: Effect of 424RSKK to ASGA substitution in NAMPT structure on growth rate.
HepG2 cell lines with stable overexpression of GFP (control), WT NAMPT (NAMPTWT), NAMPT with 424RSKK to ASGA substitution (NAMPTASGA) and NAMPT with D219A, R311A and D313A substitutions (NAMPTKO) were prepared using plasmids pT2HB-CAGGS-GFP, pT2HB-CAGGS-NAMPT (WT or carrying mutation for 424RSKK to ASGA substitution or for D219A, R311A and D313A substitutions). After transfection, selection and cloning, 3 clones were selected from each cell variant (Selection of clones: NAMPTWT, NAMPTASGA and NAMPTKO, with increased and similar level of NAMPT; Control - GFP expression and similar level of NAMPT as nontransfected cells). All 3 clones were always used for determination of other parameters.

A: Western blot analysis (representative figure and optical density evaluation) of NAMPT and GAPDH (internal control protein) protein expression of control, NAMPTWT, NAMPTASGA and NAMPTKO cells. Lines 1-3 represent individual clones. B: Activity of NAD salvage enzymes in lysates of control, NAMPTWT, NAMPTASGA and NAMPTKO cells. C: Relative quantification of exogenous NAMPT mRNA in control, NAMPTWT, NAMPTASGA and NAMPTKO cells. D: Concentration of NAD in lysates of control, NAMPTWT, NAMPTASGA and NAMPTKO cells. E: Relative quantification of endogenous NAMPT mRNA in control, NAMPTWT, NAMPTASGA and NAMPTKO cells. F: Relative quantification of NMNAT1 mRNA in control, NAMPTWT, NAMPTASGA and NAMPTKO cells. G: Indirect immunolocalization of NAMPT. The localization of NAMPT in NAMPTWT and NAMPTASGA cells was determined by immunofluorescence using antibody directed against NAMPT and fluorescence labeled secondary antibody. Figure shows representative fluorescence microscopy images and quantitative evaluation of proportion of cells containing NAMPT predominantly in nucleus (N) or in cytoplasm (C). H: Generation time of control, NAMPTWT, NAMPTASGA and NAMPTKO cells. I: Cell viability after treatment with NAMPT enzymatic activity inhibitor FK866 of control, NAMPTWT, NAMPTASGA and NAMPTKO cells. J: Histone H3 acetylation in NAMPTWT, NAMPTASGA and NAMPTKO cells. K: PARP activity in lysates of control, NAMPTWT, NAMPTASGA and NAMPTKO cells.

Data are expressed as mean ± SD, * P<0.05 compared to control; # P<0.05 compared to NAMPTWT; † P<0.05 compared to NAMPTASGA; Bar = 20 µm.
Nuclear transport of nicotinamide phosphoribosyltransferase is cell cycle dependent in mammalian cells and its inhibition slows cell growth

Petr Svoboda, Edita Krízová, Sarka Sestakova, Kamila Vapenková, Zdenek Knejzlik, Silvie Rimpelova, Diana Rayova, Nikol Volfova, Ivana Krízová, Michaela Rumlová, David Sykora, Rene Kizek, Martin Haluzik, Vaclav Zidek, Jarmila Zidkova and Vojtech Skop

*J. Biol. Chem.* published online April 11, 2019

Access the most updated version of this article at doi: [10.1074/jbc.RA118.003505](http://dx.doi.org/10.1074/jbc.RA118.003505)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/) to choose from all of JBC's e-mail alerts