The physiological role of Nicotinamide-N-methyltransferase and its evolutionary influence on NAD-biosynthesis

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## Summary

Keywords

Highlights

### Introduction

NAD metabolism represents one of the most critical links that connect cellular signal transduction and energy metabolism. According to xyz (Brenda?Kegg?) approximately 25% of all biochemical reactions (pathways?) use NAD in redox reactions in which NAD+ and NADH are reversibly interconverted. On the other hand, various signalling processes consume NAD+ by cleaving the molecule to nicotinamide (Nam) and ADP-ribose (ref). NAD+-degrading signalling reactions include poly- and mono ADP-ribosylation, NAD-dependent protein deacylation by sirtuins as well as the synthesis of calcium mobilizing molecules such as cyclic ADP-ribose. These NAD+-dependent signalling processes participate in the regulation of virtually all cellular activities. The enzymes involved in these processes are sensitive to the available NAD+ concentration, which in turn is dependent on the NAD+/NADH redox ratio. Therefore, NAD+-depending signalling can act as a transmitter of changes in the cellular energy homeostasis, for example, to regulate gene expression or metabolic activity.

The significance of NAD+-dependent signalling for NAD homeostasis has long been underestimated. However, it has now been established that inhibition of NAD biosynthesis in mammalian cells leads to a rapid decline in NAD content suggesting that NAD+-dependent signalling consumes substantial amounts of NAD. It has been estimated that the cellular NAD pool is turned over at least once per day (refs). To maintain the NAD concentration at physiological levels, NAD biosynthesis needs to proceed at an equally rapid rate. Imbalances in NAD-homeostasis have been linked to various, in particular, ageing-related diseases such as diabetes, neurodegenerative disorders and cancer. Various recent studies have demonstrated impressive health benefits of dietary supplementation with intermediates of NAD biosynthesis including NMN and nicotinamide riboside, NR (refs). Apparently, the exploitation of NAD biosynthetic routes, in addition to the use of nicotinamide as precursor (Fig. 1), results in increased NAD concentrations that stimulate NAD-dependent signalling processes, in particular, protein deacetylation by sirtuins.

In mammals, NAD biosynthesis predominantly relies on nicotinamide, a form of vitamin B3, as precursor. In the first step, Nam is converted to the mononucleotide, NMN by Nam phosphoribosyltransferase (NamPRT) using phosphoribosylpyrophosphate (PRPP) as co-substrate. The key role of this enzyme is related to the nature of NAD-dependent signalling reactions, namely, that in all these reactions Nam is cleaved off and potentially lost. If the released Nam were not recycled into NAD biosynthesis, humans would probably require a daily vitamin B3 intake of ~200g or more (ref), however, the daily recommended allowance is only ~15 mg (ref). The nearly complete recycling of Nam is achieved by an extraordinary high affinity NamPRT to Nam, the Km being in the low nM range. Surprisingly, despite the importance of its salvage, Nam can also be marked for excretion by methylation. Indeed, the presence of nicotinamide N-methyltransferase (NNMT) in vertebrates is among the most enigmatic and counterintuitive features of NAD metabolism. Why is there one enzyme (NamPRT) seemingly optimized to recycle even the faintest amounts of Nam back into NAD synthesis, while at the same time there is another one (NNMT) that seems to have no metabolic function other than to remove Nam from NAD metabolism? This puzzle becomes even more intriguing when considering that the majority of lower organisms and plants deaminated Nam to nicotinic acid (NA) before it can enter NAD biosynthesis via the Preiss-Handler pathway (Fig. 1). That is, there are three enzymes that convert Nam in three principally different reactions. However, even though the utilization of Nam is a fundamentally important process, so far, little is known about the potential interplay between these enzymes and their occurrence and evolution. Previous phylogenetic analysis show that the two Nam recycling pathways are both ancestral showing a scattered distribution in bacteria (Ref?). But NNMT has so far only been found in animals. As previous analysis where limited especially by the number of available eukaryotic genome sequences. Consequently, a comprehensive evolutionary analysis is needed to understand the phylogeny of NAD metabolism, in particular, Nam metabolizing enzymes and their relationship to signalling pathways.

Many enzymes of NAD metabolism have been carefully characterized both in vitro and in their cellular context. However, how biosynthesis and signalling are coupled and influence each other is not understood. It is widely assumed that a major driving force of NAD-dependent signalling reactions is the cellular NAD concentration. On the other hand, essentially all enzymes using NAD in signalling reactions, especially sirtuins, but also PARPs and ADP-ribosylcyclases, are subject to inhibition by nicotinamide. Therefore, to promote NAD-dependent signalling reactions, the balance between elevated NAD levels and accumulating nicotinamide concentrations needs to be kept such that the inhibitory effect of Nam does not override the kinetic stimulation by high NAD levels. Given the complex nature of the NAD metabolome, it is not trivial to predict the optimal conditions for efficient NAD-dependent signalling. However, it is obvious that Nam-converting enzymes must play a key role in this regard.

In the present study, we have conducted a comprehensive phylogenetic analysis of the genes that encode Nam-converting enzymes. The most surprising result was that with the emergence of deuterostomes, there has been a strong selection for the co-existence of NamPRT and NNMT. Moreover, this selection for the co-existence of NamPRT and NNMT was accompanied by a marked increase in the number of genes encoding NAD-dependent signalling reactions. On the other hand, based on available kinetic parameters, we built a kinetic model of NAD metabolism. This mathematical model provided a powerful tool to analyse the relationship between NAD biosynthetic and signalling fluxes when different subsets of Nam-utilizing enzymes are present. Importantly, the model demonstrated that NNMT has a critical role to maintain high NAD-dependent signalling fluxes by preventing accumulation of inhibitory Nam. At lower Nam concentrations, the impact of NNMT is minimal, because of the very high affinity of NamPRT. Taken together, our results suggest that the co-evolution of NamPRT and NNMT was a prerequisite to enable the versatile NAD-dependent signalling mechanisms present in vertebrates.

## Results

As shown in Fig. 1, NAD can be synthesized using several routes from altogether three different precursors: tryptophan, nicotinamide (Nam) and nicotinic acid (NA). Nam and NA are together known as vitamin B3 or niacin. As in humans only 1% of the tryphtophan taken up with our diet is converted into NAD, vitamin B3 is an essential part of our diet, with Nam being the major NAD precursor in humans. But Nam is also the product of NAD-consuming signalling reactions such as sirtuins (NAD-dependent histone deacetylases) or PARPs (poly ADP robosylases)

## Below old version

For example, expression changes of both NAD-consuming and –synthesising enzymes have been associated with these diseases (refs). However, the observations are in part contradictory and seem to be context-specific. These apparent inconsistencies most likely reflect the complex dynamics of the NAD metabolome with regard to both biosynthesis and signalling. It is also important to realize that there are regulatory mechanisms that modulate cellular NAD concentrations, most prominently, in conjunction with the circadian clock (ref). Obviously, understanding the dynamic behaviour of this network requires modelling approaches that take into consideration the kinetics of the enzymes involved and thei known regulatory components.

Among the most puzzling constituents of this network is the enzyme

Signal transduction and metabolism are heavily intertwined and to analyse and understand their complex dynamic interaction is still a challenge. NAD is one of the major cofactors and metabolites at the crossroad between these processes. In redox reactions it is reversible interconverted between its reduced and oxidized form, whereas NAD-dependent signalling (NAD consumption) degrade NAD to Nam and ADPR ….and convert it into its precursor and essential vitamin nicotinamide (Nam).

High turnover 🡪 both signalling and biosynthesis have to be fast

Constant replenishment of cellular NAD pools is required to retain NAD concentrations required for metabolic processes.

This holds especially true for the effect of expression changes of sirtuins (Alhazzazi et al., 2011) (a family of NAD-dependent histone deacetlyases EC 2.4.2.B15) and the Nam-N-methyltransferase (NNMT, EC 2.1.1.1) that methylates Nam and thus makes it unavailable for further salvage, eventually leading to excretion of Nam-metabolites with the urine.

NNMT has been found to be overexpressed in several types of cancer and neurodegenerative diseases (Ref?) as well as obesity (Kraus et al., 2014) and diabetes (Ref). Overexpression of NNMT leads to life span extension and increased reactive oxygen production in *Caenorhabditis elegans* (Schmeisser et al., 2013) as well as changes in protein methylation in cell culture experiments (Ulanovskaya et al., 2013). Tissues specific knockdown of NNMT in mouse white adipose tissues protects against diet-induced obesity (Kraus et al., 2014)whereas overexpression of NNMT in liver has similar effects (Hong et al., 2015).The physiological role of NNMT is still not known and the question, why we need an enzyme that degrades an essential vitamin, has not been answered yet.

of NAD biosynthesis and consumption using available enzyme kinetics. In addition, we performed a phylogenetic analysis to evaluate the potential co-evolution of NNMT with NAD-biosynthesising or -consuming enzymes. We show that NNMT helps to maintain NAD-homoestasis under different conditions and counter-intuitively enables higher NAD consumption rates. This is supported by the fact that NNMT evolution is followed by an increase in the diversity of NAD-consuming enzymes. We furthermore reveal that NNMT might have exerted an evolutionary pressure on the development of the biosynthetic enzyme NamPRT and the compartmentalisation of NAD-metabolism.

### Results

### Final: NNMT KD in HeLa 🡪 change in acetylation (SIRT1/2 inhibition)?

### NNMT (only) in organisms with Nampt

### 1) In welchem Zusammenhang stehen NNMT, Nampt, NADA 🡪 3 enzymes competing for the same substrate

### 2) Woher, wann tritt NNMT auf?

### Describe phylogenetic analyses (which signalling enzymes; explain graph components), only NNMT – obviously incomplete genomes

### Results:

### bacteria, plants fungi predominantly just NADA, bacteria less than 25% Nampt and/or NADA

### Emergence of NNMT apparently concomitant with evolution of deuterostomia, although in ecdysozoa NNMT is occasionally present, in particular in worms

### Most strikingly: with the emergence of NNMT in Deutersostomia NADA becomes minor 🡪 chordata: NADA disappears. These analyses also support the view that tunicates and branchiostomes are not part of the phylum chordata

### Interestingly, NNMT would appear to have evolved de novo in metazoan.

### It is almost exclusively present in deuterostomia in combination with Nampt as the NAD biosynthetic enzyme

### Even more strikingly: Co-existence of NNMT and Nampt also correlates with a considerable increase in NAD-dependent signalling pathways, that is, processes that degrade NAD to Nam and ADPR. (explain numbers on graph; for example ~10 signaling processes per organism in mammals vs. ~4 in protostomia).

### How can NAD-dependent signalling increase specifically under conditions where the precursor of NAD biosynthesis is directly diverted into a compound that cannot be recycled into NAD synthesis.

### Intuitively, one would expect that a decrease in precursor concentration should cause a decrease of NAD availability and consequently less active NAD-dependent signalling. Given the complexity of the NAD-metabolic network, this question is difficult to be comprehensively addressed experimentally. Therefore 🡪 model simulations

### The additional presence of NNMT in cells expressing Nampt drives NAD-dependent signalling

### To analyse the potential impact of NNMT on NAD homeostasis we built a mathematical model to simulate human cellular NAD metabolism. The model included … (List enzymes/processes) and was constructed using the known kinetic parameters (retrieved from BRENDA or original articles, see Suppl. Tab. 1). We also included substrate competition and product inhibition when relevant. NamPRT was modelled as being in its fully activated form (Schramm).

### Given the high affinity of Nampt to Nam, in cells using this activity, NAD synthesis would always proceed at a maximal rate (Nampt is rate-limiting and the enzyme is saturated at ~15nM Nam, that is ~3-fold Km). Moreover, without growth or Nam degrading activities, cells would not need any influx of Nam (NAD metabolism would be independent of externally available Nam), since all NAD consumption leads to the re-formation of Nam. Therefore, in our model we considered dividing cells that, depending on the growth rate, require a net increase of the total NAD amount, based on synthesis initiated by Nam influx.

### Describe results with/without NNMT…

As expected the presence of NNMT lowers the steady state concentration of NAD in simulations, but the effect is rather low as NAD concentrations are not even halved (Figure 2A). Counter intuitively NAD-consumption rates are increased in the presence of NNMT (Figure 2B) nearly independent of Nam availability (i.e. Nam efflux/import rate ratio).

These effects are due to two independent properties of the pathways. The remaining high NAD-concentration is due to the very high affinity of NamPRT, whereas the increased NAD-consumption rates are caused by the inhibition of NAD-consuming reactions by Nam. Thus, NNMT seems to enable higher NAD-consumption rates.

Has NNMT potentially been driving NamPRT evolution? To answer this question, we analysed the effect of the substrate affinity of NamPRT (KM) on NAD-consumption flux in the presence and absence of NNMT. This analysis revealed several aspects of NNMT-presence. In the absence of NNMT NAD-consumption flux is very dependent on Nam efflux or import (Figure 4A and Supplementary Figure 2A), whereas it is relatively stable in the presence of NNMT largely independent of Nam efflux rates (Figure 4D) and Nam import (supplementary Figure 2D), the latter only if NamPRT has a high affinity. This is true for both NAD consumption flux and NAD steady state concentrations (Figure 4D and E). Thus, NNMT seems to contribute to the maintainance of NAD-homeostasis. We furthermore see that substrate affinity changes of NamPRT have very little effect on NAD-concentration or NAD-consumption flux in the absence of NNMT (Figure 4A and B and Supplementary Figure 2A and B) or the effect is limited to particular Nam-efflux/import ratios. Only in the presence of NNMT, increased substrate affinity of NamPRT is increasing NAD-concentration and steady state fluxes of NAD-consumption (Figure 4D and E). We thus hypothesis that NNMT most likely exerted an evolutionary driving force on NamPRT and thus contributed to the development of the extremely high substrate affinity observed for human NamPRT.

Another interesting observation is that only in the presence of NNMT NAD-consumption flux is reflecting NAD-concentration under different conditions. This is important as especially sirtuins are often described as NAD-sensors relaying metabolic status to changes in signal transduction (Ref?).

In the evolutionary context and additional question arises: Why do only a few organisms, mostly less complex animals, express NADA in addition to NamPRT and NNMT. Including NADA into the simulations we see that the effect of NADA on NAD-consumption is very limited in the presence of NNMT (Figure 4F) even at high expression levels (see Suppl. Figure 1C and D) and could be compensated by increased expression of NamPRT (not shown). In the absence of NNMT, NADA has some effect on NAD-consumption under certain conditions at low expression (Figure 4C) and much stronger effect on NAD-concentration at high expression (Suppl. Figure 1A), NAD-consumption is however reduced at low efflux rates.

Taken together these observations might explain the observed phylogenetic distribution of these enzymes.

#### Compartmentalisation of NAD-biosynthesis in vertebrates

Even though the affinity of NamPRT is already very high, the question arises whether the affinity is optimal or could be further increased to increase steady state NAD-concentration and –consumption fluxes. In addition, NNMT affinity changes could have an affect on both NAD-concentration and NAD-consumption. By performing a comprehensive parameter scan we show that the affinities of both enzymes found in human (indicated by asterisks in Figure 4, D, E, G and H) appears to be close to optimal and further improvements would only have minor effects.

Until this point we have neglected compartmentalisation of the pathway. We do however know from previous studies that in early vertebrate development a compartmentalisation of the pathway has occurred reflected by a gene triplication of NMNAT and the occurrence of compartment specific domains called ISTIDs (Lau et al., 2010). Looking at the evolutionary timepoint of appearance of NNMT and the gene triplication of NMNATs, we see that NNMT occurs prior to the gene triplication and the first occurrence of ISTIDs and we have confirmed this in our own analysis (not shown).

Human NamPRT is known to be partially localised (Ref?) to the nucleus, whereas NNMT has been described to be cytosolic. We thus analysed the effect of nuclear localisation of NamPRT on nuclear versus cytosolic NAD-consumption flux. As can be seen from Figure 4I nuclear localisation of NamPRT together with NMNAT enables higher NAD-consumption flux of nuclear NAD-consumption e.g. hSirt1p.

Using multiple sequence alignments NamPRTs we then identified an insert region only occurring in deuterostomia that encode both NNMT and NamPRT (Figure 5A). The sequence stretch is part of a predicted nuclear localisation signal (NLS), and removing the insert from the sequence the NLS is no longer predicted. In all available protein structures of NamPRT (e.g.(Wang et al., 2006)) this part of the sequence is not resolved, thus representing a flexible loop at the surface of the enzyme dimer that could indeed serve as a localisation signal (Figure 5B). Mutation experiments show that the deletion of the insert partially compromises nuclear localisation (Figure 5C).

### Discussion

#### Evolutionary and physiological role of NNMT

The dynamic interaction between metabolism and signal transduction as well as gene regulation is still not very well understood. New approaches are required to disentangle the underlying network, helping us to understand how alterations affect human physiology. We here combine phylogenetic analysis and mathematical modelling of a central metabolic pathway supported by experimental analysis to reveal the likely evolutionary development of the NAD-biosynthesis and –consumption pathway and the physiological role of the methyltransferase NNMT.

We show that the vitamin B3 degrading enzyme NNMT plays a vital role for NAD-dependent signalling reactions and NAD-homeostasis. Due to the fact that NamPRT has a very high affinity for Nam whereas NNMT has a rather low affinity for it, NNMT is able to remove excess Nam that would lead to inhibition of NAD-consuming enzymes while maintaining NAD-concentrations and even increasing NAD-consumption fluxes.

The affinities measured for the human enzymes seem to be optimal and further increase in NAD-consumption rates is only enabled by compartmentalisation of NAD-biosynthesis. This process appears to have been driven by occurrence of NNMT as the sequence insertion leading to nuclear localisation of NamPRT as well as the gene triplication of the second NAD-biosynthesis enzyme NMNAT together with the occurrence of the respective localisation signals (ISTIDs) seem to have evolved later. It is unfortunately difficult to analyse the predicted affinity changes, as a lot of sequence variations have occurred in the relatively long evolutionary time frame between NNMT occurrence and NamPRT-NLS or ISTID development. This is further hampered by the fact that sequences of living organisms have independently evolved after the separation of the phyla. Thus, current sequences do not necessarily reflect earlier evolutionary developments, especially as a strong positive selection of features can be assumed based on our kinetic analysis.

#### General applicability

It is of course impossible to extend our conclusions to other metabolic processes without analysing them in more detail, but we have noted that the metabolism of other vitamins, such as pyridoxal (vitamin B6) metabolism, also contains vitamin degrading enzymes, that might thus have similar roles. Degrading enzymes do in general not receive the same attention as biosynthetic enzymes, reflected by the observation that NNMT has only recently been analysed in more detail, beside the much earlier recognition of NNMT as potential marker for some types of cancer (e.g. Ref?) and routine clinical measurements of urine methyl-Nam concentrations in the context of different diseases (e.g. Ref? )Our analysis show, however, that degrading enzymes do not solely modify substrates for better excretion, but can play a vital role both in for human physiology as well as in the evolutionary development of biological processes.

#### Limitations of our analysis and outlook

Among others we neglected the potential effects of the methyl donor S-adenosyl-methionin (SAM) and its precursor methionine in our analysis, although it is most likely contributing yet another regulatory level for Nam availability and thus an additional interaction point between gene regulation and metabolism. It has been shown earlier that in turn NNMT-expression influences protein methylation dependent on methionine availability (Ulanovskaya et al., 2013). One of the challenges in the analysis of this aspect is the availability of *in vivo* concentration measurements for SAM and the large amount of reactions using it as substrate. The same holds for the analysis of enzyme acetylation versus deacetylation that would enable prediction about enzyme activation or histone state if available.

### Experimental Procedure

#### Dynamic modelling

Kinetic parameters (substrate affinity (Km) and turnover rates (kcat), substrate and product inhibitions) were retrieved from the enzyme database BRENDA and additionally evaluated by checking the original literature especially with respect to measurement conditions. Parameter values from mammals were used if available. For yeasts specific enzymes, yeast values were used. The full list of kinetic parameter including reference to original literature can be found in Supplementary table 1. For NMNAT the previously developed rate law for substrate competition was used (Schauble et al., 2013). Despite these modifications, Henri-Michaelis-Menten kinetics were used for all reactions except the import and efflux of Nam, which were simulated using constant flux and mass action kinetics, respectively. All simulations were performed using the steady state calculation and parameter scan options provided by COPASI 4.17 (Hoops et al., 2006). The model will be available at the Biomodels database accession number #. Related figures were generated using gnuplot.

#### Phylogenetic Analysis

NADA, NamPRT, and NNMT enzymes or enzyme candidates were identified using Blastp with known enzymes against the non-redundant protein sequence database (nr). A list of functionally verified enzymes used as templates is given in supplementary table 2. This table also includes the length cut-offs for identified enzymes. The e-value cut-off was 1e-30 for all enzymes. We configure Blastp to yield maximum 20000 target sequences, using the BLOSUM62 matrix with a word size of 6 and gap opening and extension costs of 11 and 1, respectively. Low-complexity filtering was disabled. Obvious sequence contaminations were removed by manual inspection of the results. The taxonomy IDs of the species for each enzyme was derived from the accession2taxonomy database provided by NCBI. Scripts for creating, analysing, and visualising the phylogenetic tree were written in Python, using the ETE2 toolkit (Huerta-Cepas et al., 2010).

#### Cloning and mutation of NamPRT

#### Cell culture conditions and fluorescence imaging

### Figure Legends

#### Figure1

***Schematic overview of NAD-biosynthesis and consumption***. (NADA- Nam deamidase; NamPRT- Nam phosphoribosyl transferase; NAPRT-NA phosphoribosyltransferase; NMNAT- Nam mononucleotid adenylyl transferase; NADS- NAD-synthase)

#### Figure 2

***The presence of NNMT increases the NAD-consumption rates.*** NAD-concentration is reduced in the presence of NNMT but only to a limited extend (A) in contrast NAD-consumption flux is increased over a wide range of Nam efflux to import rates in the presence of NNMT (B). Only at very high Nam export rates NAD-consumption is higher without NNMT.

#### Figure3

***NNMT can mainly be found in combination with NamPRT.*** NADA is part of the Nam salvage pathway in yeast and plants but is absent in vertebrates, while NamPRT is mainly found in vertebrates together with NNMT. Other enzyme combinations are rare. The color-coding is described in the legend in the upper left corner. The circle size indicates the number of genomes analysed in the particular taxon using a logarithmic scale. The numbers below the taxon names indicate the average number of NAD-consuming enzyme families found in all sub-taxa. Only selected sub-trees are shown. In total genomes or transcriptomes of 969 eukaryotic species are included.

#### Figure 4

***Comprehensive analysis of the potential evolutionary role of NNMT and the effect of different enzyme combinations on NAD-consumption and concentration*** If NNMT is absent (A-C) NAD-consumption fluxes and NAD-concentration are very dependent on the availability of Nam (Nam efflux/import ratio). Furthermore, affinity changes of NamPRT have only very little effect on NAD-consumption flux (A) and –concentration (B) or only under specific conditions. In the presence of NNMT (D-F) NAD-consumption flux (D and F) and-concentration (E) are relatively independent on the ratio between Nam efflux and import, in contrast affinity changes of NamPRT have a large effect. The addition of NADA has very little effect (C and F), especially in the presence of NNMT (F). The substrate affinity of NNMT and NamPRT have opposite effects on NAD-consumption(G) and –concentration (H), as would be expected. The affinities previously measured for human enzymes (indicated by the white star) appears to be close to optimal as further improvements would have little or no effect on NAD-consumption or –concentration. Further improvement of NAD-consumption rates can only be achieved by compartmentalisation (I), as NNMT is localised to the cytosol, cytosolic NAD-consumption rates are lower than nuclear NAD-consumption rates if NAD-biosynthesis and-consuming reactions are present in both compartments.

#### Figure 5

***Deuterostomia that encode NNMT show a sequence insertion in the N-terminal region of NamPRT that enables nuclear localisation.***

Multiple sequence alignment of NamPRT from selected deuterostomia clearly show a sequence insertion (A) in organisms that encode NamPRT and NNMT. (Species names are given and the number in parenthesis indicates the enzyme combination encoded in these species; 3 – NADA and NamPRT, 6 – NamPRT and NNMT, 7 – NADA, NamPRT and NNMT. The nuclear localisation signal (NLS) indicated was predicted using using cNLS-mapper (<http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi> (Kosugi et al., 2009)). The inserted region is not resolved in crystal structures of human NamPRT and thus appears to be a flexible loop structure at the surface of the NamPRT-dimer. The visualisation is based on a structure prediction of SWISS-MODEL (Arnold et al., 2006; Biasini et al., 2014) of the sequence of the human NamPRT (P43490) using the model 2H3D as template (Wang et al., 2006). Deleting the respective sequence insertion results in a reduced nuclear localisation of GFP-labeled NamPRT in human cell lines. images

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