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Review

The Nudix hydrolase superfamily

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Abstract. Nudix hydrolases are found in all classes of organism and hydrolyse a wide range of organic pyrophosphates, including nucleoside di- and triphosphates, dinucleoside and diphosphoinositol polyphosphates, nucleotide sugars and RNA caps, with varying degrees of substrate specificity. Some superfamily members, such as *Escherichia coli* MutT, have the ability to degrade potentially mutagenic, oxidised nucleotides while others control the levels of metabolic intermediates and signalling compounds. In prokaryotes and simple eukaryotes, the number of Nudix genes varies from 0 to over 30, reflecting the metabolic complexity and adaptability of the organism. Mammals have around 24 Nudix genes, several of which encode more than one variant. This review integrates the sizeable recent literature on these proteins with information from global functional genomic studies to provide some insights into the possible roles of different superfamily members in cellular metabolism and homeostasis and to stimulate discussion and further research into this ubiquitous protein family.

Key words. Nudix; NUDT; MutT; nucleotide metabolism; housecleaning; antimutator; decapping.

Introduction

The Nudix superfamily (InterPro IPR000086; Pfam PF00293) is widespread among eukaryotes, bacteria, archaea and viruses and consists mainly of pyrophosphohydrolases that act upon substrates of general structure **nu**cleoside **di**phosphate linked to another moiety, **X** (NDP-X) to yield NMP plus P-X [1]. Such substrates include (d)NTPs (both canonical and oxidised derivatives), nucleotide sugars and alcohols, dinucleoside polyphosphates (Np_nN), dinucleotide coenzymes and capped RNAs. However, phosphohydrolase activity, including activity towards NDPs themselves [2–5], and non-nucleotide substrates such as diphosphoinositol polyphosphates (DIPs) [6, 7], 5-phosphoribosyl 1-pyrophosphate (PRPP) [8], thiamine pyrophosphate (TPP) [9] and dihydroneopterin triphosphate (DHNTP) [10] have also been described. Catalysis depends on the conserved 23-amino acid Nudix motif (Nudix box), Gx_5Ex_5[UA]$xREx_2EExGU$, where U is an aliphatic, hydrophobic residue (PROSITE PS00893), although several interesting examples exist with altered consensus residues. This sequence is located in a loop-helix-loop structural motif and the Glu residues in the core of the motif, REx_2EE, play an important role in binding essential divalent cations. In most cases, Mg^{2+} is likely to be the most physiologically relevant. Numerous site-directed mutagenesis studies have defined the importance of individual residues in the Nudix motif for catalysis, although there is considerable variation in the site of attack on the substrate, in the position of the catalytic base (usually a Glu, which may be outside the motif) and in the number of divalent ions involved [11, 12]. Substrate specificity is determined by side chains and motifs elsewhere in the structure, and a number of distinct families can be defined by these motifs. Together, these form part of the structural Nudix fold, an $\alpha/\beta/\alpha$ sandwich shared by the isopentenyl diphosphate isomerases [13] and the C-terminal domains of MutY-type DNA glycosylases [14] that, together with the hydrolases, form a larger Nudix suprafamily with a common evolutionary origin. Nudix hydrolases are typically small proteins (16–21 kDa), larger ones having additional domains with interactive or

other catalytic functions. Many have alkaline pH optima and they are strongly inhibited by F^-, probably due to the occupation of the position of the leaving group in the transition state by an MgF_3^- complex [11]. The structures and mechanisms of Nudix hydrolases have recently been reviewed in detail [11], so this article will concentrate on the distribution and functions of these enzymes.

In 1996, Bessman defined the Nudix hydrolases as having a 'housecleaning' function, 'cleansing the cell of potentially deleterious endogenous metabolites and modulating the accumulation of intermediates in biochemical pathways' [1]. Among the bacteria, there is a linear correlation between the number of Nudix genes and genome size, and this broadly reflects the environmental adaptability and metabolic complexity of the organism (fig. 1). At one end of the scale, the soil actinomycetes, *Streptomyces coelicolor*, *S. avermitilis* and *Frankia* sp. EAN1pec with their myriad of pathways for secondary-metabolite synthesis, have 29–33 Nudix genes; at the other, most intra- and extracellular parasites and symbionts like the mycoplasmas, *Borrelia* and *Wigglesworthia*, whose genomes have become reduced in size by eliminating unnecessary anabolic pathways, have none. Of particular note are those organisms with fewer or more Nudix genes than would be predicted from this simple relationship. For example, another soil bacterium, *Acidobacteria-3* sp., with a 10-Mbp genome size similar to the actinomycetes, has only 7 Nudix genes. In contrast, the 3.3-Mbp genome of *Deinococcus radiodurans* encodes 26, the highest number per Mbp of any bacterium sequenced so far (fig. 1). The extreme radiation and desiccation tolerance of this organism have been cited as reasons for this large number, with the additional members eliminating the wide range of oxidised or otherwise modified nucleotides that could arise from such extreme environmental conditions [16, 17]. However, radiation resistance per se does not demand multiple Nudix genes, as the highly resistant *Kineococcus radiotolerans* and *Rubrobacter xylophilus* have 14 and 8, as would be predicted from their respective genome sizes of 5.0 and 3.2 Mbp. Furthermore, we have found that the majority of *D. radiodurans* Nudix genes (17 out of 19 studied) are strongly induced upon entry into stationary phase, suggesting a possible involvement in metabolic reprogramming [J. Cartwright and A. G. McLennan, unpublished data]. Considering that *Bacillus halodurans* has 10 Nudix genes, the existence of 26–31 in the closely related *B. cereus, B. anthracis* and *B. thuringiensis* implies that specific gene amplification events occurred after the evolutionary branching of the latter group from *B. halodurans* [18]. A preliminary survey of the activities of the *B. cereus* Nudix hydrolases suggests that many of these may be NDP-sugar hydrolases [19]. The reason remains to be established.

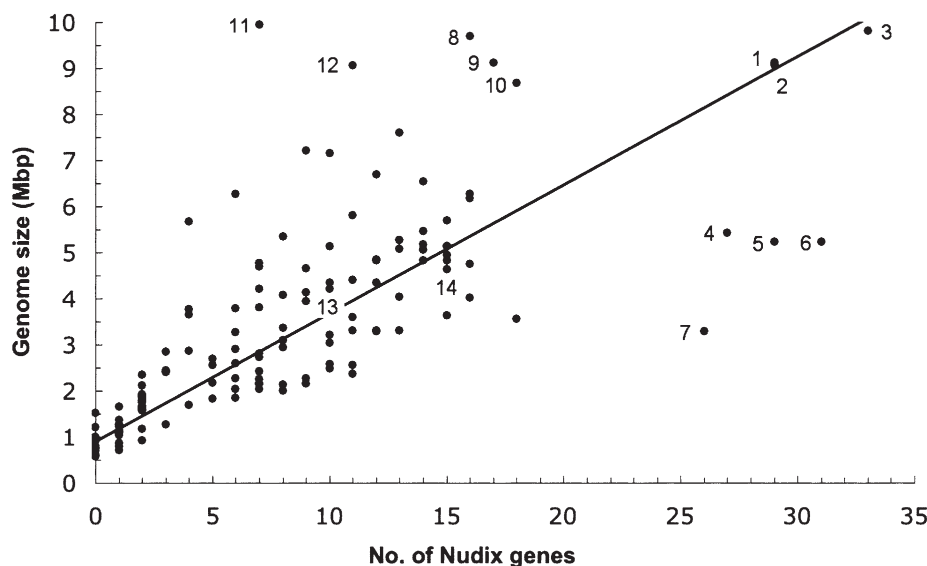

Figure 1. Relationship between number of Nudix genes and genome size in bacteria. The number of Nudix genes in each of 134 different bacterial species is taken from the Superfamily (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/index.html) [15] and Integrated Microbial Genomes (http://img.jgi.doe.gov) databases and includes related suprafamily sequences such as isopentenyl diphosphate isomerases. Only single strains of a particular species are included. Numbered species are: 1, *Streptomyces avermitilis* MA-4680; 2, *Streptomyces coelicolor* A3(2); 3. *Frankia sp.* EAN1pec; 4, *Bacillus cereus* ATCC 14579; 5, *Bacillus anthracis Ames*; 6, *Bacillus thuringiensis ser. konkukian* 97–27; 7, *Deinococcus radiodurans* R1; 8, *Burkholderia xenovarians* LB400; 9, *Bradyrhizobium japonicum* USDA 110; 10, *Burkholderia capacia* 383; 11, *Acidobacteria-3 sp.* Ellin6076; 12, *Nostoc punctiforme* PCC73102; 13, *Bacillus halodurans* C-125; 14, *Escherichia coli* K12. The line of best fit ($R^2 = 0.51$) was calculated using a y intercept of 0.9 Mbp, the mean genome size of bacteria with no Nudix genes.

Among eukaryotic microorganisms and fungi, the number of Nudix genes also increases linearly with genome size ($R^2 = 0.75$), although per Mbp DNA, there are on average only about one-fifth of the number found in bacteria. For example, *Encephalitozoon cuniculi* (2.5 Mbp) has 2, *Saccharomyces cerevisiae* (12.2 Mbp) has 7 and *Dictyostelium discoideum* (34 Mbp) has 20. In higher eukaryotes, the large amount of non-coding DNA means there is no relationship to total genome size. *Caenorhabditis elegans* has 14, *Drosophila melanogaster* has 20, mammals have about 24, while *Arabidopsis thaliana* has 32. Selective amplification or retention after genome duplication has led to the expansion of certain Nudix families, the members of which may show differences in subcellular location and/or tissue-specific expression, e.g. *A. thaliana* has 3 orthologues of the human NUDT2 Ap_4A hydrolase, 6 orthologues of NUDT6 (all of which may be ADP-ribose pyrophosphohydrolases [20]) and 7 members of the DIP phosphohydrolase (DIPP) family. In the following sections, the Nudix hydrolase complements of *E. coli*, *S. cerevisiae* and human/mouse cells are described in detail as they cover the spectrum of activities probably common to most prokaryotes and eukaryotes. A Supplementary Table comparing the hydrolases in these organisms is available at http://www.liv.ac.uk/~agmclen/nudixrev.html. However, other hydrolases that may be restricted to certain species are also discussed in this review.

E. coli Nudix hydrolases

Laboratory strains of *E. coli* have 13 Nudix hydrolase genes (table 1). The first to be studied genetically and enzymically was the 15-kDa MutT (NudA) protein, hence the original name of the Nudix family – the MutT family [1, 21]. The nuclear magnetic resonance (NMR) solution structures of free and complexed forms of MutT have been studied extensively [11, 22]. Disruption of *mutT* leads to a ~1000-fold rise in spontaneous mutation frequency due to increased AT:CG transversions [23]. This is consistent with its *in vitro* enzymic activity. MutT efficiently converts the mutagenic, oxidised nucleotide 8-OH-dGTP [a product of reactive oxygen species (ROS) attack on dGTP] to 8-OH-dGMP and PPi, thus preventing the incorporation of *syn*-8-OH-dG into DNA opposite dA [24]. MutT is also active towards the canonical (d)NTPs [25]; however, its K_m for 8-OH dGTP of 0.08-0.52 μM is some 10^3–10^4 times lower than that for dGTP [5, 26]. It also degrades 8-OH-GTP and so may prevent transcriptional errors as well [27]. The physiological relevance of 8-OH-dGTP as a MutT substrate has been questioned, since it could not be detected in *mutT*$^-$ strains using an HPLC assay whose sensitivity was such that mutagenic levels of this nucleotide should have been observed, suggesting that an alternative mutagenic substrate may exist [28]. On the other hand, direct introduction of 8-OH-dGTP into permeabilised *mutT*$^-$ *E. coli* led to a six-fold

Table 1. Summary of the Nudix hydrolases of *E. coli*.

Name	Synonyms	Known substrates[1]	Transcript abundance[2]
NudA	MutT	8-OH-(d)GTP, 8-OH-(d)GDP, other (d)NTPs	226
NudB	NtpA, YebD, orf17	dATP, dADP, 8-OH-dATP, 8-OH-dADP, other (d)NTPs	3225
NudC	YjaD, orf257	NADH, deamino-NADH, Ap_2A	1328
NudD	WcaH, YefC, gmm, orf1.9	GDP-mannose, GDP-glucose	146
NudE	YrfE, orf186	Ap_3A, Ap_2A, NADH, ADP-ribose	619
NudF	RdsA, AspP, AdpP, TrgB, YzzG, YqiE, orf209	ADP-ribose, ADP-glucose, ADP-mannose	1113
NudG	YnjG, orf135	5-Me-dCTP, (d)CTP, 5-OH-(d)CTP, 8-OH-dGTP, 2-OH-dATP	310
NudH	YgdP, orf176	Ap_5A, Ap_6A, Ap_4A	3095
YeaB		(CoA and derivatives)[3]	477
YmfB	orf153	TPP	567
YffH		(NDP-sugars)[3]	137
YfaO	orf141	?	249
YfcD	orf180	?	639

[1] Details of substrate preferences are given in the text.
[2] Relative abundance in mRNA prepared from mid-log phase cells grown in minimal medium. Data taken from Corbin et al. [38].
[3] Predicted substrates.

increase in mutation frequency compared to wild-type cells, showing that MutT can suppress mutations induced by 8-OH-dGTP *in vivo* [29]. This rather low level of induced mutagenesis may be due to the existence of additional 8-OH-dGTPase activities, such as the RibA GTP cyclohydrolase II [30]. If another MutT substrate does exist that fits the specificity of the *mutT* phenotype, it could be 8-OH-dGDP which is converted to 8-OH-dGMP and Pi with a K_m of 0.06 μM [5].

NudG (orf135) also has pyrophosphohydrolase activity towards (d)NTPs. According to k_{cat}/K_m ratios, the preferred substrates *in vitro* are 5-Me-dCTP and 5-OH-CTP, which are favoured over dCTP and CTP [31–33]. 8-OH-dGTP and 2-OH-dATP are also hydrolysed but with efficiencies some 200-fold lower than the cytidine nucleotides [33]. 5-OH-dCTP is reportedly a much poorer substrate than 5-OH-CTP (4.3%) [32], but this would still make it significantly better than 8-OH-dGTP or 2-OH-dATP. A *nudG*$^–$ strain was shown to have an increased spontaneous and H_2O_2-induced mutation frequency, due mainly to GC:AT transitions and GC:TA transversions, but with few of the AT:CG transversions characteristic of *mutT*$^–$ strains. This was interpreted in terms of reduced 2-OH-dATP elimination [34]. However, 5-OH-dCTP readily causes GC:AT and GC:TA mutations [35] and so must be considered a relevant, if not the sole, substrate for NudG *in vivo*. NudB (NtpA, orf17) is another dNTPase, originally reported to prefer dATP as a substrate [36]. Recently, it was shown to hydrolyse both 8-OH-dATP and 8-OH-dADP, though with efficiencies similar to the unoxidised nucleotides [4]. Demonstration of a possible antimutator function will require further genetic analysis. In a recent genome-wide study, NudB was the only *E. coli* Nudix hydrolase found to be essential for aerobic growth in rich media (or at least to impart a substantial fitness advantage) [37]. If this is confirmed, it would suggest that NudB has more than just an antimutator function and may be involved in regulating nucleotide pools for growth. It also has the highest transcript abundance of any *E. coli* Nudix hydrolase, at least 15-fold greater than that of *mutT* [38].

E. coli YgdP (NudH, orf176) is a 21-kDa Np_nN hydrolase. It belongs to the family of asymmetrically cleaving diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) hydrolases, first described in eukaryotes, that cleave Ap_4A to ATP + AMP [39, 40]. This is in contrast to the unrelated symmetrically cleaving Ap_4A hydrolases, like *E. coli* ApaH, that produce 2 ADPs [40]. The bacterial YgdP-type enzymes show greater sequence similarity to plant Ap_4A hydrolases than to the animal orthologues [41]. An exception is *Thermus thermophilus* Ndx1, which strongly prefers Ap_6A and is more similar to animal Ap_4A hydrolases and the DIPP family [42]. Ap_4A is synthesised by aminoacyl-tRNA synthetases and other AMP-forming ligases [43] and may have a number of roles in prokaryotes, including modulating chaperone and heat shock protein activity [44, 45] and controlling the timing of cell division [46]. If allowed to accumulate unchecked, it could also interfere with a number of ATP-dependent reactions [47]. The Nudix Ap_4A hydrolases all have a Tyr or Phe residue 17 amino acids downstream of the catalytic motif [48] that binds the adenine ring of the substrate distal to the site of nucleophilic attack [49, 50]. They hydrolyse Np_nN where n>3, always producing an NTP product. *E. coli* YgdP and the orthologues from *Salmonella typhimurium* [51], *Rickettsia prowazekii* (InvA) [52] and *Pasteurella multocida* (PnhA) [53] prefer Ap_5A over Ap_4A *in vitro*, whereas Ap_4A is preferred by the enzymes from *Bartonella bacilliformis* (IalA) [54, 55] and *Helicobacter pylori* (NudA) [56]. *E. coli* K1 YgdP and *B. bacilliformis* IalA have both been implicated in the ability of these pathogens to invade brain microvascular endothelial cells [57] and erythrocytes [58], respectively, suggesting that proper regulation of bacterial Np_nN is required for expression of the invasive phenotype. Direct evidence for this was provided by the demonstration that deletion of the *S. typhimurium ygdP* and *apaH* genes leads to synergistic increases in bacterial Ap_4N levels while decreasing cellular invasion [51]. Furthermore, *pnhA*$^–$ mutants of *P. multocida* are 1000-fold less virulent in a chick embryo animal model [53]. Disruption of *E. coli ygdP* also relieves lethal hybrid jamming, the blocking of the general protein secretory pathway caused by the expression of normally cytoplasmic proteins when fused to an N-terminal Sec signal sequence. The C-terminal portion folds before export and blocks the translocation pores [59]. This may further implicate Ap_nA, and hence YgdP, in modulating the activity of chaperone proteins. *E. coli* YgdP also appears to be part of an interaction network involving ribosomal and ribosome-associated proteins [60] and is strongly up-regulated by 1 μg/ml Cd^{2+}, a treatment that disrupts ribosomal protein production and induces several stress response systems [61]. Since the *apaH* gene is part of an operon whose genes relate to ribosome function and starvation survival, these observations strengthen the proposed link between Ap_nA and nutritional and oxidative stress [62], which may underpin the role of Ap_nA in bacterial invasion.

NudF (AspP, TrgB, orf209) is a 47-kDa dimer originally characterised as an ADP-ribose pyrophosphohydrolase [48]. Similar, high-K_m (50–200 μM) ADP-ribose hydrolases have been isolated from a number of other bacteria and have the common structural feature of a proline residue 15–16 amino acids downstream of the Nudix motif [12, 48, 63–65]. NudF is notable in that it displays domain swapping, with each active site formed by residues from both monomers [66, 67]. ADP-ribose is a potentially toxic product of NAD catabolism. In bacteria, most NAD turnover is due to the activities of DNA ligase and NAD(H) pyrophosphatase activities, while most NAD glycohydrolase activities are secreted [68]. However,

there is evidence for an intracellular NAD glycohydrolase activity that could generate free ADP-ribose [68, 69]. This reactive nucleotide-sugar is able to non-enzymically glycate protein N-terminal and lysyl amino groups and cysteinyl thiols, leading to loss of function [70]. It may also contribute to tellurite toxicity, since the *Rhodobacter sphaeroides trgB* tellurite resistance gene encodes a putative ADP-ribose hydrolase [48]. Thus, the elimination of free ADP-ribose is desirable. However, NudF is not specific for ADP-ribose and hydrolyses ADP-glucose and ADP-mannose with similar efficiencies [71]. The *E. coli nudF* gene is part of the cre regulon and is up-regulated during growth in minimal media by CreBC, a regulatory system that responds to changes in carbon supply [71, 72]. A *nudF*$^{-}$ mutant had very low ADP-glucose hydrolase activity compared to wild-type cells, but unaltered ADP-ribose hydrolase activity. This mutant also had a higher glycogen content than the wild type even when grown in minimal glucose medium, while overexpression of NudF led to a dramatic loss of glycogen [71], suggesting that NudF can regulate glycogenesis downstream of CreBC via the availability of the precursor ADP-glucose. Whether *E. coli* NudF serves the dual role of glycogen control and ADP-ribose elimination remains to be determined. In contrast, the enzyme from the archaeon *Methanocaldococcus jannaschii* is highly specific for ADP-ribose and 2′-phospho-ADP-ribose, the NADP metabolite, and confers increased tellurite resistance when expressed in *E. coli*, specifically implicating ADP-ribose as a causative factor in tellurite toxicity, although the precise reason is presently unknown [48, 73]. NudE (orf186) is also active with ADP-ribose but additionally hydrolyses the structurally related Ap_3A, NADH, Ap_2A and FAD [74]. Its function is unclear. An enzyme with broadly similar specificity is encoded by the *nudE.1* gene of bacteriophage T4 but is not required for normal phage growth under laboratory conditions [75].

NudD (WcaH, orf1.9), a 37-kDa dimer confined to enterobacteria and *Vibrio* species, is unique among the Nudix hydrolases in that it hydrolyses GDP-α-D-mannose and GDP-α-D-glucose to GDP and the corresponding β-sugar by nucleophilic substitution at the sugar C1 carbon rather than at phosphorus [11, 76, 77]. The core of the Nudix motif, RLTMAE, lacks two of the usual Mg^{2+}-binding Glu residues and this, plus a change in position of the catalytic base due to a six-residue deletion (with a neutral His preferred to the normal anionic Glu), leads to the change in mechanism [78]. *NudD* is located in the *E. coli* K12 gene cluster necessary for the production of colanic acid, an extracellular polysaccharide [79]. The enterohaemorrhagic strains O157: H7 and O111 have additional, related genes, *wbdQ* [80] and *wbdI* [81], respectively, with Nudix motifs similar to NudD. These show 63% and 45% identity to NudD and 43% identity to each other at the protein level. They are located in the gene clusters for the O-antigen component of the outer membrane lipopolysaccharide that control the synthesis of GDP-fucose, GDP-colitose and GDP-perosamine, but the precise roles of the hydrolases are not known.

NudC (YjaD, orf257) is a 60-kDa dimeric NADH pyrophosphohydrolase with a 120-fold preference for NADH over NAD^+ [82]. The sequence SQPWPFPQS located ten residues downstream of the Nudix box is found in this and all other characterised NADH hydrolases and may confer pyridine nucleotide specificity [48]. The structurally related NADPH, ADP-ribose and Ap_2A are also substrates. NudC may regulate the intracellular NAD^+/NADH ratio or generate NMNH for some as yet unknown role [82]. Disruption of *Haemophilus influenzae* NudC profoundly reduces the survival of this pathogen in an animal model [83], so YgdP appears not to be the only Nudix hydrolase involved in intracellular invasion and survival.

Another sequence motif, LLTxR[SA]x_3Rx_3Gx_3FPGG (PROSITE UPF0035), is found upstream of and contiguous with the Nudix box in the uncharacterised *E. coli* YeaB protein. As this motif is found in proteins from *D. radiodurans* and several eukaryotes that are active towards coenzyme A and its derivatives [84–87], YeaB is most likely also a CoA pyrophosphohydrolase. A screen for genes conferring resistance to bacimethrin, a thiamine analogue, identified *ymfB* [9]. YmfB (orf153) has TPP phosphatase activity but may also utilise other substrates depending on the divalent ion [88]; however, the phenotype of the *ymfB*$^{-}$ mutant implies that TPP is a substrate *in vivo* and that YmfB regulates TPP concentration.

The remaining three *E. coli* enzymes, YffH, YfaO and YfcD, have yet to be characterised. YffH has significant sequence similarity to NudF and human NUDT14, a UDP-glucose/ADP-ribose hydrolase [89], and so may be an NDP-sugar hydrolase. Genetic evidence suggests that it could be involved in remodelling extracellular polysaccharides for biofilm formation. [90]. YfaO appears so far to be unique to *E. coli* and the closely related *Shigella flexneri* and *Salmonella enterica* and may therefore have a role specific to their phylogenetic or environmental niches. It has an evolutionary retention index (ERI) of zero, where the ERI has been defined as the fraction of times a gene occurs in 33 diverse microbial genomes, and indicates uniqueness (≤0.2) or conservation and essentiality (0.4–0.7) [37]. YfcD, with an ERI of 0.03 (like NudD) is also phylogenetically restricted. It may prove difficult to find the true substrates for specialised Nudix hydrolases with low ERI values.

Other prokaryotic Nudix hydrolases

In addition to orthologues of the *E. coli* enzymes, a number of Nudix hydrolases have been isolated from other

prokaryotes with interesting specificities, properties and functions. A family of enzymes active with 5-methyl-UTP (ribo-TTP) and UTP has been found in a range of organisms including *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa* and *Caulobacter crescentus*. Activity with CTP is variable but purine nucleotides are poor substrates [91]. Two sequence motifs, L[VL]VRK and AANE, appear to define this family. In *Corynebacterium* sp., this enzyme is encoded on horizontally acquired plasmids or transposons and transcribed with transition metal ion transporters of the Nramp family [92]. The *Bacillus subtilis* YtkD protein catalyses the sequential conversion of (d)NTP substrates to (d)NMP via (d)NDP, unlike all other Nudix (d)NTPases, which produce (d)NMP and PPi [3]. The *ylgG* gene of *Lactococcus lactis* is part of the folate synthesis operon and YlgG specifically removes PPi from the folate intermediate DHNTP with a K_m of 2 μM. Inactivation of *ylgG* caused accumulation of DHNTP and a three-fold reduction in intracellular folate [10]. Cell viability was, however, retained due to non-specific phosphatases degrading DHNTP via the diphosphate. A 17-kDa DHNTP hydrolase with no NTPase activity has been isolated from *E. coli*, but not identified [93]. The *E. coli* Nudix hydrolase closest in sequence to YlgG is the 17.9-kDa YmfB TPPase with a BLAST E-value of 0.003 over 54 amino acids, but any identity between the two must be confirmed experimentally.

The ACT protein of *Bacillus methanolicus* is particularly interesting. It is required for activation of the NAD^+-dependent alcohol dehydrogenase, MDH [94]. Although it is an efficient ADP-ribose hydrolase *in vitro*, ACT hydrolyses MDH-bound NAD(H) *in vivo*, yielding NMN(H) and AMP. This alters the reaction mechanism of MDH to cofactor independence, increasing methanol turnover by 40-fold. This system provides feedback control and may help prevent accumulation of toxic formaldehyde [94]. The *pif* gene of the *Streptomyces ambofaciens* conjugative plasmid pSAM2 encodes a Nudix hydrolase, orf131, that helps maintain the plasmid in a prophage state under non-conjugal conditions [95]. Orf131 is almost identical to a hydrolase encoded in the *S. coelicolor* genome (SC6A9.30c) but its role in maintaining conjugal immunity between cells harbouring the plasmid is unknown. The *pur7* gene of *Streptomyces alboniger* is part of the puromycin biosynthetic cluster and specifically hydrolyses 3′-amino-3′-dATP, a pathway intermediate and a potent RNA polymerase inhibitor. This may prevent accumulation of this toxic nucleotide [96]. In large-scale substrate screens of enzymes from *D. radiodurans* and *B. cereus*, several activities towards UDP-sugars have been detected. The short motifs, GE and NGD, either side of the Nudix box may define a UDP-sugar family [17, 19]. Finally, a CDP-choline hydrolase is also present in *B. cereus* [19].

Nudix domains have also been observed in multidomain proteins. Proteins that are structurally separate in some organisms but which have become fused in others are likely to participate in the same biochemical pathway and have been termed Rosetta stone proteins [97]. Thus, the function of one domain may be inferred if the function of the other is known. For example, the C-terminal Nudix domain of the slr0787 protein from *Synechocystis* sp. has ADP-ribose hydrolase activity while the N-terminal domain is an NMN adenylyltransferase [98], suggesting a pivotal role for this protein and, therefore, for ADP-ribose hydrolases, in NAD^+ metabolism. The existence of a Nudix domain fused to a putative dihydroneopterin aldolase in *Parachlamydia* sp. strengthens the argument that DHNTP is a Nudix substrate *in vivo* [10]. YzdG from *Paenibacillus thiaminolyticus* has a haloacid dehalogenase (HAD) domain with pyridoxal phosphatase activity, and a Nudix domain that hydrolyses NDP-sugars and CDP-alcohols [99]; the connection here is not obvious. The *D. radiodurans* DR0603 protein has cytosine/deoxycytidylate deaminase and SAM-dependent methyltransferase domains on either side of a Nudix domain, while the DR0329 and DR0004 UDP-sugar hydrolases are unique in that each has two fused Nudix domains [16, 17], both of which in DR0004 have the characteristic GE and NGD motifs, but only one of which in DR0329 has these sequences. Other proteins are listed in the FusionDB database (http://igs-server.cnrs-mrs.fr/FusionDB/main.html) in which Nudix domains are fused to predicted phosphopantetheine adenylyltransferase, thiamine monophosphate synthase, glycerol-3-phosphate cytidylyltransferase and S-adenosylhomocysteinyl hydrolase domains. No doubt others exist.

Nudix hydrolases of *S. cerevisiae*

A study of eukaryotes reveals Nudix hydrolases with new specificities, diverse subcellular locations and tissue-specific expression appropriate to their roles in eukaryotic metabolism. *S. cerevisiae* has six Nudix hydrolases, two of which are located in peroxisomes. Npy1p (YGL067w) is an NADH hydrolase with a preference for NAD(P)H over $NAD(P)^+$, similar to *E. coli* YjaD [100, 101]. GFP-fusion and immunofluorescence experiments have located it exclusively in peroxisomes [101]. It has a C-terminal PTS1 tripeptide peroxisomal-targeting signal, deletion of which abolishes specific targeting [S. R. AbdelRaheim and A. G. McLennan, unpublished data]. Pcd1p (YLR151c) is a CoA hydrolase with a UFP0035 motif and hydrolyses CoA, CoA esters and derivatives, with a preference *in vitro* for oxidised CoA disulphide (CoASSCoA) [84]. Its experimentally established peroxisomal location may be due to a putative N-terminal PTS2 signal embedded within a predicted mitochondrial-

targeting sequence, the latter providing a limited degree of mitochondrial localisation. Given the location of Npy1p and Pcd1p in peroxisomes, the sole site of fatty acid β-oxidation in yeast, they may both participate in the maintenance and/or protection of nucleotide cofactor pools required for this process. The peroxisomal membrane is impermeable to NAD(P)(H) and CoA and so the only way to remove these molecules from this organelle, should the need arise, would be to degrade them to smaller, freely permeable products [101, 102]. This might be an aspect of normal cofactor regulation or it could be a means of eliminating non-functional, damaged nucleotides produced in the oxidising environment of the peroxisomal lumen, particularly under conditions of stress, when ROS production may increase. Unfortunately, no altered phenotype for *NPY1* and *PCD1* deletion mutants has yet been found [84, 100, 101]. Somewhat surprisingly, Pcd1p was recently reported also to hydrolyse 8-OH-dGTP and 2-OH-dATP, but not the non-oxidised dNTPs, and to suppress AT:CG transversions in a *mutT*$^-$ strain of *E. coli*. A *PCD1* deletion mutant had a moderate mutator phenotype [103]. It is not clear why such an antimutator activity would be required in peroxisomes, which lack DNA, so this may indicate an affinity for ring-oxidised nucleotides; such derivatives of CoA have not been tested. However, elimination of oxidised dNTPs could be a relevant activity of mitochondrial Pcd1p.

Ddp1p (YOR163w) is a member of the DIPP family with activity towards highly phosphorylated (di)nucleotides such as Ap_6A, Ap_5A, p_5A and p_4A but also towards the DIPs diphosphoinositol pentakisphosphate ($PP\text{-}InsP_5$) and bisdiphosphoinositol tetrakisphosphate ($[PP]_2\text{-}InsP_4$) [7, 104]. The K_m values for $PP\text{-}InsP_5$ and Ap_6A are 0.03 and 56 μM, respectively, suggesting that DIPs may be more relevant substrates *in vivo.* A similar enzyme, Aps1, has been found in *Schizosaccharomyces pombe*, and disruption of the *aps1* gene led to increases in DIPs, but not Ap_5A [105]. The DIPs have been implicated in the regulation of endocytic trafficking [106], telomere length and apoptosis [107, 108], polyphosphate accumulation [109], responses to hyperosmotic and thermal stress [110, 111] and in non-enzymic protein phosphorylation [112]. A direct involvement of Ddp1p in vesicle trafficking is supported by its reported interaction with Yif1p, an integral membrane protein required for the fusion of endoplasmic reticulum (ER)-derived COPII transport vesicles with the Golgi [113], but its overall role in regulating the levels of DIPs and the possible interplay between these and p_nA/Ap_nA have not been adequately studied. p_4A and p_5A accumulate in *S. cerevisiae* during sporulation [114], while DIPs have been shown to accumulate in spores of *D. discoideum* [115], so Ddp1p-type enzymes could have a role in degrading both classes of compound during spore germination.

For a Nudix hydrolase, Dcp2p (Psu1p) is an unusually large (109 kDa) protein. The N-terminal one-third contains a typical Nudix fold flanked by two regions, Box A and Box B, that are conserved in most Dcp2 proteins from other organisms [116]. The large C-terminal region is rich in Q, N, P and S, a feature commonly found in transcriptional regulators, and contains regions that can function as nuclear receptor interaction domains and autonomous transcriptional activation domains. Thus, Dcp2p has been proposed to have a role in ligand-dependent transactivation by nuclear receptors [117]. However, the Nudix domain functions as the catalytic part of the essential Dcp1p/Dcp2p mRNA decapping complex, generating m^7GDP and 5′-phosphorylated RNA [118, 119]. Mn^{2+} and longer mRNA substrates are preferred [119]. Dcp2p interacts with several other proteins involved in mRNA decapping and 5′→3′ degradation [118, 120] and has been localised to cytoplasmic P bodies, a proposed site for mRNA decapping and decay [121]. The C-terminal region of *S. cerevisiae* Dcp2p is not required for decapping and is poorly conserved, even in other yeasts; for example, the N-terminal region is 84% identical to the corresponding sequence of *Candida glabrata* Dcp2p but their C-terminal portions share only 22% identity. Therefore, the C-terminal region may simply be a large interaction scaffold with, at least in *S. cerevisiae*, the ability to recruit transcription factors in addition to mRNA turnover factors.

Of the remaining two yeast Nudix hydrolases, Ysa1p (YBR111c) is an NDP-sugar hydrolase with similarities to *E. coli* NudF. Cytotoxic free ADP-ribose has been proposed to be an important substrate [48] and null mutants of *YSA1* exhibit a reduced growth rate [122], but its function has not been firmly established. Finally, the uncharacterised YJR142w, with a Lys replacing the usual Arg in the core of the Nudix motif, is similar to the N-terminal half of the *S. pombe* TNR3 thiamine pyrophosphokinase (TPK). The C-terminal half of this enzyme is similar to the *S. cerevisiae* THI80 TPK [123]. The implication of the *S. pombe* Rosetta protein TNR3 is that the substrate of YJR142w (and the N-terminal half of TNR3) is likely to be TPP or possibly thiamine triphosphate [124], but this remains to be proved. *A. thaliana* has two similar genes but a likely orthologue appears to be absent from mammals. Conversely, mammals have a specific, unrelated thiamine triphosphatase that is absent from other organisms [124].

Human and mouse Nudix hydrolases

The human genome has 24 Nudix hydrolase genes and at least 5 pseudogenes (table 2). Most of the human *NUDT* gene products or their mouse Nudt orthologues have been characterised to some extent. NUDT1 (MTH1) is func-

Table 2. Summary of the human Nudix genes and hydrolases.

Name	Synonyms	Chromosomal location	Subcellular location[1]	Known substrates[2]
NUDT1	MTH1	7p22	c, n, m	8-OH-(d)GTP, 8-OH-(d)ATP, 2-OH-(d)ATP
NUDT2	APAH1	9p13	n[3], c	Np_nN ($n \geq 4$) e.g. Ap_4A, Gp_4G, Ap_5A, Ap_6A
NUDT3	DIPP1	6p21.2	c	PP-$InsP_5$, $[PP]_2$-$InsP_4$, Ap_6A, Ap_5A
NUDT4	DIPP2α and DIPP2β	12q21	c	PP-$InsP_5$, $[PP]_2$-$InsP_4$, Ap_6A, Ap_5A
NUDT4P1		1q12-q21	pseudogene	–
NUDT4P2		1p12-p13	pseudogene	–
NUDT5	hYSA1, ADPRibase-II?	10p13-p14	c	ADP-sugars, 8-OH-(d)GDP
NUDT6	GFG, FGF-AS	4q26	n, m[4]	(ADP-ribose)[4]
NUDT7		16q23.1	p[3]	CoA, CoA esters, CoASSCoA, 3′-dephospho-CoA[3]
NUDT8		11q13.2	m[4]	(CoA and derivatives)[4]
NUDT9	ADPRibase-I and ADPRibase-m	4q22.1	m, c	ADP-ribose, IDP-ribose
NUDT9L1	TRPM2, LTRPC2, TRPC7	21q22.3	pm	ADP-ribose
NUDT9P1	C10orf98	10q23.33	pseudogene	–
NUDT10	DIPP3α, hAps2	Xp11.1–11.22	c	PP-$InsP_5$, $[PP]_2$-$InsP_4$, Ap_6A, Ap_5A
NUDT11	DIPP3β, hAps1	Xp11.1–11.22	c	PP-$InsP_5$, $[PP]_2$-$InsP_4$, Ap_6A, Ap_5A
NUDT12		5q21.2	p, other	NAD(P)H, $NAD(P)^+$, Ap_2A, FAD, ADP-ribose, Ap_3A
NUDT13		10q22.3	m[3]	NAD(P)H, Ap_2A[3]
NUDT14	UGPP	14q32.33	c[3]	UDP-glucose, ADP-ribose, ADP-glucose, GDP-glucose[3]
NUDT15	MTH2	13q14.12	?	8-OH-dGTP, dGTP, (DHNTP)[4]
NUDT15P1		8q12.1	pseudogene	–
NUDT15P2		17q11.2	pseudogene	–
NUDT16	hX29	3q21.3	n	capped snoRNAs[3]
NUDT16L1	syndesmos	16p13.3	c	?
NUDT17		1q21.1	?	?
NUDT18		8p21.3	?	?
NUDT19	hRP2, hD7RP2e	19q13.11	p[4], m[4]	(CoA and derivatives)[4]
NUDT20	hDcp2	5q22.2	c (P bodies)	capped mRNAs
NUDT21	CF I_m25, CPSF5	16q12.2	n	?
NUDT22		11q13.1	c[4]	?

[1] c, cytoplasm; n, nucleus; m, mitochondria; p, peroxisomes; pm, plasma membrane.
[2] Details of substrate preferences are given in the text.
[3] Data for animal orthologue.
[4] Predicted.

tionally similar to *E. coli* MutT but with a broader substrate range: it can hydrolyse 8-OH-(d)GTP, 8-OH-(d)ATP and 2-OH-(d)ATP [125–129]. Substrate selectivity has been examined by site-directed mutagenesis, and mutants constructed that selectively lack either 8-OH-dGTPase or 2-OH-dATPase activity [130]. Multiple transcriptional and translational initiation sites are present in both the human and mouse genes, and four isoforms of 26, 22, 21 and 18 kDa have been detected in human cells [131–133]. Both p26 and p18 have potential mitochondrial-targeting signals and the leader sequence of p26 targets GFP to mitochondria [133]. p26 is only formed in individuals with the GC allele of a GT/GC polymorphism that is part of an initiation codon at the start of exon 2c [132]. Although most MTH1 activity is nucleo-cytoplasmic, mitochondrial MTH1 has been detected [134] and its level appears to be increased in the substantia nigra neurones of Parkinson's disease patients, something that may be related to the role of mitochondrial respiratory failure and oxidative stress in the aetiology of this disease [135]. *Mth1* knockout mice show an increased rate of spontaneous tumorigenesis and fibroblasts from these mice have a markedly increased sensitivity to H_2O_2, with treated cells showing pyknotic nuclei, degenerate mitochondria and an increase in 8-OH-dG in the DNA of both compartments [136, 137]. These changes are prevented by expression of recombinant wild-type human MTH1 in the null cells but are only partially suppressed using mutant MTH1 forms that are defective in either the 8-OH-dGTPase or 2-OH-dATPase activities, which would suggest that oxidised dG and dA lesions arising from OH-dNTPs are both important contributors to cell dysfunction [137]. This is confirmed by the finding that overexpression of MTH1 in embryonic fibroblasts derived from mismatch-repair-defective *msh2*$^{-/-}$ mice restores their high spontaneous mutation rate to normal [138]. An increase in MTH1 expression has been observed in renal [139], brain [140], lung [141] and several other tumours and this is consistent with the concept that cancer cells suffer from persistent oxidative stress. Interestingly, a polymorphic variant of MTH1 (V83M) has been associated with an increased frequency of stomach cancer and correlates with p53 mutation [142]. The M83 allele is closely linked to the exon 2c GC allele and the M83 form is more heat labile than V83 [132]. Hence, polymorphic variation in MTH1 may contribute to cancer predisposition, at least in the stomach.

A second 8-OH-dGTPase, NUDT15 (MTH2), has been cloned from mouse and human cells [143, 144]. It is less selective for oxidised nucleotides than MTH1, with mouse Mth2 having K_m values of 32 and 75 μM for 8-OH-dGTP and dGTP, respectively. However, it did significantly reduce the spontaneous mutation frequency when expressed in *mutT*$^-$ *E. coli* [143]. Its presence may explain the limited (two-fold) increase in spontaneous mutation frequency observed in *mth1*$^{-/-}$ cells derived from *mth1* null mice [136]. A complicating issue is that AtNUDT1 from *A. thaliana*, which is 40% identical to NUDT15, hydrolyses the structurally related DHNTP in addition to dGTP and 8-OH-dGTP and so has been proposed to be involved in tetrahydrobiopterin (BH_4) and folate synthesis [10]. Thus, NUDT15 may also be required for the conversion of DHNTP to BH_4 in mammals. Unlike humans, the mouse appears to have three apparently functional Nudt15 genes (a, b and c) on different chromosomes, although most known expressed sequence tags (ESTs) arise from the Chr14 gene. Whether substrate utilisation in the mouse has been subfunctionalised among these species is not known. Another antimutator candidate is NUDT5. This protein was originally characterised as an ADP-sugar hydrolase similar to yeast YSA1 with activity towards a number of ADP-sugars including ADP-ribose [145, 146], and probably corresponds to the high-K_m ADPRibase-II isolated from human and rat tissues [147–149]. However, it also hydrolyses 8-OH-dGDP to 8-OH-dGMP and Pi with a K_m of 0.77 μM and completely suppresses the increased mutation rate when expressed in an *E. coli mutT*$^-$ strain [150]. Since dNDPs are potent inhibitors of MTH1 [126, 151], NUDT5 could act in concert with MTH1 (and possibly NUDT15) in antimutagenesis. MTH1 and NUDT5 (but not NUDT15) may also prevent transcriptional errors and mistranslation by eliminating 8-OH-GTP and 8-OH-GDP [144]. Whether there is any meaningful physiological interaction between the hydrolysis of oxidised (d)NDPs and NDP-sugars remains to be established. NUDT5 interacts with the testis-specific small heat shock protein HSPB9 [152], and its transcription is regulated during neuronal differentiation [153, 154] and by HNF4α in hepatocytes and pancreatic islets [155]. It can also be down-regulated by tumour necrosis factor-α (TNF-α) [156] and is a candidate gene for the thymus hypoplasia/aplasia and heart defects associated with partial monosomy 10p [157]. Further study is needed to assess the significance of these observations in terms of the substrate specificity of this hydrolase.

The classical, eukaryotic asymmetrically cleaving Ap_4A hydrolase, examples of which have been isolated from many animals and plants [39, 40], is encoded by the human *NUDT2* (*APAH1*) gene. It hydrolyses Ap_nA ($n \geq 4$), always producing ATP as a product [158, 159]. Because of its greater abundance, the principal substrate of NUDT2 *in vivo* is assumed to be Ap_4A [47], although the enzyme from the brine shrimp *Artemia* – possibly the first Nudix hydrolase ever to be isolated – is believed to degrade the large Gp_4G purine store that is virtually unique to this organism [160, 161]. In eukaryotes, Ap_4A has been implicated in the control of DNA replication and repair [reviewed in ref. 47], regulation of ATP-sensitive K^+ channels [162, 163], initiation of apoptosis [164], modulation of Fhit tumour suppressor protein activity in

conjunction with Ap_3A [165], and activation of gene expression [166]. In some cells (e.g. platelets, chromaffin cells, myocytes), it is also present with other Np_nN in specialised granules from which it is exocytosed to act as an extracellular messenger [167, 168]. As in prokaryotes, the level of nucleo-cytoplasmic Ap_4A is increased by various forms of cellular stress and, since high levels may be toxic, this needs to be controlled. A major fraction of the Nudix Ap_4A hydrolase in tomato [169] and *Drosophila* cells [L. Winward, A. G. McLennan and S. T. Safrany, unpublished data] is located in the nucleus, suggesting that nuclear functions are of major importance. However, like *E. coli* YgdP, the *C. elegans* NUDT2 orthologue, Ndx-4, also appears to be ribosome associated [170]. The human *NUDT2* promoter binds the transcription factors HNF4α and HNF6 in pancreatic islets and HNF4α in hepatocytes [155] and may also bind one of the FOXO family of insulin-regulated transcription factors [171]. HNF4α and HNF6 are master regulators of hepatocyte and islet transcription; specific regulation of *NUDT2* in the pancreas may be relevant to the fine control of Ap_4A levels required in response to glucose in this tissue [172]. *NUDT2* is down-regulated in serum-deprived and contact-inhibited T98G glioblastoma cells by transcription factor E2F4 in combination with the retinoblastoma family protein p130 [173]. Ap_4A is generally found at much higher levels in proliferating compared to resting cells, so down-regulation of its hydrolase during quiescence may appear counter-intuitive. However, if resting cells are not generating Ap_4A, then reducing hydrolase activity may ensure the preservation of a required minimum level of Ap_4A. *NUDT2* is up-regulated by interferon in liver [174] and by hypoxia in cardiomyocytes [175]. The latter may explain the reduction observed in intracellular Ap_4A to 2% of the aerobic level when Ehrlich ascites cells were subjected to 2 h of anaerobiosis [176].

The *NUDT3*, *4*, *10* and *11* genes all encode members of the DIPP family and hydrolyse both DIPs and Ap_nA. Mutagenesis of active-site residues in NUDT3 (DIPP1) has shown that the same site is responsible for hydrolysis of both classes of substrate [177]. The *NUDT4* gene encodes two variants, DIPP2α and DIPP2β, that differ by only a single amino acid. DIPP2β has an additional glutamine (Q86) that arises through intron boundary skidding and which reduces the k_{cat} with PP-$InsP_5$ five-fold [178, 179]. NUDT10 (DIPP3α, hAps2) and NUDT11 (DIPP3β, hAps1) also differ by only a single amino acid (P89R) but are encoded by separate, adjacent genes on the human X chromosome. Unlike the other DIPPs, which are widely though not uniformly expressed, human *NUDT10* and *NUDT11* were reported to be expressed preferentially in testis, and to a lesser extent in brain [180]. Another study showed strong expression of *NUDT10* in brain, liver and testis and kidney, and *NUDT11* in brain, pancreas and testis [181]. Mouse Nudt10 and 11 are also encoded by adjacent genes, indicating that the causative gene duplication took place prior to the primate-rodent split. Remarkably, they are identical to each other, so no sequence divergence between the two enzymes has occurred since then [182]. Mouse Nudt10 is expressed in testis, liver and kidney while Nudt11 is mainly restricted to brain, and this differential expression may explain why both have been retained [182]. In general, the DIPPs have substantially lower K_m values for DIPs (0.004–0.088 μM) than for Ap_nAs (5.9–43 μM), giving the DIPs a perceived favoured substrate status. However, possible physiological switches in substrate preference and utilisation dependent upon divalent ion (e.g. Mg^{2+} or Mn^{2+}) or redox conditions (e.g. presence or absence of DTT *in vitro*) cannot be excluded [181, 182]. The existence of multiple, differentially expressed DIPP isoforms in mammals compared to yeast, *C. elegans* and *D. melanogaster* (each of which has one) has been proposed to provide tight regulation of the response times of the molecular-switching activities in which the DIPs are involved [179] but, as in the case of yeast, whether and how the alternative diadenosine substrates impact on these activities remain to be determined. A more direct role for Nudt3 in signalling has also been discovered. Overexpression of mouse Nudt3 negatively regulates signalling through the ERK1/2 MAP kinase pathway even if an active-site mutant is used, implying that substrate-bound Nudt3 blocks the ERK1/2 pathway by interacting with one of the components [183]. Hydrolysis of the substrate would then relieve the inhibition. This is reminiscent of the activation of Ras by GTP binding [184] and of the Fhit tumour suppressor protein by Ap_3A binding [185]. Since heat shock and osmotic stress increase $[PP]_2$-$InsP_4$ by up to 25-fold in animal cells via ERK1/2 and p38α/β pathways [111, 186], substrate-bound Nudt3 may be part of a feedback loop that controls DIP accumulation. Finally, all of the DIPPs as well as NUDT2 and other enzymes capable of hydrolysing Ap_nAs are able to convert PRPP to the glycolytic activator ribose1,5-bisphosphate and Pi *in vitro* [8] but the physiological significance of this is unknown.

NUDT6 (GFG) is encoded by an mRNA derived from antisense transcription of the 3′-untranslated region of the basic fibroblast growth factor (FGF-2) gene [187]. The full-length 35-kDa protein predominates, with low levels of 28- and 17-kDa isoforms [188], the last one appearing in a glutathionylated form in oxidatively stressed lymphocytes [189]. It has a putative mitochondrial-targeting signal and has also been reported in the nucleus but has not been fully characterised. *A. thaliana* encodes 6 similar proteins, and several of these have been cloned and shown to have activity with ADP-ribose and NADH [20], so mammalian NUDT6 may have similar abilities. Both the sense and antisense FGF-2 RNAs are co-ordinately expressed and translated and so, like FGF-2, NUDT6 may have a role in modulating proliferation in response

to growth signals through sense-antisense RNA interactions and, independently of FGF, via the protein itself [188, 190, 191]. Like *NUDT2*, the *NUDT6* gene is down-regulated by E2F4 and p130 in growth-inhibited cells [173].

Mouse Nudt7 is a peroxisomal CoA pyrophosphohydrolase with a UFP0035 motif like yeast Pcd1p but with a C-terminal PTS1. Nudt7 and the corresponding *C. elegans* Ndx-8 hydrolyse CoA and CoA esters but, unlike Pcd1p, do not have a preference for oxidised CoA [85, 86]. Expression of *Nudt7* was highest in liver, intermediate in lung and kidney, and lowest in brain and heart, and expression of human *NUDT7* showed a similar pattern [85]. Mouse liver *Nudt7* is strongly up-regulated by thyroid hormone via a TRβ receptor [192] and down-regulated in response to starvation, a condition where most enzymes involved in fatty acid β-oxidation are up-regulated [193]. This may prevent depletion of the peroxisomal pool of CoA under conditions of increased fat catabolism. It is also down-regulated in the liver of senescence-accelerated mice [194] and in mice overexpressing a mutated human keratin 18 [195], both conditions associated with increased oxidative stress and liver damage. The 23-kDa putative mouse Nudt8 is 44% identical to Nudt7 over the N-terminal 140 amino acids and also has the UPF0035 motif, suggesting activity with CoA. Human NUDT8 is very similar to mouse Nudt8 but the sequence reported in GenBank (NM_181843) lacks the C-terminal 70 amino acids due, apparently, to loss of exon 4. However, there are numerous EST clones with exon 4 present, so alternative splice forms may exist. Both mouse and human NUDT8 have potential mitochondrial- and membrane-targeting signals and the related *Drosophila* CG11095 protein has been experimentally verified as mitochondrial but with some evidence of additional nuclear localisation [S. AbdelRaheim and A. G. McLennan, unpublished data]. A mitochondrial location for a CoA hydrolase would not be surprising but both the activity and location of mammalian NUDT8 need to be confirmed.

NUDT19 (RP2) is yet another potential, but uncharacterised, CoA hydrolase in which the beginning of the Nudix motif just downstream of the UPF0035 motif is interrupted by a 45-amino-acid sequence that itself has some similarities to the UPF0035 sequence. It has a conserved C-terminal PTS1 as well as a strongly predicted mitochondrial-targeting sequence and is coregulated with known mitochondrial genes in mice [196], so it may have a dual location. RP2 was originally reported as a major testosterone-inducible protein in kidney and liver of *Mus musculus*. RP2 mRNAs were also detected but uninducible in several other tissues [197, 198]. Since androgen inducibility was absent in two other mouse species [199, 200], it is not clear how widespread this is and, therefore, whether human NUDT19 is androgen-regulated.

In eukaryotes, major sources of ADP-ribose are (i) poly(ADP-ribose) degradation by nuclear and mitochondrial glycohydrolases (though not in yeast) [201, 202], (ii) the dephosphorylation of ADP-ribose 1″-phosphate, a product of NAD$^+$-dependent tRNA splicing [203], (iii) cell surface and mitochondrial NAD glycohydrolases (ADP-ribosyl cyclases) [204, 205] and (iv) the deacetylation of O-acetyl-ADP-ribose, a product of the Sir2 family of NAD$^+$-dependent histone deacetylases [206]. Of these, the rapid turnover of nuclear poly(ADP-ribose) synthesised in response to cellular stress could lead to a very large increase in free ADP-ribose, the intracellular concentration of which has been estimated at 44–73 μM in mouse and human T cells [207] but at only 0.5 μM in anucleate human erythrocytes [208]. Higher eukaryotes have a highly specific ADP-ribose hydrolase, NUDT9, that may be responsible for ADP-ribose elimination. The *NUDT9* gene gives rise to two transcripts, *NUDT9*α and *NUDT9*β, the former of which encodes a 39-kDa monomeric, mitochondrial ADP-ribose hydrolase with a marked specificity for ADP-ribose and IDP-ribose [196, 209, 210]. Even O-acetyl-ADP-ribose is a poor substrate [206]. This enzyme corresponds to the low-K_m ADPRibase-m from rat liver [147]. The specific, low-K_m, cytoplasmic ADPRibase-I [147, 149] may be the product of the minor NUDT9β transcript, which lacks the mitochondrial signal, or it may arise from processing of NUDT9α [210]. Although the K_m of NUDT9 for ADP-ribose has been reported as 100 μM [210, 211], 180 μM [209] and 33 μM [206], we have re-examined this under conditions identical to those used to determine the kinetic parameters for ADPRibase-I and ADPRibase-m and now find a value of 0.3-0.5 μM, identical to that of human placental ADP-ribase I [A. Carloto, M. J. Costas, J. C. Cameselle, A. G. McLennan and J. M. Ribeiro, unpublished data]. The reason for the discrepancy is not clear but it does confirm the identity between NUDT9 and the low-K_m ADPRibases. NUDT9 has been shown by yeast two-hybrid analysis and pull-down assays to interact with C17orf25, a predicted mitochondrial protein with two glyoxalase domains [212]. This interaction may involve the N-terminal domain of NUDT9 that is not required for enzyme activity [210]. Glyoxalases catalyse the detoxification of methylglyoxal, a toxic 2-oxoaldehyde derived mainly from glycolytic triosephosphates that glycates proteins, nucleic acids and nucleotides and causes oxidative stress and apoptosis [213]. Depletion of NAD$^+$ by stress-activated poly(ADP-ribose) polymerase (PARP) should lead to the accumulation of triosephosphates and, hence, methylglyoxal, concomitant with the increase in free ADP-ribose. Therefore, NUDT9 and C17orf215 may form a complex designed to prevent the formation of advanced glycation end products in mitochondria following cellular stress.

Recently, an important signalling function for intracellular ADP-ribose has been observed. The TRPM2 (LTRPC2,

NUDT9L1) cation channel found in monocytes, brain and other tissues responds to oxidative and nitrosative stress with a sustained rise in intracellular Ca^{2+} leading to cell death. It has an intracellular C-terminal domain (NUDT9H) related to NUDT9 but with an altered Nudix core (RILRQE) that substantially reduces its hydrolytic activity. This channel is gated specifically by ADP-ribose [211, 214–216]. Hydrolysis of ADP-ribose is not required for gating [217] and mutating the Nudix motif of NUDT9H to make it more similar to NUDT9 abolishes gating, indicating that prolonged binding is required [218]. Slow hydrolysis then closes the channel. Activation of TRPM2 may involve stress-induced production and release of ADP-ribose from mitochondria, as overexpression of mitochondrial NUDT9 suppresses H_2O_2- and MNNG-induced TRPM2 gating [217]. Experiments with PARP inhibitors suggest that PARP-related NAD glycohydrolase activities could give rise to this ADP-ribose [217, 219, 220]. ADP-ribose also inhibits ATP-sensitive K^+ channels in myocytes [221] and may have further regulatory roles through its interaction with macro domains, structural modules found in a wide variety of proteins that bind ADP-ribose and its derivatives with high affinity [203, 222, 223].

The 52-kDa human NUDT12 protein is 59% identical to *E. coli* YjaD over a 60-amino-acid region encompassing the Nudix motif and, like Yjad, hydrolyses NADH and NADPH with 20-fold greater efficiency than NAD^+ [224]. Like yeast Npy1, it has a C-terminal PTS1, and GFP-NUDT12 fusions were found to locate to peroxisomes and larger, unidentified structures. Deletion of the PTS1 specifically abolished the peroxisomal localisation [224]. The Nudix motif is close to the C terminus and the reason for and function of the large N-terminal domain that contains a single ankyrin repeat sequence is unknown. The related Nudt13 protein (mouse) is a mitochondrial enzyme that also preferentially hydrolyses NAD(P)H of the substrates tested, although unusual divalent ion conditions were required to show this (2 mM Mn^{2+} or 50 mM Mg^{2+}) suggesting that other substrates may exist [S. AbdelRaheim and A. G. McLennan, unpublished data]. In Nudt13, the PROSITE PS01295 motif is located upstream of the Nudix motif. This motif is found in 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (e.g. *E. coli* IspD), a key enzyme of the non-mevalonate isoprenoid pathway, and is involved in substrate binding [225]. Structurally, the product of this enzyme, CDP-methylerythritol, is a typical Nudix substrate. Although this pathway is not found in mammalian cells, the IspD motif is found in other mammalian proteins and could be involved in binding the substrate(s) of Nudt13. *NUDT13* transcripts lacking the exon encoding the Nudix and IspD motifs exist in several animal species, suggesting that the N-terminal domain of this protein may have a function on its own. As with yeast Pcd1p and Npy1p, the roles of NUDT7, 8, 12, 13 and 19 may be the regulation of natural and/or the elimination of oxidized forms of NAD(P)H and CoA cofactors from peroxisomes, mitochondria and, possibly, the cytoplasm as well.

NUDT14 is an NDP-sugar hydrolase with a preference *in vitro* for UDP-glucose and ADP-ribose. A Lys replaces the usual Arg in the core of the Nudix motif. Given that the K_m for UDP-glucose of 0.6 mM is within the physiological range for this metabolite, while the K_m for ADP-ribose (1.7 mM) is far higher than the usual micromolar concentrations of this compound, NUDT14 has been proposed to be linked to the control of glycogen metabolism, like NudF in *E. coli*, and may also regulate the supply of UDP-glucose for glycoprotein and glycolipid synthesis [89]. It has been reported to interact with the N-terminal domains of the A-Raf and C-Raf protein kinases [226], suggesting that it may modulate Raf/Ras-mediated responses to extracellular signals in a nucleotide-dependent manner. The *C. elegans* 125-kDa Mdf-1/Mad-1 protein has a central region similar to NUDT14 but with the Arg in REx_2EE replaced by another Glu, a change that is likely to reduce the catalytic power of the motif. Mdf-1 is an essential component of the mitotic spindle checkpoint [227] but the Nudix domain is not found in Mdf-1 homologues, so its role in *C. elegans* is unclear. Curiously, the *D. melanogaster* CG31063 protein has a duplicated Nudix domain similar to that of *C. elegans* Mdf-1 but has no microtubule-binding HOOK domain. The NUDT14 family is clearly one that requires further investigation.

hDcp2 (NUDT20) and NUDT16 are two RNA-decapping enzymes. The 48-kDa hDcp2 is similar to yeast Dcp2p but with a much shorter C-terminal domain that lacks the sequences associated with transcriptional activation in Dcp2p. Like Dcp2p, it prefers Mn^{2+} and can only decap intact mRNA; free cap structures such as m^7Gp_3G are not substrates and methylated substrates are also preferred [116, 228, 229]. Since uncapped RNA can competitively inhibit decapping of capped mRNA, RNA binding seems to be an essential prerequisite for cap recognition. Experiments with truncated forms of hDcp2 suggest that the C-terminal end of the Nudix fold plus the adjacent Box B motif are necessary for RNA binding [229]. The interaction and colocalisation of hDcp2 with proteins involved in 5′→3′ mRNA decay and nonsense-mediated decay (NMD) and the inhibition of NMD by siRNA-mediated down-regulation of hDcp2 show the involvement of this Nudix hydrolase in both these processes [230, 231]. NUDT16 is the human orthologue of the *Xenopus laevis* nucleolar X29 protein that binds and decaps the U8 snoRNA, releasing m^7GDP. The hypermethylated $m^{227}G$ cap present on U8 *in vivo* is also removed and other small RNAs may also be substrates [232]. Thus, NUDT16 may be involved in regulating ribosome biogenesis by altering the stability of U8 and other guide RNAs. It has also been shown to interact with a nuclear protein phosphatase,

possibly in a complex with snRNP components [233]. NUDT16 is 58% identical to syndesmos (NUDT16L1), a myristylated protein that binds specifically to the cytoplasmic domain of the focal adhesion receptor syndecan-4 to promote actin stress fibre formation and cell spreading [234, 235]. Syndesmos lacks all three glutamates in the core of the Nudix motif and so may have very limited, if any, hydrolytic activity; however, the RNA- or cap-binding properties of NUDT16 may be preserved in syndesmos. Recently, RNA and RNA-binding proteins have been shown to be involved in the early stages of cell spreading and to colocalize with known focal adhesion markers [236] including paxillin, which interacts with syndesmos [235], and so the ability to bind capped RNA may be important for the function of syndesmos. Mouse has an additional gene, *Nudt16L2*, which is adjacent to *Nudt16* on Chr9 and closer in sequence to *Nudt16* than to *Nudt16L1*. Its expression is confined largely to testis.

Poxviruses encode two related Nudix hydrolases in their genomes with similarities to Dcp2 proteins, while several other double-stranded DNA animal viruses have one. The Vaccinia D10 Nudix gene was shown to inhibit cap-dependent (but not cap-independent) translation when overexpressed in mammalian cells, suggesting a role in cap metabolism [237]. When the African Swine Fever Virus g5R protein, which has strong sequence similarity to *S. pombe* Dcp2p in the region of the Nudix motif, was assayed *in vitro*, little activity with methylated cap analogues was found. Some activity with non-methylated cap analogues such as Gp_4G as well as (d)GTP, p_4A and p_5A was found but the best substrate was PP-$InsP_5$ which, although hydrolysed slowly, had a K_m of 1.2 μM [238]. PP-$InsP_5$ was also shown to be depleted in cells during virus infection, and so a role for the ER-localised g5R protein in membrane trafficking during virus assembly was proposed. However, given that Nudix decapping enzymes require an intact RNA, a role for g5R and other viral Nudix hydrolases in mRNA cap inactivation cannot yet be discounted. The possibility that DIPs could be involved in modulating decapping would be intriguing.

A further Nudix protein involved in RNA metabolism is NUDT21 (CPSF5), the 25-kDa subunit of cleavage factor (CF) I_m. CF I_m is a nuclear heterotrimer that cleaves pre-mRNAs at the 3′ end prior to polyadenylation. The 25-kDa subunit binds to RNA and interacts with poly(A) polymerase and nuclear poly(A)-binding protein [239]. As in *E. coli* NudD and human NUDT9H, the core Nudix motif sequence in CF I_m25 (RLMTEI) lacks two of the three usual Glu residues but the protein could still bind and hydrolyse a substrate. What this substrate and its role might be remain to be determined. Finally, nothing is yet known about the three remaining human Nudix hydrolases, NUDT17 (NP_001012776), NUDT18 (NP_079091) and NUDT22 (NP_115720), although the *Xenopus laevis* NUDT22 orthologue, P17F11, is believed to play an important role in specification of the head organiser and neural induction during development [240].

Conclusions

The above survey of Nudix hydrolases confirms that their functions include the originally proposed housecleaning roles of eliminating toxic metabolites and controlling the availability of pathway intermediates [1]. In particular, combating the consequences of oxidative stress appears to be a recurring theme in the activity and regulation of several hydrolases. However, activities such as RNA processing, Ca^{2+} channel gating, activation of alcohol dehydrogenase and regulation of ERK signalling show that the Nudix fold and motif have been adapted for the binding and hydrolysis of a wide range of nucleoside and other pyrophosphates for a much greater diversity of purposes and so an overall role for the Nudix family cannot easily be defined. Certainly, housecleaning is not exclusive to the Nudix family: ITPases of the Maf/HAM1 family can hydrolyse potentially mutagenic dITP [241], cap-scavenging enzymes of the HIT family remove residual cap structures [242], homotrimeric dUTPases prevent incorporation of dUMP into DNA and consequent glycosylase-mediated strand breaks [243], while another superfamily of all-α NTP pyrophosphohydrolases contains dimeric dUTPases and MazG proteins, the latter possibly including 2-OH-(d)ATP among their substrates [244]. Thus, the Nudix hydrolases are just one of many families that have evolved a chemistry suited to the hydrolysis of phosphate esters, with an architecture that accepts primarily, though not exclusively, sugar pyrophosphates, and with roles that are many and varied.

Nevertheless, if a core function can be defined, it is pertinent to ask what activities are expressed by organisms that possess only a single Nudix gene, as this may indicate the least dispensable and perhaps most primordial role. The only experimentally verified solitary bacterial Nudix hydrolase is the Ap_nA hydrolase from *R. prowazekii* [52]. Related sequences are found in the endosymbionts *Wolbachia pipientis*, *Candidatus blochmannia floridanus* and *Chlamydia* spp., suggesting that intracellular survival may depend on such an activity. However, the single hydrolase in *Buchnera aphidicola* is much more similar to *E. coli* MutT, while *Wigglesworthia glossinidia* has none and the insect-borne phytopathogen *Phytoplasma asteris* (Onion yellows strain) encodes an enzyme with the GE and NGD motifs characteristic of a UDP-sugar hydrolase [19]. Thus, while endocellular life may benefit from the fine control of Ap_nA, the Nudix complement of a small genome is clearly tailored to the specific needs of the organism. The free-living marine bacterium *Prochlorococcus marinus* ssp. *pastoris* has a probable ADP-ribose hydrolase and such an activity predominates among archaea with single

Nudix genes. The thermophilic *M. jannaschii* has a verified, highly specific ADP-ribose hydrolase [73], and related sequences are present in the hyperthermophiles *Methanopyrus kandleri*, *Archaeoglobus fulgidus* and *Pyrococcus* sp. and also the mesophilic *Methanococcus maripaludis*. It is tempting to speculate that this specific need has arisen because, like most eukaryotes, archaea have a PARP and, therefore, the potential to generate significant free ADP-ribose [245].

The promiscuous behaviour of many Nudix hydrolases with respect to substrate specificity has made it very difficult to determine the true functions of individual family members. Much has been inferred from *in vitro* assays that are by their very nature limited by the availability of substrates to test. The existence of hydrolases that appear to be active towards oxidised (d)NTPs in addition to other, unrelated substrates and which can complement *mutT*⁻ strains (e.g. NUDT5, Pcd1p) is especially curious. Even NUDT6 and NUDT3, which has no 8-oxo-dGTPase activity, can partially complement MutT deficiency [183, 187]. However, the suppression of mutation in *mutT*⁻ *E. coli* by overexpression of other hydrolases needs to be viewed with caution as in some cases this might simply enhance the removal of competitor nucleotides that inhibit the activity of other, endogenous antimutator hydrolases. Nevertheless, hydrolysis of oxidised (d)NTPs might represent a residual ability that has been retained by chance or design after duplication and subfunctionalisation of ancestral, low-specificity hydrolases. Where subcellular compartmentalisation exists, the relevant substrates and roles could even differ depending on the location, e.g. *S. cerevisiae* Pcd1p. In future, the determination of the relevant substrates and true functions of individual Nudix hydrolases will depend largely on genetic studies combined with metabolome analysis, particularly when the discrimination and sensitivity of the latter methodology is improved. In addition, structural determination combined with the modelling of compounds into binding sites should help to predict potential substrates that may not be easily available to test.

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