Is glyceraldehyde-3-phosphate dehydrogenase a central redox mediator?

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ABSTRACT

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an immensely important enzyme carrying out a vital step in glycolysis and is found in all living organisms. Although there are several isoforms identified in many species, it is now recognized that cytosolic GAPDH has numerous moonlighting roles and is found in a variety of intracellular locations, but also is associated with external membranes and the extracellular environment. The switch of GAPDH function, from what would be considered as its main metabolic role, to its alternate activities, is often under the influence of redox active compounds. Reactive oxygen species (ROS), such as hydrogen peroxide, along with reactive nitrogen species (RNS), such as nitric oxide, are produced by a variety of mechanisms in cells, including from metabolic processes, with their accumulation in cells being dramatically increased under stress conditions. Overall, such reactive compounds contribute to the redox signaling of the cell. Commonly redox signaling leads to post-translational modification of proteins, often on the thiol groups of cysteine residues. In GAPDH the active site cysteine can be modified in a variety of ways, but of pertinence, can be altered by both ROS and

RNS, as well as hydrogen sulfide and glutathione. Other redox modifications are also possible, while some redox-driven GAPDH post-translational modifications (PTMs) may occur through indirect mechanisms. With the abundance of redox mediators targeting GAPDH, and the range of moonlighting activities that might ensue, this enzyme can be considered as being central to ensuring correct redox signaling in cells in a wide range of species.

KEYWORDS | GAPDH; hydrogen peroxide; hydrogen sulfide; moonlighting; nitric oxide; post-translational modifications; reactive oxygen species; redox signaling.

ABBREVIATIONS |

APE-1; apurinic/apyrimidinic acid endonuclease-1; ARE, antioxidant response element; Arg, arginine; ATP, adenosine triphosphate;1,3 BPG,1,3 bisphosphoglycerate; CAT, catalase; cGMP, Cyclic guanosine monophosphate; Cys, cysteine; Cys-Gly, cyteinyl-glycine; γ-Glu-Cys, γ-glutamyl-cysteine; DNA-PK, DNA-activated protein kinase; E_{hc}, half-cell reduction potential; ER, endoplasmic reticulum; ETC, electron transport chain; GAP glyceraldehyde-3-phosphate; GAPDH, D-Glyceraldehyde-3-phosphate dehydrogenase; GSNO, *S*-nitrosoglutathione; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; H_2S, hydrogen sulfide; HDAC2, histone deacetylase-2; LMW, low-molecular weight; LUCA, last universal common ancestor; Lys, lysine; Met, methionine; NADH, reduced form of nicotinamide adenine dinucleotide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; Nrf2, nuclear erythroid factor-2; NSL, nuclear localization sequence; OGlcNAc, O-linked N-acetylglucosaminylation; $ONOO^-$, peroxinitrite; PTM, post-translational modifications;

RNS, reactive nitrogen species; ROS, reactive oxygen species; Ser, Serine; SIAH1, E3 ubiquitin ligase; SOD, superoxide dismutase; SSH, sulfidation; T2DM, type-2 diabetes mellitus; TCA, citric acid cycle; Thr, Threonine; Tyr, tyrosine.

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1. INTRODUCTION

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a 37kDa, 335 amino acid (in humans) enzymatic protein ubiquitous throughout nature. The processes GAPDH catalyze have been noted in all living cells, in all domains of life - Euarchaea, Eubacteria and Eukarya. As such, GAPDH is postulated to have pre-dated the last universal common ancestor (LUCA) of free-living cells [1]. The primary function of this enzymatic protein is to catalyze the sixth step in the glycolytic pathway where glyceraldehyde-3-phosphate (GAP) becomes phosphorylated, forming 1,3 bisphosphoglycerate (1,3 BPG). This is sequentially converted to pyruvate and enters the citric acid cycle (TCA), and the Calvin-Benson cycle in plants [2]. Such processes ultimately produce adenosine triphosphate (ATP), a high-energy acyl-phosphate bonded molecule used intracellularly for providing the activation energy for many endogenic cellular reactions. Therefore, without such metabolic processes, including the action of GAPDH, the energy required for basic cellular processes would be diminished and therefore would be highly unfavorable to the sustention of life.

Each GAPDH polypeptide contains a nicotinamide adenine dinucleotide (NAD^+) binding domain, rich in lysine residues, and a GAP catalytic domain with a predominant reactive cysteine residue centrally located within the peptide chain (Table 1; Supplementary material). In its role as a glycolytic enzyme, GAPDH converts GAP to 1,3 BPG, in a two-step process. In the first reaction an aldehyde moiety is oxidized to carboxylic acid in the proximal binding domain and a molecule of NAD^+ is reduced to NADH. The reducing power of NADH may subsequently be used in many metabolic processes, for example it may be oxidized by the action the mitochondrial electron transport chain (ETC), with protons being utilized as a

protonmotive force during ATP synthesis [3]. The second vacillating reaction takes place in the distal catalytic domain and involves the transference of an inorganic phosphate to the intermediary GAP, thus creating 1,3 BPG. Dorothy Needham's work originally defined this aspect of GAPDH functionality in the late 1930's [4] when it was suggested there was a coupled mechanism where phosphorylation events are accompanied by oxido-reductive reactions. The enzyme itself, however, was not characterized until two years later when Warburg and Cristian crystallized the protein using yeast as their model organism. This process allowed for formal identification of GAPDH, originally named *das oxydierende Gärungsferment* [5]. Since then GAPDH has been well established as a metabolic cytoplasmic enzyme, although subsequent possible roles in mediating the redox environment, as well as numerous other activities, are seemingly counterintuitive.

Within nature, GAPDH most commonly forms quaternary structures with four 37 kDa (human) subunits amalgamating to form a singular tetrameric protein (148 kDa). These tetramers are largely represented as either as a homotetramer formation (A_4) with each unit being identical, or as two heterodimer subunits, A_2/B_2; the latter being evident in the cytosol of both *Arabidopsis thaliana* [6] and *Anabaena* cyanobacteria [7]. Interestingly, the latter has a preference for reducing $NADP^+$ as opposed to NAD^+.

The preference of GAPDH for its nicotinamide co-factor may shed light on the endosymbiotic theory of evolution, wherein plant chloroplasts are proposed to have derived from an endocytosed cyanobacterium, forming a symbiotic relationship with the host cell. Similarly, mitochondria are postulated to have originated from a purple bacterium symbiont [1]. Fortifying this hypothesis, archaeal GAPDH amino acid sequences are neither similar to those of eukaryotic or eubacterial GAPDH ancestry

(10-15% homologues), the latter being more closely related to each other and thus suggesting eukaryotic GAPDH genes may have originated from eubacterial genetic material, most likely through horizontal gene transfer [8]. Interestingly, the variation in genetic content between the domains also suggests GAPDH pre-dates the bacterial/archaeal split. To date the structures of numerous GAPDH molecules have been resolved [9], with over fifteen hundred listed representing all domains of life, available at the Protein Data Bank [10].

A single gene encoding for Human GAPDH (*GAPD*) can be found on chromosome 12(p)13.31, with a separate isoform present in male haploid cells (*GAPDS*) located on chromosome 19(q) 13.12. *GAPDS* displays a unique N-terminal proline-rich extension of 72 amino acids in human isoforms (Figure 1). This extension allows for attachment of GAPDH to the fibrous sheath of the spermatid flagellum, highlighting the importance of this enzyme to spermatid motility. Male mice (*Mus musculus*) lacking this enzyme produce less ATP and have been noted as being infertile [11]. This is a pertinent concept as GAPDH is shown to be oxidized on reactive thiols by ROS resulting in decreased sperm mobility [12]. Contradicting evidence, however, has shown *GAPDS* expression to be unaltered between hypokinetic and normokinetic human spermatids [13]. This is intriguing as should *GAPDS* affect the motility, and therefore viability, of sperm, this enzyme could provide a focused target for male contraception. Welch *et al*. [14] have identified GAPDS as being 68% identical with somatic GAPDH (also see Figure 1) and that 11 exon intron junctions are matched between isoforms. The conserved homology of GAPDS between species demonstrates that each isoform has a similar functional role [14]. This is echoed with the alignment between active residues such as cysteine, methionine and tyrosine detailed in Figure 1. In addition, human *GAPDS* is

shown to possess two additional cysteine residues, in the C-terminal extension, increasing the possible modification targets for redox signaling.

Although in human cells two GAPDH encoding genes are observed, other eukaryotic species including wheat and yeasts (*Triticum aestivum* and *Saccharomyces cerevisiae* accordingly) have multiple isomers of this enzyme [15, 16]. In the higher plant kingdom, there are several isoforms including: cytoplasmic A_4 and A_2/B_2 (*GAPC1* and *GAPC2* {A/B respectively}); chloroplastic *GAPA1*, *GAPA2* and *GAPB*; and plastid borne *GAPCP1* and *GAPCP2*. Details of multiple organisms' genetic isoforms of GAPDH are listed in Table 1 (Supplementary material).

Although GAPDH was first identified for its rate-limiting step in glycolysis, this enzyme has also been noted to have a multitude of roles in both intracellular and extracellular compartments. GAPDH complexes have been demonstrated to undergo post-translational modifications (PTM), allowing the molecule to perform a variety of tasks [17]. Possibly the most notable of these is the frequency of cellular signaling events involving gaseous substances and intracellular redox peptides such as glutathione (GSSG/GSH) [18]. For example, GAPDH is susceptible to oxidation processes initiated by reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the superoxide radical ($O_2^{\cdot-}$), all of which have been shown to interact with catalytic cysteine residues creating sulfenic, sulfinic and sulfonic acids (SOH, SO_2H, SO_3H, respectively) [19]. The same residues are also prone to *S*-nitrosylation by reactive nitric oxide species (RNS) such as nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$); glutathionylation by glutathione peptides, and sulfidation (SSH) by non-radical compounds such as hydrogen sulfide (H_2S). All of these small adjustments to the structure of GAPDH typically inhibit glycolysis and permit other cellular functions to take place [2]. In addition, GAPDH has also been

demonstrated to accept modifications such as acetylation, O-linked N-acetylglucosaminylation (OGlcNAc) and phosphorylation on other amino acid residues including lysine, threonine and tyrosine [20]. As an example, the modifications recorded at the NBCI entry for human GAPDH (Accession number: NM_002046) are listed in Table 2 (Supplementary material).

The myriad of PTMs applicable to GAPDH support the array of functions attributed to the protein which include, but are not limited to: receptor mediated signaling (e.g. NO), protein-protein binding (e.g. plasminogen, SIAH-1), maintenance of DNA integrity (e.g. APE-1) transcriptional and post transcriptional genetic regulation (e.g.HDAC-2), response to oxidative stress (e.g. ROS/RNS), membrane trafficking, and the initiation of apoptosis via protein aggregation or nuclear translocation [21]. Each individual PTM stimulates GAPDH to perform specific functions related to cellular resistance including; drought and dehydration, disease, oxidative and nitrosative stress, malignancy, infection and ageing [22, 23]. These modifications may occur as a single addition of a given molecule, as with sulfenic acid oxidation; or in a stepwise manner with one adaptation increasing the likelihood of another modification. This is often the case with both oxidation processes and *S*-nitrosylation, commonly leading to *S*-glutathionylation or the formation of disulfide bridges for example. As the conformation of GAPDH is readily altered by numerous biological molecules this review will focus upon the PTM's associated with GAPDH translocation events and with particular attention being paid to ROS/RNS interactions upon the active cysteine thiols. Furthermore, as GAPDH has been best categorized in both plant and animal cells, observations will favor the functionality within these tissues as opposed to prokaryotic lifeforms.

2. MOONLIGHTING AND GAPDH AS A MOONLIGHTING PROTEIN

GAPDH is often considered to be one of the first enzymes discovered to have multiple functions. Moonlighting functions have been observed throughout the evolutionary tree and such pleiotropic proteins are again observed in all domains of life, suggesting an ancient origin to these adaptations [24]. The concept of protein moonlighting was first proposed in 1989 by Wistow and Piatigorsky who determined ancillary functions of water soluble crystallin proteins (e.g. lactate dehydrogenase, GAPDH) as structural compounds in the lens of the eye [25]. It was not until a decade later, however, that Constance Jeffery defined the term ‘moonlighting protein’ when studying the multifactorial nature of several enzymes (e.g. phosphoglucose isomerase, GAPDH and PutA *(Escherichia coli*)). These studies incorporated a discussion on the translocation and diverse activities of these enzymes; a notion which later abolished the assumption that one gene encodes for a protein with a single function [26]. Despite its multiple roles, however, GAPDH is consistently expressed and distributed throughout the cell in all types of organisms and is frequently referred to as a ‘housekeeping gene’ due to its necessity and abundance within cellular systems, leading to GAPDH frequently being used as a control element in transcriptomic and proteomic studies [27]. However, the levels of GAPDH expression are not constant, as might be expected for a protein that changes function, and its role as a housekeeping gene has been questioned [28].

Many proteins, however, have now been demonstrated as having the capacity for accessory functions, with many acting as structural, transcriptional or cell-surface binding proteins. Although the primary evolutionary role of GAPDH is still considered to be the essential and reversible step of GAP metabolism, contemporary studies are beginning to reveal complex interactions between this enzyme and a variety of

cellular components, such as the cytoskeleton, the Golgi apparatus and ER [24, 29], and even the extracellular environment [30]. Studies using pathogenic Streptococci have also revealed expression of GAPDH on the cell surface where it acts as a receptor for transferrin and the cellular uptake of iron [31].

Arguably, one of the most influential additional, or moonlighting, functions of GAPDH is seen within the nucleus where it has been demonstrated to affect positive transcription of genes associated with the stress response [32]. In contrast, increased oxidation of GAPDH instigates the binding to E3 ubiquitin ligase (SIAH-1), nuclear translocation and the induction of pro-apoptotic gene transcription. Additionally, aggregates of GAPDH localize to the mitochondria, disrupting the ETC, thus promoting the release of cytochrome *c* to the cytosol, and so initiating the apoptotic cascade [33] (Figure 2D).

Moonlighting proteins such as GAPDH are of particular interest when it comes to the study of pathological conditions. They have frequently been observed as having a critical role in a multitude of diseases involving the respiratory, cardiovascular, immune and neurological systems, whilst also occupying a central role in the development and proliferation of many neoplastic events [34, 35]. For instance, many studies have documented the presence of GAPDH in prokaryote species including *Streptococcus pneumoniae* and *Staphylococcus aureus*, where GAPDH provides a binding mechanism for components such as fibrinogen, plasminogen and collagen, essential elements for the adhesion, virulence and colonization, during infection [24, 36].

Many attributes of GAPDH (Table1: Supplementary material) are not a result of genetic mutations or RNA splicing or fusion events [29] but instead many of these additional behaviors have been attributed to redox modifications involving ROS, RNS

and other redox signaling molecules (Table 2: Supplementary material). Although the primary target site of ROS/RNS is often the active cysteine [37], further investigations have also identified structural amino acids such as lysine which can be modified. Such amino acids may not have a direct role in the catalytic aspects of enzyme behavior but are also widely responsible for protein-protein binding through acetylation, succinylation and OGlcNAc modification [38]. Such adaptations also have a pivotal role in the regulation of glycolytic/gluconeogenic flux [39].

3. REACTIVE OXYGEN SPECIES AND THEIR INFLUENCE ON GAPDH

Reactive oxygen species have been defined as reduced, partially reduced, or activated complexes containing oxygen (O). Although this could include RNS, all of which include an O molecule in their configuration, in scientific literature they are generally regarded separately. ROS can be further categorized into either radical (e.g. $O_2^{\cdot -}$, OH) or non-radical (e.g. H_2O_2, O_3) molecules usually having redox properties [40]. Often ROS are generated as unavoidable byproducts of metabolic processes, such as respiration [41] or photosynthesis [42]. However, they can also be produced by dedicated enzymes such as the NADPH oxidase complex [43] or xanthine oxidoreductase [44]. Production may be increased as part of a pathological response or to unfavorable conditions which create biotic stress during adverse conditions such as fluctuating temperatures, salinity, dehydration or heavy metal exposure, for example [45]. There are numerous endogenous sources of ROS, however they can also arise from exogenous origins [46]. Exogenous sources of ROS include air and water pollution caused by chemical fumes and deposits, drugs and medications (e.g. acetaminophen), pesticides, and ionizing radiation.

In mammalian systems, sources of the physiological production of ROS include phagocytic leukocytes, neutrophils and macrophages, known for the generation of an intense respiratory burst containing ROS (H_2O_2, $O_2^{\cdot-}$, $^{\cdot}OH$) during their response to infection and inflammatory processes. This particular response has been evidenced to further stimulate the release of cytokines and proinflammatory markers (e.g. IF-γ, TNFα) [47]. In plants, ROS are well known for their signaling roles when the cells are exposed to biotic or indeed, abiotic stressors [48]. For example, relatively recent studies by Noctor *et al*. [49] and Mittler [50] have demonstrated ROS regulation of seed germination, root and shoot development and stomatal closure. Therefore, ROS can be generated at several sources in and around cells, and their influence will be perceived on redox chemistry, such as Cys-based PTM of proteins, a pertinent factor to GAPDH functionality. Such modifications of proteins have been reported in multiple subcellular locations, including the cytoplasm, cellular membrane, nucleus and mitochondria. Therefore, GAPDH will be exposed to a possible varying redox status in numerous cellular locations.

An imbalance of ROS is known to generate oxidative stress within the cell, occurring due to either an overproduction of ROS in the locale of a cell, or particular cellular compartment; or alternatively, as a deficiency of antioxidant enzymes and mechanisms. Here, of particular interest are superoxide dismutase (SOD), catalase (CAT) and reduced glutathione GSH [51]. This can lead to the endogenous cellular redox status to become more oxidizing [18, 52], influencing the ability for further redox reactions and PTM of proteins, which is of relevance when considering multiple disease states. The importance of ROS are often noted in many pathological conditions including type-2 diabetes mellitus (T2DM), neurodegenerative diseases (e.g. Alzheimer’s and Parkinson’s diseases), cardiovascular disease (e.g.

298 atherosclerosis and hypertension) and also in many cancers (e.g. colorectal;
299 prostate) [53, 54].
300 Mitochondria are a major site for ROS production, involving both Complex 1
301 (NADH:ubiquinone oxidoreductase) and Complex 3 (ubiquinol:cytochrome *c*
302 oxidoreductase), where H^+ are actively transported across the inner mitochondrial
303 membrane [55]. In plants, the electron chain of the chloroplast membranes has a
304 similar influence. The superoxide anion ($O_2^{\cdot-}$), predominately generated by
305 mitochondrial complexes and NAPDH oxidase, has been demonstrated to
306 accumulate in µM concentrations (perhaps much higher if compartmentalized)
307 physiologically and readily reacts with the proximal constituents of the mitochondrion
308 and chloroplast damaging localized DNA, ETC complexes and membrane
309 phospholipids. As well as dismuting to H_2O_2 (influenced by pH and the presence of
310 SOD polypeptides), an excess of $O_2^{\cdot-}$, a relatively short-lived but comparably reactive
311 molecule, has been shown to release ferrous iron (Fe^{2+}) from ferritin, thereby
312 contributing to substrates available for the conversion of H_2O_2 into the extremely
313 damaging $^{\cdot}OH$ radical, utilizing the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + {}^{-}OH$
314 [19]. In addition, $O_2^{\cdot-}$ may also undergo the Haber-Weiss reaction in the presence of
315 H_2O_2 forming hydroxyl radicals: $O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^-$. These reactions
316 notwithstanding, the oxidative potential of $O_2^{\cdot-}$ itself is typically contained within the
317 organelle due to the intervention of the antioxidants including ascorbic acid, vitamin
318 A and mitochondrial-specific superoxide dismutase (Mn-SOD) [56]. Unsurprisingly
319 perhaps, as cytosolic GAPDH is a major contributor to the glycolytic process,
320 between 5-10% has been observed as accumulating close to mitochondria during
321 normal cellular function, at physiological pH, and is therefore in close proximity to the

major production site of the $O_2^{\cdot-}$ radical, and therefore H_2O_2 via dismutation, making GAPDH highly susceptible to oxidative modification [57].

As mentioned above, $O_2^{\cdot-}$ is known to undergo a dismutation reaction - $O_2^{\cdot-} + O_2^{\cdot-} \rightarrow H_2O_2 + O_2$ - that results in the formation of H_2O_2, although the presence of CAT further reduces the ROS to water and molecular oxygen: $2H_2O_2 \rightarrow 2H_2O + O_2$. Despite H_2O_2 not being a radical, due to the stability of its paired electron configuration, it is however a highly destructive compound even in low concentrations once the homeostatic, or tolerance, threshold has been overwhelmed. Unlike $O_2^{\cdot-}$, H_2O_2 is readily diffusible through phospholipid membranes and is known as having a proportionately long half-life, spanning minutes at 37^{o}C [58]. Reactions with cytosolic GAPDH are commonplace, a major factor contributing to H_2O_2 toxicity of GAPDH and causing extensive oxidation of sensitive thiolate anions, resulting in successive oxidative modifications and the promotion of internal disulfide bridging. These numerous adaptations effectively result in the alteration of protein configuration, inhibiting glycoltic activity and promoting the moonlighting functions of GAPDH [59].

The successive progression of oxidation states caused by increasing H_2O_2 accumulation has led to H_2O_2 being regarded as having a `Jekyll and Hyde' position in the cell. Low levels if H_2O_2 are responsible for cellular signaling events and mediation of downstream kinase activity [60]. By contrast, increasing concentrations are associated with cellular instability and disruption to membrane integrity via lipid peroxidation, inactivation of proteins (e.g. GAPDH), and equally, direct DNA and RNA nucleotide assault [61]. In addition, H_2O_2 also undergoes transformation via the aforementioned Fenton reaction into the extremely detrimental $^{\cdot}OH$ radical, having

profound influences on many bio-molecules due to its ability to undergo hydrogen abstraction [62].

The hydroxyl radical ($^{\cdot}OH$) is the most reactive of the ROS species and evidence shows frequent interactions with carbohydrates, lipids, proteins, nucleotides, and inorganic compounds including $Fe^{2/3+}$ and copper (Cu^{2+}) [63]. To illustrate, $^{\cdot}OH$ is known to modify the thiol residue of the catalytic cysteine of GAPDH establishing covalent attachment of oxygen, thus producing a sulfenic acid modification. Although highly reactive, $^{\cdot}OH$ is relatively short-lived with the half-life being determined to be as little as 1 nanosecond (~10^{-9} s) in biological systems [64] and therefore is generally not considered to be an oxidant to many proteins. However, the close proximity of cytosolic GAPDH to the mitochondria voltage-dependent anion channel (VDAC) channel allows covalent attachment to the susceptible cysteine reside [65]. Interestingly, $^{\cdot}OH$ has also been shown to induce formation of the second messenger cyclic guanosine monophosphate (cGMP) through interactions with the enzyme guanylyl cyclase [66]. Therefore it also has an essential role in many cell signaling processes with downstream effects being noted in such pathways as PKG/MAPK (responsible for smooth muscle contraction), CREB/c-src/Bad (involved in proliferation and cell survival), and cell adhesion and migration circuits under the influence of vasodilator-stimulated phosphoprotein (VASP) proteins [67].

Therefore, it can be seen that ROS will be produced in cells in multiple places and under a variety of conditions and hence can lead to the oxidation of GAPDH and the initiation of its moonlighting activities. With the oxidation of cysteine residues in GAPDH known [68], this makes it well placed to mediate redox signaling.

4. REACTIVE NITROGEN SPECIES AND GAPDH

Nitric oxide (NO), the predominant RNS involved in redox maintenance, is an uncharged radical containing an unpaired electron in its π^2 orbital. However, it can readily gain or lose an electron, fluctuating between the $NO^{\cdot}$ radical, cation nitrosonium (NO^+), and the nitroxyl anion (NO^-). Further reactions with O_2 stimulate the formation of the non-radical species nitrogen dioxide (NO_2): $2NO^{\cdot} + O_2 \rightarrow 2NO_2$ [69]. Together such nitrogen containing species are included under the term RNS, and they are instrumental in signaling, often mediating protein PTMs, as evidenced with GAPDH.

In similarity with H_2O_2, NO has been well documented as having an essential role for cell signaling events. In animals, signal transduction is mediated by the formation cGMP, but such a mechanism has been questioned in plants [70]. Another aspect of increasing intracellular $NO^{\cdot}$ is the reciprocal rise of cytosolic calcium ions (Ca^{2+}) initiated by the release of mitochondrial Ca^{2+} independently from IP_3/cGMP intracellular release mechanisms as demonstrated by Horn *et al*. [71] in human striatal neuronal cells. The effects of RNS are also notable in plant tissues. Studies have established NO involvement in seed germination and seedling development, senescence, protection against pathogenic attack, the expression of defense related genes and apoptosis [72]. Endogenously in animals, NO is synthesized by conversion of L-arginine in the presence of oxygen and NADPH by a variety of nitric oxide synthases (iNOS, eNOS, nNOS {human}) although this is disputed in plants [73]. Rather nitrate reductase is a major source in plants, as well as by a variety of other enzymes or directly by reduction of inorganic nitrate in plant tissues.

Throughout evolutionary cellular development, NO is proposed to have had a crucial function in the defense against the rising levels of oxygen in the environment,

in particular ozone (O_3), essentially neutralizing its oxidative potential and in so giving the cell a developmental advantage during hostile conditions [74]. Subsequent adaptations to the NO pathway may well have been harnessed and utilized by cells before aerobic respiration, allowing for the control of intracellular oxygen species, alongside other gaseous molecules such as H_2S, which have been demonstrated as early redox mediators [75]. It should, however, also be considered that $NO^{\cdot}$ is a toxic compound capable of binding essential metalloproteins and causing disruption to physiological function [76]. An additional negative effect of NO accumulation is that this simple compound is predisposed to reactions with ROS, in particular $O_2^{\cdot -}$ producing further detrimental RNS such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2) which are themselves biologically destructive compounds. $ONOO^-$ is associated with oxidation of all major classes of biomolecules, whilst NO_2 has been shown to favor lipid oxidation [77]. Both, however, are known to typically interact with thiol groups on cysteine residues of proteins such as GAPDH, leading to *S*-nitrosation (specific interactions with NO^+ (as defined by Heinrich [78]), *S*-nitrosylation (the addition of $NO^{\cdot}$ to sulfur); and the further addition of compounds including GSH responsible for the formation of *S*-nitrosglutathione adducts [78, 79].

Additionally, GAPDH has been reported to transnitrosylate such nuclear proteins as histone deacetylase-2 (HDAC2) and DNA-activated protein kinase (DNA-PK), effectively widening the scope of protein-protein interactions, with GAPDH being evidenced as having an essential role in multiple signal transduction pathways [80].

Looking at the NO/GAPDH interaction in more detail, using the human GAPDH as the model for amino acid numbering, NO is known to nitrosate Cys152 within the active site of the enzyme, as well as Cys247. Looking at the human

GAPDS sequence (Figure 1), although the Cys152 is conserved, as would be expected, the [IL]-x-C-x-x-[DE] motif around the Cys247 nitrosation site is not, thus suggesting that *S*-nitrosation here may not be possible in this isoform, limiting its potential interactions with RNS and subsequent signaling cascades.

It can be seen therefore that, as with ROS, RNS can have a significant influence on GAPDH PTMs, affecting its structure, functionality and subcellular location. The presence of potential PTMs within GAPDH by RNS allows GAPDH to be placed downstream of NO in signaling pathways, mediating the effects of RNS accumulation in cells. However, as discussed below, such action is not in isolation from the influence of other mediators of GAPDH PTM and further moonlighting functions.

5. THE INFLUENCE OF CELLULAR GSH/GSSG

Glutathione peroxidases are a group of enzymes that are responsible for catalyzing the degradation of H_2O_2 and organic peroxides into water and alcohol (C-OH) moieties, respectively, using glutathione as a substrate [81]. Glutathione is a tripeptide (glutamyl-cysteinyl-glycine) that possesses an exposed sulfhydryl group that is susceptible to modification by ROS/RNS. Oxidation of GSH forms a coupled disulfide-glutathione (GSSG) in a reversible reaction, readily re-reduced by glutathione reductase with NADPH as the electron donor [82]. Whereas NO reacts forming *S*-nitrosoglutathione adducts (GSNO) [83], a possible sink of NO and a way for it to be transported around organisms [84].

GSH is produced intracellularly in mM concentrations while the ratio of oxidized glutathione (GSSG) and GSH, the reduced form, are indicators of the oxidative state of the cell and thus are widely used when assessing oxidation in

biological systems [85]. The GSH:GSSG ratio has a significant effect on the redox environment of any given cell or organelle [18]. GSH:GSSG forms a biological defense against the detrimental effects of increasing oxidation, whilst GSSG:GSH is an indication of the severity of oxidative stress. Moreover, raised GSH content has been demonstrated to rejuvenate water soluble Vitamin C, and the lipid soluble Vitamin E, both of which display antioxidant qualities [86]. Therefore, it is imperative that investigations into the redox potential of any given cell or organelle discuss the influence of such antioxidant and reducing compounds as glutathione. Such compounds clearly have influential and opposing roles to redox species such as ROS. Alterations to the half-cell reduction potential (E_{hc}) of GSH/GSSG have been evidenced to parallel the same biological redox status of the whole cell observed during events including proliferation, differentiation and apoptotic stages (E_{hc} = -240mV, -200mV, -170mV respectively) [18] which may be a significant factor in processes as ageing, neurodegeneration and tumor development. More recently the notion of a “Goldilocks Zone” has been suggested [87], where the redox status of the cell has to be held within defined limits. Along with other thiol containing compounds, referred to as low molecular weight (LMW) thiols, such as cysteine (Cys), cyteinyl-glycine (Cys-Gly) and γ-glutamyl-cysteine (γ-Glu-Cys) [88], the GSH/GSSH ratios and concentrations are instrumental in making sure the cells’ interiors stay within this zone.

It is clear, therefore, that GAPDH will be forever in the presence of influential redox mediators such GSH. It is also known that glutathione can mediate protein PTM [89]. *In vitro* experiments by Zaffagnini *et al*. [90], on GAPC1 (A_4) GAPDH revealed glutathionylation of cysteine residues occurs due to direct covalent attachment of GSSG, GSNO, or in the presence of GSH and H_2O_2. This effectively

inhibits enzymatic activity by occupying the active site, whilst also reducing the likelihood of further oxidative modifications. Additionally, GSH is known to act as a scavenger of free ROS within the cell, thus reducing the overall oxidative potential of such deleterious compounds as $O_2^{\cdot -}$ and ·OH [91].

6. OTHER REDOX-RELATED SIGNALING MOLECULES – H_2S AND H_2

Hydrogen sulfide (H_2S) was first identified in the early 1700's and has long been known as a toxic substance to humans in minimal doses (>100ppm). H_2S has been demonstrated to inhibit cytochrome oxidase in the ETC, whilst also encouraging accumulation of sulfur-based compounds in the tissues [92]. Paradoxically, as sulfur has been used throughout evolution as an electron acceptor, mitochondria display a high affinity for sulfide which may be beneficial in micromolar doses (<0.0003ppm) [93]. A study supporting this theory was conducted by Budde and Roth [94] who, using H_2S gas in atmospheric chambers on the model organism *Caenorhabditis elegans*, demonstrated that nematode cultures exposed to H_2S are longer-lived, and more resistant to hypoxia than untreated animals. H_2S administration was shown to produce a decrease in metabolic rate, reduced hypertension in subjects via mediation of vascular tone, and a decline in hypoxic conditions due to reperfusion injury [94, 95]. Coinciding studies have also noted H_2S to have influence over cellular signaling mechanisms in both plant and animal tissues [51].

In contrast to many influential gaseous compounds, diatomic hydrogen (H_2) is often described as an inert gas. However studies on both plant and animal species are beginning to reveal that exposure to molecular hydrogen imbues protection against oxidative stress, suggesting H_2 is capable of influencing many cellular mechanisms, including signal modulation, protein phosphorylation and gene expression [96]. To

date, interactions of H_2 with GAPDH have yet to be well characterized and an increased understanding of the downstream effects of H_2 application on plant cells could be used as an alternative to chemical pesticides, or to extend the shelf-life of products derived from the agricultural industry, as suggested for H_2S [97]. Alternatively, further investigations into the effect H_2 has on oxidative stress may also provide a new target for new medical therapeutics with such conditions as neurodegeneration, metabolic diseases, multiple cancers [98] and others, benefiting from detailed observations and additional empirical evidence [99].

7. POST-TRANSLATIONAL MODIFICATIONS AND INLFUENCES ON GAPDH

As discussed above, PTMs are hugely influential on the functioning of proteins; various adaptations to a protein's structure have been shown to alter the molecular charge, conformation and activity of biological molecules [100], and also influence subcellular localization [101]. Furthermore, as can be seen from Table 2 (Supplementary material), a GAPDH polypeptide is able to be modified in a myriad of ways along its length. Several of the PTMs reported are mediated by redox active compounds, such as ROS or RNS, suggesting an important role for GAPDH in mediating redox signaling.

Approximately 5-20% of cytosolic GAPDH is known to pool in the cytoplasm at physiological pH and has been shown to accumulate close to the mitochondrial membrane where the end products of glycolysis are in demand [33]. However, studies have determined that the accumulation of GAPDH aggregates during oxidative/nitrosative stress cause mitochondrial dysfunction, which is likely to be responsible for subsequent autophagy and programmed cell death.

The ongoing expression of GAPDH and therefore the high quantity of available GAPDH monomers allows for identical molecules to be selected for a variety of tasks, dependent upon the PTM each unit receives [102]. For example, modifications involving fatty acids (e.g. palmitate) favor translocation of GAPDH to lipid vesicles or biological membranes, encouraging the uptake and transportation of heme containing compounds such as transferrin and lactoferrin [103]. Conversely, modification by NO has been demonstrated to inhibit heme transduction pathways, effectively regulating cellular heme metabolism [104], and suggesting GAPDH has a central role in communicating, and acting upon, fluctuations of the localized redox environment.

Many PTM's are known to promote translocation of cytosolic GAPDH by: (1) a constitutive mechanism, whereby the acquisition of a new function is preceded by localization of GAPDH; (2) by inducible means, where modification and alternative functionality occurs ahead of translocation; or (3) by semi-constitutive means involving a change of location, a gain of function and a further relocation, an action often seen in the intracellular trafficking roles of GAPDH [105]. As an example of the former constitutive pathway, GAPDH has an influential role in the maintenance of genome integrity, by both the restoration of apurinic/apyrimidinic acid endonuclease-1 (APE-1) catalysis, a critical DNA repair enzyme, and the modulation of telomere integrity, a mechanism that utilizes the NAD^+ binding site [106]. These pathways describe the migration of `free-protein' GAPDH to the nucleus where it is then recruited, via modification sequences, forming complexes such as APE-1/GAPDH which then perform a given task [107] (Figure 2A). However, the physical translocation of GAPDH in this scenario is debatable, as observations show human GAPDH genetic information is not known to possess a nuclear localization sequence

(NSL). Instead GAPDH is unusual in that it possesses a 13 amino acid CRM1 nuclear export signal within the distal catalytic domain [108], and therefore it would be logical to consider that these functions may precede the translocation of GAPDH into the cytosol.

The inducible pathway is essentially the antithesis of the constitutive pathway wherein modification leads to the attainment of alternative function before translocation events are observed. With this sequence of events, adaptations to GAPDH structure, most commonly by ROS/RNS, result in displacement of the protein from the cytoplasm, often into the nucleus, but also to the extracellular matrix and cytoskeletal structures [109] (Figure 2C). This mechanism has been well described when studying the apoptotic cascade, and more specifically, where oxidation of a thiol residue enhances disulfide bridging and protein-protein bonding of GAPDH/SIAH-1 complex (Figure 2A). Observations detail relocation of the complex to the nucleus and post binding of SIAH-1, an action possible via modification of GAPDH via *S*-nitrosylation or through oxidation. Once in the nucleus, the composite protein then initiates the degradation of nucleic proteins by accepting an acetyl moiety (Lys160) from p300/CREB acetyltransferase and activating the catalytic tendency of p300/CBP, responsible for initiation of p53 apoptotic pathway [110].

Structural analysis reveals GAPDH can undergo a whole host of modifications, many of which are mediated by redox active compounds (Figure 3). Many of these modifications are reversible and inhibit the catalytic action of the active cysteine (Cys152) through adaptations to the neutrophilic thiol (-SH) residue. For example, sulfhydration (-SSH) (typically by polysulfide compounds, although H_2S may also have an influence), of multiple cysteine residues (Cys152, Cys156, Cys247

(human)) can ultimately affect the 3-dimensional positioning of catalytic Cys152, increasing its pKa, altering the nucleophilic charge and inactivating enzymatic action [68]. This is in contrast to conflicting evidence suggesting that H_2S signaling increases glycolytic activity [111, 112].

Various interactions with the active cysteine (position variable in polypeptides from different species but within a conserved amino acid sequence) are essential for many non-metabolic processes including, regulation of mRNA stability, intracellular membrane trafficking, heme metabolism, and iron uptake and transportation [113]. The thiolate form of the cysteine residue is a nucleophilic anion attracting ROS/RNS as well as unreactive species (e.g. H_2O_2) that readily bond, occupying the active site of catalysis. Most modifications promote the cessation of glycolytic activity and repeatedly affect further modifications [114]. There is, as always, an exception to the rule, and here the most ubiquitous anomaly is the binding of H_2O_2 which Peralta et *al.* [115] have described as having a specific binding domain between Cys152 and Thr153. Affectingly, upon reaction of these compounds a proton relay is initiated with the outcome of converting $H_2O_2 \rightarrow H_2O$. The successive deprotonation of multiple residues culminates with the deprotonation of active Cys152 and was noted to be a prerequisite for H_2O_2 signaling that curiously preserves glycolytic function [115]. Contrarily, excessive H_2O_2-dependent oxidation has been shown to cause dramatic changes to the architecture of GAPDH by linking Cys152-Cys156 via disulfide bridging [116] and disrupting cytosolic activity. Interestingly, this paradox supports the theory that GAPDH is indeed a mediator and even translator of the oxidation state of the cell. Additionally, reversible oxidation of GAPDH has been shown to stimulate secretion of GAPDH into the extracellular matrix where it effectively increases the immunoreactive response acting as both a receptor for

596 transferrin/lactoferrin, thus sequestering free iron, and as an inhibitor of IL-10
597 chemokine release responsible for the inhibition of phagocytic leukocytes, further
598 widening the scope of GAPDH moonlighting functions [117, 118].
599 It can be seen therefore, that by using a host of sensitive and selective
600 mechanisms GAPDH is able to sense the redox environment and modify its activity
601 in accordance to the specific alteration, or alterations applied. For example, ROS
602 adaptations to GAPDH predominantly involve oxidation of cysteine thiols creating
603 sulfenic, sulfinic or sulfonic moieties (Figure 3). The reversible alterations of sulfenic
604 (SOH) and sulfinic (SO_2H) acids to the protein structure are known to promote
605 disulfide bonding between GAPDH molecules, forming aggregates and functional
606 compounds as GAPDH/SIAH-1 (E3 Ubiquitin ligase {protease}) [119]. Sulfenic and
607 sulfinic oxidation of the same cysteine thiolate anion is also known to enhance the
608 binding capability of nucleic GAPDH to nucleic acids in both DNA and tRNA and may
609 be an important factor in DNA repair and tRNA transportation respectively.
610 Irreversible sulfonic acid oxidation has been demonstrated to promote protein
611 deformation, autophagy and the apoptotic cascade [120]. Furthermore, stepwise
612 oxidation of GAPDH leads to significant inhibition of glycolytic activity and as a result
613 oxidative PTM have been hypothesized as having a key role in the pathology of
614 neurodegenerative disease, including Alzheimer's Disease, where GAPDH has been
615 noted to interact with such plaque-inducing proteins as amyloid β-protein precursor
616 [121].
617 Further PTMs often follow oxidation, not only promoting protein-protein
618 binding but also reversible bonding of GSH. This is seen working in a protective
619 capacity by inhibiting degradation, an action that protects the active cysteine thiol
620 from further, irreversible, oxidation processes. Glutathionylation of the sulfur of

cysteine residues essentially stabilizes oxidized GAPDH by occupying a target site of proteolytic enzymes, or by altering host protein configuration and thus masking the reactive residue [122]. Glutathionylation is observed in both plant and animal tissues, where the cysteine sulfhydryl is converted to sulfenic acid through oxidation; this moiety reacts with the reduced form of glutathione creating a mixed disulfide bond, termed *S*-glutathionylation. Excess accumulation of GSH adducts however have been demonstrated to create further reactions within the GAPDH molecule, resulting in the formation of a disulfide bridge between Cys150 and Cys154 as reported by Barinova and colleagues when investigating the effect of *S*-glutathionylation within rabbit tissues (equivalent to Cys152 & Cys 156 in humans) [123]. Further supporting these findings, in *Arabidopsis thaliana,* Zaffagnini's team report aggregation of cytoplasmic (*GAPC1*) upon increasing *S*-glutathionylation of its catalytic cysteine, Cys149, which further reacts with Cys153 in the formation of an internal disulfide bridge, altering its conformation [124]. Likewise, oxidation of a methionine residue (Met46) has also been established to cause aggregation of GAPDH. In their 2014 study Samson *et al.* [125] proposed oxidation of Met46 as a marker for irreversible aggregation, leading to apoptosis. With this in mind, it would be beneficial to assess the stepwise oxidation processes of GAPDH, possibly by molecular dynamic simulation methods, to determine the primary event of irreversible oxidation.

In a similar fashion to ROS, RNS are also able to directly affect protein configuration by *S*-nitrosylating thiols, forming an -SNO moiety on GAPDH's active cysteine (Cys152). *S*-nitrosylation occurs when a molecule of $NO^{\cdot}$ covalently attaches to the cysteine thiol group. This action is generally short-lived due to the abundance in the cell of reducing GSH, effectively generating GSNO adducts upon the cysteine residue and stabilizing the conformation of the protein [126]. As with

oxidation, *S*-nitrosylation of GAPDH also promotes protein-protein binding, however the addition of NO adducts hosts a myriad of downstream effects including trans-*S*-nitrosylation, a mechanism that allows for a signaling relay analogous to phosphorylation cascades. As an example, NO induced intramolecular disulfide bonding is not only responsible for *S*-glutathionylation; *S*-nitrosylation of Cys152 has also been demonstrated to affect GAPDH aggregation, a process that has been well evidenced in both neurodegenerative and neuropsychiatric disorders [127]. Contrastingly, disulfide bonding (-SS-) as a result of either oxidative or nitrosative stress has been demonstrated to lead to *S*-cysteinylation, converting L-cysteine of GAPDH to *S*-L-cysteine in the presence of another protein cysteine residue, or GSSG. This reversible, modification has been demonstrated to act in a protective capacity in multiple bacterial species [128].

In addition to redox modifications, Cys152 has been described as undergoing arylation by 1,2 naphthoquinone, a functional group derived from aromatic hydrocarbon. This PTM has been demonstrated to decrease catalytic activity which can be restored in the presence of GSH. Interestingly, a study by Toyama *et al.* [129] shows that further arylation of Lys4 to be irreversible, ultimately affecting the catalysis of GAP and the cellular energy process needed to maintain cellular functionality [129].

As previously eluded to, it is not only the active cysteine that is regularly modified by PTM. Arginine, lysine, methionine, serine, threonine and tyrosine residues are also prone to adaptations (Table 2: Supplementary material). Many of these modifications involve non-oxidative compounds, however, whether the redox environment is a significant factor in these adaptations has yet to be elucidated.

Of the basic amino acids mentioned above several (Lys70, Arg78, Lys84) can form stable bonds with phosphatidylserine, an acidic lipid demonstrated to associate with biological membranes [130]. Paradoxically, the increased expression of membrane bound GAPDH during intracellular iron stress has been demonstrated to reduce transferrin receptor (TR1,TR2) expression in the cell surface, instead favoring an isoform that recruits apotransferrin, a protein associated with the efflux of iron, again highlighting the adaptive capability of GAPDH [31]. Alternatively, addition of O-linked β-N-acetylglucosamine (O-GlcNAc) moieties to Lys227 has been evidenced to disrupt tetrameric formation of GAPDH and mediate nuclear translocation of individual subunits [131]. Furthermore, acetylation of Lys160 is known to promote cellular proliferation and differentiation [132]. Therefore, future studies may benefit from identifying the role of not only redox sensitive cysteine residues, but also lysine specific modifications of GAPDH, when investigating tumorigenesis and tumor progression.

Phosphorylation is another mechanism by which GAPDH acts as a signal transductor. For example, addition of inorganic phosphate to Ser122 during glucose starvation has been evidenced as instigating AMPK/Sirt1 activation, a process that leads to nuclear translocation and autophagy [133]. Phosphorylation events have also been identified in processes as RAB2 (a membrane-associated G protein) mediated retrograde trafficking (Tyr41). In addition, Akt-2 induced phosphorylation of Thr237 inhibits the formation of GAPDH/Siah-1 apoptotic ligase, decreasing nuclear translocation and downregulating the expression of GAPDH [132]. Interestingly, phosphorylation of Thr237 has been noted to decrease nucleic aggregation of GAPDH in ovarian cancer cells, therefore negating the instigation of programmed

cell death [134], possibly due to increased surface area or altered protein configuration making it unable to pass through nucleic pores.

GAPDH is not only under the direct influence of redox components for its regulation and functionality. The redox environment of the cell may also to have an indirect impact. To illustrate, a tyrosine residue near the C-terminal end (Y42 in human) may be phosphorylated. Given that H_2O_2 inhibits tyrosine phosphatases, it could be argued that increased ROS will lead to an increase in the Y42 phosphorylation state, and hence influence GAPDH function. This tyrosine is conserved in GAPDS (Figure 1) and in other GAPDH sequences from other species (BLAST of amino acids 30-50 (human) – data not shown).

8. CONCLUSION AND PERSPECTIVES

From an evolutionary perspective, GAPDH is a rate-limiting, and therefore integral, enzyme in the formation and continuation of the energy yielding glycolytic cascade. As glycolysis is a ubiquitous process across all domains of life, it is logical to assume the structural conformation of GAPDH is of great importance in its activity, providing the energy required for cellular processes. It follows that PTMs to this ancient enzyme should therefore also have an essential role in the maintenance of cellular processes with each individual modification likely to provide an alternative function for the protein. It is also worth considering that all accessory functions are dependent on the specific PTM applied and redox state of the cell or organelle [101]. As GAPDH is highly conserved and ubiquitous throughout life, it is likely that any adaptations to this enzyme are of great evolutionary consequence, a factor supported by the sequence homology of the *GAPDH* gene between species. Alterations of GAPDH and their cellular functions are summarized in Figure 3.

In the human GAPDH sequence amino acids which may be modified by ROS or RNS include Cys152, Cys156, Cys247. However, there are three caveats here. Firstly, although Cys152 and its flanking sequences are well conserved this is not true for all cysteine residues, where the [IL]-x-C-x-x-[DE] motif may be variant. Secondly, some GAPDH sequences have more cysteines, which leads to the potential for more redox based signaling. Thirdly, the same thiol groups can be modified in different ways, depending on the interacting compound. For example, Cys152 can become an ADP-ribosylcysteine, a persulfide, or a nitrosated cysteine. Alternatively, Cys152 may be oxidized or glutathionylated, actions that frequently lead to intramolecular or intermolecular disulfide bridging, with each adaptation resulting in a different functional outcome. To exemplify, modification of GAPDH by H_2O_2 often leads to inhibition of glycolytic activity [60, 115], while attack by H_2S may increase the catalysis of glucose [112]. Furthermore, redox active compounds may have a direct effect through modification of amino acids such as Met46, or indirectly through Y42.

Increasingly, protein-protein binding, as a result of oxidative and nitrosative PTM, is demonstrated to affect the translocation of GAPDH to another cellular compartment (e.g. nucleus), where one action leads to another. This favors the theory that GAPDH acts as a mediator of the redox environment [101]. Although increased expression of GAPDH has been noted in cell proliferation and tumorigenesis, oxidative modifications typically lead to a reduction in glycolytic activity, effectively leading to cellular ageing and programmed cell death.

As previously discussed, the accessory functions attributed to GAPDH are mediated through various adaptations, both oxidative and non-oxidative, to multiple residues with each modification inducing a specific protein behavior. These

744 alternative actions have been observed in many cellular compartments including the
745 cytosol, plasma membrane, ER and Golgi apparatus [135] (Figure 2B). Furthermore,
746 observations of GAPDH distribution during cellular stress events may well lead to
747 both evolutionary insights and new health interventions.

748 To gain a full comprehension of how PTM adaptation of GAPDH may be
749 relevant in health and diseased states, future investigations may benefit from
750 detailed multifunctional and structural analysis of GAPDH, assisting in the
751 identification of the moonlighting roles of GAPDH in a variety of downstream
752 pathways. These investigations may help to isolate the roles of GAPDH in numerous
753 conditions, including those associated with oxidative stress related maladies, such
754 as neurodegenerative, metabolic and neoplastic disease. To give a holistic view of
755 this protein, future studies should consider both the causes and the consequences of
756 PTM and the downstream effects these have upon the localized redox environment,
757 glycolytic activity, protein interactions and transcription events, allowing for the
758 research and development of new therapeutics.

759 Although the GAPDH polypeptide can undergo a wide range of PTMs, redox
760 active compounds are involved in many of these leaving little doubt that GAPDH has
761 a central role in mediating the downstream effects of altered accumulation of ROS
762 and RNS, and changes in cellular redox status.

763

764 **COMPETING INTERESTS STATEMENT**

765 The authors confirm that they have no competing interests.

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Figure and legends:

Figure 1: ClustalOmega alignment of human GADPH and GAPDS (testis variant).

Possible redox interacting sites on peptides: Cysteine residues in yellow; Methionine sulfatation site in green; Tyrosine phosphorylation site in blue. Note GAPDS [IL]-x-C-x-x-[DE] motif lacks the [DE] amino acid (purple). Possible phosphorylation sites (Ser/Thr) highlighted in grey.

```
CLUSTAL O(1.2.4) multiple sequence alignment

GAPDH      ------------------------------------------------------------
        0
GAPDS
MSKRDIVLTNVTVVQLLRQPCPVTRAPPPPEPKAEVEPQPQPEPTPVREEIKPPPPPLPP 60


GAPDH      ------------
MGKVKVGVNGFGRIGRLVTRAAFNSGKVDIVAINDPFIDLNYMVYMFQ      48
GAPDS      HPATPPPKMVSVARELTVGINGFGRIGRLVLRACMEKG-
VKVVAVNDPFIDPEYMVYMFK  119
                          ::.**:********** **.::.* *.:**:****** :******:

GAPDH
YDSTHGKFHGTVKAENGKLVINGNPITIFQERDPSKIKWGDAGAEYVVESTGVFTTMEKA 108
GAPDS
YDSTHGRYKGSVEFRNGQLVVDNHEISVYQCKEPKQIPWRAVGSPYVVESTGVYLSIQAA 179
           ******:::*:*: .**:**::.: *:::* ::*.:* *  .*: ********: ::: *

GAPDH      GAHLQGGAKRVIISAPSADAPMFVMGVNHEKYD-
NSLKIISNASCTTNCLAPLAKVIHDN     167
GAPDS
SDHISAGAQRVVISAPSPDAPMFVMGVNENDYNPGSMNIVSNASCTTNCLAPLAKVIHER 239
           . *:..**:**:***** **********.:.*: .*::*:******************:.

GAPDH
FGIVEGLMTTVHAITATQKTVDGPSGKLWRDGRGALQNIIPASTGAAKAVGKVIPELNGK 227
GAPDS
FGIVEGLMTTVHSYTATQKTVDGPSRKAWRDGRGAHQNIIPASTGAAKAVTKVIPELKGK 299
           ************: ********** * ******* ************** ******:**

GAPDH
LTGMAFRVPTANVSVVDLTCRLEKPAKYDDIKKVVKQASEGPLKGILGYTEHQVVSSDFN 287
GAPDS
LTGMAFRVPTPDVSVVDLTCRLAQPAPYSAIKEAVKAAAKGPMAGILAYTEDEVVSTDFL 359
           ********** :********** :** *. **:.** *::**: ***.***.:***:**

GAPDH      SDTHSSTFDAGAGIALNDHFVKLISWYDNEFGYSNRVVDLMAHMASKE- 335
GAPDS      GDTHSSIFDAKAGIALNDNFVKLISWYDNEYGYSHRVVDLLRYMFSRDK 408
           .***** *** *******:***********:***:*****: :* *::
```

Figure 2. Schematic representations of how GAPDH can be involved in moonlighting activities

A: (1) Increased oxidation/nitrosation of GAPDH leads to SIAH1/GAPDH binding, nuclear translocation and activation of proapoptotic gene transcription; (2) Increased oxidation of GAPDH arrests interaction with proteins involved in antioxidant gene transcription (APE-1; uracil-DNA glycosylase (UDG)), cell cycle regulation (SET) and telomere protection (Oct1). **B:** (1) GAPDH activation of IP_3 receptors in ER membranes increases intracellular calcium release; (2) aPK phosphorylation of GAPDH promotes interaction with vesicular tubular clusters (VTC) essential for protein trafficking between the ER and (3) Golgi apparatus; (4) Phosphorylated GAPDH interacts with aPK, Rab2, Src and VTC allowing for (5) cytoskeletal modifications and intracellular transportation. **C:** (1) GAPDH acts as a membrane receptor for plasminogen (plg) promoting cellular adhesion; (2) GAPDH enables endocytosis of iron-containing proteins (lactoferrin, transferrin); (3) Extracellular GAPDH promotes inflammatory cytokine expression (TNFα; IFγ). **D:** (1) Oxidation of GAPDH promotes aggregation and interaction with VDAC channels causing increased permeability and subsequent release of cytochrome *c* and apoptosis inducing factor (AIF) leading to cell death; (2) An increasing oxidative environment causes a decrease in mitochondrial membrane potential (Ψm) effectively increasing GAPDH glycolytic activity and ATP production and promoting autophagy of dysfunctional mitochondria avoiding the more destructive apoptotic pathway. Pictorial graphics courtesy of creative commons licensed under CC BY-SA.

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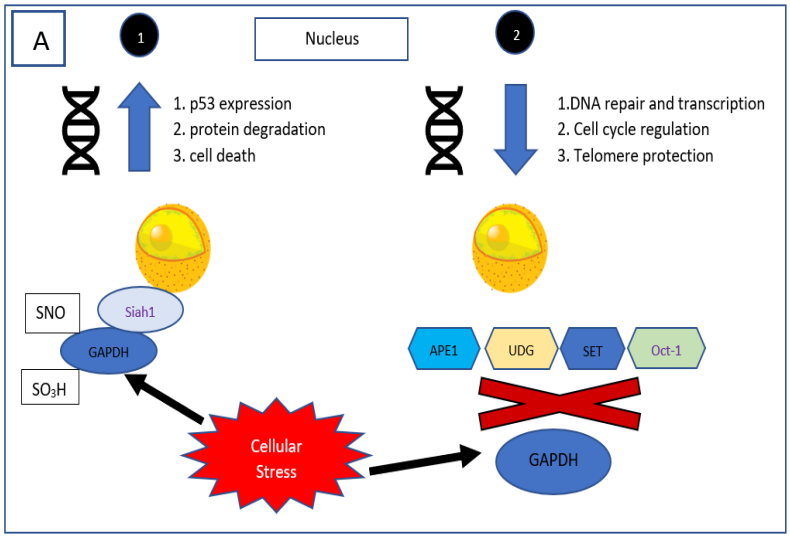
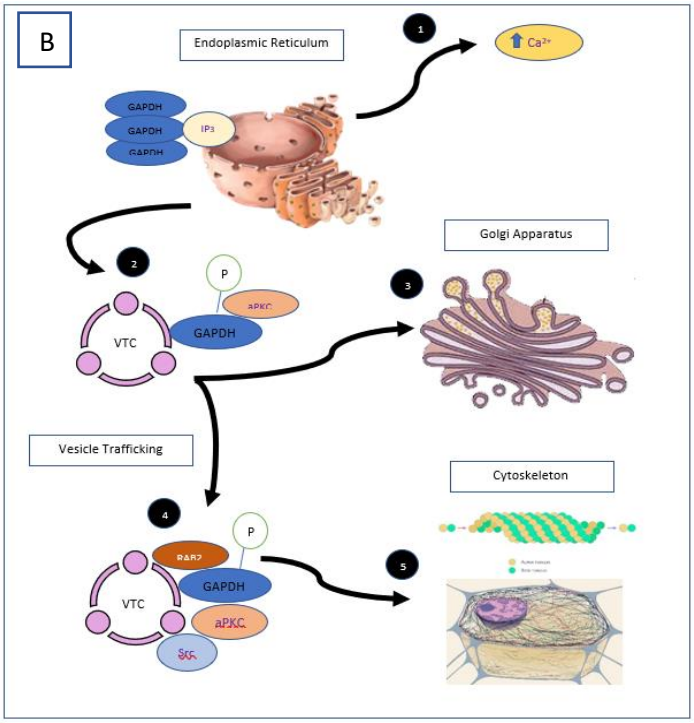
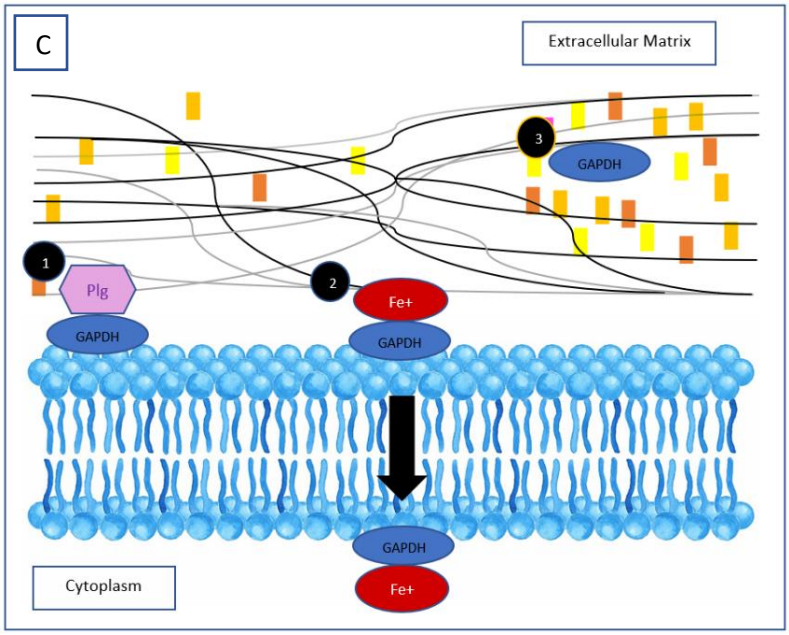
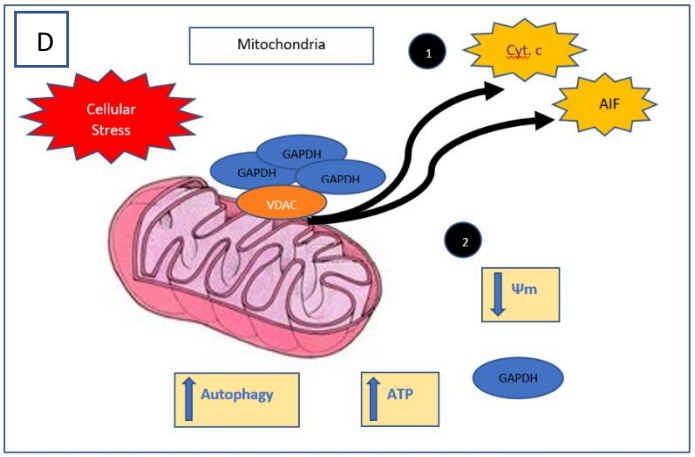

A
1
Nucleus
2
1. p53 expression
2. protein degradation
3. cell death
1.DNA repair and transcription
2. Cell cycle regulation
3. Telomere protection
SNO
Siah1
GAPDH
SO3H
Cellular Stress
APE1
UDG
SET
Oct-1
GAPDH


B
Endoplasmic Reticulum
1
Ca2+
GAPDH
GAPDH
IP3
2
P
aPKC
VTC
GAPDH
Golgi Apparatus
3
Vesicle Trafficking
Cytoskeleton
4
P
PAR2
GAPDH
VTC
aPKC
Src
5

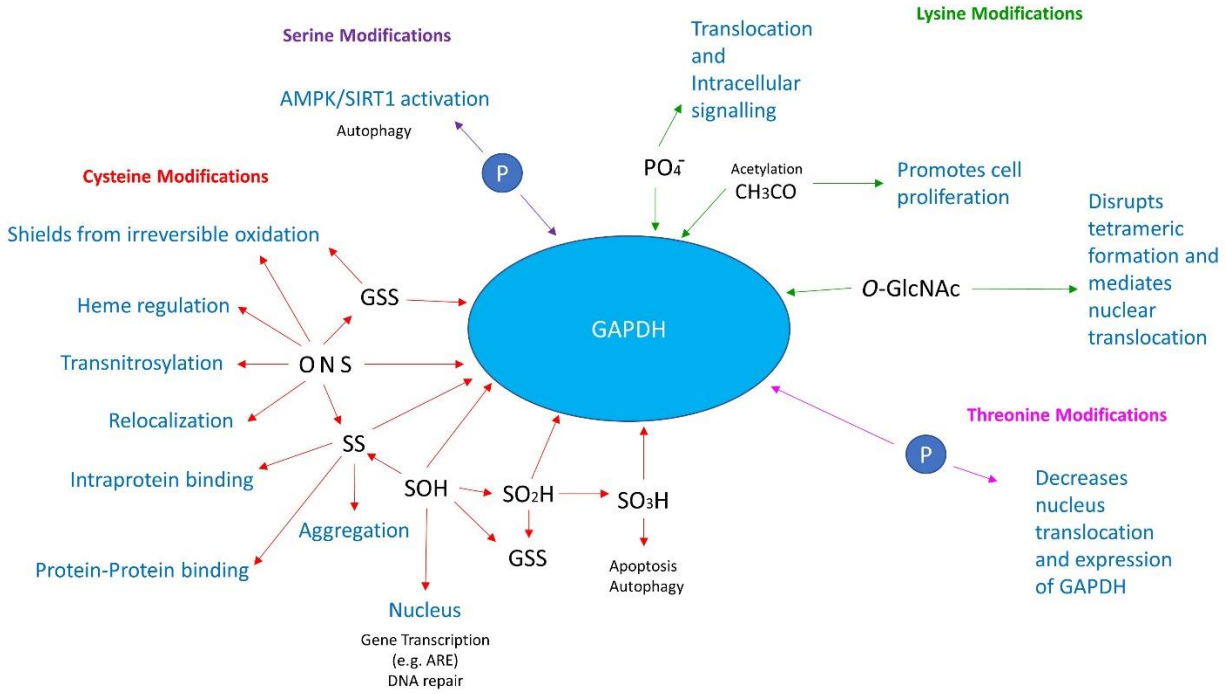

C
Extracellular Matrix
3
GAPDH
1
Plg
GAPDH
2
Fe+
GAPDH
GAPDH
Fe+
Cytoplasm


D
Mitochondria
1
Cyt. c
AIF
Cellular Stress
GAPDH
GAPDH
GAPDH
VDAC
2
Ψm
GAPDH
Autophagy
ATP

410

411

Figure 3. An overview of the post-translational modifications of GAPDH and the possible outcomes. Red: cysteine modification and the multiple pathways associated with redox signaling. Dark green: lysine adaptations. Pale green: oxidation or nitrosation of methionine residue; Purple: phosphorylation of serine; Brown: tyrosine phosphorylation; Pink: threonine phosphorylation; P: inorganic phosphate.



Supplementary material

Tables and legends:

Table 1: **Notation of multiple isoforms of GAPDH and the pleiotropic effects shown across numerous species and subcellular locations.**

Individual GAPDH isoform gene length and position of the active cysteine given for comparison, noting the similarity across domains of life and describing the highly conserved nature of GAPDH. Protein details taken from www.uniprot.org.

	GENE NAME(s)	UNIPROT IDENTIFICATION	ACCESSION NUMBER	AMINO ACID LENGTH	POSITION OF ACTIVE CYSTEINE RESIDUE	PRIMARY and ADDITIONAL FUNCTIONS with SUBCELLULAR LOCATION	REFERENCES
PROKARYOTE							
ARCHAEA							
Haloferax volcanii (Strain ATCC 29605 / DSM 3757 / JCM 8879 / NBRC 14742 / NCIMB 2012 / VKM B-1768 / DS2)	*Type1gap* *gap* *gap Type 2* *gap Type 1*	A0A384LEV9_HALVD L9ULZ2_HALVD D4GS18_HALVD D4GS21_HALVD	WP_144858775.1 AOHU01000000 WP_013035627.1 WP_004044440.1	350 324 355 350	167 137 140	Glycolysis **CYTOSOL** Gluconeogenesis only - **CYTOSOL**	[136]
BACTERIA							
Clostridium perfringens	*gapC*	A0A127EIR5_CLOPF	WP_003460339.1 ,	332	150	Glycolysis **CYTOSOL** Fibronectin binding **CELL SURFACE**	[137]
Cyanobacteria Nostoc spp. (Strain PCC 7120 / SAG 25.82 / UTEX 2576)	*gap1* *gap2* *gap3*	G3P1_NOSS1 G3P2_NOSS1 G3P3_NOSS1	WP_010996722.1 WP_010999188.1 NZ_RSCN01000008.1	343 337 337	155 154 153	Glycolysis **CYTOSOL** Binds CP12 and phosphoribulokinase (PRK) inhibiting the Calvin cycle in response to changes in light **CHLOROPLAST**	[21, 138, 139]
Lactobacillus plantarum	*gapB*	F9UM10_LACPL	WP_003643974.1	340	156	Glycolysis **CYTOSOL** Binds mucin and Caco-2 cells Recognizes A and B antigens on red blood cells **CELL SURFACE**	[140, 141]
Staphylococcus aureus (Strain: MRSA252)	*gapA1*	G3P1_STAAR	WP_100183630.1	336	151	Glycolysis **CYTOSOL** Transferrin-binding protein involved in the acquisition of transferrin-bound iron AND plasminogen binding **CELL SURFACE**	[142]
Streptococcus pneumoniae – (Strain: ATCC BAA-255/R6)	*gapA*	Q8CWN6_STRR6	WP_010976649.1	359	175	Glycolysis **CYTOSOL** Plasminogen binding **CELL SURFACE**	[143]
Streptococcus pyogenes - serotype M18 (Strain: MGAS8232)	*gap*	G3P_STRP8	WP_002986042.1	336	152	Glycolysis **CYTOSOL** Binds uPAR/CD87 receptor on human cells **CELL SURFACE** Fibronectin and plasminogen binding **EXTRACELLULAR MATRIX**	[144, 145]
EUKARYOTE							

ANIMAL							
Homo sapiens	*GAPD* *GAPDS* (Male gametes only)	G3P_HUMAN G3PT_HUMAN	NP_001276674.1 NP_055179.1	335 408	152 224	Glycolysis **CYTOSOL** Microtubule cytoskeleton organization **CYTOSKELETON** Regulation (+/-) of translation **NUCLEUS** Apoptotic process **NUCLEUS & MITOCHONDRIA** Membrane trafficking **GOLGI & ENDOPLASMIC RETICULUM**	[17]
Rabbit *Oryctolagus cuniculus*	*GAPDH* *GAPDHS*	G3P_RABIT G1T091_RABIT	NP_001075722.1 XP_002722284.1	333 426	150 221	Glycolysis **CYTOSOL** Single stranded DNA binding effects on the DNA-polymerase-alpha- primase complex **NUCLEUS**	[146]
Mouse *Mus musculus* *Mus musculus (cont.)*	*gapdh* *gapdhs*	G3P_MOUSE G3PT_MOUSE	NP_032110.1 NP_001277560.1	333 440	150 256	Glycolysis **CYTOSOL** Membrane fusion **MEMBRANES** Microtubule bundling **CYTOSKELETON** Phosphotransferase Activity Binding AU-rich element (ARE) mRNA Regulation of cyclo-oxygenase-2 (COX-2) expression **NUCLEUS**	[147]
Rat *Rattus norvegicus*	*Gapdh* *gapdhs*	G3P_RAT G3PT_RAT	NP_058704.1 NP_076454.1	333 432	150 248	Glycolysis **CYTOSOL** Microtubule binding **CYTOSKELETON** Binds Siah1, involved in apoptosis **NUCLEUS** Protein stabilization **CYTOSOL**	[148, 149]
Nematode:							
Caenorhabditis elegans	*GPD1* *GPD2* *GPD3* *GPD4*	G3P1_CAEEL G3P2_CAEEL G3P3_CAEEL G3P4_CAEEL	NP_496237.1 NP_508535.1 NP_508534.3 NP_496192.1	341	158 158 158 158	Glycolysis **CYTOSOL** Regulation of phosphoinositide signalling pathway **CYTOSOL**	[150]
YEAST							
Candida albicans (strain WO-1)	*TDH1*	G3P_CANAW	AAC49800.1	335	151	Glycolysis **CYTOSOL** Plasminogen, fibronectin and laminin binding	[151]

						CELL SURFACE & EXTRACELLULAR MATRIX	
Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	*TDH1* *TDH2* *TDH3*	G3P1_YEAST G3P2_YEAST G3P3_YEAST	NP_012483.3 NP_012542.1 NP_011708.3	332 332 332	150 150 150	Glycolysis **CYTOSOL** Cell-cell adhesion molecule (fibronectin and laminin binding) **CELL MEMBRANE**	[152]
PLANT							
Arabidopsis thaliana	*GAPC* *GAPC2* (Cytosolic)	G3PC1_ARATH G3PC2_ARATH	NP_187062.1 NP_172801.1	338 338	156 156	Glycolysis **CYTOSOL** Associates with FBA6 on the outer mitochondrial membrane leading to actin binding and bundling **MITOCHONDRIA** DNA binding	[153.154]
Arabidopsis thaliana (cont.)	*GAPA1* *GAPB* *GAPCP1* *GAPCP2* (Chloroplastic)	G3PA1_ARATH G3PB_ARATH G3PP1_ARATH G3PP2_ARATH	NP_566796.2 NP_174996.1 NP_178071.1 NP_173080.1	396 447 422 420	213 235 236 234	**NUCLEUS** Involved in the photosynthetic reductive pentose phosphate pathway (Calvin-Benson cycle) **STROMA OF CHLOROPLAST**	
Triticum aestivum	*GAPC8* *GAPC6* *GAPC* *GAPC3* *Gap3dp* *GAPC1*	W5GYX5_WHEAT W5GM69_WHEAT A5YVV3_WHEAT A0A096UTL2_WHEAT C7C4X1_WHEAT A0A1B1V4R0_WHEAT	ABS59297.1 ALE18233.1 ALE18232.1 ABQ81648.1 ANW11921.1 ANW11922.1	337 358 337 337 337 335	154 175 154 154 154 153	Glycolysis **CYTOSOL** RNA-binding protein stabilization **CYTOSOL** Telomere protection Regulation of cell survival **NUCLEUS** Regulation of apoptosis **NUCLEUS & MITOCHONDRIA**	[100]

Table 2: Details of possible post-translational modifications of human cytosolic GAPDH

Cysteine residues on the sequence are highlighted in green (active site) and yellow (non-active site). Under possible PTMs, a possible phosphotyrosine is highlighted in purple, while Ser/Thr phosphorylations are in green. The histidine (179) which activates Cys152 in the active sight is in bright blue. Redox mediated modifications are in red. All details obtained from NCBI (NM_002046).

Protein	*Homo sapiens* glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	
Accession number	NM_002046	
Amino acid sequence	MGKVKVGVNG FGRIGRLVTR AAFNSGKVDI VAINDPFIDL NYMVYMFQYD STHGKFHGTV KAENGKLVIN GNPITIFQER DPSKIKWGDA GAEYVVESTG VFTTMEKAGA HLQGGAKRVI ISAPSADAPM FVMGVNHEKY DNSLKIISNA SCTTNCLAPL AKVIHDNFGI VEGLMTTVHA ITATQKTVDG PSGKLWRDGR GALQNIIPAS TGAAKAVGKV IPELNGKLTG MAFRVPTANV SVVDLTCRLE KPAKYDDIKK VVKQASEGPL KGILGYTEHQ VVSSDFNSDT HSSTFDAGAG IALNDHFVKL ISWYDNEFGY SNRVVDLMAH MASKE	
Gene Length	1285 bases	
Coding region	77 - 1084	
Amino acid length	335	
Position of active site Cys residue	152	All GAPDH peptides have a conserved Cys
Total number of Cys residues	3 (αα:152; αα:156; αα: 247)	
Amino acid position	Amino acid modification	Comment
5	N6,N6-dimethyllysine	methylation site
9	Deamidated asparagine	amidation site
42	Phosphotyrosine	phosphorylation site
46	Methionine sulfoxide *(in vitro)*	sulfatation site
61	N6-acetyllysine	acetylation site
64	Deamidated asparagine	amidation site
66	N6,N6-dimethyllysine	methylation site
70	Deamidated asparagine	amidation site
75	Phosphothreonine	phosphorylation site
83	Phosphoserine	phosphorylation site
122	Phosphoserine	phosphorylation site
148	Phosphoserine	phosphorylation site
149	Deamidated asparagine	amidation site
151-153		Glyceraldehyde 3-phosphate binding
151	Phosphoserine	phosphorylation site
152	ADP-ribosylcysteine	
152	Cysteine persulfide	sulfatation site
152	S-nitrosocysteine	nitrosylation site

153	Phosphothreonine	phosphorylation site
155	Deamidated asparagine	amidation site
177	Phosphothreonine	phosphorylation site
179	Histidine	Activates thiol group during catalysis
182	Phosphothreonine	phosphorylation site
184	Phosphothreonine	phosphorylation site
194	N6,N6-dimethyllysine	methylation site
194	N6-acetyllysine	acetylation site
194	N6-malonyllysine	
211-212		Glyceraldehyde 3-phosphate binding
211	Phosphothreonine	phosphorylation site
215	N6,N6-dimethyllysine	methylation site
215	N6-malonyllysine	
219	N6-acetyllysine	acetylation site
225	Deamidated asparagine	amidation site
227	N6,N6-dimethyllysine	methylation site
227	N6-acetyllysine	acetylation site
229	Phosphothreonine	phosphorylation site
237	Phosphothreonine	phosphorylation site
241	Phosphoserine	phosphorylation site
245-250		[IL]-x-C-x-x-[DE] motif
247	S-nitrosocysteine	nitrosylation site
254	N6-acetyllysine	acetylation site
260	N6,N6-dimethyllysine	methylation site
263	N6,N6-dimethyllysine	methylation site
312	Phosphoserine	phosphorylation site
316	Deamidated asparagine	amidation site
333	Phosphoserine	phosphorylation site
334	N6,N6-dimethyllysine	methylation site

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