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Reconsidering the role of protein glycation in disease

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Abstract

Protein glycation has long-been considered a toxic consequence of carbohydrate metabolism. Yet recent evidence demonstrates tight regulation for these non-enzymatic post-translational modifications, pointing to a broader role in cell biology rather than simply serving as a biomarker for toxicity.

Post-translational modifications (PTMs) provide a rapid response to regulate protein structure and function, expanding the functional proteome to maintain homeostasis. Although many PTMs are added to proteins through controlled, enzyme-catalyzed reactions, there is mounting evidence that many PTMs occur by non-enzymatic means[1]. One of the most well-documented cases of a catalysis-free PTM is the covalent attachment of a reducing sugar to a nucleophilic amino acid[1]. Since its initial discovery, protein glycation has become an important facet of biology and is routinely measured in diabetics as a biomarker for long-term glycemic control (that is, glycated hemoglobin, HbA_{1c}). Ensuing decades of biomedical research have now implicated these non-enzymatic PTMs in nearly every disease state, including diabetes, obesity, aging and cancer[1,2].

As the metabolic dependence on sugar is essential for life, the intracellular concentrations of hexoses, including glucose, often reside in the millimolar range, with the ring-opened, electrophilic isomers comprising around 1% of the total pool. As hexose catabolism proceeds, a wide array of glycating species begin to present themselves, including glycolytic intermediates (such as 1,3-bisphosphoglycerate), pentoses (such as ribose), and metabolic by-products (such as 3-deoxyglucosone and methylglyoxal (MGO))[1,3,4] (Fig. 1). Consequently, cells are continuously exposed to high concentrations of reactive sugars, giving rise to a chemically diverse profile of PTMs that are present under basal, non-stressed conditions[1,5,6]. However, despite considerable advancements in the detection of these PTMs, our understanding of protein glycation in the broader scheme of cell biology and disease remains weak. Sensitive assays and stably labeled isotopic standards have been developed to quantify many glycation-derived PTMs in clinical and non-clinical settings[4-7]. Even so, the identification of target proteins and their sites of modification from complex

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Competing interests

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samples remains a major roadblock, which has hindered our understanding of how these PTMs regulate biological processes. In addition, a lack of physiologically relevant study designs, relying on bolus dosing of glycating agents, have plagued the literature, implicating glycation solely as a causative agent in cell death and disease. This has led to a debate about the role of glycation in physiology: are these PTMs merely a toxic consequence of sugar metabolism or are they crucial components for homeostatic signaling?

In this Comment, we present an emerging paradigm that supports a hormetic role for protein glycation, extending beyond its designation as a simple measure of 'sugar toxicity'. This field has drawn notable parallels to that of protein oxidation, which was long believed to serve as a measure of oxidative stress and protein damage[8]. It was not until appropriate study designs were established that reactive oxygen species (ROS) gained an appreciated homeostatic role in the regulation of numerous cellular processes, including metabolic flux and transcriptional responses to cellular redox status[8]. Here, we opine that glycation should be evaluated in the same vein as other canonical PTMs, including protein oxidation, in which dose and context should be taken into consideration.

A historical perspective

In the early twentieth century, there was growing interest in the physiological role of α-oxoaldehydes and their metabolic regulation. These reactive intermediates were initially proposed to be toxic by-products of metabolism owing to their reactivity with DNA, RNA and proteins[9]. Arguably, the most prevalent α-oxoaldehyde is MGO, an electrophilic by-product of glycolysis that is produced in nanomolar to low micromolar concentrations in eukaryotic cells[2]. Interest in MGO began in the early 1900s, when Dakin and Dudley established that it was enzymatically oxidized to lactic acid, hypothesizing it to be a key intermediate in the breakdown of glucose[9]. Of course, this was later discredited following the full elucidation of glucose metabolism to pyruvate and subsequently L-lactate through the Embden–Meyerhof pathway (that is, glycolysis). This seminal work on MGO was not ignored, however, and a complete picture of its metabolism by the glyoxalase cycle was subsequently described in 1951, showing the conversion of MGO to D-lactate through an acyl-glutathione intermediate (lactoylglutathione, LGSH) catalyzed by the enzyme, glyoxalase 1 (GLO1)[9] (Fig. 2). As MGO concentrations are reflective of triose flux, it was hypothesized that GLO1 inhibition may serve as a selective metabolic liability in highly glycolytic cancer cells[9], increasing MGO to lethal concentrations in tumors. Since this initial hypothesis, interest in targeting GLO1 for the treatment of cancer and other metabolic disorders has remained a focal point in biomedical research[10].

Perhaps the first use of protein glycation as a marker for disease was the discovery of an 'unusual' hemoglobin in individuals with diabetes. HbA_{1c}, or glucose-modified hemoglobin, is now routinely used as a biomarker for long-term glycemic control in diabetics[2]. These initial studies laid the foundation for protein glycation as a marker for excess sugar exposure and subsequent reports have revealed notable correlations with various protein glycation products, including those derived from MGO (such as MG-H1 or CEL), in diabetes[11]. Given the importance of the glyoxalase cycle in the regulation of α-oxoaldehydes (notably MGO), the logical assumption is that reduced GLO1 activity may be a causative factor for

the pathogenesis of diabetes and other metabolic diseases. Indeed, mice with reduced *Glo1* expression following short hairpin RNA (shRNA) knockdown show spontaneous generation of diabetic nephropathy and increased MGO-derived PTMs[12]. It should be noted, however, that these results have not been reproduced in *Glo1*-knockout mice, which display no overt phenotype nor elevations in MGO[12]. This has also been corroborated in red blood cells isolated from patients with and without type 1 and type 2 diabetes, where no significant differences in GLO1 activity are observed[11]. The implied correlation between GLO1 or MGO and metabolic disease has since been a driving force for subsequent investigations, despite a notable lack of mechanistic studies.

In addition to metabolic disorders, protein glycation has been implicated in the progression of neurodegeneration and aging: increases in free MG-H1 adducts have been detected in cerebrospinal fluid of individuals with Alzheimer's disease[13]. Similarly, reduced GLO1 activity is observed in the brains of aged individuals[13]. Although the mechanisms that mediate cognitive decline and aging are unknown, one hypothesis is that protein glycation mediates cytoskeletal crosslinking, resulting in reduced neuronal function, although this has not been critically evaluated[14]. These correlative associations highlight a notable lack of sound mechanistic studies in the fields of aging and neurodegeneration. A strong exception to this is a report revealing regulation of the $Na_V1.8$ channel by MGO modification at Arg43 in the channel's activation gate[15]. This modification facilitates nociceptive neuronal firing and hyperalgesia in a mouse model for diabetic neuropathy. Clinically, patients experiencing pain were also found to have higher levels of plasma MGO (~900 nM) compared to those without pain (~500 nM)[15]. This was confirmed in mice, showing altered thermal and mechanical hyperalgesia in *Glo1*$^{+/-}$ or wild-type mice dosed with the GLO1 inhibitor, *S*-*p*-bromobenzylglutathione cyclopentyl diester (BBGC)[15]. This study represents a rare mechanistic evaluation as to how glycation affects physiology, and it is pertinent for future studies to follow a similar path and provide more physiologically relevant context in their investigations.

MGO has long been classified as a toxic by-product of metabolism[13]. Perhaps the driving factor behind this notion stems from decades of supraphysiological investigations. As noted, the intracellular concentrations of MGO often reside in the nanomolar to micromolar range[2,5]. Despite this established physiological range, studies consistently rely on bolus administration of glycating agents, often exceeding the median lethal dose (LD_{50}) by more than 1 order of magnitude[16]. Thus, to gain meaningful information into the role of protein glycation in biology, future investigations must be designed with physiological or pathophysiological conditions in mind, such that the correlative versus causative role becomes evident[2]. Although this shift in thought may seem obvious, these supraphysiological study designs have perhaps impeded a more provocative hypothesis: that glycation serves an essential role in cell signaling and metabolic flux[2].

Protein glycation as a homeostatic PTM

Protein oxidation by ROS was long believed to be associated with protein dysfunction and disease pathogenesis[8]. Improvements in analytical measurements and physiologically relevant study design have now identified these diverse PTMs as crucial components for

protein folding, cell signaling and transcriptional responses to cell stress[8]. This shift in our understanding of how non-enzymatic PTMs regulate cellular processes has begun to be appreciated in the field of protein glycation: 1,3-bisphosphoglycerate, a reactive primary glycolytic intermediate, can modify protein Lys residues, yielding the PTM, phosphoglycerylLys[3] (Fig. 1). Despite its non-enzymatic origins, these modifications display relative specificity, being enriched on glycolytic enzymes and reducing metabolic flux to restore homeostasis[3]. Similarly, lact(o)ylLys, which is derived from a non-enzymatic S-to-N acyltransfer from the glyoxalase cycle intermediate, lactoylglutathione (LGSH), is also enriched on glycolytic enzymes[6,17]. This was determined using an alkyne analog of MGO and click-based chemistry, relying on the cell's natural metabolism of MGO to generate physiologically relevant concentrations of LGSH[6]. Like phosphoglycerylLys, these PTMs are responsive to glucose concentrations and reduce glycolytic output, serving as a form of non-enzymatic metabolic feedback[6,17]. Collectively, these mechanistic studies are perhaps some of the first indications that protein glycation may serve a broader purpose in cell biology beyond functioning as a biomarker of disease.

There is perhaps no stronger link between metabolic flux and feedback than in the nucleus where histone glycation has garnered considerable attention in the regulation of chromatin function. MGO-hydroimidazolone 1 (MG-H1) and carboxyethylArg (CEA), both MGO-derived PTMs (fig. 1), are increased on histones after perturbations in GLO1, altering chromatin structure and function[5,18]. These PTMs are ubiquitously present and have pervasive effects on transcription, rather than facilitating canonical stress responses typically associated with toxicity (for example, heat shock or ER stress)[5,18]. These studies also highlight the range of effects MGO has on chromatin compaction and function, in which lower doses or shorter exposures result in chromatin decompaction and higher doses or longer exposures lead to crosslinking and notable compaction, likely resulting in cell death[18]. MGO-derived PTMs also occupy the space normally dedicated to canonical histone PTMs (such as Arg methylation), which further alters the histone code and transcriptional profile of cells exposed to increased glycation[5,18]. These findings are not limited to MGO: histones have now been identified as targets for modification by ribose, 3-deoxyglucosone, and lactate (such as lact(o)ylLys)[4,5,18]. Although additional mechanistic work is needed for many of these PTMs to understand their role in cell fate, lact(o)ylLys modifications have been identified as crucial PTMs that facilitate transcriptional responses to inflammation, inducing homeostatic genes involved in wound healing and macrophage polarization[19]. In addition to the non-enzymatic mechanism highlighted above with LGSH, these PTMs are also proposed to result from lactyl-CoA and an enzyme-catalyzed reaction (that is, histone acyltransferase). Regardless of the metabolic source, these glycolysis-derived PTMs provide yet another example of regulatory feedback, dictating cellular responses to metabolism.

In addition to histones, protein glycation also regulates gene expression through the modification of transcriptional machinery. KEAP1 sequesters the transcription factor NRF2 in the cytosol. Canonical activation of the antioxidant response occurs through modification of KEAP1 on conserved Cys residues by electrophilic metabolites, resulting in the release of NRF2 and its translocation to the nucleus. These Cys residues on KEAP1 are prone to modification by MGO, resulting in NRF2 translocation to the nucleus and activation of the antioxidant response[20]. NRF2 is also a target of glycation through glucose-derived

fructosylLys modifications (Fig. 1), resulting in reduced NRF2 stability and defective transcriptional activation of the antioxidant response[21]. As the non-enzymatic origins of these PTMs may seem uncontrolled, especially in the context of highly glycolytic tumors, the deglycase fructosamine-3-kinase (FN3K) serves a regulatory role in glycation-mediated NRF2 responses through removal of fructosylLys PTMs, restoring stability and activation of the antioxidant response[21]. This context-specific regulation is also likely to facilitate metabolic feedback as NRF2 activates genes involved in carbohydrate metabolism.

The hormetic effects of glycation have been elegantly described in a series of recent studies. In contrast to early work suggesting GLO1 as a metabolic liability in cancer, knockdown of GLO1 in breast cancer cells results in increased tumor growth and metastasis through glycation of Hsp90 and activation of the pro-tumorigenic transcription factor YAP1[22]. This was corroborated in patients, in which protein glycation correlates with increased nuclear localization of YAP1[22]. In a follow-up to this work, low-dose administration of MGO was found to promote tumor growth, while supraphysiological administration (>500 μM), perhaps not surprisingly, slowed tumor growth[16]. This highlights the biphasic nature of MGO, in which bolus administration of supraphysiological concentrations mediate toxic responses and controlled, metabolically relevant concentrations as achieved by genetic knockdown, often yield a completely different outcome. As the intracellular concentrations of dicarbonyls (such as MGO) rarely exceed low micromolar levels, even in highly glycolytic tumors, one may question the validity and conclusions drawn from studies dosing with supraphysiological bolus administration of glycating agents.

Considerable efforts have been made to understand the role of GLO1 and MGO in neuropharmacology: copy number variations leading to increased *Glo1* expression, and consequently reduced MGO in the brain, result in increased anxiety-like behaviors in mice[23]. By mechanistic investigations, MGO has been identified as a partial $GABA_A$ agonist, thus explaining its anti-anxiolytic properties. Furthermore, GLO1 also mediates ethanol withdrawal severity in mice[24]. Here, increased *Glo1* expression is associated with severity of withdrawal symptoms, whereas pharmacological inhibition of GLO1 reduces these effects[24]. These studies, in addition to the investigations on $Na_V1.8$ function, highlight the diverse nature of MGO and perhaps protein glycation as a whole, demonstrating a wide array of neuropharmacological effects that are likely to be context specific.

Outlook

The discovery of HbA_{1c} as a biomarker for diabetes provided the first evidence that protein glycation may be a toxic response to sugar overload. Further correlative studies have supported this notion, showing increased dicarbonyls and glycation products in various disease states including cancer, diabetes, aging and neuropathies[11,13,14,22]. Although these investigations have no doubt enhanced our understanding of disease pathogenesis, there is a notable lack of comprehensive mechanistic studies that demonstrate the effect of glycation on protein function. One cannot help but draw parallels to the field of oxidative stress, in which ROS were long believed to be a toxic consequence of cell metabolism. This was largely propagated by studies using bolus dosing of ROS, eliciting a myriad of non-specific

reactions and cellular outcomes[8]. This, of course, has been re-evaluated and ROS now have established roles in cell biology.

Protein glycation is present in all living organisms surveyed so far. This ubiquitous, low-level of glycation suggests a potentially broader role in physiology, crucial to cell survival and homeostasis. Exposure to low, basal levels of protein glycation may be necessary to condition cells to periods of increased glycolytic flux and exposure to glycating agents (such as hormesis). Thus, we opine that glycation, at low levels, has an important role in metabolic homeostasis. To test this hypothesis, it is important to focus efforts on physiologically relevant study designs using genetic manipulations, hyperglycemic conditions, and properly vetted inhibitors (Fig. 3). The application of click chemistry using tagged analogs (such as alkMGO or azidoribose) also provides the opportunity to monitor protein glycation by relying on endogenous cell metabolism, generating reactive precursors in a dose and location-controlled manner (Fig. 3). Advances in genetic code expansion have also enhanced our understanding of how these PTMs function in the cell, by precisely placing PTMs at one site and using functional read-outs to determine their role in cell biology (Fig. 3). This has been achieved with lact(o)ylation[17], a PTM that is heavily enriched on glycolytic enzymes and facilitates metabolic feedback and glycolytic output[6]. Using non-natural amino acid incorporation, regulatory versus non-regulatory sites of modification have been established on enolase 1, where Lys343, and not Lys326, alters protein function[17]. These approaches highlight an important consideration; perhaps not all PTMs alter protein function, but instead serve as agnostic sites of modification to simply store excess carbon.

Considerable attention has been paid to the therapeutic feasibility of targeting GLO1 for the treatment of cancer, with the goal of increasing aldehyde concentrations above the lethal threshold. The most widely reported classes of GLO1 inhibitors are designed as competitive inhibitors with a glutathione (GSH) scaffold (such as BBGD or BBGC)[10]. Although these inhibitors have shown efficacy against high GLO1 expressing cancer cell lines in vitro and in xenograft models, little progress has been made with clinical trials. This perhaps could have been predicted, as *Glo1*-knockout cells and mice are viable and the LD_{50} of MGO in most cell lines (high micromolar to low millimolar range) is likely unattainable in physiology[5,12,16]. The reported efficacy of these inhibitors may, in part, be explained through a myriad of off-target effects, as the GSH scaffold is likely to inhibit far more than just GLO1 and, to our knowledge, the specificity of these compounds (that is BBGC or BBGD) has not been determined. Although in vitro data may suggest a therapeutic potential for these inhibitors, the considerable lack of data regarding specificity and pharmacokinetic properties will continue to impede their advancement into clinical trials.

So far, a putative role for glycation has been implicated in a host of biological processes including metabolic flux[3], proliferation[16,22], signaling[20,22] and chromatin structure or function[4,5,18]. Important questions remain, however. Do these PTMs serve as molecular handles for protein-protein interactions? Is a toxic threshold of dicarbonyls achievable with small molecule inhibitors? Do all sites regulate enzyme function? Are these PTMs enzymatically removed? FN3K is now reported as a protein deglycase, capable of regulating the protein glycation abundance.[4,21]. Although the origins of these PTMs may be non-enzymatic, the abundance, and potentially site-specificity, are likely under control through

concerted enzymatic regulation via their removal. By contrast, DJ-1 has previously been reported as a deglycase; however, more recent evidence suggests that it directly converts MGO to lactate[25]. Although not considered a true deglycase, it still has an active role in the regulation of intracellular MGO and by proxy, MGO-derived PTMs. This clearly highlights the need for thorough mechanistic studies on the role of protein glycation in cell health. Collectively, glycation has outcomes that exceed a simple measure of toxicity and further investigations using physiologically relevant and mechanistically thorough studies will determine new roles in homeostatic regulation by these chemically diverse PTMs.

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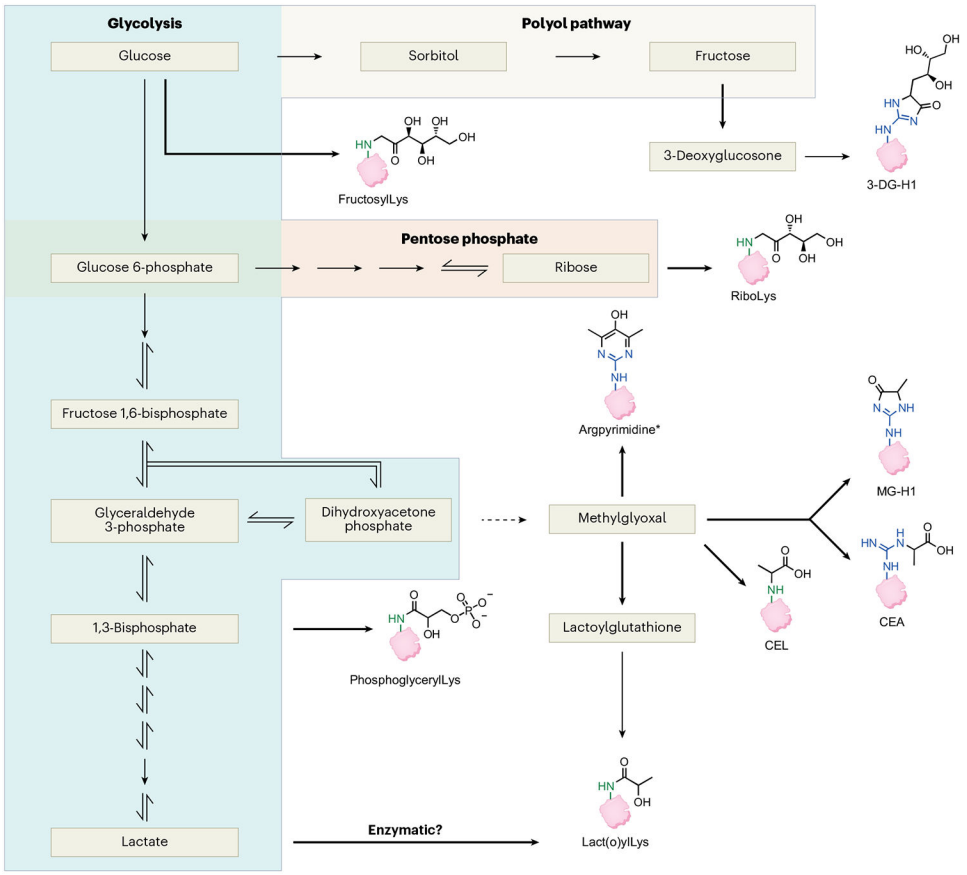

Fig. 1 |. Metabolic sources of protein glycation.

Non-enzymatic PTMs are derived from metabolic intermediates In glycolysis and the polyol pathway. Note the proposed mechanism of argpyrimidine formation and the metabolic source of 3-deoxyglucosone have not been fully determined.

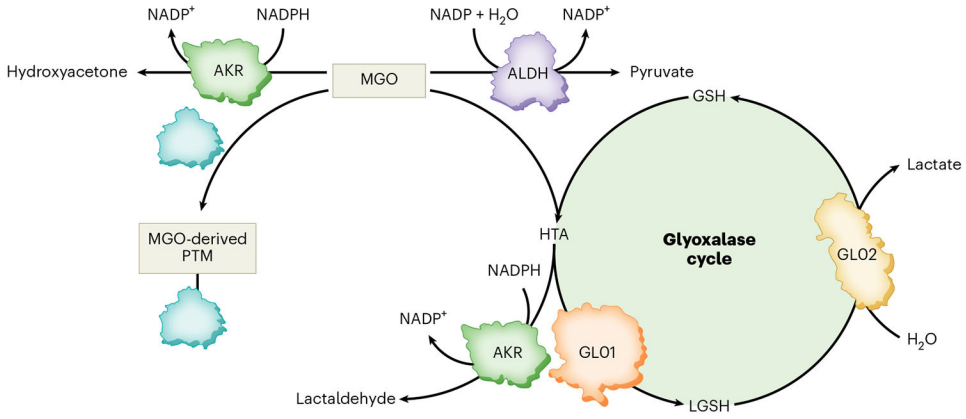

Fig. 2 | Methylglyoxal metabolism.

The bulk of MGO (~99%) is detoxified by the glyoxalase cycle, consisting of GLO1 and GLO2. Aldo-keto reductases (AKRs) and aldehyde dehydrogenases (ALDHs) also have a minor role in MGO metabolism, yielding hydroxyacetone and pyruvate, respectively. MGO, methylglyoxal; GSH, glutathione; LGSH, lactoylglutathione; HTA, hemithioacetal.

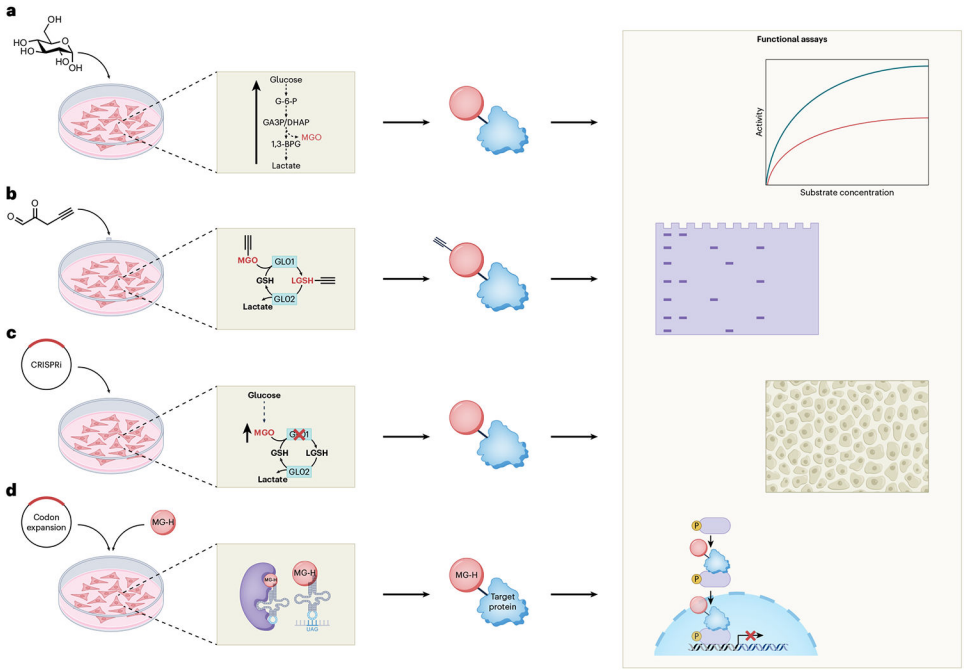

Fig. 3 | Towards relevant study designs in protein glycation.

a, Hyperglycemic conditions mimic pathophysiologic concentrations of glycating agents. **b**, Clickable analogs of glycating agents can be used to monitor targets of protein glycation. These probes can be pulsed into cells, relying on endogenous metabolism to yield physiologically relevant concentrations and locations of the probes. **c**, Genetic manipulations (such as CRISPRi) provide local, controlled increases in reactive species. **d**, The use of non-natural amino acids (such as MG-H1 or fructosylLys) and genetic code expansion provides precise incorporation of PTMs on target proteins, yielding mechanistic insight into their role in enzyme function and cell fate.