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The pentose phosphate pathway in health and disease

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Abstract

The pentose phosphate pathway (PPP) is a glucose-oxidizing pathway that runs in parallel to upper glycolysis to produce ribose 5-phosphate and nicotinamide adenine dinucleotide phosphate (NADPH). Ribose 5-phosphate is used for nucleotide synthesis, while NADPH is involved in redox homoeostasis as well as in promoting biosynthetic processes, such as the synthesis of tetrahydrofolate, deoxyribonucleotides, proline, fatty acids and cholesterol. Through NADPH, the PPP plays a critical role in suppressing oxidative stress, including in certain cancers, in which PPP inhibition may be therapeutically useful. Conversely, PPP-derived NADPH also supports purposeful cellular generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) for signalling and pathogen killing. Genetic deficiencies in the PPP occur relatively commonly in the committed pathway enzyme glucose-6-phosphate dehydrogenase (G6PD). G6PD deficiency typically manifests as haemolytic anaemia due to red cell oxidative damage but, in

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Competing interests

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severe cases, also results in infections due to lack of leucocyte oxidative burst, highlighting the dual redox roles of the pathway in free radical production and detoxification. This Review discusses the PPP in mammals, covering its roles in biochemistry, physiology and disease.

The PPP is a glucose-metabolizing pathway that comprises two branches. The input of the oxidative branch (oxPPP) is glucose 6-phosphate, which also feeds glycolysis. The oxPPP converts glucose 6-phosphate into ribose 5-phosphate while making two molecules of NADPH. The non-oxidative branch (non-oxPPP) reversibly interconverts ribose 5-phosphate with glycolytic intermediates. In most healthy mammalian cells in unstressed conditions, the PPP exhibits much lower flux than glycolysis, for example, by 10–100-fold[1,2]. Nevertheless, the PPP carries higher flux than most pathways outside central metabolism. Moreover, it can be transiently activated in response to oxidative stress or during the phagocyte oxidative burst to meet urgent NADPH demands.

One function of the PPP is to provide biosynthetic precursors, with ribose 5-phosphate an essential nucleotide building block and NADPH used for biosynthesis of fatty acids, cholesterol, proline, tetrahydrofolate and deoxyribonucleotides. From bacteria to mammals, the oxPPP is a major source of cytosolic NADPH to support these processes[3,4]. However, alternative routes for cytosolic NADPH production exist, including isocitrate dehydrogenase 1 (IDH1), malic enzyme 1 (ME1), methylenetetrahydrofolate dehydrogenase and formyltetrahydrofolate dehydrogenase, and can often compensate to generate the required NADPH for biosynthesis.

The PPP also supports cellular defence against oxidative stress, a driver of cardiovascular disease, neurodegeneration and aging[5]. To prevent damage from ROS and RNS, cells rely on antioxidant systems including glutathione and thioredoxin. Both use NADPH to regenerate reduced thiols from disulfides. The oxPPP may be uniquely suited for acute antioxidant responses compared to other NADPH-production routes due to its having excess capacity that is not used in the basal state but stands ready for times of enhanced demand[6] (reserve flux capacity). Indeed, PPP flux upregulation keeps yeast cells alive when they encounter oxidative substances, protects skin cells from the consequences of UV irradiation and allows cancer cells to tolerate increased oxidant levels during detachment.

While often considered bad actors, ROS and RNS also play physiological roles in mammals and are produced by dedicated enzymes that use NADPH as the reductant: NADPH oxidase (NOX) or nitric oxide synthase (NOS)[7]. Modest amounts of ROS and RNS play signalling and biochemical roles. Larger quantities contribute to immune defence by killing invading pathogens and thereby preventing life-threatening bacterial and fungal infections. In activated phagocytes, the PPP fuels this process, shifting flux away from glycolysis to generate large amounts of NADPH[8].

In this Review, we will cover the basic principles of the PPP and its regulation as well as current technical approaches to determine metabolic flux through this pathway. We will then discuss its implications in physiology and pathology in the context of red blood cells (RBCs), immune cells, tissue homoeostasis and cancer.

Basic principles of the PPP

Biochemical modes

The oxPPP pathway produces two NADPH molecules per glucose-6-phosphate molecule through two dehydrogenase steps, resulting in the formation of ribulose 5-phosphate (Fig. 1a). The first committed step, which is catalysed by G6PD, generates an initial NADPH molecule. Mutations in G6PD are the most common enzymatic defect in humans, leading to fragile RBCs, but also providing protection against malaria[9]. Next comes 6-phosphogluconolactonase (6PGL). Mammals also have an alternate enzyme, hexose-6-phosphate dehydrogenase (H6GD), localized in the endoplasmic reticulum, which can catalyse the first two steps of the oxPPP pathway to produce NADPH for processing corticosteroids. The final step of the oxPPP pathway, which produces the second molecule of NADPH, is catalysed by 6-phosphogluconate dehydrogenase (6PGD).

The non-oxPPP pathway allows for interconversion of various important molecules, including the oxPPP product ribulose 5-phosphate, the key nucleotide precursor ribose 5-phosphate and the glycolytic intermediates fructose 6-phosphate and glyceraldehyde 3-phosphate. These reversible reactions are catalysed by a series of enzymes, including ribulose-5-phosphate epimerase, ribose-5-phosphate isomerase, transaldolase (TALDO) and the thiamine-dependent enzyme transketolase (TK; Fig. 1a). Another intermediate in the non-oxPPP pathway, erythrose 4-phosphate, is used by microbes and plants to synthesize essential amino acids, but mammals are unable to produce these and instead obtain them through their diet. Accordingly, in mammals, the non-oxPPP pathway primarily functions to provide flexible carbon flow between pentoses and glycolysis.

The two branches of the PPP can function together in three different modes based on cellular requirements: pentose insufficiency, pentose overflow and pentose cycling (Fig. 1b). In pentose-insufficiency mode, the non-oxPPP pathway produces ribose 5-phosphate, as it is insufficiently supplied by the oxPPP pathway. In pentose-overflow mode, the non-oxPPP pathway consumes excess ribose 5-phosphate produced by the oxPPP pathway and feeds it back into glycolysis. In pentose-cycling mode, glycolytic intermediates made from excess ribose 5-phosphate are redirected up the glycolysis pathway to regenerate glucose 6-phosphate. This glucose 6-phosphate is then reused by the oxPPP pathway to produce more NADPH. Theoretically, if the gluconeogenic enzyme fructose 1,6-bisphosphatase is active, pentose cycling could lead to complete oxidation of glucose into six CO_2 molecules, generating 12 NADPH molecules (Fig. 1). Although this total oxidation has been postulated for several decades, it has not yet been observed in mammalian systems. However, partial pentose cycling appears to be crucial for the oxidative burst of immune cells, which aids in pathogen killing. The PPP mode depends on the cellular context. In proliferating cells, both ribose and NADPH are consumed in parallel to support proliferation, with the PPP mode determined by whether cellular demand for nucleotides or lipids is greater. In non-proliferating cells, on the other hand, demand for ribose is typically low, while NADPH is still needed for redox and/or biosynthetic purposes (for example, fat synthesis in adipocytes), resulting in pentose overflow as seen in quiescent fibroblasts[10] or pentose cycling as seen in immune cell oxidative burst[11].

Regulation

PPP activity correlates with NADPH demand (Fig. 2) and depends on the provision of glucose 6-phosphate, for which it competes with other pathways (foremost glycolysis). Such coordinated metabolic activity is achieved substantially through levels of the pathway substrate NADP (in part relative to the product NADPH). NADPH consumption makes NADP, which promotes G6PD activity and thus oxPPP flux both (1) as a substrate and (2) via an allosteric binding site on G6PD[12,13] (Fig. 3). The non-oxPPP in turn adapts to consume or produce pentose phosphates, as controlled by metabolite levels, which determine reaction thermodynamics and thus flux direction.

Alignment between NADPH demand and oxPPP flux is also achieved via transcriptional regulation. One major demand is de novo lipogenesis (Fig. 2). OxPPP flux is much higher in lipogenic yeast than in baker's yeast[14]. In mammals, expression of G6PD is high in most lipogenic tissues including the lactating mammary gland and brown adipose tissue (but not the liver, which makes NADPH in other ways)[15–17]. Such activity is supported by a master transcription factor that promotes conversion of carbohydrates to fat: sterol regulatory element-binding protein (SREBP)[18–22]. Another important NADPH demand is oxidative stress[23]. The nuclear respiratory factor 2 (NRF2) family of transcription factors activate many genes involved in responding to oxidative stress including multiple PPP enzymes (G6PD, 6PGD, TK and TALDO)[24,25]. Oxidative stress turns on NRF2 in part by suppression of its Kelch-like ECH-associated protein 1 (KEAP1)-mediated degradation by oxidation of KEAP1 cysteine residues[26,27]. ROS-induced DNA damage can also simulate G6PD expression through the combined activities of ataxia-telangiectasia mutated (ATM) serine/threonine kinase and heat shock protein 27 (HSP27)[28–31].

Rapid oxPPP responsiveness, on the timescale of seconds, is physiologically important for dealing with acute oxidative stress and to support oxidative burst for pathogen killing by phagocytes. Such responsiveness is enabled by reserve flux capacity, which is defined as excess enzyme relative to that required to catalyse basal pathway flux. Reserve PPP capacity has been seen in a wide variety of contexts from *Escherichia coli* to mammalian cells[3,6,11,23,32,33]. Basal activity in these cells is limited by low NADP and/or high NADPH. When NADP levels increase due to oxidative stress, the pathway nearly instantaneously turns on[3,11,33].

Mechanisms that enhance the supply of glucose 6-phosphate can complement such NADP-mediated regulation. One way to enhance glucose 6-phosphate is to slow glycolysis. Oxidative stress reduces glycolytic flux by oxidation of the active site cysteine of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, which inactivates the enzyme within seconds of oxidative stress, or in response to endogeneous increases in oxidant levels, for instance, upon anchorage-independent growth of cancer cells[23,34–37]. Also, cysteine oxidation of pyruvate kinase M2 (PKM2), the last enzyme in glycolysis, decreases PKM2 activity and has been reported to correlate with increased PPP flux[38]. In support of glycolysis and the PPP competing for glucose 6-phosphate, in bacteria, knocking out the gene encoding the upper glycolytic enzyme glucose-6-phosphate isomerase redirects carbon flux to the PPP[39], and, in yeast, reducing the activity of triosephosphate isomerase (TPI) results in increased NADPH formation in the PPP and

increased oxidant tolerance[6]. Furthermore, rendering GAPDH oxidation-resistant in cancer cells mitigates an increase in PPP flux (for instance, upon matrix detachment)[37]. In mammals, increased consumption of hexose phosphates by phosphofructokinase (PFK) driven by small-molecule PFK agonists can decrease NADPH levels in immune cells, while knockdown of PFK can increase NADPH[40,41]. Furthermore, the p53-induced phosphatase TIGAR, which supports intestinal oxidative defence and nucleotide synthesis[42,43], may promote PPP flux by dephosphorylating the PFK activator fructose 2,6-bisphosphate and/or the downstream glycolytic metabolite 2,3-biphosphoglycerate[44]. Collectively, these data support the possibility that suppressed glycolysis is a physiological means of turning on the PPP in mammals.

Measuring the PPP

Intermediates of the PPP can now be routinely measured using metabolomic methods based on liquid chromatography–mass spectrometry, with proper choice of quenching solvent crucial for measuring NADP(H). For NADP and NADPH, enzyme assay kits are also available but may be less accurate[45].

To measure PPP flux rather than metabolite levels, most methods use isotope-labelled glucose. A classic approach compares release of radioactive CO_2 from [1-^{14}C]glucose to [6-^{14}C]glucose, with glucose carbon atoms 1 and 6 metabolically similar except for selective C1 release by the PPP[46]. This method can measure absolute PPP flux in cell culture and at the whole-body level by detecting expired $^{14}CO_2$[47].

Stable isotope tracers offer greater biochemical and tissue specificity without concerns about radioactivity but typically measure relative flux (for example, the ratio of glycolytic to pentose-overflow flux) rather than absolute flux (for example, moles of glucose passing through oxPPP per unit time). The [1,2-^{13}C]glucose isotope tracer distinguishes glycolytic intermediates directly made through glycolysis, which retain both labelled carbon atoms, versus from pentose overflow, in which C1 is lost[48,49]. It also measures the relative contribution of oxPPP versus non-oxPPP to ribose 5-phosphate and can be applied both in cell culture and in vivo[50,51]. As a relative measurement, results using this tracer method do not always mirror absolute fluxes; for example, increased glycolysis would misleadingly decrease the relative oxPPP flux. This deficiency can be addressed by making a complementary absolute measurement such as total glucose uptake[52,53]. OxPPP flux can also come from glycogen stored within cells rather than glucose directly, which can be monitored based on glucose-6-phosphate labelling[54]. Together, these carbon-tracing strategies provide valuable quantitative tools for probing oxPPP activity.

Deuterium-labelled glucose ([1-^{2}H]glucose or [3-^{2}H]glucose) provides a perspective complementary to carbon tracing by measuring the fraction of NADPH that is produced by the oxPPP, as opposed to by other enzymes such as IDH1, ME1 and aldehyde dehydrogenase 1 family member L1 (ALDH1L1) (refs. 17,55–59). A challenge with this method is that the active hydrogen on NADPH can undergo enzyme-catalysed exchange with water, which leads to loss of labelling, but it is feasible to account for such losses by measuring the exchange rate between NADPH and water[60]. Deuterium labelling can also be followed downstream into products such as fatty acid molecules synthesized by

de novo lipogenesis, the labelling of which specifically reflects cytosolic NADPH and can be measured not only by mass spectrometry but also with subcellular spatial resolution by Raman spectroscopy[58,61]. The merit of these strategies is in revealing the contribution of the PPP to NADPH in different contexts.

To more directly visualize PPP activity in vivo, nuclear magnetic resonance imaging holds potential but currently lacks the sensitivity to detect the above tracers. To address this, investigators are exploring hyperpolarized [1-^{13}C]gluconolactone[62,63], which feeds into the middle of the oxPPP. Up to now, however, in vivo measurements are limited to initial uptake and phosphorylation of this tracer and do not yet probe the pathway's pivotal redox or ribose-5-phosphate-producing steps[62,63]. We expect continued technological advances, including more effective in vivo tracing, to drive further understanding of the PPP.

Role in physiology and disease

The role of the PPP in redox regulation and biosynthesis makes it important in many contexts, including in development and differentiation[64–69]. In this Review, we will focus on four settings in which the PPP plays a role relevant to human health and disease: (1) RBCs, (2) the immune system, (3) tissue maintenance, damage and repair, and (4) cancer.

RBCs

RBCs are the cell type most sensitive to oxPPP loss. Mature RBCs live for roughly 120 d without fresh protein synthesis under conditions of high oxygen exposure. Due to their lack of mitochondria, they have limited supplies of many metabolites, including tricarboxylic acid cycle (TCA) intermediates, which are required for NADPH production via the main alternative pathways IDH1 and ME1. Thus, the PPP plays an outsized role in RBC health.

Many mutations in PPP enzymes in humans result in haemolytic anaemia[67]. The *G6PD* gene on the X chromosome in humans has over 300 variants and 150 unique mutations that result in G6PD deficiency, the most common human enzyme defect[70]. The frequency of *G6PD* mutation geographically overlaps with areas where malaria has been historically endemic, and people who are heterozygous carriers of a *G6PD* mutation or homozygous deficient are protected from severe disease and mortality from malaria[9,71–74]. G6PD deficiency can be asymptomatic until patients are exposed to oxidative stress from infection, certain drugs or fava beans causing haemolysis[75]. Mutations that impact protein stability cause G6PD deficiency in RBCs, especially as the red cells age, while other cell types maintain sufficient levels of the enzyme due to continued protein synthesis.

Mutations are broken down into classes based on clinical phenotype and enzyme activity in RBCs: class I mutations have less than 10% activity and result in chronic anaemia; class II mutations also have less than 10% activity and result in intermittent haemolytic anaemia; class III mutations have 10–60% activity with haemolytic anaemia only after oxidative stress; and class IV mutations have more than 60% enzymatic activity and are asymptomatic[76] (Fig. 4). Consistent with *G6pdx* knockout being embryonic lethal in mice[77], no null mutations have been observed clinically. In vitro studies have found that oxPPP

activity is important for the emergence of erythroid progenitors from the haemogenic endothelium, which may contribute to the lethality of null mutations[78].

G6PD activity is highest in the homotetramer form; it is active as a homodimer and inactive as a monomer[79]. Tetramers and dimers are also more stable[80]. Many of the class I mutations fall along the dimer interface and in the allosteric NADPH-binding site[13,81,82]. Class II and III mutations are more randomly distributed throughout the protein and impact stability and activity[83] (Fig. 4). Mice with a common class II mutation, the Mediterranean mutation, have been generated, and they recapitulate the human phenotype[84]. Thus, across species, G6PD deficiency manifests in RBCs.

Immune system

The PPP plays a key role in the immune system, impacting immune cell fate and functions, such as cytokine production and the oxidative burst. Different types of immune cells have unique metabolic requirements. Phagocytic immune cells, which include macrophages and neutrophils, produce an oxidative burst for killing pathogens. T cells, on the other hand, require a large amount of biosynthesis and energy for rapid proliferation and cytokine production upon activation. This leads to different roles of the PPP in different cell types.

Both intracellular signalling and killing of pathogens during phagocyte inflammatory responses involve the production of superoxide and nitric oxide by the enzymes NADPH oxidase (NOX) and inducible NOS (iNOS), respectively. Both NOX and NOS enzymes use NADPH, with the PPP as a major source. Due to defective pathogen killing in phagocytes, deficiency of NOX causes chronic granulomatous disease, which is characterized by serious recurrent bacterial and fungal infections[85]. Severe *G6PD* mutations phenocopy chronic granulomatous disease and put patients at risk for sepsis[86–88].

Macrophages.—Macrophages are large mononuclear phagocytic cells that play an important role in fighting infection, maintaining tissue homoeostasis and removing foreign substances. They can be classified along a spectrum from classically activated pro-inflammatory macrophages (‘M1’ like) to alternatively activated macrophages that support tissue repair and wound healing (‘M2’ like)[89–91].

In response to a classical activation signal such as lipopolysaccharide, the polarization of naive macrophages into a pro-inflammatory state requires coupled metabolic remodelling[92,93]. Within 1 h of activation, glucose uptake and glycolytic flux increase. The contribution of glucose to PPP metabolites via the oxidative branch also increases, as determined by [1,2-^{13}C]glucose tracing[94]. This increased flux supports NADPH production for NOX and iNOS activity (Fig. 5a). Perturbing oxPPP activity modulates ROS and RNS production[11,95,96]. In some studies but not others, perturbation of the oxPPP also had broader impacts such as changes in expression of iNOS or cytokines such as interleukin (IL)-6 (refs. 96–98). The extent to which these broader pro-inflammatory phenotypes depend on ROS, RNS or the changes in redox state is not well understood.

Macrophages are also important for haem clearance. Haem-loaded macrophages require the PPP for haem detoxification, probably to provide NADPH as a cofactor for haem

oxygenase (HMOX)1, which metabolizes haem to biliverdin and carbon monoxide, and biliverdin reductase, which metabolizes biliverdin to bilirubin (Fig. 5b). Haem loading of macrophages induces glucose uptake and increases oxPPP enzyme activity. This oxPPP increase can be phenocopied by treating cells with the carbon monoxide-producing molecule tricarbonylchloro(glycinatro)ruthenium, with release of carbon monoxide by HMOX1 seeming to promote PPP activity, although the biochemical mechanism remains unknown[99].

In contrast to the upregulation of glycolysis upon M1 polarization, M2 polarization favours respiratory ATP generation. In addition, M2 polarization by IL-4 upregulates a recently discovered mammalian enzyme, sedoheptulose kinase (SHPK). SHPK, also known as CARLK, converts sedoheptulose, a seven-carbon sugar that is found in various fruits and vegetables, to sedoheptulose 7-phosphate, a non-oxPPP metabolite[100,101]. Via an unclear mechanism, SHPK overexpression suppresses the M1 phenotype and glycolytic metabolism while promoting M2 polarization[94].

The importance of metabolism in macrophage function raises the question of how common metabolic diseases such as obesity and diabetes may affect macrophages. Hypercholesterolaemia suppresses macrophages' NRF2 antioxidant response, decreasing 6PGD expression. Thus, hypercholesterolaemia may lower oxPPP activity, impairing the macrophage immune response[102]. Such interactions may be medically important, given the central role of macrophages in atherosclerotic plaques and the health toll of atherosclerosis[103].

Neutrophils.—Neutrophils are abundant, short-lived, mobile phagocytic granulocytes that play an important role in fighting bacterial and fungal infections. As part of their inflammatory response, neutrophils, like macrophages, produce an oxidative burst, releasing large quantities of superoxide anion via NOX, which requires NADPH for its activity. Patients with G6PD deficiency have less ROS release from neutrophils[104]. Pharmacological G6PD inhibition did not alter neutrophil cytokine production but impaired the oxidative burst[98].

During NOX-dependent oxidative burst, which can occur within minutes of stimulation, neutrophils shunt most of their glucose flux toward oxPPP. Remarkably, to maximize NADPH yield from glucose 6-phosphate, flux through the glycolytic enzyme glucose-6-phosphate isomerase is reversed to enable pentose cycling. The main molecular trigger of these events appears to be NADPH oxidation by NOX, which stimulates oxPPP flux[11]. Similar to directly inhibiting the oxPPP, indirectly disrupting pentose cycling by either knocking out the genes encoding the non-oxPPP enzymes TK or TALDO or pharmacologically activating the glycolytic enzyme PFK suppresses oxidative burst in neutrophils[11,40]. Pharmacologic inhibition of the neutrophil oxidative burst by PFK activators presents a potential therapeutic strategy for inflammatory diseases.

In addition to release of superoxide, neutrophils can also release chromatin traps to capture and kill microorganisms ('neutrophil extracellular traps' (NETs)). Superoxide production triggers NET release. NOX is a main source of NET-inducing superoxide. NADPH from the oxPPP fuels NOX. Consistent with this pathway, inhibiting oxPPP suppresses

ROS-dependent NET release[11,105]. In severe coronavirus disease 2019 (COVID-19) infection, decreased neutrophil glyceraldehyde-3-phosphate dehydrogenase activity and increased oxPPP flux may contribute to aberrantly increased NET release and pathogenic inflammation[106].

The importance of oxPPP-derived NADPH during the oxidative burst and NET release is now becoming clear. These connections raise the question of whether diet or other upstream steps impacting glucose metabolism can affect neutrophil function[107]. Diabetes, a disease that causes high levels of circulating glucose, impairs wound healing. While neutrophils play an important role in enabling wound healing, overactivity of neutrophil NETs can also result in tissue damage. Neutrophils from patients with either type 1 diabetes or type 2 diabetes are primed for NET release, which probably contributes to impaired wound healing[108]. These observations lead to the intriguing hypothesis that increased glucose levels may increase PPP flux and cause the observed phenotype. It will be interesting to determine whether low-carbohydrate or ketogenic diets can prevent unwanted inflammatory responses such as impaired wound healing, gout or infection-induced cytokine storms and whether the PPP may be one pathway moderating these effects.

T cells.—T cells exist in a variety of subpopulations with different immune functions. One fundamental division is between cytotoxic $CD8^+$ T cells that kill pathogen-infected or malignant cells and helper $CD4^+$ T cells. Either can exist in an immunosuppressive regulatory T cell (T_{reg}) form, in addition to the traditional invader-fighting form. Upon antigen stimulation, all these T cell types switch from a quiescent to a highly proliferative state. This switch requires corresponding metabolic changes, including upregulation of both glycolysis and mitochondrial functions[109–111]. Similar to immunosuppressive macrophages, anti-inflammatory T_{reg} cells rely more on fatty acid oxidation than their infection-fighting counterparts[112–114].

Similar to macrophages and neutrophils, cytotoxic and helper T cells both increase oxPPP flux in response to activation[98,115]. ROS levels are increased in T cells with G6PD inhibition[98], and glucose restriction in $CD8^+$ T cells is sufficient to cause a decrease in the glutathione/glutathione disulfide ratio. These observations suggest a major role for oxPPP in T cell antioxidant defence[116].

Consistent with these findings, the oxPPP is functionally important in T cells. G6PD inhibition during T cell activation does not block proliferation but leads to decreased production of pro-inflammatory cytokines including IFNγ[99]. T cells from patients with rheumatoid arthritis are hyperproliferative and manifest increased G6PD expression and decreased levels of ROS, consistent with upregulation of oxPPP flux. Such flux may facilitate ongoing proliferation and promote differentiation into pro-inflammatory type 1 and 17 helper T cells that cause excessive inflammation in rheumatoid arthritis[117,118]. Thus, the oxPPP plays an important role in infection-fighting and cancer-killing $CD4^+$ and $CD8^+$ T cells as well as in T cells driving autoimmunity.

The functional importance of the oxPPP carries over into T_{reg} cells. Such cells express the transcription factor FOXP3, which is required for their immunosuppressive function.

The FOXP3 transcriptional programme inhibits glycolytic and oxPPP flux, measured by [1-^{14}C]glucose, and promotes oxidative metabolism[113]. While this might suggest limited importance of the oxPPP in T_{reg} cells, knockout of *Pgd* specifically in T_{reg} cells (driven by *Foxp3*-Cre) causes severe T_{reg} dysfunction in mice, which die because of autoimmunity between days 20 and 30 of life[119]. Similarly, T_{reg}-specific knockout of the gene encoding an enzyme from non-oxPPP, TK, also causes lethal autoimmunity[120]. Moreover, knockout of *Pgd* across all $CD4^+$ and $CD8^+$ T cells promoted antitumour immunity (rather than suppressed it, as one might expect if the defect was primarily in pro-inflammatory T cells). Data from this pan-T cell knockout raise the possibility that the in vivo effect of oxPPP loss is mediated primarily through its effect on T_{reg} cells and outweighs the effects of its loss in pro-inflammatory T cells[121].

Currently, interpretation of these results is complicated by different oxPPP enzymes being modulated and different aspects of immunity being probed. For example, severe systemic G6PD deficiency predisposes to bacterial and fungal infections, while *Pgd* knockout in T cells putatively promotes antitumour immunity[121]. Is the difference due to G6PD's role also in phagocytes? Is it because G6PD is pro-immune and 6PDG is immunosuppressive, even though they are both oxPPP enzymes? Resolving these questions is pressing because the PPP could potentially be an appealing target for new immunosuppressive or immuno-oncology agents.

Tissue maintenance, damage and repair

Tissue maintenance and repair involve both redox balance and biosynthesis. The PPP probably plays a role in the homoeostasis of many vital organs, including heart, kidney, brain, skin and intestine[67,122–127]. Here, we focus on the latter two as well as ischaemia–reperfusion injury, a medically important tissue-damage process with a large redox component.

Skin.—As the body's primary barrier, the skin is exposed to damaging agents such as chemicals and UV radiation[128]. These can cause oxidative stress, the control of which depends in part on the PPP. Some patients with G6PD deficiency can have haemolysis with exposure to normally tolerable chemicals such as those found in the plant-derived dye henna[129], probably due to oxidant chemicals interacting with RBCs in skin capillaries. But the role of the PPP in skin extends beyond its vasculature.

G6PD levels in the skin decrease in psoriasis and vitiligo[130–132]. Treatment of psoriasis with 6-aminonicotinamide, which inhibits the 6PGD reaction in the oxPPP, decreased itching and cleared some lesions[133]. While these effects may be through interactions with immune cells within the skin microenvironment, the PPP also seems to play a role in skin fibroblasts and keratinocytes. Kinetic ^{13}C-glucose experiments revealed that PPP flux increases in fibroblasts and keratinocytes in response to oxidative stress from hydrogen peroxide or UV exposure. This flux was dependent on non-oxPPP enzymes; thus, oxidative stress activates pentose overflow in skin[128]. The importance of the PPP in the many cell types within the skin may be due to the hazardous nature of this microenvironment.

Intestine.—The PPP can play an important role in homoeostasis of proliferative tissues due to the requirement of nucleotides for cell replication. The intestine is one of the most replicative, high-turnover tissues in the body. The PPP is upregulated during intestinal regeneration. Whole-body *Tigar* knockout, which may decrease PPP flux, causes no obvious developmental problems but leads to reduction in intestinal crypt regeneration[43,134]. Crypt formation could be rescued by either antioxidants or nucleotides, suggesting that knockout cells lacked both NADPH and ribose 5-phosphate from the PPP[43].

Inflammatory bowel disease involves chronic autoimmune inflammation of the gastrointestinal tract, such as colitis. In mice, knockout of the gene encoding the non-oxPPP enzyme TK in intestinal epithelial cells results in spontaneous colitis with massive immune cell infiltration. Intestinal epithelial cells lacking TK are hyperproliferative and prone to apoptosis. These phenotypic changes occur without alteration of the $NADP^+$/NADPH ratio but with accumulation of the terminal oxPPP product (and non-oxPPP substrate) ribose 5-phosphate as well with decreased levels of glycolytic metabolites, possibly due to impaired pentose overflow[135]. Knockout of *Tigar* in intestinal epithelial cells also confers greater sensitivity to colitis[43]. Thus, both arms of the PPP and their putative regulator TIGAR are important for intestinal health.

Ischaemia–reperfusion injury.—Ischaemia occurs when blood flow and thus oxygen supply to a tissue is compromised. If blood flow is then restored, exposure to oxygen during reperfusion causes a burst of ROS production that damages the tissue. Such damage can be further exacerbated by neutrophil activation at the injury site.

Ischaemia–reperfusion injury is a major clinical problem, contributing to the pathogenesis of both heart attack and stroke. In mouse models of ischaemic brain injury, G6PD overexpression decreases oxidative damage and improves survival and recovery[136]. As prophylactic G6PD overexpression is not clinically feasible, scientists have searched for ways to drive such expression. One strategy involves ‘post-conditioning’ after stroke with bouts of mild hypoxia. Such post-conditioning increased G6PD activity, NADPH and glutathione levels and neuronal cell survival[137].

The oxPPP appears to suppress ischaemia–reperfusion injury also in the heart, as knockout of *G6pd* results in impaired cardiac relaxation and contractile performance after ischaemia–reperfusion injury in mice[138]. Glucose flux was also seen to increase through both glycolysis and the PPP in mouse hearts during low-flow ischaemia[139]. Thus, oxPPP flux protects against ischaemia–reperfusion injury.

Cancer

A key role for the PPP in cancer cells has long been postulated due to the proliferative nature of cancer and the biosynthetic role of the PPP. Epidemiologic evidence suggests that patients with *G6PD* mutations are protected from certain types of cancers, including endometrial and colon cancers, but not others[140,141]. This complexity parallels the dual roles of oxidative stress in cancer. ROS can cause DNA damage and thus cancer. After transformation, however, ROS can suppress both tumour growth and metastasis[142,143]. In

addition, the PPP can support biosynthesis and proliferation through both NADPH and ribose-5-phosphate production.

To more specifically assess the oxPPP's role in cancer, recent studies have turned to genetics, finding that the oxPPP (specifically G6PD) can, remarkably, transform cells when overexpressed but nevertheless is not essential for tumour growth[144,145]. Upon oxPPP loss, other cytosolic NADPH-producing enzymes, ME1 and IDH1, increase NADPH production to compensate. These alternative routes, however, are insufficient to maintain as high an NADPH/NADP ratio as the oxPPP, leading to downstream consequences including altered folate redox status due to impairment of dihydrofolate reductase activity by NADP[57]. Thus, among various NADPH-production routes, the PPP particularly effectively supports nucleotide synthesis in proliferative cells such cancer cells[57].

Alternative NADPH-production routes to the oxPPP also probably lack the dynamic flexibility to turn up NADPH production quickly. As a result, cancer cells lacking the oxPPP may fare poorly during bouts of high oxidative stress such as during metastasis[146,147]. Levels of PPP enzymes and metabolites are increased at metastatic sites relative to primary tumours[148,149]. In mouse models of melanoma metastasis, G6PD deficiency decreases circulating melanoma cells and the metastatic burden[149]. Efficient metastasis is also associated with higher levels of the lactate transporter MCT1. For reasons that remain unclear, MCT1 inhibition led in melanoma xenografts to decreased oxPPP flux (relative to glycolysis) and increased ROS levels[50]. Furthermore, the rapid PPP activation upon anchorage independent growth, which involves the production of increased levels of oxidant, might support metastasis formation[37]. Combined, these studies suggest that inhibition of the oxPPP may be effective in decreasing metastasis.

Oncogenic context.—The functional importance of the oxPPP is increased in tumours that upregulate the master antioxidant defence transcription factor NRF2. Such upregulation most frequently occurs via loss-of-function mutations in KEAP1, the E3 ubiquitin ligase that degrades NRF2. *KEAP1* mutations occur in about 20% of non-small-cell lung carcinoma, where they are associated with poor outcomes including immunotherapy resistance[150–153]. NRF2 induces expression of antioxidant genes including those encoding PPP enzymes[26,154]. In a CRISPR screen of metabolic enzymes, G6PD stood out for being selectively essential in *KEAP1*-mutant cancer cells[26] (Fig. 6a). In agreement with the screen data, *G6PD* knockdown suppressed preferentially *KEAP1*-mutant lung tumour growth. Thus, the oxPPP is a validated target in a type of lung cancer with high unmet need.

There are additional contexts in which inhibiting the PPP may be advantageous. In acute myeloid leukaemia cell lines harbouring mutations in the receptor tyrosine kinase FLT3, knockdown of *G6PD* was observed to overcome resistance to FLT3 inhibitors[155]. More generally, certain oncogenes increase demand for cytosolic NADPH and therefore would logically be expected to augment oxPPP dependency. For example, phosphoinositide-3-kinase (PI3K)–Akt signalling can increase NRF2 nuclear availability, promoting increased PPP enzyme expression[25]. Similarly, oncogenic mutations in *IDH1* (mtIDH1) change the protein from being an NADPH producer to an NADPH consumer that makes the oncometabolite 2-hydroxyglutarate. This NADPH stress suppresses the rate of lipogenesis

and increases sensitivity to oxidative stress in mtIDH1 cells in culture[156], despite mtIDH1 cells exhibiting increased oxPPP flux[157]. Experiments are needed to assess whether these flux changes lead to a functional dependence on the oxPPP (Fig. 6b).

Another logical way to inhibit tumour growth is to induce ribose limitation by inhibiting the PPP. The oncogene *BCR-ABL1*, which is a fusion of two genes (breakpoint cluster region (*BCR*) and abelson 1 (*ABL1*)), can decrease oxPPP flux, leading to dependence on non-oxPPP for ribose-5-phosphate synthesis. A thiamine analogue, oxythiamine, which inhibits the thiamine-dependent enzyme TK, is effective in mouse models of *BCR-ABL1*-driven cancer[158]. Oxythiamine has also been seen to be effective in multiple types of metastatic cancers. Further work is needed to determine whether this activity occurs via ribose limitation or redox effects[159–161].

Combination therapies.—Cancer treatments, including radiation, chemotherapy and immunotherapy, have the potential to cause oxidative stress. Chemotherapeutic agents have a wide variety of mechanisms of action and therefore generate various levels of ROS. Anthracyclines, which include doxorubicin and daunorubicin, generate high ROS levels[162]. Increased G6PD activity is observed in cancer cells exposed to doxorubicin, and knockdown of *G6PD* kills doxorubicin-resistant cells[163,164]. Alkylating agents and platinum coordination complexes, including cisplatin and oxaliplatin, also cause ROS production[165]. Decreased PPP activity, induced by a variety of indirect methods, enhances killing of colorectal cancer cells by platinum agents[166–169].

Radiotherapy causes the production of ROS and RNS. There is evidence that levels of PPP intermediates increase in response to radiation in culture[170]. But, despite many papers suggesting that radiotherapy should be used in combination with PPP inhibition, experimental evidence is missing. Experimental testing of the safety and efficacy of oxPPP inhibition in combination with radiotherapy is merited.

In addition to chemotherapy and radiotherapy, targeted metabolic inhibitors may synergize with oxPPP blockade. Logical synthetic lethal targets include the non-oxPPP to cut off its contribution to ribose supply during pentose insufficiency or IDH1 and ME1, the enzymes that compensate for loss of NADPH produced by oxPPP. IDH1 inhibitors were developed as part of the process of targeting mutant IDH1, with the Food and Drug Administration-approved mutant IDH1 inhibitor ivosidenib having activity also against the wild-type enzyme. Ivosidenib has more potent cytotoxic effects under conditions of low glucose, consistent with cytotoxic activity occurring when IDH is blocked and the PPP is limited by substrate availability. In mouse models of pancreatic cancer, ivosidenib prevented tumour growth, and this was reversed by oral glucose supplementation[171].

Unfortunately, quality ME1 inhibitors for use in vivo are not yet available. But increased ME1 flux can be targeted indirectly. ME1 activation consumes TCA cycle four-carbon units. In both *KEAP1*-mutant tumours and in some metastatic melanomas, loss of G6PD led to depletion of TCA cycle intermediates. A major means of generating TCA cycle four-carbon units is glutamine catabolism, and its importance increases with G6PD inhibition. This leads to increased sensitivity to the glutaminase inhibitor CB-839, with the combination of

G6PD loss and glutaminase inhibitor being synergistic[149,172]. Thus, combination regimens incorporating agents targeting the PPP hold potential as cancer treatments.

Perspective and future directions

Current perspective

Despite alternative NADPH-production routes, the PPP plays a key role in redox metabolism in a variety of biological contexts. This reflects its often being the fastest NADPH producer, with the greatest acute responsiveness to increased NADPH demand and with the capacity to generate the highest NADPH/NADP ratio[57]. In addition to supporting biosynthesis, NADPH produced in the oxPPP can be either antioxidant or pro-oxidant. NADPH's antioxidant function involves the reduction and removal of ROS, RNS and their downstream products, such as oxidized and nitrosylated thiols in proteins[173]. Its pro-oxidant role involves purposeful production of ROS and RNS for signalling, antimicrobial killing and other purposes[174–178]. This dual role of the PPP and NADPH mirrors the duelling biological impact of free radicals. For instance, ROS at lower doses stimulates cancer cell migration but at higher doses is inhibitory[179]. ROS can promote tumorigenesis by increasing genetic instability during cancer initiation, but, after the tumour is established, ROS can limit cancer cell survival and growth.

To make sense of the PPP and of redox metabolism more generally, it is key to move from generalizations (for example, ROS cause cancer) to time-dependent, location-dependent and molecule-specific roles. For example, soon after classical activation, macrophages produce large amounts of ROS powered by the oxPPP and NOX. This ROS is largely directed to the phagosome and extracellular space. This localization promotes pathogen killing and limits host oxidative stress. After the initial response resolves, repair of oxidatively damaged cellular components also requires NADPH. Accordingly, manipulating PPP at different times may have vastly different effects. Thus, to develop therapies, spatial and temporal understanding will be critical.

Moving forward

How can we achieve spatial and temporal understanding? Progress rests on three pillars: genetics, pharmacology and analytics. Genetics and pharmacology enable PPP manipulation (Box 1). Genetic methods for cell type-specific enzyme manipulation in mice are readily available. Their systematic application to the PPP, for example, to manipulate G6PD and 6PGD in T cells, should be highly informative, especially as initial data suggest that these oxPPP enzymes may sometimes have opposite immunological functions. Genetics can also help to inform the roles of non-oxPPP reactions, including newly discovered orphan reactions connected to non-oxPPP such as sedoheptulose kinase.

Pharmacology is an important complement to genetics, due to its capacity for partial enzyme inhibition, dynamic target engagement and eventual clinical translation. Given the shifting roles of the PPP during disease processes, timed pharmacological interventions could be particularly informative, but unfortunately many PPP enzymes continue to lack specific

inhibitors, and existing inhibitors require further optimization to enable routine in vivo application. Better compounds are needed.

The ability to make dynamic and spatially resolved in vivo measurements is also critical. Historically, such measurements of metabolism have been limited, but capabilities are rapidly advancing. The major intermediates of the PPP as well as NADP and NADPH can now be routinely measured from tissue extracts by liquid chromatography–mass spectrometry. Excitingly, imaging mass spectrometry, in which a tissue slice is coated with matrix and then ionized by a laser pulse (MALDI), offers the prospect of visualizing them at single-cell resolution[180,181], although proof of principle for PPP metabolites remains to be established. Other technologies, such as water gas cluster ion beam secondary ion mass spectrometry hold potential for subcellular resolution but remain farther from widespread application[181–183]. Mass spectrometry approaches can also be coupled to isotope tracing to gain insights into flux, for example, using [1,2-^{13}C]glucose and searching by imaging mass spectrometry for glycolytic intermediates with one ^{13}C atom to capture oxPPP overflow flux at the single-cell level.

Fluorescent methods are an important complement to mass spectrometry. Fluorescent sensors of $NADP^+$ and NADPH can be used to study the PPP at the subcellular level[184–186], with potential also for dynamic intravital analyses[184,187]. Sensors also exist for glucose but not yet for any dedicated PPP metabolite[188]. The development of such sensors would be valuable. Better measurements of specific ROS and RNS species and their covalent modifications of lipids and macromolecules is another important frontier. Ultimately, systematic application of genetics combined with improved pharmacological and measurement tools will help drive understanding of the PPP temporally and spatially. In so doing, it will also hopefully open the doors to new diagnostics and therapeutics.

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BOX 1

Manipulating the PPP

Both genetics and pharmacology can be used to manipulate the PPP. The development of CRISPR–Cas9 has enabled easy knockout of genes encoding various PPP enzymes in cultured cells and a faster way to make genetically engineered mouse models. In addition to targeting enzymes in the PPP, NADPH levels can be genetically manipulated through an engineered enzyme called triphosphopyridine nucleotide oxidase (TPNOX)[189], which converts NADPH and oxygen to NADP and water. NADPH can similarly be depleted by a small molecule, phenazine methosulfate. TPNOX and phenazine methosulfate are valuable tools for determining the importance of NADPH in regulating PPP flux.

Several inhibitors are also available to modulate the oxPPP. Dehydroepiandrosterone has been used extensively to target G6PD, but its cellular effects may be largely off target[98,190]. The molecule 6-aminonicotinamide broadly targets NAD(P) enzymes, with strong engagement of 6PDG in the oxPPP. Despite its lack of specificity, it is a useful tool. A new G6PD inhibitor (G6PDi1) with effective cellular target engagement has recently been developed[98]. A small-molecule activator (AG1) has also been identified[191,192]. Development of improved tool compounds, including ones with better specificity and with pharmacokinetics enabling in vivo application, will be valuable in advancing our understanding of the pathway.

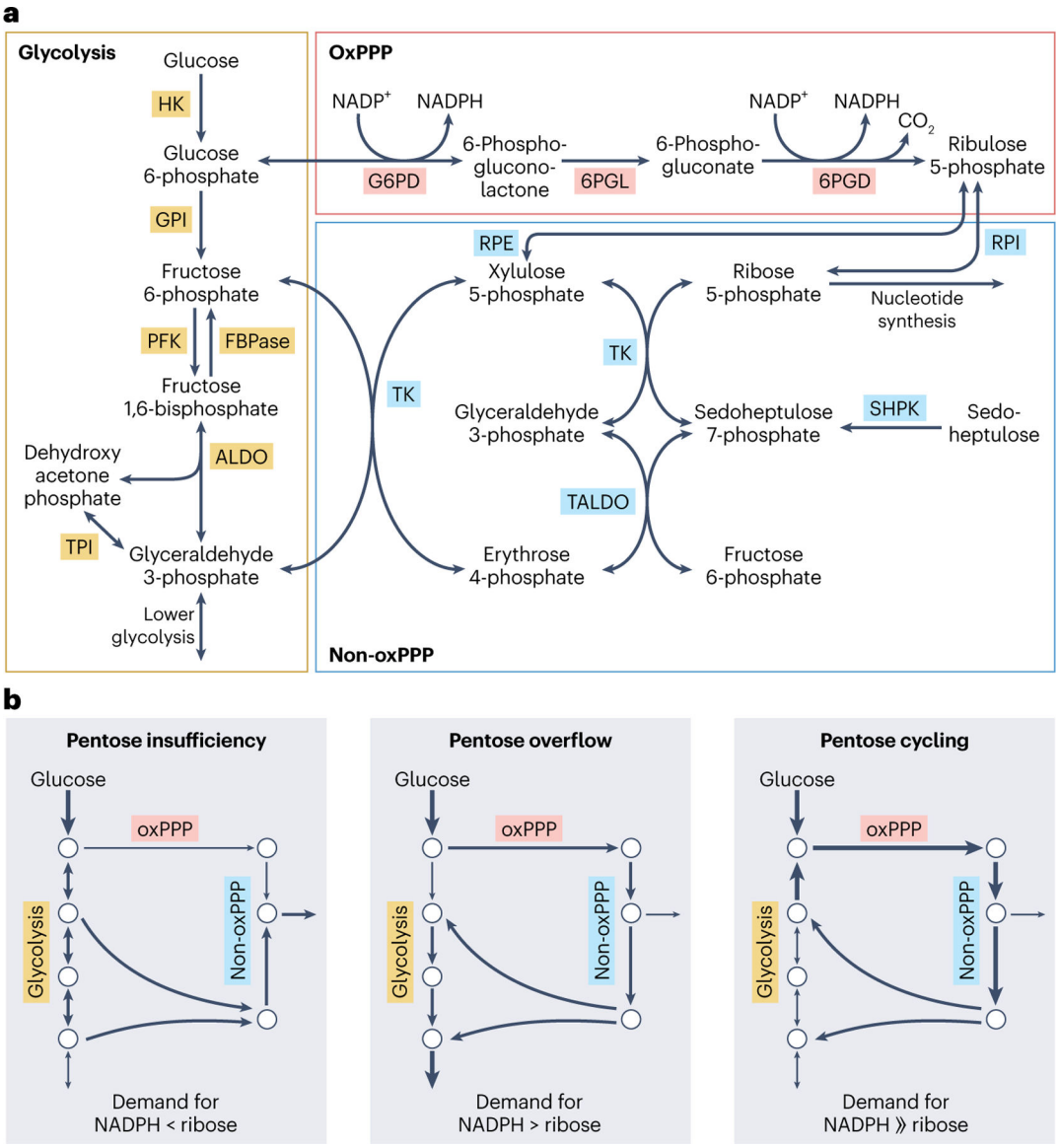

Fig. 1 |. The PPP and its modes of operation.

a, Overview of the oxPPP, the non-oxPPP and their connections to glycolysis. Each glucose that goes through the PPP can generate two NADPH molecules and one ribose-5-phosphate molecule. Abbreviations: HK, hexokinase; GPI, glucose-6-phosphate isomerase; RPE, ribulose-phosphate 3-epimerase; RPI, ribose-5-phosphate isomerase; FBPase, fructose 1,6-bisphophatase; ALDO, fructose-bisphosphate aldolase. **b**, Modes of PPP operation. Unmet ribose demand (that is, pentose insufficiency) leads to net non-oxPPP flux toward ribose-5-phosphate synthesis. Higher NADPH demand than ribose demand (after accounting for 2:1 pathway stoichiometry) causes non-oxPPP flux in the opposite direction, from ribose 5-phosphate toward glycolysis (that is, pentose overflow). Very high NADPH demand can lead to pentose cycling, in which the glycolytic enzyme 6-phosphate isomerase runs in reverse to make additional glucose 6-phosphate to feed the oxPPP.

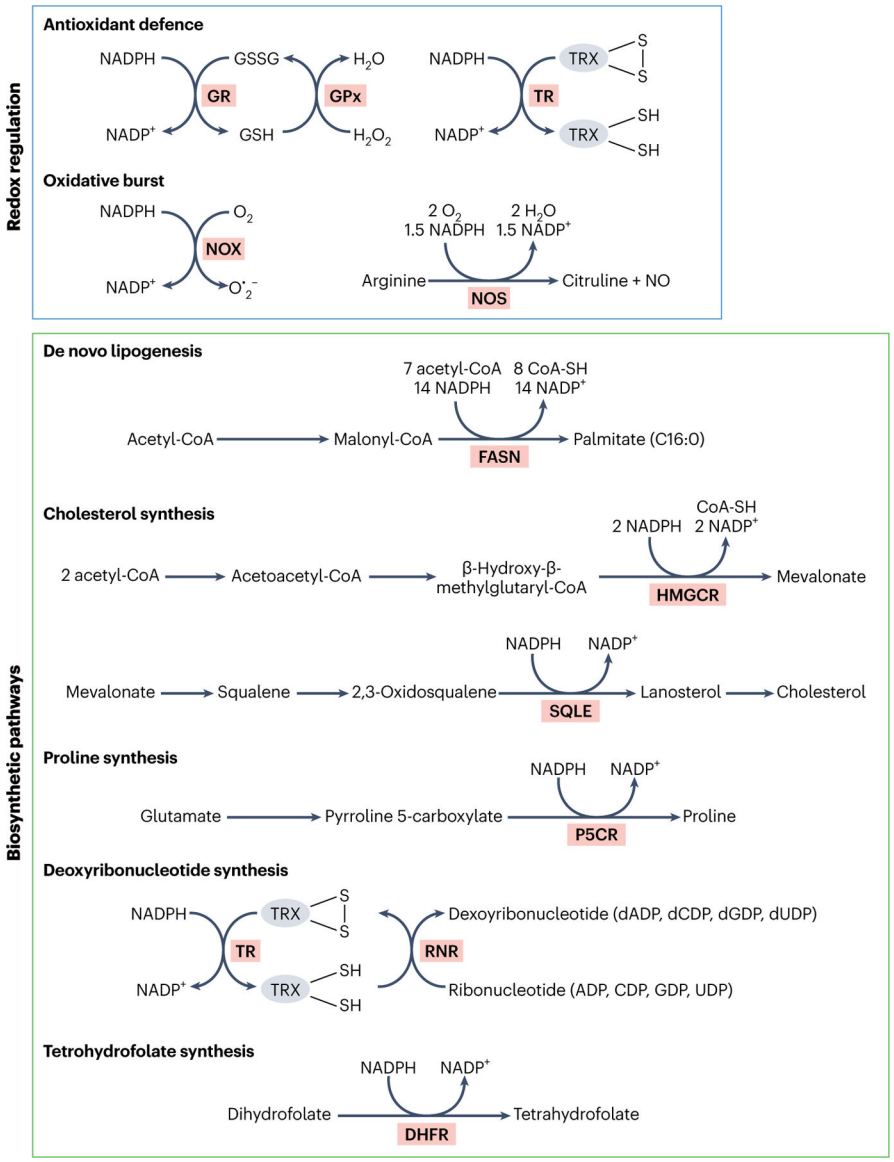

Fig. 2 |. Major NADPH-consuming pathways.

Abbreviations: CoA, coenzyme A; GR, glutathione reductase; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; TR, thioredoxin reductase; FASN, fatty acid synthase; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; SQLE, squalene epoxidase; P5CR, pyrroline-5-carboxylate reductase; RNR, ribonucleotide reductase; DHFR, dihydrofolate reductase; TRX, thioredoxin.

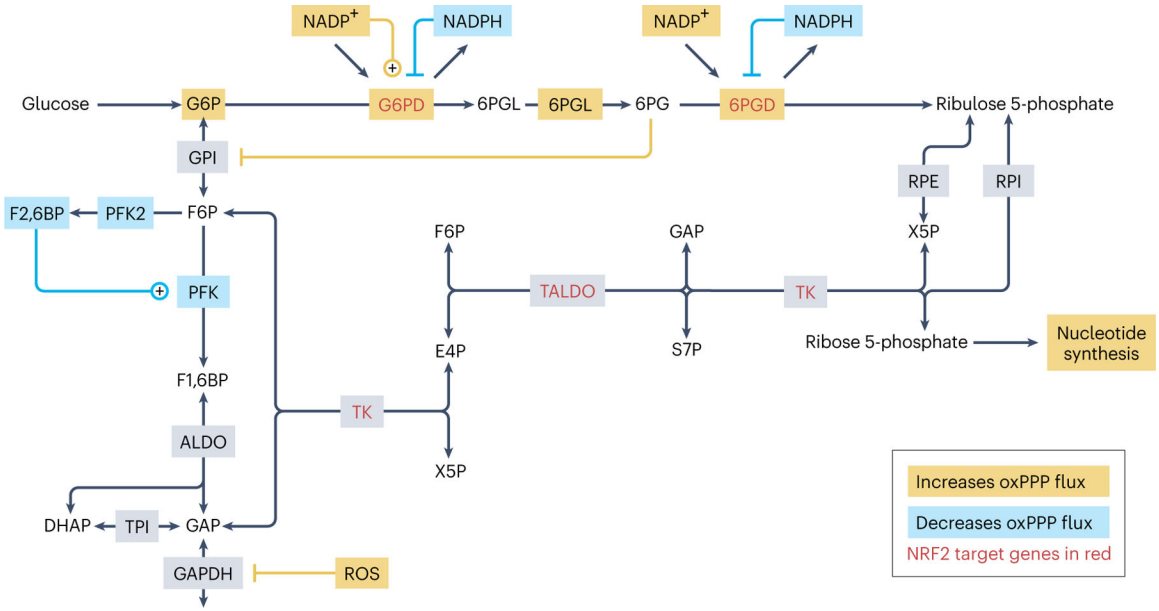

Fig. 3 |. Regulation of the PPP.

The PPP and glycolysis compete for carbon flux. Factors that increase oxPPP flux are highlighted in yellow, and those that decrease it are in blue. Names of enzymes induced by NRF2 are in red. E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; F1,6BP, fructose 1,6-biphosphate; F2,6BP, fructose 2,6-biphosphate; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; S7P, sedoheptulose 7-phosphate; X5P, xylulose 5-phosphate.

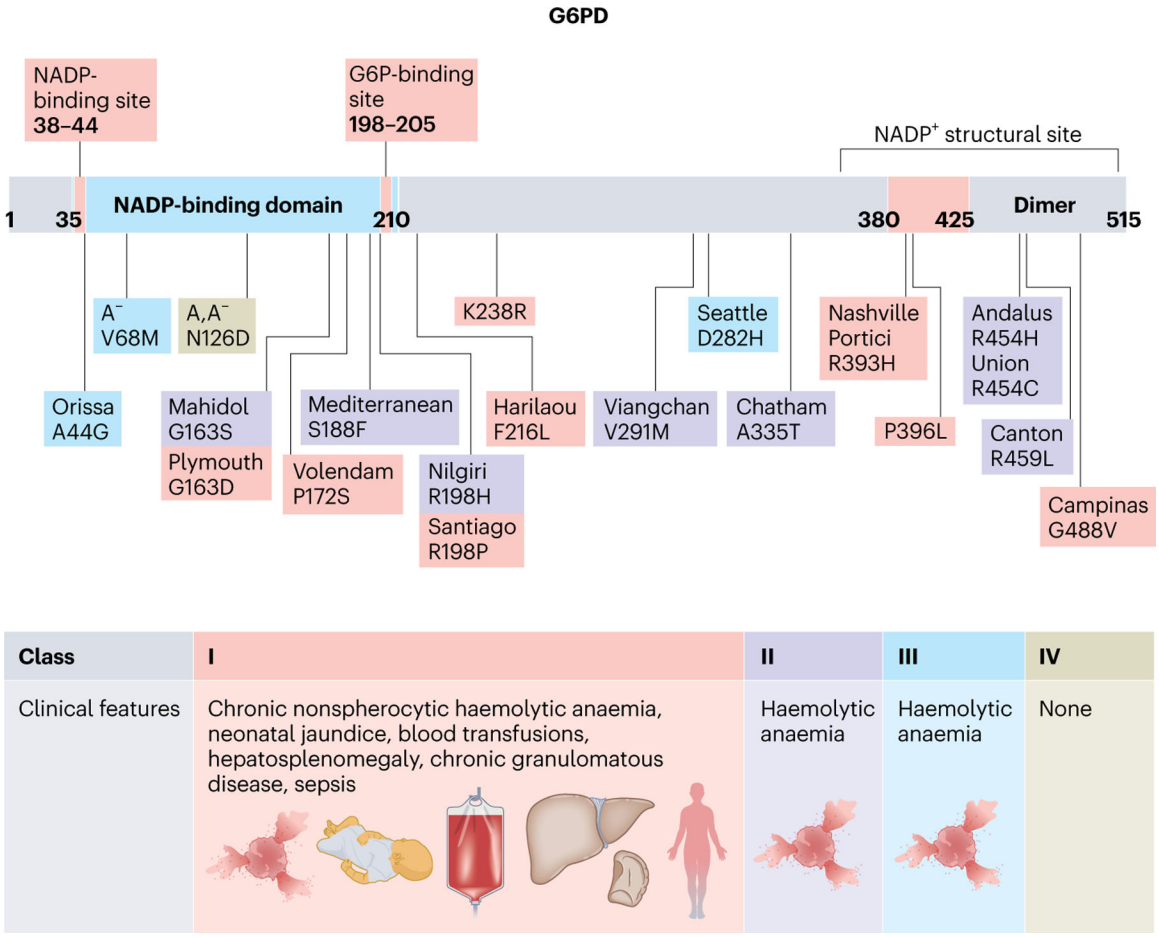

Fig. 4 |. G6PD deficiency leads to RBC and immune dysfunction.

The best-studied mutations in G6PD and their locations within the protein. Mutations are coloured according to their clinical phenotype from most to least severe: class I mutations in red, class II in purple, class III in blue and class IV in beige. The most severe class 1 mutations cluster around the glucose-6-phosphate (G6P) binding site, the dimer interface and the $NADP^+$ structural site that is involved in allosteric activation and homotetramer formation.

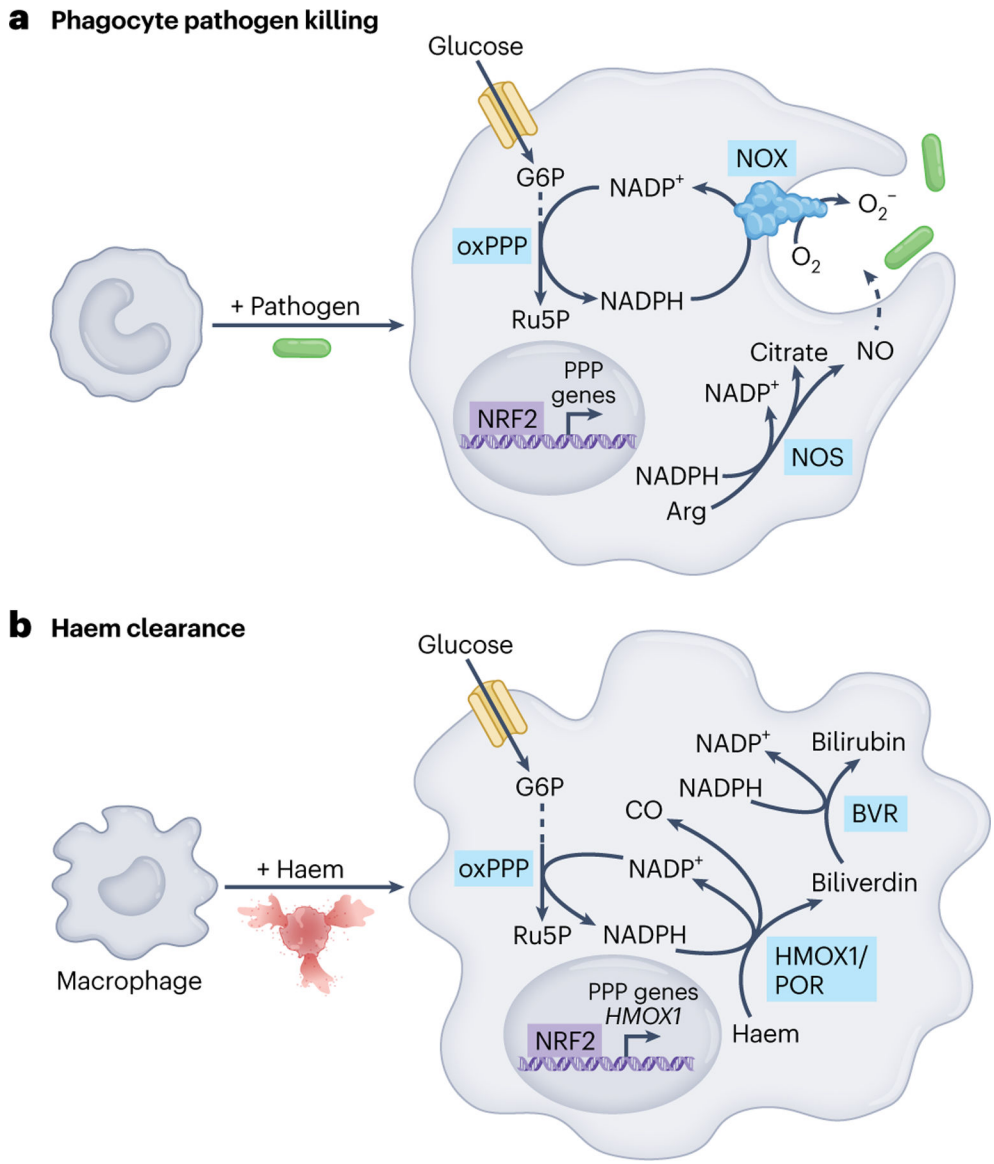

Fig. 5 |. The role of oxPPP-produced NADPH in phagocyte function.

a, In phagocytic cell types including macrophages and neutrophils, NADPH production by the oxPPP supports production of superoxide by NOX and nitric oxide by NOS for killing pathogens in the phagosomes and extracellular space. Ru5P, ribulose 5-phosphate. **b**, In macrophages involved in haem clearance, NADPH supports the breakdown of haem into biliverdin by HMOX with the help of p450 oxoreductase (POR) and biliverdin into bilirubin by biliverdin reductase (BVR).

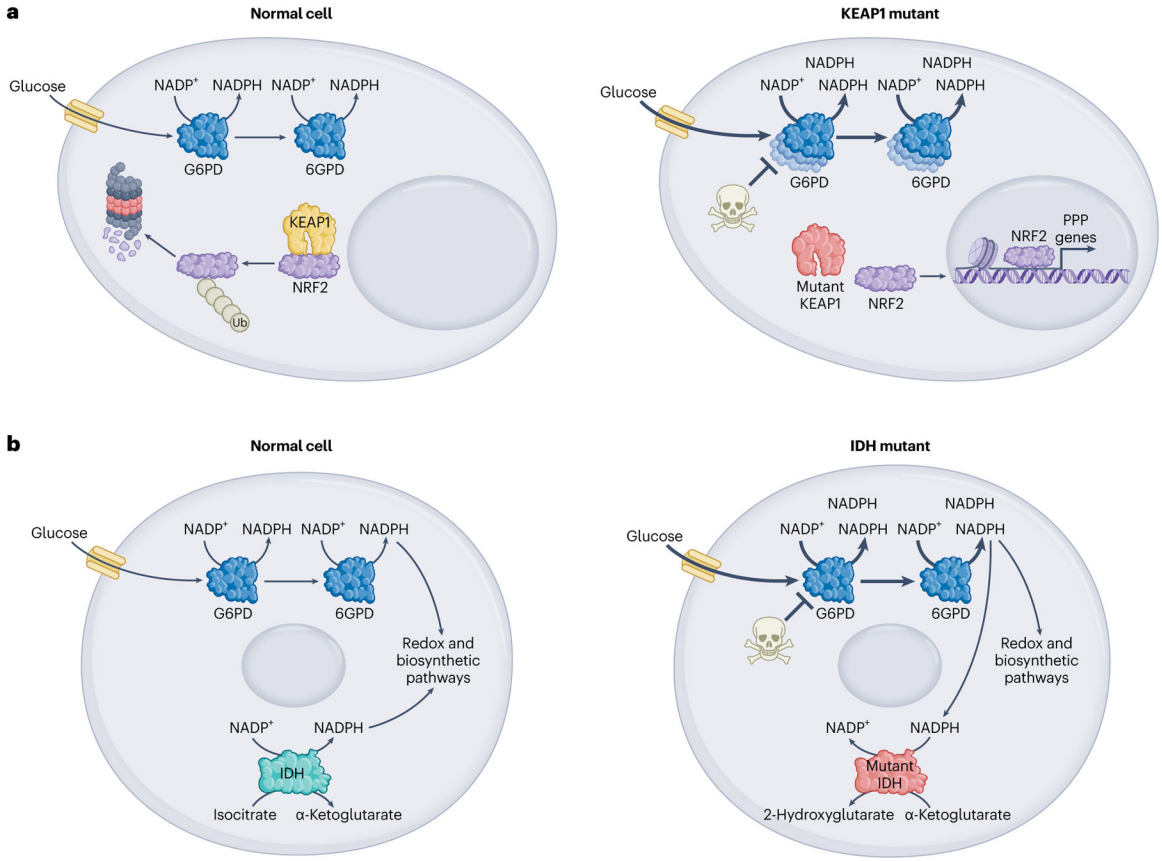

Fig. 6 |. Oncogenic contexts for targeting the PPP.

a, *KEAP1* mutations lead to stabilization of NRF2, which promotes transcription of PPP genes and leads to dependency on their enzyme activity. Ub, ubiquitin. **b**, Mutations in IDH1 convert IDH from an NADPH producer into a consumer. Therefore, the oxPPP becomes a more important source of NADPH with mutant IDH.