**Abstract**

The mammalian retina converts most glucose to lactate rather than catabolising it completely to carbon dioxide via oxidative phosphorylation, despite the availability of oxygen. This unusual metabolism is known as aerobic glycolysis or the Warburg effect. Molecules and pathways that drive aerobic glycolysis have been identified and thoroughly studied in the context of cancer but remain relatively poorly understood in the retina. Here, we review recent research on the molecular mechanisms that underly aerobic glycolysis in the retina, focusing on key glycolytic enzymes including hexokinase 2 (HK2), pyruvate kinase M2 (PKM2) and lactate dehydrogenase A (LDHA). We also discuss the potential involvement of cell signalling and transcriptional pathways including phosphoinositide 3-kinase (PI3K) signalling, fibroblast growth factor receptor (FGFR) signalling, and hypoxia-inducible factor 1 (HIF-1), which have been implicated in driving aerobic glycolysis in the context of cancer.

**Aerobic glycolysis/the Warburg effect in the retina**

In most non-dividing mammalian cells, pyruvate produced by glycolysis is predominantly oxidised in mitochondria via the tricarboxylic acid (TCA) cycle, fuelling efficient ATP production by oxidative phosphorylation (OXPHOS). However, in proliferating cells, notably in many types of cancer cells, glucose uptake is increased, the rate of glycolysis is elevated, and most pyruvate is instead reduced to lactate which is exported from the cell, a much less efficient method of generating ATP[1,2]. This metabolic reprogramming occurs even in cells with functional mitochondria and ample oxygen availability, and is hence termed aerobic glycolysis, or the Warburg effect[3,4].

The mammalian retina is one of few non-proliferative tissues to exhibit aerobic glycolysis[4-8]. For example, in adult rat retinal explants, approximately ninety percent of the glucose that is consumed is exported as lactate, even when the explants are incubated in highly oxygenated medium[8]. High rates of aerobic lactate production and export have also been demonstrated *ex vivo* in species including mouse[9], rabbit[10,11], cattle[12] and ox[13]. Similar findings *in vivo* in anesthetised cats and pigs, by measurement of retinal arteriovenous lactate, glucose and oxygen differences, have confirmed the physiological translation of observations of aerobic glycolysis made in explants[14,15].

Many studies have investigated the effects of glycolytic inhibition on retinal function and survival. Ames and Gurian showed the importance of aerobic glycolysis for retinal function with the omission of glucose from medium leading to a rapid decrease in electrical responses of rabbit retinas *ex vivo*[16]. Likewise, Winkler found in rat retinas that glucose deprivation, or treatment with the glycolysis inhibitors iodoacetate or 2-deoxyglucose, caused the loss of fast PIII wave amplitude[7,8]. In mouse explants, Chertov et al. showed that glycolysis was necessary for photoreceptor survival, and that the supply of alternative mitochondrial fuels could delay but not prevent the increased cell death caused by glucose deprivation[17]. Altogether, these data demonstrate that glycolysis is essential for retinal function and survival, and why disruption of retinal glucose metabolism is an expected cause of visual disease. There are now growing bodies of evidence to suggest that altered glucose metabolism contributes to the pathology of age-related macular degeneration and retinitis pigmentosa[18-23].

It should be recognised that oxygen is rapidly consumed by the retina, and is essential for ATP production and retinal function regardless of glucose availability[11,16]. In vascular mammalian retinas, such as those of the cat, pig and rat, microelectrode measurements of oxygen tension throughout the retina have demonstrated that the photoreceptors are well-oxygenated by the choroid, and that the inner layers are oxygenated by the retinal vasculature[24-27]. This is consistent with lactate production via aerobic glycolysis. Furthermore, the fact that rat retinas incubated *ex vivo* with medium saturated with 95% oxygen convert approximately 90% of glucose to lactate is further evidence that lactate

production in the rat retina is not predominantly driven by hypoxia[8]. Similarly, experiments *in vivo* in anesthetised cats showed that only a portion of retinal lactate production was lost under conditions of hyperoxia compared to normoxia, supporting the conclusion that most lactate is produced aerobically in normoxia[14].

However, not all of the retina is well oxygenated. For example, the outer nuclear and outer plexiform layers may reach almost anoxic conditions in darkness, when oxygen consumption by photoreceptors is highest. Similarly, in avascular retinas such as those of the rabbit and guinea pig, the choroid supplies oxygen to the whole retina via diffusion, so the distant inner retinal layers operate at low oxygen tension[28]. It is therefore possible that a small proportion of lactate production in vivo is due to anaerobic glycolysis. In this case, hypoxia-inducible factors (HIFs) would be the most likely candidates in driving lactate production. Their putative role in retinal glycolysis, discussed in this review, is relevant when considering conditions of either hypoxia or normoxia, and would only differ in their mode of activation.

It is now well-established that aerobic glycolysis is essential for normal retinal function, but the benefits that it confers to offset its apparent inefficiency remain incompletely resolved. Possible reasons for aerobic glycolysis in the retina include the generation of metabolites for the biosynthesis of photoreceptor outer segments[5,9], supplementary ATP production to meet demand that may exceed oxidative capacity[29], production of lactate as a fuel for particular cell types, including Müller glia or retinal pigment epithelial cells[30], or rapid ATP production to meet fluctuating energy demand in stimulated neurons, as has been postulated in the brain[31]. This fundamental question may be more conclusively addressed with improved understanding of the molecular mechanisms that drive aerobic glycolysis in the retina.

In this review, we discuss selected molecules and signalling pathways that commonly drive aerobic glycolysis in cancer cells, and what is known regarding the involvement of these pathways in retinal metabolism and function, with a focus on recent findings. Several studies have begun to reveal or call into question roles for key molecules in driving retinal glycolysis, filling in important pieces of the puzzle, but we still lack a complete picture. Understanding the drivers of aerobic glycolysis may provide the basis for development of novel therapeutic approaches for retinal diseases of metabolic cause. Additionally, if the roles of these pathways are conserved between cancer and the retina, then the implications for cancer therapies designed to target glucose metabolism need to be carefully considered in light of potential damage to the retina.

**Drivers of aerobic glycolysis in the retina**

A switch in glucose metabolism from oxidative phosphorylation to aerobic glycolysis typically involves increased expression and activity of glucose transporters, glycolytic enzymes, enzymes converting pyruvate to lactate, and lactate exporters, together with a decrease in the activity of enzymes converting pyruvate into acetyl CoA and promoting its entry into the TCA cycle, in mitochondrial activity and oxygen consumption. Expression and activity of individual proteins directly involved in these pathways, as well as their control by various signalling pathways, specific kinases and transcription factors have been postulated to control aerobic glycolysis in cancer (reviewed in refs[1,32]), and a number of these have been directly examined for their roles in retinal glycolysis or visual function. The following discussion focusses on analyses of some of these postulated drivers in the context of aerobic glycolysis in the mammalian retina.

**Lactate dehydrogenase A (LDHA)**

Lactate dehydrogenases (LDH) are enzymes that catalyse the reduction of pyruvate to lactate, which is coupled to the oxidation of NADH to $NAD^+$. They can also catalyse the reverse reaction. The two most widely-expressed LDH isoforms in mammals are LDHA and LDHB, which are encoded by separate genes[33,34]. LDHA and LDHB homo- or hetero-tetramerise to form five possible complexes distinguished by the number of A and B subunits. Some evidence indicates that LDHA, to a greater extent than LDHB, favours the reduction of pyruvate to lactate over the reverse reaction, enabling it to support higher rates of glycolysis through faster regeneration of $NAD^+$, especially at high pyruvate concentrations[35-37]. In general, expression of LDHA is high in highly glycolytic physiological tissues, such as skeletal muscle, whereas LDHB dominates in aerobic tissues, such as the heart[38]. LDHA is also commonly upregulated in cancers, often due to HIF-1-mediated transcription as a result of intratumoural hypoxia or aberrant HIF activation[33,39].

By immunohistochemistry (IHC) and in situ hybridisation (ISH), LDHA expression has been detected in all layers of the mouse retina, except the inner plexiform layer, with strongest expression in the photoreceptors, especially their inner segments[9,40]. The same pattern was observed in rat, marmoset and human retinas and, assuming that LDHA expression correlates with lactate production, suggests that photoreceptors are the dominant, although possibly not exclusive, producers of lactate in the retinas of most tested mammals[41]. The concept of photoreceptors being the main source of lactate production is supported by the work of Lindsay et al. who found that retinas from $AIPL1^{-/-}$ mice, which lack photoreceptors, contained 74% less lactate than retinas from normal mice[42]. Surprisingly however, in one study LDHA was not detected in the rod photoreceptors of the avascular rabbit retina[41].

In rats with inherited retinal degeneration, it was shown that there was a shift in the proportions of tetrameric LDH isozymes from LDHA-dominant to LDHB-dominant forms[43]. This isozyme-shift slightly preceded both a decrease in the overall retinal LDH activity and the onset of morphological signs of retinal degeneration, indicating that it may have been a causative factor in the degeneration. The regional or cell type-specific requirement for LDHA in this study was unclear. Recently, Chinchore et al. performed a series of experiments that tested the function of several potential drivers of aerobic glycolysis in mouse retinas, including LDHA[9]. Pharmacological inhibition specific for LDHA in retinal explants decreased lactate production by approximately 70%. The researchers also used the LMOPC1 mouse[44], which harbours Cre recombinase driven by a 4.1 kb rhodopsin promoter, to achieve the knockout of LDHA in 50-90% of rod photoreceptors. They found that lactate production in retinal explants from these mice was approximately half that of control mice. These data show that LDHA is necessary for normal lactate production in the mouse retina, and confirm the notion that rods are prodigious lactate producers. There were also morphological consequences of LDHA disruption. *In vivo* electroporation of shRNA targeting LDHA caused a large decrease in rod outer segment (OS) length over 45 days, and maintenance of the OS was dependent on LDHA catalytic activity[9].

It is evident that LDHA is required for preservation of photoreceptor morphology and consequently probably retinal function, although to our knowledge retinal function after LDHA inhibition has not been directly tested. The necessity of LDHA in particular cell types, especially of the inner retina, remains relatively unexplored, as do the factors that control its expression. Furthermore, while high LDHA expression is required for lactate production, LDHA expression alone is not thought to be sufficient to drive aerobic glycolysis, which is dependent on upregulated glucose uptake and conversion of glucose to pyruvate. In cancer cells, these processes are contingent upon transcriptional and cell signalling programs that direct the expression and activities of numerous upstream

components of glucometabolic pathways, in addition to LDHA. So, although LDHA is necessary for aerobic glycolysis in the retina, it is unlikely a driver per se.

**Hexokinase 2 (HK2)**

Hexokinases (HKs) catalyse the ATP-dependent phosphorylation of glucose to form glucose-6-phosphate in the first step of glycolysis (Figure 1). There are four hexokinase isoforms in mammals, denoted HK1-4. HK1-3 have a high affinity for glucose, and especially HK1 and 2 are able to support the elevated rates of glucose phosphorylation demanded by highly glycolytic tissues[45,46]. This efficiency of HK-mediated glucose phosphorylation by HK1 and HK2 is largely due to their ability to bind voltage-dependent anion channels (VDAC) on the outer mitochondrial membrane, an interaction which spatially links glycolysis and OXPHOS by providing HK1 and HK2 with immediate access to mitochondrially-produced ATP (Figure 1)[47]. The interaction is governed in part by phosphoinositide 3-kinase (PI3K)/Akt signalling[48,49].

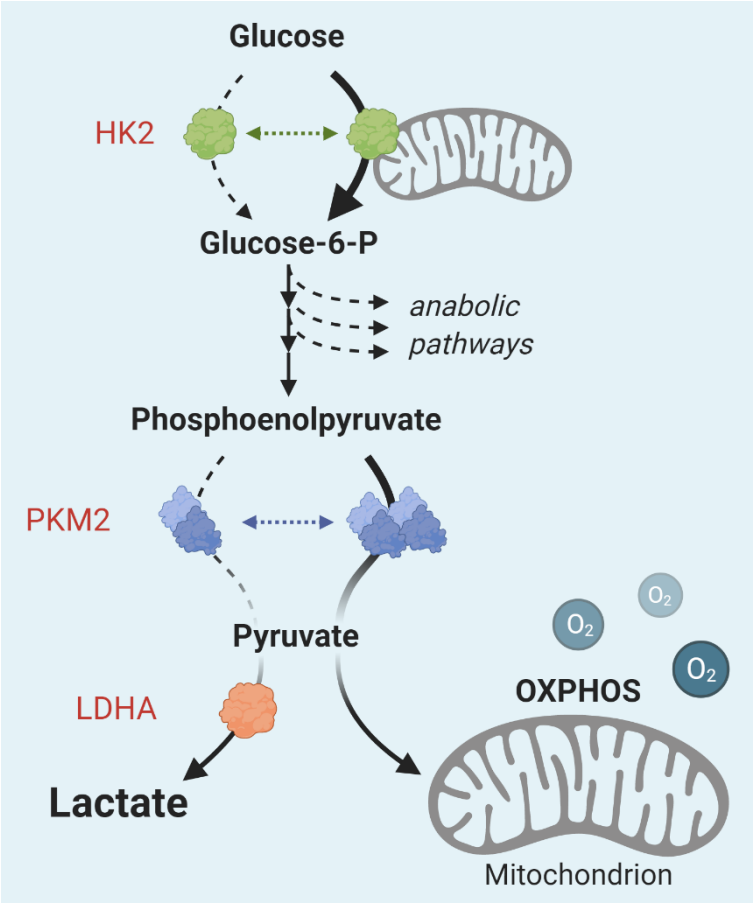


Figure 1. Glycolysis can be modulated by multiple mechanisms. Mitochondrial localisation of hexokinase 2 (HK2) can increase glucose phosphorylation. The glycolytic enzyme pyruvate kinase M2 (PKM2) can adopt a highly active tetrameric form or a less active dimeric form. The dimeric form is, somewhat paradoxically, associated with increased rates of aerobic glycolysis in cancer cells. It can promote the siphoning of upstream glycolytic metabolites into anabolic pathways for the production of nucleotides, lipids and amino acids. Tetramerisation of PKM2 is positively regulated by the glycolytic metabolite fructose-1,6-bisphosphate, and negatively regulated by high tyrosine kinase activity. Downstream of PKM2, expression of lactate dehydrogenase A (LDHA) can favour lactate production over oxidative phosphorylation (OXPHOS).

HK1 is the isoform most widely expressed in adult mammals, with expression of HK2 being much more restricted, generally to insulin-sensitive tissues[45]. HK2, however, is most commonly upregulated in cancer, where it drives aerobic glycolysis. In various cancer types including glioblastoma multiforme, medulloblastoma, and pancreatic ductal adenocarcinoma, HK2 expression has been shown to promote lactate production and tumour growth[50-52]. HK2, given that it catalyses the first step of the glycolytic pathway, cannot via its known enzymatic functions directly divert the fate of pyruvate from mitochondrial oxidation to reduction to lactate. Rather, it facilitates aerobic glycolysis by promoting high glycolytic flux. The differential expression of HK2 between normal and cancerous tissues and its role in cancer metabolism and growth render it a promising target for selective anti-cancer therapies.

In the mouse and rat retina, HK2 is most strongly expressed in the inner segments of both rod and cone photoreceptors, and also the perinuclear region specifically in cones[21,40,53,54]. This pattern is consistent with localisation to mitochondria. Reports of HK2 expression in the outer plexiform layer vary from as strong as its expression in photoreceptors[40], to weak[53], or undetectable[21,54]. HK1 is the dominant HK isoform in the inner retina, expressed from the outer plexiform layer to the ganglion cell layer, but like HK2 it is also detectable in photoreceptor inner segments, albeit relatively weakly[40,53].

Petit et al. used the iCre75 mouse[55] which, similar to the LMOPC1 mouse, expresses Cre under the control of a 4 kb rhodopsin promoter, to conditionally knock out HK2 in mouse rod photoreceptors[21]. They achieved knockout in almost 90% of rods. Explants of heterozygous and homozygous knockouts produced 25% and 33% less lactate than control retinas, respectively. They did not display decreased glucose uptake however, supporting a genuine shift in the metabolic balance from aerobic glycolysis to OXPHOS. Mechanistically, this shift may have been enabled by the observed increase of greater than four-fold in the number of mitochondria in the outer nuclear layer, specifically the rod perinuclear regions. Expression of Glucose Transporter 1 (GLUT1) and HK1 were also higher in total retinal extracts of homozygous rod HK2 knockouts[21].

Functionally, the rod HK2 knockout mice displayed diminished scotopic a- and b-wave amplitudes, and the b-wave implicit time was higher compared to controls, evident after one month of age[21]. HK2 is therefore required for normal rod function. However, neither rod survival nor morphology was affected by HK2 knockout over a period of twelve months. This was surprising given the earlier report of Chinchore et al. which, although not directly studying HK2, demonstrated the importance of aerobic glycolysis in the maintenance of rod outer segments[9]. The discrepancies may be reconcilable in the activation of compensatory mechanisms unique to disruption of particular genes. An example of such a putative compensatory mechanism is the increase in rod perinuclear mitochondria in the HK2 knockout mice[21]. Petit et al. proposed that these mitochondria provide an alternative means of NADPH production to the pentose phosphate pathway (PPP), and they may provide an alternative source of energy for rod terminal processes which have been proposed to normally be supplied with ATP from glycolysis[21,40,56]. These hypotheses will require direct investigation to confirm or rule out.

Cone-specific knockout of HK2 in mice by utilising Cre under the control of the human red/green pigment promoter (HRGP) did not induce cone cell death, or result in any detectable functional change in the retina[21,57]. HK2 is therefore required for normal function in rods but not cones. Interestingly, simultaneous deletion of HK2 from rods and cones resulted in reduced cone function[21]. This is consistent with the hypothesis that cones rely on lactate produced by rods, but the exclusion of other possible mechanisms requires further research. For example, cone function could conceivably be diminished due to altered availability of another metabolite such as glutamine, decreased oxygen availability due to the increased OXPHOS activity in rods, or impaired production of rod derived cone viability factor (RdCVF), a protein secreted by rods that stimulates aerobic glycolysis in cones[20].

Rajala et al. found that in both light- and dark-adapted rat and mouse retinas, HK2 associated with mitochondria, and more HK2 was present in mitochondrial extracts from light-adapted than dark-adapted retinas[54]. The light-associated increase in mitochondrial HK2 was dependent on insulin receptor signalling, which is primarily mediated by PI3K. Consistent with this, treatment with the PI3K inhibitor, LY294002, decreased the amount of HK2 in mitochondrial extracts from both light- and dark-adapted retinas. Further experiments elucidated a mechanism involving activation of Akt1 followed by deactivation of GSK-3β, dephosphorylation of VDAC, and therefore increased mitochondrial HK2 binding. It should be noted that LY294002 has inhibitory activity against multiple PI3K-related kinases including GSK3 at the concentration used[58]. Interestingly, the authors found less mitochondrial HK2 in type 1 diabetic mouse retinas compared to wild type. It remains to be confirmed whether this observation contributes to retinopathy in the mouse model, or indeed whether the subcellular localisation of HK2 has a significant effect on retinal metabolism, for example in modulating the glycolytic rate or fate of glucose.

Given that its enzymatic role is far upstream of the point at which pyruvate diverges to either lactate or OXPHOS, HK2 cannot alone be considered a driver of aerobic glycolysis. However, HK2 is necessary to maintain a normal level of aerobic glycolysis in mouse rod photoreceptors. It is not required in cones for function or survival, and its metabolic role in cones remains unclear. Its necessity in other retinal cell types and mammalian species is also unknown.

**Pyruvate kinase M2 (PKM2)**

Pyruvate kinases (PKs) are enzymes that catalyse the final step of glycolysis, the conversion of phosphoenolpyruvate to pyruvate (Figure 1), which is coupled to the phosphorylation of ADP to ATP. There are four PK isoforms in mammals, PKM1 (muscle 1), PKM2 (muscle 2), PKL (liver) and PKR (erythrocytes). Expression of PKL is confined to the liver and kidney, whereas PKR is expressed exclusively in erythrocytes. PKM1 and 2 are alternatively spliced products of the same gene, *Pkm*, that differ by a single exon[59], but they nevertheless have broad but unique expression patterns and significant functional differences[60,61]. PKM2 expression is widespread during embryonic development, and in most adult tissues, whereas PKM1 is expressed predominantly in highly catabolic tissues such as heart, brain and skeletal muscle. PKM1 and 2 both exist as enzymatically active tetramers, but PKM2 can also exist in a much less active dimeric form[62]. The enzymatic activity of PKM2 is lower than PKM1, and is also subject to complex regulation. For example, tetramerisation of PKM2 is allosterically promoted by the glycolytic metabolite fructose-1,6-bisphosphate (FBP) and negatively regulated by high tyrosine kinase activity due to the capacity of PKM2 to bind phospho-tyrosine residues, as well as direct phosphorylation of PKM2[62-65]. In proliferating cells, the low activity of the dimer is proposed to promote growth by facilitating the accumulation of glycolytic metabolites which can be siphoned into pathways for the biosynthesis of macromolecular precursors such as amino acids and nucleotides, whereas expression of PKM1 even in the presence of PKM2 can inhibit growth[66]. In the retina, it has been proposed that PKM2 promotes anabolic activity necessary for the maintenance of photoreceptor outer segments, which are shed and re-synthesised at a rate of approximately 10% of their length per day[9,41,67].

PKM2 is the dominant PKM isoform expressed in most cancers, consistent with a role in supporting anabolism. Christofk et al. knocked down PKM2 in highly glycolytic cancer cell lines and then expressed either recombinant PKM1 or PKM2[68]. Compared to PKM1, PKM2 expression resulted in higher lactate production, lower oxygen consumption, and lower dependence on OXPHOS for growth. It seems paradoxical that PKM2 expression is associated with increased lactate production given that is acts upstream of LDH and is less active than PKM1. This can be explained in part by the ability of PKM2 to act as a coactivator of HIF-1α and other transcription factors independently of its enzymatic activity,

thereby promoting lactate production over OXPHOS through activation of pro-glycolytic target genes[69,70].

The roles of PK isoforms in the retina are complex (recently reviewed by Rajala[71]). Both PKM1 and PKM2 are expressed in mouse retinas. In mouse total retinal extracts Lindsay et al. observed over five-fold more PKM2 than PKM1 on a molar basis, with PKM levels approximately 25% of rhodopsin, the most abundant photoreceptor protein[42]. In mouse and rat retinas, PKM2 is most highly expressed in photoreceptor inner segments, but is also detectable in the inner nuclear layer in a population of bipolar cells, and in the inner plexiform layer[9,41,42,72]. In marmosets PKM2 localisation is similar to rodent, except for weak expression in Müller cells which was not detectable by immunohistochemical analysis in mice, and for a lack of expression in the inner nuclear layer[41]. PKM1 expression in mouse and rat retinas is, however, more widespread than PKM2, especially in the inner retina. An important point given its potential to inhibit anabolic pathways is whether PKM1 is co-expressed with PKM2 in photoreceptors; indeed the overexpression of PKM1 in the presence of endogenous PKM2 has been demonstrated to slightly reduce OS length[9]. Most studies show relatively weak PKM1 expression in photoreceptors based on IHC[41,42,73,74], with one reporting it as undetectable by IHC and ISH but detectable in rods by qPCR[9]. In all cases, expression of PKM2 in the photoreceptor layer was much stronger, and this observation was consistent in both rod and cone-dominant retinas[41,73].

Wubben et al. crossed the LMOPC1 mouse with floxed PKM2 mice to knock out PKM2 in rods[74]. Only the PKM2-specific exon 10 of the *Pkm* gene is floxed in these mice, which enables PKM2 to be knocked out in rod photoreceptors, while leaving the PKM1-encoding sequence intact. No obvious functional consequences were observed upon rod-specific PKM2 deletion except for a slight decrease in scotopic a-wave amplitude after 24-28 weeks[74]. The researchers described a minor degenerative phenotype characterised by a small reduction in outer retinal volume and cell number, but no significant TUNEL staining indictive of cell apoptosis. Chinchore et al. had previously used the LMOPC1 mouse to generate rod-specific PKM2 knockout mice and most importantly only found a small but significant decrease in lactate production in retinal explants in knockout mice compared to controls[9]. This small decrease is consistent with only a minor role for PKM2 in aerobic glycolysis.

Rajala et al. used the iCre75 mouse to also knock out PKM2 in rods[55,72]. These knockout mice showed decreases in scotopic a and b-wave amplitudes at five months old. Similar to the other studies, there was only a slight morphological change noted in the form of decreased thickness of the photoreceptor end tips. Whilst in this model there was significantly more TUNEL staining in the photoreceptor layer of the knockout compared to control, only a minute fraction of photoreceptors stained positively in either. Importantly, lactate production in the rod PKM2 knockout retinas was slightly lower than in control retinas, consistent with the earlier observation that PKM2 plays a minor role in aerobic glycolysis in the mouse retina[9,72].

When the PKM2-specific exon 10 is deleted, PKM1 expression often dramatically increases, an observation common to all discussed rod PKM2 knockout studies[9,72,74]. However, there was considerable variation in this regard between the results of each mouse model. Wubben et al. directly measured total PK activity in retinal homogenates and found that it was higher in PKM2 knockouts due to increased PKM1 expression[74]. In contrast, Rajala et al. inferred decreased total PK activity from measurements showing higher levels of glycolytic metabolites and lower levels of TCA cycle metabolites in the knockout, but PK activity was not directly measured[72]. The effects of PKM2 knockout on the balance of anabolic activity and oxidative energy production may therefore be specific to each mouse model, and interpretation requires careful consideration of the residual PK activity. It is not clear why either an increase or apparent decrease in PK activity in each case resulted in a decrease in lactate production. Taken together, the results show that either heightened or

lowered PK activity in rods can be damaging, and this circumstantially indicates that PKM2, being highly regulatable, may be preferred to dynamically control the glycolytic rate.

In addition to their knockout, Chinchore et al. also used electroporation of shRNA to specifically target PKM2, and surprisingly in this case found a much greater decrease in rod OS length compared to PKM2 knockout. The utility of functional analyses following knockdown is limited by inefficient and mosaic construct penetrance inherent to the technique, but the substantial morphological changes hint that PKM2 may play a more significant role in photoreceptors than is apparent in the knockouts. As shRNA-mediated knockdown acts post-splicing, it did not result in an increase in PKM1 levels. PKM1 may therefore be able to partly compensate for the absence of PKM2 in the knockouts, resulting in milder phenotypes. However, the OS loss caused by knockdown of PKM2 could not be rescued by co-electroporation of transcript encoding PKM1, so either PKM1 alone is not protective when PKM2 is acutely lost in mature rods, or is only protective when present within a discrete range of protein expression levels which by chance or regulation was reached in the knockouts[9]. Alternatively, there may be other compensatory effects that protect photoreceptors when PKM2 is absent throughout rod development, which would not be present when shRNA is used acutely on adult mice. For example, Wubben et al. observed increased expression of glycolytic, PPP and TCA cycle genes in their homozygous PKM2 knockouts[74]. Finally, the phenotypic differences between PKM2 knockout and shRNA-mediated knockdown might due to off-target activities of the PKM2 shRNA. The severity of morphological changes to rods upon PKM2 disruption therefore remains unclear.

PKM2 has also been conditionally knocked out in cones using the HGRP-Cre mouse[57,75]. This resulted in an age-related decrease in photopic b-wave amplitude and flicker-ERG amplitude, which was used to specifically assess cone function. The number of S- and M-opsin cones was also significantly lower in the knockout, evident at 12 weeks of age and more widespread at 28 and 56 weeks. A surprising observation was that total retinal *Pkm2* RNA in the knockout was less than 40% of wild type levels, and RNA expression of a number of genes involved in glycolysis and fatty acid synthesis was also lower in the PKM2 knockout by a magnitude of approximately 50%. Cones only comprise 2.8% of photoreceptors in mice[76]. Therefore, either cones account for over 60% of retinal *Pkm2* RNA, or cone PKM2 is required for normal metabolism and PKM2 expression in other retinal cell types. In favour of the second hypothesis, PKM2 RNA expression does not appear to be elevated in cones, as evidenced by ISH[9]. In any case, from this study there is a clear cell-autonomous requirement of PKM2 in cones for their survival, as the observed decrease in the number of cones did not occur in rod-specific knockout models. Due to the small proportion of cones in the mouse retina, no analysis of rates of lactate production and aerobic metabolism were reported for the PKM2 cone-specific knockouts, so the role in cone-mediated aerobic glycolysis is unclear.

In cancer cells, tyrosine phosphorylation of PKM2 at position Y105 decreases PK activity by sterically hindering binding of the PKM2 activator FBP, and thereby promotes anabolism[65]. Fibroblast growth factor receptor 1 (FGFR1) is able to directly catalyse this phosphorylation[65]. Chinchore et al. showed that FGFR signalling may modulate Y105 phosphorylation and lactate production in mouse explants, although a causal link between PKM2 phosphorylation state and retinal lactate production remains unconfirmed[9]. Rajala et al. had earlier demonstrated light-induced PI3K-dependent Y105 phosphorylation of PKM2 and found that PK activity was lower in retinas of light-adapted compared to dark-adapted mice[73]. Whether light-dependent and FGFR-dependent PKM2 phosphorylation are related processes is unknown. It should be noted that an earlier study reported no difference in PK activity or PKM2 phosphorylation between light-adapted and dark-adapted retinas[42]. Clearly, further research is needed to establish whether PKM2 phosphorylation is functionally important in the control

of retinal metabolism, but if so then it is likely a regulatory node at which multiple upstream pathways converge to modulate the glycolytic rate and the fate of glucose.

The specific role of PKM2 in retinal metabolism and function is difficult to distinguish from the role of PKM1 as the knockout of a single exon results in artificial changes to PKM1 expression. The fact that knockout of PKM2 in rods resulted in functional and morphological changes to the retina in multiple studies utilising different mouse strains is consistent with a role for PKM2 in retinal function and morphology. The observed decreases in lactate production and changes in metabolic pathways upon PKM2 knockout in rods suggest that PKM2 also plays a role in glucose metabolism in rods. However, the fact that changes in retinal lactate production were only small upon knockout supports the notion that, while it may contribute to aerobic glycolysis, it is not a major driver of aerobic glycolysis in rods. Its role in aerobic glycolysis in other cell types of the inner retina is largely unexplored.

**Phosphoinositide 3-kinase (PI3K)/Akt signalling**

Phosphoinositide 3-kinases comprise a large family of proteins that phosphorylate inositol-containing lipids in the plasma membrane and mediate signal transduction in many growth factor signalling pathways[77]. There are three classes of PI3K in mammals. Extensively studied and most relevant to metabolism are the class I PI3Ks which are heterodimers comprising a catalytic and a regulatory subunit (detailed reviews of structure and subunit composition in refs[78,79]). Class I PI3Ks are activated upon binding of growth factors to cognate receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) (Figure 2). Active class I PI3K phosphorylate the lipid substrate phosphatidylinositol-4,5-bisphosphate ($PIP_2$), generating the second messenger phosphatidylinositol-3,4,5-trisphosphate ($PIP_3$), which then recruits a subset of pleckstrin homology (PH) domain-containing effector proteins to the plasma membrane[80]. The dominant, but not exclusive, effector protein is the serine/threonine kinase Akt[77,81,82]. There are three isoforms in mammals, Akt1, 2 and 3. Upon binding to $PIP_3$, Akt is specifically phosphorylated on two residues, T308 and S473 on Akt1 or homologous residues on Akt2 and Akt3[83]. T308 phosphorylation is catalysed by phosphoinositide-dependent kinase 1 (PDK1) and is sufficient for partial activity of Akt[84]. S473 phosphorylation, which is catalysed by the mammalian target of rapamycin complex 2 (mTORC2), completes activation of Akt and stabilises T308 phosphorylation[85]. Many target proteins with roles in diverse aspects of cell biology including cell growth, survival and metabolism are modulated in response to phosphorylation by Akt[77].

The PI3K signalling pathway is one of the most commonly mutated pathways in cancer and its aberrant activation is cancer-promoting in diverse ways, including via the direct and indirect upregulation of glycolysis[77,86]. There is direct evidence in multiple cancer cell types for the requirement of active PI3K/Akt signalling for aerobic glycolysis and growth[87-89]. Active Akt can induce the expression of the glucose transporter GLUT1 and its translocation to the plasma membrane, facilitating increased glucose uptake[90,91]. It can increase the binding of HK1 and HK2 to mitochondria via multiple mechanisms[48,49]. Akt also phosphorylates and activates phosphofructokinase 2 (PFK2), increasing production of fructose-2,6-bisphosphate, a positive regulator of the glycolytic enzyme phosphofructokinase 1 (PFK1)[92]. Phosphorylation of tuberous sclerosis complex 2 (TSC2) by Akt relieves inhibition of mTORC1, leading to a diverse set of effects including a context-dependent increase in HIF-1 levels[93-95]. HIF-1, as discussed below, is able to reprogram metabolism toward increased glycolysis and lactate production.

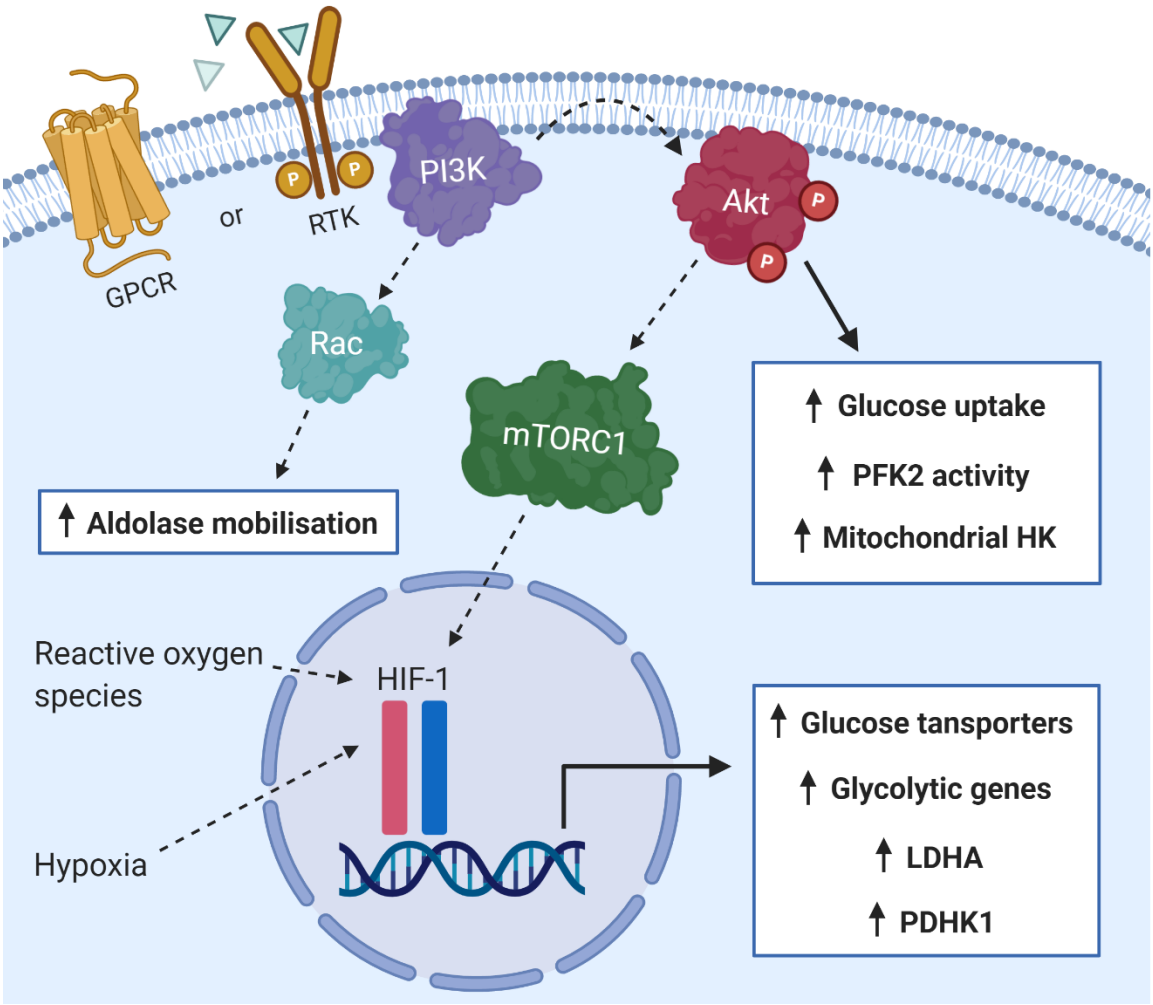


Figure 2. In cancer and proliferating cells, signalling through phosphoinositide 3-kinase (PI3K) can promote glycolysis by various means, both dependent on and independently of the serine/threonine kinase Akt or hypoxia-inducible factor 1 (HIF-1). It remains unknown whether signalling through any of these pathways is required to drive glycolysis in the mammalian retina. GPCR: G protein-coupled receptor, RTK: receptor tyrosine kinase, PFK2: phosphofructokinase 2, HK: hexokinase, mTORC1: mammalian target of rapamycin complex 1, LDHA: lactate dehydrogenase A, PDHK1: pyruvate dehydrogenase kinase 1.

Phosphoinositide signalling in the mammalian retina is active and has been well studied (reviewed by Rajala[96]). In mice, the global knockout of insulin receptor substrate 2 (IRS-2), an activator of PI3K signalling downstream of the insulin receptor, resulted in loss of up to 50% of photoreceptors within two weeks after birth, and secondary age-related retinal degeneration[97]. All three isoforms of Akt are expressed in the mouse retina and global knockout of Akt2 was shown to potentiate rods to light-stress induced degeneration. Although in contrast, knockout of Akt1 caused no obvious change to the retina, indicating the existence of isoform-specific roles of Akt in the retina[98]. In rod OS, the insulin receptor is activated by physiological light independently of insulin by a mechanism requiring photobleaching of rhodopsin, leading to the activation of PI3K and Akt[99]. These studies suggest that the activation of PI3K signalling is neuroprotective in light stress and decreases apoptosis.

There is evidence that PI3K may be particularly important for function, survival and glucose metabolism in cones. Knockout of either the p110α catalytic subunit or all isoforms of the p85α regulatory subunit of PI3K in mouse cones using the HGRP-Cre mouse resulted in cone degeneration and significant defects in cone function[100,101]. In each case, cones developed normally, with degeneration commencing after one month of age, indicating a requirement for PI3K signalling in adult cones. Rajala et al. also utilised neural retina leucine zipper (NRL) knockout mice, which only develop cone photoreceptors, to assess the role of PI3K in cones. HGRP-Cre mediated knockout of the p85α subunit of PI3K in the cone-dominant $NRL^{-/-}$ mouse retina resulted in significant defects in cone survival and function[102]. Importantly, further study showed that the $NRL^{-/-}$ $p85\alpha^{-/-}$ mice exhibited decreased levels of NADPH, and diminished expression of many glycolysis-related genes at the mRNA level, including those encoding GLUT1, HK2, PKM2 and HIF-1α, compared to $NRL^{-/-}$ mice[73]. These studies support a role for PI3K in driving glycolysis in cones, however no data on lactate production or other measures of glycolytic flux were reported.

Although there has been minimal investigation, there is presently no evidence that PI3K signalling drives aerobic glycolysis more broadly throughout the retina. One study in rat retinal explants showed that pharmacological inhibition of pan-Akt resulted in no change in lactate production rate, although Akt3 inhibition caused decreased protein synthesis[103]. Given that these experiments were performed after 2.5 h of treatment with inhibitors, indirect effects mediated by HIF-1-dependent transcription and protein translation, for example, would only be minimal over this relatively short incubation. Even if Akt does not play a role, PI3K may be able to drive aerobic glycolysis via Akt-independent mechanisms. For example, an important mechanism through which PI3K can upregulate glycolysis is by freeing the glycolytic enzyme, aldolase, from the actin cytoskeleton. This is mediated through the small GTPase, Rac, independently of Akt[104] (Figure 2).

To summarise, PI3K/Akt signalling is basally active in the retina and is upregulated by light. Circumstantial evidence in favour of a role in modulating aerobic glycolysis includes the PI3K-dependent phosphorylation of PKM2, high levels of mitochondrial HK2 and, as discussed below, the possible involvement of FGFR signalling. Evidence against the role includes the reported lack of effect of Akt inhibition on lactate production with retinal explants. PI3K may be a driver of aerobic glycolysis specifically in cones, but the lack of direct evidence for changes in lactate production following disruption of PI3K signalling renders this unconfirmed. A definitive conclusion on the role of PI3K signalling in aerobic glycolysis in the retina, and any differences in its roles between cell types and species, will ultimately require more direct investigation.

**Fibroblast growth factor receptor (FGFR)**

There are four transmembrane RTKs in the FGFR family in mammals, denoted FGFR1-4, involved in cellular processes including development, growth, survival, and metabolism[105]. Binding of a fibroblast growth factor (FGF) to the extracellular domain of an FGFR induces dimerization, cytoplasmic autophosphorylation and activation of the FGFR. There are eighteen FGFs known to directly bind and activate FGFRs with both overlapping and distinct expression patterns, receptor specificities and downstream effects. Most FGFs act in an autocrine or paracrine manner, and three are secreted endocrine hormones. Active FGFRs are able to context-dependently activate the MAPK, PI3K/Akt, PLCγ/PKC, and JAK/STAT signalling pathways, giving rise to their diverse battery of cellular roles. FGFR signalling is commonly dysregulated in cancer, and is a therapeutic target[106]. It is therefore important to understand its potential roles in normal retinal physiology.

FGFRs can drive aerobic glycolysis, and there are context-specific mechanisms through which they control glucose metabolism. As discussed above, FGFR1 can phosphorylate residue Y105 on PKM2

which inhibits its tetramerisation and thereby decreases PK activity to promote anabolism[65]. FGFR can phosphorylate four tyrosine residues on LDHA, which increases its stability and activity[107,108]. FGFR1 can phosphorylate and activate pyruvate dehydrogenase kinase 1 (PDHK1) which negatively regulates pyruvate dehydrogenase (PDH), resulting in decreased pyruvate entry into OXPHOS[109]. In human lymphatic endothelial cells, FGFR1 knockdown decreased levels of glycolytic metabolites and lactate, and treatment with FGF2 had the opposite effect[110]. These observations were attributed to changes in the expression of HK2, which was induced by FGF2 and lowered by knockdown of FGFR1 in a manner transcriptionally dependent on c-Myc. Treatment of FGF2-stimulated squamous cell lung cancer cells with inhibitors of FGFR decreased glucose uptake and expression of GLUT1, reduced PK activity, and hampered HIF-1α induction in both hypoxia and normoxia[111]. In this context, the PI3K/Akt/mTOR pathway was the key mediator of the effects of FGFR signalling. In human prostate cancer cells, ablation of FGFR1 decreased glucose uptake and lactate production, and increased oxygen consumption, consistent with a switch from glycolysis to oxidative phosphorylation[108]. Expression of LDHB mRNA and protein was higher whereas expression of LDHA protein was lower but mRNA was unchanged, indicating no link between FGFR1 and LDHA transcription in this context. Similar results were obtained in mouse embryonic fibroblasts (MEFs). The endocrine hormone FGF19 has been shown to modulate glucose metabolism in the brain, lowering glucose levels independently of insulin by increasing conversion of glucose to lactate[112]. Altogether, these studies highlight the diverse nature of the mechanisms through which FGFRs can control glucose metabolism. One study in cancer associated fibroblasts even presented evidence for FGF2 driving OXPHOS and downregulating glycolysis[113].

Chinchore et al. reported that FGFR drives aerobic glycolysis in the mouse retina[9]. The researchers treated mouse retinal explants with the FGFR inhibitor PD173074 or the multi-kinase inhibitor TKI258, and found significantly lower lactate production rates and less of the phosphorylated forms of LDHA and PKM2. It should, however, be noted that PD173074 was used at concentrations orders of magnitude above that which is typically effective in cell culture[114-117]. Leaving the RPE attached to the retina raised the lactate production rate by a similar amount to supplementing medium with FGF2, leading to the authors' proposal that FGF2 secreted by the RPE stimulates aerobic glycolysis in the retina[9]. Some evidence is consistent with a role for FGFRs in retinal neuronal survival and therefore potentially glucose metabolism. A study in mice found that expression of a dominant negative form of FGFR2, but interestingly not FGFR1, under the control of an exogenous rhodopsin promoter resulted in progressive photoreceptor degeneration[118]. On the other hand, global knockout of FGF2 in mice reportedly causes no morphological changes to the retina[119,120]. Possibly there is redundancy with other FGFs in driving retinal glycolysis. Interestingly, FGFR1 and FGF2 are upregulated and neurotrophic in response to light damage or retinal detachment, but whether modulation of glycolysis is important for this effect is unclear[121-124]. Altogether, current data show that exogenous FGF2 can drive aerobic glycolysis in the mouse retina, while a role in glucose metabolism in the basal state is plausible but will require a more detailed investigation to confirm.

**Hypoxia-inducible factor 1 (HIF-1)**

HIF-1 is a heterodimeric transcription factor comprising an oxygen-regulated α subunit, HIF-1α, and a stable β subunit, HIF-1β, more commonly known as the aryl hydrocarbon receptor nuclear translocator (ARNT)[70]. In most cells, HIF-1α and ARNT are constitutively transcribed. However, when oxygen is available, HIF-1α is enzymatically hydroxylated on specific proline residues and an asparagine, which mark it for rapid proteasomal degradation and prevent interaction with transcriptional coactivators respectively[125-127]. In hypoxia, the hydroxylation reactions cannot proceed and so HIF-1α escapes degradation, translocates to the nucleus, binds to ARNT, and activates the

expression of hundreds of target genes. These genes function to maintain oxygen homeostasis by various mechanisms including directing the metabolic switch from OXPHOS to glycolysis. HIF-1α is able to orchestrate this switch by initiating transcription of genes encoding glucose transporters such as GLUT1, and an array of glycolytic enzymes including HK2 and LDHA[128]. HIF-1α also activates the transcription of PDHK1 which phosphorylates and decreases the activity of PDH, thereby limiting entry of pyruvate into the TCA cycle[129]. A second HIF-α isoform, HIF-2α, is regulated by hypoxia in a similar manner to HIF-1α, although it has a more discrete set of target genes and plays less of a role in regulating metabolism.

In cancers, HIF-1α has been found to be stable and transcriptionally active and can, among other tumour-promoting effects, drive aerobic glycolysis[70]. Stabilisation of HIF-1α in the context of cancer occurs in a number of ways. Hypoxic microenvironments in solid tumours facilitate stabilisation via the mechanism described above. Genetic mutations in key components of the regulatory machinery such as the von Hippel-Lindau protein (VHL), which facilitates HIF-1α proteasomal degradation, can cause aberrant stabilisation in normoxic conditions. HIF-1α can also be stabilised in the presence of oxygen by high levels of reactive oxygen species such as nitric oxide, and its stability can be modulated context-dependently, including in some cancer cells, via signalling pathways such as the PI3K/Akt/mTORC1 axis[94,130,131]. Interestingly, a feed forward mechanism also exists whereby high levels of pyruvate and lactate can contribute to HIF stabilisation[130]. Given its key role in driving glycolysis and lactate production in cancer, it is logical that HIF-1α could drive aerobic glycolysis in the retina, especially given the established involvement of many of its transcriptional targets.

The role of HIF-1α in retinal lactate production in the adult retina to our knowledge has not been directly tested but evidence indicates that it may not be an essential player. Whilst HIF-1α is important in certain cell types for retinal vascular development, there is limited evidence to support the idea that it is stably expressed and required in the normal adult mammalian retina, especially in the photoreceptors which appear largely responsible for lactate production. Studies have shown that HIF-1α is expressed at the RNA level but protein is scarcely detectable by western blot in normoxic mouse retinas, consistent with rapid degradation[132-134]. Okano et al. showed that expression of HIF-1α rapidly declines in mouse retinas after birth, correlating with increased oxygenation[135]. HIF-1α expression was retained in the ganglion cell layer, however. A separate study that compared HIF-1α expression in adult normoxic and hypoxic mouse retinas found similar results, reporting weak expression in normoxia that was limited to the ganglion cell layer[133]. One study presented immunohistochemical evidence for widespread HIF-1α expression in rat and human retinas, but it is difficult to confidently interpret without validation of antibody specificity or a negative control for expression[136]. It is possible that there are species-specific differences in expression, but together the evidence does not support high levels of HIF-1α protein expression in photoreceptor cells in aerobic conditions.

Importantly, conditional knockout of HIF-1α in mouse rod or cone photoreceptors resulted in no detectable functional change under non-stressed conditions, nor did dual knockout of both HIF-1α and HIF-2α. These results suggest that either aerobic glycolysis is not required for normal retinal function, or more likely that the HIFs are not required for aerobic glycolysis in the mature mouse retina, although this has not been directly confirmed as lactate production or other aerobic analyses of glycolysis were not assessed[137,138]. Given that HIF-1α has been implicated in the pathogenesis of several ischaemic retinal diseases where stabilisation results in neovascularisation and neurodegeneration, its inhibition or deletion is a promising avenue for disease treatments[139,140]. It is worth noting that the converse is possibly true during development, however. In a hyperoxic mouse model of retinopathy of prematurity, elevated aerobic glycolysis in response to pharmacological HIF

activation may contribute to retinal neuroprotection[141,142]. Whether endogenous HIF is important in driving glycolysis during development is unclear.

It is possible that HIF-1 plays a minimal or redundant role in retinal metabolism in the mature retina, but this needs to be directly analysed. The current evidence is convincing that ablation of HIFs in normal mouse photoreceptors is not overtly damaging, and provisionally rules out HIF-1 as a key driver of aerobic glycolysis in the mammalian retina, pending direct metabolic investigations.

**Concluding remarks**

Recent findings have highlighted the importance of the expression of LDHA, PKM2 and HK2 in the maintenance of aerobic glycolysis in the retina, and in general the roles of these enzymes in the retina mirrors their roles in cancer cells. Whether the reported post-translational modifications or changes in subcellular localisation of these enzymes are important for retinal metabolism or function is still unclear, and the upstream signalling pathways or transcriptional programs that control their expression and regulation are also largely unknown. Evidence for the PI3K/Akt pathway, FGFRs and HIF-1 driving aerobic glycolysis in the retina is less convincing, with more specific investigations required to determine whether they also make an important contribution. Finally, it is possible that other factors yet to be investigated may prove to be key drivers, including factors common to cancer cells or novel photoreceptor-specific drivers. Ultimately, the identification and characterisation of the key drivers of aerobic glycolysis will enable a clearer understanding of normal retinal function, as well as provide valuable tolls to decipher the role of altered metabolism in retinal disease.

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