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Neuronal apoptosis by prolyl hydroxylation: implication in nervous system tumours and the Warburg conundrum

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Abstract

Oxygen-sensing mechanisms are often dysfunctional in tumours. Oxygen sensing is mediated partly *via* prolyl hydroxylation. The EglN prolyl hydroxylases are well characterized in regulating the hypoxia inducible factor α (HIF-α) hypoxic response, but also are implicated in HIF-independent processes. EglN3 executes apoptosis in neural precursors during development and failure of EglN3 developmental apoptosis can lead to certain forms of sympathetic nervous system tumours. Mutations in metabolic/mitochondrial enzymes (*SDH*, *FH*, *IDH*) impair EglN activity and predisposes to certain cancers. This is because the EglNs not only require molecular oxygen to execute hydroxylation, but also equally require the electron donor α-ketoglutarate, a metabolite from the Krebs cycle. Therefore EglN enzymes are considered oxygen, and also, metabolic sensors. α-Ketoglutarate is crucial for EglN hydroxylation activity, whereas the metabolites succinate and fumarate are inhibitors of the EglN enzymes. Since EglN activity is dependent upon metabolites that take part in the Krebs cycle, these enzymes are directly tied into the cellular metabolic network. Cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis), an observation that was first made by Otto Warburg in 1924. Despite the striking difference in ATP production, cancer cells might favour aerobic glycolysis to escape from EglN hydroxylation, resulting in the accumulation of oncogenic HIFα and/or resistance to EglN3-mediated apoptosis.

Keywords: prolyl hydroxylase EglN • PHD • neuronal apoptosis • phaeochromocytoma • neuroblastoma • gliomaș • Otto Warburg • Krebs cycle TCA • succinate dehydrogenase SDH • isocitrate dehydrogenase IDH

Introduction

Oxygen-sensing mechanisms enable the cell to adapt to low oxygen environments and are also critical for normal development and apoptosis. Disruptions of oxygen-sensing pathways can lead to the development of certain forms of cancer [1–15]. Oxygen sensing is mediated partly *via* the EglN hydroxylases (EglN1/EglN2/EglN3) that are dependent upon molecular oxygen, iron (II) and α-ketoglutarate to perform proline hydroxylation on their target [16–20]. Therefore these enzymes are considered oxygen and also metabolic sensors. The availability of molecular oxygen is absolutely required for the hydroxylation reaction, because it donates the oxygen atom to the hydroxyl group [21]. But equally important for the hydroxylation reaction is the electron donor α-ketoglutarate, a metabolite from the Krebs cycle. Since EglN is dependent upon metabolites that take part in the Krebs cycle, they are directly tied into the cellular metabolic network (Fig. 1).

The first identified substrate for the EglN prolyl hydroxylases is the transcription factor hypoxia inducible factor α (HIF-α) [16, 17]. The identification of HIF-α as a direct hydroxylation substrate provided the first and direct link between tumour suppressor function and oxygen sensing. The tumour suppressor von Hippel

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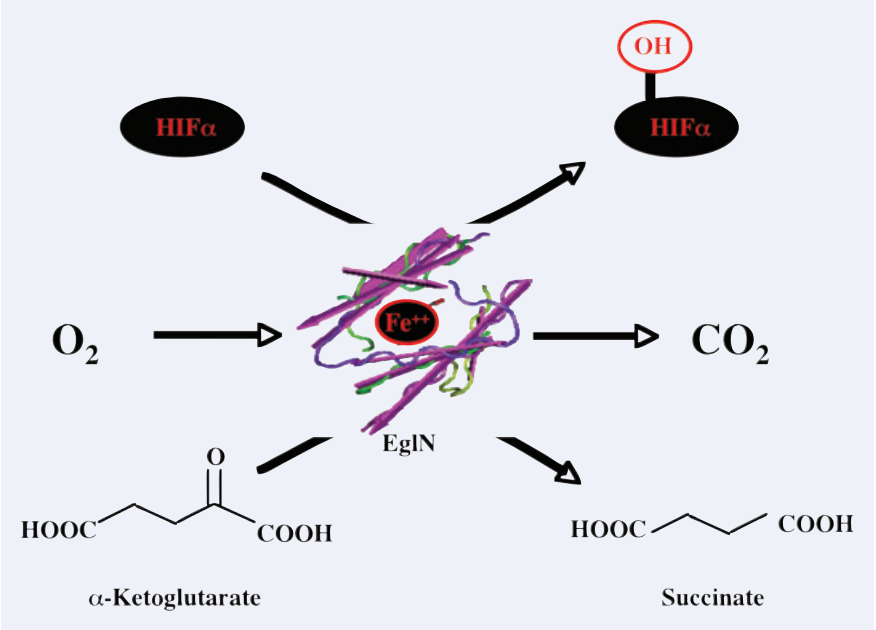

Fig. 1 Schematic representation of the EglN prolyl hydroxylase catalytic action. EglN prolyl hydroxylases (also referred to as PHD) belong to the 2-oxoglutarate-dependent oxygenase superfamily. The requirement of dioxygen as cosubstrate demonstrates function in cellular oxygen sensing. Two histidine residues at the catalytic site coordinate a non-heme iron. The reaction proceeds *via* the incorporation of dioxygen into the prolyl residue forming hydroxyproline at the substrate. As electron donor for this reaction serves α-ketoglutarate (=2-oxoglutarate) which is oxidized to succinate and CO_2. The dependency of the metabolite α-ketoglutarate for catalytic activity makes these enzymes sensitive to α-ketoglutarate levels and ties them directly into the metabolic network.

Lindau (VHL) acts as an ubiquitin ligase by targeting hydroxylated HIF-α for degradation when oxygen is available, as where under low oxygen environments, HIF-α escapes hydroxylation and subsequently escapes VHL recognition [3–5, 16, 22–27]. The escape from VHL recognition allows HIF-α to accumulate and to transactivate its target genes that are important for adaptation to low oxygen environment including energy metabolism and angiogenesis [28–30]. Both HIF-dependent and HIF-independent VHL functions contribute to VHL-defective tumorgenesis [31–34]. The VHL disease is caused by inactivating germline mutations of the VHL gene and predisposes to a variety of tumours including haemangioblastoma of the retina and nervous system, clear cell renal carcinomas (RCC; the most common form of kidney cancer) and phaeochromocytomas/paragangliomas, tumours of the sympathoadrenal nervous system. HIF-α deregulation appears to have a causal role in VHL-defective clear cell RCC and in VHL-defective blood vessel tumours haemangioblastomas (HB) [33]. Although HIF-α regulation has been a major focus of VHL research, the genotype–phenotype correlation in the VHL disease gave insight into HIF-independent VHL function (Table 1). Specific VHL germline mutations corresponding to a specific subset of tumour phenotypes have a different relationship to HIF-α deregulation (categorized in type I/IIA/IIB/IIC disease) [35–37]. The type I disease associates with RCC and HB, and their tumours reflect deregulated high HIF-α expression, whereas a very different clinical outcome is observed in the type 2C-VHL disease. The type 2C disease does not develop RCC and HB instead develops phaeochromocytomas only. More importantly, HIF-α is not deregulated and maintains in low levels in the type 2C tumours [31, 32]. These findings suggest that HIF-α deregulation is not necessary for phaeochromocytma development in VHL disease.

Interestingly, a second hydroxylation target of the prolyl hydroxylases EglN has recently been identified [38]. The β_2-adrenergic receptor is a prototypic G protein-coupled receptor, which is hydroxylated by EglN3 and also oxygen dependent degraded by pVHL. The discovery of another hydroxylation substrate by the EglN enzymes not only broadens the functionality of prolyl hydroxylation, but also expands our understanding of cellular response to oxygen and its relationship to disease.

Therefore, the identification of other EglN substrates and the clues from the genotype–phenotype correlation that emerged in the VHL disease clearly indicates HIF-independent VHL functions. Of great insights are studies from EglN3-mediated neuronal apoptosis during sympathetic neuronal development, which shed light onto the genesis of phaeochromocytoma by presumably HIF-independent pathways [9].

Lessons from a rare disease

Phaeochromocytomas are rare, with only five to eight cases diagnosed per million people a year [39]. They are neoplasias of neural crest origin arising from the adrenal medulla. Phaeochromocytomas can also develop in extra-adrenal sympathetic ganglia 10% of the time. Extra-adrenal phaeochromocytomas are sometimes referred to as paragangliomas [40]. In short, these tumours are sympathetic nervous system tumours. The predisposing germline mutations lend clues to the pathogenesis of this disease. The most frequent causes of phaeochromocytoma susceptibility are *VHL* missense mutations, activating

Table 1 Genotype–phenotype correlation in the VHL disease

Type	Mutation	Manifestation	HIF-α
I	Loss of VHL function: loss, truncated or missfolded	Haemangioblastoma, Renal cell carcinom	↑
II A/B	VHL missense mutations	Haemangioblastoma, Renal cell carcinom and phaeochromocytoma	↑
II C	VHL missense mutations	Phaeochromocytoma only	↓

mutations in *c-Ret*, mutations in neurofibromatosis type 1 (*NF1*) and mutations in succinate dehydrogenase subunits of mitochondrial complex II (*SDH B/C/D*). Loss of NF1 has been reported to promote survival of embryonic sympathetic neurons in the absence of the nerve growth factor NGF [41]. Further, mutations in subunits of mitochondrial complex II suggest impairment of mitochondria-mediated apoptosis and have led to the early hypothesis that failure of apoptosis in neuroendocrine precursor cells could result in the development of phaeochromocytoma and paraganglioma [40]. Given the evidence that NF1 promotes sympathetic neuronal survival and further that these neoplasias originate from the sympathetic nervous system, a closer look into the sympathetic neuronal development reveals important clues to the genesis of these tumours [9].

Since the discovery by Rita Levi-Montalcini and Viktor Hamburger of the neurotrophic factor NGF, our understanding of developmental apoptosis in the sympathetic nervous system has greatly increased [42]. During neuronal development, NGF is required for survival but is also limiting. Neurons that successfully compete for NGF during development survive whereas unsuccessful neurons die. Competition for NGF is an important developmental process for matching the size of a neuronal population, especially in the peripheral nervous system [43]. As much as 50% of neurons produced during embryogenesis die by apoptosis during neuronal development. Abnormal NGF signalling has been linked to paediatric nervous system tumours such as neuroblastoma [44, 45] and disease-associated mutations such as NF1 have been shown to enhance signalling by NGF receptors and promote neuronal survival in the absence of NGF [41]. In the last decades it became evident that JNK/c-Jun signalling is required for apoptosis when NGF is limiting in the developing nervous system [46–50]. Interestingly, the prolyl hydroxylase EglN3 has recently been implicated in this pathway. EglN3 is induced in sympathetic neurons deprived of NGF and further has pro-apoptotic activity when overexpressed [51]. Given that EglN3 is known to hydroxylate HIF-α and has been implicated in developmental apoptosis in sympathetic neurons, the following questions arise: (*i*) Does EglN3-mediated apoptosis depend upon its hydroxylation activity? (*ii*) Does it depend upon HIF hydroxylation or does it involve hydroxylation of unknown substrates? (*iii*) Is failure of EglN3-mediated apoptosis implicated in the genesis of phaeochromocytomas and other tumours arising from the neural crest origin?

Specificity of function within the EglN prolyl hydroxylases

The ability of EglN3 to induce neuronal apoptosis appears to be unique among the EglN family members [9]. Induction of apoptosis is dependent upon EglN3 hydroxylation activity, because catalytic impaired EglN3 fails to induce apoptosis [9, 52]. Importantly, EglN3-induced apoptosis is not diminished in the presence of stable HIF1α or HIF2α variants that cannot be hydroxylated on their prolines [9]. This suggests that hydroxylation targets of EglN3 other than HIF-α are crucial for apoptosis function.

EglN2 and EglN3 are induced by hypoxia and dampen the HIF-α response under chronic hypoxia [53–57]. However, EglN1 appears to be the primary HIF prolyl hydroxylase under normal conditions [58]. Consistent with this, mice lacking EglN2 or EglN3 are viable and grossly normal whereas mice lacking EglN1 are not viable [59]. Conditional inactivation of EglN1 in mice leads to polycythemia due to HIF-α stabilization and increased transcription of HIF target genes including erythropoietin [60, 61]. Further, patients carrying EglN1 mutations have been reported to develop polycythemia concluding that EglN1 couples HIF-α stability *in vivo* [13, 62, 63].

Consistent with the unique role of EglN3 in neuronal apoptosis are studies obtained from the EglN3-deficient mice that clearly demonstrates the requirement for EglN3-mediated apoptosis during the sympathetic neuronal development. EglN3 deficient sympathetic neurons are resistant to apoptosis after NGF withdrawal, as well as certain neurotoxins [64]. Consistently, EglN3$^{-/-}$ mice have an increased number of cells in the super cervical ganglia and in the adrenal medulla and show abnormalities in the sympathoadrenal system including systemic hypotension [65]. In fact, it appears that some of the adrenal abnormalities are caused through deregulation of the β_2-adrenic receptor (β_2-AR) [38]. EglN3 specifically hydroxylates β_2-AR, whereas EgN1 and EglN2 fail to do so. This leads to subsequent ubiquitination and degradation mediated by pVHL. Loss of EglN3 results in β_2-AR up-regulation and accordingly, hypoxia stabilizes the β_2-AR. This is consistent with the observations from the type 2C VHL patients that develop phaeochromocytoma. Type 2C patients often show excessive secretion of catecholamines (endogenous β_2-AR ligands) and increased sympathonervous system activity [66].

In summary, this points towards distinct and unique functions within the family of EglN prolyl hydroxylases. Identification of

novel EglN hydroxylation targets will open new oxygen sensing pathways independent of what we have learned from HIF-α.

Failure of EglN3-mediated apoptosis in the genesis of phaeochromocytoma

Given the evidence that (*i*) phaeochromocytomas are sympathetic nervous system tumours and that (*ii*) EglN3 is critical for apoptosis during sympathetic neural development, an attractive hypothesis emerged in which failure of EglN3-mediated apoptosis during sympathetic development predisposes to the genesis of phaeochromocytomas and perhaps other neoplasia arising from neural crest origin [9]. Interestingly, apparently unlinked phaeochromocytoma lesions (*VHL*, *NF1*, *c-RET* and *SDH*) all appear to act on a single common pathway by decreasing EglN3-mediated apoptosis at the time during development when levels of NGF become limiting (Fig. 2) [9]. During this developmental window, the sympathetic precursors undergo c-Jun-dependent apoptosis [67–70]. EglN3 appears to act downstream of the c-Jun in the NGF signalling pathway [9]. Therefore, a single pathway was established in which the genetic phaeochromocytoma defects act either directly on EglN3 or upstream of EglN3 to impair apoptosis (Fig. 2). For instance, the genetic defect in *SDH* acts directly on EglN3-mediated apoptosis function. SDH inhibition results in accumulation of intracellular succinate, which in turn can product-inhibit the EglN prolyl hydroxylases [9, 71]. This succinate-mediated inhibition not only results in HIFα stabilization, but also importantly inhibits EglN3-mediated apoptosis. Further, SDH inhibition blunts neuronal apoptosis when NGF is limiting [9]. The succinate-mediated product-inhibition of EglN3 was overcome by re-addition of α-ketoglutarate restoring NGF-mediated apoptosis [9]. This suggests that SDH inhibition acts on the prolyl hydroxylases *via* succinate and not as alternatively suggested through the generation of reactive oxygen species.

In addition to the predisposing *SDH* genetic lesion, other phaeochromocytoma lesions have been implicated upstream of EglN3 to promote neuronal survival in the NGF signalling pathway. In the case of *VHL* predisposing lesion, loss of pVHL promotes survival through up-regulation of JunB. pVHL suppresses JunB and all VHL mutants tested (including type IIC VHL disease mutant) abrogate its ability to do so [9]. JunB acts as an antagonist of c-Jun and increased JunB levels attenuate the induction of c-Jun-mediated apoptosis in sympathetic neurons deprived from NGF.

Finally, previous evidence indicated that RET (the receptor for glial-derived neurotrophic factor) and NF1 could act through this pathway by modulating the action of the NGF receptor TrkA. Loss of NF1 promotes NGF-independent survival of embryonic peripheral neurons [41]. In addition, c-RET can crosstalk with the NGF receptor TrkA [72]. Activation of c-Ret, like loss of pVHL, leads to the induction of JunB and attenuates apoptosis when NGF becomes limiting [9].

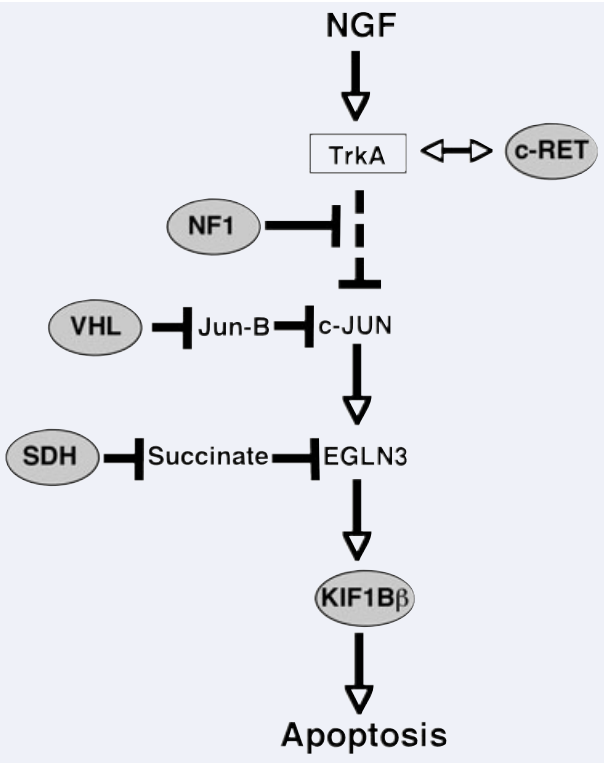

Fig. 2 Model linking familial phaeochromocytoma genes to NGF-dependent apoptosis. During sympathetic neuronal development, NGF is required for neuronal survival but is also limiting. Neurons that successfully compete for NGF survive whereas unsuccessful neurons undergo c-Jun-dependent apoptosis. C-Jun transcriptionally activates prolyl hydroxylase EglN3. As NGF levels become limiting, familial phaeochromocytoma mutations (*VHL*, *NF1*, *c-RET*, *SDH* and *KIF1Bβ*) all decrease apoptosis mediated by EglN3.

Thus, when mutated, all the genetic phaeochromocytoma lesions (*SDH*, *VHL*, *c-RET* and *NF1*) impair NGF-mediated apoptosis in neuroendocrine precursors during development. These findings provide an explanation as to why the mutations in tumour suppressors that are found in familial phaeochromocytoma are rare in sporadic phaeochromocytoma. This is because the pathway is no longer critical once development is completed.

In summary, all the genetic lesions associated with phaeochromocytoma act on a single common pathway that impinges upon EglN3 apoptotic activity during sympathetic neuronal development. Mutations in *SDH*, *VHL*, *c-RET* and *NF1* would allow sympathetic neuronal precursors to escape from developmental apoptosis and set the stage for their neoplastic transformation. It will be interesting to determine not only if *EglN3* is mutated in these neoplasias, but also, if failure of EglN3 developmental apoptosis plays a broader role in paediatric cancers and other forms of hereditary cancer.

Understanding the mechanistic basis of EglN3 killing

EglN3-mediated apoptosis is hydroxylation dependent, but independent of HIF-α hydroxylation [9]. An important challenge is to identify the link between this enzyme and apoptosis, which presumably involves hydroxylation of a protein other than HIF-α.

Early studies indicated that EglN3 mRNA and protein expression (at that time referred as SM-20) increases shortly after removal of NGF in primary sympathetic neurons and peaks between 10 and 15 hrs at a time when cells undergo apoptosis [51]. Overexpression of EglN3 is sufficient to promote cell death in NGF-maintained sympathetic neurons [51, 73]. Cell death is caspase dependent and accompanied by an increase of cytochrome c in cytosolic and mitochrondria-enriched subcellular fractions [74]. The mechanism underlying EglN3-induced cytochrome c is not known although it appears to involve an increase in cytochrome c mRNA. Other studies have identified EglN3 as a growth factor inducible gene in vascular smooth muscle cells [75] and later proposed, that it might function in growth arrest, differentiation and cell death during muscle differentiation [76, 77]. Expression of EglN3 was also reported in fibroblasts upon activation of a temperature-sensitive form of p53 [78]. However, EglN3-induced apoptosis appears not to be impaired in cells lacking p53 or expressing a dominant negative p53 protein, indicating that p53 might not function downstream of EglN3 to induce apoptosis [9]. In addition, a recent study indicated that EglN3's ability to induce apoptosis correlates with the formation of aggresome-like structures [52].

Recently, an unbiased genome-wide approach has been undertaken to understand how EglN3 causes neuronal cell death. A short interference RNA library was used to identify genes that suppress EglN3-induced apoptosis [64]. This led to the identification of the kinesin KIF1Bβ as a downstream effector of EglN3. KIF1B, a member the kinesin 3 family, consists of two major splice variants α and β. KIF1Bα and KIF1Bβ are motor proteins implicated in anterograde transport of mitochondria and synaptic vesicle precursors, respectively [79, 80]. The recent study, which identified KIF1Bβ as an EglN3 downstream target showed how KIF1Bβ is both necessary and sufficient for neuronal apoptosis when NGF becomes limiting, but it remained unclear how EglN3 regulates KIF1Bβ [64]. Interestingly, KIF1Bβ maps on to the chromosomal region 1p36.2, a region of the genome that is frequently deleted in neural crest-derived tumours including neuroblastomas [81]. The existence of one or more human tumour suppressor genes on chromosome 1p has been suspected for decades [82–84]. Further suggestion that KIF1Bβ acts as a 1p36 tumour suppressor comes from the current model for phaeochromocytoma development. Phaeochromocytomas develop when sympathetic neuronal precursors escape from EglN3-mediated developmental apoptosis, suggesting that mutations in KIF1Bβ may be relevant to phaeochromocytoma and other tumours of neuronal origin. Indeed, inherited loss-of-function KIF1Bβ missense mutations have been identified in phaeochromocytomas and neuroblastomas and an acquired loss-of function mutation in a medullablastoma, arguing that KIF1Bβ is a pathogenic target of these deletions [64]. Nonetheless, it has been further reported that the remaining KIF1Bβ allele in 1p deleted tumours and cell lines is often wild-type, contrary to the Knudson TwoHit scenario [64, 85–88]. Perhaps KIF1Bβ haploinsufficiency is adequate for tumorgenesis in some contexts. Also, the existence of multiple neuroblastoma and phaeochromocytoma suppressor genes on 1p has been suggested [89, 90]. Additional studies are needed to address how often it is deregulated, epigenetically or genetically, in cancer. A spotlight is now on understanding the mechanistic basis of how EglN3 regulates KIF1Bβ and how this translates into cell death. KIF1Bβ appears not be a direct EglN3 hydroxylation target, but EglN3 hydroxylation activity is required for KIF1Bβ regulation. Therefore EglN3 presumably involves hydroxylation of a protein that in turn might regulate KIF1Bβ to induce apoptosis.

Connecting EglN activity to the Warburg conundrum

In 1924, Otto Warburg observed that cancer cells are highly glycolytic in the presence of oxygen and have reduced rates of oxidative phosphorylation [91]. Recent studies have argued that cancer cells might benefit from this persistence of high lactate production in the presence of oxygen, referred to as aerobic glycolysis or pseudo-hypoxia [92, 93]. From a bioenergetic perspective, it remains a conundrum how highly metabolic proliferative cancer cells undergo a pathway that results in 10 times less ATP production compared to their normal counterparts that oxidize their glycolysis endproduct within the mitochondria *via* the Krebs cycle (pyruvate conversion into Acetyl-CoA, Fig. 3). Interestingly, three major enzymes of the Krebs cycle (SDH, FH, IDH) have been recently identified as bonafide tumour suppressors, but more importantly, inactivation in either of them directly affects EglN activity [9, 10, 14, 71]. This is because the EglN activity is not only dependent upon molecular oxygen to perform their hydroxylation reaction, but also require the Krebs cycle metabolite α-ketoglutarate as electron donor, which ties them directly into this metabolic network. Therefore an attractive hypothesis arises: cancer cells favour aerobic glycolysis to inhibit EglN activity in order to escape either certain oncogenic apoptotic signals and/or activate oncogenic HIF-α. About 70% or more of low-grade gliomas bear loss of function mutation in IDH1 and IDH2 [94, 95]. Loss of function mutation in IDH1 results in a decrease of intracellular α-ketoglutarate. Subsequently EglN prolyl hydroxylases are inhibited in their hydroxylase activity with the consequences of HIF-α stabilization [14]. Presumably EglN3-mediated apoptosis might be impaired in these settings as well, but this has not been investigated yet. Further, these studies follow the discoveries that germline mutation in SDH are linked to phaeochromocytoma and that FH mutation lead to leiomyosarcoma and renal cell carcinoma, both of which affect also the EglN prolyl hydroxylase activity [9, 10, 71]. This is because loss of function mutation in SDH and

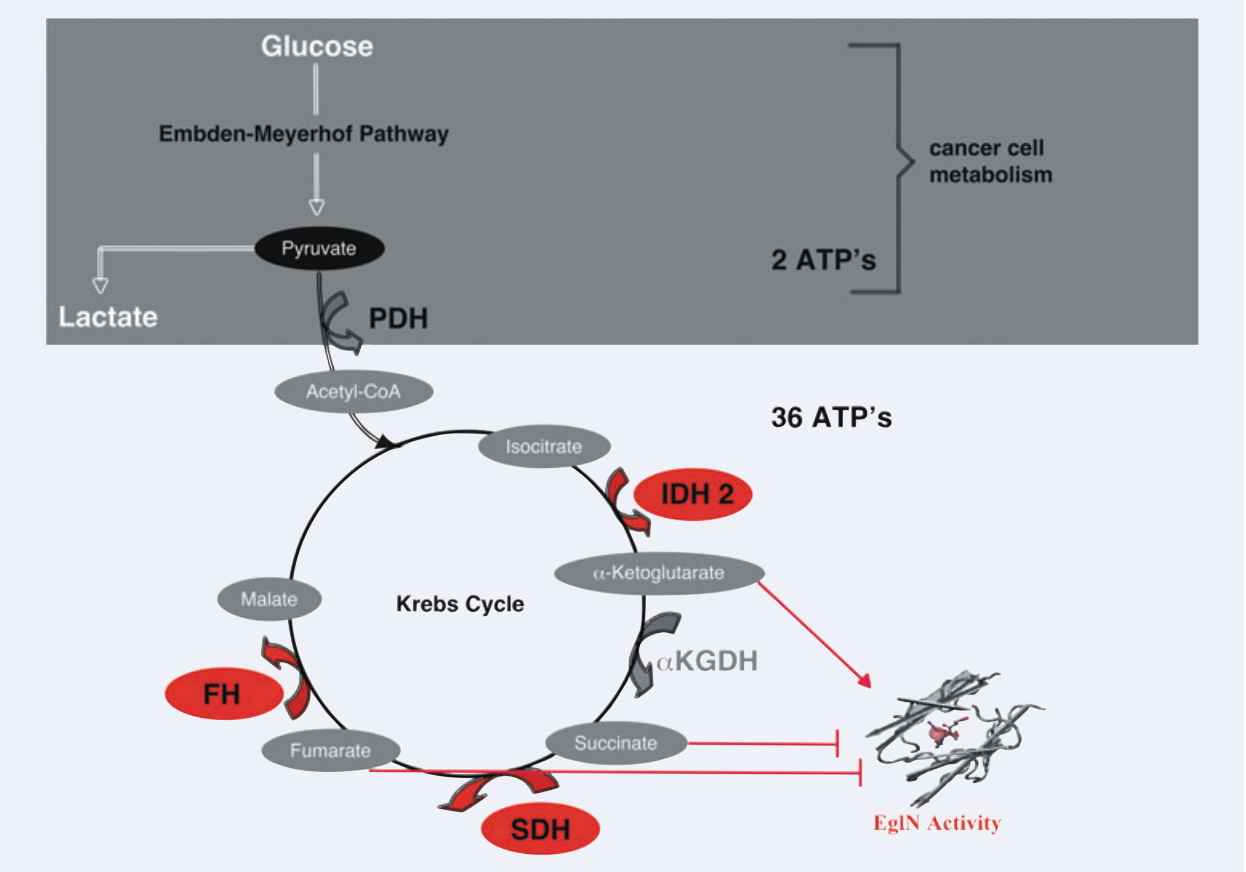

Fig. 3 The Krebs cycle enzymes (*SDH*, *FH* and *IDH*) are tumour suppressors and linked to EglN activity. Inactivation of metabolic enzymes *SDH*, *FH* or *IDH* directly affects EglN activity. The EglN prolyl hydroxylases are dependent upon the Krebs cycle metabolite α-ketoglutarate as electron donor for hydroxylation activity. Loss of function mutation in *IDH* decreases α-ketoglutarate levels and subsequently impairs EglN function. On the other hand, mutation in *SDH* or *FH* leads to accumulation of succinate or fumarate respectively, which in turn product-inhibits EglN activity. Loss of function of *SDH*, *FH or IDH* would lead to impaired Krebs cycle activity and impaired oxidative phosphorylation with the consequence of enhanced glycolysis (Embden–Meyerhof pathway) to generate ATP. The generated pyruvate during glycolysis is redirected away from the Krebs cycle by conversion into lactate. Cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis), an observation that was first made by Otto Warburg in 1924. Despite the striking difference in ATP production, cancer cells might favour aerobic glycolysis to escape from EglN hydroxylation, resulting in the accumulation of oncogenic HIFα and/or resistant to EglN3-mediated apoptosis.

FH increases the accumulation of succinate and fumarate respectively. The excess of these metabolites inhibits the proline hydroxylases with the consequences in either accumulation of oncogenic HIF-α and/or blunting EglN3-mediated apoptosis [9, 10, 71, 96].

Future directions

The recently identified mutations in the metabolic/mitochondrial enzymes (FH, SDHB/C/D, IDH1/2) provide convincing evidence that alteration in cellular metabolism contributes to the pathogenesis of cancer. Hence, 43 years later we are beginning to learn the depth of Otto Warburg's foresight that (in his words) 'cancer cells should be interpreted as a mitochondrial dysfunction' and that 'the prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar' [97, 98]. Even if this is the prime cause for some cancers, the underlying mechanistic basis remains controversial. However, recently it has become more evident that the inactivation of the EglN prolyl hydroxylases is a downstream effector of these mitochondrial alterations. Consequently, EglN inhibition results in activation of oncogenic HIFα and/or blunting EglN3-mediated apoptosis. The identification of SDH and FH mutations has already led to the development of cell permeable α-ketoglutarate derivates with the potential to suppress the transforming effects of these mutations through reactivation of the EglNs [99]. The exciting part of identifying metabolic-enzyme mutations in specific cancers is that they are 'druggable'. They

might provide new opportunities as therapeutic targets that would be more susceptible and more effective than existing cancer therapies. Future work is needed to further elucidate the direct impact of cancer metabolism in prolyl hydroxylase functioning.

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