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REVIEW

Cytoglobin: biochemical, functional and clinical perspective of the newest member of the globin family

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Abstract Since the discovery of cytoglobin (Cygb) a decade ago, growing amounts of data have been gathered to characterise Cygb biochemistry, functioning and implication in human pathologies. Its molecular roles remain under investigation, but nitric oxide dioxygenase and lipid peroxidase activities have been demonstrated. Cygb expression increases in response to various stress conditions including hypoxia, oxidative stress and fibrotic stimulation. When exogenously overexpressed, Cygb revealed cytoprotection against these factors. Cygb was shown to be upregulated in fibrosis and neurodegenerative disorders and downregulated in multiple cancer types. *CYGB* was also found within the minimal region of a hereditary tylosis with oesophageal cancer syndrome, and its expression was reduced in tylotic samples. Recently, Cygb has been shown to inhibit cancer cell growth in vitro, thus confirming its suggested tumour suppressor role. This article aims to review the biochemical and functional aspects of Cygb, its involvement in various pathological conditions and potential clinical utility.

Keywords Cytoglobin · Nitric oxide dioxygenase · Hypoxia · Oxidative stress · Fibrosis · Cancer

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Cytoglobin ancestry—globin family genes

Globins are small, respiratory proteins, which are expressed in a wide range of organisms, from archaea to animals [1, 2]. They have a compact helical conformation and an ability to attach haem that allows reversible binding of gaseous, diatomic molecules, including oxygen (O_2), nitric oxide (NO) and carbon monoxide [3]. Four globins have been discovered in higher vertebrates: erythrocyte-specific haemoglobin (Hb), myoglobin (Mb) localised in muscles, neuroglobin (Ngb) characteristic for neurons and neuroendocrine tissue, and cytoglobin (Cygb) detected ubiquitously in various organs [4–8]. The paradigmatic functions of the globins are related to O_2 transport and storage. However, some globins sense O_2, detoxify sulphide or conduct enzymatic redox reactions like NO dioxygenation, nitrite reduction and lipid peroxidation [9–13].

Biochemical aspects of Cygb

Protein structure

Cygb is a 21-kDa protein consisting of 190 amino acids [14]; however, in contrast to other globins, mammalian Cygb is extended at both its termini [4]. Cygb forms a homodimer [15] stabilised by electrostatic interactions, hydrogen bonds, [16] and inter-subunit disulfide bridge [17, 18]. It is of note, however, that only a small fraction of dimer might be formed in near-physiological conditions in vitro [17]. Its tertiary structure exhibits typical globin-like fold comprising 8 helices (from A to H) arranged in three-over-three sandwich configuration [18]. Conserved amino acids include residues within the dimerisation interface,

extended hydrophobic haem pocket, and distal (E7) and proximal (F8) histidines (His) responsible for iron binding [19].

Cygb is a hexacoordinated protein [8]. In the absence of an external ligand, iron creates a bond with six nitrogen molecules—four residing within haem and two derived from imidazole rings of HisF8 and HisE7. This arrangement is similar to Ngb and non-symbiotic plant globins, but distinguishes Cygb from pentacoordinated globins, i.e. Hb and Mb. In hexacoordinated globins, an external ligand (e.g. oxygen, O_2) must compete with HisE7 to attach to iron. Nonetheless, similarly to Mb, Cygb exhibits high intrinsic affinity to oxygen [18, 20]. Cygb contains two cysteines, which might form intramolecular or intermolecular disulfide bridge [15, 17]. Their substitution or reduction diminished Cygb affinity to O_2. This indicates that cellular redox state may influence the protein structure by S–S bond formation or cleavage, thus affecting O_2 binding. It has been proposed that conformational changes alter E-helix position and, subsequently, HisE7 affinity to iron [15, 17]. Amino acids in close spatial vicinity to haem create an apolar environment [7], which renders Cygb stable in the oxy-state [20].

There has been no evidence to support the participation of Cygb in any protein–protein interactions. Previous studies using yeast two-hybrid assay and immunoprecipitation/mass spectrometry did not discover any interacting partners of Cygb [21]. Moreover, Cygb amino acid sequence does not contain any of the known reticulum or membrane retention signals, motifs targeting peroxisomes or RNA, DNA, actin and ribosome binding sequences [14]. However, it has been shown that human Cygb binds to lipids, which enforces its transition from hexa- to pentacoordination [22].

Organ, tissue and cellular distribution

Mammalian Cygb has been shown in multiple studies to be expressed ubiquitously across a broad spectrum of vertebrate organs including liver, heart, brain, lung, retina, gut, oesophagus and many others [4, 8, 23–28]. Cygb has been detected at varying levels among various tissues and its concentration is relatively low (i.e. μM range) [29]. Tissue type-specific distribution of Cygb is still a matter of debate. Cygb was found predominantly in fibroblasts of connective tissue and in fibroblast-related cell lineages, such as chondroblasts, osteoblasts, hepatic stellate cells (HSCs) and myofibroblasts [14, 26–28, 30, 31]. Several studies have failed to detect the protein in the organ-specific cells [14, 26, 27]. However, a number of investigations have demonstrated that Cygb localises in neurons [23–25, 27, 31–34], macrophages, muscles [28], hepatocytes [23, 28] and epithelium (including specialised epithelial types, e.g. endothelium) [23, 28, 30, 35]. Cygb localisation in the cells of connective tissue [27] and epithelium explains its ubiquitous expression among various organs. Despite few contradictory reports, a certain consensus seems to have been reached regarding subcellular Cygb localisation. Cygb has been shown to localise in the cytoplasm of fibroblasts and other mesenchymal cells [14, 26–28, 31], neurons [24, 27, 31, 33, 34] and epithelium [28, 30, 35]. Nuclear distribution has been demonstrated mainly in neurons [23, 24, 27, 31, 33]. However, Cygb has also been detected in the nucleus in various epithelial cells [23, 30], as well as in some hepatocytes [23, 28] and connective tissue cells [30, 31]. Moreover, cytoplasmic and nuclear localisation has been demonstrated after enforced overexpression of Cygb [21, 26, 27], supporting the possibility of Cygb translocation to the nucleus.

Specific Cygb expression profile in neurons suggests that the globin may have divergent role in this cell population compared to mesenchymal cells [27], but none has yet been defined. It has been proposed that Cygb is implicated in collagen synthesis in fibroblasts and other fibroblast-like cells [27], and this possibility will be discussed in more detail below. The presence of Cygb in the nucleus may contribute to gene regulation and/or tighter control of the concentration of its substrate/product molecules. This might be particularly true for neurons, which have the highest nitric oxide (NO) synthesis compared to other tissues [36]. Due to its NO dioxygenase role (as described below), Cygb may modulate gene expression or protein/DNA modifications induced by NO and/or reactive nitrogen species (RNS). Moreover, Cygb may protect nuclear components from toxic effect of nitrosative stress. The small size of Cygb and the lack of a nuclear transport motif in its sequence led to the hypotheses that passive diffusion, cell-specific factors and/or cytophysiological conditions (e.g. stress) may stimulate globin translocation or retention within a nucleus [21, 23, 27].

At the moment, cytoplasmic and nuclear Cygb localisation is thought to occur mainly in neurons, while strictly cytoplasmic Cygb expression is attributed to fibroblasts and other mesenchymal cells. The general view on Cygb distribution originates from the reproducible results from several research groups [24, 26, 27, 31, 33]. The discrepancies between the studies on the cell-type and subcellular localisation of Cygb might have arisen due to technical issues related to the specificity of the antibodies used, immunodetection methods applied and endogenous Cygb expression level. In order to avoid cross-reactivity with the proteins of variable abundance between the cells or cellular compartments, it is advocated to utilise a few immunostaining procedures and antibodies targeting various epitopes of the protein of interest.

Gene structure

Human *CYGB* is a single copy gene mapped at the 17q25.3 chromosomal segment. Evolutionary studies suggest its high homology with *Mb* and it is thought that *CYGB* emerged during a large-scale duplication event [4]. *CYGB* gene has the lowest mutation rate among vertebrate globins [19, 37], which indicates that not only individual residues but also large sections of the protein are crucial for Cygb functioning [37]. The mammalian *CYGB* gene harbours two introns at positions B12-2 and G7-0 (i.e. before the first nucleotide in the 7th codon of helix G), which are typical for all globins. Genes encoding hexacoordinated globins carry an additional third intron. Although it is usually located at position E11-0, the third intron of *CYGB* uniquely occupies position H36-2 [8]. There is also the putative *FM8* gene transcribed from the reverse strand, which overlaps with the *CYGB* gene. *FM8* does not harbour any specific open reading frame and shows high variability in alternative splicing. Hypothetically, it may serve as a negative regulator of *CYGB* utilising RNAi pathways. However, functional evidence is still required to confirm this hypothesis [38].

Gene regulation

Interspecies alignment of *CYGB* genes revealed high conservation of some non-coding fragments. The *CYGB* promoter has no TATA box, contains a 1.4-kb-long CpG island and several possible transcription start sites. The *CYGB* non-coding sequence contains multiple conserved regions characteristic for the genes associated with cellular response to hypoxia. This includes hypoxia-responsive elements (HRE), hypoxia-inducible protein binding sites (HIPBS) and recognition sites required for the attachment of a number of hypoxia-related transcription factors [37]. HREs comprise the consensus core motif 5′-RCGTG-3′, which is prerequisite for binding of hypoxia-inducible factor 1 (HIF1) [39]. HIFs (HIF1, HIF2) are considered to be the key regulators of cellular response to O_2 deprivation [40], which regulate transcription of a plethora of genes implicated in various metabolic pathways including apoptosis, angiogenesis, proliferation [41], pH regulation [42], glycolysis [43], Fe^{2+} and O_2 homeostasis [44] and genetic instability [45]. HIPBSs contain attachment sites for proteins involved in transcript stabilisation upon hypoxia [46].

Several transcription factor binding sites have been identified within the *CYGB* promoter including HIF1, stimulatory protein 1 (SP1), activator proteins (AP1, AP2), nuclear factors (NF1, NFκB, NFAT), CCAAT/enhancer binding protein (C/EBP), and cellular erythroblastosis virus E26 oncogene homolog 1 (cETS-1) [37, 47]. The SP1 and NFκB binding sites are contained within the CpG island in *CYGB* promoter, which implies epigenetic control of Cygb expression. NFκB, AP1 and C/EBP factors are known to play a role in signal transduction related to hypoxia, oxidative stress and inflammation [36, 48–51]. ETSs are proto-oncogenes implicated in stem cell development and senescence pathways [52], as well as in tissue remodelling as observed in asthmatic patients [53].

Five transcription factors have been shown to physically bind to and transactivate the *CYGB* promoter [47, 54]. The minimal promoter region required for efficient transcription initiation was ascribed to the fragment located between −1,113 and −10 nucleotides upstream of the start codon. Site-directed mutagenesis and electrophoretic mobility shift assays demonstrated that one c-ETS-1 and three SP1 binding sites are required for efficient *Cygb* transcription in normoxia [47]. In hypoxia, a similar approach provided evidence for HIF1 interaction with *CYGB* promoter, while site-directed mutagenesis in HIF1- and EPO-binding motifs revealed that these sequences are crucial for Cygb regulation [54]. Recent studies have demonstrated that NFAT and AP1 induce Cygb expression, thus mediating calcineurin signalling [55]. Moreover, Cygb expression has been found as a downstream target of tumour growth factor β (TGF-β), platelet-derived growth factor, protein kinase C [26] and EPO signalling [56]. The Cygb signalling pathway is illustrated in Fig. 1.

Putative functions of Cygb

Oxygen storage, transport and sensor

Cygb possesses intrinsic O_2-binding capacity. Due to its homology with Mb, Cygb was proposed to participate in transport and facilitated diffusion of intracellular oxygen [8, 14]. Although Cygb binds O_2 with high affinity and remains stable in Fe^{2+}–O_2 form, low intracellular abundance of Cygb argues against its role in O_2 delivery to the mitochondrial respiratory system [29, 57]. Another hypothesis proposes that Cygb may serve as an oxygen reservoir. Micro-aerobic conditions promote accumulation of reductive agents (H+, NADH), which in turn modify cellular redox state. This may enforce reduction of the disulphide bridge, a shift in Cygb structure and concomitant O_2 release [15]. Since O_2-binding imposes conformational changes, Cygb may putatively act as a signal transducer into the pathways associated with oxygen sensing [6, 20, 23]. For example, in the nucleus, Cygb might transfer the signal into the transcriptional machinery and, consequently, affect gene expression [23]. As yet, there is no experimental evidence demonstrating direct signal transduction by Cygb. However, due to its involvement in NO biology, which will be discussed in

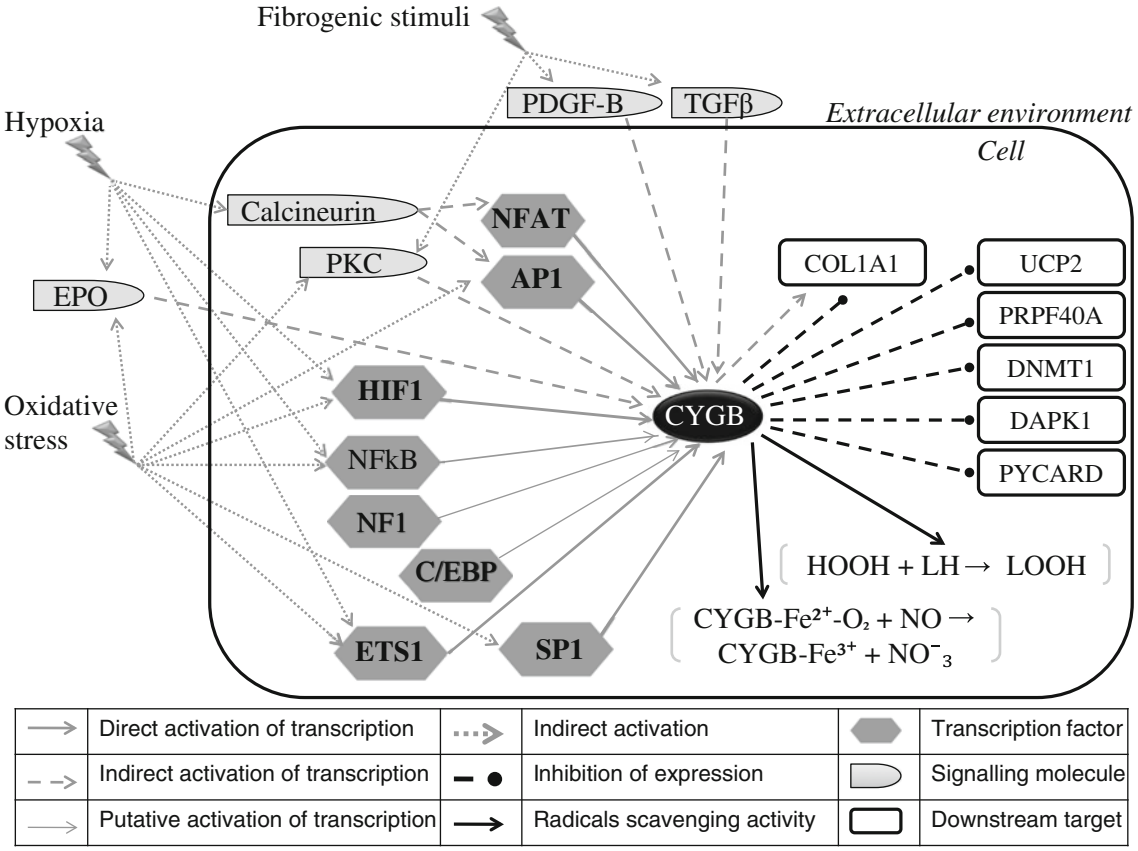

Fig. 1 Upstream regulation and downstream targets of Cygb. *AP1* activator proteins, *C/EBPα* CCAAT/enhancer binding protein, *COL1A1* collagen 1α1, *Cygb* cytoglobin, *DAPK1* death associated protein kinase 1, *DNMT1* DNA-methyltransferase 1, *EPO* erythropoietin, *ETS1* v-ets erythroblastosis virus E26 oncogene homolog 1, H_2O_2 hydrogen peroxide, *HIF1α* hypoxia-inducible factor α, *NF1* nuclear factor 1, *NFAT* nuclear factor of activated T-cells, *NFκB* nuclear factor-kB, *NO* nitric oxide, *PDGF-B* platelet-derived growth factor, *PKC* protein kinase C, *PRPF40A* RNA-binding and pre-mRNA processing factor, *PYCARD* PYD and CARD domain containing protein, *SP1* stimulatory protein 1, *TGF-β* transforming growth factor β, *UCP2* uncoupling protein 2

more detail in the next section, Cygb may contribute to O_2 sensing indirectly through modulation of NO level. This role of Cygb remains to be tested in the future. It is of note that from the functional point of view, Cygb O_2 binding capacity is utilised in NO dioxygenation reaction [58, 59].

Involvement in nitric oxide biology

Oxygen transport and storage, which are well described in the case of Hb and Mb, are in fact relatively novel functions acquired during globin evolution. The common feature within the globin family is nitric oxide dioxygenase (NOD) activity (EC 1.14.12.17) that is hypothesised to be the primary role of globins, including Cygb [9, 13, 60]. Indeed, Smagghe and colleagues demonstrated that oxyferrous Cygb dioxygenates NO (Fig. 1). While this effect was observed in vitro, Cygb substitution in the place of flavoHb (a known NO scavenger) in *E. coli* did not afford cytoprotection upon exposure to the NO donor. This suggested that the presence of appropriate reductive systems is necessary for efficient NOD activity [61]. Further studies in murine fibroblasts engineered to silence and re-express Cygb confirmed that this globin contributes to O_2-dependent NO removal and nitrate production (a feature of NOD activity) in vivo. It has also been demonstrated that Cygb protected against NO-mediated suppression of cellular respiration and growth [59]. The recent report by Gardner and colleagues provided detailed biochemical characterisation of Cygb-NOD activity in vitro and in rat hepatocytes in vivo, which included identification of native reductants supporting Cygb-related NOD function. These comprised ascorbate and reduced cytochrome b_5 as the most efficient reductants, as well as less effective NAD(P)H, NADPH-cytochrome P450 oxidoreductase and cytochrome b_5 reductase. The K_M values for NO and O_2 in the Cygb-NOD reaction were estimated to be 40 nM and ~20 μM, respectively, while its maximal turnover rate achieves ~0.5 s^{-1} in the presence of ascorbate. NO was demonstrated to suppress Cygb-NOD activity at NO:O_2 ratios higher than 1:500. This suggests that, in

physiological conditions, Cygb may function as a NO signalling modulator rather than protector against NO accumulation, while higher levels of Cygb may be required to confer the latter role [58]. Cygb involvement in NO biology is also supported by the findings of Cygb co-localisation with neuronal nitric oxide synthase (nNOS) in certain neuron populations in the mouse brain [24].

While a detailed description of a complex NO biology is not within the scope of this review, it is relevant to note that, depending on the concentration and local microenvironment, NO might act as a messenger and/or cytotoxic molecule [62]. Aerobic NO production is catalysed by various isoforms of NOS and depends on the O_2 concentration [63]. Due to its high affinity to ferrous haem, NO binds to iron-containing proteins, including soluble guanylate cyclase, prolyl hydroxylases and cytochrome c oxidase (CcO) [64]. In normoxic conditions, NO inhibits HIF1α prolyl hydroxylases thereby attenuating HIF1α proteolysis and promoting non-hypoxic HIF1 signalling [65–67]. NO competes with O_2 to bind to the CcO haem, which leads to inhibition of respiratory chain and, consequently, to increased O_2 availability within a cell and higher superoxide production [68]. NO-mediated cytotoxicity is largely conferred through reaction with superoxide, which leads to generation of highly reactive peroxynitrite ($ONOO^-$). Accumulation of peroxynitrite and other nitrosative molecules results in nitrosative stress, which might target tyrosine residues, metalloproteins, lipids and nucleic acids [36, 64]. Cygb was demonstrated to diminish the inhibitory effect of NO on cellular respiration, growth [59] and protect NO-sensitive aconitase [58]. Given Cygb-NOD activity and the versatility of NO biology, it would of interest to test to what extent Cygb might modulate downstream effects of NO in various pathological conditions.

Involvement in oxidative stress

Endogenous Cygb expression was demonstrated to become elevated in response to oxidant exposure. In the investigation on the influence of various stress factors on Cygb expression, hydrogen peroxide (H_2O_2), but not hypoxia (5%), calcium, kainic acid, heat shock or high osmolarity, increased Cygb level in the N2a neuroblastoma cell line [69]. Cygb was also upregulated in the H_2O_2-treated MCF7 breast cancer cell line [70]. Exogenous Cygb overexpression appears to protect the cells subjected to oxidative injury. In particular, oxidant-treated neuronal cell lines (SH-SY5Y, TE671), primary HSCs and kidney fibroblasts overexpressing Cygb has demonstrated higher survival rates [21, 71, 72], ability to scavenge reactive oxygen species (ROS) and lipid-derived radicals [21, 71–73] and lower DNA damage [21, 74]. Moreover, in the remnant kidney fibrosis model, Cygb overexpression in transgenic rats alleviated accumulation of nitrotyrosine and 8-hydroxy-2′-deoxyguanosine, the markers of oxidative and nitrosative stress, thus preserving kidney functioning and reducing fibrotic changes [73]. Inversely, in the cells exposed to oxidative stress, Cygb silencing caused higher apoptosis rate, reduced ROS removal [75] and decreased proliferation rate [59, 69].

The molecular mechanisms responsible for the antioxidant properties of Cygb remain unclear. Cygb, like other hexacoordinated globins, may scavenge reactive oxygen species (ROS) utilising haem and thiol residues [75, 76]. Cygb manifested antioxidant (peroxidase and superoxide dismutase) properties in vitro [14, 74]. However, while being >15,000-fold lower when compared to other known enzymes with a similar role, these activities were subsequently excluded as plausible physiological functions of Cygb. It remains to be tested, nonetheless, whether other substrates and/or supporting systems may facilitate these reactions in vivo. Cygb was shown, for instance, to be potently reduced by the reductive enzymatic system from *E. coli* [74]. Moreover, a recent study revealed that, upon binding to a lipid molecule, Cygb promotes its peroxidation in vitro. Hypothetically, this may lead to the formation of lipid-based radicals that signal an antioxidant response [22]. Previous observations, however, have shown that Cygb overexpression in rat HSCs decreased the formation of H_2O_2-induced lipid radicals, such as malondialdehyde and 4-hydroxy-2-nonenal [72]. Thus, the mechanisms, in vivo validation and biological importance of Cygb-mediated lipid peroxidation need further investigation. Due to its NO dioxygenase activity [58, 59], Cygb might reduce intracellular NO concentration, which in turn may prevent accumulation of peroxynitrite, a strong oxidant formed from the reaction between superoxide and NO [36]. In contrast to peroxidase and superoxide dismutase activities, Cygb-NOD activity is estimated to be approximately 30- to 200-fold lower than that of microbial flavoHb [58, 77]. Future studies should be directed towards the identification of downstream effectors of the globin in stress conditions.

Collagen synthesis

Extensive collagen deposition is one of the characteristic events occurring during organ fibrosis [78]. Cygb was first identified in hepatic stellate cells in fibrotic liver, and its fibrosis-induced expression correlated with the upregulation of collagen I protein [14]. Upon fibrogenic stimulus, pancreatic stellate cells and renal interstitial cells also expressed higher Cygb level and, simultaneously, synthesised more collagen [73, 79]. Moreover, exogenous overexpression of the globin in combination with TGF-β

enhanced collagen production in rat fibroblasts in vitro [26]. This positive association was further proposed to be supported by the observation that Cygb is detected in fibroblasts, chondroblasts and osteoblasts. These cells actively secrete constituents of extracellular matrix in contrast to their mature forms (chondrocytes, osteocytes), which have considerably lower Cygb level [27]. In addition, while hypoxia upregulates Cygb expression, it also triggers organ fibrosis, wound healing, osteogenesis and chondrogenesis, which require excessive, O_2-dependent collagen production. Cygb has been proposed to act as a putative O_2 donor for collagen hydroxylation reactions or to activate signalling pathways leading to enhanced collagen synthesis [6]. It is of note, however, that while Cygb revealed anti-fibrotic properties, its enforced overexpression was also linked to reduced deposition of collagen protein in fibrotic liver [72] and kidney [73]. Also, in vitro data indicate that exogenous Cygb overexpression decreases collagen mRNA and protein in kidney cell lines [73] and collagen mRNA in lung and breast cancer cell lines [80]. Interestingly, Cygb knock-down did not affect collagen expression in kidney cell lines [73]. It seems, therefore, that the relationship between Cygb and collagen expression is complex and likely to be dependent on cellular type, genetics and, more importantly, molecular environment. The mechanisms involved in this association have not yet been identified; however, it would be of interest to test whether they are related to the interplay between Cygb, NO and ascorbate. While ascorbate serves as a cofactor for collagen hydroxylation, NO inhibits collagen production via, e.g., inhibition of prolyl hydroxylase activity and upregulation of matrix metalloproteinases [81–83]. NO-related induction of collagen synthesis in wound healing has also been demonstrated [84, 85]. The association between Cygb and collagen production requires further investigation, as it implies that the globin may be involved in tissue regeneration processes and, thus, may be useful as a potential pharmacological target in the future.

Engagement in cellular response to hypoxic stress

Maintenance of aerobic energy production involves development of protective systems, which defend against oxygen deficits. Proteins with O_2-binding capacity are part of this machinery [86]. Indeed, despite a few inconsistent observations, a growing number of studies support the notion that Cygb is implicated in hypoxia-related pathways. Oxygen deficiency caused significant Cygb augmentation in most of the animal and tissue culture models studied. Cygb was upregulated in hypoxic liver, heart [27, 87], muscle [87], to a smaller extent in brain [25, 87] and, after cobalt chloride-induced chemical hypoxia, in kidney [73]. In vitro experiments have demonstrated hypoxia-driven Cygb upregulation in neuronal HN33 [87], transformed bronchial epithelial BEAS-2b, cervical cancer HeLa [54], and glioblastoma multiforme cell lines [30]. The increase of Cygb expression depended on duration and severity of hypoxia as well as the tissue type examined [75, 88]. However, in some studies, neuronal concentration of Cygb in vivo was not affected following O_2 limitation [33, 69] or ischaemia and reperfusion episodes [88]. An ischaemic neuroblastoma model revealed that exposure to ischaemia induced a modest downregulation of Cygb, while anoxia evoked significant Cygb upregulation [75]. Anoxia also promoted Cygb upregulation in head and neck [89], and in hepatic and renal cancer cell lines [35]. It has been recently shown that high Cygb mRNA levels correlated with characteristic features of tumour hypoxia, namely with HIF1α upregulation and tumour aggressiveness in head and neck cancer tissues [89]. Cygb has also been reported to be co-expressed with carbonic anhydrase IX, another marker of tumour hypoxia, in breast, liver, bladder, thyroid, ovary and brain tumours [30]. Interestingly, Cygb has been shown to have a cytoprotective effect upon exposure to hypoxia or ischaemia. In transplanted β-islet cells undergoing ischaemic injury, Cygb overexpression afforded higher survival, function maintenance and reduced immunorejection [90]. In another study, exogenous overexpression of Cygb supported cell survival, while Cygb silencing augmented cell death in ischaemic conditions [75].

The exact mechanism of Cygb action in hypoxia remains to be elucidated. Several hypotheses were proposed, however, including Cygb role in O_2 storage [15], O_2 delivery to HIF1α hydroxylation reactions [29] and, owing to changes in Cygb conformation upon O_2 release, signal transduction to the pathways associated with hypoxia response [6, 20, 23]. Cygb may also diminish cytotoxic accumulation of reactive oxygen and nitrogen species (ROS/RNS) occurring during hypoxia–reperfusion episodes [75]. Another issue requiring clarification is Cygb impact on NO concentration in hypoxic conditions. NO might modulate HIF1α signalling in normoxia and hypoxia in a dose-dependent manner due to its regulatory effect on O_2 concentration and sensing [91]. In normoxia, NO transiently inhibits HIF1α-related prolyl hydroxylases resulting in stabilisation of HIF1α and activation of hypoxia-responsive pathways [65, 66]. In the later stage of HIF1α signalling, NO enhances upregulation of prolyl hydroxylases providing a negative feedback loop for HIF1α degradation [65, 92, 93]. Importantly, NOS-associated NO production becomes limited at low O_2 tension, whereas haem-containing proteins, including globins, might reduce nitrite to NO to modulate cell respiration and hypoxic signalling [10, 11, 94]. Although hexacoordinated haemoglobins were suggested to have too high an affinity towards

O_2 to conduct nitrite reduction [95], such activity has been demonstrated for NGB, but not Cygb [76]. In the study conducted by Petersen and colleagues, Cygb-associated nitrite reduction capacity was investigated in vitro in anoxic conditions [76]. Their results argue against a nitrite reductase (NR) function of Cygb. However, in order to determine Cygb function in hypoxia, it would be necessary to directly compare the NOD and NR activities of Cygb in vivo at physiological O_2 and NO concentrations characteristic for hypoxic conditions.

Due to its responsiveness to and protection against certain injuries, Cygb functions might stay on the "crossroads" of the pathways implicated in the adaptation to various stress conditions. It is well established that hypoxia generates ROS/RNS, which in turn control responses to O_2 deprivation [91, 96], while these stresses are associated with tissue fibrosis [97, 98]. Moreover, recent expression profiling studies have established collagen type 1 (COL1A1), RNA-binding and pre-mRNA processing factor (PRPF40A) and uncoupling protein 2 (UCP2) genes as immediate downstream targets of Cygb, which are downregulated following transient Cygb overexpression in cancer cell lines [80]. These genes have already been linked to pathways related both to hypoxia and oxidative stress [99], which further supports the involvement of Cygb in various stress-related pathways.

Disorders associated with Cygb

Increasing amounts of data indicate Cygb involvement in the pathogenesis of multiple diseases. It seems likely that Cygb participates in the maintenance of normal phenotype by implementing a homeostatic effect, which counteracts stress conditions imposed on a cell. Endogenous increase of Cygb has been detected in glaucoma regions [34], fibrotic liver [14], gastroesophageal reflux disease [38], putaminal neurons and glia in patients with hereditary ferritinopathy [100] and cytoplasmic inclusions in the neocortex of patients with psychomotor retardation and/or epilepsy [101]. Conversely, reduced Cygb expression has been found in diverse cancer types including oesophagus, lung and head and neck [38, 80, 89, 102, 103]. Interestingly, a common link of all these disorders is their association with hypoxic, oxidative or/and nitrosative stress events.

CYGB in neurodegenerative disorders

Cygb upregulation in neurodegenerative disorders might indeed be connected with the stress-related conditions. Unstable blood flow within the retina generates ischaemia (and peroxynitrite accumulation) leading to subsequent nerve damage and glaucoma development [104]. Hereditary ferritinopathy is linked to the mutation within the ferritin gene, which results in pathological iron accumulation imposing acute oxidative injury and, consequently, apoptosis [100]. Recently, immunohistochemistry on brain specimens obtained from five patients with epilepsy and/or psychomotor retardation [101], and one out of two epileptic patients [105], showed positive Cygb staining within astrocytic inclusions. Although a cause–effect relationship between inclusions and seizures is unlikely [105], it cannot yet be excluded [101]. Mitochondrial malfunctioning (and therefore oxidative stress) might contribute to a seizure-prone phenotype, whereas free radicals accumulate as a consequence of epileptic seizures [106]. Neuronal hyperactivity reduces energy, oxygen and glucose level and increases lactic acid and glutamate concentration [101]. It also leads to NFκB-induced nNOS upregulation and production of NO that together with superoxide promote nerve damage [36, 107]. Therefore, it might be speculated that Cygb overexpression is induced by redox/oxygen imbalance, possibly via NFκB signalling. Alternatively, susceptibility to seizures might be elevated if functional Cygb is depleted due to its capture within the inclusions. This would impede maintenance of redox homeostasis, resulting in chronic oxidative/nitrosative stress and higher risk of seizures.

CYGB in fibrosis

The first experiments on Cygb demonstrated its upregulation in the activated rat HSCs after fibrosis stimulation [14]. In normal physiological conditions, these cells are responsible for blood flow control and vitamin A storage. In injured liver, activated HSCs undergo transformation into myofibroblasts and promote deposition of extracellular matrix components leading to organ fibrosis [108]. This activation might be induced with extensive ROS production, growth factor (e.g. TGF-β1) and cytokine secretion, which is mediated by neighbouring cells and an inflammatory response [109]. Two-dimensional SDS-PAGE coupled with mass spectrometry analysis revealed that upon treatment with a fibrogenic agent HSCs expressed higher level of Cygb. It has consequently been proposed that elevated Cygb expression might serve as a biomarker for fibrotic tissue [14]. Fibrosis/myofibroblasts-specific higher levels of Cygb have also been found in other research models in fibrotic liver [31, 108, 110, 111], kidney [73] and pancreas [26, 79]. It is of note that Cygb upregulation frequently coincided with collagen upregulation [26, 73, 79]. Furthermore, liver fibrogenesis was prevented and reversed upon delivery of Cygb transgene prior and after the fibrogenic stimulation, respectively. Exogenous Cygb overexpression preserved liver architecture and

histology, reduced liver necrosis and dysfunction and suppressed fibrotic changes, including diminished collagen synthesis and HSC activation [72]. In the study conducted by Mimura et al. [73], Cygb overexpression in transgenic rats attenuated the effect of subtotal nephrectomy by decreasing loss of renal function, kidney fibrosis, collagen deposition, fibroblast activation, macrophages infiltration and oxidative and nitrosative stress. Although overexpression of Cygb protects the cell against certain stresses, endogenous Cygb upregulation is insufficient to counterbalance the trauma [72].

How Cygb exerts cytoprotective effect after overexpression is as yet unknown. Hypothetically, it may provide an antioxidant defence, since antioxidant treatment has anti-fibrotic properties [51, 109] and Cygb overexpression was demonstrated to reduce oxidative stress in fibrotic models [72, 73]. However, ROS removal capacity remains a controversial function of the globin [74]. Neither Cygb NO dioxygenation activity will be a simple explanation due to bimodal function of NO in fibrosis, which includes both anti-fibrotic properties at the early stage and a pro-fibrogenic effect via RNS formation depending on the concentration and environment [51, 83, 109]. Also, Cygb may prevent collagen production to contribute to fibrosis prevention, but the molecular association between these two molecules is still poorly characterised. An alternative explanation for CYGB cytoprotective features may emerge from the linkage between NO and oxidative stress. Apart from peroxynitrite generation, NO and/or its derivatives augment oxidative stress via inhibition of antioxidant enzymes, such as catalase [112, 113], peroxidase [114, 115], cytochrome P450 [116, 117] and haem oxygenase [118]. These mechanisms may explain the antioxidant role of CYGB, which has very low enzymatic activity directed against reactive oxygen species [74]. In order to define anti-fibrotic mechanisms of Cygb functioning, these issues need to be addressed in future studies. It would also be of interest to determine whether Cygb knock-out provides higher susceptibility to or augments fibrogenesis in animal model.

CYGB in cancer

Deregulation of Cygb expression has been shown in multiple malignancies (Table 1). The first genetic association between Cygb and tumour was demonstrated in the tylosis with oesophageal cancer (TOC) syndrome [38]. Tylosis (palmoplantar keratoderma) manifestations include pathological thickening of the palmoplantar area of the skin frequently resulting from aberrant production of keratins and cohesion molecules [119]. TOC is an autosomal dominant hereditary disorder associated with higher risk of oesophageal cancer and oral lesions [120]. Chromosome mapping, which involved three affected families, revealed the minimal region (42.5 kb) responsible for TOC occurrence. The TOC minimal region bears regulatory elements for two genes, which showed no implication in disease development, and the entire *CYGB* gene sequence. No disease-specific mutations within the *CYGB* gene have been identified [121]; however, its expression was found to be downregulated in tylotic oesophageal biopsies when compared with normal controls. While the Cygb silencing mechanism in TOC has not yet been defined, low levels of Cygb in sporadic oesophageal cancer have been associated with promoter hypermethylation and loss of heterozygosity (LOH) at chromosome 17q25 [38]. Low levels of Cygb have also been linked to the 17q25 LOH in ovarian cancer [122].

Similar mechanisms of Cygb downregulation, namely LOH and promoter hypermethylation, have also been reported in non-small cell lung cancer (NSCLC) [103]. Significantly lower Cygb expression was found in 54% of the lung cancers in comparison to adjacent normal tissues, whereas promoter hypermethylation was detected in 48% of the tumour samples. Methylation status of the *CYGB* promoter inversely correlated with Cygb mRNA levels. LOH at 17q25 chromosome was also demonstrated to be a frequent event in NSCLC (in 67% of the tumours). However, it showed a considerable impact on Cygb mRNA level reduction only when combined with the hypermethylation data. Furthermore, the study provided evidence that Cygb downregulation is more distinct in poorly differentiated tumours, while *CYGB* promoter methylation is higher in adenocarcinoma samples [103]. The epigenetic control of Cygb expression was further confirmed in the in vitro experiments, in which treatment with a demethylating agent restored Cygb expression in the cell lines with hypermethylated *CYGB* promoter [80, 89, 103].

CYGB promoter hypermethylation and the associated mRNA downregulation have also been shown in head and neck squamous cell carcinoma (HNSCC) [89]. Hypermethylation within the *CYGB* promoter has been independently observed in two additional studies in HNSCC [102, 123], as well as in oral epithelial dysplasia [124], leukaemia, breast, colon and bladder cancers [80]. The high frequency of *CYBG* promoter hypermethylation across various tumour types and stages implies that this phenomenon might be an early event during carcinogenesis [80, 124]. This advocates usage of *CYGB* methylation status as an epigenetic biomarker for cancer detection [123]. Cancer-related promoter hypermethylation frequently affects expression of tumour suppressor genes (TSGs) [125]. Recent findings indicate that, indeed, Cygb might serve this role. Liquid colony formation assay showed that restoration of Cygb expression in lung cancer and breast cell lines reduces their growth capacity on the selection plate. Conversely, Cygb silencing increased the

Table 1 *CYGB* expression and methylation status in various human cancers

Cygb status	Cancer site (patient number)	Results	Ref.
Promoter hypermethylation and low mRNA level	Oesophagus (10)	Higher MtI ($p = 0.02$) in tumour samples (avg MtI = 19%) than in adjacent normal tissue (avg MtI = 7%);	[38]
		LOH of TOC minimal region	
		Low mRNA level in 5 cancer cell lines in comparison to normal oesophageal biopsy	
Promoter hypermethylation	Head and neck (80)	Higher MtI ($p = 0.002$) in tumour than in normal tissue	[102]
		MtI > 5% in 65% of tumours (52/80, max MtI = 55%) and 52% of normal tissue samples (11/21, max MtI = 14.9%)	
Promoter hypermethylation and low (and high) mRNA level	Non-small cell lung cancer (NSCLC) (52)	Twofold increase of MtI ($p < 0.001$) in 48% tumours (avg MtI = 0.159 versus 0.056 in adjacent normal tissue)	[103]
		Lower mRNA expression ($p = 0.001$) in 54% of tumours (Cygb/TBP = 0.76 ± 0.1) versus 1.8 ± 0.2 in normal adjacent tissue; high MtI correlated with low mRNA level ($p = 0.009$); 8/48 samples showed Cygb upregulation	
		LOH of 17q25 region in 67% of tumour samples	
		Cygb upregulation in lung cancer cell line with high MtI% for *CYGB* promoter after 5'azacytidine treatment	
Promoter hypermethylation and in vitro TSG properties	Breast (20) Bladder (41) Colon (20) Leukaemia (27) Lung (30)	High MtI in 75% of breast tumours, 27% bladder tumours, 40% colon tumours and 25% leukaemias	[80]
		24 CpG sites in *CYGB* promoter methylated in 8/10 lung cancer cell lines and 4/4 breast cancer cell lines, but not in non-malignant epithelial cell lines, Cygb mRNA level increased after treatment with 5′azadeoxycytidine in 3 lung cancer cell lines with high MtI for *CYGB* promoter	
		High MtI in 63% of NSCLC versus 18% of adjacent normal tissue, in 30% (4/13) of sputa from NSCLC patients versus 0% (0/25) in COPD patients ($p < 0.008$), 87% (26/30) breast cancer versus 40% (13/30) adjacent normal tissue, MtI level discriminated between cancer and normal tissue (AUC = 0.91)	
		Cygb silencing increased the number of colonies in liquid colony formation assay, and Cygb overexpression—decreased number of colonies	
High protein level	Lung and brain metastases of alveolar soft-part sarcoma (2)	Cygb overexpression in pulmonary and brain metastases of ASPS in contrast to the stroma as assessed with immunohistochemical staining	[128]
Promoter hypermethylation and high and low mRNA level	Head and neck (37)	High MtI (≥25%) in 11/37 and MtI <3% in 10/37 in tumour samples	[89]
		Relative Cygb expression in tumour ranged between 0.14- and 10.7-fold difference compared to normal epithelium	
		Cygb downregulation correlated with promoter hypermethylation	
		Cygb upregulation correlated with HIF1a expression ($p < 0.01$) and with tumour aggressiveness	

The table shows the site of the cancer, number of patients included in the study, major findings related to *CYGB* status and relevant reference (*Ref.*)

colony formation ability of a lung cancer cell line. Moreover, Cygb overexpression reduced the level of several genes [COL1A1, PRPF40A, UCP2, death-associated protein kinase 1 (DAPK), PYD and CARD domain containing (PYCARD) and DNA-methyltransferase 1 (DNMT1)], which play a role in the maintenance of a cancerous phenotype [80].

It remains to be elucidated what are the exact molecular mechanisms conferring TSG activity of Cygb. Taking into account the scavenging capacity of the globin, it might be speculated that Cygb reduces the carcinogen-mediated burden of oxidative and nitrosative stresses, thereby protecting the cells from damage to DNA, proteins and membranes. It may also alleviate the upregulation of redox-sensitive signalling pathways implicated in carcinogenesis [126]. Alternatively, loss of Cygb might impair regeneration in the tissue exposed to multiple microinjuries and environmental insults (as it happens frequently in the case of upper aerodigestive tracts). This might subsequently evoke fibrosis, which is frequently associated with inflammatory

response and aberrant epithelial–mesenchymal interactions. Inflammatory stimulation results in increased NO synthesis (and peroxynitrite formation), which affects p53 and mitogen-activated protein kinase pathways and promotes angiogenesis, migration, invasion and DNA damage in a manner dependent on NO concentration, cellular type and genetic background [36, 127]. Moreover, inflammation can induce oxidative stress and abnormal cytokine secretion leading to mutations and augmented cellular proliferation, respectively [98]. These events would support carcinogenesis in affected cells (Fig. 2).

Most of the data published so far demonstrate Cygb silencing in malignant tissues. However, certain studies have shown that Cygb might also be up-regulated in a cancer-specific manner. Although the majority of NSCLC cases had reduced expression of Cygb, there was a subset of samples (8/48) with high Cygb mRNA content [103]. Immunohistochemical studies in alveolar soft-part sarcoma also revealed Cygb overexpression in lung and brain metastases, but not in the stromal myofibroblasts or surrounding normal tissue. Tumour cells also expressed prolyl-4-hydroxylase, which is a prerequisite for collagen cross-linking, and, like Cygb, is upregulated by oxygen deficit [128]. Furthermore, almost one-third of the HNSCC tissue specimens exhibited elevated Cygb expression, which correlated to HIF1α mRNA levels. High Cygb mRNA concentration was also negligibly associated with other characteristics of tumour hypoxia, such as greater aggressiveness, more profound invasion and nodal metastasis [89]. In the tissue microarrays study utilising tumours of various histological origins and matched normal tissue samples, it has been demonstrated that Cygb expression is elevated in the cancer sections when compared to normal tissue. Interestingly, Cygb protein was co-expressed in the similar tumour sections as carbonic anhydrase IX; however, only in a few cancer types [30]. Furthermore, differential Cygb expression in various tumour types was recently reported by Gorr and colleagues. In their study, Cygb protein level was generally lost in breast carcinomas when compared to normal samples, although some of the tumour samples revealed high Cygb expression. In the mRNA analysis in a small number of samples, Cygb was found downregulated only in lung adenocarcinomas, while other tumour types tested did not differ in the level of Cygb from the normal tissues. In agreement with other studies, Cygb protein correlated with the expression of hypoxia marker genes, including HIF1α, HIF2α, CAIX and fatty acid synthase, but not with glucose transporter 1 [35]. Hypothetically, in developed malignancy, Cygb may be upregulated in response to oxygen deficiency in order to maintain inner homeostasis [89]. Stress-related adaptive machinery in cancer frequently corresponds to modulation of signalling pathways that control cell survival, apoptosis, angiogenesis and resistance to chemo- and radiotherapy [129]. Cygb as NOD may also modulate NO concentration [58] in a cancer setting and thus affect HIF1α stability and signalling [65, 67, 92, 93]. If Cygb function is to counteract hypoxia, oxidative and/or

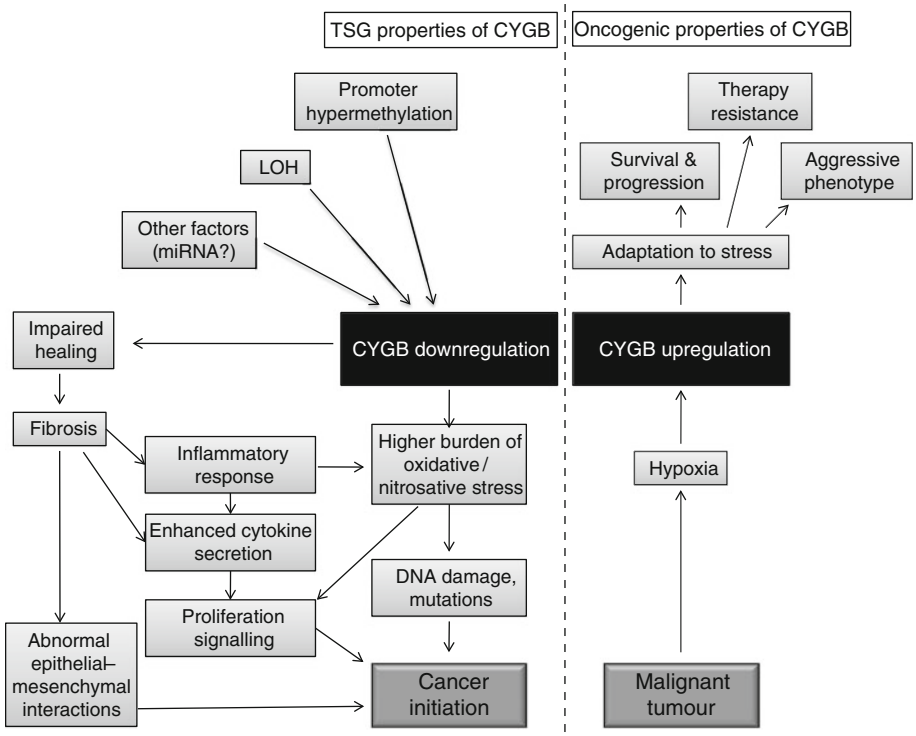

Fig. 2 Putative oncogenic and tumour suppressor activities of Cygb

nitrosative stress, then it may also be implicated in the adaptive responses supporting more aggressive phenotype in cancer (Fig. 2). If this is correct, Cygb, like a few other genes (e.g. TGF-β) [130], could exhibit both tumour suppressor and oncogene features. TSG capacity and cytoprotective features in stress-related events favour Cygb as a potential therapeutic target. However, further investigations are required in order to fully characterise molecular and cellular functions of Cygb.

Conclusions and future directions

Since the discovery of Cygb a decade ago, growing amounts of data have been gathered to characterise Cygb biochemistry, molecular function and implication in human pathologies. Cygb is a hexacoordinated, heam-containing protein able to bind O_2, NO and carbon monoxide. Its affinity to external ligand is redox-regulated and reduction of the intramolecular disulfide bridge decreases Cygb-O_2 binding. Many downstream effects are associated with Cygb, including tumour suppression and cytoprotection against oxidative and nitrosative stress, hypoxia, ischaemia and fibrotic stimulation. From the functional point of view, the most convincing evidence to date indicates that at the molecular level Cygb acts as a NO dioxygenase. Through NO-related reaction, Cygb may affect various pathways of multifaceted NO biology and serve as part of the cellular O_2-sensing machinery. Cygb-NOD function partly explains its cytoprotection towards stress conditions, in which NO signal transduction plays an important role. It is of note, however, that both NO and ROS signalling might be involved in all of the Cygb-related effects outlined above, and Cygb, similarly to other globins, may have multiple molecular functions [13]. Therefore, it should be investigated in the future whether modulation of NO level is the only mechanism of a Cygb-mediated homeostatic effect or it is one of the possibilities in the repertoire of globin functions. In particular, the Cygb molecular mode of action (including NO dioxygenation and lipid peroxidation) under stress conditions, like hypoxia and oxidative stress, should be thoroughly tested.

The clinical utilisation of Cygb in various aspects of patient management is under investigation. *CYGB* promoter methylation may be of value in cancer diagnosis [123], while Cygb protein expression may serve as a fibrosis biomarker [14, 31]. It has been recently proposed that Cygb overexpression through gene therapy methods may be a useful tool assisting transplantation, cancer and fibrosis treatment [131]. However, this approach will require thorough analysis of the Cygb molecular and cellular function to assist circumvention of potential side effects, such as a hypoxia-related aggressive phenotype [89]. Large-scale, comprehensive exploration of Cygb downstream proteomic or metabolomic targets in normal and stress conditions may provide an insight into specific pathways which are under Cygb control. If Cygb is involved in NO homeostasis during pathogenesis, then targeting NO metabolism may prove more beneficial than the introduction of the *CYGB* gene. Nevertheless, it is clear that further investigations are required to explain the mechanism by which Cygb contributes to the molecular pathogenesis of human diseases and to rationalise the utilisation of Cygb in the clinical management of relevant disorders.

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Conflict of interest None.

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