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Review

Tansley insight

Hydrogen sulphide as a guard cell network regulator

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Summary

Hydrogen sulphide (H_2S) is an endogenously produced gasotransmitter that has rapidly emerged as an active signalling component of several plant processes, stomatal movement regulation among them. The guard cells (GCs), pairs of cells that neighbour the stomatal pores, transduce endogenous and environmental signals, through signalling network, to control stomatal pore size. In this complex network, which has become a model system for plant signalling, few highly connected components form a core that links most of the pathways. The evidence summarized in this insight, on the interplay between H_2S and different key components of the GC networks, points towards H_2S as a regulator of the GC core signalling pathway.

I. Introduction

The developments of high-throughput technologies that generate massive amounts of data have emphasized the true complexity of biological signalling systems. In this new scenario, some players that were usually out of the scene have now emerged as key components of different signalling processes. Such is the case of a group of low molecular weight gases called gasotransmitters. Initially labelled as natural or anthropogenic toxic compounds, these molecules, including nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S), have now become mainstream signalling components (Aroca *et al.*, 2020). In this insight we present H_2S as a modulator of some of the components of this core signalling network.

II. Hydrogen sulphide (H_2S) in plants

The first reports on the biological action of H_2S in plants were linked to the effect of sulphur fertilization (Rennenberg, 1983, 1989). Those early works indicated that plants supplied with excess sulphur were capable of releasing H_2S into the atmosphere in a light-dependent manner (Kok *et al.*, 1986; Maas *et al.*, 1988; Rennenberg, 1989). H_2S production as a consequence of sulphur fertilization was reported to confer resistance to fungal pathogens, a process called sulphur-induced resistance (Schiitz *et al.*, 1991; Bloem *et al.*, 2007). Later works linked H_2S to other signalling processes in plants, mainly associated with oxidative stress (He

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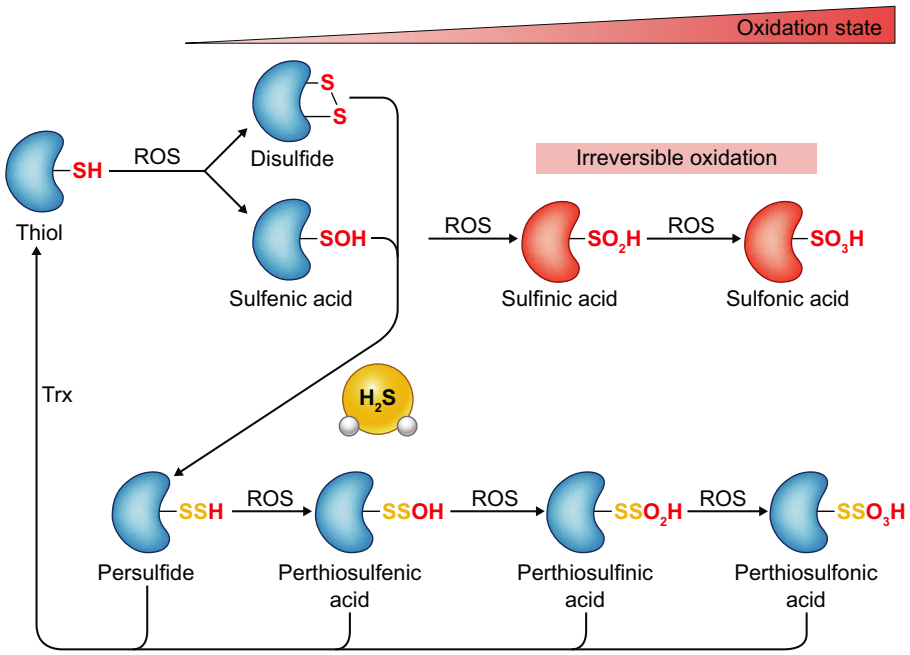
et al., 2019), and exactly a decade ago, H_2S finally broke into the stomatal signalling system (García-Mata & Lamattina, 2010; Lisjak *et al.*, 2010).

III. H_2S synthesis in plants

Plants have several H_2S sources, in different subcellular compartments, which can be grouped according their enzymatic activity, as summarized in (Fig. 1):

- Cysteine desulphydrases (CDES). These produce H_2S in the first step of the cysteine catabolic way. L/D-CDES activity was reported in different plant species. Arabidopsis genome has *L-CDES* (At3g62130) and *D-CDES* (At1g48420) isoforms. Moreover in 2010 a member of the O-acetyl-Ser(thiol)lyase (OASTL) protein family, with cytosolic localization, was characterized as a true L-CDES, and named L-cysteine desulphydrase 1 (*DES1*, At5g28030) (Gotor *et al.*, 2019).
- Sulphite reductases. In the process of inorganic sulphur assimilation, sulphur is stored in the vacuoles or reduced in the chloroplasts through a series of reactions that constitute the photosynthetic sulphate reduction pathway. In the last step of the chloroplastic way, H_2S is produced from sulphite reduction by the enzyme sulphite reductase (*SIR*, At5g04590) (Gotor *et al.*, 2019).
- β-cyanoalanine synthases (CAS). These mitochondrial enzymes catalyse the conversion of cyanide (CN^-) and cysteine to β-cyanoalanine and H_2S. In Arabidopsis there are three CAS genes: *CAS-C1* (At3g61440, also known as *Cys-C1*); *CYS-D1* (At3g04940); and *CYS-D2* (At5g28020) (Jost *et al.*, 2000). *CAS-C1*, also a member of OASTL family, is the isoform that shows higher expression (García *et al.*, 2010).
- Cysteine desulphurases. These enzymes belong to the family of nitrogen fixation S (NIFS)-like enzymes and catalyse the passage from cysteine to alanine and elemental sulphur in a PLP-dependent way. Arabidopsis has two genes coding for NIFS-like proteins, a

Box 1



Under physiological temperature and pH conditions, hydrogen sulphide (H_2S) is a highly reactive molecule. It can react with oxidized cysteine residues producing a post-translational modification on peptides called persulphidation, through which it modulates the activity and/or conformation of the target proteins.

Protein persulphidation has been proposed to act as a protective mechanism under oxidative stress conditions. On one hand, in the presence of reactive oxygen species (ROS), the thiol (RSH) moieties from cysteines can be reversibly oxidized to sulphenic acid (RSOH) or disulphide bonds (RSSR). If the oxidative state is persistent, RSOH can undergo an irreversible oxidation (overoxidation) to sulphinic acid (RSO_2H) and sulphonic acid (RSO_3H), producing the inactivation or degradation of the protein; on the other hand, H_2S is able to react with RSOH or RSSR, leading to the formation of persulphides (RSSH). As these molecules are more nucleophilic than their corresponding thiols, they are prone to react faster with ROS, producing perthiosulphenic acid (RSSOH), perthiosulphinic acid ($RSSO_2H$) and perthiosulphonic acid ($RSSO_3H$). All of the persulphidated species can be restored to free thiols after having been reduced by thioredoxins (Trx).

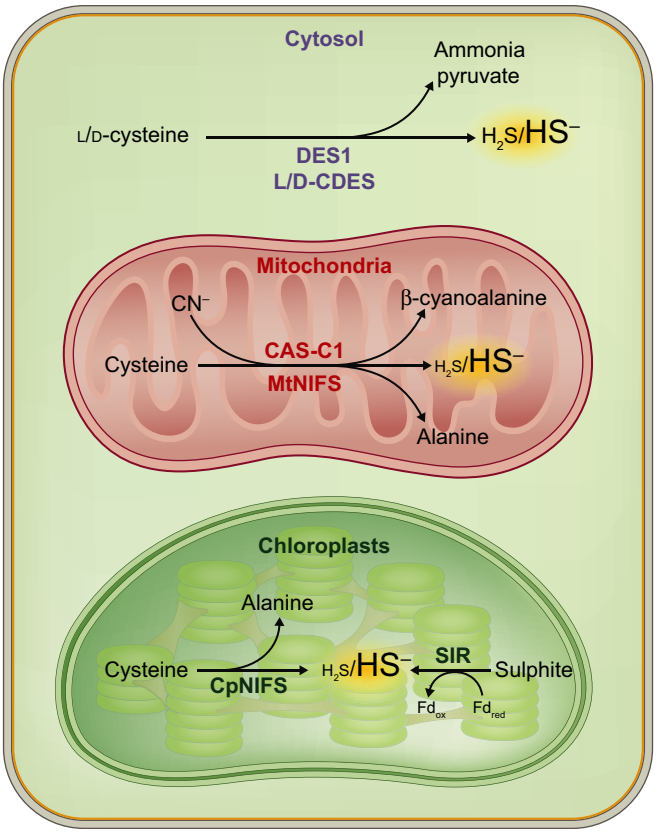


Fig. 1 Schematic representation of different enzymatic hydrogen sulphide (H_2S) sources in plants. In cytosol, cysteine desulphydrase 1 (DES1), L-cysteine desulphydrase (L-CDES) and D-cysteine desulphydrase (D-CDES) catalyse the hydrolysis of L/D cysteine to H_2S, pyruvate and ammonia. In mitochondria, β-cyanoalanine synthase C1 (CAS-C1) synthesizes β-cyanoalanine and H_2S from cysteine and cyanide (CN^-) and mitochondrial nitrogen fixation S (NIFS) (MtNIFS) catalyses the passage from cysteine to alanine and H_2S. In chloroplasts, chloroplastic NIFS (CpNIFS) catalyses the production of H_2S and alanine from cysteine, and sulphite reductase (SIR) reduces sulphite into H_2S oxidizing ferredoxins. Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidated ferredoxin. Different font size from the H_2S/HS^- equilibrium denotes the proportion of each species.

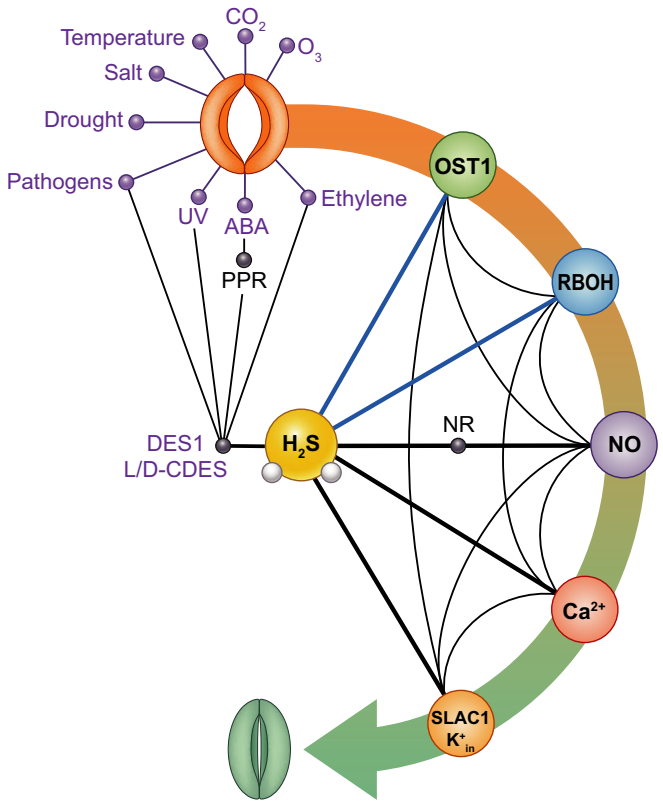


Fig. 2 Guard cell core signalling components modulated by hydrogen sulphide (H_2S) (coloured circles) pathway leading to stomatal closure (coloured arrow). Stomatal guard cells sense multiple environmental and endogenous stimuli (purple) and transduce the signal through complex signalling networks that contain a few highly connected nodes, considered hubs (coloured circles), that compose a core signalling pathway. Hydrogen sulphide is produced in guard cells in response to stomatal closing stimuli and modulates the activity of several hubs that constitute the core signalling pathway. Blue lines indicate proven target regulation (persulphidation); black lines indicate interactions requiring further study. PPR, ABA receptor complex (PYR/PYL/RCAR); DES1, L-cysteine desulphydrase 1; L/D CDES, L- or D-cysteine desulphydrase; OST1, open stomata 1; RBOH, respiratory burst oxidase homologue, isoforms D and F; NR, nitrate reductase; NO, nitric oxide; Ca^{+2}, calcium; SLAC1, slow anion-associated channels 1; K^+_{in}, inward-rectifying K^+ channels.

chloroplastic *AtCpNIFS* (At1g08490) and a mitochondrial *AtMtNIFS* (At5g65720) (Gotor *et al.*, 2019).

IV. H_2S mechanisms of action

Hydrogen sulphide is a weak acid (pKa1 = 6.98, pKa2 = 12.5–17 at 25°C) and therefore, in solution, it is found as an equilibrium of H_2S and the deprotonated ion HS^- in a 1 : 3 proportion, while the conversion to S^{-2} is negligible (Benchoam *et al.*, 2019). The form that has the greatest biological significance is yet to be determined, although HS^- is a good nucleophile and a strong reductant and is therefore more reactive. In this insight, as in the bibliography, the term H_2S generally refers to the mixture of H_2S and HS^- (Benchoam *et al.*, 2019). The mechanisms through which H_2S exerts its biological function are still under debate: H_2S can reduce or reversibly coordinate metalloprotein centres and also can react with reactive oxygen (ROS) or nitrogen (NRS) species (Filipovic *et al.*, 2018). However, recent evidence points towards persulphidation (formerly *S*-sulphydrilation), a post-translational modification (PTM) of oxidized Cys residues, as the main mechanism of action (Gotor *et al.*, 2019). H_2S can modulate the activity and/or the conformation of the target proteins through persulphidation. In addition, it has been proposed that persulphidation might confer tolerance to protein irreversible oxidation (overoxidation). Under prolonged oxidative stress, thiols can undergo oxidation to sulphinic acid (RSO_2H) and sulphonic acid (RSO_3H), generally leading to protein inactivation or degradation (Filipovic *et al.*, 2018). The reaction between H_2S and Cys, forms persulphides, which can react faster with ROS, producing different species that can be then reduced back, avoiding the overoxidation of proteins (Box 1). In fact, two recent proteomic analyses have shown that *c.* 5% of the total Arabidopsis leaf proteins undergo persulphidation under basal conditions (Aroca *et al.*, 2015, 2017).

V. H_2S and guard cell signalling network

Guard cells (GCs) are pairs of specialized cells that form the stomatal pores. Stomata function as tiny valves that regulate gas exchange between plants and the environment. Through stomatal pore variations, plants modulate CO_2 uptake for photosynthesis and restrict the transpirational water loss. Besides, stomatal pores are natural entry gates for microorganisms (Melotto *et al.*, 2017; Lawson & Matthews, 2020).

Stomatal pore size is determined by the volume of the GCs that surround it. H^+-ATPase-dependent hyperpolarization of the GC plasma membrane induces K^+ uptake, through the activation of inward-rectifying K^+ (K^+_{in}) channels and other osmotically active solutes. This generates the turgor pressure that drives water influx and GC swelling. The anisotropic growth of the GCs, determined by the disposition of the cellulose microfibrils, separates GC inner walls, increasing the pore size. Conversely, depolarization of the membrane potential inactivates K^+_{in}, activates outward-rectifying K^+ (K^+_{out}) channels and slow anion-associated channels 1 (SLAC1), generating a net loss of solutes, water efflux and the closure of the pore (Jezek & Blatt, 2017).

In order to control total gas exchange area, GCs are permanently sensing endogenous and environmental cues (e.g. light, CO_2, humidity, temperature, phytohormones and pathogens), and processing them through a complex signalling network to adjust stomatal pores locally and/or systemically. The topology of this coordinated and dynamic network, which commands stomatal movement, resembles that of a 'scale free' network in which the pathways triggered by most stimuli are linked through a few highly connected components (Hetherington & Woodward, 2003), which can be regarded as the core of the GC signalling network. Among the endogenous signals, ABA is transduced by the GC core signalling pathway (open stomata 1: OST1; respiratory burst oxidase homolog D and F: RBOHD/F, SLAC1 and K^+ channels) to induce stomatal closure (Fig. 2). When cytosolic ABA concentration increases, it binds to the cytosolic receptor PYR/PYL/RCAR (PPR) and forms a complex with the negative regulator ABA-insensitive 1 (ABI1), releasing OST1 and its downstream signalling cascade (Cotelle & Leonhardt, 2019). ABA-dependent response is commonly used as the first option when trying to demonstrate the participation of new components within GC signalling, and H_2S was not the exception to the rule. Two independent reports introduced H_2S into GC signalling, showing that it both promotes ABA-dependent stomatal closure (García-Mata & Lamattina, 2010) and inhibits ABA response by diminishing endogenous NO concentrations (Lisjak *et al.*, 2010). These apparently contrasting outputs agreed with a later work indicating a biphasic response depending on the timing of the H_2S treatment (Honda *et al.*, 2015). Subsequent genetic approaches demonstrated that ABA induces *DES1* expression preferentially in GCs, and that DES1 is activated by persulphidation in response to ABA, hence increasing H_2S production (Scuffi *et al.*, 2014; Shen *et al.*, 2020; Chen *et al.*, 2020). Interestingly, atmospheric H_2S, produced from environmental sources, seems to have no effect on stomatal movement regulation (Ausma & De Kok, 2019). Altogether, the results indicated that H_2S would be just another of the many components of the ABA-dependent signalling pathways. However, H_2S is also involved in the responses of other stomatal closure stimuli, such as ethylene, drought and pathogens (Hou *et al.*, 2016; Scuffi *et al.*, 2018), indicating that the role of H_2S in GC signalling is not limited to the responses triggered by ABA.

VI. H_2S and the components of the guard cell signalling core

OST1 is a member of the group 3 of the sucrose nonfermenting-1 (SNF1)-related protein kinase 2 (SnRK2) (Kulik *et al.*, 2011). In the absence of ABA, OST1 is dephosphorylated and inhibited by ABI1. Once in the cytosol, ABA binds to receptor and forms a complex that sequesters ABI1; OST1 is then auto/phosphorylated and activates several downstream components, among them ion channels, RBOHF and transcription factors (Kulik *et al.*, 2011).

OST1 is also susceptible to other PTMs, apart from phosphorylation, like *S*-nitrosylation, ubiquitinylation and, as recently reported, persulphidation (Chen *et al.*, 2020). Positive regulation of OST1 by H_2S occurs through the persulphidation of residues Cys131 and Cys137, both of them in an adjacent position to the catalytic loop and close to the S175 residue, which is crucial for phosphorylation of this kinase (Belin *et al.*, 2006; Chen *et al.*, 2020) (Fig. 2). Several other stimuli, such as CO_2, O_3, wounding and pathogens, were also reported to transduce the signal via OST1 (Merilo *et al.*, 2013; Melotto *et al.*, 2017), suggesting that H_2S might also be involved in the responses generated by other signals.

RBOH is a protein family of NADPH oxidases that transfer electrons from NADPH to molecular oxygen to produce superoxide anion (O_2^-) which dismutates to H_2O_2 (Kwak *et al.*, 2003). There are two isoforms, RBOHF and RBOHD, preferentially expressed in GCs and both of them are required for H_2S-dependent stomatal closure (Scuffi *et al.*, 2018) (Fig. 2). Although both ROBHF and D participate in ABA signalling pathways (Kwak *et al.*, 2003), the former is generally associated with the ABA-dependent response and is phosphorylated by OST1, while the latter forms plasma membrane protein complexes with pattern recognition kinase receptors (PRRs) as EFR and FLS2 for the recognition of bacterial elicitors EF-T and flagellin, respectively. In the presence of pathogens, or pathogen elicitors, the PRR complex activates the plasma membrane associated kinase BIK-1, which in turn phosphorylates RBOHD (Melotto *et al.*, 2017). In addition, the activity of RBOHD has also been linked to the responses triggered by ABA and H_2S and the systemic response, among others (Kwak *et al.*, 2003; Mersmann *et al.*, 2010; Devireddy *et al.*, 2018; Scuffi *et al.*, 2018; Hu *et al.*, 2020).

As an activator of OST1, H_2S would therefore be expected to affect RBOH activity. Interestingly, H_2S directly modulates RBOHD via the persulphidation of Cys825 and Cys890 residues, increasing its activity in response to ABA (Fig. 2). Both residues that undergo PTMs are conserved along the RBOH family, suggesting that H_2S might be a key regulator of other RBOH-dependent processes (Shen *et al.*, 2020).

Calcium (Ca^{2+}) is a key component of different signalling processes, among them stomatal opening and closure. The

specificity of Ca^{2+} signal is coded by the oscillations on cytosolic Ca^{2+} concentrations (Ca^{2+} signatures) given by the transport from and to the apoplast and subcellular stores (Dodd *et al.*, 2010). As a hub itself Ca^{2+} links several signalling pathways, but evidence of the interplay between H_2S and Ca^{2+} in GCs is scarce. Recently, H_2S was shown to induce Ca^{2+} influx currents, a process dependent on OST1 persulphidation (Chen *et al.*, 2020) (Fig. 2).

Ion channels are responsible for the redistribution of osmotically active solutes that drive GC volume changes. Both K^+ and anion currents were already reported to be modulated by H_2S. On the one hand, electrophysiological data indicate that H_2S inactivates K^+_{in} (Fig. 2), but has no effect on K^+_{out}. Although there is no precise information yet on how H_2S is modulating these channels, the presented data are consistent with a PTM-like regulation (Papanatsiou *et al.*, 2015). On the other hand, SLAC1 currents are activated by H_2S (Wang *et al.*, 2016).

In this case, OST1 and Ca^{2+} are required for the activation of SLAC1. Considering Chen's data on persulphidation-dependent activation of OST1, it can be concluded that H_2S persulphidates and activates OST1, which in turn activates anion currents by phosphorylating SLAC1 (Wang *et al.*, 2016; Chen *et al.*, 2020).

Nitric oxide is now recognized as an active component of the GC signalling network (Scuffi *et al.*, 2016). DES1 is required for ABA-dependent NO production (Scuffi *et al.*, 2014). The expression levels of the two genes that code for cytosolic NO source, nitrate reductase (*NIA1* and *NIA2*), increase in response to H_2S. Moreover both of them are persulphidated at basal conditions (Álvarez *et al.*, 2012; Aroca *et al.*, 2017) (Fig. 2), suggesting that this might be the mechanism through which H_2S regulates GC NO concentrations. Long H_2S treatments, more than 2 h, diminish endogenous NO and cause stomatal reopening (Lisjak *et al.*, 2010).

Stomatal closure induction in response to ethylene also involves the interplay between NO and H_2S; however, in contrast with the ABA-dependent pathway, in this case H_2S acts downstream of NO (Liu *et al.*, 2006, 2011).

In animal systems, H_2S and NO can react chemically, at physiological pH, to generate bioactive intermediates among which nitroxyl (HNO) is the most studied (Cortese-Krott *et al.*, 2015; Ivanovic-Burmazovic & Filipovic, 2019). In GCs, H_2S and NO do not induce stomatal closure when added together. Moreover, the HNO donor Angeli's salt has no effect on stomata closure induction, suggesting that both gases can react with each other (Scuffi *et al*, 2016). However, further evidence is needed in order to determinate the entrance or the formation of HNO within plant cells.

VII. Conclusions and perspectives

Hydrogen sulphide is now established as an active component of plant signalling processes, including GCs. Although current knowledge shows that H_2S regulates the activity of core components of the GC network leading to stomatal closure, there are limitations that must be overcome in order to have a full understanding of H_2S's role in stomatal movement regulation. Despite the fact that the main endogenous sources are characterized, the challenge now is to determine if there is a source-specific production in response to different endogenous or environmental stimuli.

Another key aspect is the visualization and measurement of *in vivo* H_2S production. The widely used colorimetric assay has technical limitations for determining GC-specific H_2S variations. Recent work showed *in vivo* H_2S production in GCs using a fluorescent probe (Shen *et al.*, 2020; Chen *et al.*, 2020), although the development of a ratiometric sensor is needed in order to follow H_2S variation through time and, if possible, to determine the production at a subcellular location. The later point becomes particularly interesting as mitochondrial matrix and plastidial stroma have relatively basic pH and therefore most of the H_2S should be in its ionic form, HS^-, which is not prone to cross membranes freely.

Finally, persulphidation is starting to look like the main mechanism of action of H_2S, and it has been seen that, through this PTM, H_2S modulates some of the hubs of GC signalling. However, given that stomata occupy no more than 5% of the leaf surface, GC proteins consequently represent just a minor proportion of whole leaf protein extracts. Therefore, proteomic analysis performed in GC-enriched extracts are needed in order to identify specific H_2S targets in this cell type, and to obtain a more accurate view of the role of H_2S as a regulator of the main components of GC networks.

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Author contributions

RP and DS wrote the article and created Fig. 1 (DS) and Box 1 (RP). RP and DS contributed equally to this work. CGM conceived the project, wrote the article and created Fig. 2.

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