Correction

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Correction for "A respiratory chain controlled signal transduction cascade in the mitochondrial intermembrane space mediates hydrogen peroxide signaling," by Heide Christine Patterson, Carolin Gerbeth, Prathapan Thiru, Nora F. Vögtle, Marko Knoll, Aliakbar Shahsafaei, Kaitlin E. Samocha, Cher X. Huang, Mark Michael Harden, Rui Song, Cynthia Chen, Jennifer Kao, Jiahai Shi, Wendy Salmon, Yoav D. Shaul, Matthew P. Stokes, Jeffrey C. Silva, George W. Bell, Daniel G. MacArthur, Jürgen Ruland, Chris Meisinger, and Harvey F. Lodish, which appeared in issue 42, October 20, 2015, of *Proc Natl Acad Sci USA* (112:E5679–E5688; first published October 5, 2015; 10.1073/pnas.1517932112).

The authors note that on page E5683, left column, first full paragraph, lines 3–4, "Fig. S3 *D* and *E*" should instead appear as "Fig. S2 *D* and *E*"; and in the same paragraph, line 9, "Fig. S3*D*" should instead appear as "Fig. S2*D*."

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A respiratory chain controlled signal transduction cascade in the mitochondrial intermembrane space mediates hydrogen peroxide signaling

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Contributed by Harvey F. Lodish, September 11, 2015 (sent for review June 19, 2015)

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) govern cellular homeostasis by inducing signaling. H_2O_2 modulates the activity of phosphatases and many other signaling molecules through oxidation of critical cysteine residues, which led to the notion that initiation of ROS signaling is broad and nonspecific, and thus fundamentally distinct from other signaling pathways. Here, we report that H_2O_2 signaling bears hallmarks of a regular signal transduction cascade. It is controlled by hierarchical signaling events resulting in a focused response as the results place the mitochondrial respiratory chain upstream of tyrosine-protein kinase Lyn, Lyn upstream of tyrosine-protein kinase SYK (Syk), and Syk upstream of numerous targets involved in signaling, transcription, translation, metabolism, and cell cycle regulation. The active mediators of H_2O_2 signaling colocalize as H_2O_2 induces mitochondria-associated Lyn and Syk phosphorylation, and a pool of Lyn and Syk reside in the mitochondrial intermembrane space. Finally, the same intermediaries control the signaling response in tissues and species responsive to H_2O_2 as the respiratory chain, Lyn, and Syk were similarly required for H_2O_2 signaling in mouse B cells, fibroblasts, and chicken DT40 B cells. Consistent with a broad role, the Syk pathway is coexpressed across tissues, is of early metazoan origin, and displays evidence of evolutionary constraint in the human. These results suggest that H_2O_2 signaling is under control of a signal transduction pathway that links the respiratory chain to the mitochondrial intermembrane space-localized, ubiquitous, and ancient Syk pathway in hematopoietic and nonhematopoietic cells.

fostamatinib | dasatinib | rotenone | Btk | PTPN6

The accumulation of oxygen on earth not only enabled aerobic respiration but also forced life to adapt to its toxic effects. Molecular oxygen (O_2) inevitably forms reactive oxygen species (ROS) such as superoxide (O_2^-) and the more stable hydrogen peroxide (H_2O_2) due to its proclivity to react with univalent electron donors, such as flavin enzymes of the respiratory chain or NADPH oxidase that releases H_2O_2 into the extracellular space. Relevant other sources of extracellular and intracellular H_2O_2 include environmental exposure to ozone, UV light, and ionizing radiation (1, 2). Among the most conserved defense mechanisms against the oxidizing effects of H_2O_2 are detoxifying enzymes, such as catalase, as well as aquaporins that control the influx of H_2O_2 into the cell (3–7). These mechanisms are highly efficient and adaptable, and provide an explanation for the great variation in susceptibility to H_2O_2 among cell types despite the generally low intracellular concentrations across bacteria, plants, and mammals (3, 8, 9). Indeed, it requires up to 10 mM exogenous H_2O_2 to induce a measurable signaling response and more than 30 mM H_2O_2 to induce necrotic blebbing in some mammalian cells, while plant tissues can contain up to 100 mM H_2O_2 (10–13).

Life further evolved to communicate and convert the presence of H_2O_2 into diverse cellular responses appropriate to the amount of intracellular and extracellular H_2O_2 the organism is faced with (9, 14, 15). In the metazoan lineage, it appears that such mechanisms were usurped to amplify receptor-mediated signaling: Engagement of a large number of plasma membrane resident receptors, as well as extracellular H_2O_2, results in increased intracellular ROS production by the respiratory chain, and much of such induced cellular responses, including inflammasome signaling, NF-κB activation, and B-cell receptor (BCR)–induced proliferation, is ROS-dependent (9, 16–22). It is thus clear that extracellular and intracellular H_2O_2 is an ancient and essential mediator of cellular homeostasis linked to a wide range of physiological and pathological responses and numerous diseases (23, 24). However, the underlying mechanisms and their evolutionary purpose remain largely elusive.

A critical question is how the cell translates an encounter with H_2O_2 into a distinct cellular response. Early work demonstrated that H_2O_2 reversibly oxidizes deprotonated cysteine residues, and thereby inactivates protein tyrosine phosphatases. Thus

Significance

Both the mitochondrial respiratory chain and reactive oxygen species (ROS) control numerous physiological and pathological cellular responses. ROS such as hydrogen peroxide (H_2O_2) are thought to initiate signaling by broadly and nonspecifically redox-modifying signaling molecules, suggesting that H_2O_2 signaling may be distinct from other signal transduction pathways. Here, we provide evidence suggesting that H_2O_2 signaling is under control of what appears to be a typical signal transduction cascade that connects the respiratory chain to the mitochondrial intermembrane space-localized conserved Syk pathway and results in a focused signaling response in diverse cell types. The results thus reveal a mechanism that allows the respiratory chain to communicate with the remainder of the cell in response to ROS.

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inactivated phosphatases were hypothesized to shift the equilibrium of inactive to active kinases resulting in enhanced kinase activity (18, 25, 26). By now, a large number of redox modifications in phosphatases, kinases, adapters, receptors, and transcription factors have been proposed to modulate signaling, among them multiple components of the BCR signaling pathway such as tyrosine-protein kinase Lyn, tyrosine-protein kinase SYK (Syk), tyrosine-protein phosphatase nonreceptor types 6 and 11 (SHP1/PTPN6; SHP2/PTPN11), phosphatase and tensin homolog, and a mitogen activated protein (MAP) kinase serine/threonine phosphatase (27–36). However, it has been challenging to prove or disprove the physiological relevance of such a mechanism, and results have been difficult to reconcile as a whole (3, 26, 35). Nevertheless, these findings have resulted in the view that ROS signaling fundamentally differs from classical signal transduction because it broadly targets signaling molecules and thus assumes a relative independence of each redox-modified component in promoting signaling.

Although not excluding a contribution of cysteine modifications to ROS signaling, it is conceivable that H_2O_2 signaling, in fact, resembles other signaling pathways characterized by a cascade of phosphorylation events that originate locally and control a distinct signaling response in all tissues and species responsive to the ligand. Compatible with the existence of a few upstream mediators of H_2O_2 signaling, limited evidence indeed exists supporting that kinases, such as MAP kinases and Syk, are critical intermediaries of H_2O_2-induced signal transduction in yeast, plants, and chicken cells, respectively (13, 15, 37). However, the concept remains currently unexplored, in part, because it implies the existence of a “ROS receptor” that initiates signaling, a notion that has been dismissed previously (13, 18). Here, we provide evidence in support of such a model, demonstrating that H_2O_2 signaling is under control of the respiratory chain and the mitochondrial intermembrane space-localized Syk pathway leading to a circumscribed signaling response in B lymphocytes and fibroblasts.

Results

Syk Is Critical for H_2O_2-Induced Activation of Bkt, PLCγ2, JNK, and Akt. Receptor-activated signal transduction cascades typically use the same intermediaries to induce a cellular response in all tissues responsive to the ligand and are often conserved in different species (38). Because H_2O_2 induces signaling across many tissues and species, we speculated that Syk is a critical mediator of H_2O_2 signaling in both hematopoietic and nonhematopoietic cells, as well as in different vertebrate species. To test this idea, we H_2O_2-stimulated ex vivo harvested mouse splenic B cells and freshly derived mouse embryonic fibroblasts (MEFs) briefly pretreated with the Syk inhibitor R406 (39, 40), as well as Syk-deficient DT40 B cells, which tolerate genetic Syk deficiency well, unlike primary B cells, and are derived from the genetically stable chicken DT40 B-cell line (41–44).

Both Syk inhibition with R406 in B cells and MEFs, as well as genetic Syk deficiency in DT40 B cells, resulted in strongly decreased H_2O_2, as well as anti-IgM–induced phosphorylation of tyrosine-protein kinase BTK (Btk), phospholipase Cγ2 (PLCγ2), and c-Jun N-terminal kinase (JNK) and some reduction in extracellular-signal regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38) phosphorylation (Fig. 1 *A* and *B*). Consistent with decreased PLCγ2 phosphorylation, H_2O_2-induced calcium flux was greatly reduced in Syk-deficient DT40 B cells (Fig. 1*C*). However, H_2O_2 resulted in no or minor loss of phosphorylation at inhibitory Lyn Tyr507 and intermediate reduction of phosphorylation at activating Lyn Tyr396 and of SHP1 in Syk-inhibited and Syk-deficient cells (Fig. 1*A*). These findings suggest

Fig. 1. Syk is critical for H_2O_2-induced activation of Btk, PLCγ2, JNK, and Akt. (*A* and *B*) Immunoblots of mouse splenic B cells and primary MEFs pretreated with 2 μM R406 and Syk-deficient DT40 B cells stimulated with 1 mM H_2O_2 for 5 min or 50 μg/mL anti-mouse IgM for 3 min (B cells), 5 mM H_2O_2 for 10 min (MEFs), and 5 mM H_2O_2 for 5 min or 10 μg/mL anti-chicken IgM for 3 min (DT40 B cells). (*C*) Calcium flux of Syk-deficient DT40 B cells stimulated with 5 mM H_2O_2 or 10 μg/mL anti-IgM as determined by the emission ratio of Indo-1. (*D*) Immunoblots of mouse splenic B cells and primary MEFs pretreated with 2 μM R406 and Syk-deficient DT40 B cells stimulated as indicated for 10 min.

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that Syk mainly acts downstream of Lyn in H_2O_2 signaling similar to signal transduction by the BCR but also has a role in feedback regulation of Lyn in response to H_2O_2. Stimulation with 0.1–0.6 mM H_2O_2, but not higher concentrations, revealed decreased Akt phosphorylation in Syk-inhibited B cells and MEFs and in Syk-deficient DT40 cells (Fig. 1*D*), highlighting a role for Syk also at low H_2O_2 concentrations and expanding on similar observations in DT40 B cells (45). Syk is thus critical for Btk, PLCγ2, JNK, and Akt but not, or less so, for Lyn and SHP1 activation in response to high and low extracellular H_2O_2 concentrations across tissues and species.

Syk Controls Tyrosine Phosphorylation of Major Pathways Involved in Basic Cellular Processes. Cellular signal transduction induced by an external stimulus generally results in a circumscribed signaling response mediated by a few upstream kinases that reversibly phosphorylate downstream effectors (38). Syk inhibition with R406 in B cells and MEFs, as well as genetic Syk deficiency in DT40 B cells, resulted in strongly decreased H_2O_2-induced tyrosine phosphorylation of numerous protein species, suggesting that these proteins are direct or indirect tyrosine phosphorylation targets of Syk (Fig. 2*A*). To determine their identity, we performed label-free quantitative proteomics of phospho-Tyr–enriched lysates of H_2O_2-stimulated Syk-deficient DT40 B cells and H_2O_2-treated controls. The abundance of one-third of all phosphopeptides mapping to 455 homologous human genes was more than 2.5-fold decreased in H_2O_2-stimulated Syk-deficient DT40 B cells compared with controls (Fig. 2*B* and Dataset S1), suggesting that Syk is a major regulator of protein tyrosine phosphorylation in the presence of H_2O_2. These phosphopeptides included multiple peptides mapping to Btk and PLCγ2, consistent with decreased H_2O_2-induced phosphorylation of these proteins as judged by Western blotting (Fig. 1*A* and Dataset S1). Another 57 unique human homologs were identified that displayed an exclusive increase in phosphorylation in Syk-deficient cells, consistent with differential regulation by Syk (Fig. 2*B* and Dataset S1). Eighty-two percent of all Syk-regulated genes were found to be part of a network of proteins with known interactions and associations, suggesting a functional relationship (Fig. 2*C*). Closer examination revealed that the network components clustering around Syk contained numerous members of major signaling pathways related to the Syk, NF-κB, MAPK, PI3 kinase, JAK/STAT, and rho/ras/rac signaling pathways (Fig. 2*C* and Dataset S1), some of which are known Syk targets in response to immune receptor engagement (46). Further, the identified Syk targets were greatly enriched for basic cellular processes. They broadly fell into categories such as transcription, translation, protein folding, metabolism, cell cycle regulation, and tumor suppression, and they contained numerous functionally important and well-studied proteins, many of which have been implicated in ROS signaling (Table 1 and Dataset S1). In summary, these findings suggest that Syk is a critical mediator of a distinct signaling response to extracellular H_2O_2 focused on the regulation of basic cellular processes.

Of note, the cellular models used in this study were remarkably resistant to the effects of H_2O_2 in our hands, consistent with some previous results (47, 48). Stimulation with a minimum of 1 mM H_2O_2 for 5 min or 5 mM for 10 min was required in primary B cells and MEFs, respectively, to detect robust H_2O_2-induced

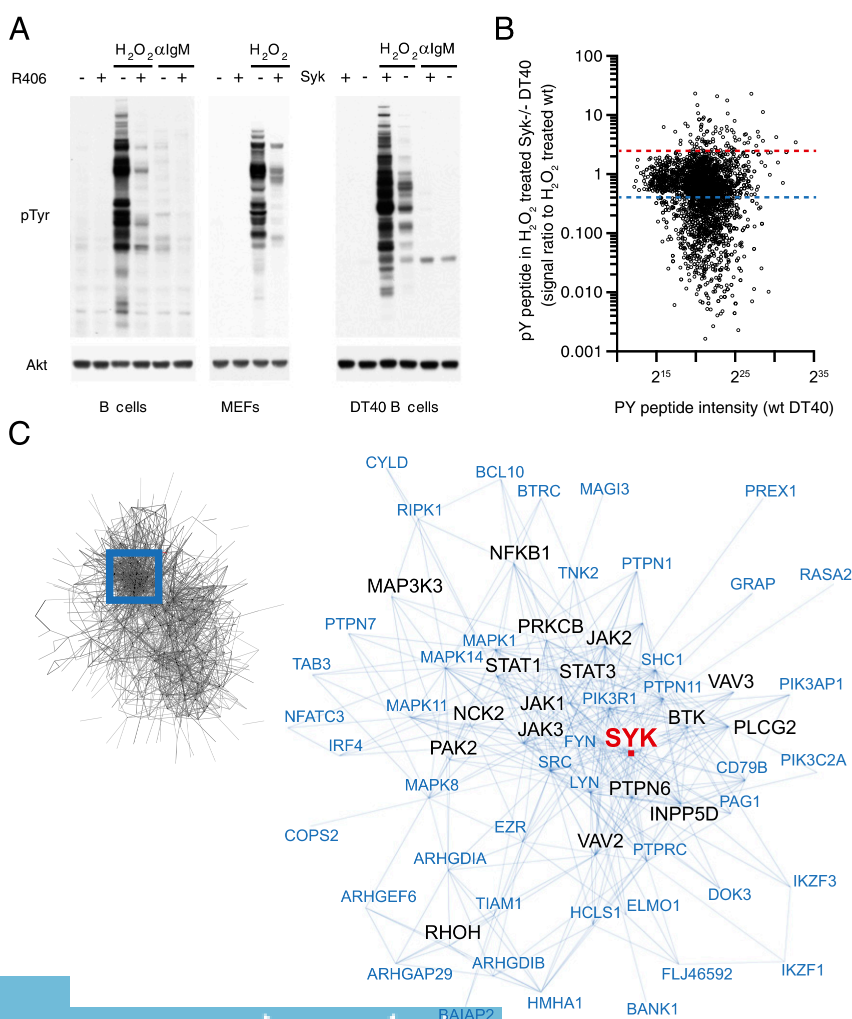

Fig. 2. Syk is a major regulator of protein Tyr phosphorylation in the presence of H_2O_2. (*A*) Immunoblots of mouse splenic B cells and primary MEFs pretreated with 2 μM R406 and Syk-deficient DT40 B cells stimulated with 1 mM H_2O_2 for 5 min or 50 μg/mL anti-mouse IgM for 3 min (B cells), 5 mM H_2O_2 for 10 min (MEFs), and 5 mM H_2O_2 for 5 min or 10 μg/mL anti-chicken IgM (DT40 B cells). (*B*) Phosphorylated peptide abundance determined by label-free quantitative MS following enrichment for protein Tyr phosphorylation in H_2O_2-treated Syk-deficient DT40 B cells compared with H_2O_2-treated DT40 controls (5 mM H_2O_2 for 5 min). Red and blue dotted lines denote 2.5-fold increase, and decrease in pY peptide abundance, respectively. (*C*) Known network interactions of phosphorylated proteins regulated by Syk identified in the experiment described in *B* as determined by algorithms of the string database (87) (*Left*) and an extract (boxed) from this network focusing on signaling pathways clustering around Syk (*Right*). Key pathway members are highlighted in black.

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tyrosine phosphorylation of Syk pathway members Lyn, Syk, SHP1, Btk, and PLCγ2, as well as of many other proteins (Fig. 1*D* and Fig. S1*A*). These concentrations were well below saturation in primary B cells (Fig. S1*A*) and did not result in signs of disintegration after the stimulation period as suggested by intact cellular and mitochondrial ultrastructure in B cells and MEFs (Fig. S1*B*). In MEFs, H_2O_2 stimulation resulted in a dose-dependent decrease in cell recovery that was partially rescued by pretreatment with R406 and less so by cyclosporin A, an inhibitor of mitochondrial transition pore opening and H_2O_2-induced apoptosis (49) (Fig. S1 *C* and *D*). DT40 B cells required stimulation with 5 mM H_2O_2 for 5 min to detect Syk phosphorylation (Fig. 1*D*). Similar to MEFs treated with H_2O_2 and R406, these treatment conditions resulted in decreased cell recovery of DT40 B cells that were partially rescued by Syk deficiency (Fig. S1*E*). Further, we observed that the signaling response to H_2O_2 and susceptibility to H_2O_2-induced cell death were greatly affected by culture conditions. For example, H_2O_2 but not BCR signaling in B cells almost completely disappeared in response to prior serum deprivation for 2 h (Fig. S1*F*), suggesting that metabolic health is a critical determinant of H_2O_2 signaling. Overall, these results suggest that the high H_2O_2 concentrations used in parts of this study reflect physiological doses of extracellular H_2O_2 to B cells and MEFs because they were either greatly below saturation of the signaling response or elicited regulated "programmed" cellular responses.

Lyn but Not Protein Tyrosine Phosphatases Are Required for H_2O_2-Induced Syk Activation. Signal transduction cascades are characterized by hierarchical signaling events, in which upstream mediators diversify and amplify the signaling input (38). Protein tyrosine phosphatases were previously proposed to initiate and promote H_2O_2 signaling as a result of redox-mediated inactivation (18, 25, 26). We therefore hypothesized that protein tyrosine phosphatases might be upstream activators of Syk, and that inhibition or loss of relevant phosphatases should therefore diminish H_2O_2 signaling in a cellular context. To address this question as it relates to the Syk pathway, we pretreated primary B cells and MEFs with the general protein tyrosine phosphatase inhibitor sodium orthovanadate (Na_3VO_4) (50), followed by stimulation with H_2O_2. Na_3VO_4 had little effect on protein tyrosine phosphorylation in the absence of H_2O_2 in B cells and MEFs (Fig. 3*A*), suggesting that decreasing protein tyrosine phosphatase activity is not sufficient to induce signaling. Consistent with these findings, cell recovery was normal after overnight culture of MEFs with Na_3VO_4 (Fig. S2 *A* and *B*). However, H_2O_2 combined with prior Na_3VO_4 treatment led to strongly enhanced phosphorylation of Syk pathway members Lyn, Syk, Btk, PLCγ2, and many other proteins, as well as increased phosphorylation of ERK, JNK, and p38. Enhanced cellular signaling in H_2O_2-stimulated Na_3VO_4-pretreated MEFs correlated with reduced cell recovery after overnight culture similar to treatments with higher concentrations of H_2O_2 that induce increased Syk pathway activation (Figs. S1 *A* and *C* and S2 *A* and *B*). Genetic deficiency of SHP1 and SHP2, the main phosphatases dephosphorylating Syk (51), resulted in normal or slightly enhanced phosphorylation of the Syk pathway following H_2O_2 stimulation in DT40 B cells (Fig. 3*A*). These results are thus consistent with a nonessential role of SHP1, SHP2, and other protein tyrosine phosphatases in H_2O_2-induced Syk pathway activation.

Lyn is a membrane-bound Src family kinase critical for Syk activation downstream of the BCR (52, 53). We therefore reasoned that Lyn might be critical for H_2O_2-induced activation of the Syk pathway in hematopoietic and nonhematopoietic cells as well. Treatment of primary B cells and MEFs with the Src family kinase inhibitor dasatinib (39) resulted in a reduction of both H_2O_2 and anti-IgM–induced tyrosine phosphorylation of Lyn, consistent with Lyn inhibition, as well as reduced Syk, SHP1, Btk, and PLCγ2 phosphorylation and reduced general protein tyrosine phosphorylation. Similarly, Lyn-deficient and Lyn/Syk-doubly deficient DT40 B cells treated with H_2O_2 exhibited reduced phosphorylation of Syk and its downstream target proteins, thus adding to earlier results (48) (Fig. 3*B*). Dasatinib treatment and genetic deficiency of Lyn also led to a reduction in H_2O_2- and anti-IgM–induced JNK and ERK but not p38 phosphorylation (Fig. 3*B*). In summary, these results suggest that Src family kinases, and Lyn in particular, are upstream regulators of Syk and SHP1 activation in response to H_2O_2 as well as BCR engagement.

Interference with the Respiratory Chain Selectively Diminishes H_2O_2-Induced Activation of Lyn and the Syk Pathway. In addition to producing ATP through aerobic respiration, the mitochondrial respiratory chain is involved in signal transduction in the absence of apparent stimuli as well as in response to ROS-inducing stressors and extracellular H_2O_2 (54–57). To test whether the respiratory chain might also play a role in H_2O_2-induced activation of Lyn and the Syk pathway, primary B cells, MEFs, and DT40 B cells were treated with 50 nM rotenone, a complex I inhibitor (58). Consistent

Table 1. Examples of tyrosine-phosphorylated proteins regulated by Syk in the presence of H_2O_2 grouped by biological process as identified by label-free quantitative proteomics of phospho-Tyr–enriched lysates of H_2O_2-stimulated Syk-deficient DT40 B cells and H_2O_2-treated controls

Biological process	No. of Syk-regulated pY proteins	Examples
Epigenetic regulation	14	MLL/KMT2A, DNMT1, KDM4A, PBRM1
Transcription	24	POLR1A, POLR2B, POLR3E, MED1, HMGB1, YY1, BACH1
Posttranscriptional regulation	35	DICER1, HNRNPK, SF3A1, SF3B, SMG1, SYNCRIP, SNRPC
Nuclear import/export	8	NUP98, NUP210, IPO7, DDX3X
Translation	34	EIF3A, EIF5, EEF1D, EEF2K, AARS, RPL26, RPL35A, RPS2, STAU1, DHX29, FXR1, UPF1
Protein folding	10	HSPA8, HSP90AA1, HSPD1, DNAJA1 CDC37
Proteolysis	34	PSMA3, PSMB5, ADAM17, ANAPC1, BTRC, CYLD, USP9X, USP16, *ANXA2*
Metabolic pathways	34	IGF2R, GAPDH, PDH1A, FDPS, ADSL, MDH2, SREBF2, FASN, LSS, CYP51A1, HGS, TOMM34, *IRS1, GSK3B, LDHB*
Cell cycle/tumor suppression	64	POLA1, TK1, KIF11, KIF15, DCTN2, *CDC7, CDK1, CDK2, CDK5,* LATS1, MLH1, RAD51, MGMT, VCP, BUB1, WEE1, STK4/MST1, DYRK2, NEDD9, PAK2, BCL11A, PDCD4, AXIN1
Redox regulation	4	SESN1, PRDX1, HVCN1, NCF4

Italicized names denote an increase in tyrosine phosphorylation of these proteins in H_2O_2-treated Syk-deficient DT40 cells, whereas tyrosine phosphorylation of all other proteins was decreased in the Syk-deficient cells.

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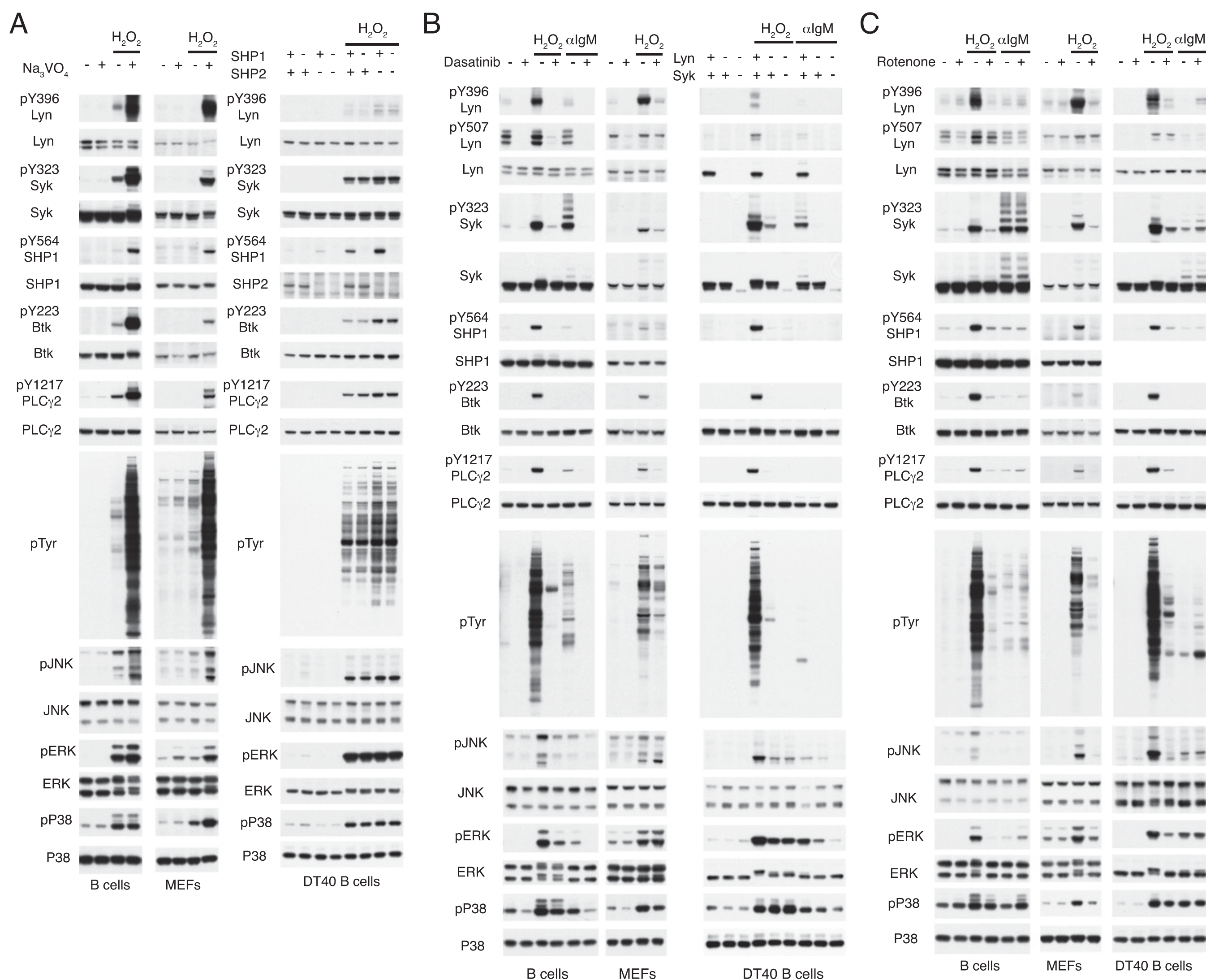

Fig. 3. Lyn and the respiratory chain, but not protein tyrosine phosphatases, are required for H_2O_2-induced activation of the Syk pathway. (*A*) Immunoblots of H_2O_2-stimulated mouse splenic B cells (0.5 mM H_2O_2 for 5 min) or primary MEFs (2 mM H_2O_2 for 10 min) pretreated with the phosphatase inhibitor Na_3VO_4 (100 μM) and H_2O_2 stimulated SHP1- and/or SHP2-deficient DT40 cells (2 mM for 5 min). Stimulation of mouse splenic B cells and primary MEFs pretreated with 30 nM dasatinib (*B*) or 50 nM rotenone (*C*) and Lyn- and Lyn/Syk-deficient DT40 B cells treated with 1 mM H_2O_2 for 5 min and 50 μg/mL anti-mouse IgM for 3 min (B cells), 5 mM H_2O_2 for 10 min (MEFs), and 5 mM H_2O_2 for 5 min and 10 μg/mL anti-chicken IgM for 3 min (DT40 B cells).

with results in other cell types (59), culture of MEFs with this concentration of rotenone and high-glucose culture medium for 16 h did not impair viability (Fig. S2*C*).

Treatment with rotenone alone for 30 min did not induce activation of the Syk pathway at a concentration of 50 nM and higher despite induction of mitochondrial ROS (Fig. 3*C* and Fig. S3 *D* and *E*). It thus appears that ROS is not sufficient to induce Syk signaling. However, addition of H_2O_2 to rotenone-pretreated cells resulted in almost complete loss of phosphorylation at activating Lyn Tyr396 but normal phosphorylation at inhibitory Lyn Tyr507 and only partial or no loss of p38 phosphorylation (Fig. 3*C* and Fig. S3*D*). These findings suggest that the mitochondrial respiratory chain has a selective role in H_2O_2-induced activation but not inhibition of Lyn nor H_2O_2-induced activation of p38 and that rotenone combined with short-term H_2O_2 treatment does not reduce cellular ATP to levels prohibiting ATP-dependent kinase signaling. Consistent with decreased Lyn activity, rotenone also led to a strong reduction of H_2O_2-induced tyrosine phosphorylation of the Syk pathway members Syk, SHP1, Btk, PLCγ2, JNK, ERK, and many other proteins (Fig. 3*C*). In contrast, rotenone treatment did not impair BCR-mediated activation of the Syk pathway in primary B cells and DT40 B cells, suggesting that BCR-induced activation of this pathway is independent of the respiratory chain (Fig. 3*C*). Similar results were obtained when mitochondrial respiratory chain function was perturbed directly or indirectly with the ATP synthase inhibitor oligomycin and electron transport chain uncoupler carbonyl cyanide m-chlorophenyl hydrazine, thus further consistent with a critical role of the respiratory chain in H_2O_2 signaling (Fig. S2*F*). Taken together, the mitochondrial respiratory chain thus has a selective role in H_2O_2-induced activation of the Syk pathway but not in activation of this pathway in response to extracellular ligand-mediated receptor engagement. Further supporting the notion that H_2O_2-induced activation of Syk and BCR-induced activation of Syk are distinct processes, BCR ligation but not H_2O_2 induced the appearance of slower migrating Syk-positive bands consistent with ubiquitinated Syk (60) (e.g., Fig. 3*C*). Overall, H_2O_2-mediated signal transduction is thus characterized by a series of hierarchical signaling events placing the respiratory chain upstream of Lyn, Lyn upstream of Syk and SHP1, and Syk upstream of a distinct signaling and cellular response in hematopoietic and nonhematopoietic cells.

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H_2O_2 Induces Phosphorylation of Mitochondria-Associated Lyn, Syk, and Many Other Proteins. Signaling induced by the BCR and other plasma membrane-bound receptors initially clusters at the site of the engaged receptors (38, 61). Given our results that H_2O_2-mediated Syk signaling is controlled by the respiratory chain, we reasoned that Lyn and Syk activation occurs, at least in part, in association with the mitochondria. Immunopurification of mitochondria and associated membranes showed that H_2O_2 induced robust phosphorylation of Lyn, Syk, and many other distinct proteins in the mitochondrial fraction of both primary B cells and MEFs (Fig. 4*A*). Consistent with these findings, H_2O_2 treatment of B cells induced Lyn and Syk phosphorylation overlapping with complex I staining as judged by confocal imaging (Fig. 4 *B* and *C*). In contrast, Syk phosphorylation induced by BCR cross-linking on the B-cell surface was found exclusively at the outer rim of the cell, consistent with cap formation and localized signaling from the plasma membrane (61, 62) (Fig. 4 *B* and *C*). Furthermore, 13 Syk targets are known mitochondrial proteins among them essential metabolic enzymes, in support of a role for Syk in H_2O_2-mediated mitochondrial regulation (Dataset S1). H_2O_2 induced activation of the Syk pathway thus appears to take place in part though not exclusively associated with the mitochondria in line with the view that the respiratory chain is an upstream component of an H_2O_2-induced signal transduction cascade mediated by Lyn and Syk.

A Pool of Cellular Lyn and Syk Localizes to the Mitochondrial Intermembrane Space. To determine the precise spatial relationship of Syk pathway members with the mitochondria, we performed submitochondrial fractionation of mouse spleen mitochondria. A large portion of Lyn, Syk, and SHP1 remained associated with the mitochondria

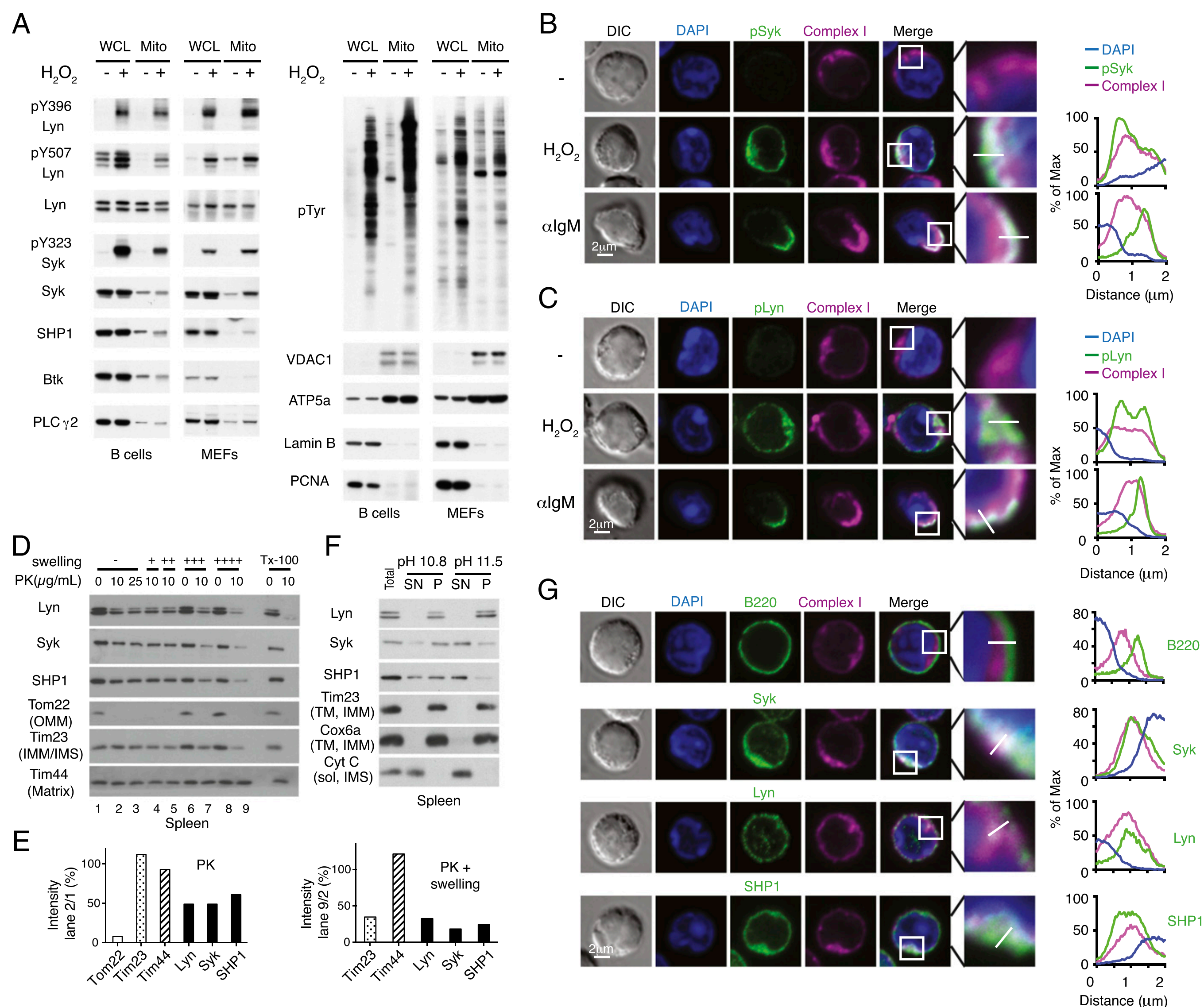

Fig. 4. H_2O_2 induces phosphorylation of mitochondria-associated Lyn and Syk, which localize to the differential interference contrast mitochondrial intermembrane space and are transiently and stably associated with the mitochondrial membrane compartment. (*A*) Tom22-mediated mitochondrial immunopurification of B cells and MEFs stimulated with H_2O_2 (B cells: 1 mM H_2O_2 for 5 min, MEFs: 5 mM H_2O_2 for 10 min). Mito, mitochondrial fraction; WCL, whole-cell lysate. (*B* and *C*) Immunofluorescence staining and confocal images of mouse splenic B cells stimulated with 1 mM H_2O_2 for 5 min and 50 µg/mL anti-IgM for 3 min. DIC, differential interference contrast; Max, maximum. Mitochondrial subfractionation of mouse spleen mitochondria treated with hypoosmotic (swelling) buffer and PK (*D*) and quantitation of signal intensity of immunoblots (*E*). IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane. (*F*) Carbonate extraction of mouse spleen mitochondria separating membrane integral (P) and soluble (SN) proteins. sol, soluble; Tm, transmembrane. (*G*) Confocal images of resting mouse splenic B cells stained as indicated.

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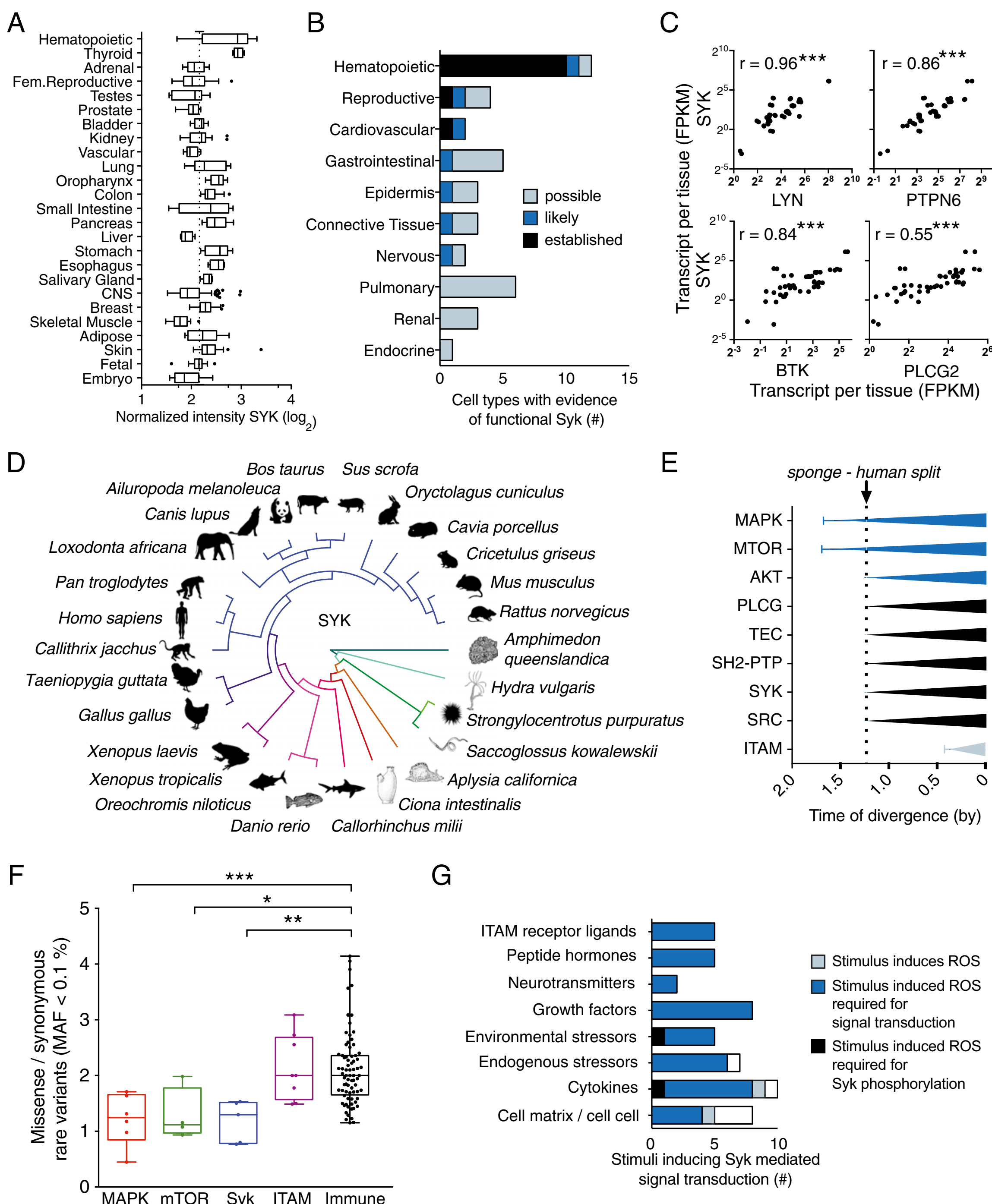

Fig. 5. The Syk pathway is coexpressed, is evolutionary ancient, and displays low missense variation in the human. (*A*) Microarray analysis of normal human tissues showing *SYK* transcript expression plotted as a box plot with Tukey whiskers (n = 688). The dotted line represents the median of all samples across tissues. (*B*) Categorization of evidence for a critical function of Syk in different cell types: established, abundant evidence using different cellular models and a combination of pharmacological and genetic approaches or pharmacological in vivo evidence demonstrating a critical role; likely, abundant evidence using different cellular models and a combination of pharmacological and genetic approaches or pharmacological in vivo evidence demonstrating a critical role; possible, at least one study in a cellular model using a combination of pharmacological and genetic approaches demonstrating a critical role. (*C*) Correlation of mRNA expression across different normal human tissues derived from mRNA sequencing datasets ($n = 48$). r, Pearson correlation coefficient. (*D*) Rooted phylogenetic tree of Syk orthologs in the animal kingdom. (*E*) Estimated evolutionary age of the Syk pathway in billion years (by). (*F*) Ratios of rare missense to rare synonymous variants for individual genes (dots) extracted from exomes of the Exome Aggregation Consortium. MAF, minor allele frequency. (*G*) Number of stimuli known to induce Syk-dependent signaling per category. Colors denote the number of stimuli per category with evidence that the stimulus induces cellular ROS (light blue), that the stimulus induces ROS and signals in a ROS-dependent manner (blue), and that ROS is required for stimulus-induced Syk phosphorylation (black). *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

after digestion with proteinase K (PK), which resulted in degradation of outer mitochondrial membrane proteins as indicated by loss of Tom22 (Fig. 4 *D* and *E*). Lyn, Syk, and SHP1 almost disappeared after incubation in hypotonic buffer, rupturing the outer mitochondrial membrane and resulting in PK-mediated degradation of the intermembrane space-facing domain of inner

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mitochondrial membrane protein Tim23 (Fig. 4 *D* and *E*), overall suggesting localization of a sizable fraction of Lyn, Syk, and SHP1 in the mitochondrial intermembrane space. Separation of soluble and membrane-integrated mitochondrial proteins showed that Syk and SHP1 were partially associated with and Lyn was exclusively associated with the mitochondrial membrane fraction (Fig. 4*F*), thus paralleling the transient activating association of Syk and SHP1 with plasma membrane-bound phosphotyrosine motifs and the stable plasma membrane association of Lyn (53, 63). Consistent with mitochondrial localization, Lyn, Syk, and SHP1, but not the plasma membrane-bound phosphatase receptor-type tyrosine-protein phosphatase C (B220/CD45/Ptprc), overlapped with complex I in splenic B cells by confocal imaging (Fig. 4*G*). Lyn, Syk, and SHP1 were also detected in liver mitochondria from which endoplasmic reticulum remnants tethered to mitochondria were removed by Percoll gradient centrifugation (Fig. S3*A*). In line with these findings, Syk and phosphorylated Syk also overlapped with liver mitochondria by tissue immunofluorescence staining and confocal imaging, overall supporting mitochondrial localization also in nonhematopoietic cells (Fig. S3*B*). Taken together, these results suggest that a portion of cellular Lyn, Syk, and SHP1 localizes to the mitochondrial intermembrane space and appears to be transiently (Syk and SHP1) or stably (Lyn) associated with the mitochondrial membrane compartment.

The Syk Pathway Is of Early Metazoan Origin, Is Coexpressed Across Tissues, and Shows Evidence of Evolutionary Constraint in the Human. Given the critical role of ROS signaling across biology, we finally reasoned that evidence may exist compatible with a function of Syk beyond linking immunoreceptor tyrosine–based activation motif (ITAM)-bearing receptors of the immune system to downstream pathways (46, 64). Indeed, database mining of large microarray and mRNA sequencing datasets, and our confirmatory quantitative PCR assay and immunohistochemistry of normal human and mouse tissues, showed that Syk transcript and protein were detectable in every tissue examined, although smaller amounts relative to total RNA and protein were found in most nonhematopoietic cells (Fig. 5*A* and Fig. S4 *A*–*D*). Analysis and categorization of the available 3,078 indexed research articles mentioning Syk suggested that Syk is functional in cell types derived from every organ system, although conclusive genetic evidence for a critical in vivo role exists only for hematopoietic tissues and mammary and vascular endothelial cells (Fig. 5*B* and Table S1). Further, expression of *LYN*, *PTPN6*, *BTK*, and *PLCγ2*, was correlated with *SYK* expression in a wide range of human tissues, whereas there were minor, no, or negative correlations with expression of the BCR-associated adapter *CD79A* (Igα), related family members, and other Syk targets as judged by both mRNA sequencing and microarray data (Fig. 5*C* and Table S2). These results suggest a constant stoichiometry of Syk with Syk pathway members, consistent with the idea that these proteins interact and form functional units or "signalosomes" in many different tissues.

We identified known and predicted Syk orthologs in every vertebrate examined, as well as in evolutionarily distant groups of extant metazoans, including a member of the earliest group of metazoans, the sponge *Amphimedon queenslandica* (65), but not in yeast, plants, and bacteria (Fig. 5*D*, Fig. S4*E*, and Table S3). These findings add to earlier observations identifying Syk orthologs in the tunicate *Hydra vulgaris* and highlight a distribution of Syk orthologs throughout the animal kingdom (66). Similarly, orthologs of the Syk pathway members Lyn, SHP1, Btk, and PLCγ2 were found in the sponge *A. queenslandica* but not in premetazoan species. In contrast, all known ITAM-containing immune receptor-associated adapters were detected only in evolutionarily recent vertebrates. These findings thus suggest an evolutionary origin of the Syk pathway ~1.2 billion y ago, closer to the evolutionary origins of members of the MAPK and mammalian target of rapamycin (mTOR) pathways than to the evolutionary origins of the ITAMs of the immune system (Fig. 5*E* and Table S4).

A low ratio of nonsynonymous to synonymous rare variants in humans and other species suggests purifying selection, thus allowing an estimate of the effects of missense variation in a given gene on reproductive fitness. Similar to genes of the MAPK and mTOR pathways, *LYN*, *SYK*, *PTPN6*, *BTK*, and *PLCG2* displayed low ratios of rare missense variants to synonymous variants compared with the known ITAM-bearing immune adapters and many other immune-related genes as judged by mining exomes of 60,706 individuals assembled by the Exome Aggregation Consortium (Fig. 5*F*). Syk and the Syk pathway may thus also have a critical function in normal human physiology.

Literature curation revealed that 45 diverse stimuli ranging from hormones and growth factors to endogenous stressors such as high glucose induce signaling in a Syk-dependent manner (Fig. 5*G* and Table S5). Thirty-eight of these diverse stimuli are also known to induce signaling in a ROS-dependent manner, raising the possibility that a unifying mechanism of Syk activation by many stressors might be its activation by endogenous ROS (Fig. 5*G* and Table S5). In support of such a notion, osmotic stress and TNF induce Syk phosphorylation in a ROS-dependent manner (67, 68), suggesting that Syk critically mediates signaling not only in response to extracellular ROS but possibly also in response to intracellular ROS. Taken together, the ubiquitous expression of Syk, coexpression of Syk interaction partners in different tissues, occurrence of Syk across the animal kingdom, origin of the Syk pathway early in metazoan evolution, evidence for Syk signaling in numerous ROS-mediated processes, and signs of evolutionary constraint on the pathway in the human suggest a much broader role for Syk than currently appreciated and are compatible with a role in ROS signaling.

Discussion

Here, we provide evidence suggesting that H_2O_2 signaling has multiple distinguishing features of a signal transduction cascade. It is characterized by a sequence of events culminating in a distinct signaling response: The upstream respiratory chain selectively activates Lyn, resulting in activation of downstream Syk, which, in turn, controls tyrosine phosphorylation of pathways critically involved in signaling, transcription, translation, metabolism, and cell cycle regulation. Its upstream components reside and are active in physical proximity: H_2O_2-mediated Lyn and Syk activation occurs, at least in part, in proximity to the respiratory chain, and a pool of cellular Lyn and Syk localizes to the mitochondrial intermembrane space. Finally, it is controlled by the same mediators in different species and tissues responsive to H_2O_2: The respiratory chain and the conserved and ubiquitous Syk pathway mediate H_2O_2 signaling in diverse cell types that include mouse and chicken B cells as well as fibroblasts. The results thus provide a framework to conceptualize ROS signaling and offer a rationale for numerous avenues of investigation.

ROS and mitochondrial dysfunction have been linked to a large number of biological processes and diseases, including adipogenesis, neurodegeneration, cardiovascular disease, inflammation, and the aging process itself (69–74). Although only extracellular H_2O_2 was used in this study, it seems likely that intracellular H_2O_2 induced by receptors and other stressors also uses this pathway, overall suggesting that the immune kinase Syk might be critical for many more cellular responses and disorders than currently appreciated. Exploring how modulation of Syk activity and gene dosage affects different disease states will be particularly relevant for ongoing drug development efforts currently focused only on hematological malignancies and autoimmune disease (64, 75).

The finding that the respiratory chain is required for H_2O_2-induced Syk activation raises the intriguing possibility that an ITAM in one of the more than 100 largely uncharacterized mammalian respiratory chain subunits binds and activates mitochondrial intermembrane space-localized Syk. Although the present results implicate the respiratory chain in signal transduction, identification of a subunit with a functional ITAM would establish the respiratory chain as a bona fide signal transducer. Such a subunit might also mediate some of the many functions of the respiratory chain described that are independent of its ability to produce ATP (59, 76–78). The present data suggesting mitochondrial intermembrane space localization of the Syk pathway also raise the question of whether Syk and Lyn might directly tyrosine-phosphorylate

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and modulate respiratory chain function. Indeed, there is precedence for a Src family kinase to modulate complex II activity, consistent with the observation that the respiratory chain is extensively posttranslationally modified (79, 80).

Our proposed model that H_2O_2 signaling resembles canonical signal transduction implies the existence of an upstream ROS receptor that recognizes H_2O_2 with much higher sensitivity than its surroundings. The most intuitive location of such a sensor might be in the respiratory chain itself, which may have evolved to sense H_2O_2 at the site of its production and transmit a signal to the cell via mitochondrial intermembrane space localized signaling pathways. Although the reaction constant for oxidation of the abundantly present cysteine residues is generally low, iron and iron clusters display much higher reactivity with H_2O_2, thus offering a limited number of candidates as exquisitely sensitive receptor modules (1, 81, 82). In support of such a possibility, multiple iron cluster-containing proteins induce transcriptional changes in bacteria, and thus might represent ancestral ROS-sensing signal transducers (83).

Further, one might speculate that this pathway represents a mechanism of mitochondrial control over ROS-induced cellular processes such as differentiation and proliferation, or senescence and programmed cell death. Indeed, communicating mitochondrial health to the cell might be a critical prerequisite to the successful implementation of ROS-stimulated energetically demanding cellular processes. Further, the mitochondria might also activate the Syk pathway to induce growth arrest and/or programmed cell death as the present results suggest. Consistent with utilization of this pathway for a spectrum of cellular responses, Syk and mitochondrial dysfunction have both been implicated in cellular differentiation and proliferation, as well as in tumor suppression (15, 64, 75, 84).

Finally, it is striking that a kinase of early metazoan origin such as Syk is so critical for H_2O_2 signaling in vertebrate cells, given that cellular responses to ROS first evolved in bacteria (1). Perhaps the occurrence of the Syk pathway along with multicellularity reflects an adaptation specific to metazoan life that allows the integration of metabolic signals from the mitochondria with other extracellular and intracellular cues transmitted and amplified by ITAMs (85). Indeed, the existence of several hundred ITAM-containing proteins across biological processes has been suggested (86), which might fulfill this function linking tissue- and context-specific inputs to the basic and ubiquitous Syk pathway.

Materials and Methods

Cell Culture. Primary B cells were isolated by depletion of CD43-positive cells from mouse spleen. Primary MEFs derived from embryonic day 14.5 embryos were cultured for 2–4 d before use. DT40 cell lines were imported from RIKEN and cultured in RPMI medium at 39.5 °C. All procedures were performed according to protocols approved by the Committee on Animal Care at the Massachusetts Institute of Technology and the University of Freiburg.

Phosphoproteomics. A label-free quantitative liquid chromatography-tandem MS analysis was performed using an LTQ-Orbitrap-ELITE mass spectrometer (Thermoscientific), electrospray ionization–collision-induced dissociation, and SEQUEST search results following immunoprecipitation with Tyr phosphorylation motif antibody pY-1000 (Cell Signaling Technology).

Mitochondrial Subfractionation. Crude mitochondria were resuspended in normosmotic buffer or hypotonic buffer [20 mM Hepes/KOH (pH 7.6)] and incubated for 15 min, followed by PK (Roche) digestion for 15 min.

Statistical Methods. All statistical analyses were performed using Prism 6 (GraphPad Software, Inc.). Statistical significance was indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

Supplementary materials and methods, including antibodies used in this study and listed in Table S6, are included in *SI Materials and Methods*.

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