Integrated signaling and transcriptome analysis reveals Src-family kinase individualities and novel pathways controlled by their constitutive activity

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Abstract

The enzymatic activity of Src-family kinase (SFKs) members Lck and Lyn is crucial to lymphocyte development and function. Lck has a T-cell restricted pattern of expression, however its ectopic presence has been documented in numerous pathological conditions. Amongst those, the erroneous presence of Lck in Chronic Lymphocytic Leukemia (CLL) B-cells has been suggested as a relevant factor for disease pathophysiology. However, the exact impact of Lck on the B-cell signaling apparatus remains unclear. In this work we sought to investigate the mechanistic basis of ectopic Lck in B-cell activation events. We examined the onset of proximal B-cell Receptor (BCR) signaling pathways and transcriptome alterations under the influence of ectopic Lck and compared it to events elicited by the predominant B-cell SFK member, Lyn.

Our studies pinpointed signalling pathways exclusively governed by the constitutive activity of the two SFKs, excluding interferences from receptor/co-receptor engagement, influence of the microenvironment, alterations on their stereochemical integrity, or modulation of their regulators such as Csk or CD45, which also affect additional substrates.

Lck- and Lyn-transduced signals sufficed to drive signalling events reminiscent of B-cells found in an activated state. We further discovered that the two kinases display differential susceptibility to mechanisms designed to prevent the onset of unscheduled signalling events, by keeping global levels of SFK activity under control. Finally, our analyses revealed a yet unrecognized role of SFK-driven signals in tipping the balance of cellular stress response regulation.

Introduction

Src-family kinases (SFKs) are ancient and evolutionarily conserved molecules. Their enzymatic activity is mandatory for several survival and activation-dependent biological processes in the context of many different tissues and cell types. Consequently, deregulated SFK activity has been described as an underlying cause for several neoplastic transformations, with the prototype of the family, c-Src, being the first ever described proto-oncogene[1].

SFK enzymatic efficiency is regulated by reversible phosphorylation events. Particularly relevant is a conserved tyrosine residue within their catalytic center, Y416 (Src numbering, used throughout this report). Trans-autophosphorylation of Y416 serves to stabilize the activation loop for optimal substrate binding and constitutes a conserved mechanism for SFK activation. Several protein tyrosine phosphatases (PTPs) have been attributed a role in keeping cellular levels of SFK activity at bay by dephosphorylating Y416 including CD45, SHP-1 and PTPN22[1].

SFK members show ubiquitous and tissue-restricted expression, with Lck and Lyn being preferentially relevant for T- and B-lymphocyte function, respectively. Lck's primary job is the phosphorylation of ITAM (Immunoreceptor Tyrosine -based Activation Motif) tyrosines located in the CD3 and ζ chains of the T-cell Receptor (TCR) complex and the consequent triggering of signalling responses after TCR engagement. Lyn performs a similar function on the B-cell Receptor (BCR) ITAMs[1].

Lck is found constitutively active in resting T cells, and the levels of the preactivated pool are necessary but also sufficient to transduce TCR-driven signalling responses without a need for further augmentation[2]. Ectopic presence of Lck has been recorded in different types of malignant cells, however the functional relevance of this erroneous expression has almost exclusively been investigated in the context of CLL B-cells. Several studies have documented the concurrence between ectopically expressed Lck in CLL, and enhanced BCR-driven activation[3]. Further to that, our previous work[4] designated Lck overexpression a marker of CLL B-cell subpopulations with heightened constitutive signaling profiles. Although these studies indicate the involvement of Lck in triggering BCR signals, several unmapped factors that can be present within neoplastic cells, prohibit the direct affirmation of a cause-effect relationship.

With the present work we sought to determine whether ectopic Lck could indeed ignite signaling reactions in B-cells, and if those would potentially differ from the conventional pathways triggered by the endogenous B-cell SFK, Lyn. Our data showed that alike Lyn, Lck can initiate autonomous B-cell signaling and alter the gene expression landscape of resting B-cells, albeit with a seemingly compromised efficacy. We further found that enzymatic promiscuity of SFKs can be buffered by their differential susceptibility to regulatory control mechanisms designed for keeping global SFK activity levels under strict control, thus preventing the initiation of unscheduled signaling. Finally, transcriptome analysis uncovered a yet unrecognized role of SFK signaling in cellular stress responses by favoring the onset of autophagy in the expense of proteasome-mediated degradation and the unfolded protein response (UPR).

Results

Ligation-independent BCR signaling is triggered by Lck overexpression

To enable studies that would provide evidence for a direct impact of Lck on BCR signaling pathways, we developed a stable B-cell line model. We used the Burkit lymphoma cell line, BJAB and introduced Lck via lentiviral gene transfer. To avoid counterselection and/or signaling pathway counterbalance events, often occurring as a result of the stable expression of signaling mediators in cell lines, we utilized a Doxycycline (Dox)-inducible system for Lck expression. Lck was invariably untraceable in non-Dox treated cultures (designated -Dox henceforth). Supplemental Text 1 and Fig. S1 describe selection criteria and characterization of the B-cell model system. Similar to its physiological T-cell environment, Lck was also constitutively active within BJAB cells. Importantly, the introduction of Lck in BJAB significantly increased the baseline levels of SFK activity (Fig. 1A upper panel, and S2A) as determined by staining with a-pY416, an antibody specifically recognizing the phosphorylated form of the activation loop tyrosine of SFKs and thus widely used as an indicator of pan-SFK activity[2,5]. The first step of B-cell activation from the BCR, is the phosphorylation of the Igα and Igβ ITAM tyrosines by Lyn[6]. Lck performs exactly the same job on the TCR ITAMs[3]. Staining with an Ab recognizing the phosphorylated Igα ITAM Y182 (designated pCD79a henceforth) revealed that Lck expression in BJAB resulted in BCR ITAM phosphorylation without a requirement for receptor ligation. pCD79a was abrogated after treatment with a specific Lck inhibitor, Lcki, or by expression of LckΔSH4, a mutant lacking the moiety responsible for anchoring at the inner leaflet of the plasma membrane[3]. Therefore, BCR ITAM phosphorylation required Lck's intact enzymatic activity and its juxtaposition to the receptor (Fig. 1A).

2D FACS plots showed a dose-response relationship between Lck expression/activity and pCD79a induction and exposed a prerequisite for surpassing an expression threshold for receptor-independent ITAM phosphorylation to occur. Further to pCD79a, introduction of Lck to BJAB cells increased basal levels of global

tyrosine phosphorylation, again in the absence of receptor or co-receptor ligation (Fig. 1B) implying that Lck's effect on the BCR ITAMs is "productive" enough to propagate to downstream pathways. Importantly, these phosphorylation events were unrelated to Lyn or other SFKs, since we could not detect any shifts on their activation status, unpredictably resulting from the ectopic presence of Lck (Fig. S2B). Unlike BJAB cells, Lck overexpression in HEK293T failed to alter the global phosphotyrosine landscape, whereas Src and to a lesser extend Lyn and Fyn did (Fig. S2C), indicating a preference of Lck for antigenic receptor signaling.

Lck and Lyn differ in the efficiency for signal ignition and in their susceptibility to regulatory mechanisms in B-cells

In light of these observations, we asked whether ignition of autonomous signaling results from failure of B-cell regulatory mechanisms to restrain an "alien" kinase, or if this is a general outcome of increased SFK activity levels, overruling the processing capacity of inhibitory pathways. To answer this conundrum, we deduced that a comparison between ectopic Lck and "resident B-cell SFK" Lyn, would prove informative. This type of analyses required a means of readily comparing expression levels between the two SFKs. We thus introduced a GFP-tag on their C-termini. When expressed in BJAB cells, the GFP-tagged proteins behaved identically to their non-GFP versions, with regard to pY416 levels and their ability to induce constitutive pCD79a (Fig. S3A), confirming that the presence of GFP did not interfere with their biological function and thus the tagged constructs could be reliably used for the continuation of our studies. To obtain as homogeneous populations as possible, cell lines were sorted for GFP^+ cells (Fig. S3B).

During the course of these experiments, we faced an insuperable difficulty to obtain Lyn expression levels equally high to those of Lck (Fig. S3B and S3C). This appeared to be a problem with Lyn protein stability within the lymphocyte environment since transient transfection of the two SFK constructs in HEK 293T cells resulted in comparable expression (Fig. S3D). The longer splice variant of Lyn, LynA, is particularly susceptible to ubiquitin-mediated breakdown, thus we used the degradation-resistant isoform (LynB), lacking the region which flags the kinase for Cbl-mediated polyubiquitination[6]. However, even LynB could not reach expression levels comparable to those of Lck, a trend that was reversed after treatment with the proteasome inhibitor MG132 (Fig. 1C). Thus, the proteasomal degradation machinery constrains the abundance of Lyn, but not Lck, within B-cells.

Nonetheless, the presence of the GFP tag allowed gating and direct comparison of cell populations with equal levels of protein expression (Fig. S3E). Similar to Lck, Lyn was also constitutively active in B cells, as confirmed by pY416 staining, and its expression augmented SFK activity levels (Fig. S3C). However, the BJAB-Lck line not only contained higher amounts of global SFK activity, due to the surplus in Lck protein expression (Fig. S3C), but also higher pY416 when compared with Lyn on an equivalent protein expression basis (cells gated for equal GFP) (Fig. 1D). 2min treatment with the Tyrosine Phosphatase (PTP) inhibitor Pervanadate sufficed to drastically increase Lyn-pY416 whereas Lck-pY416 remained unaffected. Appreciable Lck-pY416 increases required much longer incubation times, at least 5-10 min of pervanadate treatment (Fig. 1D and data not shown). This differential sensitivity of the two SFKs towards pervanadate strongly suggests that Lyn is subjected to stricter PTP control on the phosphorylation dynamics of its activation loop tyrosine.

Following antigen recognition and ITAM phosphorylation, propagation of BCR signaling requires the recruitment and activation of Syk and subsequent phosphorylation of its substrates. BCR ligation also activates the PI3K/Akt pathway, which is essential for B-cell function[7]. Dox-induced expression of both Lck and Lyn drove the phosphorylation of signal transducers pCD79a, Syk and Akt, increased the global phosphotyrosine content in unstimulated BJAB cells (Fig. 2A and S4B) and provided a relative enhancement in BCR-triggered responses after a-IgM stimulation (Fig. S4). Consistent with the higher content of SFK activity, the phosphorylation fingerprint of downstream signaling was significantly more intense in the BJAB-Lck line. However, analysis of cells with equivalent SFK protein expression (equal GFP gates), revealed the superior efficiency of Lyn in BCR signaling, since Lck had to be expressed in much higher amounts in order to elicit responses of comparable magnitude (Fig. 2B).

Collectively these data show that the BCR signaling machinery is more responsive to the action of Lyn, at the same time imposing stricter regulation on its expression and activity levels. On the other hand, the case of Lck shows that despite reduced efficiency for the B-cell signaling apparatus, when global SFK activity levels surpass a certain threshold, autonomous activation can occur. Therefore, SFK protein individualities and the total amount of SFK activity within a cell, are equally contributing factors in fine-tuning signaling thresholds for antigenic receptors.

Constitutive triggering of the CD22/SHP-1 inhibitory axis by Lck and Lyn overexpression

Further to their necessity for ITAM phosphorylation Lyn, and to a lesser extent Lck, are capable of triggering parallel inhibitory pathways. CD22 is an extensively characterized inhibitory receptor in B-cells. CD22 ITIM phosphorylation by Lyn, creates docking sites for the recruitment of the PTP of SHP-1[6]. Furthermore, Lck and Lyn can phosphorylate Y564 of SHP-1 which results in enhanced phosphatase activity[6,8]. Dox-induced expression of both SFKs was followed by enhanced constitutive and a-IgM induced phosphorylation of CD22 and SHP-1Y564 (Fig. 2C and 2D). Accordingly, stronger co-immunoprecipitation of CD22 with SHP-1 (and pSHP-1) was detected in SFK-expressing BJAB cells compared to the -Dox counterpart. This association, as well as pSHP-1 phosphorylation receded to baseline levels upon treatment with the pan-SFK inhibitor PP2 (Fig. 2D), demonstrating the interdependence of intact SFK kinase activity and upregulation of the CD22/SHP-1 inhibitory axis. Therefore, in addition to igniting signaling downstream the BCR, ectopic Lck can trigger ITIM-induced responses from CD22.

SFK overexpression modifies the Transcriptome profile of B-cells

Further to their influence on proximal BCR signaling, we investigated the net-result of SFK-triggered pathways on the gene expression program. RNA extracted from BJAB lines overexpressing Lyn or Lck, 48h after Dox addition, and the non-Dox-treated counterparts were used to produce corresponding cDNA libraries for NGS and Bioinformatics analysis. Differentially expressed genes (DEG) were selected using two criteria: log fold change >0.5 or <-0.5 (for upregulated and downregulated DEGs respectively) relative to the -Dox sample, and $p<0.05$. We identified 1264 and 956 differentially expressed genes in the BJAB-Lyn and BJAB-Lck cell lines, respectively (GEO Accession number: GSE212759). Lyn overexpression coincided with upregulation of 759 genes and Lck with 517. 321 upregulated genes were common for the two SFKs. Likewise, the expression of 505 and 439 genes was repressed as a consequence of Lyn and Lck overexpression respectively, 219 of which were commonly downregulated by both SFKs (Fig. 3A and 3B).

Gene ontology (GO) enrichment analysis assigned differentially expressed genes to several functional outcomes ranging from processes reminiscent of B-cells that have received BCR engagement signals (B-cell activation, Ig and cytokine synthesis, Ag presentation) to regulation of apoptosis, autophagy and the ER stress response (Fig 3C.). Unique genes upregulated or downregulated by each SFK could not be assigned to any specific biological function by GO enrichment analysis, so at this point we cannot deduce that there is discrimination on the signaling pathways originating from Lyn or Lck overexpression. Although GO analysis of Lck-induced genes did not reveal any significant enrichment in biological processes other than cell adhesion/migration, a more thorough inspection showed Lck's involvement in the same biological processes as Lyn (Fig. 3D and 3E).

SFK overexpression was followed by induction of class I and II HLA genes, the transcriptional co-activator CIITA and accessory molecules (CTSH, TAP1, TAPBP, RAB4B and SPPL3) essential for the formation and presentation of pMHC complexes (Fig. 3D, corresponding heat map). Several genes encoding constant and variable Immunoglobulin regions were significantly induced (Fig. 3D, corresponding heat map). In addition to the two BCR isotypes, IgHM and IgHD, mRNAs encoding the constant regions of IgA and IgE were strongly upregulated. Counterintuitively, expression of Activation-induced cytidine deaminase (AICDA/AID), the key initiator of class switch recombination (CSR), was repressed whereas the E3 Ubiquitin ligase responsible for AID protein degradation, CUL7[9] was upregulated. Concomitantly, DNA repair factors acting alongside AICDA

were differentially expressed. PARP3 a negative CSR regulator [10] was upregulated, whereas UNG and HMCES, both of which are required for efficient CRS[11,12], were downregulated. Notably the transcriptional activator UBP1 (LBP-1a) was repressed. Suppression of UBP1 has been shown to preferentially promote IgA (but not IgG1) in B-lymphocytes induced to undergo CSR[13], which interestingly coincides with increase of IgHA but not IgHG mRNA levels.

Consistent with B-cells' recognized contribution to the cytokine milieu, several cytokine encoding genes were induced together with the cytokine-activator member of the Caspase-1 family, CASP-4 (Fig. 3D, corresponding heat map).

The expression level of genes encoding proteins involved in cell migration and adhesion were also strongly upregulated (Fig. 3D, corresponding heat map). Amongst those were members of the integrin family and their regulators (PPM1F, LIMS2/Pinch-2), adhesion molecules (CEACAM1, ICAM3) and the adhesion G-protein coupled receptor ADGRA2, as well as the chemoattractant receptor CCR7, upregulation of which is one of the earliest events following BCR engagement[14].

Numerous B-cell co-stimulatory receptors, activation markers and signaling mediators[15] were also induced (Fig. 3D, corresponding heat map). Amongst those were, BAFFR (TNFRSF13C), one of the major pro-survival receptors in B-cells, CD40, CD48, CD69, CD52, CD20, CD27, the ITIM containing CD72 and the BCR-induced exhaustion marker, PD1[16] as well as members of the FCRL family. mRNA levels of the cell-death receptor FAS were also upregulated, a feature characteristic of activated B-cells[17].

Expression profiles of several B-cell activation-relevant transcription factors and transcriptional regulators[18] were altered as a result of SFK-triggered signals in BJAB cells (Fig. 3D, corresponding heat map). Induced genes common between the two SFKs included ID2, an E2A and Pax5 antagonist, the EBF family member EBF4, the AP-1 transcriptional coactivator CRTC1, the NF-kB subunit REL (in parallel to downregulation of the NF-kB inhibitor, NF-kBIB), STAT2, SOX11 as well as the early growth response genes EGR1 and EGR2. BTG2, a transcriptional coregulator important for B-cell development and differentiation was also upregulated. In parallel, we detected repression of pro-apoptotic mediator/tumor suppressors ID4 and ARID5B, downregulation of which correlates with enhanced acute lymphoblastic leukemia (ALL) cell proliferation[19]. The Lyn-specific signature comprised induction of Oct-2, ATF5, the AP-1 member JUNB, AP4 and NAB2 (a BCR-induced transcriptional co-activator of IL-2 production), with concomitant repression of CTCF (a BCR-induced growth suppressor). Lck-expressing cells preferentially upregulated CREB1, and SMAD2, an activator of IgA class switching. There appeared to be opposing regulation of two TFs belonging to the ETS family, namely downregulation of ETS-2 (an IL-2 mRNA repressor) and upregulation of EBV6 (a PAX5 suppressor). mRNA levels for E4F1, an inhibitor of cell proliferation and PER2, a negative regulator of lymphoid immune functions, were uniquely downregulated in response to Lck expression in BJAB cells.

Alterations in the balance between pro-survival versus pro-apoptotic transcriptome landscape appeared more complex (Fig. 3D, corresponding heat map). SFK-triggered pathways led to the induction of inhibitors of apoptosis BIRC7 and CFLAR. Repressed genes included AIMP2 an enhancer of TNF-mediated cell death and the apoptosis-promoting molecules DRAK1 (a member of the death-associated protein kinases - DAPK) and the transcriptional repressor BCLAF1. Expression patterns of genes belonging to the BCL2 family revealed upregulation of the key anti-apoptotic regulator Bcl2 and pro-apoptotic protein Bim whereas in Lck-expressing cells mRNA levels of the pro-apoptotic mediators NOXA and BAK1 were downregulated. Lyn expression was accompanied by repression of the VDAC2 gene, encoding for a voltage-dependent anion channel responsible for maintaining BAK1 in an inactive state.

Expression of several E3 Ubiquitin ligases were induced upon SFK overexpression, whereas ubiquitin modifiers and conjugating enzymes as well as mRNAs encoding for ubiquitins B and C were downregulated. Similar pattern of repression was observed for genes encoding a number of proteasome subunits and proteasome assembly and maturation proteins (POMP, PSMG1) (Fig. 3D, corresponding heat map).

A number of positive regulators of autophagy were induced. Amongst them were TP53INP2, EPG5, RUFY4 and TOLLIP whereas mRNA levels of autophagy enhancer RUBCNL/PACER were downregulated (Fig.

3D, corresponding heat map). Notably, genes encoding the ER-phagy receptors RETREG1/FAM134, CCPG1 and CALCOCO1 and facilitators GABARAP and SEC23 were also induced.

Rather unexpectedly a significant proportion of repressed genes were related to the endoplasmic reticulum (ER) stress and the Unfolded Protein Response (UPR) pathways (Fig. 3D, corresponding heat map). These included a wide range of ER chaperones and enzymes (PDI4, HSPA1, HSPA9), UPR target genes and downstream regulators (HSPA5, GRP94, CHOP, CHAC1, SESN2, HERPUD1 and others), as well as UPR-related transcription factors XBP1, ATF3.

Altered mRNA levels of key genes discovered by the transcriptome analysis were validated by qPCR (Fig. 3E). Importantly we verified that the recorded gene expression alterations were unrelated to the addition of Dox to the cell cultures (Fig. S5).

A novel role of SFKs in ER homeostasis

The abundance of downregulated genes assigned to ER homeostasis responses, and given the central role of the endoplasmic reticulum on the B-cell secretory pathway, prompted us to investigate whether SFK-induced signals would influence processes related to the excessive demand for protein synthesis and folding. BJAB-Lck and BJAB-Lyn cells were removed from the culture at regular time intervals ranging from 0-48h after Dox addition, stained with ER tracker and analysed by FACS, at the same time monitoring SFK expression by GFP fluorescence intensity (Fig. 4A). Increases in SFK expression levels correlated with ER expansion. Importantly, ER size receded to basal levels after 18h incubation with the Src kinase inhibitor PP2, affirming that the observed ER expansion was a consequence of SFK-induced signals. Aliquots of cells stained with ER tracker for each corresponding time point, were analysed by qPCR for the expression of UPR mediators (Fig 4B). It has been documented that XBP1, sXBP1, HSPA5 and CHOP mRNA levels significantly increase by 6h of chemically induced ER stress and remain elevated after to 12h[20]. However, at no point we could detect upregulation of any of the four genes, despite the recorded ER expansion.

These data indicate augmented protein load reaching the ER, forcing it to increase its volume in order to meet demands, but surprisingly this process seems to be accompanied by a parallel blocking of the UPR.

Discussion

In this work we examined disruptions in B-cell signaling homeostasis resulting from the overexpression of endogenous (Lyn) and ectopic (Lck) SFKs. Similar to Lck's behavior in T cells[2], both kinases are constitutively active in B-cells and elicit phosphorylation events in a dose-dependent fashion without any requirement for receptor engagement. However, aberrances in baseline B-cell signaling required the surpassing of a certain expression, and concomitant SFK activity, threshold.

A role for Lck in BCR ITAM phosphorylation has been suggested by studies in CLL cells treated with Lck inhibitors[21] and experiments demonstrating co-localization of the kinase with the CD79 chains, under the imposition of BCR ligation[22] or in the context of unstimulated subpopulations expressing exceedingly high levels of ectopic Lck[4], all of which may represent coincidental events. Here, we demonstrate a direct cause-effect relationship between ectopic Lck and BCR ITAM-driven ignition of signaling responses, which conclude to alterations in the gene expression program. Interestingly, in contrast to Src, Fyn and to a lesser extent Lyn, Lck did not affect the global phosphotyrosine profile of HEK293T cells, indicating a dedication to ITAM/ITIM-containing receptors. This preferential behaviour questions the biological/pathological relevance of ectopic Lck outside the strict leukocyte environment.

Comparative analysis between Lck and Lyn designate two parameters governing the threshold of responses downstream antigenic receptors, which are not mutually exclusive. On one hand there is the total amount of SFK activity within the cell, and on the other the individuality of SFK family members, dictated by intrinsic molecular features. When cells were presented with excessive amounts of active SFKs due to the

enforced Lck or Lyn overexpression, unscheduled signaling was initiated and progressed all the way to the nucleus, indicating quantitative overwhelming of the mechanisms designed to keep SFK activity at bay. Nonetheless, for comparable protein levels, Lyn appears more efficient in driving BCR ITAM and downstream effector phosphorylation. This could be due to Lyn displaying higher substrate affinity and/or facilitated juxtaposition to the BCR, dictated by its SH4 and adaptor domains. In parallel however, Lyn is subjected to stricter regulatory restrictions. Even the degradation-resistant LynB isoform appears more susceptible than Lck to proteasome-mediated proteolysis and thus not allowed to exceed strictly delimited expression levels. Furthermore, PTP inhibition resulted in the acute augmentation of Lyn's but not Lck's activation loop tyrosine phosphorylation. This could be due to a kinetic advantage of Lck trans-autophosphorylation versus dephosphorylation, when compared to Lyn, and/or its preferential segregation in "PTP-free" membrane subregions. The second scenario seems more plausible since Lck pY416 could appreciably increase but required longer pervanadate treatments, implying a delayed rate of encounter between Lck and PTPs. In any case, the time gap of response to pervanadate between Lck and Lyn, implies that the phosphorylation status of Lyn's activation loop tyrosine is kept under stricter surveillance by PTPs.

Overexpression of both Lck and Lyn generated signaling events capable of altering the gene expression landscape of BJAB cells. We identified a plethora of upregulated mRNAs associated with B-cell activation. Induced genes included HLA molecules, Immunoglobulin constant and variable regions, surface markers and co-receptors, cytokines and their receptors, adhesion and migration molecules, anti-apoptotic mediators and several transcription factors. This induced-gene profile is in accordance with the augmented phosphorylation of proximal BCR signaling transducers and highlights an ability of both SFKs to, at least partially, drive B-cells towards an activation mode in the absence of additional stimulatory signals. Both the GO enrichment analysis and our own comparison of upregulated mRNAs, leaves a general impression that Lck is not as effective as Lyn in the induction of B-cell activation-related genes. This was despite the more robust phorsphorylation of CD79a, Syk, Akt and global phosphotyrosine content recorded in the Lck-BJAB cells. Further and more detailed studies are required in order to clarify whether this is due to qualitative signaling differences (i.e., incapability of Lck in triggering parallel stimulatory pathways) or due to the more intense activation of SHP-1/CD22 negative regulatory axis and perhaps additional inhibitory pathways. Lyn also triggers both ITAM and ITIM phosphorylation in B-cells, thus simultaneously participates in activatory and inhibitory pathways, but to which side the scales are tipped remains debatable. Lyn-deficient B-cells display hyperresponsive BCR signaling[23], implying a net inhibitory effect. On the other hand, expression of a constitutively active Lyn transgene (Y508F) resulted in exaggerated positive proximal BCR signaling[24]. This phenotype was mimicked by enforced SFK activation via Csk inhibition and revealed that additional B-cell expressed SFKs efficiently triggered inhibitory signaling in the absence of Lyn[5]. The latter studies suggest a preferential positive influence on B-cell signaling responses. On a similar note, our data showed that the mere elevation of wild-type Lyn expression levels sufficed to elicit proximal BCR signaling and shift the gene expression program towards responses reminiscent of B-cell activation, in the presence of concomitant triggering of the CD22/SHP-1 axis.

A somehow curious observation from the transcriptomics data was the pronounced upregulation of IgHA and IgHE mRNAs and the parallel suppression of AID and accessory DNA repair factors essential for CSR. This was not a peculiarity of the cell line model, since BJAB cells do not constitutively express IgA or IgE isotypes and are used by several groups as a model to study CSR in response to different stimuli. Not surprisingly, many of these reports have documented the concurrence of class switching with AID upregulation[25]. However, Hauser et al. reported that a-IgM stimulation of mouse splenic B-cells induced to undergo CSR by a 48h LPS/IL-4 treatment, caused sharp downregulation of AID transcripts[26]. Furthermore, AID gets downregulated as B-cells exit the germinal centers and becomes completely absent upon terminal differentiation[27]. It was thus proposed that, high-intensity BCR signals shut off AID expression as a means of halting mutagenesis of the antibody genes once good affinity of immunoglobulins has been reached. In our system, cells are in a state of interminable ITAM-transmitted signals, the intensity of which could progressively increase in a time-dependent fashion, in correlation to the ongoing increases in SFK expression levels. The emergence of

switched isotype mRNAs inevitably requires AID upregulation at some point, which could possibly have occurred relatively early after SFK expression. Therefore, AID downregulation (detected 48h after Dox addition) may designate a state reminiscent to termination of the switch process. Although based solely on these data, we cannot claim a role of SFKs as tuners of class switching or B-cell terminal differentiation, we strongly believe it is a hypothesis deserving more in-depth exploration.

SFK overexpression affected several genes involved in the apoptotic process. Of note, we noticed parallel upregulation of two members of the BCL2 family with opposing actions, Bcl2 and Bim (BCL2L11). It has been shown in T-cells, that these proteins mutually affect each other's expression[28]. Notably, high amounts of Bcl2 increased Bim mRNA levels and was proposed that this codependency has a role in tipping the balance between survival and the timely elimination of activated T-cells in order to restore T-cell homeostasis. Our data implies that a similar mechanism may be in effect for activated B-cells.

Transcriptome analysis of SFK-overexpressing BJAB cells revealed a somehow discordant profile of upregulated Immunoglobulin mRNAs and ER expansion accompanied by marked downregulation of important UPR transducers. To cope with the excessive demand for protein synthesis and folding, antibody-producing B-cells employ mechanisms that increase ER output and alleviate the stress created by the vast accumulation of unfolded/misfolded protein cargo. Accordingly, activation of the UPR signaling pathway drives the upregulation of several target genes that enhance ER functionality, including transcription factors and numerous chaperones and folding enzymes[29]. An additional mechanism acting in parallel or independently of the typical UPR is the physical expansion of the organelle[30]. Indeed, activated B-cells can expand their ER volume up to threefold during the differentiation route to plasma cells[31]. It has been reported that Lyn overexpression can block the induction of CHOP and HSPA5 in airway epithelial cells[32]. Although in this model Lyn signaling per se did not directly affect the UPR, but rather participated in limiting its intensity under conditions of inflammation-induced ER stress, these data are in line with our observations of a suppressive role of Lyn and Lck in UPR signaling. Lyn-mediated suppression of the UPR in the aforementioned study resulted in inhibition of mucus hypersecretion, it was thus credited with a protective role during allergic reactions in the lung. What might then be the biological value of UPR downregulation in our system? It has been demonstrated that T-cells from an Slfn2 mutant mouse (*elektra* mouse model) display a semi-activated phenotype, which is combined with chronic ER stress and UPR activation, under resting conditions[33]. As a consequence, *elektra* T-cells, display compromised viability via the intrinsic apoptotic pathway. In this model, knockdown of the major UPR mediators XBP1 or CHOP sufficed to restore viability, thus connecting suppression of the UPR with improved survival. This concept could also apply to our cells. Further to antigen recognition by the BCR, full B-cell activation requires additional signals from the engagement of stimulatory and inhibitory co-receptors, cytokines, and help from T-cells. None of these events take place in our system, nonetheless SFK overexpression triggered BCR proximal signaling events and modulated the expression of several genes characteristic of activated B-cells. Based on these facts, one could hypothesize that SFK-overexpressing BJAB cells display what could be characterized as a semi-activated phenotype, which could ultimately compromise their viability as in the case of *elektra* T-cells. In this scenario, the simultaneous UPR downregulation by SFK signaling might serve as a means of preserving survival and reveal a previously unrecognized SFK-mediated mechanism for protecting B-cells from apoptosis under conditions of "inadequate" activating signals. Interestingly, in contrast to Lyn and Lck, the activity of c-Src was essential for UPR activation (XBP1 and HSPA5 induction) in human breast cancer cell lines[34] revealing differential involvement of SFKs in stress responses.

An additional noteworthy observation on the transcriptome profiles of SFK-overexpressing BJAB cells was the suppression of genes involved in the Ubiquitin/Proteasome degradation machinery. Assuming that the recorded ER expansion is a consequence of increased protein load delivered to the organelle, one would expect that the proportion of newly synthesized proteins failing to attain their native conformation and thus be destined for degradation should also increase, calling for a reinforcement of the proteasomal machinery. Indeed, augmented protein synthesis is accompanied by a global induction of genes encoding proteasomal

subunits[35], whereas in yeast, protein misfolding was shown to drive the expression of genes responsible for proteasome biogenesis[36]. However, a similar to ours paradoxical imbalance between protein production load and cellular degradation capacity has been documented in "short-lived" plasma cells. In this model, differentiating B-cells started downregulating proteasomal activity and subunit expression despite the high rates of Immunoglobulin production. The disproportional proteasomal load to capacity ratio was linked with the onset of apoptosis and was proposed as a one of the mechanisms responsible for controlling plasma cell lifespan[37]. The Ubiquitin-Proteasome system is functionally coupled to the UPR and autophagy[38]. Inhibition of the former frequently results in compensatory autophagic mechanisms for protein degradation. Our transcriptomics analysis revealed induction of several autophagy-related genes implying that SFK-transduced signals favor autophagic catabolic processes in the expense of proteasomal degradation and the UPR. Of particular interest was the induction of genes encoding the ER-phagy receptors RETREG1/FAM134, CCPG1 and CALCOCO1 and facilitators GABARAP and SEC23. The notion of selective ER-phagy acting as a rheostat between optimal Immunoglobulin production, ER stress responses and viability has been suggested by studies in Atg5 deficient plasma cells[39]. In this model, autophagic regulation of the ER ensured that Immunoglobulin synthesis, XBP1, HSPA5 expression and ER expansion, would not exceed levels that would be intolerable by the plasma cell.

In conclusion, our model provides an unbiased picture of the signaling machineries set in motion by Lck and Lyn and uncovers several interesting aspects about SFK regulation and function. We show that the WT forms of both kinases are constitutively active in B-cells and can autonomously emit post-translational and transcriptome changes reminiscent of BCR activation. Constitutive ignition of signaling pathways occurred once a certain threshold of SFK expression/activity was surpassed, highlighting limitations in the amplitude of the machinery designed to keep SFKs under control. We further discovered that Lyn was a more effective signal transducer in B-cells and identified specialized control mechanisms designed to keep Lyn, but not Lck, activity levels under strict control. These data may signify that SFKs have been evolutionarily diversified to best suit the needs of the cellular environment they are expressed in and provides an interesting prospect to study whether this trend will be maintained or reversed when Lck and Lyn are overexpressed in T-cells. Finally, we show a previously unrecognized role of Lck and Lyn in tipping the transcriptome balance towards autophagy and in parallel suppressing the proteasome machinery and UPR, which opens new avenues for studying the role of SFKs in the regulation of stress responses especially in the context of lymphocyte differentiation into effector cells.

Materials and Methods

Detailed description of Materials and Methods in supplementary information.

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Additional Information

The manuscript has not been accepted or published elsewhere.

All authors have read and approved the manuscript.

The authors declare no competing financial interests.

Data Availability

All raw sequencing data are available at the GEO Repository under the Accession Number: GSE212759.

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Fig.1

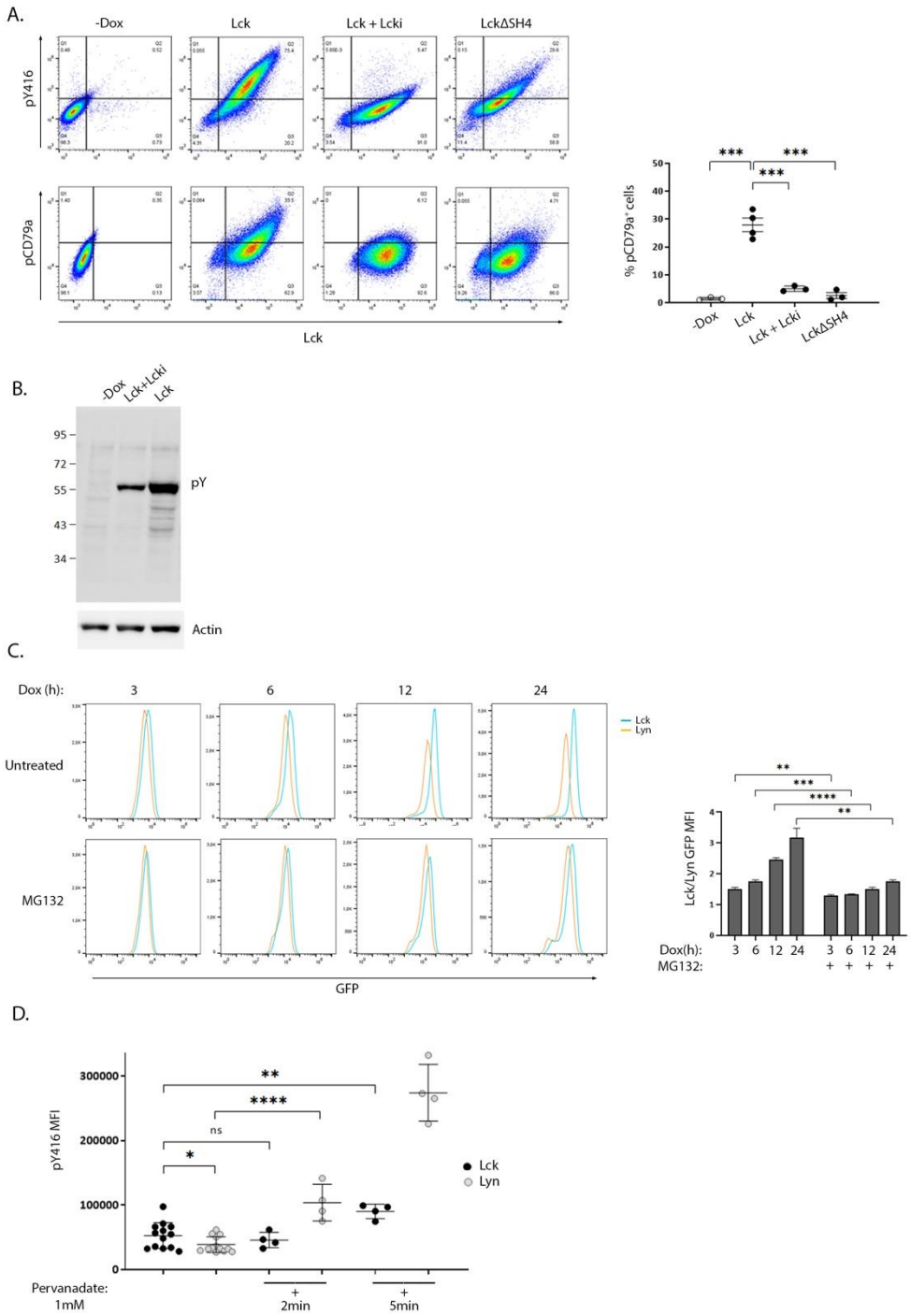

Figure 1. Differential susceptibility to regulatory control mechanisms, sets the threshold for SFK activity and ligation-independent BCR signaling

A. High expression of ectopic Lck in B-cells drives constitutive BCR ITAM phosphorylation.

BJAB cells transduced with LckWT or the deletion mutant ΔSH4 were cultured in the absence or presence of Dox for 48h. LckWT-expressing cells were further treated with 10 µM of the specific Lck inhibitor Lcki for 10 min. Samples were stained for Lck, its' active form (pY416) or pCD79a and are presented as 2D FACS plots of Lck^+ gated cells, to better visualize the dose-response relationship between Lck expression and its active form

(upper panels) or ignition of constitutive BCR ITAM phosphorylation (lower panels). Quadrant gating was set on the corresponding negative populations (-Dox samples). The right-hand panel graph shows the frequency of cells in the upper right quadrant (double-positive for Lck and pCD79a) (n≥3, Unpaired Student t test; mean +/- standard deviation [SD]; ***P < 0.001).

B. **Ectopic Lck in B cells ignites signaling pathways downstream the BCR**

Anti-phosphotyrosine (pY) clone 4G10 western blot of total cell lysates from LckWT-expressing cells untreated or incubated with 10 µM of Lcki for 10 min and the -Dox treated sample. Actin blot (lower panel) verifies equal sample loading.

C. Lyn shows significantly higher predisposition to proteasomal degradation than Lck

Cells expressing GFP-tagged Lck or Lyn were either left untreated or incubated with 1 µM of the proteasome inhibitor MG132. Samples were removed from the culture at the indicated time points after Dox addition and were analysed by FACS to monitor SFK protein production via GFP fluorescence intensity.

Graph shows the ratios of Lck/Lyn GFP Mean Fluorescence Intensities (MFIs) in the absence or presence of MG132 at each indicated time point (n=3, Unpaired Student t test; mean +/- SD; **P < 0.01, ***P < 0.001, ****P < 0.0001).

D. The phosphorylation status of the activation loop tyrosine of Lyn is under stricter control by PTPs

Cells expressing GFP-tagged Lck or Lyn were either left untreated or incubated with 1mM Pervanadate for the indicated times. Graph shows pY416 MFI values within cell populations gated for comparable SFK expression levels (equal GFP fluorescence)

(n=13 for untreated and n=4 for pervanadate treated cells, Unpaired Student t test; mean +/- SD; *P < 0.05, **P < 0.01, ****P < 0.0001; ns: not significant).

Fig 2.

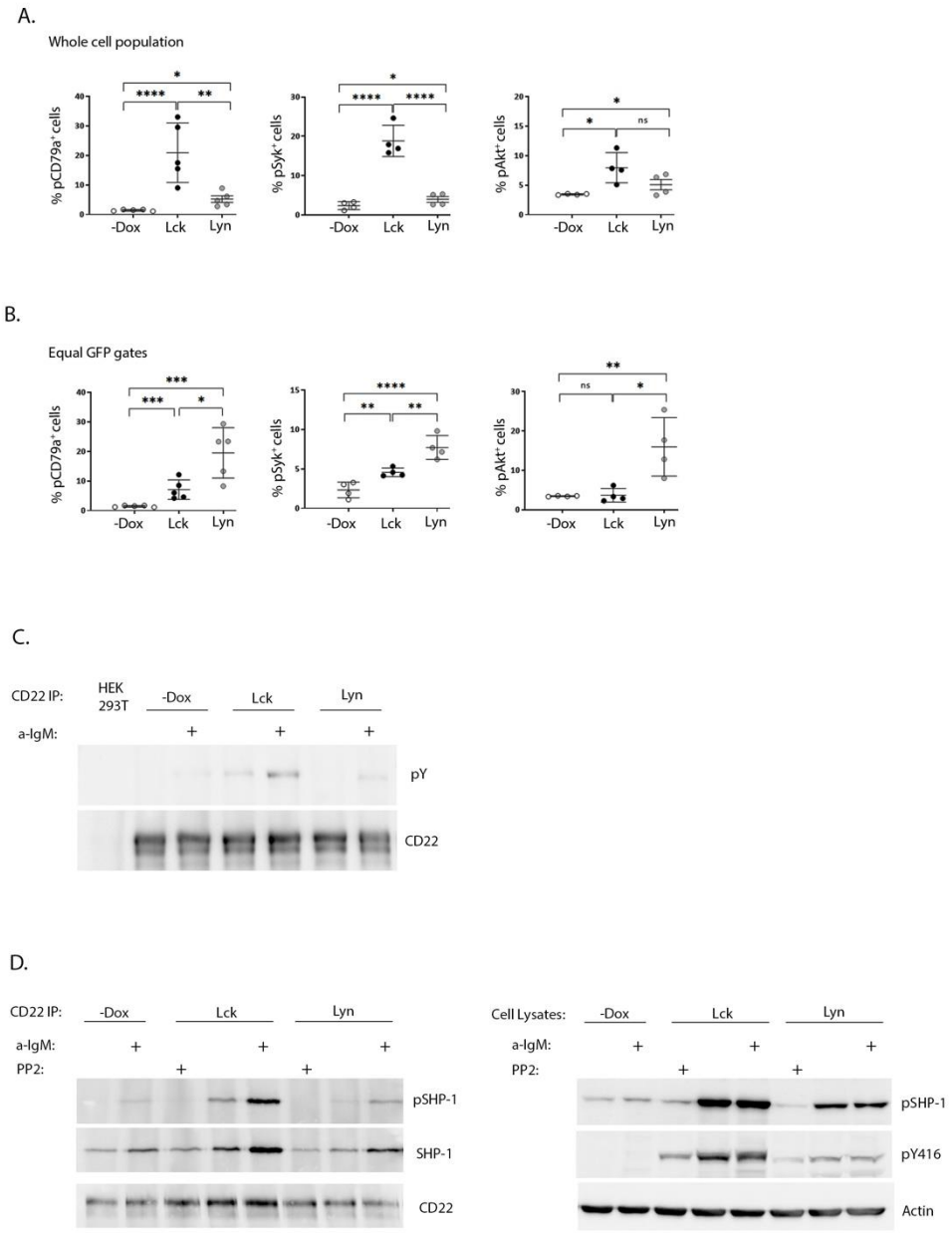

Figure 2. SFK overexpression suffices to drive ligation-independent positive and negative signaling pathways

A. Lck-expressing cells have more robust signaling responses on a whole cell population basis

Resting Lck- or Lyn-expressing cells were stained for pCD79a, pSyk (Y525/526) and pAKT (Ser473) and analysed by FACS. Graphs display the frequency of cells double-positive for GFP and pCD79a, pSyk and pAKT respectively (Gating as in Figure 1A, % of cells in the upper right quadrants) (n≥4, Unpaired Student t test; mean +/- standard deviation [SD]; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns: not significant).

B. Lyn is more efficient than Lck in igniting phosphporylation of BCR signaling mediators on an equal protein expression level

Same as in A, but for equivalent levels of SFK expression (Fig. S3E for description of equal GFP gating strategy).

C. Enhanced CD22 tyrosine phosphorylation in response to SFK overexpression

CD22 was immunoprecipitated from the Lck- and Lyn-BJAB cell lines and their -Dox counterparts in the absence or presence of stimulation with 10 µg/mL a-IgM for 10min. IPs were analysed by western blotting with a-pY 4G10 (upper panel) and a-CD22 (lower panel) antibodies. HEK293T cells (devoid of CD22 expression) were used as a negative control for the IPs.

D. SFK overexpression ignites the CD22/SHP-1 inhibitory pathway and this depends on their intact kinase activity

Left-hand panels. CD22 IPs as in C, but with additional samples treated for 10 min with 100 µM of the pan-SFK inhibitor PP2, as indicated. IPs were analysed by western blotting with a-pSHP-1 Y564 (upper panel), total a-SHP-1 (middle panel) and a-CD22 (lower panel) antibodies.

Right-hand panels. Total cell lysates from the same experiment, analysed by western blotting with a-pSHP-1 Y564 (upper panel) and a-pY416 (middle panel) to verify the PP2-induced reduction in SFK activity. Actin blot (lower panel) attests equal sample loading.

Shown are representative blots of two independent experiments.

Fig.3

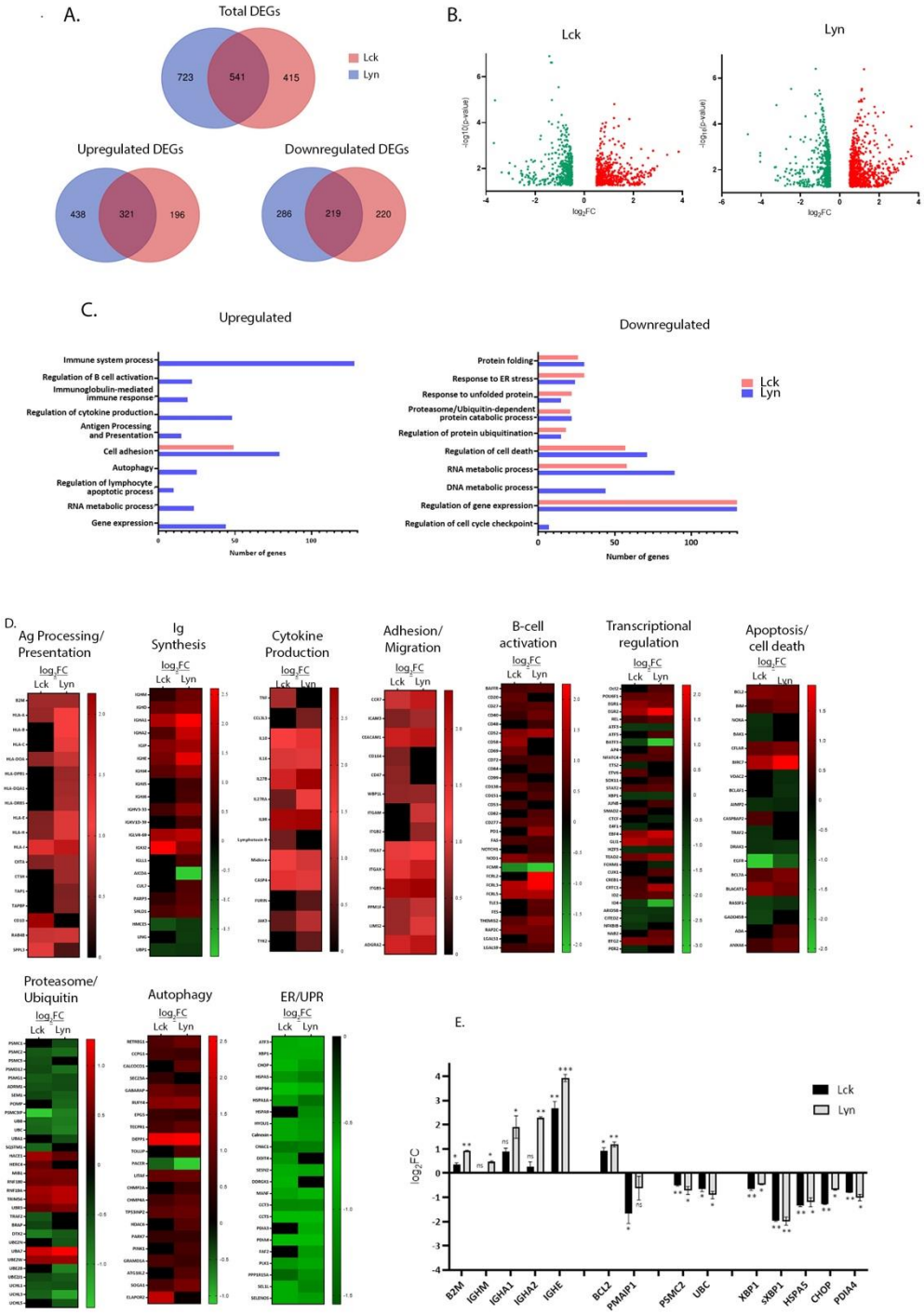

Figure 3. SFK overexpression suffices to change the gene expression landscape of unstimulated BJAB cells

A. Lck and Lyn alter the expression profile of common and unique genes

Venn diagram representation of the RNAseq data from Lck- (pink) and Lyn- (blue) expressing cells. Top panel, number of total differentially expressed genes (DEGs). Lower panels, number of upregulated (left) and downregulated (right) DEGs. The numbers in the common intersecting areas designate overlapping DEGs.

Thresholds for DEG selection: log_2 fold change (log_2FC) >0.5 and <-0.5 for upregulated and downregulated genes, respectively and adjusted p-value <0.05 (calculated using limma).

B. Distribution of upregulated versus downregulated DEGs in SFK overexpressing cells

Volcano plots of DEGs from Lck- (left) and Lyn-expressing cells (right). The graphs present the relationship between log_2FC, and statistical significance ($-log_{10}$(p-values)), using a scatter plot view.

C, and D. Lck and Lyn modulate DEG signatures assigned to the regulation of several biological processes

C. Graphical representation of Gene Ontology (GO) enrichment analysis of upregulated (left) and downregulated (right) DEGs in Lck-BJAB (pink) and Lyn-BJAB (blue) cells. The bar graphs illustrate the most representative enriched (FDR (false discovery rate) < 0.05) biological function classes and the number of genes corresponding to each GO term. **D.** Heatmaps illustrating transcript abundance (log_2FC) for DEG sets involved in the indicated biological processes. Downregulated genes are marked green and upregulate red. Black denotes genes that are not differentially expressed. (n = 2 for each cell line).

E. RT-qPCR based validation of representative RNA-seq derived DEGs.

The y-axis shows the log_2FC of the gene expression between Lck-BJAB (black)/-Dox and Lyn-BJAB (grey)/-Dox (n=2, Multiple unpaired Student t test; mean +/- SD; *P < 0.05, **P < 0.01, ***P < 0.001; ns: not significant).

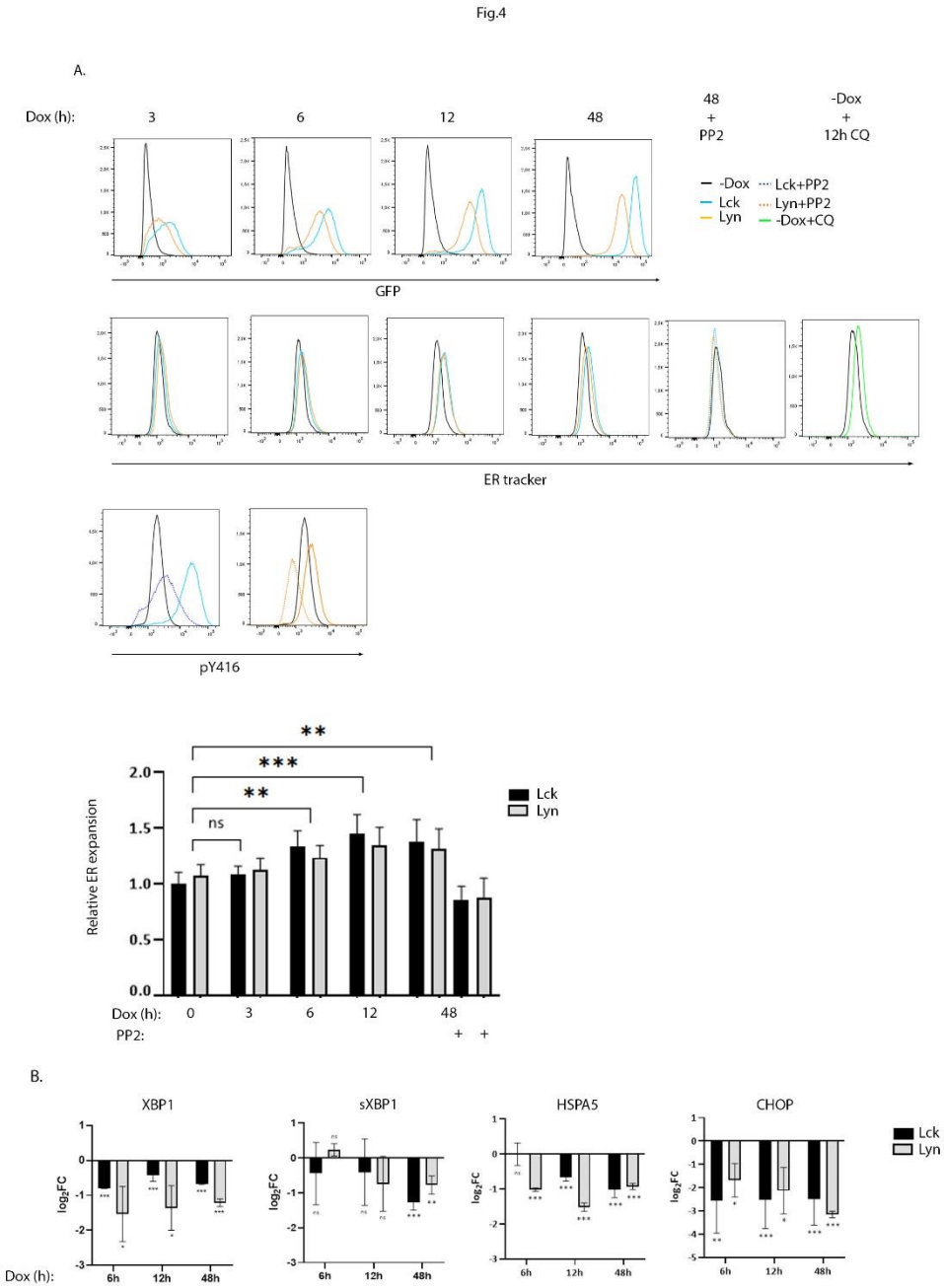

Figure 4. Role of SFKs in the regulation of ER homeostasis

A. SFK overexpression suffices to induce ER expansion in an enzymatic activity-related fashion. Lck- or Lyn-expressing cells and the -Dox counterparts were stained with ER tracker Blue-White DPX at the indicated time points after Dox addition to the cell cultures and analysed by FACS. ER expansion measured by increases of ER tracker dye fluorescence (lower panel histograms) is correlated to increases in SFK protein levels (GFP fluorescence, upper panel histograms). 18h incubation with the pan SFK inhibitor PP2 reduced ER expansion to baseline (-Dox) levels. The correspondent reduction of pY416 after PP2 incubation for each SFK, is shown in the bottom-panel histograms. Treatment with 50 µM of the ER-stress inducer Chloroquine (CQ) for 12h was used a positive control for the expected function of the ER-tracker reagent. Graph shows the ratio of ER tracker dye MFI from Lck- or Lyn-expressing cells to -Dox treated cells. Since essentially there was no difference

between the Lck and Lyn samples, statistics were calculated for the mean values between each time point (n=4, Unpaired Student t test; mean +/- SD; **P < 0.01, ***P < 0.001; ns: not significant).

B. SFK-driven ER expansion is not concurrent with upregulation of UPR mediators

RT-qPCR of the indicated UPR modulators. Each time point corresponds to samples stained with ER tracker shown in A. RT-qPCR data are shown log_2FC of the gene expression between Lck-BJAB (black)/-Dox and Lyn-BJAB (grey)/-Dox (n=2, Multiple unpaired Student t test; mean +/- SD; *P < 0.05, **P < 0.01, ***P < 0.001; ns: not significant).