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Rac1/p21-activated kinase pathway controls retinoblastoma protein phosphorylation and E2F transcription factor activation in B lymphocytes

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Small GTPases of the Ras superfamily are capable of activating E2F-dependent transcription leading to cell proliferation, but the molecular mechanisms are poorly understood. In this study, using immortalized chicken DT40 B cell lines to investigate the role of the Vav/Rac signalling cascade on B cell proliferation, it is shown that the proliferative response triggered by B cell receptor activation is dramatically reduced in the absence of Vav3 expression. Analysis of this proliferative defect shows that in the absence of Vav3 expression, retinoblastoma protein (RB) phosphorylation and the subsequent E2F activation do not take place. By combining pharmacological and genetic approaches, phosphatidylinositol-3-kinase and phospholipase Cγ2 (PLCγ2) were identified as the key regulatory signalling molecules upstream of the Vav3/Rac pathway leading to RB phosphorylation and E2F transcription factor activation. Additionally, *vav3*$^{-/-}$ and *plcγ2*$^{-/-}$ DT40 B cells were not able to activate the RB–E2F complex wild-type phenotype when these genetically modified cells were transfected with constitutively active forms of RhoA or Cdc42. However, when these knockout cells were transfected with different constitutively active versions of PLCγ, Vav or Rac1, not only activation of the RB–E2F complex wild-type phenotype was recovered but also the cellular proliferation. Furthermore, by evaluating the effect of two known effector mutants of Rac1 (Rac1$^{Q61L/F37A}$ and Rac1$^{Q61L/Y40C}$), the RB–E2F complex activation dependency on p21-activated kinase (PAK) and protein kinase Cε (PKCε) activities was established, being independent of both actin cytoskeleton reorganization and Ras activity. These results suggest that PAK1 and PKCε may be potential therapeutic targets to stop uncontrolled B cell proliferation mediated by the Vav/Rac pathway.

Abbreviations
BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester); BCR, B cell receptor; CDK, cyclin-dependent kinase; DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; PAK, p21-activated kinase; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PKD, protein kinase D; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; RasGRP, RAS guanyl releasing protein; RB, retinoblastoma protein.

Introduction

T and B lymphocytes are particularly interesting cell types for the study of intracellular signalling events leading to DNA synthesis and cell proliferation. In B cells, cellular proliferation is stimulated by the activation of its specific B cell receptor (BCR). Upon BCR activation the intracellular immune receptor tyrosine-based activation motifs (ITAMs), located in the cytosolic domains of the antigen-binding transmembrane Igα–Igβ heterodimer (IgM), are rapidly phosphorylated [1]. This molecular structure and its potential to establish intermolecular interactions result in a complex and flexible platform for both recognition and processing information as well as the transduction of signals to the cell cytosol and the nucleus.

B lymphocytes may be maintained in a quiescent state and stimulation of BCR leads to potent activation of signalling molecules, such as the small GTPases of the Ras superfamily [2–5] mediating the activation of intracellular signalling cascades [2–9]. Briefly, BCR stimulates the activity of both the Syk [10,11] and the Src family of protein tyrosine kinases [12] leading to the activation of the B cell signalosome, which includes the adapter protein BLNK (B cell linker-protein), Bruton's tyrosine kinase (BTK) and phospholipase Cγ2 (PLCγ2) [13,14]. PLCγ2 in its active state, hydrolyses the membrane component phosphatidylinositol-4,5-bisphosphate (PIP_2), resulting in the second messengers inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 receptors in specialized areas of the endoplasmic reticulum triggering Ca^{2+} release [15], while DAG remains attached to the inner region of the plasma membrane and serves to recruit and contribute to activating the cytosolic protein kinase C (PKC) [16]. Additionally, DAG cooperates with the Vav/Rac pathway in order to facilitate the localization of RAS guanyl releasing protein (RasGRP) (guanine nucleotide exchange factors (GEFs) for Ras) family members into actin juxtamembrane structures, thereby facilitating Ras activation [2,17].

The Ras GTPase, in addition to participating in the establishment of memory B cells in response to antigen-dependent lymphocyte activation [18,19], also contributes to promoting cell proliferation in many cell types. Ras switches on a gene transcription programme that induces the expression of early genes such as *myc* and *fos* that cooperate with the MAP kinase pathway to promote progression through the G1 phase of the cell cycle [6]. Similar to Ras, small GTPases of the Rho family also contribute to promoting progression through the G1 phase of the cell cycle. But differently from Ras, Rho GTPases inhibit the expression of the cell cycle negative regulator p21 on the one hand, and on the other, stimulate the expression of the cyclin D1 and RB hyperphosphorylation. In turn, these events increase the transcription mediated by E2F transcription factor family members, an essential step for DNA replication and cell proliferation [20–22].

In DT40 B and Jurkat cells, it has been established that Ras activation and the regulation of the MAP kinase pathway is controlled by Rac1 GTPase [2,3]. DT40 B cells expressing the BCR are stimulated with a specific antigen (α-IgM), and thereby initiate a complex signalling cascade that leads to concomitant cellular proliferation and differentiation [23]. In order to examine whether the BCR controls the RB–E2F pathway and cell proliferation through GTPases of the Rho family and their regulatory molecules, the DT40 B cellular system (wild-type) with its knockout variants ($vav3^{-/-}$ and $plc\gamma2^{-/-}$) were used. Here we show that BCR activation of RB hyperphosphorylation and E2F-mediated transcription and DT40 B cell proliferation depend on both PLCγ2 and Vav3 but are independent of Ras. This signal transduction pathway characterization identified PLCγ2, Vav, Rac1, PAK1 and PKCε as potential target molecules to block B lymphocyte proliferation in deregulated conditions, such as in B cell leukaemias.

Results

The Vav/Rac1 pathway regulates the disassembly of the RB–E2F complex in B cells

A functional role for Vav in the Ras/MAP kinase pathway in BCR-activated B cells has not been previously established. Therefore, this putative role of the Vav/Rac1/Ras pathway in B cell proliferation upon IgM stimulation was first investigated by comparing the proliferation between wild-type (WT) DT40 B and $vav3^{-/-}$ DT40 B cells. As shown in Fig. 1A, there was an 11-fold increase in DT40 B (WT) cell number present in each well at 96 h after starting the proliferation assay. This proliferation index was dramatically reduced from 11 to around 5 in the absence of *vav3* expression.

In order to establish the degree of phosphorylation of RB protein following BCR activation in the presence or in the absence of Vav expression, both wild-type and $vav3^{-/-}$ DT40 B cells were serum-starved for 24 h and subsequently stimulated with 10 $\mu g \cdot mL^{-1}$ IgM for 5 and 8 h. Cells were lysed and lysates were

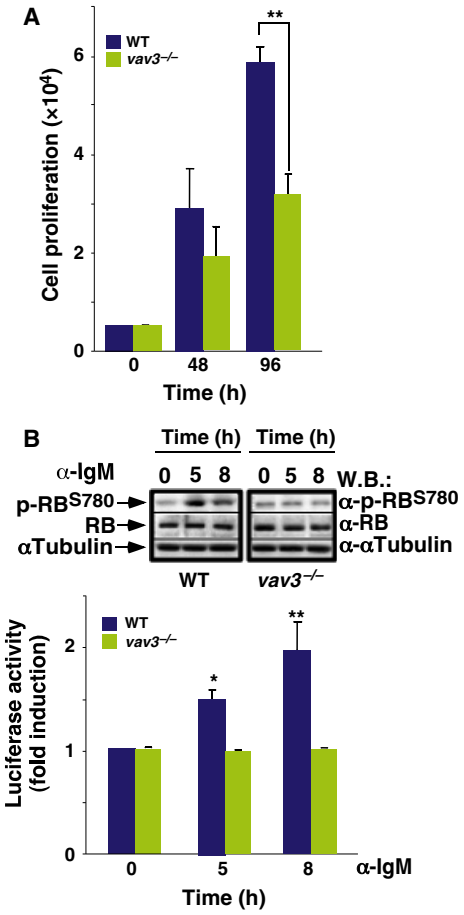

Fig. 1. Vav-dependent DT40 B cells proliferation, RB phosphorylation and E2F transcription activation. (A) DT40 (WT) and *vav*$^{3-/-}$ DT40 B cells stained with PKH26 were cultured as described in Materials and Methods. Fluorescence was analysed before α-IgM stimulation (0 h) and after every 48 h incubation with 10 μg·mL^{-1} α-IgM for two consecutive days. Results represent the mean ± SD of three independent experiments carried out with triplicates and the statistical analysis showed a significant difference (**$P < 0.001$). (B) DT40 (WT) and *vav*$^{3-/-}$ DT40 B cells were deprived for 24 h and stimulated or not with 10 μg·mL^{-1} α-IgM for 5 and 8 h and lysed, and RB phosphorylation and total RB in cell lysates were analysed by western blotting using specific antibodies as indicated. In parallel, DT40 (WT) and *vav*$^{3-/-}$ DT40 B cells were cotransfected with 100 ng reporter plasmid (pGL3-6xE2F) and 10 ng phRL-CMV plasmid (containing the cDNA encoding *Renilla* luciferase). After 36 h, transfected cells were stimulated or not with 10 μg·mL^{-1} α-IgM for 5 and 8 h and transcriptional activity was measured. Results represent the mean ± SD of the ratio Renilla/Firefly expressed as fold induction obtained from three independent experiments carried out with duplicates, and the statistical analysis showed a significant difference (*$P < 0.05$, **$P < 0.001$).

analysed by western blot. Immunoreactive bands were visualized with specific antibodies as indicated. As shown in Fig. 1B, RB phosphorylation reached its maximum after DT40 B cells (WT) were exposed to IgM for 5 h, and this phosphorylation state was maintained for up to 8 h. In contrast, *vav3*$^{-/-}$ DT40 B cells showed an absolute deficit in RB protein phosphorylation. The second panel of Fig. 1B shows total RB protein expression in whole cell lysates.

Next, the role of Vav in mediating transcriptional activation of E2F was examined. Wild-type and *vav3*$^{-/-}$ DT40 B cells were transfected with a plasmid that drives expression of the luciferase gene containing 6xE2F sites in its promoter (pGL3-6xE2F). After 24 h, cells were serum-starved for an additional 24 h and subsequently stimulated with 10 μg·mL^{-1} IgM for 5 and 8 h, and then the transcriptional activity was measured. As shown in Fig. 1B, while DT40 B cells (WT) stimulated with IgM exhibited a time-dependent progressive increase in E2F transcriptional activity, *vav3*$^{-/-}$ DT40 B stimulated with IgM cells exhibited no activity.

To confirm that this deficiency in RB phosphorylation and the subsequent lack of E2F transcriptional activation was due to the absence of Vav3 expression, reconstitution of Vav in the *vav3*$^{-/-}$ DT40 B cell line was achieved both by transfecting Vav (WT) and the constitutively active form of Vav (oncoVav or Vav Δ1–66) or as a negative control the empty vector (mock control) and cotransfecting these plasmids with the plasmid encoding the luciferase reporter gene containing 6xE2F sites in its promoter. Cell cultures were then further processed as described above. Vav (WT) overexpression not only rescued RB phosphorylation, but also E2F transcriptional activity (Fig. 2A), being much more robust when Vav Δ1–66 was overexpressed (Fig. 2A).

Next, the involvement of small GTPases of the Rho family as positive regulators of RB–E2F complex activation was examined. To this end, *vav3*$^{-/-}$ DT40 B cells were transfected with the constitutively active forms of RhoA (RhoA^{Q63L}), Rac1 (Rac1^{Q61L}), or Cdc42 (Cdc42^{Q61L}), or with empty vector (mock control). And these plasmids were cotransfected with the plasmid encoding the luciferase reporter gene containing 6xE2F sites in its promoter. As expected, IgM stimulation of DT40 B cells (WT) was able to induce both RB phosphorylation and E2F transcriptional activation whereas IgM stimulation of *vav3*$^{-/-}$ DT40 B cells was not (Fig. 2B). RhoA^{Q63L} overexpression did not restore either RB phosphorylation or E2F-mediated transcriptional activity (Fig. 2B). Although Cdc42^{Q61L} partially restored RB phosphorylation (Fig. 2B), this effect did not translate into any E2F transcriptional activity (Fig. 2B). In contrast, as occurred with Vav overexpression, Rac1^{Q61L} fully restored both RB phosphorylation and E2F-mediated

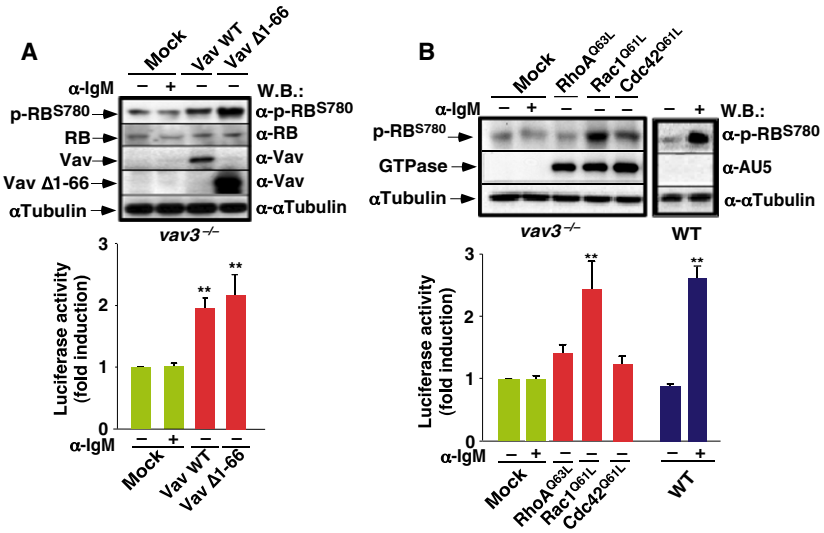

Fig. 2. Overexpression of Vav or Rac rescues both RB phosphorylation and E2F transcriptional activation. (A, B) *vav3*$^{-/-}$ DT40 B cells were transfected with empty vector (mock control) or with plasmids encoding Vav (WT), constitutively active forms of Vav (Vav Δ1–66), AU5-tagged RhoA (RhoA^{Q63L}), AU5-tagged Rac1 (Rac1^{Q61L}) and AU5-tagged Cdc42 (Cdc42^{Q61L}) and cotransfected with pGL3-6xE2F and phRL-CMV. After 36 h, transfected cells were stimulated or not with 10 μg·mL^{-1} α-IgM for 5 h and RB phosphorylation, total RB and protein expression levels were analysed by western blotting using specific antibodies as indicated. DT40 B cells (WT) were used as a positive control. Results are representative of four independent experiments. Bar graphs represent the mean ± SD of the ratio Renilla/Firefly expressed as fold induction obtained from five independent experiments carried out with duplicates and the statistical analysis showed a significant difference (*$P < 0.05$, **$P < 0.001$).

transcriptional activity compared with IgM-stimulated DT40 B cells (WT) (Fig. 2B).

Rac1 pathway controls the disassembly of the RB–E2F complex in B cells through PAK1

Given that Rac1 can activate several signalling cascades simultaneously [24], a putative intermediary effector pathway leading to the disassembly of the RB–E2F complex was investigated. In order to address this issue, the experimental procedure consisted of using two constitutively active Rac1 effector mutants (Rac1$^{F37A/Q61L}$ and Rac1$^{Y40C/Q61L}$) that discriminate between different signalling routes. Rac1$^{F37A/Q61L}$ is able to activate PAK and JNK but does not signal to the cytoskeleton, and it is not involved in cellular transformation processes [25,26]. On the other hand, Rac1$^{Y40C/Q61L}$ is involved in the control of cytoskeletal changes and tumorigenesis without mediating the activation of PAK or JNK kinases [25,26]. This control over the cytoskeleton by Rac1$^{Y40C/Q61L}$ promotes also RasGRP translocation and H-Ras activation. In contrast, Rac1$^{F37A/Q61L}$ is significantly less efficient in both responses [2].

Accordingly, *vav3*$^{-/-}$ DT40 B cells were transfected with plasmids encoding Rac1^{Q61L}, Rac1$^{F37A/Q61L}$, or Rac1$^{Y40C/Q61L}$, or empty vector (mock control). And these plasmids were also cotransfected with the luciferase reporter gene containing 6xE2F sites in its promoter. As shown in Fig. 3A, IgM-stimulated *vav3*$^{-/-}$ DT40 B cells did not show either RB phosphorylation or E2F transcriptional activation. Overexpression of the constitutively active form of Rac1 (Rac1^{Q61L}) induced strong RB phosphorylation as well as a robust increase in E2F transcriptional activity. This result was similar to that obtained with the overexpression of Rac1$^{F37A/Q61L}$ mutant. In sharp contrast, Rac1$^{Y40C/Q61L}$ overexpresion was unable to induce either RB phosphorylation or E2F transcriptional activation (Fig. 3A). In order to verify that this Rac1$^{Y40C/Q61L}$ mutant was active, the phosphorylation state of ERK1 in *vav3*$^{-/-}$ DT40 B cells overexpressing Rac1$^{Y40C/Q61L}$ was investigated. To determine the phosphorylation state of ERK1 induced by Rac1$^{Y40C/Q61L}$ mutant is an indirect manner to measure the activation of Ras [2]. As shown in Fig. 3A, this Rac1 mutant induced ERK phosphorylation to an extent similar to that obtained by overexpressing constitutively active Rac1. Furthermore, although Rac1$^{F37A/Q61L}$ restored ERK phosphorylation, this was less robust than ERK phophorylation induced by either Rac1^{Q61L} or Rac1$^{Y40C/Q61L}$ overexpression. A similar pattern is achieved when Ras activation is measured when these Rac1 effector mutants are overexpressed in *vav3*$^{-/-}$ DT40 B cells [2]. This suggests that this weak ERK phosphorylation depends

directly on a serine–threonine kinase activity. In fact, when Rac1 mediated PAK serine-threonine kinase phosphorylation state, as an indicator of its activation, was examined both constitutively active Rac1 as well as $Rac1^{F37A/Q61L}$ induced robust PAK phosphorylation. In contrast, $Rac1^{Y40C/Q61L}$ was unable to significantly restore PAK phosphorylation as compared to either $Rac1^{Q61L}$ or $Rac1^{F37A/Q61L}$ overexpressing cells (Fig. 3A). These results strongly suggest that the putative Rac1 effector pathway responsible for the disruption of the RB–E2F complex is mediated by PAK1 and ERK independently of Ras.

In order to examine this possibility, *vav3*$^{-/-}$ DT40 B cells were transfected with plasmids encoding PAK1 wild-type (WT) and $PAK1^{T423E}$ (constitutively active form of PAK1) or empty vector (mock control). And these plasmids were cotransfected with the luciferase reporter gene containing 6xE2F sites in its promoter. Overexpression of either PAK1 (WT) or $PAK1^{T423E}$ induced both RB phosphorylation and E2F transcriptional activation as well as ERK1 phosphorylation, as compared with control cells (Fig. 3B).

Signalling molecules upstream of Vav/Rac regulate RB phosphorylation and E2F transcriptional activation in B cells

Considering that Inabe and Kurosaki described that the *vav3*$^{-/-}$ DT40 B cell line showed deficient activation of PLCγ2, PI3K and Ca^{2+} release when BCR was activated [27], here it was investigated whether the absence of PLCγ2 expression in IgM-stimulated DT40 B affected RB phosphorylation. To this end, *plcγ2*$^{-/-}$ DT40 B cells were transfected with a plasmid encoding a constitutively active form of PLCγ1 (PLCγ1-palm) or empty vector (mock control). After 24 h, cells were serum-deprived for an additional 24 h and finally cells were stimulated or not with IgM for 5 h. Cells were lysed and the lysates analysed by western blot. Immunoreactive bands were visualized using specific

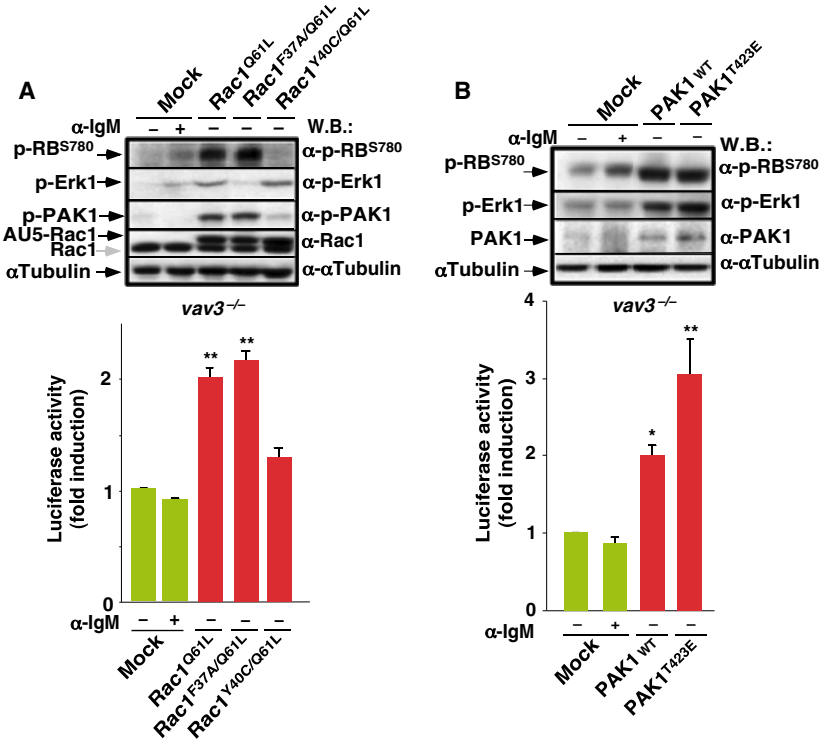

Fig. 3. PAK1-dependent RB phosphorylation and E2F transcriptional activation in DT40 B cells. (A) *vav3*$^{-/-}$ DT40B cells were transfected with empty vector (mock control) (A, B) or with plasmids encoding $Rac1^{Q61L}$, $Rac1^{F37A/61L}$ and $Rac1^{Y40C/61L}$ (A), PAK1 (WT) or constitutively active PAK1 ($PAK1^{T423E}$) (B) and cotransfected with pGL3-6xE2F and phRL-CMV to measure transcriptional activity. After 36 h, transfected cells were stimulated or not with 10 μg·mL^{-1} α-IgM for 5 h and RB, ERK1, PAK1 phosphorylation, and protein expression levels of Rac1 [black arrow (Rac1 ectopic expression) and grey arrow (Rac1 endogenous expression)], PAK1 and α-tubulin were analysed by western blotting using specific antibodies as indicated. Results are representatives of three independent experiments. Bar graphs represent the mean ± SD of the ratio Renilla/Firefly expressed as fold induction obtained from four independent experiments carried out with duplicates and the statistical analysis showed a significant difference (*$P < 0.05$, **$P < 0.001$).

antibodies as indicated. As shown in Fig. 4A, in the absence of PLCγ2 expression, BCR activation stimulated by IgM was unable to induce RB phosphorylation. However, when *plcγ2*$^{-/-}$ DT40 B cells ectopically overexpressed a constitutively active form of PLCγ1, these transiently transfected cells restored the capacity of IgM to stimulate RB phosphorylation (Fig. 4A). In addition, it was examined whether Rac1 activation induced by IgM was dependent on PLCγ expression. *plcγ2*$^{-/-}$ DT40 B cells were cotransfected with a

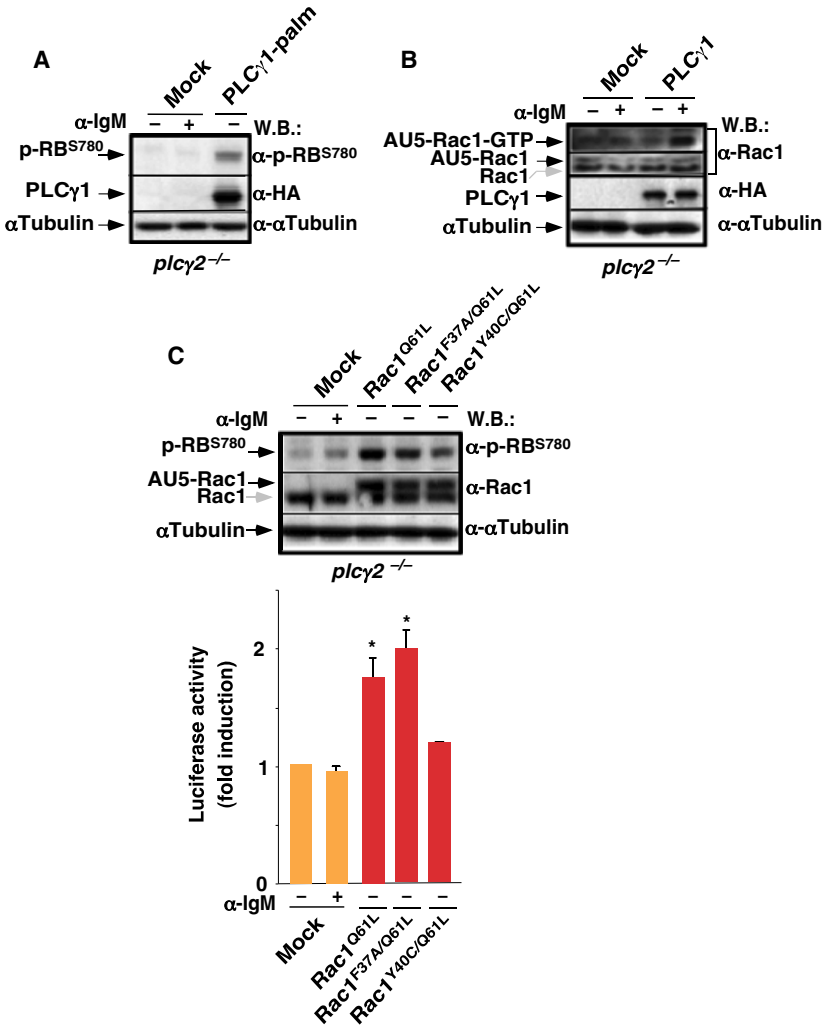

Fig. 4. PLCγ-dependent RB phosphorylation and E2F transcriptional activation in DT40 B cells. (A) *plcγ2*$^{-/-}$ DT40 B cells were transfected with empty vector (mock control) or with a plasmid encoding the constitutively active form of PLCγ1 (PLCγ1-palm). After 36 h, transfected cells were stimulated or not with 10 μg·mL^{-1} αIgM for 5 h and RB phosphorylation, PLCγ1 expression level, and α-tubulin were analysed by western blotting using specific antibodies as indicated. Results are representatives of three independent experiments. (B) *plcγ2*$^{-/-}$ DT40 B cells were transfected with empty vector (mock control) or with a plasmid encoding PLCγ1. After 36 h, transfected cells were stimulated or not with 10 μg·mL^{-1} αIgM for 10 min and lysed. Cell lysates were used to measure Rac1 activation by pull-down assay. Precipitated active [Rac1-GTP), total Rac1 (black arrow (Rac1 ectopic expression) and grey arrow (Rac1 endogenous expression)] and PLCγ1 expression levels were analysed by western blotting using anti-Rac1 and anti-HA antibodies, respectively. Results are representative of three independent experiments. (C) *plcγ2*$^{-/-}$ DT40 B cells were transfected with empty vector (mock control) or with plasmids encoding Rac 1^{Q61L}, Rac1$^{F37A/61L}$ and Rac1$^{Y40C/61L}$, or cotransfected with these plasmids and pGL3-6xE2F and phRL-CMV to measure transcriptional activity. After 36 h, transfected cells were stimulated or not with 10 μg·mL^{-1} αIgM for 5 h and RB, phosphorylation, and protein expression levels of Rac1 [black arrow (Rac1 ectopic expression) and grey arrow (Rac1 endogenous expression)] and α-tubulin were analysed by western blotting using specific antibodies as indicated. Results are representatives of four independent experiments. Bar graph represents the mean ± SD of the ratio Renilla/Firefly expressed as fold induction obtained from four independent experiments carried out with duplicates and the statistical analysis showed a significant difference (*$P < 0.05$).

plasmid encoding Rac1 and a plasmid encoding PLCγ1 (WT) or empty vector (mock control). After 24 h, cells were serum-deprived for an additional 24 h and finally cells were stimulated or not with IgM for 10 min. The Rac1 activated state was measured by the pull-down assay, as decribed in Materials and Methods. As shown in Fig. 4B, in the absence of PLCγ expression, IgM was unable to induce Rac1 activation. However, transient transfection of PLCγ1 (WT) in *plcγ2*$^{-/-}$ DT40 B cells restored Rac1 activation after IgM stimulation. Figure 4B also shows PLCγ1 expression level as determined by western blotting and total Rac1 shows that equivalent protein amounts were loaded in all lanes.

As shown previously in Fig. 3A, Rac1$^{F37A/Q61L}$ overexpression in *vav3*$^{-/-}$ DT40 B cells restored both RB phosphorylation and E2F transcriptional activation. In line with these results, it was also investigated whether the constitutively active form of Rac1 was able to rescue both RB phosphorylation and E2F transcriptional activation in *plcγ2*$^{-/-}$ DT40 B cells. To this end, *plcγ2*$^{-/-}$ DT40 B cells were transfected with plasmids encoding Rac1^{Q61L}, Rac1$^{F37A/Q61L}$, or Rac1$^{Y40C/Q61L}$, or empty vector (mock control). And these plasmids were also cotransfected with the luciferase reporter gene containing 6xE2F sites in its promoter. As shown in Fig. 4C, IgM-stimulated *plcγ2*$^{-/-}$ DT40 B cells did not induce either RB phosphorylation or E2F transcriptional activation. Overexpression of the constitutively active form of Rac1 (Rac 1^{Q61L}) exhibited strong RB phosphorylation as well as a significant increase in E2F transcriptional activity. This result is similar to that obtained with overexpression of the Rac1$^{F37A/Q61L}$ mutant. However, Rac1$^{Y40C/Q61L}$ mutant overexpression was not able to rescue either RB phosphorylation or E2F transcriptional activity (Fig. 4C).

Given that *vav3*$^{-/-}$ DT40 B cells show both deficient PI3K activation and impairment in intracellular Ca^{2+} release in response to BCR activation [27], the roles of PI3K and of PKCs on RB phosphorylation in our cellular model were examined. DT40 B cells (WT) were serum-starved for 24 h and then pretreated or not with

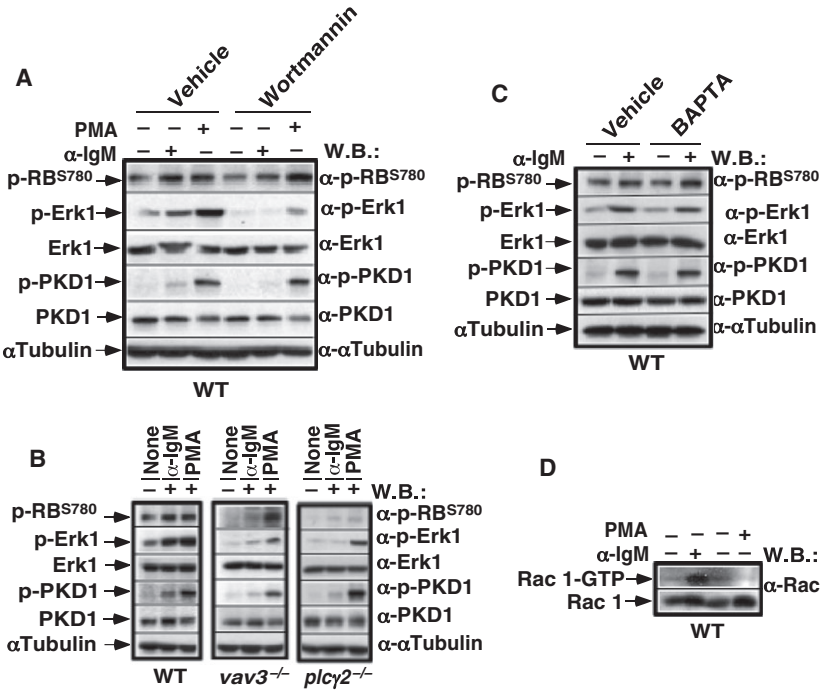

Fig. 5. Role of PI3K and PKCs on RB phosphorylation in DT40 B cells. (A) Serum-deprived DT40 B cells (WT) for 24 h were pretreated with vehicle or 100 nM Wortmannin for 1 h and unstimulated (–) or stimulated (+) with 10 μg·mL^{-1} αIgM or 1 μM PMA for 5 h. (B) Serum-deprived WT, *vav3*$^{-/-}$ and *plcγ2*$^{-/-}$ DT40 B cells for 24 h were unstimulated (–) or stimulated (+) with 10 μg·mL^{-1} αIgM or 1 μM PMA for 5 h. (C) Serum-deprived DT40 B cells for 24 h were pretreated with vehicle or 30 μM BAPTA for 1 h and unstimulated (–) or stimulated (+) with 10 μg·mL^{-1} αIgM for 5 h. RB, ERK and PKD1 phosphorylation and protein expression level of ERK, PKD1 and α-tubulin were analysed by western blotting using specific antibodies as indicated. Results are representatives of four independent experiments. (D) Serum-deprived DT40 B cells for 24 h were unstimulated (–) or stimulated (+) with 10 μg·mL^{-1} αIgM or 1 μM PMA for 10 min and lysed. Cell lysates were used to measure Rac1 activation by affinity precipitation assay. Precipitated active (Rac1-GTP) and total Rac1 expression levels were analysed by western blotting using anti-Rac1 antibody. Results are representative of three independent experiments.

100 nM wortmannin, a specific inhibitor of PI3K, for 1 h and further stimulated with 10 $\mu g \cdot mL^{-1}$ IgM or 1 μM phorbol 12-myristate 13-acetate (PMA; a natural analog of DAG that directly activates PKCs) for 5 h. Results show that wortmannin blocked IgM-stimulated RB phosphorylation (Fig. 5A). Wortmannin also blocked ERK1 and PKD1 phosphorylation (Fig. 5A). Protein kinase D (PKD) is a serine–threonine kinase that is a substrate of the protein kinase C (PKC) family members [28] and, therefore, PKD phosphorylation serves as a proxy for PKC activation [29]. On the other hand, wortmannin was not able to block PMA-stimulated phosphorylation of RB, ERK1 or PKD1 (Fig. 5A). These results not only implicate PI3K in IgM-stimulated phosphorylation of RB, ERK1 and PKD1 but also position PI3K upstream of PKCs and the MAP kinase pathway.

Next it was examined whether PKC-affected RB phosphorylation was modified in *vav3*$^{-/-}$ and *plcγ2*$^{-/-}$ DT40 B cell lines. In parallel experiments, DT40 B cells (WT) were used as controls. Cells were serum-deprived for 24 h and then stimulated or not with 10 $\mu g \cdot mL^{-1}$ IgM or 1 μM PMA for 5 h. As shown in Fig. 5B, both IgM and PMA induced RB, ERK1 and PKD1 phosphorylation in wild-type cells. In contrast, while PMA was able to stimulate phosphorylation of RB, ERK1 or PKD1 in *vav3*$^{-/-}$ DT40 B cells, IgM was not. However, in the absence of PLCγ2 expression neither IgM nor PMA was able to stimulate RB phosphorylation while PMA-stimulated phosphorylation of ERK1 and PKD1 were unaffected (Fig. 5B).

Given that intracellular Ca^{2+} release in response to BCR activation is also defective in the *vav3*$^{-/-}$ DT40 B cell line [27], it was investigated whether intracellular calcium release induced by the BCR activation was necessary to modulate RB phosphorylation. To address this issue, DT40 B cells (WT) were serum-starved for 24 h and then treated or not with 30 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA/AM) for 1 h in order to deplete intracellular Ca^{2+} stores and subsequently stimulated or not with 10 $\mu g \cdot mL^{-1}$ IgM for 5 h. As shown in Fig. 5C, BAPTA was not able to block IgM-stimulated phosphorylation of either RB, or ERK1 or PKD1.

The nPKC and Vav3/Rac1 pathways cooperate in the control of RB phosphorylation and E2F transcriptional activation in B cells

Our recent results demostrating that novel PKCs mediate Rac1 activation in neurons [29] taken together with previously described results prompted us to investigate whether PKCs mediate the signalling pathway leading to the RB–E2F complex disassembly via Rac1 activation. To this end, PMA activation of Rac1 in DT40 B cells (WT) was examined first. Cells were serum-starved for 24 h and stimulated or not with IgM or PMA for 10 min. Rac1 activation state was measured by pull-down assay, as described in Materials and methods. As shown in Fig. 5D, in contrast to IgM, PMA treatment was unable to stimulate Rac1 activation.

In order to determine whether PKCs were located downstream of Vav3/Rac1 in this signal transduction pathway leading to RB–E2F complex disassembly, *vav3*$^{-/-}$ DT40 B cells were transfected with plasmids encoding $Rac1^{Q61L}$, $Rac1^{F37A/Q61L}$, or $Rac1^{Y40C/Q61L}$, or empty vector (mock control). After 24 h, cells were serum-starved for an additional 24 h and pretreated with 1 μM GF109203X, a broad spectrum inhibitor of PKCs [30], for 1 h prior to being stimulated or not with IgM or PMA for 5 h. As expected, IgM was unable to stimulate RB phosphorylation in the abscence of Vav3 expression (Fig. 6A). Overexpression of the constitutively active forms of Rac1 ($Rac1^{Q61L}$ or $Rac1^{F37A/Q61L}$) mutants, unlike that of $Rac1^{Y40C/Q61L}$ mutant, also restored the RB phosphorylated state. Additionally, GF109203X did not affect Rac1-induced RB phosphorylation in *vav3*$^{-/-}$ DT40 B cells (Fig. 6A).

This scenario led us to investigate the role of PKCα and PKCε, as representative members of the classical (cPKCs) and novel PKC (nPKCs) subfamilies, respectively, in E2F transcriptional activation in our cellular model. *vav3*$^{-/-}$ DT40 B cells were cotransfected with plasmids encoding PKCα constitutively active mutant ($PKC\alpha^{A25E}$), PKCε wild-type (WT), PKCε inactive mutant ($PKC\varepsilon^{K437R}$), or PKCε constitutively active mutant ($PKC\varepsilon^{A159E}$), or empty vector (mock control), and these plasmids were also cotransfected with the luciferase reporter gene containing 6xE2F sites in its promoter. After 24 h, cells were serum-starved for an additional 24 h and after this time cells were stimulated or not with 10 $\mu g \cdot mL^{-1}$ IgM for 5 h and E2F transcriptional activity was measured. As shown in Fig. 6B, in the absence of Vav3 expression, BCR activation did not lead to E2F transcriptional activation. Likewise, $PKC\alpha^{A25E}$ overexpression also was unable to rescue E2F transcriptional activity. In contrast, $PKC\varepsilon^{A159E}$ was able to mediate E2F transcriptional activation upon BCR activation. Unlike $PKC\varepsilon^{A159E}$, PKCε (WT) and its inactive mutant ($PKC\varepsilon^{K437R}$) were unable to mediate E2F transcriptional activation (Fig. 6B).

In order to verify the involvement of PKCε on this E2F transcriptional activation, DT40 B cells (WT) transfected with the luciferase reporter gene containing 6xE2F sites in its promoter (pGL3-6xE2F) and with

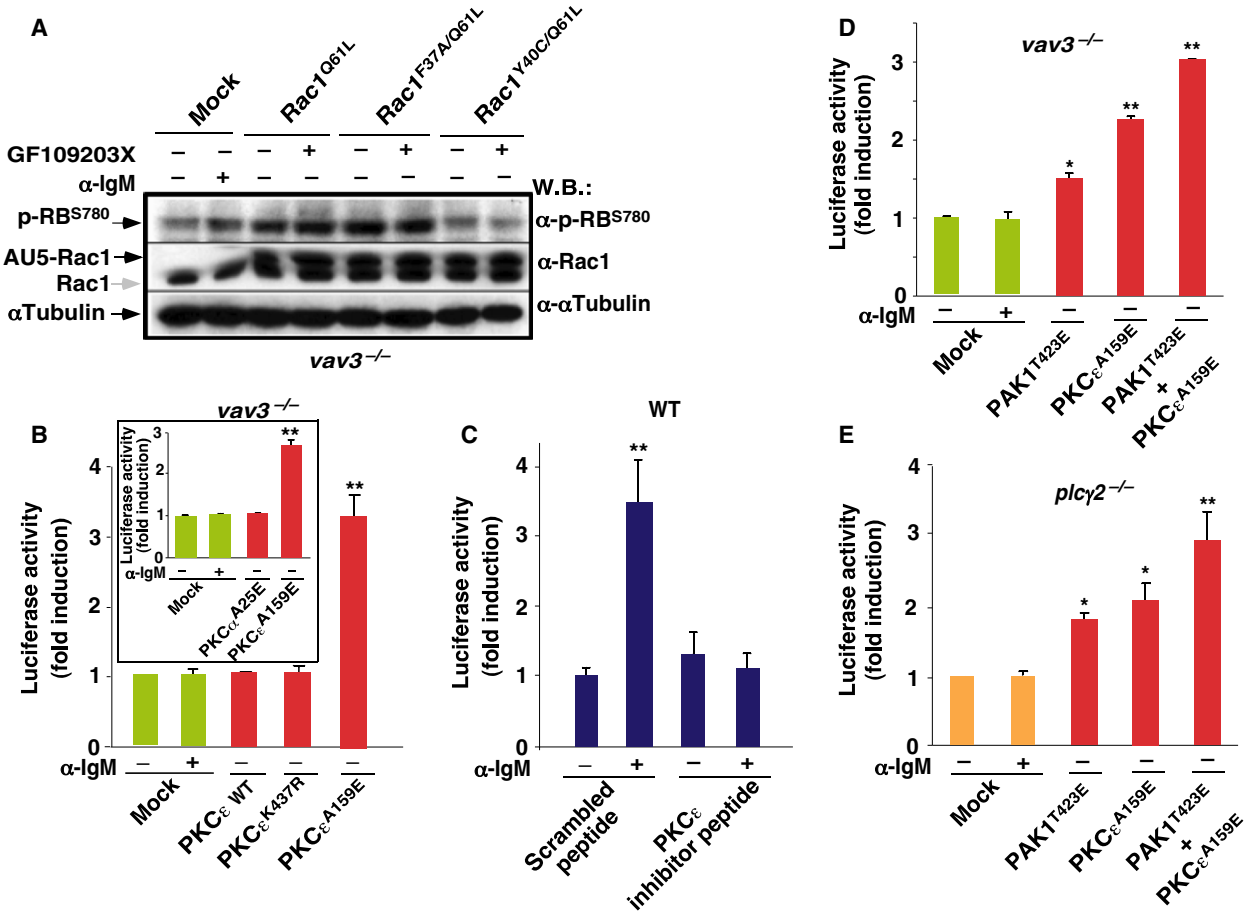

Fig. 6. PAK1 and PKCε pathways cooperate to activate E2F transcription. (A) $vav^{3-/-}$ DT40 B cells were transfected with empty vector (mock control) or with plasmids encoding $Rac1^{Q61L}$, $Rac1^{F37A/61L}$, and $Rac1^{Y40C/61L}$. After 36 h, transfected cells were pretreated or not with the PKC inhibitor GF109203X (1 μM) for 1 h and stimulated or not with 10 μg·mL^{-1} αIgM for 5 h. RB phosphorylation, Rac1 and α-tubulin expression levels were analysed by western blotting using specific antibodies as indicated. Results are representatives of three independent experiments. (B) $vav^{3-/-}$ DT40 B cells were transfected with empty vector (mock control) or with plasmids encoding $PKC\alpha^{A25E}$ (constitutively active), different forms of PKCε, PKCε (WT), $PKC\varepsilon^{K437R}$ (dominant negative) and $PKC\varepsilon^{A159E}$ (constitutively active), $PAK1^{T423E}$ (constitutively active) and with pGL3-6xE2F and phRL-CMV to measure transcriptional activity. After 36 h, transfected cells were stimulated or not with 10 μg·mL^{-1} αIgM for 5 h and transcriptional activity was measured. Results represent the mean ± SD of the ratio Renilla/Firefly expressed as fold induction obtained from four independent experiments carried out with duplicates and the statistical analysis showed a significant difference (*$P < 0.05$, **$P < 0.001$). (C) DT40 B cells (WT) were transfected with pGL3-6xE2F and phRL-CMV and after 46 h were transfected with PKCε inhibitor peptide or scrambled peptide (as negative control). After 2 h, treated cells were stimulated or not with 10 μg·mL^{-1} α-IgM for 5 h and transcriptional activity was measured. Results represent the mean ± SD of the ratio Renilla/Firefly expressed as fold induction obtained from two independent experiments carried out with duplicates and the statistical analysis showed a significant difference (**$P < 0.001$). (D, E) $vav^{3-/-}$ DT40 B cells (D) and $plc\gamma2^{-/-}$ DT40 B cells (E) were transfected with empty vector (mock control) or with plasmids encoding $PKC\varepsilon^{A159E}$ (constitutively active), or $PAK1^{T423E}$ (constitutively active), or by transfecting both ($PAK1^{T423E}$ + $PKC\varepsilon^{A159E}$), together with pGL3-6xE2F and phRL-CMV to measure transcriptional activity. After 36 h, transfected cells were stimulated or not with 10 μg·mL^{-1} α-IgM for 5 h and transcriptional activity was measured. Results represent the mean ± SD of the ratio Renila/Firefly expressed as fold induction obtained from four independent experiments carried out in duplicates and the statistical analysis showed a significant difference (*$P < 0.05$, **$P < 0.001$).

PKCε translocation inhibitor peptide (EAVSLKPT), which functions as a selective and reversible inhibitor of PKCε translocation to intracellular membranes, and consequently blocks its activation [31], or scrambled peptide as the negative control (LSETKPAN). Two hours later, cells were stimulated or not with 10 μg·mL^{-1} IgM for 5 h and E2F transcriptional activity was measured. As shown in Fig. 6C, PKCε inhibitor peptide blocked E2F transcriptional activation stimulated by IgM. These results suggest that PKCε

may also control the E2F transcriptional activation in haematopoietic B cells.

Next, the possibility that Vav/Rac and PKCε pathways could be synergistically mediating the disassembly of the RB–E2F complex was examined. To test this hypothesis *vav3*$^{-/-}$ DT40 B cells were cotransfected with plasmids encoding the constitutively active mutant of PAK1 (PAK1^{T423E}), constitutively active mutant PKCε (PKCε^{A159E}) or empty vectors (mock control), and these plasmids were also cotransfected with the luciferase reporter gene containing 6xE2F sites in its promoter. After 24 h, cells were serum-starved for an additional 24 h and stimulated or not with 10 μg·mL^{-1} IgM for 5 h, and subsequently E2F transcriptional activity was measured.

As expected, BCR activation in the absence of expression of Vav3 did not modify E2F transcriptional activity (Fig. 6D). However, the individual overexpression of both the constitutively active form of PAK1 as well as of PKCε led to E2F transcriptional activation. This transcriptional activity response was potentiated when both active forms were cooverexpressed (Fig. 6D). Equivalent results were obtained when the same assay was performed on *plcγ2*$^{-/-}$ DT40 B cells (Fig. 6E). Taken together, these results suggest that the Vav/Rac1/PAK1 and PKCε pathways may synergize in order to disassemble the RB–E2F complex and to subsenquently promote the cellular response.

Vav and Rac1 overexpression rescue *vav3*$^{-/-}$ DT40 B cell proliferation

Once it was identified that the lack of Vav expression in DT40 B cells was directly related to a deficit in the phosphorylation of RB and E2F transcriptional activity as well as in cell proliferation, cell proliferation of Vav and Rac overexpressing *vav3*$^{-/-}$ DT40 B cells stimulated by IgM was examined. Cell proliferation was analysed after flow cytometry by monitoring the decrease in fluorescence of the incorporated membrane dye PKH26, which is diluted approximately twofold with each cell division. PKH26-labeled cells were stimulated with IgM every 24 h for 4 days. In DT40 B cells (WT) cultured for 4 days, IgM stimulation resulted in an approximately 2.3-fold increase in cell number (Fig. 7, first bar) when compared with IgM-stimulated *vav3*$^{-/-}$ DT40 B cells (Fig. 7, second bar). Remarkably, the IgM-stimulated proliferation capacity of Vav or Rac1^{Q61L} overexpressing *vav3*$^{-/-}$ DT40 B cells recovered when compared with DT40 B cells (WT) (Fig. 7). Interestingly, unstimulated Rac1^{Q61L} overexpressing *vav3*$^{-/-}$ DT40 cells proliferated as much as IgM-stimulated *vav3*$^{-/-}$ DT40 B cells (Fig. 7). Taken together, these results suggest an important role for Vav/Rac1 pathway in IgM-stimulated cell proliferation.

Discussion

Small GTPases of the Ras superfamily are components of a signalosome, participating actively in the integration of the extra- and intracellular signals. In this way, they control a wide variety of cellular processes among which are the actin cytoskeleton reorganization, the formation of integrin complexes, cell adhesion, gene transcription, cell cycle progression, and cell proliferation [4,5,32–34]. In fact, the control of CDKs by the activation of the growth factor-dependent Ras/MAP kinase pathway [35] leading to RB phosphorylation,

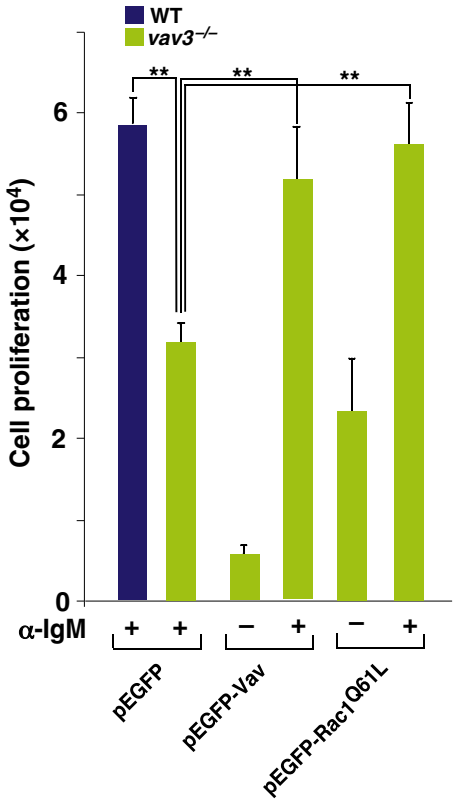

Fig. 7. Overexpression of Vav or Rac rescues *vav*$^{3-/-}$ DT40B cell proliferation. Proliferation analysis with the vital dye PKH26 of transfected DT40 B (WT, purple bar) and *vav*$^{3-/-}$ DT40B cells (green bars). Cells transfected with empty vector pEGFP-C2 or the different gene-expressing vectors, pEGFPVav and pEGFP Rac1^{Q61L}, were maintained in the serum-free medium for 24 h and stained with PKH26. Fluorescence was analysed before adding 10 μg·mL^{-1} α-IgM (0 h) and after 96 h incubation with α-IgM. Results show the mean ± SD of three independent experiments, and the statistical analysis showed a significant difference (**$P < 0.001$).

E2F activation and subsequent changes in gene transcription [36] and entry into the S phase of the cell cycle [37] is of great importance. However, this control over E2F-mediated transcription is not limited only to the Ras/MAP kinase pathway. For example, constitutively active forms of the GTPases Rac and Cdc42, but not Rho, were reported as being potent inducers of the activation of this transcription factor family culminating in S phase entry and DNA synthesis in both Swiss 3T3 cells and Rat-1 R2 cells. However, there exist additional requirements for NIH 3T3 cells, and E2F activation is not enough for entry into S phase and DNA synthesis [21].

In B and T lymphocytes, there is a hierarchy of GTPases activation in which Rac controls the activity of the Ras/MAP kinase pathway [3,38]. Our finding opened new prospects of studying the Rho family of GTPases in relation to the mechanisms that control cell proliferation in haematopoietic cells. In the present study, we show that the Vav/Rac pathway signals to the RB–E2F complex via the serine–threonine kinases, PAK1 and PKCε independenly of Ras.

Vav belongs to a family of proteins that act as guanine nucleotide exchange factors (GEFs) for small GTPases of the Rho family and play key roles in intracellular signalling, in lymphoid development, and in immune responses [39]. It has been reported that in T lymphocytes Vav or Rac knockouts manifest abnormal proliferation after T cell activation [40,41]. We have observed that B cells reproduce the same anomaly; the absence of Vav expression in *vav3*$^{-/-}$ DT40 B cells results in a striking proliferative defect after BCR activation. This defect due to the absence of Vav expression is a consequence of altered intracellular signalling that reflects a lack not only of Rac and Ras activation [2], but also of downstream signalling elicited by RB phosphorylation and E2F transcription factor transcriptional activity. RB phosphorylation state and transcriptional activity of E2F wild-type phenotype were rescued when *vav3*$^{-/-}$ DT40 B cells overexpressed either oncogenic Vav or the constitutively active forms of Rac1. In contrast, results obtained with RhoA and Cdc42 showed that these GTPases do not control the dynamics of the RB–E2F complex activation. Even when Cdc42 achieved a limited ability to phosphorylate RB, it was not enough to result in E2F transcriptional activation. To release E2F from the RB–E2F complex, RB needs to be hyperphosphorylated [42] and in our cellular model, Cdc42 was unable to hyperphosphorylate RB in comparison with Rac1. This phenomenon is not exclusive of Cdc42; Pruitt *et al.* reported that Raf regulates RB hyperphosphorylation and activation of E2F-mediated gene expression in NIH 3T3 and in RIE-1 epithelial cells. Raf regulated positively E2F transcriptional activation after RB hyperphosphorylation in NIH 3T3 cells; even when RB phosphorylation took place via Raf in RIE-1 cells, this phosphorylation was not enough to activate E2F-mediated transcription [6]. Therefore, our results obtained from Rac and Cdc42 presented here highlight the importance of the Rac-dependent pathway in the activation of E2F-mediated transcription in B cells. This hierarchy of Rac and Cdc42 in the control of B cell transcriptional activity is probably established by Vav RhoGEF, which does not seem to act as efficiently as a Cdc42 GEF to activate Cdc42 under conventional physiological catalytic/substrate ratio conditions.

Genetic and biochemical studies have shown that Rac interacts with its target proteins through multiple effector sites [24,43]. The Rac1 effector domain mutant F37A signals to PAK and JNK, but does not signal either to the actin cytoskeleton or to the Ras, and Y40C is unable to signal to PAK and JNK but does signal to both the actin cytoskeleton and to Ras [26]. We have found that in DT40 B cells, the action of Rac on RB–E2F complex signalling requires the participation of PAK, independently of both the Ras GTPase and actin cytoskeleton reorganization. There is some controversy regarding the role of PAK1 in mediating transcription activation and DNA synthesis. For some cellular models, such as Swiss 3T3, Cos-1 and REF-52 cells, it was reported that PAK1 activity is not required for mediating DNA synthesis [25,26,44]. In contrast, in other mammalian cells, besides being involved in the regulation of mitosis [45,46], PAK1 has emerged as a key element controlling the upregulation of cyclin D1 during the transition from the G1 to the S-phase of the cell cycle [47,48].

We postulate that the conection between PAK and the RB–E2F complex is set via ERK. In fact, Park *et al.* reported a new Ras-independent ERK activation pathway in which PAK controls ERK phosphorylation via MEK1 [49]. In agreement with this, neither ERK nor RB phosphorylations were detected (data not shown) when DT40 B cells (WT) were treated with MEK inhibitors. This suggests that the Vav/Rac pathway acts on the RB–E2F complex via the PAK/MEK/ERK pathway, independently of Ras. In parallel fashion, BCR activation stimulates activation of PI3K and PLCγ, in turn leading to the activation of downstream signalling molecules, such as PKCs, which can also influence the entry into the G1 phase of the cell cycle by regulating the CDK–cyclin complex activity or RB phosphorylation state [50,51].

In conclusion, our findings reveal that the Vav/Rac pathway requires PLCγ integrity in order to stimulate

E2F transcription activation and that it also integrates the PKCε signalling cascade in order to potentiate ERK activation. Indeed, the study of different cell models, such as NIH 3T3 fibroblasts, Rat 6, colonic epithelial cells, PC12 or HEK 293 demonstrated that PKCε controls the MAP kinase pathway at the level of Raf-1 [52]. Therefore, BCR activation in B cells regulates two Ras-independent signalling pathways leading to DNA synthesis and cell proliferation, i.e. the Vav/Rac and PKCε pathways, and both signalling pathway are integrated at the level of different components of the MAP kinase pathway where the control of RB–E2F complex disassembly is attributed to ERK. In summary, the proposed signalling pathways that are responsible for the Vav-mediated regulation of the RB–E2F complex activation are shown in Fig. 8. Future studies will allow us to evaluate the possible role of these signalling pathways in haematopoietic deregulated proliferative processes, such as the hairy cell leukaemia, an uncommon haematological malignancy characterized by an accumulation of abnormal B lymphocytes.

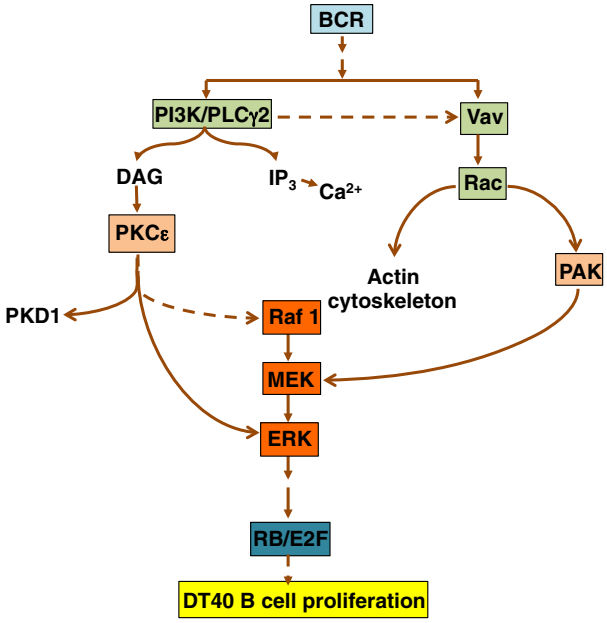

Fig. 8. Signalling model in which the Vav/Rac1 pathway controls the mechanism of the RB–E2F dissociation and mediates DT40 B cells proliferation independently of Ras.

Materials and methods

Antibodies and reagents

Mouse monoclonal antibody to chicken IgM was from Southern Biotech, mouse monoclonal anti-α-tubulin and anti-HA antibodies were obtained from Sigma-Aldrich (Dorset, UK) and Covance (Berkeley, CA, USA), respectively. Mouse monoclonal antibodies to Rac1 and Vav were purchased from Merck Millipore (Darmstadt, Germany). PKCε translocation inhibitor peptide (PKCε inhibitor peptide, EAVSLKPT) and PKCε translocation inhibitor peptide, negative control (scrambled peptide, LSETKPAN) were obtained from Calbiochem, Merck Millipore. Rabbit polyclonal antibodies to PKD, phospho-PKD, phospho-Raf, ERK, phospho-ERK and phospho-RB^{S780} were purchased from Cell Signaling Technology (Danvers, MA, USA), and the rabbit polyclonal anti-RB antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish-conjugated secondary antibodies to rabbit and mouse IgGs were obtained from GE Healthcare (Pittsburgh, PA, USA). Phorbol 12-myristate 13-acetate (PMA), PKC inhibitor GF109203X and Wortmannin were obtained from Sigma-Aldrich.

Plasmid construct and site-directed mutagenesis

pcDNA3-HA-PAK1^{T423E}, single mutated construct was generated by PCR according to the manufacturer's instructions (QuickChange Lightning Site-Directed Mutagenesis Kit; Stratagene) using pcDNA3-HA-PAK1 WT as template. Oligonucleotides used for T423E mutation were 5′-AGC AAA CGG AGC GAA ATG GTA GGA ACC-3′ (forward) and 5′-GGT TCC TAC CAT TTC GCT CCG TTT GCT-3′ (reverse). pcDNA3-PKCα^{A25E}, pcDNA3-PKCε^{A159E}, and pcDNA3-PKCε^{K437R} single mutated constructs were generated by PCR according to the manufacturer's instructions (QuickChange Lightning Site-Directed Mutagenesis Kit; Stratagene) using pcDNA3-PKCα and pcDNA3-PKCε as templates. Oligonucleotides used for A25E mutation were 5′-CGC AAA GGG GCG GAG AGG CAG AAG-3′ (forward) and 5′-CTT CTC CCT CAG CGC CCC TTT GCG-3′ (reverse), for A159E mutation were 5′-AGG AAG CGG CAG GGG GCC GTC AGG CGC AGG-3′ (forward) and 5′-CCT GCG CCT GAC GGC CCC CTG CCG CTT CCT-3′ (reverse) and for K437R were 5′-GTA TAT GCT GTG AAG GTC TTA AAG AAG GCA-3′ (forward) and 5′-GTC CTT CTT TAA GAC CCT CAC AGC ATA TAC-3′ (reverse). Plasmids encoding either wild-type (pCIneo-PLCγ1) or palmitoylated (pCIneo-palm PLCγ1) HA-tagged PLCγ1 were obtained from E. Bonvini (MacroGenics, Rockville, MD, USA). Mouse oncogenic Vav1 (Δ1–66 mutation), AU5-Rac 1, AU5-RhoA, and AU5-Cdc42 proteins have been previously described [2,3].

Cell culture and DNA transfections

Wild-type and mutant DT40 B cells (*vav3*$^{-/-}$ and *plcγ2*$^{-/-}$) were obtained from the Cell Bank, Riken Bioresource Center (Ibaraki, Japan) and cultured in RPMI 1640 med-

ium supplemented with 10% fetal bovine serum, 1% chicken serum, 2 mM β-mercaptoethanol, 1% glutamine, 100 ng·mL^{-1} streptomycin and 100 units·mL^{-1} penicillin (all from Life Technologies, Thermo Fisher Scientific). For transient transfection, cells (2×10^7) were washed twice, resuspended in 200 μL of serum-free medium, and placed in an electroporation cuvette (0.4 mm; Sigma-Aldrich) containing 10–20 μg of DNA. Cells and DNA mixture were electroporated at 260 V, 950 μF in a Gene Pulser Xcell Electroporator (Bio-Rad). The cuvette content was collected into 10 mL of complete RPMI 1640 medium and cultured for 24 h at 37 °C. Cell cultures transfected with vectors encoding the indicated proteins were verified as described before [2,3]. For cell stimulation, DT40 cells were suspended in RPMI 1640 media at a density of 5×10^6 cells·mL^{-1} and then treated with either 10 μg·mL^{-1} αIgM or 1 μM PMA for the indicated periods of time. Inhibition of specific signalling pathways was carried out by incubation of DT40 cells with indicated inhibitors for 1 h at 37 °C prior to cell stimulation. To inhibit PKC activities, DT40 B cells were incubated with 1 μM GF109203X for 1 h. To inhibit PI3K activity, DT40 B cells were incubated with 100 nM wortmannin for 1 h. After the indicated stimulation conditions, cells were disrupted by vortexing in a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Igepal® CA-630, 1 mM NaF, 100 μM Na_2VO_4, and a mixture of protease inhibitors (Complete, Roche Applied Science, Barcelona, Spain). The resulting cell lysates were cleared by centrifugation, diluted 1 : 1 in SDS/PAGE sample buffer, and subjected to western blot analysis with appropriate antibodies. Immunoreactive bands were visualized using a chemiluminescence detection system (Bio-Rad, Alcobendas, Madrid, Spain).

Rac1 activation assay

DT40 B cells were lysed in a buffer containing 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 10 mM $MgCl_2$, 1% Igepal® CA-630, 1 mM phenylmethylsulfonyl fluoride, 10 μg·mL^{-1} leupeptin and 10 U·mL^{-1} aprotinin. Cell lysates were collected after centrifugation and incubated for 1 h at 4 °C with specific GST-RBD (Rac Binding Domain of PAK 1) coupled to glutathione–sepharose beads. Precipitated proteins were eluted, electrophoresed by SDS/PAGE and analysed by western blotting with specific antibodies. Immunoreactive bands were visualized using ECL.

Luciferase assays

To measure transcriptional activity, cells were transfected by electroporation with reporter plasmid (pGL3-6xE2F) and vectors encoding the indicated proteins and a phRL-CMV plasmid (containing the cDNA encoding *Renilla* luciferase). After 36 h of transfection, cells were stimulated or not with 10 μg·mL^{-1} αIgM for the indicated times and lysed. The lysates were collected and assayed for luciferase activity by using the Dual Luciferase Reporter Assay System (Promega). Luciferase activity from untreated control cells was used for the background signal. All luciferase values were normalized to the *Renilla* luciferase readout values and expressed as fold induction relative to unstimulated cells.

PKCε inhibitor peptide transfection

DT40 B cells (WT) were transfected with the luciferase reporter gene containing 6xE2F sites in its promoter (pGL3-6xE2F). After 24 h, cells were serum-starved for 22 h and after this time cells were transfected with PKCε inhibitor peptide or scrambled peptide as control, using the Xfect Protein Transfection Reagent from Clontech Laboratories, Inc. (Takara Bio). Two hours later, cells were stimulated or not with 10 μg·mL^{-1} α-IgM for 5 h and E2F transcriptional activity was measured as indicated above.

Cell proliferation measurement

DT40 B and *vav3*$^{-/-}$ DT40 B cells (10^6 cells·mL^{-1}), previously serum-deprived overnight, were seeded in 24-well plates in complete RPMI 1640 medium and incubated with 4 μM PKH26 following the manufacturer's instructions (Sigma-Aldrich). A sample (10^4 cells) was taken as the start control, another sample (10^4 cells) was left untreated, and remaining cells were incubated in the presence of 10 μg·mL^{-1} α-IgM. For EGFP-expressing transfectants, 40×10^6 cells were transfected with EGFP-expressing constructs as shown in Fig. 7. The day after transfection, live cells were purified by Ficoll gradient following the manufacturer's instructions (GE Healthcare). Purified cells were maintained in serum-free medium for 24 h, and after that were incubated with PKH26 as explained above. A sample was taken from the non-stimulated control, and the remaining cells were grown in the presence of 10 μg·mL^{-1} α-IgM added every 24 h. Fluorescence was measured at 48 and 96 h (Fig. 1) and at 96 h (Fig. 7) in EGFP-construct transfected cells to monitor cell division rate on a FACSCalibur (Becton Dickinson) flow cytometer. Data obtained were analysed using MODFIT LT 3.0 software (Verity Software House, Topsham, ME, USA).

Statistical analysis

Student's *t*-test for the mean of two paired samples was used to determine the significance between data means (**$P < 0.05$, ***$P < 0.001$).

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Author contributions

N.Z., L.A.P. and J.L.Z. planned the experiments, N.Z., F.L., A.A. and P.G. performed the experiments, N.Z., F.L., A.A., P.G., H.M.L., L.A.P. and J.L.Z. analysed the data, and J.L.Z. wrote the paper.

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